MASARYKOVA UNIVERZITA

Přírodovědecká fakulta

Úloha proteinů rodiny Anterior gradient v lidských nádorech

Habilitační práce

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Poděkování

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Obsah

1. Úvod

1.1. Proteiny rodiny Anterior gradient

Rodina proteinů Anterior gradient (AGR) je u člověka tvořena třemi členy: AGR1 (ERp18), AGR2 a AGR3. Geny kódující proteiny Anterior gradient byly původně identifikovány při studiu vývoje embrya drápatky vodní (*Xenopus laevis*) [1]. Jako nejvýznamnější a tudíž i nejvíce prostudovaný se v současnosti jeví gen *XAG-2*, homolog lidského genu kódujícího protein AGR2, jenž hraje důležitou roli při vývoji embrya drápatky [2]. Zvýšená exprese *XAG-2* vede k diferenciaci cementové žlázy, jejíž buňky produkují mukózní sekret potřebný k přichycení čerstvě vylíhnutého embrya v období, než je pulec schopen sám plavat. Další práce zaměřené na studium AGR2 u *Notophthalmus viridescens* (čolek zelenavý), který je označovaný též jako nAG, prokázaly jeho nezastupitelnou úlohu při regeneraci končetin [3]. Regenerační proces začíná dediferenciací fibroblastů, svalových buněk a Schwannových buněk nervové tkáně. Zesílená proliferace těchto buněk následně vede ke vzniku značného množství mezenchymálních nediferencovaných buněk označovaných jako blastém, který je nezbytný pro vznik regenerované tkáně. nAG je při regeneraci exprimován především Schwannovými buňkami nervového obalu a následně pak i žlázovými buňkami epidermis poraněné tkáně, kde slouží jako mitogen podporující proliferaci buněk blastému [3].

1.2. Nomenklatura AGR proteinů

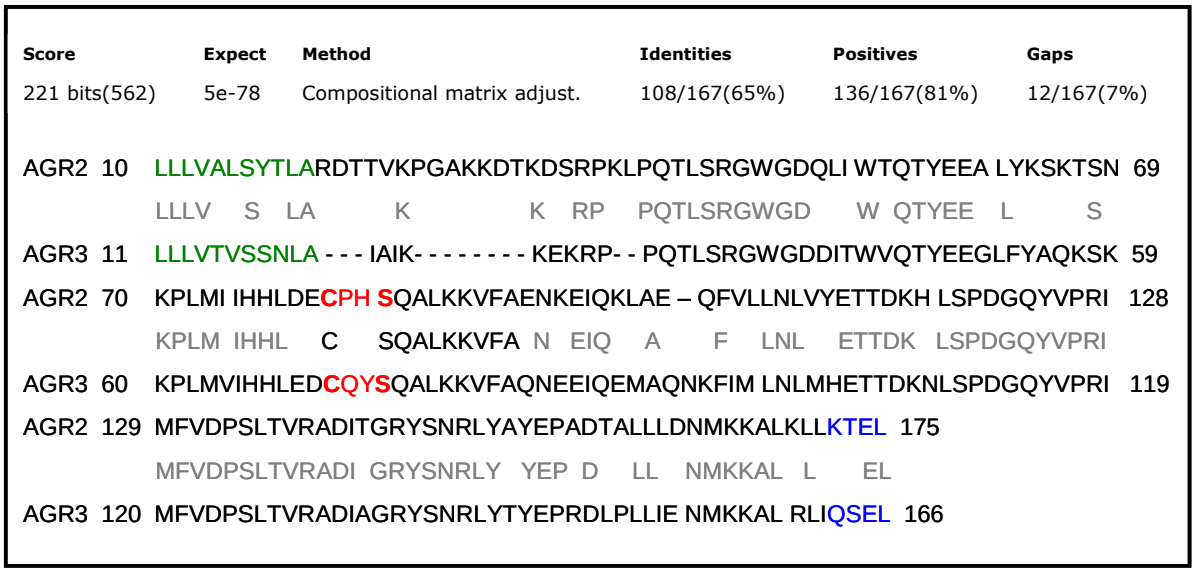
Z biochemického hlediska se všechny AGR proteiny řadí mezi tzv. protein disulfid isomerázy (PDI). Jsou typicky lokalizovány v endoplazmatickém retikulu (ER) prostřednictvím tzv. retenční sekvence. Jedná se o motiv typický pro většinu PDI, tvořený sekvencí aminokyselin lysin (K) – kyselina asparagová (D) – kyselina glutamová (E) – leucin (L), tedy K-D-E-L [4]. Retenční sekvence slouží k retrográdnímu transportu proteinů endoplazmatického retikula uplatňujících se při exportu sekrečních proteinů ven z buňky. Proteiny nesoucí K-D-E-L nebo jemu podobný motiv interagují se specifickými (KDEL) receptory v intermediárních kompartmentech Golgiho aparátu. Tato vazba způsobuje konformační změnu KDEL receptoru a nasměruje jej i s navázaným ligandem do váčků, které se vrací zpět do endoplazmatického retikula. Vyšší pH v endoplazmatickém retikulu pak vede k disociaci K-D-E-L peptidu z KDEL receptoru a jeho následné recyklaci [5]. V případě

AGR1 se můžeme na C-konci setkat se sekvencí E-D-E-L, v případě AGR2 je na C-konci K-T-E-L a u AGR3 Q-S-E-L motiv.

Ze strukturního a především funkčního hlediska je pro PDI důležitý tzv. thioredoxinový motiv, který se vyskytuje v oblasti thioredoxinové domény a obvykle obsahuje dva aktivní cysteinové zbytky oddělené od sebe dvěma různými aminokyselinami C-X-X-C. Právě s využitím těchto cysteinových zbytků se PDI v buňce podílí na oxidačně/redukčních reakcích, které mají zásadní význam pro vznik a správné uspořádání intramolekulárních a intermolekulárních disulfidových vazeb při zrání proteinů v endoplazmatickém retikulu. U AGR1 se můžeme setkat s plně funkčním thioredoxinovým motivem v pozici aminokyselin 66-69: C-G-A-C. Naopak v případě AGR2 a AGR3 se musíme spokojit pouze s motivem C-X-X-S (tzv. „*thioredoxin like*“ motiv), který obsahuje cysteinový a serinový zbytek oddělený dvěma libovolnými aminokyselinami. Přestože CXXS obsahuje pouze jeden cysteinový zbytek a ve srovnání s CXXC doménou vykazuje logicky nižší aktivitu, z hlediska reorganizace disulfidových vazeb může i tato doména přispívat k isomerizaci disulfidových vazeb v endoplazmatickém retikulu [6].

1.3. Geny kódující AGR proteiny

Kódující sekvence *AGR1* se u člověka nacházejí na chromosomu 1. Velikost genu je přibližně 36 kb, je tvořen celkem 7 exony, které mohou vést ke vzniku 4 různých transkripčních (sestřihových) variant, přičemž translací největšího transkriptu o velikosti 2396 bp vzniká protein tvořený 172 aminokyselinami o molekulové hmotnosti 19,206 kDa. Naopak geny *AGR2* a *AGR3* jsou lokalizovány v těsné blízkosti na krátkém raménku chromosomu 7. Jejich kódující sekvence vykazují značnou míru podobnosti (téměř 72 %) [7]. *AGR2* představuje oblast o velikosti přibližně 42 kb genomové DNA, ve které se vyskytuje celkem 8 exonů. Celkem bylo predikováno 9 různých transkripčních variant, z nichž pouze 5 dává vznik funkčnímu proteinu. Nejčastěji se vyskytující sestřihovou variantou je *AGR2-001*, jejíž translací vzniká polypeptid o velikosti 175 aminokyselin s předpokládanou molekulovou hmotností 19,979 kDa (**obr. 1**). V případě genu *AGR3*, který je tvořen taktéž 8 exony, je predikována možnost vzniku 4 rozdílných transkriptů, z nichž pouze 3 kódují funkční protein. Nejběžnější variantou je protein AGR3-001 sestávající ze 166 aminokyselin o molekulové hmotnosti 19,171 kDa (**obr. 1**).



Score	Expect	Method	Identities	Positives	Gaps
221 bits(562)	5e-78	Compositional matrix adjust.	108/167(65%)	136/167(81%)	12/167(7%)

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AGR2  10   LLLVALSYTLARDTTVKPGAKKDTKDSRPKLPQTLSRGWGDQLI WTQTYEEA LYKSKTSN  69
           LLLV  S  LA       K          K  RP   PQTLSRGWGD    W QTYEE  L      S
AGR3  11   LLLVTVSSNLA - - - IAIK- - - - - - - - KEKRP- - PQTLSRGWGDDITWVQTYEEGLFYAQKSK  59
AGR2  70   KPLMI IHHLDECPH SQALKKVFAENKEIQKLAE – QFVLLNLVYETTDKH LSPDGQYVPRI  128
           KPLM IHHL   C    SQALKKVFA N EIQ   A    F   LNL   ETTDK LSPDGQYVPRI
AGR3  60   KPLMVIHHLEDCQYSQALKKVFAQNEEIQEMAQNKFIM LNLMHETTDKNLSPDGQYVPRI  119
AGR2  129  MFVDPSLTVRADITGRYSNRLYAYEPADTALLLDNMKKALKLLKTEL  175
           MFVDPSLTVRADI GRYSNRLY  YEP D   LL  NMKKAL  L    EL
AGR3  120  MFVDPSLTVRADIAGRYSNRLYTYEPRDLPLLIE NMKKAL RLIQSEL  166
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Obr. 1: Primární sekvence proteinů AGR2 a AGR3. *Zeleně jsou znázorněny signální peptidy, červeně thiredoxin like domény a modře retenční sekvence endoplazmatického retikula.*

1.4. Funkce AGR proteinů v endoplazmatickém retikulu

Přítomnost AGR proteinů v endoplazmatickém retikulu naznačuje, že by se tyto proteiny mohly podílet na některých z funkcí této buněčné organely. ER představuje významnou součást sekrečních buněčných drah, neboť umožňuje transport látek do perinukleárního prostoru jádra, ale také směrem z buňky ven do extracelulárního prostoru. Mezi další nezastupitelné funkce ER patří významný podíl na regulaci i) metabolismu lipidů, ii) hladiny vápenatých iontů a především pak iii) proteosynézy včetně oligomerizace nascentních polypeptidů. Vlivem celé řady vnějších signálů nebo působením specifických podmínek buněčného mikroprostředí může docházet k narušení funkce endoplazmatického retikula. Buňka se těmto tlakům snaží čelit vyvoláním procesu, který je označovaný jako stres endoplazmatického retikula, tzv. „*unfolded protein response"* neboli UPR (pro více informací viz práci: „*Endoplasmic reticulum stress and apoptosis*", **příloha 1**).

Higa a kol. provedli proteomickou analýzu, jejímž hlavním cílem byla identifikace proteinů, které jsou asociovány s ribozomy drsného ER. V rámci této studie byl mimo jiné identifikován i protein AGR2, který byl lokalizován v lumen ER, kde byla prokázána jeho interakce s ribozomy. Následně byla této interakci přiřazena důležitá úloha v rámci procesu translokace nascentních polypeptidů [8]. Následovaly další práce, ve kterých byla prokázána přímá spojitost mezi UPR a AGR2 [9]. Význam exprese AGR2 pro nádorové buňky a především pak fundamentální úloha AGR2 při udržování homeostázy ER a v rámci odpovědi ER na stres jsou shrnuty v přehledovém článku nazvaném „*Emerging roles for the pro-oncogenic anterior gradient-2 in cancer development*" (**příloha 2**).

2. AGR2 a AGR3 u nádorových onemocnění

Ve vztahu k nádorovým onemocněním byly proteiny AGR2 a AGR3 poprvé popsány u nádorových buněčných linií odvozených od karcinomu mléčné žlázy. Zajímavé bylo především zjištění, že tyto proteiny jsou exprimovány spolu s funkčními estrogenovými receptory (EsR) [7,10], které představují významný prognostický a prediktivní faktor při léčbě zhoubných novotvarů mléčné žlázy. Poté následovalo několik dalších prací, které se zaměřovaly především na protein AGR2 a možnosti jeho využití jako prognostického nebo prediktivního markeru u karcinomů mléčné žlázy [11-13].

2.1. Analýza exprese AGR2 a AGR3 u pacientek s karcinomem mléčné žlázy

V návaznosti na tyto práce byl započat výzkum zaměřený na úlohu proteinů rodiny anterior gradient i na našem pracovišti. Jako první byl analyzován konsekutivní soubor 109 pacientek s primárním karcinomem mléčné žlázy, které byly hospitalizovány v Masarykově onkologickém ústavu v letech 2004-2005. V rámci standardizovaného zpracování byly všechny vzorky obdrženy do 20 minut po chirurgickém zákroku a okamžitě zhodnoceny patologem. Nádorová tkáň byla rozkrájena na kousky přibližně 3×3×8 mm, které byly následně zamraženy v parách tekutého dusíku nebo uloženy ve fixačním roztoku vhodném pro stabilizaci a protekci RNA. Souběžně byla část nádorové tkáně zfixována, dehydratována a z takto upravené tkáně byly připraveny parafinové bloky. Parafinové bloky byly použity pro přípravu tkáňových arrays umožňujících imunohistochemické barvení několika desítek vzorků současně na jednom skle.

Exprese *AGR2* byla analyzována ve vztahu k rutinně stanovovaným klinicko-patologickým parametrům a to na úrovni mRNA pomocí RT-qPCR (**tab. 1**) i proteinu imunohistochemicky (**tab. 2**).

Tab. 1: Stanovení hladiny exprese AGR2 a AGR3 na úrovni mRNA pomocí RT-qPCR.

Parametr	Celkem vzorků	exprese *AGR2* (p-hladina)		exprese *AGR3* (p-hladina)	
		Všechny vzorky	EsR-pozitivní vzorky	Všechny vzorky	EsR-pozitivní vzorky
Histologický grade					
G1	29				
G2	38	0,233 [2]	0,565 [2]	**0,001** [2]	0,177 [2]
G3	41				
Uzlinové metastázy					
Negativní	41	0,488 [1]	0,672 [1]	0,265 [1]	0,600 [1]
Pozitivní	64				
Velikost tumoru	109	0,388 [3] Rs 0,084	0,312 [3] Rs 0,108	0,273 [3] Rs 0,107	0,187 [3] Rs 0,143
EsR status					
Negativní	19	**0,034** [1]	-	**<0,001** [1]	-
Pozitivní	90				
PR status					
Negativní	24	**0,015** [1]	-	**<0,001** [1]	-
Pozitivní	85				
Her2 amplifikace					
Negativní	87	0,451 [1]	0,738 [1]	0,948 [1]	0,657 [1]
Pozitivní	13				
CD1 amplifikace					
Negativní	83	0,350 [1]	0,280 [1]	0,702 [1]	0,436 [1]
Pozitivní	14				
CD1 exprese (%)	96	**0,005** [3] Rs 0,285	**0,010** [3] Rs 0,290	**<0,001** [3] Rs 0,386	**0,020** [3] Rs 0,268
PCNA exprese					
1 (slabá)	19				
2 (střední)	39	0,310 [2]	0,638 [2]	0,347 [2]	0,571 [2]
3 (silná)	36				
Ki67 exprese (%)	109	0,525 [3] Rs 0,062	0,075 [3] Rs 0,189	0,816 [3] Rs -0,023	0,169 [3] Rs 0,189

[1] Mannův-Whitneyův U test

[2] Kruskalův-Wallisův ANOVA test

[3] Spearmannova neparametrická korelace

Tab. 2: Imunohistochemické stanovení klinicko-patologických parametrů ve vztahu k expresi proteinu AGR2.

Parametr	Celkem vzorků	AGR2 negativní (%)	AGR2 nízká (%)	AGR2 silná (%)	p-hladina	p-hladina*
Histologický grade						
G1	28	5	15	8		
G2	35	8	20	7	0,161 [4]	0,275 [4]
G3	34	14	11	9		
Uzlinové metastázy						
Negativní	39	16	15	8	0,075 [4]	0,070 [4]
Pozitivní	56	11	29	16		
Velikost tumoru	98	27	47	24	0,202 [2]	0,310 [2]
EsR status						
Negativní	17	15	2	0	**<0,001 [4]**	-
Pozitivní	81	12	45	24		
PR status						
Negativní	19	14	5	0	**<0,001 [4]**	-
Pozitivní	79	13	42	24		
Her2 amplifikace						
Negativní	87	26	41	20	0,308 [4]	0,272 [4]
Pozitivní	11	1	6	4		
CD1 amplifikace						
Negativní	84	25	42	17	0,115 [4]	0,205 [4]
Pozitivní	13	2	5	6		
CD1 exprese (%)	97	27	46	24	**0,018 [2]**	0,568 [2]
PCNA exprese						
1 (slabá)	22	4	14	4		
2 (střední)	38	5	22	11	**0,015 [4]**	0,365 [4]
3 (silná)	36	16	11	9		
Ki-67 exprese (%)	97	27	46	24	0,061 [2]	0,151 [2]

*pouze EsR-pozitivní vzorky
[2]Kruskalův-Wallisův ANOVA test
[4]Pearsonův Chi-square test

Exprese AGR3 byla analyzována pouze pomocí PCR v reálném čase, neboť tehdy ještě nebyla k dispozici vhodná AGR3 specifická protilátka. V případě exprese na úrovni mRNA bylo možné u *AGR2* a *AGR3* pozorovat obdobný expresní profil ($p<0.001$), což poukazuje na podobné mechanismy uplatňující se při regulaci exprese *AGR2* a *AGR3*. Exprese obou studovaných genů pak korelovala s přítomností EsR, progesteronových

receptorů (PR) a expresí cyklinu D1 (**tab. 1)**. Jediný rozdíl bylo možné pozorovat při analýze exprese vzhledem ke stupni diferenciace nádoru, neboť histologický grade statisticky významně koreloval pouze s expresí *AGR3* (p=0,001). V případě imunohistochemického stanovení AGR2 vzhledem k rutinně stanoveným klinicko-patologickým parametrům byla opět potvrzena korelace mezi hladinou exprese AGR2 a přítomností EsR, PR a expresí cyklinu D1. Lze tedy uzavřít, že byl potvrzen trend obdržený v rámci analýzy exprese na úrovni mRNA. Navíc bylo možné pozorovat statisticky významnou asociaci s expresí PCNA (p=0,015) a hraničně s Ki-67 (0,061), tedy markery zvýšené proliferace (**tab. 2**).

Postupně byla přítomnost AGR proteinů, především pak AGR2, detekována u řady dalších malignit. Po karcinomu mléčné žlázy jsou co do počtu nejčetnější práce zaměřené na studium AGR2 u karcinomů prostaty. V souvislosti s touto malignitou se na jednu stranu můžeme setkat s publikacemi ukazujícími korelaci mezi zvýšenou expresí AGR2 a zhoršenou prognózou onemocnění [14], ale naopak i publikacemi, které deklarují, že AGR2 u tohoto onemocnění žádný prognostický význam nemá [15]. Z hlediska regulace a vlastností AGR2 je důležitá práce prokazující regulaci exprese AGR2 prostřednictvím androgenních receptorů [5,16], což je v souladu s našimi výsledky získanými studiem AGR2 u karcinomů mléčné žlázy a se současným obecným názorem, že exprese AGR2 je převážně regulována působením hormonů. V podstatě všechny práce se shodují a diskutují možnosti využití AGR2 jako biomarkeru karcinomu prostaty [17]. Kromě výše zmíněných karcinomů mléčné žlázy a prostaty byla exprese AGR2 popsána u řady dalších malignit včetně nádorů jícnu, vaječníků, slinivky břišní, jater, žaludku, střev, plic, ledvin a dalších. Podrobnější informace o expresi proteinu AGR2 v různých typech tkání, a to nejen nádorových, ale i normálních jsou shrnuty v přehledovém článku s názvem *„Anterior gradient 2: a novel player in tumor cell biology“* (**příloha 3**).

V současné době je onkopatology u převážné většiny případů karcinomu mléčné žlázy, spolu s klasickými klinicko-patologickými parametry, rutinně stanovována pouze přítomnost funkčních estrogenových (konkrétně estrogenový receptor α), progesteronových a HER2 receptorů. Toto je v současné době realita, a to i navzdory neutuchající snaze nalézt nové vhodné biomarkery, které by byly využitelné při diagnostice či léčbě tohoto onemocnění. Tato praxe spolu se skutečností, že přibližně 70-80 % karcinomů mléčné žlázy exprimuje v době diagnózy estrogenové receptory, jež představují nejdůležitější a dnes jediný standardně vyšetřovaný prediktivní faktor pro hormonální léčbu, iniciovaly náš zvýšený zájem o geny *AGR2* a *AGR3*, jejichž zvýšená exprese byla pozitivně asociována právě s přítomností estrogenových receptorů. Skutečně jsme prokázali, že exprese genů *AGR2* i *AGR3* významně

koreluje s přítomností funkčních estrogenových receptorů, a to jak *in vitro* na modelových buněčných liniích, tak i na klinických vzorcích získaných z resekátů odebraných v rámci chirurgických výkonů prováděných v Masarykově onkologickém ústavu v Brně, což dokládají i publikované práce: „*The pro-metastatic protein anterior gradient-2 predicts poor prognosis in tamoxifen-treated breast cancers*“ (**příloha 4**) a „*Anterior gradient protein 3 is associated with less aggressive tumors and better outcome of breast cancer patients*“ (**příloha 5**).

3. Mechanismy zodpovědné za regulaci exprese AGR2

3.1. Klasický mechanismus účinku EsR

Skutečnost, že exprese *AGR2* a *AGR3* na úrovni mRNA prokazatelně koreluje s expresí EsR, však ještě nemusí nutně znamenat, že hladina proteinů AGR2 a AGR3 je přímo regulována působením estrogenů. Prvním krokem proto bylo alespoň nepřímo potvrdit funkční vztah mezi aktivitou EsR a expresí AGR2. Do kultivačního média, ve kterém byla kultivována buněčná linie MCF-7 (EsR+, AGR2+), bylo přidáváno různé množství 17-β estradiolu (dále jen estrogenu) a analyzovány změny hladiny AGR2. Tento jednoduchý experiment prokázal, že hladinu proteinu AGR2 lze pozitivně modulovat přítomností estrogenu, který představuje přirozený ligand EsR (**příloha 4**). V návaznosti na získané výsledky byla provedena *in silico* analýza potenciální promotorové sekvence *AGR2*, v rámci které byly identifikovány ERE (*estrogen response element*) konsensus sekvence, na které se mohou specificky vázat EsR a přímo tak regulovat transkripci cílových genů. Pomocí chromatinové precipitace byla skutečně potvrzena vazba EsR na promotorové sekvence *AGR2*. Následně byly promotorové sekvence *AGR2* naklonovány do pGL3 plazmidu před sekvenci kódující luciferázu. Získaný konstrukt byl kotransfekován spolu s pSG5-ERα (plazmid exprimující EsR) do buněk H1299 (EsR-, AGR2-). Po přidání estrogenu a tamoxifenu bylo možné pozorovat zvýšenou tvorbu luciferázy, což potvrdilo, že exprese *AGR2* je skutečně regulována na úrovni transkripce pomocí EsR (**příloha 4**).

Po vstupu do cytoplazmy se estrogen váže na EsR, což vede k uvolnění receptoru z komplexu s chaperony, především Hsp90. EsR je fosforylován, dochází k jeho dimerizaci, čímž je v podstatě plně aktivován a translokován do jádra, kde plní funkci transkripčního faktoru, neboť se následně váže na promotory genů obsahujících ERE místa. Význam funkčních EsR v etiologii karcinomu mléčné žlázy dokládá řada prací. V rámci expresního profilování zaměřeného na geny regulované EsR bylo pomocí čipové analýzy odhaleno více

než 400 genů, u kterých docházelo k významným změnám v expresi v rámci odpovědi na působení estrogenu, a co je zajímavé, u většiny z nich (cca 70%) byla exprese snížena [18]. Především se jednalo o proapoptotické geny, antiproliferační geny a geny kódující transkripční represory. Naopak zvýšená exprese byla pozorována především u genů uplatňujících se při pozitivní regulaci buněčné proliferace, jako jsou růstové faktory či regulátory buněčného cyklu umožňující jeho progresi. I z této práce a mnoha dalších vyplývá, že aktivované EsR indukují expresi proonkogenů a expresi tumor supresorových genů naopak potlačují.

Je tedy nasnadě, že vzhledem k frekvenci výskytu EsR u karcinomů mléčné žlázy a zvýšené hladině hormonů představuje inhibice exprese EsR významný terapeutický přístup. Léčba tamoxifenem je standardní léčebnou modalitou karcinomu mléčné žlázy již od 70. let minulého století a je úspěšně používána až doposud právě u EsR-pozitivních nádorů, které představují více jak 70 % všech případů tohoto onemocnění [19]. Mechanismus účinku tamoxifenu spočívá v kompetitivní inhibici s ligandy, které se váží na EsR, tedy především estrogeny. Nicméně i přes jednoznačný přínos tamoxifenu v léčbě nádorových onemocnění mléčné žlázy, který se odráží především v delším celkovém přežití pacientek, se u řady případů EsR-pozitivních karcinomů po čase objevuje rezistence k tamoxifenu a vyskytují se i případy, kdy bezprostředně po nasazení tamoxifenu nebyl zaznamenán žádný pozitivní léčebný efekt. Jinými slovy se můžeme setkat i s případy, kdy jsou nádory k tamoxifenu rezistentní *de novo*. Dále se odhaduje, že asi u 40 % případů dochází v rámci adjuvantní terapie založené na tamoxifenu ke vzniku rezistence, která vede k relapsu onemocnění a případně i k úmrtí. Možnost predikovat rezistenci k tamoxifenu by tedy mohla významně přispět ke zvýšení účinnosti léčby. O to zajímavější a překvapivější bylo naše zjištění, že působením tamoxifenu dochází k razantnímu zvýšení exprese *AGR2 in vitro* i *in vivo*, a to jak na úrovni mRNA, tak i proteinu (**příloha 4**). Ve světle funkčních studií, které potvrdily, že AGR2 má schopnost inhibovat nádorový supresor p53 [20,21], může být zodpovědný za tvorbu metastáz [22] a přímo se podílet na zvýšené proliferaci a migraci nádorových buněk [23] a spolu s naším zjištěním, že tamoxifen indukuje expresi AGR2, lze spekulovat, že by se AGR2 mohl přímo uplatňovat při mechanismech zodpovědných za rezistenci karcinomů mléčné žlázy k tamoxifenu a jeho zvýšená exprese by mohla predikovat sníženou citlivost právě k léčbě tamoxifenem.

Analýza exprese *AGR2* na úrovni mRNA pomocí RT-qPCR na konsekutivním souboru pacientek s EsR-pozitivním karcinomem mléčné žlázy léčených tamoxifenem potvrdila, že pacientky s nižší hladinou *AGR2* vykazují významně delší bezpříznakové přežití v porovnání

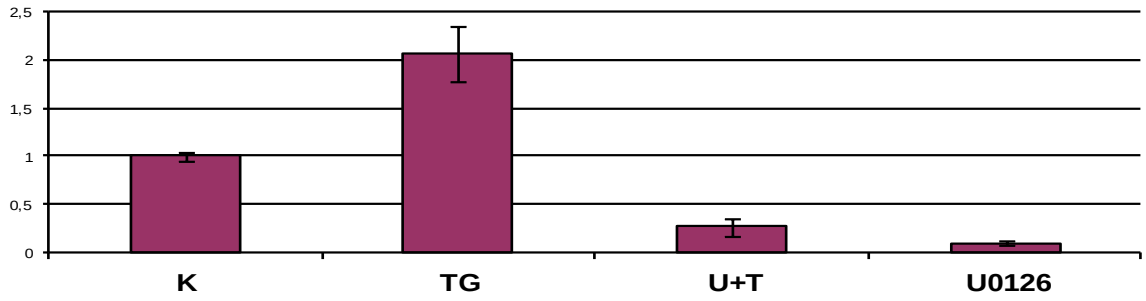
s pacientkami se zvýšenou expresí *AGR2* (**příloha 4**). Možnou úlohu AGR2 v mechanismech zodpovědných za rezistenci k tamoxifenu dokresluje i naše navazující práce *„AGR2 predicts tamoxifen resistance in postmenopausal breast cancer patiens“* (**příloha 6**), v rámci které byla provedena retrospektivní analýza exprese AGR2 v „tru-cut“ jehlových biopsiích odebraných od pacientek s EsR-pozitivním karcinomem mléčné žlázy, které nemohly být z důvodu vysokého věku nebo celkové sešlosti operovány a byl jim pouze v rámci adjuvantní léčby podáván tamoxifen. Na tomto souboru postmenopauzálních pacientek bylo možné přímo sledovat odpověď na podávanou terapii vzhledem k expresi AGR2 v nádorové tkáni, která byla stanovena nejen na úrovni mRNA pomocí RT-qPCR, ale i na úrovni proteinu pomocí imunohistochemického barvení. Tato studie skutečně naznačila možnost využití AGR2 jako prediktivního markeru v rámci endokrinní terapie karcinomu mléčné žlázy, neboť na základě snížené hladiny mRNA genu *AGR2* bylo možné identifikovat skupinu pacientek, které odpovídaly na terapii, a tudíž docházelo k regresi onemocnění (HR 0,57; 95% IS 0,31 - 0,96; p = 0,0366) a na druhou stranu u pacientek se zvýšenou expresí AGR2 se v mnohem vyšší frekvenci objevovaly případy, kdy léčba tamoxifenem nebyla účinná, nebo dokonce docházelo i k progresi onemocnění (p = 0,0011) [24]. Právě v těchto případech by pak zvýšená hladina mohla predikovat rezistenci k tamoxifenu a pacientkám by měly být indikovány jiné terapeutické režimy.

3.2. Neklasický mechanismus účinku EsR

Kromě genomového tzv. klasického mechanismu účinku EsR se můžeme setkat i s tzv. neklasickým mechanismem účinku, kdy receptor může interagovat s jiným transkripčním faktorem, nejčastěji Sp-1 (*specific protein-1*) nebo AP-1 (*activator protein-1*) a zesilovat tak intenzitu transkripce cílových genů, jejichž promotorové sekvence nemusí nutně obsahovat ERE místo [25]. Skutečnost, že v promotoru genu *AGR2* bylo *in silico* predikováno vazebné místo pro transkripční faktor AP-1, umocnila naše spekulace, že by se tento transkripční faktor mohl podílet na regulaci exprese *AGR2* ať už v komplexu s EsR nebo na tomto streroidním receptoru nezávisle a iniciovala naši snahu prokázat nebo naopak vyvrátit tento potenciální regulační mechanismus. AP-1 je transkripční faktor indukovatelný působením řady hormonů a růstových faktorů. K jeho vytvoření dochází dimerizací transkripčních faktorů Fos a Jun mechanismem leucinového zipu. Při modulaci transkripční aktivity AP-1 se uplatňuje řada signálních drah, mezi nejčastější patří ERK1/2, JNK či p38 MAPK. Je zajímavé, že právě zablokováním MEK1/2 respektive ERK1/2 signální dráhy pomocí inhibitoru PD98059 lze výrazně potlačit expresi proteinu AGR2 [26]. Tuto skutečnost jsme

potvrdili i pomocí inhibitoru MAP kináz MEK1/2 označeného U0126. Popsané výsledky nepřímo naznačují na možnou regulaci exprese *AGR2* působením aktivované ERK1/2 signální dráhy, která může úzce souviset s aktivací AP-1 právě v rámci neklasického mechanismu účinku EsR.

Prvním krokem při snaze prokázat predikovanou vazbu AP-1 na promotor genu *AGR2* bylo provedení chromatinové imunopreciptace (ChIP). Pomocí 1% formaldehydu byl vytvořen „cross-link" mezi DNA a proteiny, následovala lýze buněk a sonikace vedoucí k fragmentaci DNA. Buněčný lyzát byl inkubován s c-Jun specifickou protilátkou s cílem precipitovat komplex AP-1 s navázanými fragmenty DNA. Pomocí PCR v reálném čase byla identifikována a současně kvantifikována *AGR2* promotorová sekvence, na kterou se navázal AP-1. Pro experiment byla použita buněčná linie MCF-7, jenž byla vystavena působení thapsigarginu (indukce ER stresu vedoucí ke zvýšené expresi AGR2), U0126 (inhibitor MEK1/2) a kombinaci obou látek (**obr. 2**).

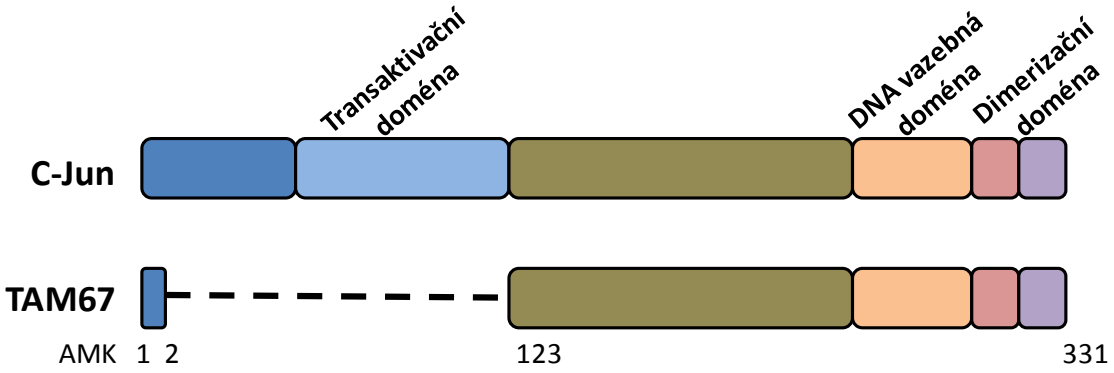


Obr. 2: Analýza vazebné afinity AP-1 k promotoru AGR2 pomocí ChIP. *Změny v intenzitě vazby transkripčního faktoru AP-1na promotor genu AGR2 u buněčné linie MCF-7, která byla vystavena působení thapsigarginu (TG), inhibitoru MEK1/2 (U0126) a jejich kombinaci (U+T) byly relativně kvantifikovány vzhledem ke kontrolním buňkám (K).*

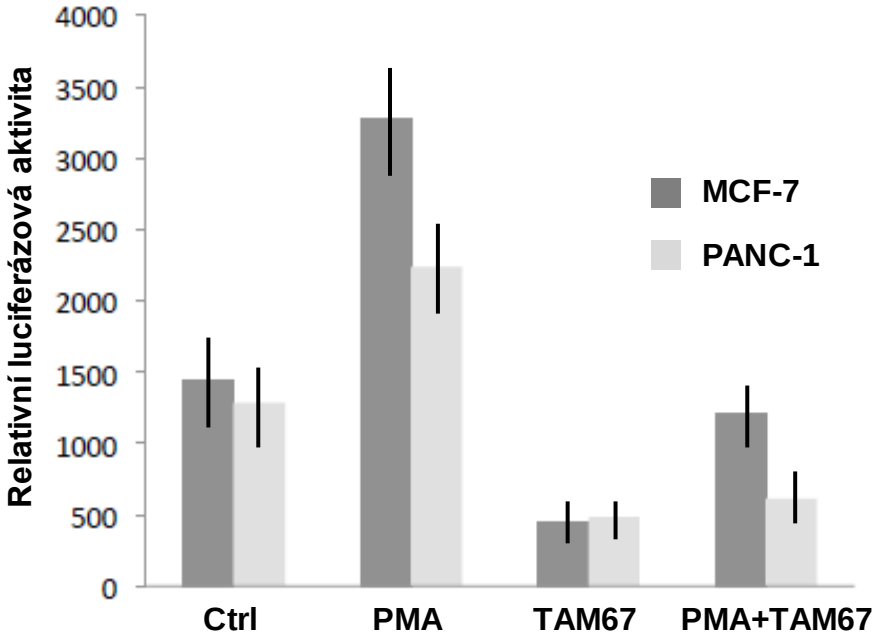
Jak vyplývá z graficky zobrazených výsledků, při indukci stresu ER působením thapsigarginu docházelo k výraznému zvýšení afinity AP-1 transkripčního faktoru k promotoru *AGR2*. Naopak po působení U0126 se AP-1 transkripční faktor vázal na *AGR2* promotor v mnohem nižší míře v porovnání s kontrolními buňkami. Stejně tak kombinované působení thapsigarginu a U0126 vedlo ke snížení hladiny AP-1, respektive jeho vazebné afinity (**obr. 2**).

S cílem funkčně potvrdit vztah mezi AP-1 a expresí *AGR2* jsme připravili expresní vektor pcDNA3.2-GW-TAM67 nesoucí nekompletní kódující sekvenci proteinu c-Jun, postrádající N-koncovou transaktivační doménu (**obr. 3**). Vzniklý mutovaný protein označený

jako TAM67 vykazuje dominantně negativní efekt vůči *wild-type* c-Jun, který se projevuje inhibicí aktivity AP-1 komplexu [27]. Schopnost TAM67 inhibovat AP-1 byla následně ověřena pomocí reportérového luciferázového systému (**obr. 4**).

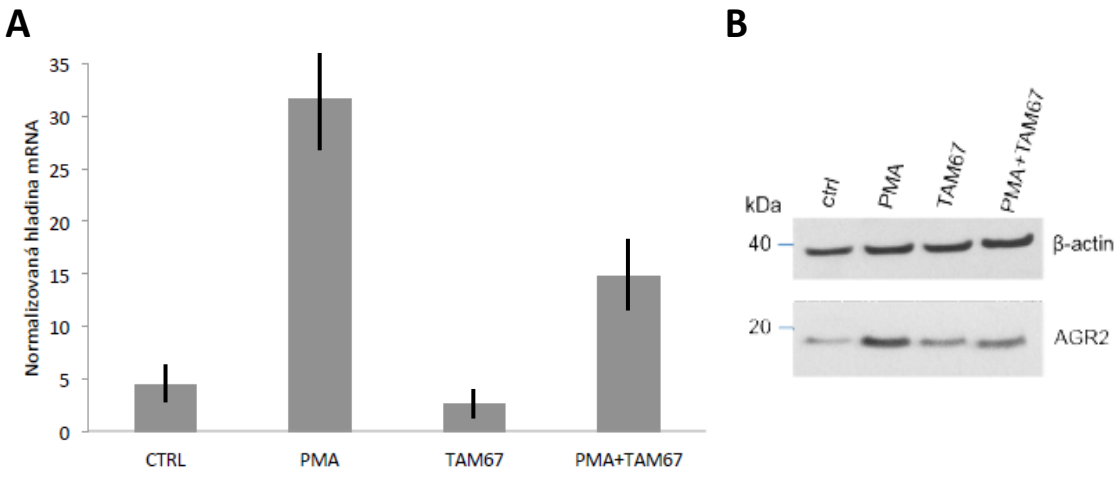


Obr. 3: Struktura proteinů c-Jun a TAM67. *Dominantně negativní mutant proteinu c-Jun postrádající N-koncovou transaktivační doménu TAM67 má celkovou velikost 210 aminokyselin (AMK) v porovnání s wild-type proteinem c-Jun, jenž má velikosti 331 aminokyselin.*



Obr. 4: Stanovení transkripční aktivity AP-1. *Transkripční aktivita byla ověřena reportérovým testem na buněčných liniích MCF-7 a PANC-1. Buňky byly tranzientně transfekovány p3TPluc plazmidem nesoucím gen kódující luciferázu, před kterým je umístěna regulační sekvence, na níž se specificky může vázat transkripční faktor AP-1 a aktivovat tak expresi genů nacházejících se v bezprostřední blízkosti. Relativní luciferázová aktivita vyjadřuje transkripční aktivitu AP-1 responzivního místa, která je aktivována 20 nM PMA (forbolmyristátacetát) a inhibována transfekcí 1 μg plazmidu pcDNA3.2-GW-TAM67.*

Dalším krokem bylo studium vlivu transkripčního komplexu AP-1 na expresi genu *AGR2* s cílem prokázat případné zapojení AP-1 v regulaci exprese tohoto genu. Jako modelový systém byla vybrána buněčná linie MCF-7, do které byl transientně transfekován plazmid pcDNA3.2-GW-TAM67 umožňující expresi AP-1 dominantně negativního mutanta TAM67 nebo pouze prázdný plazmid pcDNA3.2-GW. Poté byly transfekované buňky vystaveny působení PMA (forbolmyristátacetát), který působí mj. i jako aktivátor transkripce prostřednictvím AP-1. Změny v expresi *AGR2* byly stanoveny pomocí RT-qPCR po 24 hod kultivaci (**obr. 5A**) a imunochemicky po 72 hod (**obr. 5B**). PMA indukoval expresi *AGR2* na úrovni mRNA i proteinu. Avšak v případě, že v buňkách docházelo k produkci AP-1 dominantně negativního mutanta TAM67, jsme pozorovali výrazně nižší hladiny AGR2.



Obr. 5: Exprese AGR2 v důsledku ovlivnění aktivity AP-1 transkripčního komplexu. *Stanovení hladiny AGR2 na úrovni mRNA (A) a proteinu (B) na buněčné linii MCF-7.*

3.3. Negenomová aktivita EsR

Ačkoli přítomnost funkčních EsR sama o sobě představuje významný prediktor efektivity endokrinní terapie, funkční interakce mezi EsR a dalšími signálními dráhami, které se uplatňují při regulaci buněčného růstu, při odpovědi na stresové podmínky, reakci na zvýšenou hladinu cytokinů apod. byly též popsány jako významný mechanismus vedoucí/přispívající k rezistenci k endokrinní terapii. Hovoříme o tzv. negenomové aktivitě EsR, kdy je receptor obvykle asociován s plazmatickou membránou a vytváří komplexy s dalšími proteiny, především tyrosinkinázovými receptory nebo proteiny G. Tyto interakce obvykle vedou k aktivaci proproliferačních buněčných signálních drah, jako jsou např.

MAPK/ERK nebo PI3K/AKT [28]. Klinická relevance této formy komunikace EsR se signálními dráhami aktivovanými působením růstových faktorů byla potvrzena i prospektivními klinickými studiemi na souborech pacientek s metastatickým onemocněním. Tyto studie potvrdily spojení mezi rezistencí k tamoxifenu a zvýšenou hladinou tyrosinkinázových receptorů HER2 a EGFR [29].

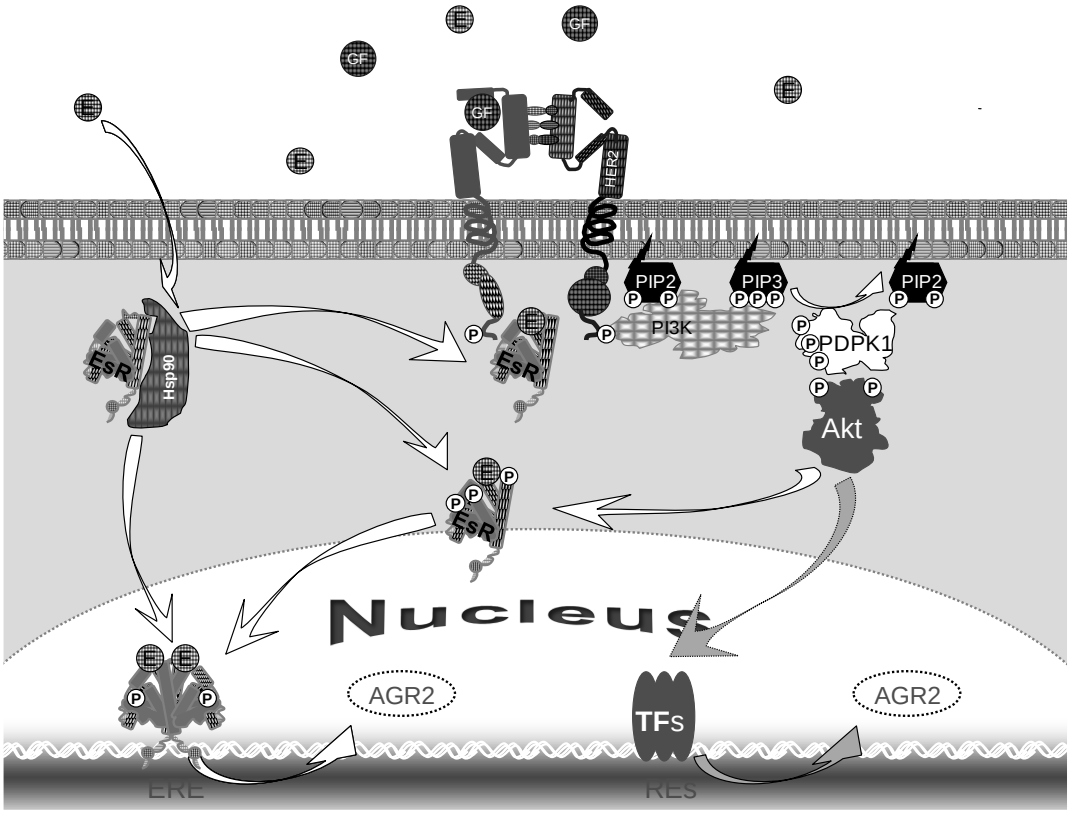
Na možnou spojitost mezi expresí AGR2 a EGFR poukázali ve své práci Dong a kol., kteří testovali hypotézu, zda může protein AGR2 ovlivňovat buněčnou signalizaci řízenou receptory EGFR [30]. V souvislosti s tím byla zjištěna korelace mezi expresí AGR2 a hladinou proteinu AREG (amfiregulin) v buňkách pocházejících z různých typů lidských adenokarcinomů. AREG představuje významný ligand EGFR receptoru, po jehož vazbě dochází k fosforylaci receptoru a iniciaci signalizace aktivující další signální dráhy včetně PI3K/AKT. Posléze se podařilo potvrdit, že AGR2 se přímo uplatňuje při regulaci hladiny AREG, a to jak na úrovni mRNA, tak i proteinu a tudíž může tímto způsobem aktivovat EGFR a PI3K/AKT signální dráhu [30].

Možnost regulace exprese AGR2 i některým z alternativních mechanismů naznačuje i již výše zmíněná práce Zweitzig a kol., v níž autoři ukazují, že exprese AGR2 může být u karcinomů mléčné žlázy ovlivňována i fyziologickým stresem, jako je např. hypoxie či hladovění a že při inhibici ERK1/2 dochází k signifikantní redukci hladiny AGR2 [26].

Další indicií byly výsledky skríningu potenciálních nízkomolekulárních inhibitorů různých buněčných signálních drah v kombinaci s genetickým skríningem pomocí RNA interference, který provedli Iorns a kol. s cílem nalézt nové inhibitory a identifikovat signální dráhy, které se uplatňují při vzniku rezistence k tamoxifenu [31]. Pomocí tohoto přístupu byly skutečně nalezeny dvě sloučeniny triciribin a tetrandrin, které způsobovaly sensitizaci nádorových buněk k tamoxifenu, přičemž byly následně verifikovány jako inhibitory PDPK1-AKT signální dráhy.

Vzhledem k našim předchozím výsledkům potvrzujícím důležitou úlohu AGR2 při rezistenci k tamoxifenu a v návaznosti na uvedené práce jsme se zaměřili na analýzu vztahu mezi působením tamoxifenu, expresí AGR2 a AKT signální dráhou. Prvním krokem byl průkaz vztahu mezi expresí AGR2 a fosforylací AKT na serinu 473 působením tamoxifenu a v kombinaci s působením AKT specifických inhibitorů v čele s triciribinem, terandrinem a Akti-1/2. Při tomto pokusu jsme dle předpokladu pozorovali indukci exprese AGR2 v rámci buněčné odpovědi na expozici tamoxifenu, přičemž míra fosforylace AKT na serinu 473 zůstávala nezměněna. Avšak aplikace AKT inhibitorů vedla nejen k významnému snížení fosforylace AKT na serinu 473, ale i k redukci hladiny AGR2, což ostatně dokládá práce

nazvaná: „*Identification of an AKT-dependent signalling pathway that mediates tamoxifen-dependent induction of the pro-metastatic protein anterior gradient-2*“ (**příloha 7**). Naše výsledky potvrdily, že exprese AGR2 může být přímo regulovaná AKT signální dráhou. Toto tvrzení podpořil i další pokus, v rámci kterého jsme se zaměřili na modulaci hladiny PDPK1 kinázy, která je přímým aktivátorem AKT kinázy. Při indukci AGR2 působením tamoxifenu jsme mohli pozorovat signifikantní snížení hladiny AGR2 při umlčení exprese PDPK1 působením specifických siRNA (**příloha 7**). Funkční vztah mezi AKT signální dráhou a AGR2 pak potvrdila i retrospektivní studie na souboru 94 primárních karcinomů mléčné žlázy, v rámci které byla prokázána statisticky významná korelace mezi expresí AGR2 a fosforylací AKT (**příloha 7**). Schematické zobrazení modulace exprese AGR2 v rámci komunikace mezi EsR a AKT signální dráhou viz **obr. 6**.

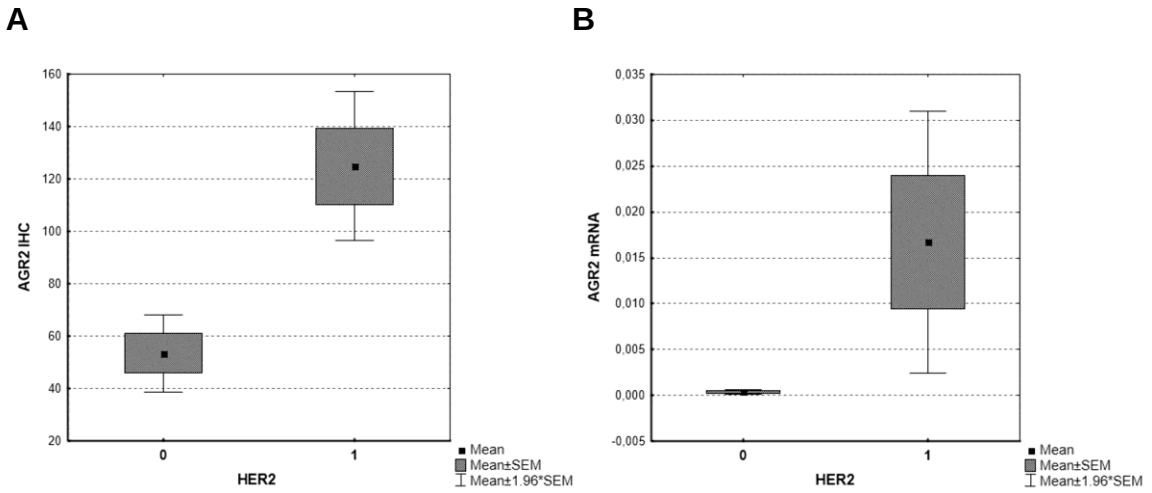


Obr. 6: Potenciální mechanismy regulace exprese AGR2. *AGR2 exprese může být regulována: i) přímo (genomicky) vazbou EsR na ERE místa a indukcí transkripce, ii) negenomicky aktivací pro-onkogenních buněčných signálních drah.*

Další důkaz o úloze AGR2 při změně fenotypu nádorových buněk z tamoxifen-senzitivních na tamoxifen-rezistentní přináší práce Wright a kol., kteří studovali vazbu komplexu tamoxifen-EsR na promotor genu *AGR2* a následnou indukci exprese AGR2 [32]. V souladu s našimi výsledky ukázali, že zvýšená exprese AGR2 indukuje buněčnou proliferaci buněk nejen citlivých k tamoxifenu, ale i rezistentních. V souvislosti s tím zjistili, že v případě tamoxifen-senzitivních buněk je zvýšená exprese AGR2 přímo závislá na přítomnosti EsR a zvýšená hladina AGR2 následně zprostředkovává zvýšenou expresi EsR. Naopak u tamoxifen-rezistentních buněk není exprese AGR2 závislá na funkčních EsR, avšak EsR stále vyžadují přítomnost AGR2, což naznačuje, že u tamoxifen-rezistentních buněk dochází k deregulaci zpětnovazebné smyčky EsR-AGR2-EsR. Autoři dále ukazují, že v případě tamoxifen-rezistentních nádorů se na aktivaci exprese AGR2 podílí transkripční faktor FOXA1, který obecně hraje důležitou úlohu ve fyziologii buněk odvozených od karcinomů mléčné žlázy a který je schopen udržovat zvýšenou expresi AGR2 nezávisle na expresi EsR [32].

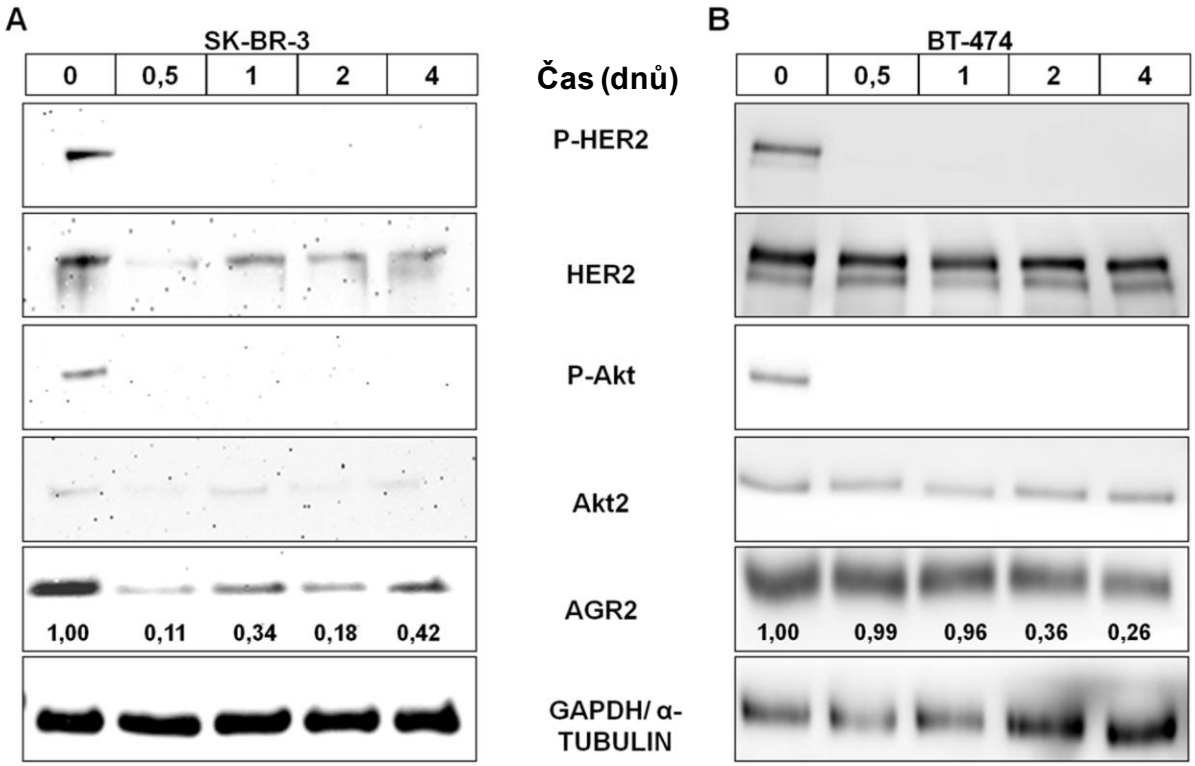
3.4. Exprese AGR2 u karcinomů mléčné žlázy nezávislá na EsR

Možnou spojitost mezi expresí receptorů HER2 a proteinem AGR2 již naznačily výsledky v naší výše zmiňované práci „*Identification of an AKT-dependent signalling pathway that mediates tamoxifen-dependent induction of the pro-metastatic protein anterior gradient-2“* (**příloha 7**), kde popisujeme mimo jiné i marginální korelaci mezi imunohistochemicky stanovenou expresí AGR2 a přítomností receptorů HER2. Receptory HER2 byly determinovány z toho důvodu, že patří mezi možné regulátory PDPK1/AKT signální dráhy. Dalším podpůrným faktem pro započetí studia vztahu mezi AGR2 a HER2 byla skutečnost, že exprese proteinu AGR2 byla prokázána i u karcinomů mléčné žlázy (byť méně často), které nedisponují funkčními EsR. Zaměřili jsme se tudíž i na studium karcinomů mléčné žlázy, kde funkční EsR nebyly přítomné. AGR2 exprese byla prokázána u přibližně 60 % z celkového počtu 211 analyzovaných vzorků. Následující statistická analýza odhalila významnou korelaci ($p<0,001$; Mannův-Whitneyův U test) mezi expresí AGR2 a přítomností receptoru HER2 (**obr. 7**).



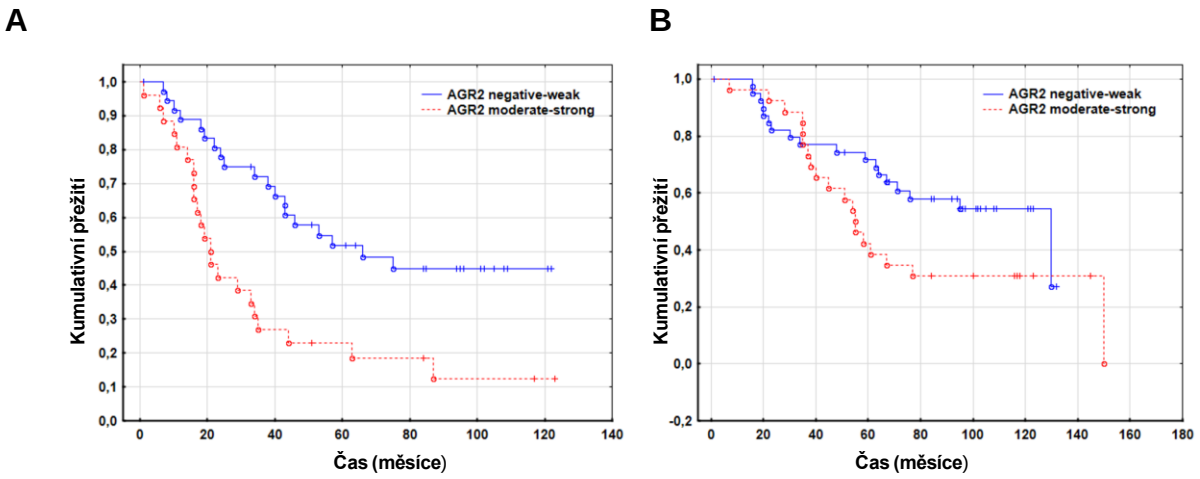
Obr. 7: Analýza hladiny AGR2 ve vztahu k expresi receptorů HER2. *(A) Exprese AGR2 i HER2 byla stanovena pomocí imunohistochemického barvení. V případě AGR2 byla exprese definována pomocí tzv. histoskóre, které bylo vypočítáno jako součin intenzity barvení ve škále 0-3 a procenta pozitivních buněk pohybujícího se v rozmezí hodnot 0-100. Exprese HER2 byla stanovena rutinně pomocí HercepTestu ve škále 0-3. Pro vlastní analýzu byly použity případy HER2 0-1 vs. 2-3, přičemž u všech případů stanovených jako „2" byla ještě provedena validace HER2 amplifikace pomocí FISH. (B) Analýza HER2 stanoveného pomocí IHC ve vztahu k expresi AGR2 na úrovni transkripce.*

Následná analýza hladiny mRNA genu *AGR2* vzhledem k expresi HER2 jednoznačně potvrdila trend pozorovaný na úrovni proteinu ($p<0,001$; Mannův-Whitneyův U test). Na základě dat získaných z této retrospektivní studie bylo naším dalším cílem prokázat funkční vztah mezi HER2 receptory a AGR2. Pro tyto účely byla zvolena buněčná linie SK-BR-3, u které byla dříve prokázána tvorba proteinu AGR2. Tato linie byla ustavena z karcinomu mléčné žlázy, který byl EsR a PR negativní, ale HER2 pozitivní. Jako alternativní model byly použity buňky BT-474, které mají funkční EsR, avšak současně jsou i HER2-pozitivní. Obě buněčné linie byly vystaveny působení lapatinibu, nízkomolekulárnímu inhibitoru EGFR a HER2. Z předložených imunochemických analýz je jasně patrné, že působením lapatinibu docházelo k inhibici HER2 receptorů, AKT signální dráhy a současně i k viditelnému snížení exprese AGR2, a to především u buněčné linie SK-BR-3 (**obr. 8A**). V případě buněčné linie BT-474 byl podobný efekt pozorován až v pozdějších časových intervalech. Potenciálním důvodem mohou být aktivní EsR, které představují alternativní možnost pozitivní regulace AGR2 nezávisle na HER2 receptorech (**obr. 8B**).



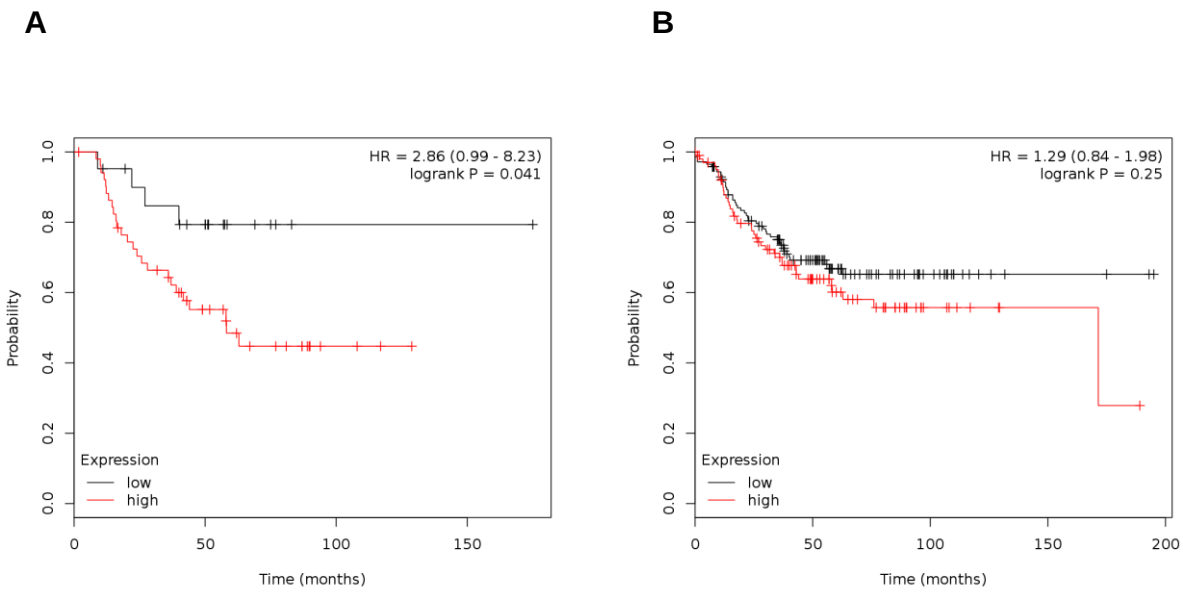
Obr. 8: Vliv inhibice receptoru HER2 na buněčnou signalizaci a expresi AGR2. *Buněčné linie (A) SK-BR-3 a (B) BT-474 byly vystaveny působení 1 nM lapatinibu po dobu uvedenou v horní části obrázku. Poté byly buňky sklizeny a imunochemicky stanovena exprese vybraných proteinů.*

Uvedené výsledky naznačují, že aktivní receptory HER2 se mohou přímo uplatňovat při regulaci exprese AGR2, zřejmě aktivací PI3K/AKT signální dráhy, což do značné míry vysvětluje i výskyt relativně vysoké exprese AGR2 u karcinomů mléčné žlázy negativních pro EsR a PR, ale pozitivních pro HER2. Vzhledem ke skutečnosti, že AGR2 protein vykazuje proonkogenní vlastnosti, byla provedena statistická analýza s cílem zjistit, zda se zvýšená exprese AGR2 u tohoto molekulárního subtypu (EsR-, PR-, HER2+) nějakým způsobem promítá do bezpříznakového (Disease Free Survival, DFS) či celkového (Overal Survival, OS) přežití pacientek (**obr. 9**).



Obr. 9: Exprese AGR2 vzhledem k přežití pacientek s EsR-, PR-, HER2+ karcinomem mléčné žlázy. *Kaplanova-Meierova analýza (A) bezpříznakového a (B) celkového přežití, kdy modrá křivka znázorňuje případy, kdy AGR2 exprese byla stanovena jako negativní nebo slabá (histoscore <50), naopak červená křivka reflektuje případy se zvýšenou hladinou AGR2 (histoscore ≥50).*

Naše výsledky zřetelně ukazují, že zvýšená hladina AGR2 představuje negativní prognostický faktor u karcinomů mléčné žlázy, které nemají funkční estrogenové a progesteronové receptory a současně disponují receptory HER2. Paralelní analýza hladiny *AGR2* na úrovni transkripce s využitím on-line webové databáze nazvané „Kaplan-Meier Plotter“ (http://kmplot.com/analysis/index.php?p=service) pak pouze potvrdila spojitost mezi zvýšenou hladinou AGR2 a zhoršeným přežitím pacientek s EsR-, PR-, HER2+ karcinomem mléčné žlázy (**obr. 10**). Z obrázku je jasně patrný význam přítomnosti HER2 receptorů, která se s velkou pravděpodobností promítá do pozitivní regulace exprese AGR2 a zvýšená hladina exprese *AGR2* pak negativně ovlivňuje délku bezpříznakového přežití pacientek. Jinými slovy i tato analýza provedená na nezávislém souboru (n = 73) prokázala, že zvýšená exprese AGR2 může významně přispívat k negativní prognóze pacientek s karcinomy mléčné žlázy, které nemají funkční EsR a PR receptory, ale naopak mají pozitivní HER2 receptory.



Obr. 10: Metaanalýza vlivu exprese AGR2 na prognózu onemocnění. *Kaplanova-Meierova analýza u kohorty (A) EsR-, PR-, HER2+karcinomů mléčné žlázy, p =0,041 a (B) EsR-, PR- karcinomů mléčné žlázy bez ohledu na status HER2 receptorů, p = 0,25.*

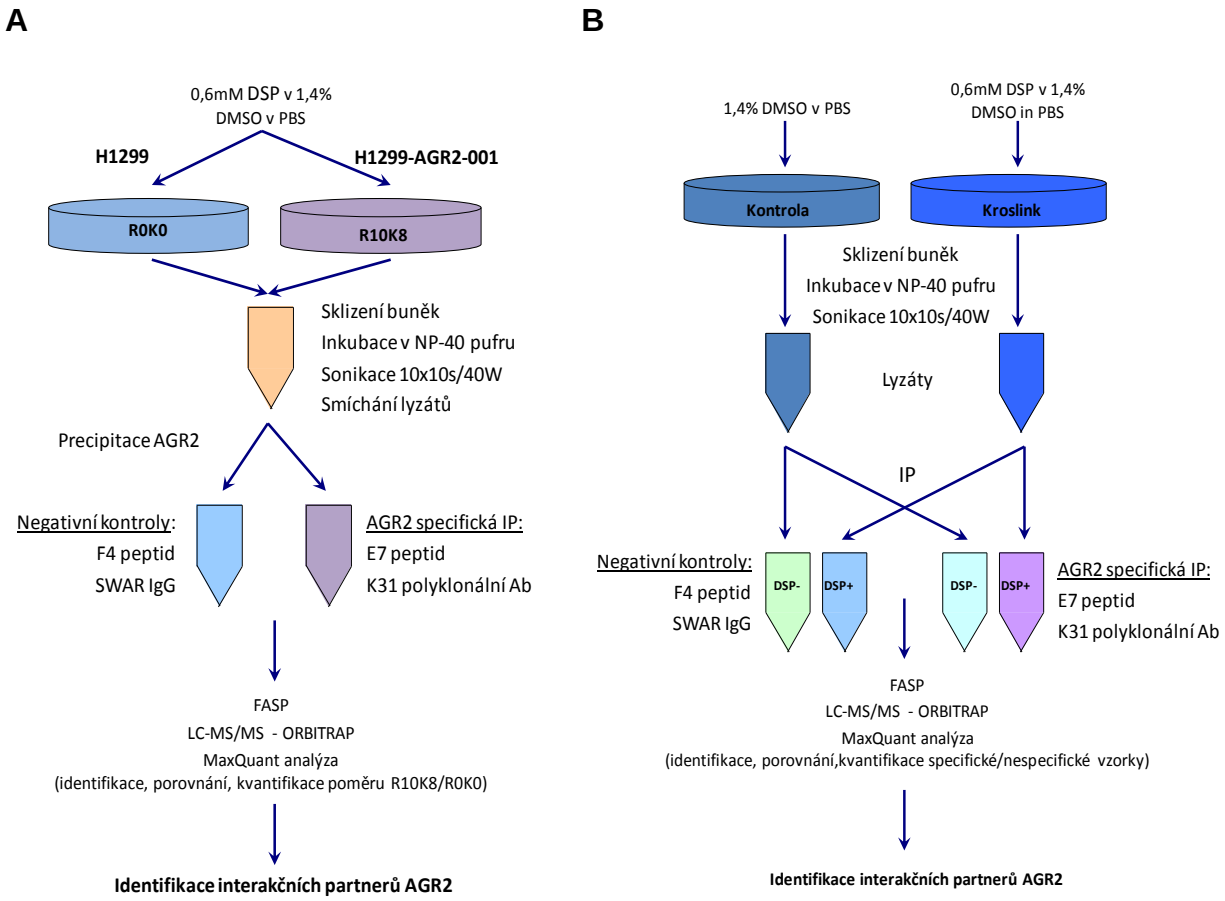
4. Studium funkce proteinů AGR2 a AGR3 na buněčné (molekulární) úrovni

Informace týkající se signálních drah či faktorů zodpovědných za zvýšenou expresi AGR2/3 v nádorových buňkách jsou prozatím značně omezené. Doposud byl podrobněji charakterizován především protein AGR2. Mezi stěžejní patří práce Liu a kol., ve které autoři studují AGR2 ve vztahu k metastazování a v podstatě potvrzují signifikantní úlohu AGR2 při tvorbě metastáz [22]. V návaznosti na tuto práci pak Wang a kol. pomocí *in vitro* i *in vivo* modelů prokázali přímý vliv zvýšené exprese AGR2 na iniciaci neoplastické transformace a zesílenou schopnost buněk migrovat [23]. Tato zjištění jsou mj. i v souladu s výsledky práce ukazující, že protein AGR2 interaguje s C4.4a a DAG-1, o kterých je známo, že se výraznou měrou uplatňují při tvorbě metastáz [33]. Tuto skutečnost podtrhuje i práce, ve které je popsán vývoj nové terapeutické protilátky, pomocí které je možné zablokovat interakci mezi extracelulárně produkovaným proteinem AGR2 a C4.4a receptorem, což ve svém důsledku vedlo k výrazné redukci růstu nádorů a metastazování *in vivo* na testovaných myších xenograftech [34].

4.1. Identifikace interakčních partnerů proteinu AGR2

Přestože protein AGR2 představuje poměrně zajímavý terapeutický cíl a skutečnost, že na svém C-konci nese tzv. ER retenční sekvenci, která předznamenává přítomnost tohoto proteinu v endoplazmatickém retikulu, kde se podílí na skládání a zrání řady proteinů, nejsou k dispozici žádné další práce, které by mapovaly AGR2 interakční partnery. Jedinou výjimku tvoří soubor několika prací, ve kterých je popisována spojitost mezi expresí AGR2 a tvorbou mucinů, konkrétně MUC2, MUC5AC, MUC5B a MUC1 [35-37].

Provedli jsme tedy LC-MS/MS analýzu s cílem najít další interakční partnery proteinu AGR2. Paralelně byly použity dva na sobě nezávislé modelové systémy a dva různé přístupy. V prvním případě byla použita buněčná linie H1299 (AGR2 negativní) a z ní odvozená buněčná linie H1299-AGR2-001 (buňky H1299, do kterých byl stabilně vnesen expresní vektor umožňující produkci AGR2). Buňky H1299 byly kultivovány v lehkém médiu (R0K0), zatímco H1299-AGR2-001 byly kultivovány v těžkém médiu (R10K8). Před samotným sklizením a následnou lýzou byly takto kultivované buňky inkubovány s DSP (Dithiobis[succinimidylpropionát]) s cílem vytvořit mezi buněčnými proteiny reverzibilní kroslinky. Získané buněčné lyzáty byly smíchány v hmotnostním poměru 1:1 a následovala imunoprecipitace pomocí „*in house*“ připravené králičí polyklonální protilátky rozpoznávající AGR2. Druhou alternativou bylo použití biotinylovaného peptidu pracovně nazývaného E7 (biotin-SGSGPTTIYY), který se specificky váže na AGR2 [38] (**obr. 11A**). V rámci druhého přístupu byla použita buněčná linie T-47D, pro kterou je typická silná endogenní exprese AGR2. Pomocí tohoto experimentu byl současně testován efekt kroslinkování na zachycení transientních málo stabilních interakcí, respektive identifikaci falešně pozitivních interakčních partnerů. Polovina buněk byla opět vystavena působení DSP a druhá byla použita jako kontrola. Následně probíhala imunoprecipitace ve stejném schématu jako v předchozím experimentu (**obr. 11B**).

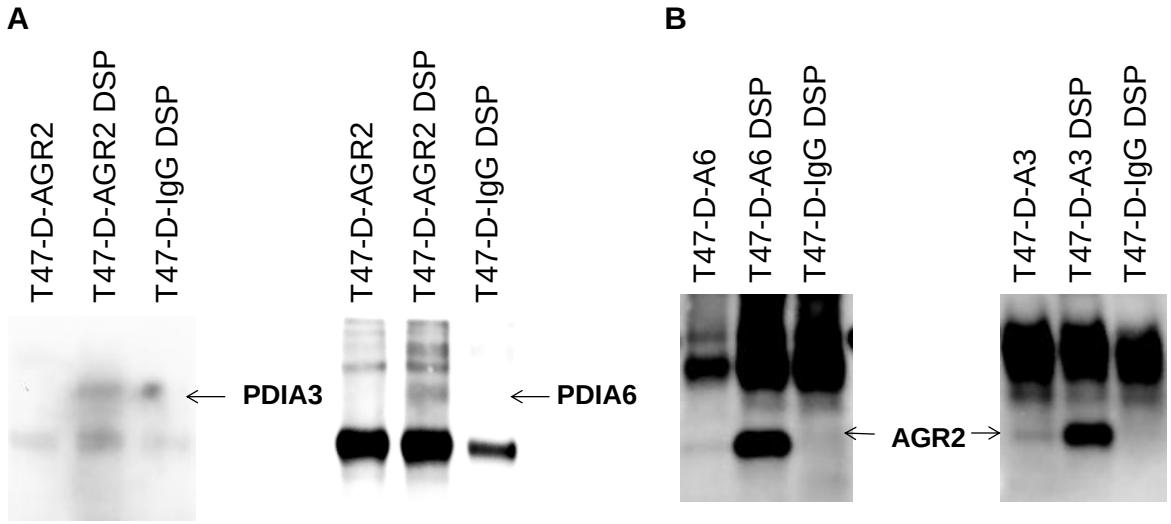


Obr. 11: Schematické znázornění protokolu identifikace interakčních partnerů proteinu AGR2. *(A) SILAC analýza buněčných linií H1299 a H1299-AGR2-001, (B) „Label-free" analýza buněčné linie T-47D.*

Pomocí obou protokolů se podařilo precipitovat protein AGR2, což bylo potvrzeno imunochemicky s využitím polyakrylamidové gelové elektroforézy (PAGE) následované Westernovým přenosem a imunochemickou identifikací pomocí specifických protilátek. Oba přístupy byly plně kompatibilní s FASP (*Filter-Aided Sample Preparation*) protokolem a následnou LC-MS/MS analýzou. U T-47D buněk bylo identifikováno cca 1600 proteinů, u H1299-AGR2-001 přibližně 200. Jednoznačně byly odhaleny 2 proteiny interagující s AGR2: PDIA3 (ERp57) a PDIA6 (TXNDC7), a to jak v buňkách T-47D, tak i H1299-AGR-001. Skutečnost, že oba nově identifikované proteiny interagující s AGR2 patří taxonomicky do rodiny PDI a nacházejí se v ER, naznačuje, že spolu s AGR2 mohou vytvářet multichaperonové komplexy, které se pravděpodobně podílejí na regulaci homeostázy ER.

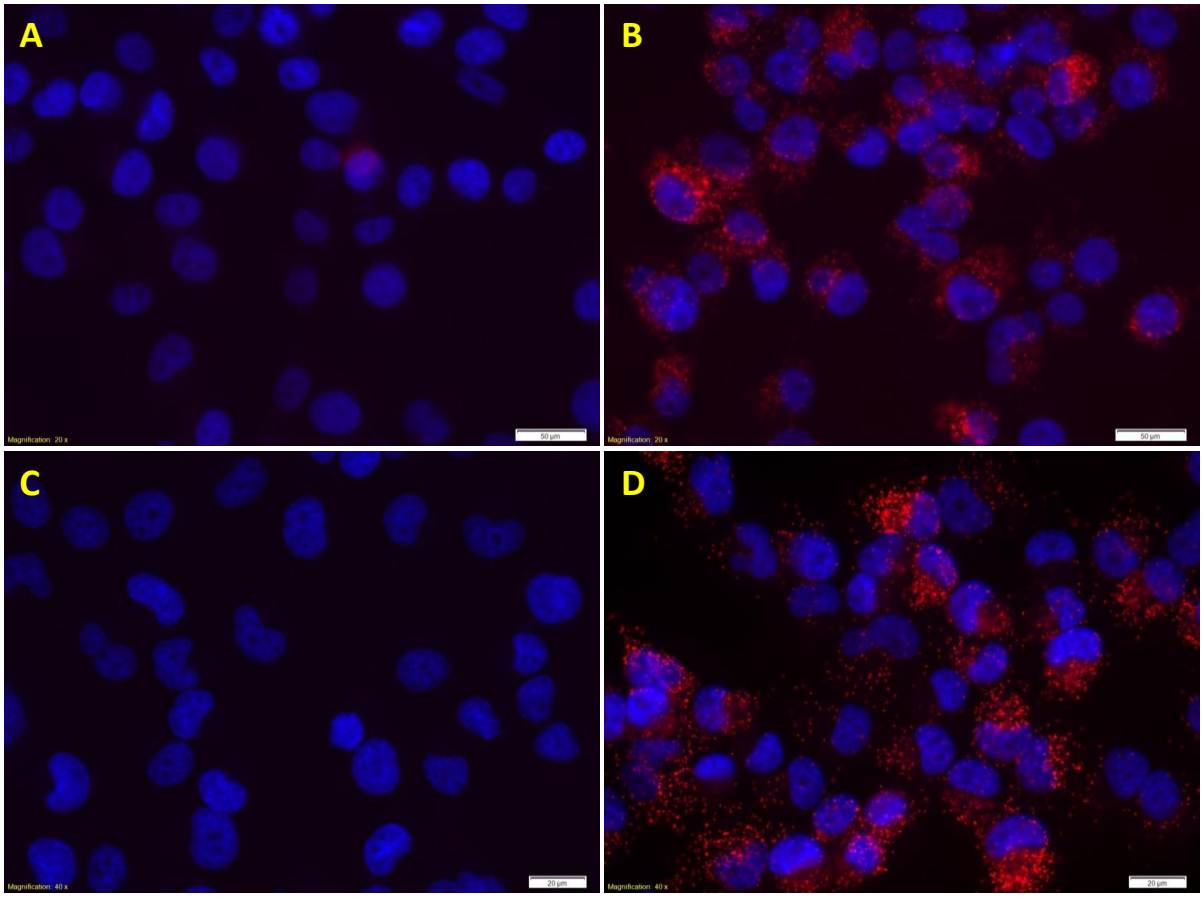
Interakce PDIA3 a PDIA6 s AGR2 byla následně potvrzena i pomocí imunoprecipitace. Proteiny interagující s AGR2 byly precipitovány pomocí AGR2 specifického peptidu E7, respektive AGR2 specifické protilátky, následovala elektroforéza

v polyakrylovém gelu a Westernový přenos. Imunochemicky byla prokázána přítomnost proteinů PDIA3 i PDIA6, které byly precipitovány v komplexu s AGR2 (**obr. 12A**). Pokus byl proveden i opačně, tedy k precipitaci byly použity protilátky rozpoznávající PDIA3, respektive PDIA6 a detekována byla přítomnost proteinu AGR2 (**obr. 12B**).



Obr. 12: Validace AGR2 interakčních partnerů pomocí imunoprecipitace. *Detekce PDIA3 respektive PDIA6 vázaných na AGR2 (A). Detekce AGR2 vázaného na PDIA6 a PDIA3 (B).*

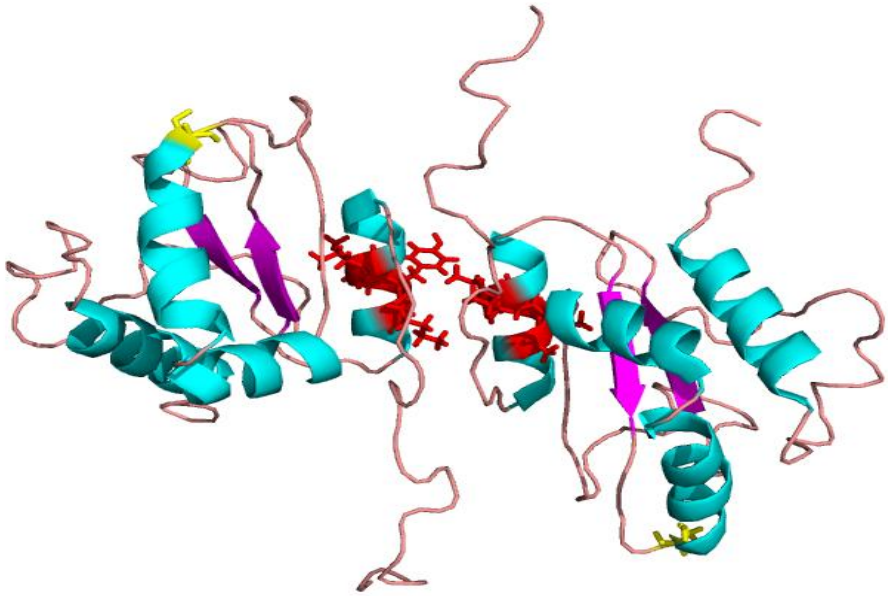
S cílem prokázat, že k interakci nedochází náhodně, respektive nespecificky při sklízení a lýze buněk, jsme použili *in situ* metodu PLA (*Proximity Ligation Assay*). Jedná se o relativně novou techniku umožňující přímou vizualizaci proteinů, jejich hladin, modifikací a především interakcí ve fixovaných buňkách a tkáních. Sondy pro PLA jsou tvořeny specifickými protilátkami s navázaným oligonukleotidem, který slouží jako reportérová molekula. Pokud dojde k navázání sond v těsné blízkosti, právě díky interakci protein – protein, následuje vznik kružnicové DNA, která slouží jako templát pro PCR amplifikaci otáčivou kružnicí. Amplifikační reakce umožňuje vizualizaci sledované interakce [39]. V návaznosti na identifikaci partnerů AGR2 jsme se tedy zaměřili na průkaz interakce AGR2 s PDIA3 a PDIA6 (**obr. 13**).



Obr. 13: Vizualizace interakcí AGR2-PDIA3 a AGR2-PDIA6 pomocí PLA in situ. *Pro analýzu interakcí byla použita buněčná linie H1299 (AGR2 negativní) a z těchto buněk derivovaný klon H1299-AGR2 se stabilně začleněným expresním plazmidem nesoucím kódující sekvenci pro AGR2, jenž umožňují tvorbu proteinu AGR2. Buňky H1299 sloužily jako negativní kontrola pro interakci AGR2 s PDIA3 (A) respektive PDIA6 (C). Pozitivní signály prokazující protein-proteinovou interakci bylo možné pozorovat u linie H1299-AGR2, a to jak v případě PDIA3 (B), tak i PDIA6 (D).*

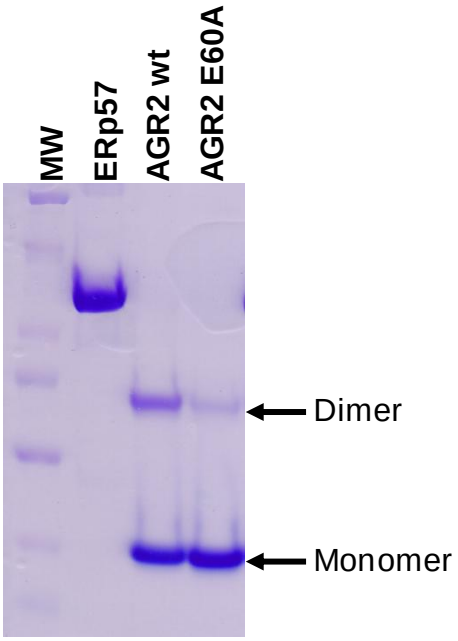
Další zajímavostí je, že pomocí MS analýzy buněk T-47D byl statisticky významně identifikován jako interakční partner proteinu AGR2 i protein AGR3. V případě buněk H1299-AGR2-001 tak tomu nebylo, neboť jsou AGR3 negativní. Možnost, že AGR2 může skutečně tvořit s AGR3 heterodimery, popř. i složitější komplexy (multimery), podporuje značná míra homologie mezi oběma proteiny a především je v souladu s recentně publikovanými pracemi, ve kterých je prokázáno, že protein AGR2 se *in vitro* vyskytuje v rovnovážném stavu mezi monomery a dimery (K(d) = 8,83 μM) [40,41]. Na základě NMR (*Nuclear Magnetic Resonance*) stanovené struktury jsme vytvořili model dimeru AGR2, v rámci kterého bylo možné identifikovat oblast obsahující aminokyselinové zbytky E60 a

K64, které umožňují tvorbu solných můstků a jsou pro vznik a stabilizaci dimeru nezbytné (**obr. 14**).



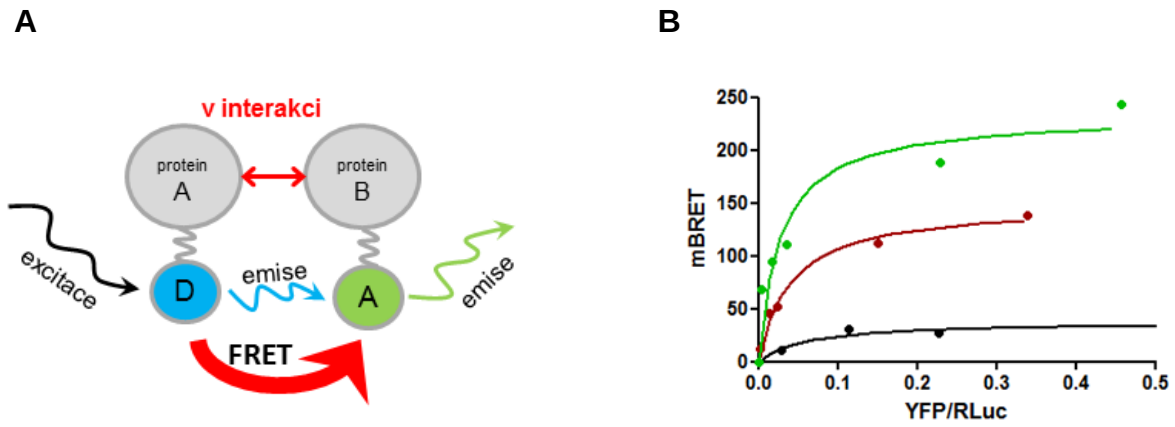
Obr. 14: Model dimeru AGR2. *Červeně jsou vyznačeny aminokyseliny E60 a K64, které hrají hlavní roli při vzniku a stabilitě dimeru.*

Dimerizaci AGR2 jsme následně prokázali i na našem pracovišti s využitím purifikovaného proteinu AGR2, přičemž vzniklé dimery proteinu byly kroslinkovány pomocí glutaraldehydu a následně vizualizovány pomocí barvení Coomassie Brilliant Blue (**obr. 15**). Na obrázku je též zřetelně vidět, že v případě použití proteinu AGR2 nesoucího mutaci E60A (AGR2 E60A), tedy oblasti důležité pro vznik dimeru, byla schopnost AGR2 tvořit dimery významně redukována.



Obr. 15: Průkaz dimerizace AGR2 v podmínkách in vitro. *20 μl 0,3 mg/ml proteinu AGR2 bylo 5 min inkubováno v pufru (pH=7,4) obsahujícím 0,23% glutaraldehyd, 25 mM Hepes, 0,1 M NaCl, 50 mM octan draselný. Poté byla reakce zastavena přidáním 2,5 μl 1 M TrisCl, pH=8,0.*

Funkční analýza založená na využití mutovaných proteinů AGR2 v pozici E60A, které nejsou schopné vytvářet dimery, odhalila, že vznik dimerů nemá zásadní vliv na oxido-redukční vlastnosti proteinu AGR2, ale významně ovlivňuje jeho adhezivitu [41]. V naší práci jsme se zaměřili na potvrzení interakce mezi AGR2 a AGR3 a tedy i možné tvorby heterodimerů AGR2-AGR3. Vzájemnou interakci mezi těmito proteiny se skutečně podařilo potvrdit nejen pomocí imunoprecipitace, ale i PLA *in situ*. S cílem blíže studovat tvorbu AGR2-AGR2 homodimerů a AGR2-AGR3 heterodimerů byla na pracovišti zavedena metoda BRET (*Bioluminescence Resonance Energy Transfer*), která umožňuje zachytit a studovat protein-proteinové interakce v živých buňkách. Tato metoda je založená na přenosu energie mezi donorovu a akceptorovou molekulou v případě, že se tyto molekuly nachází v dostatečné blízkosti (do 10 nm). Jako donorová molekula je využíván enzym luciferáza Rluc, jako akceptorová molekula slouží žlutý fluorescenční protein YFP. K vyvolání reakce je využíván luciferin (světlo emitující molekula), coelenterazine-h slouží jako substrát pro luciferázu, která jej degraduje a dochází tak k uvolnění modrého světla (energie). Ta je zachycena akceptorovou molekulou (YFP) a následně emitována ve formě světla o vyšší vlnové délce (dle emisního spektra akceptoru). Toto uvolněné světlo je zachyceno detektorem a je tak potvrzena interakce mezi proteiny (**obr. 16A**). Pro studium dimerizace AGR proteinů byly do expresních vektorů pRluc-C1 a pEYFP-C1 naklonovány kódující sekvence *AGR2* a *AGR3* (**obr. 16B**). Z obrázku je jasně patrné, že protein AGR3 tvořil přednostně homodimery, v menší míře však prokazatelně vznikaly i heterodimery. Nicméně otázky týkající se úlohy a funkce těchto heterodimerů v buňce zůstávají nezodpovězeny. Stejně tak bude zajímavé zkoumat, zda přítomnost mutace v kyselině glutamové v pozici 60, která se nachází v oblasti nezbytné pro dimerizaci proteinu AGR2, může ovlivňovat i vznik heterodimerů AGR2-AGR3.



Obr. 16: Využití metody BRET při studiu interakcí mezi AGR2 a AGR3. *(**A**) Znázornění mechanismu rezonančního přenosu energie. (**B**) Dimerizace AGR3 je znázorněna zelenou křivkou (pRluc-C1_AGR3 + pEYFP-C1_AGR3), vznik heterodimerů AGR2-AGR3 zachycuje červená křivka (pRluc-C1_AGR3 + pEYFP-C1_AGR2), negativní kontrolu představuje černá křivka (pRluc-C1_AGR3 + pEYFP-C1_empty).*

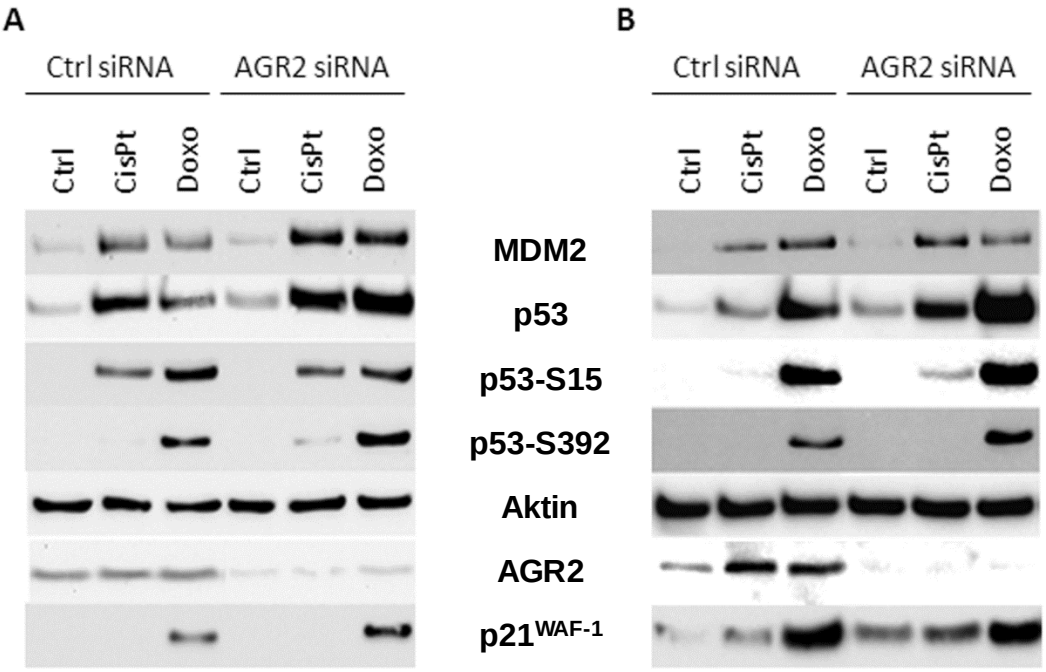
Další metodický přístup pro studium vztahů mezi proteiny včetně identifikace partnerů při protein-proteinových interakcích představuje kvasinkový dvouhybridový skríning (*Yeast Two-Hybrid screening, Y2H*) [42]. Tento systém jsme využili při hledání nových proteinů potenciálně interagujících s AGR2 a podařilo se nám identifikovat poměrně velké množství proteinů včetně již dříve dvou popsaných prometastatických receptorů C4.4A a DAG1. Překvapivě se mezi identifikovanými proteiny objevil i Reptin (RUVB2), nukleární protein uplatňující se při regulaci řady biologických procesů, jako jsou reparace DNA, regulace transkripce, sestavování snoRNP či metastazování [43]. I díky skutečnosti, že existuje celá řada prací ukazujících zvýšenou expresi Reptinu v primárních nádorech [44,45], se analýza interakce tohoto proteinu s AGR2 stala jednou z priorit, viz publikace nazvaná: „*A divergent substrate-binding loop within the pro-oncogenic protein anterior gradient-2 forms a docking site for Reptin*“, (**příloha 8**). Nejdříve jsme prokázali společnou expresi Reptinu s AGR2 na panelu primárních karcinomů mléčné žlázy a následně jsme potvrdili i jejich interakci pomocí imunoprecipitace. Zajímavým zjištěním bylo, že přítomnost a zvyšování koncentrace ATP významně snižuje stabilitu komplexu tvořeného AGR2 a Reptinem. Dalším krokem byl pokus identifikovat oblast AGR2, na kterou se Reptin váže. Byla připravena peptidová knihovna odvozená z proteinové sekvence AGR2, která byla tvořena biotinem značenými peptidy o velikosti 15 aminokyselin, jejichž sekvence se navzájem překrývaly v 10 aminokyselinách. Jednotlivé peptidy byly navázány na streptavidinem značené agarózové kuličky a následně inkubovány s buněčným lyzátem obsahujícím Reptin. Pomocí Westernové analýzy byl imunochemicky detekován Reptin. Takto se podařilo jednoznačně identifikovat specifickou oblast AGR2 (104-FVLLNLVY-111), která je nezbytná pro vazbu Reptinu (**příloha 8**). Je

zajímavé, že tato aminokyselinová sekvence je součástí smyčky, která je typická pouze pro AGR2 a u ostatních členu rodiny AGR proteinů se nenachází. To znamená, že interakce AGR2 s Reptinem představuje specifickou vlastnost proteinu AGR2, který se tímto mechanismem může významně podílet na regulaci řady biologických funkcí Reptinu.

4.2. AGR2 jako negativní regulátor proteinu p53

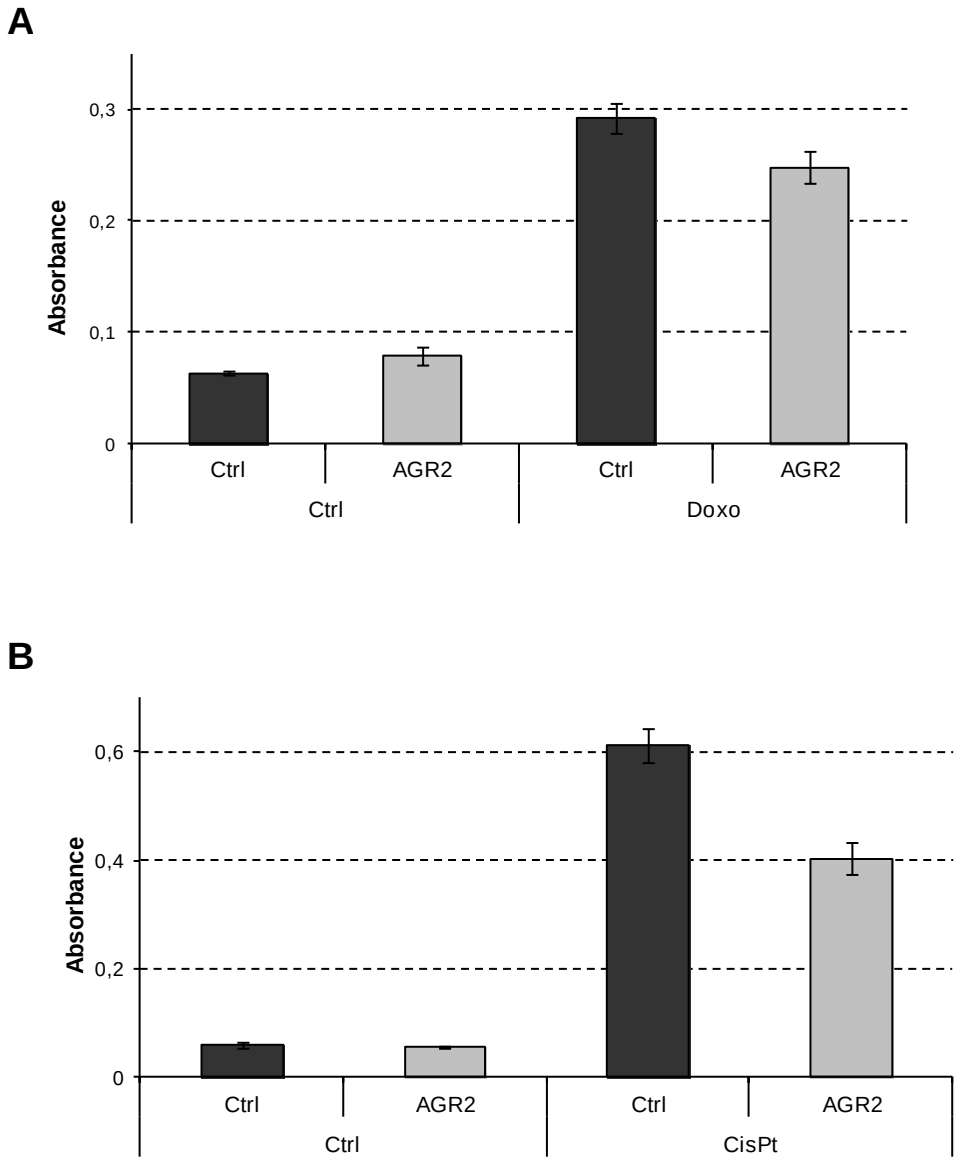
Při proteomické analýze epitelu Barretova jícnu byla mimo jiné detekována i zvýšená hladina proteinu AGR2. Podstatou vzniku Barretova jícnu je chronické působení refluxátu na sliznici jícnu, které způsobí, že se vrstevnatý dlaždicový nerohovějící epitel jícnu začne měnit na jednovrstevný cylindrický epitel intestinálního typu a vzniká tzv. intestinální metaplazie. Ve své podstatě se jedná o unikátní mikroprostředí, kde je v poměrně vysoké frekvenci pozorován výskyt různých mutací v genu *TP53*. Protein p53 je jeden z nejintenzívněji studovaných nádorových supresorů vůbec. Z tohoto úhlu pohledu se jedná o poměrně zajímavý model umožňující studium mechanismů, které přeživší buňky aktivují právě v kontextu přítomnosti různých variant mutovaného nebo *wild-type* proteinu p53. Pozorování, že zvýšená exprese AGR2 byla detekována především u buněk s *wild-type* p53, naznačila, že se AGR2 může aktivně podílet na inhibici aktivity nádorového supresoru p53 a přispívat tak k maligní transformaci buněk bazální vrstvy dlaždicového epitelu jícnu [21]. Současně Pohler a kol. ukázali, že zvýšená exprese AGR2 skutečně vede ke snížení fosforylace p53 na Ser 392 indukované UV zářením.

V návaznosti na uvedenou publikaci jsme se zaměřili na studium mechanismu, kterým AGR2 inhibuje aktivitu p53, z čehož rezultovala práce nazvaná *„AGR2 oncoprotein inhibits p38 MAPK and p53 activation through a DUSP10-mediated regulatory pathway“* (**příloha 9**). Jako modelové byly zvoleny buněčné linie, pro které jsou typické vysoké hladiny AGR2: A549 (odvozená od karcinomu plic, EsR-negativní, wt p53) a MCF-7 (odvozená od karcinomu mléčné žlázy, EsR-pozitivní, wt p53). Pomocí specifické siRNA byla umlčena exprese AGR2 a následně sledována hladina a transkripční aktivita proteinu p53 (**obr. 17**).



Obr. 17: Studium vlivu exprese AGR2 na protein p53. *Buněčné linie A549 (A) a MCF-7 (B) byly transientně transfekovány kontrolní siRNA (Ctrl siRNA), respektive siRNA specifickou proti AGR2 (AGR2 siRNA). 32 hodin po transfekci byly buňky vystaveny působení cisplatiny (CisPt) nebo doxorubicinu (Doxo) po dobu 16 hodin. Poté byly buňky sklizeny do lyzačního pufru a podrobeny imunochemické analýze.*

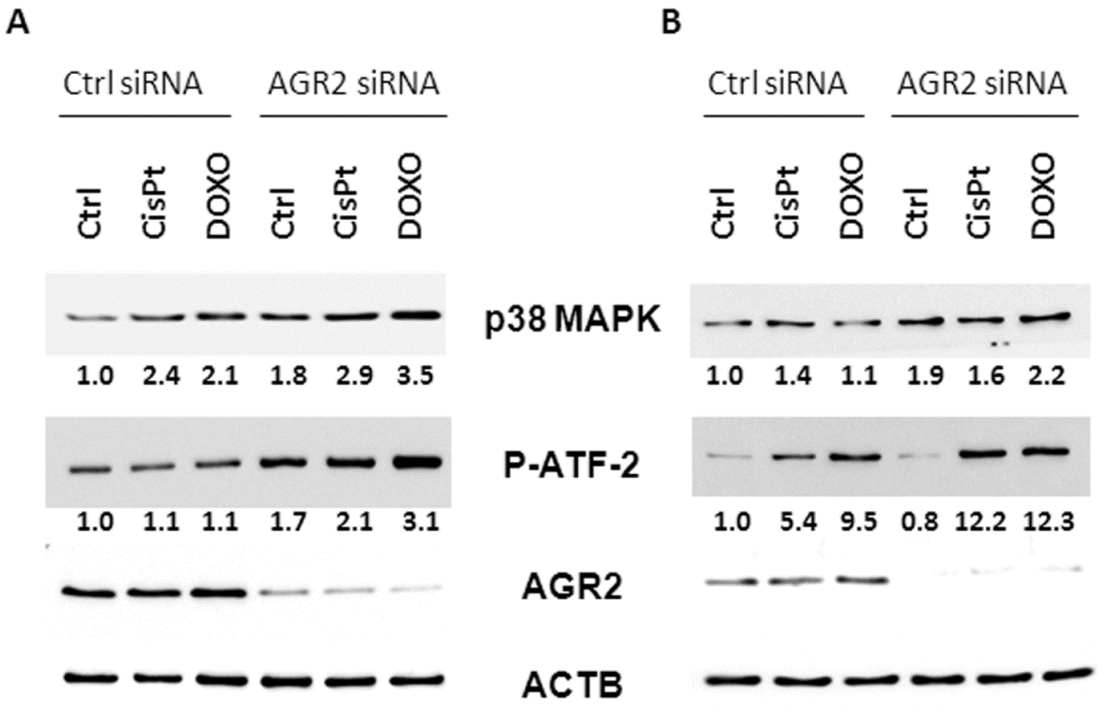
Jak vyplývá z obrázku 17, umlčení exprese AGR2 vedlo nejen ke zvýšení hladiny proteinu p53, ale i k intenzivnější fosforylaci tohoto proteinu na Ser 15 a Ser 392. Stejně tak docházelo k indukci exprese p53 regulovaných genů *mdm2* a *p21*$^{WAF-1}$. Uvedené výsledky zřetelně naznačují, že přítomnost proteinu AGR2 vede k redukci posttranslačních modifikací proteinu p53, které jsou nezbytným předpokladem pro jeho aktivaci. S cílem prokázat, že se zvýšená hladina proteinu AGR2 přímo podílí na inhibici transkripční aktivity p53, byl použit modelový systém umožňující testovat transaktivaci p53 (**obr. 18**). Konkrétně byla použita buněčná linie ARN8, jenž nese stabilně začleněný konstrukt tvořený kódující sekvencí pro β-galaktosidázu, před kterou se nachází regulační oblast obsahující vazebnou, tzv. konsensus, sekvenci pro protein p53 [46]. V případě, že dojde k aktivaci p53, protein p53 se naváže na konsensus sekvenci a dochází k indukci exprese β-galaktosidázy.



Obr. 18: Stanovení transkripční aktivity proteinu p53. *Buněčná linie ARN8 byla transientně transfekována prázdným plazminem (Ctrl) a plazmidem umožňujícím expresi AGR2 (AGR2). Poté byly buňky vystaveny působení doxorubicinu (A) respektive cisplatiny (B) a kolorimetricky stanovena hladina resp. aktivita β-galaktosidázy.*

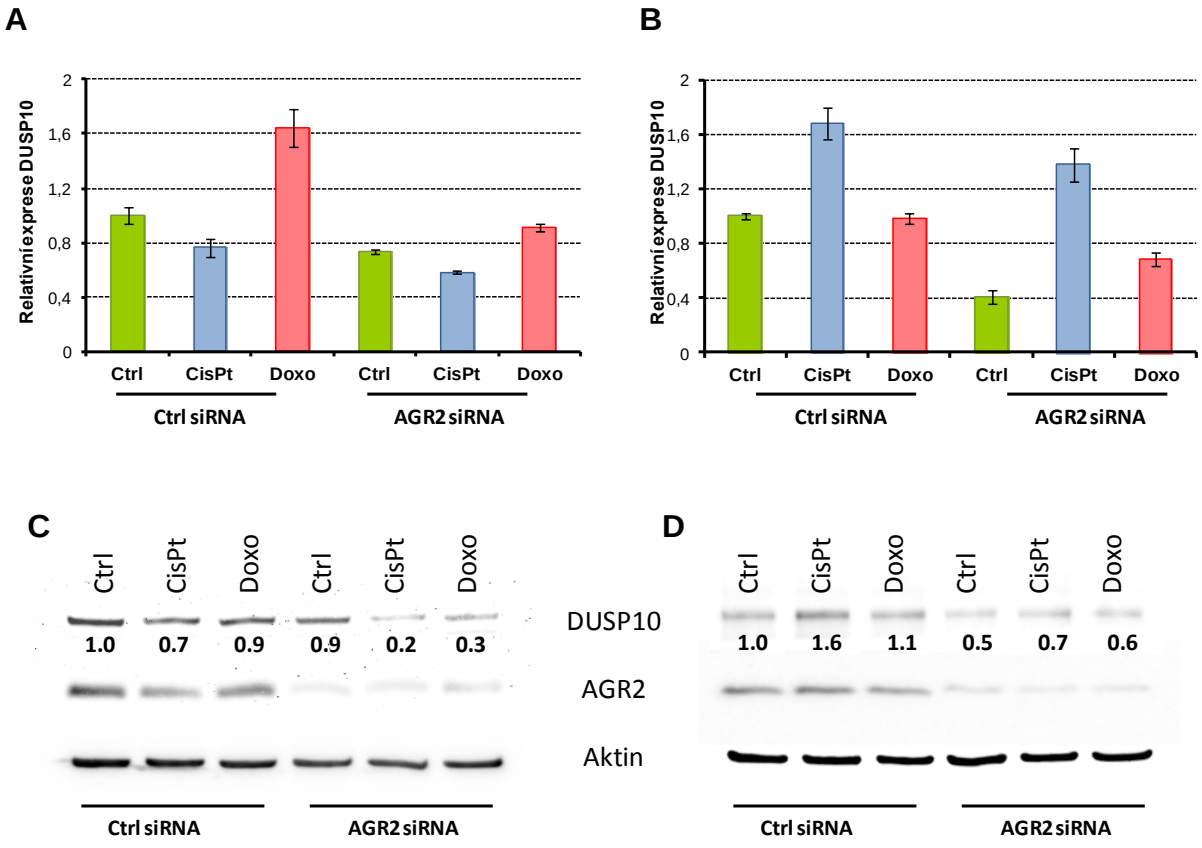
Skutečnost, že zvýšená hladina proteinu AGR2 jednoznačně vede k inhibici transkripční aktivity p53, iniciovala naši snahu identifikovat mechanismus, kterým AGR2 reguluje p53. Vzhledem k pozorovaným změnám v intenzitě fosforylace na Ser 15 a Ser 392 jsme se zaměřili především na kinázy, o kterých je známo, že se na modifikaci p53 v těchto pozicích podílejí. Zatímco v případě ATM/ATR nebyla jednoznačně prokázána přímá spojitost mezi expresí AGR2 a specifickými fosforylacemi p53, při analýze p38 MAP kinázy (p38 MAPK), která se aktivně účastní při fosforylaci na obou studovaných serinech, jsme

skutečně potvrdili, že aktivita p38 MAPK je modulována přítomností proteinu AGR2 (**obr. 19**).



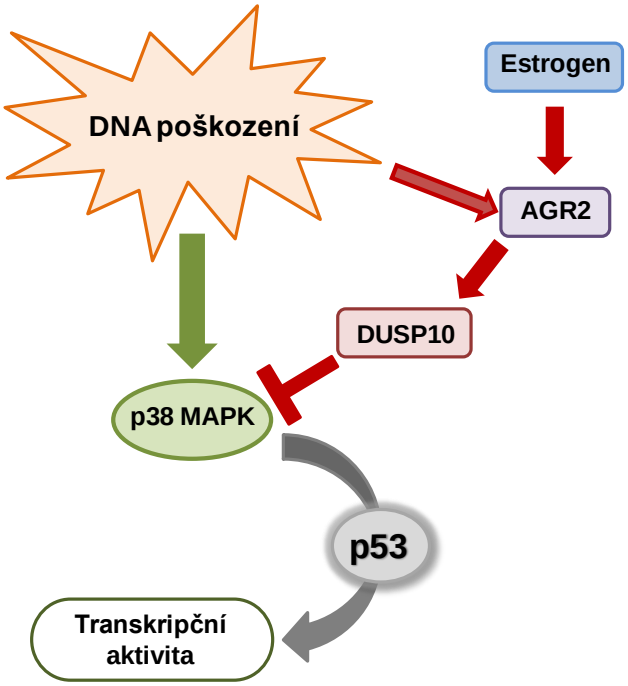
Obr. 19: Úloha p38 MAPK při AGR2 regulované inhibici proteinu p53. *Buněčné linie A549 (A) a MCF-7 (B) byly transientně transfekovány kontrolní siRNA (Ctrl siRNA), respektive siRNA specifickou proti AGR2 (AGR2 siRNA). 32 hodin po transfekci byly buňky vystaveny působení cisplatiny (CisPt) nebo doxorubicinu (Doxo) po dobu 16 hodin. Následně byla imunochemicky analyzována hladina p38 MAPK a především stanovena kinázová aktivita p38 MAPK pomocí detekce fosforylace ATF fúzního proteinu sloužícího jako substrát pro aktivovanou p38 MAPK.*

Nicméně mechanismus, kterým AGR2 reguluje aktivitu p38 MAPK zůstával i nadále neobjasněn. Proto byla s využitím technologie mikrofluidních karet paralelně analyzována exprese 92 nejvýznamnějších genů, které se uplatňují při MAP-kinázové signalizaci. Zajímavým zjištěním bylo, že při umlčení exprese AGR2 docházelo u obou buněčných linií k signifikantní změně exprese (snížení) pouze u jednoho genu *DUSP10* (*Dual Specificity Phosphatase 10*) též známého jako *MKP5* (*MAP Kinase Phosphatase 5*) (**obr. 20A, B**). Tato skutečnost byla potvrzena i dalším nezávislým experimentem na úrovni mRNA pomocí RT-qPCR a stejně tak na úrovni proteinu (**obr. 20C, D**).



Obr. 20: Identifikace DUSP10 jako další důležité komponenty AGR2 řízené anti-p53 signální dráhy. *Buněčné linie A549 a MCF-7 byly transientně transfekovány kontrolní siRNA (Ctrl siRNA), respektive siRNA specifickou proti AGR2 mRNA (AGR2 siRNA). 32 hodin po transfekci byly buňky vystaveny působení cisplatiny (CisPt) nebo doxorubicinu (Doxo) po dobu 16 hodin. (A) Stanovení mRNA hladina DUSP10 u A549. (B) Stanovení mRNA hladiny DUSP10 u MCF-7. (C) Imunochemická analýza DUSP10 u A549. (D) Imunochemická analýza DUSP10 u MCF-7.*

Na základě obdržených výsledků pak bylo možné schematicky znázornit AGR2 regulovanou signální dráhu (**obr. 21**). Tato dráha se aktivuje především v návaznosti na genotoxický stres a v případě karcinomů mléčné žlázy dochází v úzké kooperaci s aktivovanými EsR k indukci AGR2, což vede ke zvýšení exprese DUSP10. Následkem toho dochází k defosforylaci a tudíž inaktivaci p38 MAPK a v konečném důsledku pak ke snížení transkripční aktivity nádorového supresoru p53.



Obr. 21: Grafické znázornění mechanismu zodpovědného za inhibici proteinu p53.

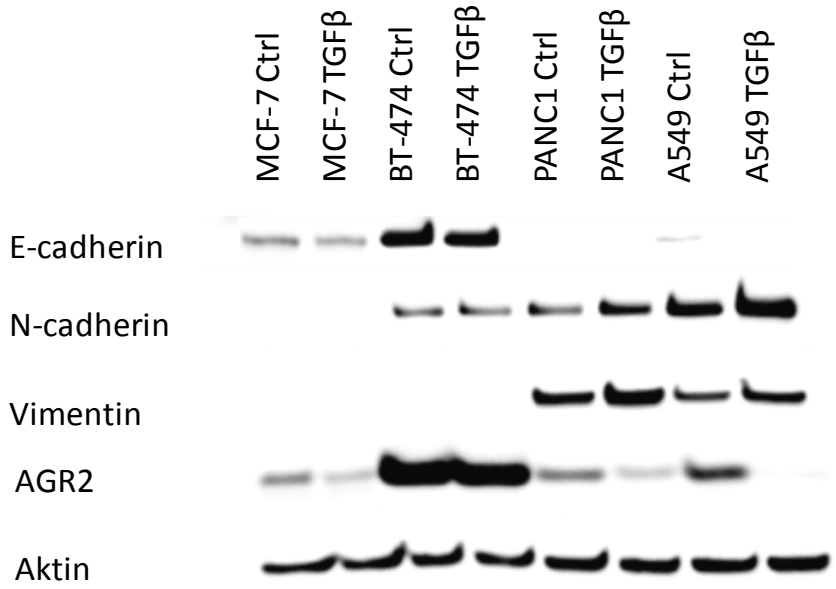
4.3 Úloha proteinu AGR2 při vzniku metastáz

Vzhledem ke skutečnosti, že exprese proteinu AGR2 je v případě karcinomu mléčné žlázy silně závislá na funkčních EsR, které představují pozitivní prognostický faktor tohoto onemocnění, bylo značně překvapivé, když byla publikována práce deklarující schopnost AGR2 indukovat tvorbu metastáz [40]. Konkrétně byly krysí benigní mammární buňky Rama 37 transfekovány expresním plazminem obsahujícím kódující sekvence *AGR2* a po jejich vnesení do syngenních krys docházelo ke vzniku metastáz, a to především v plících. Toto zjištění spolu s výsledky nedávno publikované práce identifikující s metastazováním často spojované proteiny C4.4a a extracelulární alfa-dystroglycan jako interakční partnery proteinu AGR2 [33] vedlo ke zvýšenému zájmu o AGR2 v rámci klinických studií zaměřených na studium metastazování. Výsledky získané napříč klinickými studiemi řady nádorových onemocnění jsou však značně heterogenní a jednoznačně nepotvrdily přímou úlohu AGR2 při vzniku metastáz.

Obecně vzato mají pro metastazování nádorových buněk zásadní význam buněčná migrace a invazivita, tedy děje, v rámci kterých se u nádorových buněk aktivně uplatňuje i AGR2 [23]. Tyto procesy však hrají zásadní úlohu i v řadě dalších biologických procesů, jako jsou embryogeneze, imunitní odpověď, hojení ran, morfogeneze či zánět, přičemž u řady těchto jevů byla aktivní úloha proteinu AGR2 popsána nebo alespoň predikována [3].

Tvorba metastáz probíhá multifaktoriálně během vícestupňového procesu, jehož prvním krokem bývá oddělení nádorové buňky od primárního nádoru [47]. Toto je umožněno díky zvýšené expresi proteáz štěpících extracelulární matrix. Jednotlivé uvolněné nádorové buňky následně pronikají bazální membránou a mohou prostupovat do stromatu. Tento proces se nazývá epiteliálně-mezenchymální tranzice (EMT) a v jeho důsledku dochází ke ztrátě buněčné adheze, epiteliální polarity a ke zvýšení migrační a invazivní schopnosti nádorových buněk [48]. Buňky s mezenchymálním fenotypem pak tzv. intravazací snadno pronikají do cévního řečiště, které jim umožní dostat se až do oblastí vzdálených orgánů, tzv. extravazaci, a následně dochází k opačnému procesu tzv. mezenchymálně-epiteliální tranzici (MET) a vzniká sekundární nádor neboli metastáza [48].

V naší práci jsme se zaměřili na studium úlohy AGR2 v procesu EMT. Jedním z hlavních důvodů byla skutečnost, že exprese AGR2 byla detekována především u epiteliálních buněk a pokud byly epiteliální nádorové buňky vystaveny působení TGF-β (*Transforming growth factor beta*), docházelo k intenzívnímu potlačení exprese AGR2. TGF-β představuje snad nejčastěji používaný induktor EMT u nádorových buněčných linií [49,50]. Po vazbě TGF-β na TGF-β receptory dochází k aktivaci dvou na sobě nezávislých signálních kaskád obecně označovaných jako „Smad-závislé“ a „Smad-nezávislé“. Působením TGF-β docházelo nejen k inhibici exprese AGR2, ale i ke změně buněčné morfologie a potlačení exprese E-cadherinu a naopak indukci exprese mezenchymálních markerů vimentinu a N-cadherinu (**obr. 22**).

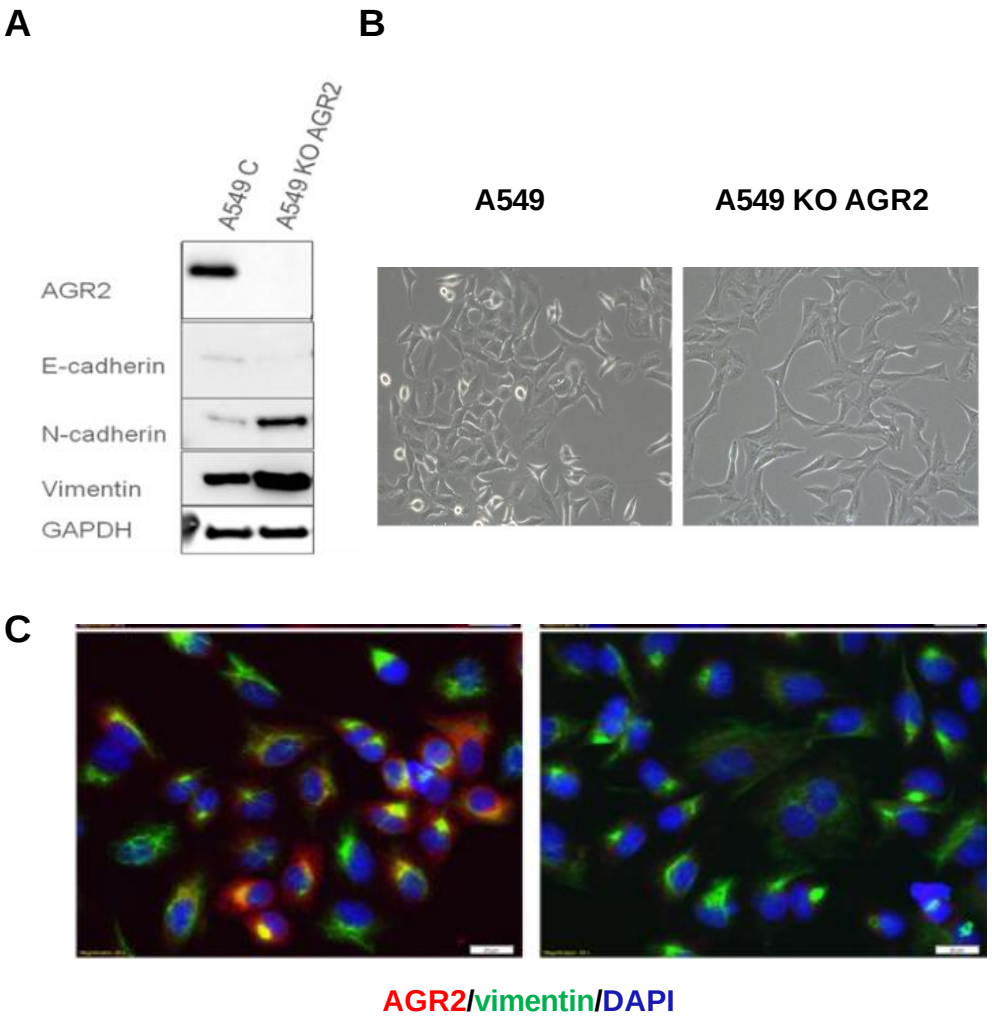


Obr. 22: Vliv TGF-β na hladinu vybraných proteinů. *Změny v expresi vybraných proteinů byly detekovány pomocí imunochemické analýzy u vybraných buněčných linií. Jako kontrola množství nanášeného celkového proteinu byl použit aktin.*

V souvislosti s indukcí EMT jsme analyzovali, zda se snížení hladiny AGR2 aktivně uplatňuje při navozování mezenchymálního fenotypu, nebo je pouze vedlejší konsekvencí tohoto procesu buněčné transformace. Nejdříve jsme u nádorových buněk přirozeně exprimujících AGR2 potlačili expresi tohoto proteinu pomocí specifické siRNA. Analogicky jako v případě expozice buněk působení TGF-β jsme pozorovali snížení hladiny E-cadherinu a naopak mírnou indukci vimentinu a N-cadherinu.

Vzhledem ke skutečnosti, že působením siRNA nedocházelo k úplnému potlačení exprese AGR2, byl pomocí TALEN technologie z buněčné linie A549 připraven klon (A549 KO AGR2) s umlčenou expresí AGR2. Stanovení exprese markerů EMT pak jasně potvrdilo předchozí výsledky, neboť oproti parentální linii A549 došlo ke ztrátě E-cadherinu a současně bylo pozorováno zvýšení hladin N-cadherinu a vimentinu (**obr. 23A**). Stejně tak bylo možné pomocí invertovaného světelného mikroskopu pozorovat změny v morfologii buněk (**obr. 23B**). Pomocí imunofluorescence byly vzhledem k přítomnosti, respektive nepřítomnosti AGR2 studovány změny v hladině vimentinu a především jeho buněčné lokalizaci. V případě buněk A549 se vimentin nacházel především v perinukleárním prostoru, zatímco po zablokování exprese AGR2 docházelo k jeho difúzní delokalizaci do cytoplazmy (**obr. 23C**).

Naše výsledky jednoznačně ukazují, že expozice TGF-β vede u studovaných buněčných linií k indukci EMT a je spojena i s výrazným snížením hladiny proteinu AGR2. Funkční analýzy prokazují, že protein AGR2 představuje jednu z klíčových molekul procesu EMT, neboť v případě manipulace s expresí AGR2 dochází k navození epiteliálně mezenchymální tranzice nezávisle na jakémkoli dalším vnějším podnětu. Naše výsledky dále potvrzují, že protein AGR2 ve své podstatě představuje epiteliální marker a v případě, že dojde k jeho odstranění resp. inhibici exprese, dochází ke snížení hladiny E-cadherinu a naopak indukci exprese N-cadherinu a vimentinu. Nádorová buňka mění svůj fenotyp na mezenchymální, což je provázeno zvýšenou motilitou a invazivitou nádorových buněk a představuje iniciální impuls vedoucí ke vzniku metastáz. Je však nezbytné si uvědomit, že u primárních nádorů dochází k potlačení exprese AGR2 zřejmě pouze transientně. Naše předběžná data naznačují, že během následného procesu MET, kdy se cirkulující mezenchymální buňka snaží kolonizovat vzdálené tkáně, dochází k reexpresi proteinu AGR2, který se významně podílí na schopnosti buněk adherovat [22,51] a především signifikantně zintenzivňuje jejich proliferaci [30,52], čímž podporuje tvorbu sekundárních nádorů.

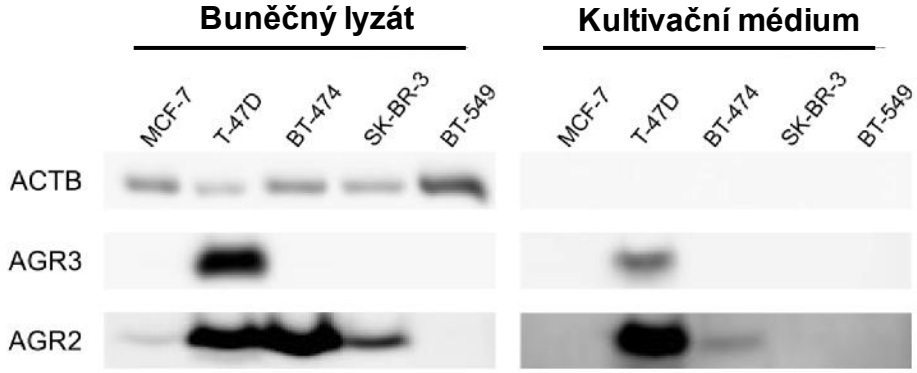


Obr. 23: Stanovení exprese vybraných proteinů po úplném umlčení exprese genu AGR2. *(A) Imunochemická validace exprese AGR2 a stanovení dalších EMT markerů. Exprese GAPDH byla použita jako kontrola stejného množství nanášeného celkového proteinu. (B) Ukázka změn v morfologii buněk s vyřazenou expresí AGR2 vzhledem k parentální linii A549. (C) In situ analýza exprese AGR2 a vimentinu pomocí imunofluorescenčně značených protilátek, vlevo parentální linie A549, vpravo A549 KO AGR2.*

4.4. AGR2 a AGR3 jako sekretované proteiny

AGR2 a AGR3 byly již v roce 2003 identifikovány v rámci proteomické analýzy jako proteiny nacházející se nejen v plazmatické membráně nádorových buněk pocházejících z karcinomu mléčné žlázy, ale i v endozomech. Tato skutečnost dávala za pravdu hypotézám vytvořeným na základě analýzy aminokyselinové sekvence, které i přes přítomnost retenční sekvence predikovaly, že se pravděpodobně jedná o proteiny, které mohou být sekretovány ven z buněk [10]. Následně se objevilo několik dalších prací, které tuto skutečnost potvrdily. Za zmínku stojí především práce autorů Tsuji a kol., která ukazuje schopnost extracelulárního

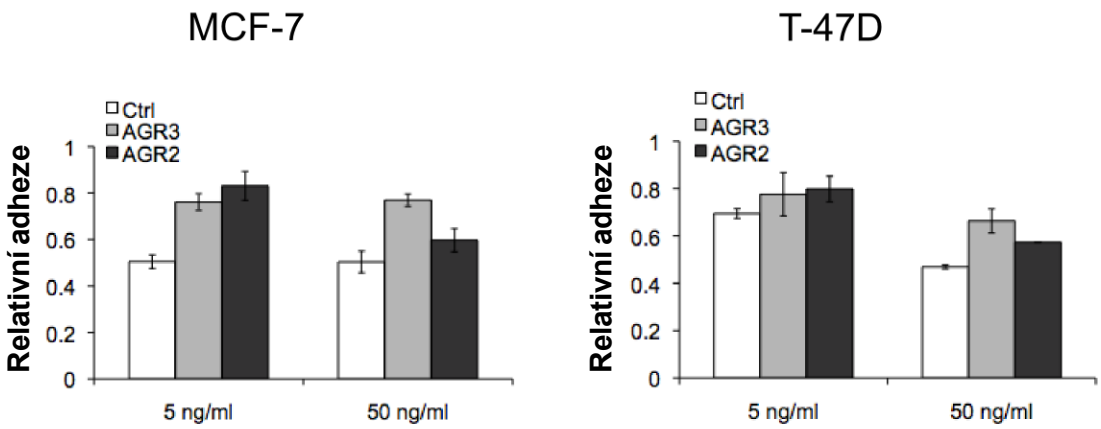
AGR2 aktivovat fibroblasty ve stromatu a iniciovat tak invazi nádorových buněk [53]. Klíčová je pak práce zaměřená na studium úlohy AGR2 v etiologii karcinomů pankreatu, kde bylo prokázáno, že dochází nejen k indukci zvýšené exprese AGR2, ale současně i sekreci tohoto proteinu [54]. Na tuto publikaci pak v podstatě stejní autoři navazují další významnou prací, kde se jim podařilo u duktálních karcinomů pankreatu identifikovat receptor C4.4A jako cílovou molekulu, se kterou extracelulární AGR2 může interagovat, a iniciovat tak zvýšenou buněčnou proliferaci, migraci, invazivitu a naopak potlačovat indukci apoptózy a obecně vzato tak přispívat ke zvýšené agresivitě nádorových buněk díky autokrinní signalizaci. V návaznosti na toto zjištění pak vyvinuli specifické monoklonální protilátky proti AGR2 i C4.4A, které jim umožnily efektivně zablokovat interakci mezi těmito dvěma proteiny. Následná aplikace těchto protilátek vedla k nejen účinnému potlačení schopnosti nádorových buněk migrovat, ale současně je i senzitizovala k indukci apoptózy. V rámci *in vivo* studií pak při použití zmiňovaných protilátek na myších xenograftech docházelo nejen k regresi primárních tumorů, ale současně i k redukci počtu metastáz [34]. Možnost využití AGR2 jako nádorového biomarkeru analyzovatelného přímo v tělních tekutinách pak dokládá několik recentních publikací, a to jak v krevním séru [55,56], tak i v moči [17,57,58]. Podobně pak byla zvýšená hladina proteinu AGR3 detekována v krevním séru pacientek s karcinomem mléčné žlázy [59].



Obr. 24: Imunochemická analýza. *Hladiny proteinů AGR2 a AGR3 byly stanoveny v buněčném lyzátu a současně kultivačním médiu odebraném před sklízením a lýzou buněk. Aktin (ACTB) byl použit nejen jako kontrola množství nanášeného vzorku, ale i skutečnosti, že v odebraném médiu nebyly přítomny buňky.*

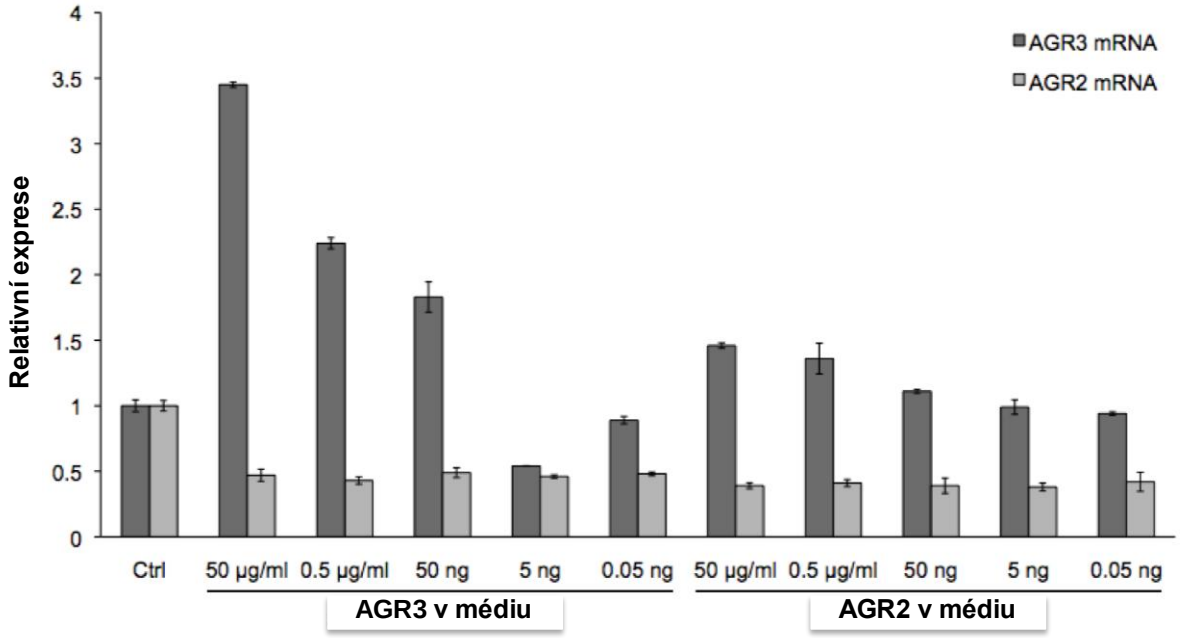
Parakrinní/autokrinní signalizací proteinů AGR2 a AGR3 se zabýváme i na našem pracovišti. Prvním krokem byla analýza exprese AGR2 a AGR3 na panelu vybraných buněčných linií odvozených od karcinomu mléčné žlázy a současně pak detekce přítomnosti

AGR2 a AGR3 v kultivačním médiu, ve kterém byly tyto buněčné linie kultivovány po dobu 72 hodin (**obr. 24**). Sekrece proteinu AGR2 byla pozorována u buněčných linií T-47D a BT-474 a AGR3 pouze u buněk T-47D, pravděpodobně především proto, že u zmíněných linií byla pozorována silná exprese AGR proteinů. Naše předběžné výsledky jednoznačně potvrzují schopnost extracelulárně přítomných proteinů AGR2 nebo AGR3 významným způsobem ovlivňovat fenotyp nádorových buněk, a to především prostřednictvím signálních drah uplatňujících se při regulaci buněčné adheze (**obr. 25**). Bližší analýza buněčné signalizace pomocí kinázových testů u buněčných linií MCF-7 a T-47D kultivovaných s rekombinantním proteinem AGR2 respektive AGR3 přidaným do média prokázala, že dochází k modulaci aktivity řady významných buněčných kináz, které jsou ve vztahu k extracelulárním AGR proteinům v současné době na pracovišti intenzívně studovány.



Obr. 25: Vliv extracelulárního AGR2 a AGR3 na buněčnou adhezi. *Ukázka nakolik jsou rekombinantní proteiny AGR2 a AGR3 přidané do kultivačního média k daným buněčným liniím schopny zvýšit jejich adhezivitu ve srovnání s buňkami standardně kultivovanými (Ctrl).*

Funkční analýza účinku rekombinantního proteinu AGR2 přidaného do média, ve kterém byly nádorové buňky kultivovány, ukázala, že dochází zhruba ke dvojnásobné redukci exprese *AGR2*, zatímco efekt na expresi *AGR3* byl v podstatě zanedbatelný. Naopak v případě parakrinního působení AGR3 na okolní nádorové buňky docházelo k masivní indukci exprese *AGR3*, zatímco exprese *AGR2* byla inhibována (**obr. 26**).



Obr. 26: Vliv extracelulárního AGR2 a AGR3 na hladinu mRNA AGR2 a AGR3. *Změny v expresi AGR2 a AGR3 byly analyzovány na úrovni transkripce u buněčné linie T-47D po působení rekombinantního proteinu AGR3 respektive AGR2. Protein GST byl použit v rámci kontrolního experimentu (Ctrl).*

5. Studium exprese AGR2 u vybraných lidských malignit

Aberantní exprese AGR2 byla kromě karcinomů mléčné žlázy prokázána i u řady jiných malignit (**Příloha 3**). Protein AGR2 je uvažován jako jeden z potenciálních biomarkerů karcinomu pankreatu, což je nádorové onemocnění, jehož incidence je téměř totožná s mortalitou („*American Cancer Society*“ udává, že za posledních pět let se přežití pacientů prodloužilo v podstatě jen zanedbatelně [60]). Velmi špatná prognóza a pozdní manifestace onemocnění podporují nemalé úsilí nalézt specifické a spolehlivé biomarkery a pochopit přesné molekulární mechanismy uplatňující se při vzniku invazivního karcinomu pankreatu. Doposud však neexistuje dostatečně specifický a senzitivní biomarker, který by jednoznačně přispíval k detekci tohoto onemocnění v raných stádiích, nebo predikoval odpověď na podávanou terapii. Zvýšená exprese AGR2 byla detekována v prekancerózních lézích, nádorových buňkách, buněčných liniích odvozených od karcinomu pankreatu i v cirkulujících nádorových buňkách [61,62]. Na molekulární úrovni AGR2 stimuluje buněčný růst a proliferaci, podporuje invazivitu a především schopnost přežívání pankreatických nádorových buněk a významně tak může přispívat k rezistenci vůči podávané léčbě [54].

5.1. Analýza exprese AGR2 u duktálních adenokarcinomů pankreatu

Na souboru 135 nádorových biopsií pocházejících z karcinomů pankreatu jsme pomocí imunohistochemického barvení paralelně analyzovali expresi proteinů AGR2 a mucinu 4. Výsledky jsou detailně popsány v práci *„Anterior gradient 2 and mucin 4 expression mirrors tumor cell differentiation in pancreatic adenocarcinomas, but aberrant anterior gradient 2 expression predicts worse patient outcome in poorly differentiated tumors“* (**příloha 10**). Vzhledem ke skutečnosti, že IHC barvení proteinu AGR2 vykazovalo heterogenní charakter, byly analyzovány celé tkáňové řezy. V případě, že bylo možné v jednotlivých řezech pozorovat různě diferencované komponenty, byla každá komponenta hodnocena zvlášť. I díky tomuto přístupu jsme jednoznačně prokázali, že hladina exprese proteinu AGR2 odpovídala stupni diferenciace nádorové tkáně pankreatu. Zajímavější výsledky jsme pozorovali u méně diferenciovaných nádorů pankreatu, kde zvýšená hladina AGR2 naopak predikovala horší odpověď pacientů k terapii a kratší přežití. Výsledky práce Ramachandran a kol. ukazují, že pokud dojde k umlčení exprese AGR2, dochází k signifikantnímu zefektivnění léčby gemcitabinem. V souladu s tím a na základě našich výsledků by tedy bylo možné v případě málo diferencovaných duktálních adenokarcinomů pankreatu uvažovat o léčebném protokolu, jenž by zahrnoval cílenou terapii proti AGR2 v kombinaci s podáváním gemcitabinu.

6. Studium exprese proteinu AGR3 u vybraných nádorových onemocnění

Oproti proteinu AGR2, jehož funkce v nádorové buňce a exprese u různých typů nádorů je poměrně komplexně popsána, se protein AGR3 v odborné literatuře objevuje jen velmi omezeně. Dosavadní poznatky o tomto proteinu jsme shrnuli v přehledovém článku *„The role of AGR2 and AGR3 in cancer: similar but not identical“*, který je zaměřen především na porovnání vlastností AGR3 vzhledem k mnohem více studovanému proteinu AGR2 (**příloha 11**). V souvislosti s lidskými nádorovými onemocněními byly oba proteiny objeveny u buněčné linie MCF-7 [7]. Později byl protein AGR3 objeven pomocí proteomických analýz v membránách u studovaných nádorových linií odvozených od karcinomu mléčné žlázy [10] a následně identifikován i u dalších nádorových onemocnění (**příloha 11**). Přestože jsou oba proteiny u řady nádorových onemocnění koexprimovány, *in silico* analýza pomocí programu MatInspector [63,64] ukázala rozdílná spektra transkripčních faktorů potenciálně se vázajících na promotor *AGR2*, respektive *AGR3*, přičemž řada

transkripčních faktorů byla predikována jako exkluzivně vázajících se na promotor genu *AGR2* nebo *AGR3* (**příloha 11**).

Vedle regulace exprese *AGR2* a *AGR3* byly pomocí webové aplikace Genevestigator [65] identifikovány geny, které jsou koexprimovány spolu s *AGR2* a/nebo *AGR3*. Vedle skupiny genů, k jejichž expresi dochází simultánně s oběma geny, byly identifikovány i geny k jejichž ko-expresi dochází pouze s jedním či druhým. V případě *AGR3* bych zmínil především *claudin 3* (*CLDN3*), jehož produkt byl již dříve popsán jako faktor, který se významně uplatňuje při rezistenci nádorů vaječníku k cisplatině. Tato skutečnost je v souladu s výsledky naší práce nazvané: *„Anterior Gradient-3: a novel biomarker for ovarian cancer that mediates cisplatin resistance in xenograft models“*, kde mimo jiné ukazujeme, že zvýšená hladina exprese proteinu AGR3 může úzce souviset s rezistencí nádorových buněk k cisplatině (**příloha 12**). Pro tuto práci, která vznikla ve spolupráci s Edinburgh Cancer Research Centre, jsme vyvinuli panel monoklonálních protilátek specifických proti proteinu AGR3, které byly následně využity k vývoji ELISA testu umožňujícího detekci AGR3. Současně byla na našem pracovišti imunohistochemicky analyzována exprese AGR3 na souboru karcinomů vaječníku. Naše studie odhalila, že protein AGR3 je exprimován nezávisle na estrogenních receptorech a především, že ke ko-expresi mezi AGR2 a AGR3 dochází pouze u mucinózních karcinomů vaječníku, zatímco v případě serózních, endometrioidních a světlobuněčných byla pozorována rozdílná exprese AGR2 a AGR3 (**příloha 12**). V kontextu těchto výsledků byla započata nová klinická studie na souboru téměř 300 karcinomů vaječníku s cílem analyzovat rozdílné expresní profily AGR2 a AGR3 vzhledem k prognóze pacientek s karcinomem vaječníku a ve vztahu k rozdílné buněčné signalizaci uplatňující se při karcinogenezi daného histologického subtypu.

Zhoubná onemocnění jater patří ve světě k nejčastějším malignitám, a jejich výskyt je častější u mužů nežli u žen. Rizikovými faktory jsou infekce virem hepatitidy typu B a C a hepatotoxické látky, např. aflatoxin. Dalším rizikovým faktorem je jaterní cirhóza, kterou má až 90 % pacientů. Kromě vlastního zhoubného onemocnění představují játra časté místo metastáz ostatních zhoubných nádorů, nejčastěji nádorů plic, mléčné žlázy, tračníku a konečníku. Důvodem je především výrazná vaskularizace, přičemž přibližně 25 % objemu krve vydaného srdcem protéká játry. Nejčastějším primárním nádorem jater je hepatocelulární karcinom (HCC), který tvoří 90 % všech primárních nádorů jater. Druhým nejčastějším nádorem jater je cholangiogenní karcinom (CC), který postihuje intrahepatální žlučovody a obvykle se vyznačuje velmi špatnou prognózou. Terapeutické přístupy u obou jaterních malignit se značně liší, a tudíž maximálně specifické diagnostické přístupy umožňující

rozlišení obou subtypů jsou nanejvýš žádoucí. Mezi nejčastěji používané imunohistochemické markery HCC patří Hep Par 1 (*hepatocyte paraffin antigen-1*), CEA (karcinoembryonální antigen), CD10, AFP (alfa-fetoprotein), β-katenin, Hsp70, a GPC-3 (glypican-3). Nicméně žádný z výše uvedených biomarkerů není univerzálně použitelný a 100% specifický. Při imunohistochemickém stanovení přítomnosti proteinu AGR3 na pilotním souboru karcinomů jater jsme zjistili, že se tento protein nachází specificky u cholangiogenních karcinomů. Proto jsme se v práci nazvané *„Differential expression of anterior gradient protein 3 in intrahepatic cholangiocarcinoma and hepatocellular carcinoma“* rozhodli rozšířit původní soubor a otestovat AGR3 jako potenciální biomarker specifický pro CC (**příloha 13**). Celkem bylo imunohistochemicky analyzováno 74 případů, z toho 52 mužů (70,3 %) a 22 žen (29,7 %), diagnosticky se jednalo o 26 CC a 48 HCC. Imunohistochemická analýza jasně prokázala, že protein AGR3 se vyskytuje specificky u CC ($p < 0{,}00001$). Jako referenční bylo použito IHC barvení GPC-3, který se naopak specificky vyskytoval u HCC ($p = 0{,}00013$). ROC (*receiver operating characteristic*) analýza pak demonstrovala, že pro diferenciální diagnostiku mezi HCC a CC je AGR3 v porovnání s GPC-3 vhodnější ($AUC_{AGR3} = 0{,}801$ oproti $AUC_{GPC\text{-}3} = 0{,}701$). Kombinace AGR3 a GPC-3 se pak ukázala jako nejvýhodnější s AUC = 0,859 (**příloha 13**). Na základě získaných výsledků lze konstatovat, že i přes ne zcela homogenní imunohistochemické stanovení AGR3 ve smyslu slabě pozitivního barvení AGR3 u několika HCC a nepřítomnosti AGR3 u 6 CC tento protein v porovnání se stávajícími rutinně používanými biomarkery představuje poměrně atraktivní alternativu při diferenciální diagnostice hepatocelulárního a cholangiogenního karcinomu.

Exprese proteinu AGR3 byla důsledně studována pomocí IHC i na souboru 129 primárních karcinomů mléčné žlázy (**příloha 5**). Přítomnost proteinu AGR3 byla prokázána u přibližně 80 % studovaných případů. Dle očekávání byla prokázána statisticky signifikantní korelace s přítomností estrogenových a progesteronových receptorů a dále pak s histologickým gradem a inverzní korelace s expresí markeru proliferace Ki-67. Jinými slovy zvýšená exprese AGR3 byla typická pro dobře diferencované slabě proliferující EsR-pozitivní tumory. Tento výsledek by tedy naznačoval, že zvýšená exprese AGR3 v případě karcinomů mléčné žlázy souvisí s lepší prognózou tohoto onemocnění, což v podstatě naznačila i Kaplanova-Meierova analýza přežití, kdy pacientky se zvýšenou hladinou AGR3 vykazovaly signifikantně delší bezpříznakové přežití ($p = 0{,}037$), celkové přežití pak naznačovalo podobný trend ($p = 0{,}1111$). Avšak Coxův model proporcionálních rizik, jenž umožňuje zjistit vztah mezi dobou přežití a možnými vysvětlujícími proměnnými, prokázal, že expresi AGR3 nelze označit jako samostatný nezávislý prognostický faktor karcinomu mléčné žlázy na

rozdíl od velikosti tumoru a statutu HER2 receptorů v případě bezpříznakového přežití nebo velikosti tumoru a stupně diferencovanosti v případě celkového přežití pacientek (**příloha 5**).

7. Závěr

Rodina proteinů Anterior gradient tvoří evolučně poměrně širokou skupinu proteinů, které se vyznačují významnými, ale doposud z velké míry ne zcela prostudovanými funkcemi. Předložená práce je zaměřena na lidské homology AGR2 a AGR3, jejichž přítomnost byla popsána u řady onkologických onemocnění, kde se přímo podílejí na maligní transformaci, rezistenci k léčbě a přispívají tak ke zvýšené agresivitě daného nádorového onemocnění. Poměrně vysoká míra homologie mezi AGR2 a AGR3 predikuje podobné vlastnosti obou proteinů, což se projevuje i v obdobném expresním profilu typickém pro řadu nádorů. Naše výsledky, včetně doposud nepublikovaných, potvrzují v podstatě identický expresní profil AGR2 a AGR3 např. u EsR pozitivních karcinomů mléčné žlázy a naznačují možnou kooperaci mezi oběma proteiny. Toto je mimo jiné podpořeno i skutečností, že AGR2 a AGR3 spolu mohou vytvářet komplexy, čímž se mohou významně podílet na progresi nádorových onemocnění. Otázky týkající se významu interakcí mezi AGR2 a AGR3 a především jejich důsledků na molekulární úrovni v kontextu buněčných procesů, ať už za fyziologických či stresových podmínek, však doposud zůstávají převážně nezodpovězeny.

Na druhou stranu řada nejnovějších poznatků naznačuje existenci exkluzivních funkcí či vlastností typických pouze pro protein AGR2, respektive AGR3. Toto dokládá i naše práce zaměřená na analýzu exprese proteinu AGR3 u karcinomů vaječníku, kde jsme pozorovali podobnou expresi AGR2 a AGR3 pouze u mucinózních karcinomů, zatímco u ostatních typů byla exprese proteinů AGR2 a AGR3 značně heterogenní. V souladu s výše uvedeným, funkční analýza účinku extracelulárních proteinů AGR2 a AGR3 na nádorové buňky ukázala, že odlišně ovlivňují expresi genů *AGR2* a *AGR3* na úrovni transkripce. Souhrnně lze tedy konstatovat, že expresní profily obou genů *AGR2* a *AGR3* jsou značně podobné, nikoli však identické, což mimo jiné odráží i obrovskou diverzitu maligních onemocnění a signálních drah, které se uplatňují při regulaci buněčných procesů v nádorové buňce, v rámci kterých hraji nezanedbatelnou úlohu i proteiny rodiny Anterior gradient.

Mezi nejvýznamnější výsledky získané v rámci téměř desetiletého výzkumu zaměřeného na proteiny AGR bych zařadil verifikaci estrogenových receptorů, jako klíčových regulátorů exprese AGR2 u karcinomů mléčné žlázy. V návaznosti na tuto skutečnost se pak podařilo prokázat, že zvýšená hladina AGR2 může sloužit jako negativní prediktivní faktor při adjuvantní léčbě tamoxifenem. Následně pak byly u karcinomů mléčné žlázy objeveny i další faktory uplatňující se při regulaci exprese AGR2, především se jedná o PDPK-1, AKT a

receptory HER2, čímž se alespoň částečně vysvětlila skutečnost, že zvýšená exprese AGR2 bývá vzácněji pozorována i u EsR negativních karcinomů mléčné žlázy.

Funkční analýzy zaměřené na funkci proteinu AGR2 v nádorových buňkách odhalily mechanismus, kterým se protein AGR2 negativně podílí na regulaci hladiny a především aktivity proteinu p53 a připívá tak k agresivnějšímu fenotypu EsR pozitivních nádorů mléčné žlázy, u kterých nedošlo k mutaci genu *TP53*. Nemalé úsilí v současné době věnujeme studiu úlohy AGR2 při EMT indukované působením TGF-β. Při tomto procesu dochází k evidentnímu snížení hladiny proteinu AGR2, což jak se zdá, významně napomáhá k navození mezenchymálního fenotypu původně epiteliálních buněk a přispívá tak k jejich invazivitě a schopnosti tvořit metastázy.

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9. Seznam použitých zkratek

AFP	Alfa-fetoprotein
AGR	Anterior gradient
AKT	Protein kináza B
AMK	Aminokyseliny
AP-1	*Activator protein-1*
AREG	Amfiregulin
ATM	*Ataxia Telangiectasia Mutated*
ATR	*Ataxia Telangiectasia and Rad3-related protein*
BRET	*Bioluminescence Resonance Energy Transfer*
C4.4a	*GPI-anchored metastasis-associated protein C4.4A homolog*
CC	Cholangiocelulární karcinom
CD1	Cyklin D1
CD10	Společný antigen akutní lymfoblastické leukemie (CALLA)
CEA	Karcinoembryonální antigen
CisPt	Cisplatina
c-Jun	Jun proto-onkogen
CLDN3	Claudin 3
Ctrl	Kontrola
DAG-1	Dystroglykan 1
DFS	Bezpříznakové přežití (disease free survival)
Doxo	Doxorubicin
DSP	Dithiobis[succinimidylpropionát]
DUSP10	*Dual Specificity Phosphatase 10*
EGFR	Receptor pro epidermální růstový faktor
ELISA	*Enzyme-linked immunosorbent assay*
EMT	Epiteliálně-mezenchymální tranzice
ER	Endoplazmatické retikulum
ERE	Vazebná místa pro estrogenový receptor (*estrogen response element*)
ERK1/2	*Extracellular signal-regulated kinase 1/2*
ERp57	*Endoplasmic reticulum resident protein 57*
EsR	Estrogenový receptor

FOXA-1	*Forkhead box A1*
GPC-3	Glypikan-3
GST	Glutathion-S-transferáza
HCC	Hepatocelulární karcinom
Hep Par 1	*Hepatocyte paraffin antigen-1*
HER2	Receptor pro lidský epidermální růstový faktor. (human epidermal growth factor receptor 2)
hod	Hodin
HR	*Hazard ratio*
Hsp90	*heat shock protein 90*
ChIP	Chromatinová imunopreciptace
IHC	Imunohistochemické barvení
IS	Interval spolehlivosti
JNK	Janusova kináza
K(d)	Disociační konstanta
Ki67	A*ntigen identified by monoclonal antibody Ki-67*
Mdm2	*Mouse double minute 2*
MEK1/2	Mitogeny aktivovaná protein-kináza 1/2
MET	Mezenchymálně-epiteliální tranzice
MKP5	*MAP Kinase Phosphatase 5*
mRNA	mediátorová RNA
mTOR	*Mammalian target of rapamycin*
MUC1	Mucin 1
MUC2	Mucin 2
MUC5AC	Mucin 5AC
MUC5B	Mucin 5B
NMR	*Nuclear Magnetic Resonance*
OS	Celkové přežití (*overall survival*)
$p21^{WAF-1}$	Protein p21 (*cyclin-dependent kinase inhibitor 1A*)
p38 MAPK	Mitogeny aktivovaná protein kináza p38
PCNA	*Proliferating cell nuclear antigen*
PDI	Protein disulfid isomeráza
PDIA3	*Protein disulfide isomerase family A3*
PDIA6	*Protein disulfide isomerase associated 6*

PDPK1	Phosphatidylinositol 3-kinase/phosphatidylinositide-dependent protein kinase 1
PI3K	*Phosphoinositide 3-kinase*
PLA	*Proximity Ligation Assay*
PR	Progesteronový receptor
ROC	*Receiver Operating Characteristic*
RT-qPCR	Reversní transkripce kombinovaná s kvantitativní polymerázovou řetězovou reakcí
RUVB2	Reptin
Ser 15	Serin 15
Ser 392	Serin 392
SILAC	*Stable isotope labeling by amino acids in cell culture*
siRNA	*Short interfering RNA*
snoRNP	*Small nucleolar ribonucleoproteins*
Sp-1	Specific protein-1
TGF-β	Transformující růstový faktor beta (*transforming growth factor beta*)
TXNDC7	*Thioredoxin domain containing 7*
UPR	*Unfolded Protein Response*
wt	*wild-type*

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Příloha 1

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ENDOPLASMIC RETICULUM STRESS AND APOPTOSIS

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Abstract: Cell death is an essential event in normal life and development, as well as in the pathophysiological processes that lead to disease. It has become clear that each of the main cellular organelles can participate in cell death signalling pathways, and recent advances have highlighted the importance of the endoplasmic reticulum (ER) in cell death processes. In cells, the ER functions as the organelle where proteins mature, and as such, is very responsive to extracellular-intracellular changes of environment. This short overview focuses on the known pathways of programmed cell death triggering from or involving the ER.

Key words: Endoplasmic reticulum, Apoptosis, p53, Scotin

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Abbreviations used: ATF6 – activating transcription factor 6; BAP31 – B-cell receptor-associated protein 31; BiP – luminal binding protein; C/EBP – CCAAT/enhancer-binding proteins; eIF-2α – eukaryotic translation initiation factor; EOR – ER overloaded response; ER – endoplasmic reticulum; GADD 153 – growth arrest and DNA damage; GCN2 – general control of amino acid biosynthesis kinase; GRPs – glucose-regulated protein family; GSK3β – glycogen synthetase kinase-3β; HRI – hemin-regulated inhibitor of protein synthesis; IP3 – inositol 1,4,5-trisphosphate; IRE1 – inositol-requiring gene-1; IRF-1 – interferon regulatory factor 1; MEFs – mouse embryonic fibroblasts; NF-κB – nuclear factor-κB; PDI – protein disulphide isomerase; PERK – PKR-like ER kinase; PKR – interferon-inducible; dsRNA-activated protein kinase; PS1, 2 – presenilin-1, -2; SREBPs – sterol-regulatory element-binding proteins; SRP 72 – 72 kDa component of SRP (signal recognition particle); UPR – unfolded protein response; XBP1 – X-Box protein-1

ENDOPLASMIC RETICULUM

The endoplasmic reticulum (ER) is the cytoplasmic compartment where proteins and lipids are synthesized and modified. The ER provides a unique oxidizing compartment for the folding of membrane and secretory proteins that are transported to the cell surface, and to the lysosomes and the Golgi compartments. Due to the presence of high concentrations of protein, numerous protein chaperones exist in the ER to maintain proteins in a folding-competent state and prevent protein folding intermediates from aggregating. Proteins that do not mature properly are retained in the ER and eventually re-translocated to the cytosol for degradation by the 26S proteasome [1] (Fig. 1).

In response to a range of cytotoxic conditions, including hypoxia, nutrient deprivation, pH change, Ca^{2+} depletion from the ER lumen, inhibition of asparagine (N)-linked glycosylation, reduction of disulfide bonds and overexpression of some proteins, protein misfolding occurs, leading to unfolded proteins accumulating and aggregating in the ER. These abnormalities in the ER are collectively called ER stress. In order to overcome this, the organelle has

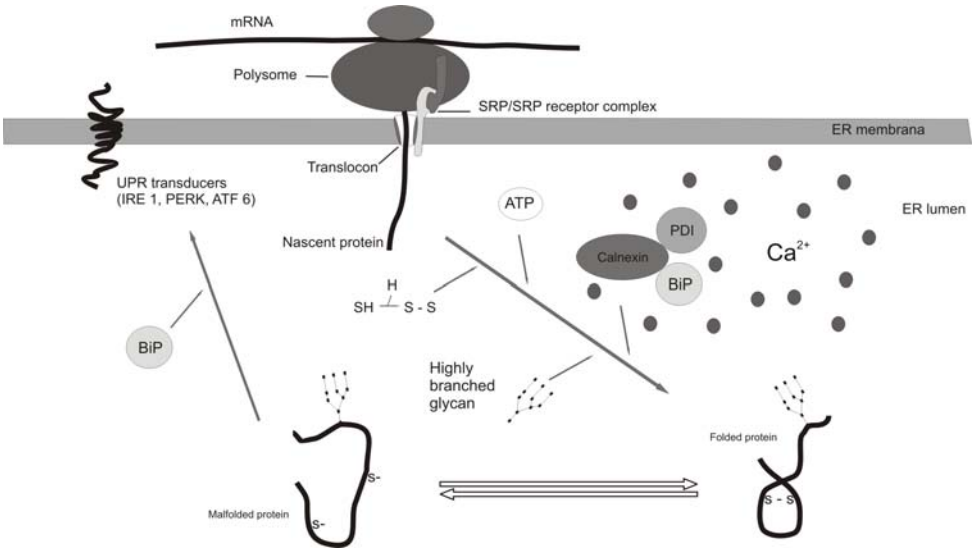

Fig. 1. Schematic representation of endoplasmic reticulum functions. Proteins are translocated into the endoplasmic reticulum lumen through proteinaceous channels in the ER membrane called translocons. In the environment of the ER lumen, resident chaperones like BiP, calnexin and protein disulphide isomerase (PDI) serve to facilitate the proper folding of the nascent protein by preventing aggregation, monitoring the processing of the highly branched glycans, and forming disulphide bonds. Changes in the ER environment shift the balance from normal to improper folding, leading to the accumulation of unfolded proteins in the ER. This activates ER-stress sensors (IRE1, PERK and ATF6), which initiate the unfolded protein response.

specific signalling pathways that have been termed (i) the UPR (unfolded protein response) and (ii) the EOR (ER overloaded response). There is substantial overlap in these responses, both in terms of the activating signals and the response [2, 3].
Recent observations indicate that the ER cooperates with other organelles that are important in the processes of apoptosis, like the mitochondria and the nucleus, and it is suggested that the ER also plays a role in autophagic pathways [4].

THE UNFOLDED PROTEIN RESPONSE (UPR)

The UPR is a signal transduction cascade which serves to limit the accumulation of unfolded proteins. It results in the reduction of general protein synthesis and selectively activates the expression of proteins facilitating chaperone activities. The primary effects of UPR activation are designed to protect the ER, but they also serve to limit damage to other organelles and protect the organism by eliminating cells experiencing prolonged stress. Under conditions of severe ER stress that cells are unable to adapt to, the UPR triggers apoptosis [3, 5].
The UPR is essential for a range of normal physiological and developmental processes. These include the regulation of insulin secretion by pancreatic islet β-cells [6], the differentiation of immunoglobulin-secreting plasma cells [7], B-cell differentiation [8], and possibly the proliferation of hepatocytes [9]. In these cases, activation of the UPR is essential to maintain homeostasis in the cell, and genetic disruption of the UPR can lead to diabetes, a block in plasma-cell differentiation, or hepatocellular death [3]. The UPR was first studied in yeast; it was later found that mammals have preserved the basic components of the yeast UPR and greatly expanded it.

ER stress and the survival response

In mammals, three ER transmembrane proteins, IRE1, ATF6 and PERK, respond to the accumulation of unfolded proteins in the lumen. They are normally kept in an inactive state through an association between their N-terminal luminal domains and the chaperone BiP (luminal binding protein). A number of experiments suggest that BiP negatively regulates the UPR. In both yeast and mammalian cells, overexpression of BiP down-regulates the UPR, and reduction of BiP levels is sufficient to induce the UPR [5].
BiP was identified as a member of a glucose-regulated protein family (GRPs) that is expressed at a high level in virally transformed cells and under conditions of glucose deprivation. BiP is present in the ER in monomeric and oligomeric states, and its oligomers are modified by phosphorylation and ADP [10, 11]. BiP interacts transiently with exposed hydrophobic areas on protein-folding intermediates, and is thought to prevent their aggregation while maintaining the protein in a folding-competent state. BiP interaction ensures that only properly folded and assembled proteins exit the ER compartment. Under conditions of ER stress, BiP dissociates (allowing binding to unfolded proteins), and IRE1 and

PERK undergo homo-oligomerization and stimulate trans-autophosphorylation within their serine/threonine kinase domains [5].

IRE1 (inositol-requiring gene-1) exists in two homologues; IRE1α and IRE1β [12, 13]. An activated IRE1 cytosolic endonuclease domain cleaves XBP1 (X-Box protein-1) mRNA. This act allows the synthesis of a highly active bZIP transcription factor that stimulates the transcription of ER chaperone genes [14-16]. The mRNA for IRE1α appears to be auto-regulated in that the endonuclease activity of IRE1α degrades its own mRNA. IRE1α was shown to colocalize with RanGAP1, a component of the nuclear pore complex, suggesting that IRE1 is an ER membrane protein preferentially localized to the nuclear envelope. Overexpression of wild-type IRE1α or β constitutively activates the UPR [12].

Activated PERK (PKR-like ER kinase) phosphorylates eIF-2α (eukaryotic translation initiation factor), resulting in an inhibition of general protein synthesis and G1 arrest [17, 18]. PERK is also required for NF-κB (nuclear factor-κB) activation, which positively regulates anti-apoptotic proteins like BCL-2 during ER stress, thus contributing to the balance between survival and death signals [19].

ATF6 (activating transcription factor 6) contains an ER luminal stress-sensing domain and a cytosolic domain that encodes a transcription factor. During ER stress, BiP dissociates from its luminal domain and allows ATF6 to traffic to the Golgi, where it is cleaved by two proteases, S1P and S2P [20]. The cytosolic transcription-factor domain is uncovered and migrates to the nucleus, where it transactivates downstream promotors. ATF6 induces XBP1 transcription, which is then spliced by the activated IRE1 endonuclease domain and stimulates ER chaperone gene transcription [21, 22] (Fig. 2).

The solid arrows represent the survival response, in which the specific transmembrane sensors ATF 6, IRE 1 and PERK (and PKR) are activated by dissociation of the chaperone BiP, caused by the accumulation of unfolded/misfolded proteins in the ER. ATF6 is proteolytically cleaved after the dissociation of BiP, and acts as a transcription factor in the nucleus, where it transactivates the transcription of XBP1, among others. The cytosolic endonuclease domain of IRE1 initiates XBP1 mRNA splicing into an activated transcription factor, bZIP. PERK and PKR are protein kinases promoting the phosphorylation of eukaryotic initiation factor 2 (eIF-2), which suppresses ER protein synthesis, decreases delivery of newly formed proteins to the ER lumen, and attenuates the unfolding stress, thus preserving nutrients and energy. PERK also activates the transcription factor NFκB. All three ER transmembrane receptors are thus responsible for initiating the expression of proteins that enable the cell to survive in stress conditions.

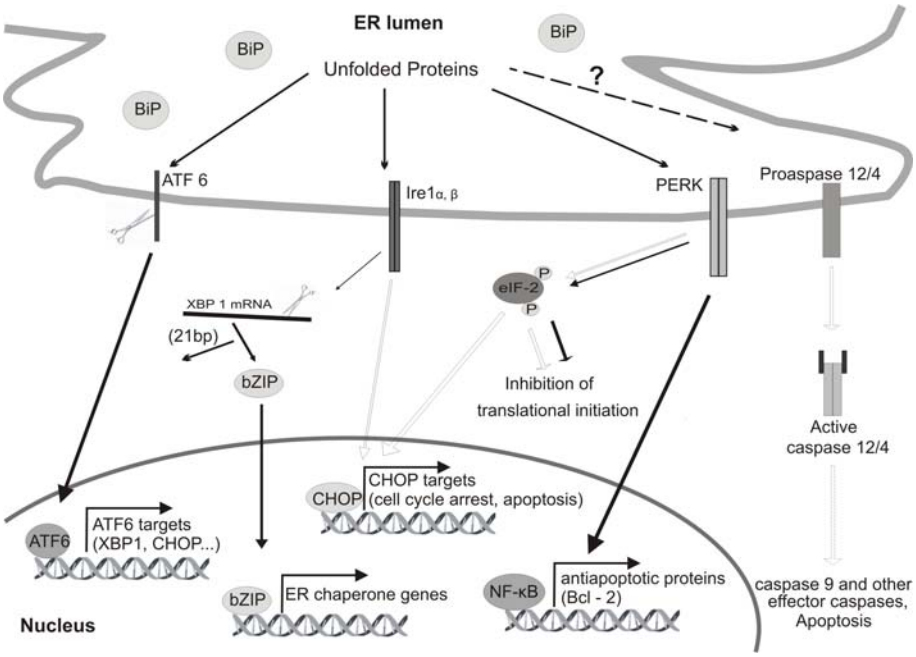

Fig. 2. Unfolded protein response pathways. When the conditions of ER stress persist, apoptotic pathways are activated (empty arrows). eIF-2 phosphorylation by PERK outbalances NFκB activation. All three signaling pathways (ATF 6, PERK and IRE 1) can initiate CHOP transcription. CHOP is a nuclear transcription factor that negatively regulates cell growth, leading to cell cycle arrest and apoptosis. Moreover, evidence has been presented for the involvement of caspase-12. A proposed model suggested that caspase-12 (probably represented by caspase-4 in humans) is activated by IRE1 and triggers the activation of a downstream cascade of effector caspases, but more recent data questions the role of caspase-12 in ER stress-dependent apoptosis (see text for details).

ER stress and the death response

When stress conditions persist, apoptotic signalling pathways are initiated and damaged cells are eliminated. During ER stress, three death-inducing signals are generated. The first triggers the transcriptional induction of CHOP/GADD153, the second is mediated through phosphorylation of the translation initiation factor eIF-2, and the third involves activation of caspases.

ER stress and CHOP/GADD153

CHOP was identified as a member of the C/EBP (CCAAT/enhancer-binding proteins). It serves as a dominant negative inhibitor of the C/EBP, and is also known as a gene induced on growth arrest and DNA damage (GADD 153) [23, 24]. CHOP is a nuclear transcription factor that represses the BCL-2 promotor

and may sensitize mitochondria to the proapoptotic effects of BH3-only proteins. Overexpression of CHOP can lead to cell cycle arrest and apoptosis [25, 26].
Expression of CHOP is mainly regulated at the transcriptional level, and it is one of the most highly induced genes during ER stress. For maximal induction of CHOP, all three ER stress-signalling pathways are required, but the PERK/eIF2α signalling pathway plays an essential role and is dominant over the ATF6 and Ire1/XBP-1 pathways [6, 27, 28]. CHOP is also regulated at the post-translational level by MAP kinase phosphorylation, which enhances transcriptional activation and elicits a maximal apoptotic effect of CHOP [29].
Overexpression of CHOP leads to cell cycle arrest, elevated BiP attenuates the induction of CHOP in ER stress, and $CHOP^{-/-}$ mice exhibit reduced levels of apoptosis in response to ER stress. Thus, it is supposed that CHOP plays an important role in ER stress-induced apoptosis [30, 31].

ER stress-signalling through eIF-2α kinases
In addition to changes in the transcriptional machinery, the immediate response to the accumulation of unfolded proteins in the ER occurs at the translational level with the inhibition of translational initiation, thereby protecting cells from further accumulation of unfolded proteins, as well as preserving nutrients and energy. The most frequently used mechanisms for translation control are the reversible phosphorylation of eIF-4E and eIF-2 [1]. The original observations demonstrated that treating cells with agents that disrupt ER function leads to protein synthesis inhibition, which correlates with increased eIF-2α phosphorylation. Small increases in the phosphorylation of eIF-2α immediately inhibit additional initiation events [32]. The status of eIF-2α phosphorylation is controlled by several Ser/Thr protein kinases that primarily recognize eIF-2α as a substrate. Four specific eIF-2α kinases have been identified: HRI (hemin-regulated inhibitor of protein synthesis), GCN2 (general control of amino acid biosynthesis kinase), PKR (interferon-inducible, dsRNA-activated protein kinase), and PEK/PERK [17, 18]. Although all known eIF-2α kinases are activated by different cellular stress conditions, current evidence indicates that PERK and possibly PKR respond to stress in the ER [17].
PKR is the interferon-inducible, dsRNA-activated protein kinase that is ubiquitously expressed in all mammalian cells [33]. The transcription of PKR is induced upon treatment with interferon α and β [34]. PKR displays two well-known activities: autophosphorylation, and phosphorylation of its physiological substrate, the α subunit of eIF-2. As a consequence of PKR activation, viral replication and cell growth are inhibited and cells may undergo differentiation. Increasing evidence supports an additional role for PKR in the transcriptional regulation of dsRNA-activated genes such as interferon β through activation of IRF-1 (interferon regulatory factor 1) and NF-κB [35, 36]. PKR is associated with rough ER membranes, and this localization suggested that PKR might signal in response to ER stress [37-39].

Activation of caspases

Caspase-12 is located in the ER membrane and participates in apoptosis under ER stress. After the initiation of apoptotic pathways, procaspase-12 is cleaved and becomes activated by an unknown mechanism. In mice, procaspase-12 is localized to the cytosolic face of the ER membrane, placing it in a position to respond to ER stress as a proximal signalling molecule [40]. Fischer *et al.* [41] reported that the human caspase-12 gene has acquired deleterious mutations that prevent the expression of a functional protein. Despite these discoveries, two reports showed that antibodies against murine caspase-12 detect an appropriately sized protein in human cells that is processed following ER stress. Caspase-12 is remarkably specific to factors that elicit ER stress, and is not proteolytically activated by other death stimuli. Recent data indicates that in humans, there is perhaps a caspase-4 that is homologous to murine caspase-12, and that it is activated in an ER stress-specific manner [42].

It is suggested that caspase-12 activation is linked to IRE1 signalling. The cytosolic tail of IRE1 can recruit TRAF2 [43], and when overexpressed, TRAF2 can interact with caspase-12 and weakly induce its oligomerization and cleavage [44]. Activated caspase-12 (lacking its protodomain) may directly process downstream caspases in the cytosol, or it may target other as-yet unidentified substrates that influence the progression of apoptosis. Two groups reported that caspase-12 can directly trigger caspase-9 activation and apoptosis independently of the mitochondrial cytochrome c/Apaf-1 pathway [45, 46]. However, the exact mechanism of caspase-12 function is still unknown. There is some contradictory evidence about its function in the latest literature. Several recent papers have confirmed that caspase-12 is activated in ER stress conditions in the caspase-$12^{-/-}$ mouse model, and in cortical neurons [47, 48]. On the other hand, using the same model, Saleh *et al.* [49] demonstrated the role of caspase-12 in modulating the inflammatory response by the inhibition of caspase-1. Moreover, they argued against an apoptotic caspase-12 function, proposing that caspase-11 rather than caspase-12 might have a role in ER stress-induced apoptosis in some settings [50].

THE ER OVERLOADED RESPONSE (EOR)

Very similar signals to those induced by the UPR are triggered during viral infection when viral glycoproteins are produced in massive amounts. The ER then sends a signal to activate the transcription factor NF-κB to induce expression of inflammatory genes and immune response genes, such as interferons and cytokines. This signalling pathway was termed the ER overloaded response (EOR), because it is activated by the accumulation of membrane proteins in the ER [51, 52]. A number of stimuli that disrupt protein folding, such as 2-deoxyglucose, tunicamycin and calcium ionophores, activate both the UPR and the EOR [1].

THE ER AND OTHER ORGANELLES INVOLVED IN APOPTOSIS

It is no exaggeration to say that cells can die by a thousand deaths, as current knowledge on cell death pathways shows. A pattern emerges, however, that each major cellular structure can initiate its own set of unique signals to induce apoptosis. These signals are often associated with specific damage or perturbation to the organelle involved. Although the ER can trigger unique apoptotic pathways as a reaction to different stress signals, there is evidence that the ER can also cooperate with other apoptotic pathways [4].

The ER and mitochondria

The BCL-2 family and the ER

Members of the BCL-2 family are also located on the ER, and several lines of evidence suggest that mitochondria are important components of the ER stress-induced apoptotic pathway.

1. ER stress agents cause mitochondrial release of cytochrome c and loss of mitochondrial transmembrane potential [53, 54].
2. BCL-2/BCL-XL inhibit ER stress-induced apoptosis [53, 55, 56].
3. *Bax-/-* and *Bak-/-* MEFs (mouse embryonic fibroblasts) are resistant to tunicamycin (which inhibits N-linked glycosylation), thapsigargin (which disrupts ER Ca^{2+} stores) and brefeldin A (which blocks ER to Golgi transport), three pharmacological agents that can induce ER stress [57].

The ER is the main intracellular storage compartment for Ca^{2+}, which is an important secondary messenger that is required for numerous cellular functions. Apoptosis occurs upon the perturbation of cellular Ca^{2+} homeostasis, such as cytosolic Ca^{2+} overload, ER Ca^{2+} depletion, and mitochondrial Ca^{2+} increase. The close physical contact of mitochondria and the ER results in the mitochondria being exposed to more Ca^{2+} than the rest of cytosol when Ca^{2+} is released from the ER. BAX and BAK localized to the ER can directly affect ER Ca^{2+} stores [58].

When a cell is committed to apoptosis, BAX and BAK undergo conformational changes, leading to their oligomerization and localization in the mitochondrial outer membrane [59]. This process is followed by the release of cytochrome c from the mitochondria to the cytosol. Cytochrome c, once released into the cytosol, interacts with APAF-1, leading to the activation of caspase-9 proenzymes. Active caspase-9 then activates the rest of the caspase cascade [57, 60, 61]. ER stress probably induces conformational changes and oligomerization of BAX and BAK on the ER membrane, which may lead to damage of ER membrane integrity. Expression of BAK in the ER results in ER Ca^{2+} depletion and ultimately cell death. These findings indicate that, in addition to their roles in the mitochondria, BAX/BAK proteins may also be involved in initiating apoptosis from the ER [61].

The anti-apoptotic properties of BCL-2 and BCL-X_L have been attributed to their ability to antagonize BAX/BAK by forming heterodimers that prevent their oligomerization and apoptosis initiation. Bcl-2 expression also regulates

intracellular Ca^{2+} homeostasis and thus contributes to the protective role of this oncogene against programmed cell death [62]. At normal physiological levels, Ca^{2+} released from the ER during cell activation is taken up by mitochondria to stimulate oxidative phosphorylation and enhance ATP production. Sustained and complete release of ER Ca^{2+} can initiate Ca^{2+}-dependent forms of apoptosis by triggering the opening of the mitochondrial permeability transition pore.

Foyouzi-Youssefi *et al.* [63] showed that Bcl-2 decreases the free Ca^{2+} concentration within the ER lumen by increasing the Ca^{2+} permeability of the ER membrane, and thereby protects cells from apoptotic stimuli. Conversely, Bax enhances the loading of the ER Ca^{2+} store and thus boosts the Ca^{2+} load to which the apoptotic effector systems (including the mitochondria) are exposed upon physiological and/or pathological challenges. This effect of Bax coincides with gross perturbation of mitochondrial structure and function and finally results in apoptotic progression [64].

In mitochondria-initiated apoptosis, BAX and BAK activation involves members of the BCL-2 family named BH3-only proteins. The presence of several BH3-only proteins, such as tBID, BIM, NOXA and PUMA, in the mitochondria when apoptosis is triggered suggests that their activation of BAX/BAK may take place at this site [65, 66]. Recent models suggest that the BH3-only subset of the proapoptotic BCL-2 family senses diverse death signals and initiates caspase activation [67]. BH3-only molecules may achieve this by binding and inhibiting anti-apoptotic BCL-2 family members or by directly activating pro-apoptotic BAX and BAK. Most BH3-only proteins localized to the mitochondria trigger cytochrome c release, which in all cases is inhibited by BCL-2 overexpression [68].

It is possible that some of the BH3-only, such as BIK/NBK, are localized to the ER and trigger BAX and BAK activation at the ER. BIK mRNA and protein are induced by p53 in response to DNA damage or oncogenic stress, and induce cytochrome c release independent of an association with the mitochondria or zVAD-sensitive caspases. BIK activates factors in both the ER and cytosol to induce mitochondrial transformations [69]. This activity of BIK is influenced by BCL-2. High BIK:BCL-2 ratios change the set of proteins that BCL-2 interacts with at the ER, resulting in Ca^{2+} release, cytochrome c release from the mitochondria, and apoptosis. ER-localized BCL-2 is able to protect against BIK-induced apoptosis [5].

ER stress-induced cytochrome c release is apparently dependent on the c-ABL tyrosine kinase. c-ABL is relocalized from the ER to the mitochondria, which parallels an increase in its kinase activity. c-ABL may function with JNK kinases, which are recruited and activated by IRE1 during ER stress, and are essential for mediating cytochrome c release in other cell death pathways [43, 70].

Therefore, stress in the ER evokes either mitochondrial-dependent apoptosis or mitochondrial-independent pathways that include activation of relevant caspases. These two arms of the ER stress response apparently operate independently of each other.

Caspase substrates located at the ER

Several ER proteins have now been identified as caspase substrates. The majority seem to be caspase-3 targets; therefore, their cleavage likely contributes to the coordinated shutdown of normal cellular processes during the execution phase of apoptosis.

They are:

- BAP31 (B-cell receptor-associated protein 31), which has been suggested as a possible caspase-8 substrate. Breckenridge *et al.* reported that BAP31 regulates ER-mediated apoptosis through the promotion of membrane fragmentation and cytochrome c release from the mitochondria [71-73].
- SREBPs (sterol-regulatory element-binding proteins). Caspase-3 cleavage of SREBPs upregulates sterol response genes, which may affect lipid rearrangements and/or morphological changes of the plasma membrane during apoptosis [74].
- SRP 72 (72-kDa component of SRP (signal recognition particle)), which is thought to help translocation of polypeptides across the ER membrane. Caspase cleavage of SRP 72 might shut down or alter the translation of secretory proteins during apoptosis [75, 76].
- IP_3R-1, -2 and -3 (inositol 1,4,5-trisphosphate receptors 1, 2 and 3). IP_3R1 and 2 are cleaved by caspase-3 and IP_3R-3 undergoes calpain proteolysis during apoptosis, which removes IP_3R-induced Ca^{2+} flow. Caspase-mediated proteolysis might represent functional down-regulation of this channel during the execution phase of apoptosis. Similarly, calpain proteolysis of the Ca^{2+}-binding ER chaperone GRP94 may also affect Ca^{2+} signalling during apoptosis [77, 78].
- PS1 and 2 (presenilin-1 and -2), which are highly homologous integral membrane proteins of the ER and Golgi, and which are mutated in most aggressive early-onset forms of familial Alzheimer's disease (AD). Caspase cleavage of presenilins creates an apoptotic amplification cycle, accelerating cell death in the neurons of AD patients [79].

p53 and stress in the ER

ER stress was found to inhibit p53. Qu *et al.* [80] demonstrated that ER stress, induced by ER-specific protein folding inhibitors such as thapsigargin and tunicamycin, or by glucose starvation, inhibits the ability of p53 to induce apoptosis triggered from the nucleus in response to DNA damage. ER stress targets wild-type p53 into the cytoplasm, where it is degraded, providing a mechanistic basis for the inhibition of the apoptotic activity of p53 by ER stress. A pathway by which ER stress modulates p53 intracellular localisation and function involves GSK3β (glycogen synthetase kinase-3β), which can phosphorylate p53 on Ser 376 and 315. In *GSK3β -/-* cells, p53 did not translocate to the cytoplasm in response to ER stress, and its apoptotic activity was not inhibited. The effect of ER stress on the p53 phosphorylation of Ser 376 is opposite to that of the DNA damage checkpoint, which facilitates

dephosphorylation and activation of p53 function. In the absence of ER stress, wild-type p53 localized predominantly in the nucleus. Thus, Ser 315 and Ser 376 phosphorylation appear to dictate the intracellular localization of p53 only in cells with ER stress, implying that some other post-translational modification is required. It is concluded that inactivation of p53 is a protective mechanism utilized by cells to adapt to ER stress [81, 82].

A further link between the ER, p53 and apoptosis proceeds from the recent identification of a novel p53-regulated ER membrane protein named scotin. Further studies showed that scotin is conserved between mouse and human and is detected in the ER and in the nuclear envelope, but not in the Golgi apparatus or the mitochondria. Scotin is directly transactivated by p53 in response to stresses that evoke apoptosis [83]. It would be interesting to know whether scotin acts downstream of p53 in a transcription-dependent or independent manner. Another question is how scotin activates caspases, because the predicted protein does not contain a specific domain crucial for the activation of caspases. In addition, the inhibition of endogenous scotin protein expression reduced the p53-dependent apoptosis induced by UV irradiation strongly, but not completely, suggesting that other proapoptotic pathways were activated. It also appears that the human scotin gene has several splice variants that are selectively expressed in response to different stress conditions, or occur in neoplastic human tissues, suggesting specific roles in cancer development (author's unpublished data).

CONCLUSION

Although it is well known that ER stress can lead to apoptosis, very little is known about the mediators involved in ER-controlled apoptosis. It is clear that the ER has its own complement of apoptotic accessories that independently activate caspases and mitochondrial dysfunction. A better understanding of the ER in apoptosis may come after the clarification of BCL-2, scotin and p53 function at this location. Since deregulation of BCL-2 family members at the ER affects cell survival outcomes, an understanding of their functions at this site could be important for developing new therapeutics for treating diseases such as cancer.

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Příloha 2

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REVIEW

Emerging roles for the pro-oncogenic anterior gradient-2 in cancer development

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Clinical studies have defined the core 'genetic blueprint' of a cancer cell, but this information does not necessarily predict the cancer phenotype. Signalling hubs that mediate such phenotype have been identified largely using OMICS platforms that measure dynamic molecular changes within the cancer cell landscape. The pro-oncogenic protein anterior gradient 2 (AGR2) is a case in point; AGR2 has been shown using a range of expression platforms to be involved in asthma, inflammatory bowel disease, cell transformation, cancer drug resistance and metastatic growth. AGR2 protein is also highly overexpressed in a diverse range of human cancers and can be secreted and detected in extracellular fluids, thus representing a compelling pro-oncogenic signalling intermediate in human cancer. AGR2 belongs to the protein disulphide isomerase family with all the key features of an endoplasmic reticulum-resident protein—this gives clues into how it might function as an oncoprotein through the regulation of protein folding, maturation and secretion that can drive metastatic cell growth. In this review, we will describe the known aspects of AGR2 molecular biology, including gene structure and regulation, emerging protein interaction networks and how its subcellular localization mediates its biological functions. We will finally review the cases of AGR2 expression in human cancers, the pathophysiological consequences of AGR2 overexpression, its potential role as a tumour biomarker that predicts the response to therapy and how the AGR2 pathway might form the basis for drug discovery programmes aimed at targeting protein folding/maturation pathways that mediate secretion and metastasis.

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INTRODUCTION

In recent years, an increasing number of reports have pointed towards intracellular organelles as playing major roles in cancer development and progression. This is particularly true for the endoplasmic reticulum (ER), which has appeared as a major player in early carcinogenesis and in tumour growth.[1,2] The ER is the first compartment of the secretory pathway controlling the cell's calcium fluxes, lipid homeostasis and the biogenesis of transmembrane and secretory proteins.[3] The latter is achieved through the coordinated action of several molecular machines, including protein synthesis and translocation, protein folding, export, degradation and ER stress signalling (Figure 1). Genes/proteins that constitute these molecular machines have been shown to significantly contribute to the development and progression of cancers.[2,4] This is also true for oxidative protein folding components, which are altered in various cancers, thereby leading to cell damages and altered protein secretion that contributes to adaptation to the local tumour micro-environment, tissue remodelling and metastatic cell growth. Major actors in oxidative protein folding comprise the family of protein disulphide isomerases (PDIs; Benham[5]), which is characterized by the presence of the thioredoxin motif CXXC. The roles played by PDIs in cancer remain unclear. Although few somatic mutations were found in those genes,[6] changes in their expression landscape were identified from proteomics screens. Recently, PDI-like proteins have been identified that present incomplete or evolutionarily divergent CXXC motifs,[7] but their molecular functions remain enigmatic. Anterior gradient 2 belongs to this category (AGR2). In the present review, we provide an in-depth description of AGR2 in the context of the AGR family, the previously reported functions of this protein and how the AGR2 pathway might be targeted to develop new therapeutic strategies to inhibit cancer growth.

THE AGR GENE FAMILY

AGR2

The AGR family is composed of three proteins, including AGR1, AGR2 and AGR3. *AGR2* gene was first identified in *Xenopus laevis*. *Xenopus* anterior gradient-1 and -2 (XAG-1 and XAG-2) comprise one class of cement gland-specific genes based on their spatial expression patterns.[8,9] Studies showed that XAG-2 function in specifying the fate of the dorso-anterior ectoderm that forms the cement gland and in inducing the forebrain's fate. During early development, XAG-1 and XAG-2 are expressed within ectoderm-derived organs like the cement gland, which is a mucus-secreting organ located at the extreme anterior end of the frog's head.[10] The cement gland forms a cone of columnar epithelium and is

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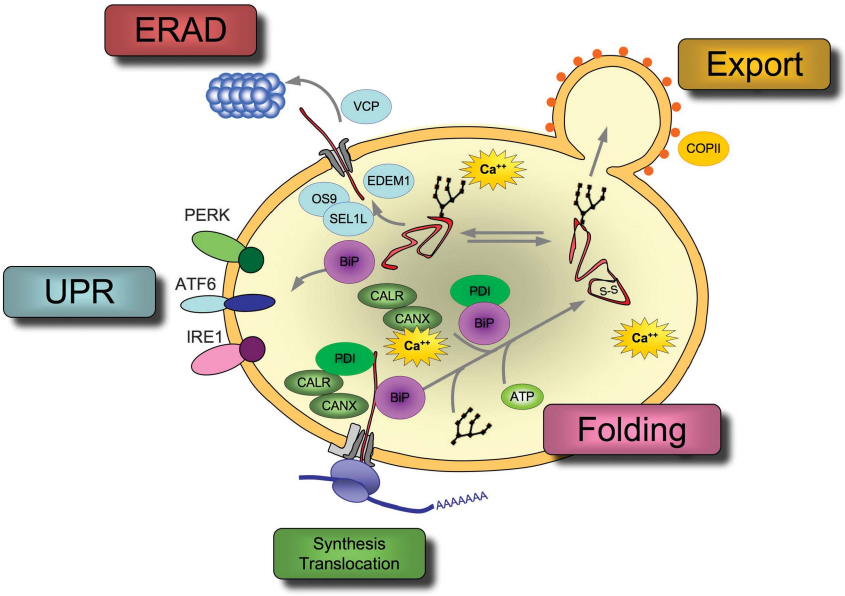

Figure 1. ER molecular machines that control protein homeostasis. Five major molecular machines, namely translation/translocation (green), folding (pink), export (yellow), degradation (ER-associated degradation, red) and ER stress signalling (blue) control ER protein homeostasis.[1]

involved in the attachment of the frog embryo to a solid support.[11] In mouse embryos, *XAG-2* homologous gene (Gob4/MAgr2) was also identified as a highly expressed mRNA biomarker of murine gut.[12] Mouse AGR2 is localized in mucus-secreting cells in these tissues by *in situ* hybridization. The majority of proteomics or transcriptomics screens identified AGR2 as a dominant outlier and hence most research was focused on validating this protein; however, the other homologues like AGR3 or ERp29 are beginning to be identified in other systems.

AGR3

AGR3 was first identified in a proteomic analysis of purified membrane preparations from multiple human breast tumour-derived cell lines in efforts to identify oncogenic membrane signalling proteins. Adam and co-workers[13–15] identified a unique protein BCMP11 that was homologous to AGR2 and whose expression correlates with oestrogen receptor (ER) in human breast cancers. In view of their high degree of sequence identity (71%), BCMP11 was named AGR3. The function of AGR3 is not known, but AGR3 has since been identified to be expressed in ER-negative human ovarian cancers[16] and to mediate cisplatin resistance in tumour xenograft studies. In the latter study, it is interesting to note that many ovarian cancers that express AGR3 can be AGR2-negative, indicating that their expression can be uncoupled. The functions of the two genes might not be redundant, as AGR2 and AGR3 do not similarly colocalize to the ER[16] and AGR3 has generally not been identified in the many OMICS screens in human cancer, suggesting a more critical role for AGR2 in carcinogenesis. However, a recent study using exome sequencing to identify the mutant cancer gene landscape in cancer has indicated that the *AGR3* gene is mutated in approximately 10% of kidney tumours examined,[17] suggesting that AGR3 perturbation can also contribute to cancer progression.

AGR1

The third member of the AGR family was highlighted in a bioinformatics study[18] that identified AGR2 and AGR3 as highly similar to ERP18/19, also named AGR1, which could be considered the founding gene of the AGR family given its higher similarity to the classic thioredoxin fold containing the CXXC motifs and its presence in invertebrates.[19,20] The function of ERP18 involves its ability to form mixed disulphides with client proteins in the ER;[21] however, ERP18 has not been identified in OMICS screens in human cancer, thus its role in human cancer remains unknown.

Future studies on dissecting the role of the AGR family in biology and disease: genetic models that define CXXC/S motif protein function

A key missing methodology thus far in analysing the AGR-1/2/3 protein family is the lack of powerful animal models to dissect signalling *in vivo* using classic genetics. This is mainly due to the fact that, although the three genes belong to the same family (Figure 2), the *AGR2* and *AGR3* genes emerged during the evolution of chordates and thus far appear confined to vertebrates, whereas AGR1/ERP18 seems to be the founding member of this trio and is found within complex invertebrates, like *Caenorhabditis elegans*. There has been no expansion of the *AGR2* and *AGR3* genes as their appearance in vertebrates, suggests that strict selection pressures exist in maintaining essential functions during evolution by natural selection. Similar selection pressures have apparently maintained the closest orthologues of the AGR2 family, TRX1 and TRX2 (Figure 2). Other non-catalytic PDIs have also been identified as ERp44 (PDIA10) and ERp29, 27 (PDIA9, PDIA8), and as reported for the AGR family their functions remain poorly understood.[7,22,23] Although these proteins can form mixed disulphide bonds with their clients, their functions in the control of cell's secretome still remains equivocal. One research avenue to shed light on the fundamental role of the CXXC/S PDI family would be to use animal models such as *C. elegans* to dissect genetically protein folding pathways. For example, if conditional or constitutive phenotypes could be identified for an AGR1/ERP18 allele in *C. elegans*, then suppressor screens could be initiated to define genetic pathways important to overcome such bottlenecks. In addition, in vertebrates the *AGR2* and *AGR3* gene functions could be similarly dissected using the powerful gene knock-in TALEN technologies in zebrafish and in murine systems to evaluate specific motif mutations on biological signalling *in vivo*. The development of genetic models in vertebrates, for example,

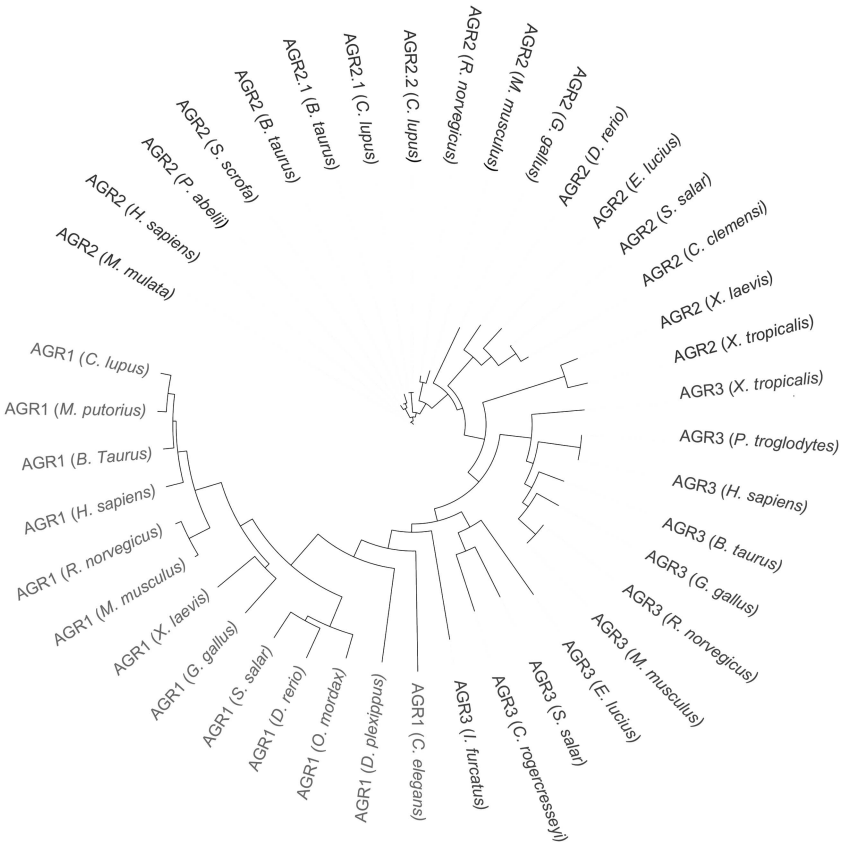

Figure 2. The AGR family tree. Protein sequences from 41 AGR family members were aligned using ClustalW and the corresponding matrix represented as a tree using the iTOL website (http://itol.embl.de/). Blue: AGR2; green: AGR3; red: AGR1/ERP18. A full colour version of this figure is available at the *Oncogene* journal online.

creating a CXXS to CXXC (or SXXS) allele conversion of AGR2, would add significant understanding of fundamental aspect in the CXXC/S family.

AGR2: GENE ARCHITECTURE AND EXPRESSION

Genomic structure

The detailed genomic structure for human AGR2 was determined on the basis of its mRNA sequences and genomic DNA clone by using the Spidey program.[24] The *AGR2* gene spans a region of 50 kb in genomic DNA. Two transcripts were also detected and both mRNA isoforms contained eight exons and seven introns. Both cDNA clones of the two mRNA isoforms were fully sequenced and were found to contain the same open-reading frame. The putative translation start site for AGR2 contained the KOZAK consensus sequence.[24] In their study, they reviewed the public databases and the Celera Discovery Systems to establish a gene model for AGR2, and annotated an additional 5′ exon, confirmed by reverse transcription–polymerase chain reaction in a tissue panel.[25] Moreover, using radiation hybrid analyses and FISH, Petek and co-workers[26] mapped the gene encoding human AGR2 to chromosome 7p21.3.

Developmental regulation of the AGR2 promoter

In situ hybridization and reverse transcription-polymerase chain reaction analyses revealed that *AGR2* mRNA is strongly expressed in endoderm-derived organs that contain mucus-secreting cells. Zheng and co-workers[25] also investigated the promoter activity of the human *AGR2* gene independent of oestrogen responsiveness. Using a luciferase reporter gene construct driven by the *AGR2* promoter, they observed that co-transfection with the forkhead box transcription factors FOXA1 and FOXA2, which have been implicated in maintaining goblet cell function, led to a significantly increased luciferase activity in HEK293 cells. In addition, Zheng and co-workers[25] described that there is a binding site for hepatic nuclear factor 1, which belongs to the same family as FOXA1 and FOXA2, in the *AGR2* promoter region at SNP 07AGRNP53. Furthermore, using a global chromatin immunoprecipitation approach in three tumour cell lines (MCF7, SW480 and Ntera2), Krig and co-workers[27] identified *AGR2* promoter as a binding site MCF7-specific to the oncogene ZNF217. Finally, using the UCSC genome database (http://genome.ucsc.edu), relevant NF-κB and SOX9 binding sites were found on the *AGR2* promoter.

AGR2 promoter regulation by oestrogen

Historically, using the technique of suppression subtractive hybridization as a forerunner to transcriptomics, the Weigel laboratory reported the identification of 29 gene fragments that were expressed in the ER-positive MCF7 breast carcinoma cell line that were absent or minimally expressed in the ER-negative MDA-MB-231 breast carcinoma cell line.[28] One of these gene fragments, DEME2 (GenBank EST accession no. AA506763), exhibited an expression pattern that correlated with the expression of ER in a panel of eight breast carcinoma cell lines. DEME-2 was named hAG-2 (for example, AGR2) based on sequence similarity. AGR2 was significantly upregulated in response to oestradiol. Indeed, transplantation of epithelium and stroma of normal breast tissue

into female nude mouse treated with oestradiol revealed significant upregulation of *AGR2* mRNA upon oestradiol treatment.[29]

Regulation of AGR2 promoter by androgens

Zhang and co-workers[24] have reported that *AGR2* mRNA is androgen inducible. This induction is time and dose dependent, with a maximum fold increase more than a 10-fold in the level of *AGR2* mRNA, and requires the presence of the androgen receptor. Also, this induction was completely abolished by cycloheximide, suggesting that newly synthesized proteins play a role in the induction process. In this study, they also determined the effects of various agents, including protein kinase inhibitors and activators, on *AGR2* induction.

Regulation of the AGR2 promoter by (patho)physiological stress conditions

When the breast cancer cell line MDA-MB-231 was subjected to serum depletion alone or combined with oxygen depletion to mimic conditions reflecting tumour cell-associated stress, Zweitzig and co-workers[30] found that *AGR2* mRNA is induced about fivefold. Specific inhibitors for ERK1/2, JNK, p38 and PI3K were tested in this model and only ERK1/2 inhibition was associated with a block of *AGR2* mRNA induction.[30] It was demonstrated that tunicamycin, an inhibitor of N-linked glycosylation, promoted the induction of *AGR2* mRNA expression.[24] *AGR2* mRNA was indeed induced upon ER stress using various inducers such as tunicamycin, the reducing agent dithiothreitol or the sarcoplasmic/endoplasmic reticulum calcium pump inhibitor thapsigargin.[31] Moreover, ER-stress-mediated upregulation of *AGR2* mRNA mainly resulted from activation of the ATF6 and IRE1 arms of the unfolded protein response. This was demonstrated using small interfering RNA-mediated silencing of PERK, ATF6 or IRE1 and measuring *AGR2* mRNA expression upon ER stress, thereby suggesting the presence of an ERSE-like element on its promoter.[31] Thus far, the role of ATF6 and IRE1 was not investigated on *AGR1* or *AGR3* mRNA expression.

Future research areas on AGR family gene expression: biochemical dissection of the AGR transcriptional machine

Although clinical studies have shown that AGR2 and AGR3 are expressed *in vivo* within human cancers by both hormone-dependent and hormone-independent pathways, we know very little about the architecture of the dynamic multi-protein complexes that drive these promoters. This is a very compelling area of research, as AGR2 is induced by the anti-oestrogen Tamoxifen and thus provides a mechanism to account for induced or acquired resistance to this drug.[32] AGR2 is the only compelling Tamoxifen-induced gene with a pedigree in diverse biological systems, including colitis, limb regeneration, metastatic growth and p53 inhibition. Thus, identifying the components that co-ordinate the Tamoxifen signal provide potential targets for sensitizing AGR2-positive Tamoxifen-resistant cancers. A more fundamental question also remains provocative—as the *AGR2* and *AGR3* genes are contiguous on the same chromosome, but are often expressed in a mutually exclusive manner;[16] what signalling pathways and multi-protein complex assemblies mediate this selective promoter usage *in vivo*?

CORE BIOCHEMICAL FUNCTIONS OF AGR2

The ER landscape of AGR2: intracellular biochemical function within the ER

One of the most conspicuous motifs in AGR2 is the thioredoxin fold that was initially identified by Persson and co-workers.[18] Although the thioredoxin activity of AGR2 has never been demonstrated either *in vitro* or *in vivo*, the potential implication of this motif in AGR2 biology has been suggested in some instances.[16,33] It is curious that the apparent 'founding' gene in this family (ERP18) contains the classic 'thioredoxin fold (W-CXXC-K)', but has not been an outlier OMICS biomarker in the many screens reviewed in this article. Rather, AGR2 contains the divergent thioredoxin fold E/D-CXXS-Q that has been implicated in disease development. Thus, the role of the CXXS-containing PDIs in vertebrate biology will likely be a key focus in understanding the protein folding function of this family and how this effects disease progression. In the case of ERP18, synthetic mutants containing a mutation of the CXXC motif to CXXS resulting in the covalent trapping of dithiothreitol-sensitive covalent intermediates[21] and similar approaches might be used to trap physiological substrates in other CXXS-containing PDIs. AGR2 contains putative CXXS TX-domain motifs also found in two other PDIs/ERps, Eug1p and ERP44.[22,34]

Functional aspects for the CXXS motif proteins have been suggested, such as interactions with intermediates in redox reactions during folding[22] and retrograde transport in the secretory pathway.[35] The CXXS motif possesses lower activity associated with disulphide bond reorganization, but may, however, contribute to isomerization of already existing disulphide bridges and possibly perform specialized functions in the ER.[35,36] A cysteine residue within this domain forms mixed disulphide bonds with mucin-2, indicating a direct role for AGR2 in mucin processing.[33] Mice lacking AGR2 were viable, but were highly susceptible to colitis, indicating a critical role for AGR2 in protection from disease.[33] Another study reported that AGR2$^{-/-}$ intestine has also decreased goblet cell mucin-2, dramatic expansion of the Paneth cell compartment, abnormal Paneth cell localization, elevated ER stress, severe terminal ileitis and colitis.[37] Another study also highlighted the ability of allergens to induce AGR2 and mucin-5 secretion; this secretion of mucin-5AC/B is attenuated in AGR2$^{-/-}$ cells.[38] The role of AGR2 in the development of airway goblet cells was confirmed and was demonstrated to be under the control of the transcription factors Foxp1 and Foxp4, most likely to achieve mucus secretion.[39] Finally, ER functions of AGR2 were shown to contribute to this compartment's homeostasis through the signalling of the unfolded protein response[31] (Figure 5).

The intracellular and extracellular landscape of AGR2

The 'degenerate ER retrieval motif' of AGR2: AGR2 protein harbours a canonical cleavable signal sequence for targeting into the secretory pathway in addition to the thioredoxin fold, and a non-optimal ER retention motif at its C terminus (KTEL) (Figure 3). This is likely to have a significant impact on how AGR2 protein is trafficked to different compartments and functions in cells. In addition, the ability of AGR2 to mediate protein folding of pro-metastatic secretory molecules is also likely to be regulated by its residence time in the ER. Although the *Xenopus* homologue AGR2 protein (XAG-2) was shown to be secreted,[10] it appears that hAGR2 can be found in various cellular compartments, including the ER,[31] the nucleus,[40] the cell surface and the extracellular milieu.[41]The majority of PDIs/ERPs harbours a typical H/KDEL ER retrieval signal and localizes to the ER. Both AGR2 and AGR3 ER retrieval signals, and although atypical, were able to bind to KDEL

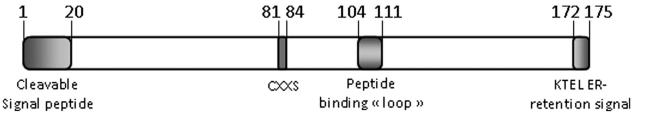

Figure 3. AGR2 protein. Primary structure of the AGR2 protein. The identified functional domains and amino acids implicated in regulation of its function are indicated by grey shaded boxes.

receptors to be retrieved in the ER.[42] It was also shown that AGR2 can escape the ER retrieval machinery and be secreted to play an autocrine/paracrine role.[41,43] Subcellular fractionation approaches revealed that AGR2 can also localize to the nucleus.[44]

Altogether, these observations lead to the speculation that the divergence of the highly conserved KTEL sequence of AGR2 from the canonical KDEL may lower the affinity of AGR2 to the KDEL receptor protein and allow AGR2 to exit the ER at a higher rate when overexpressed. Although mainly localised in the ER, AGR2 was reported to play other intracellular roles. For instance, AGR2 was shown to regulate p53 signalling,[45] induce the EGFR ligand amphiregulin (AREG)[46] and interact with the AAA$^+$ protein Reptin,[47] thereby suggesting regulatory functions in gene expression. This part of AGR2 biology remains to be further studied to understand (1) how AGR2 can localize to the cytosol and (2) what could be its true functions in this compartment (Figure 5). However, these results may help to explain the observations that show that AGR2's ability to induce the expression of either CDX2 (transcription factor) or AREG is lost when either the C terminus is deleted or mutated to KDEL.[40] Taken together, these data suggest that AGR2's tumour-promoting ability could be due to the combined action of its activity within the cell and that achieved as a secreted protein.

Because of the similarity between AGR2 with the XAG proteins, it may be speculated that AGR2 may be involved in the proliferation of mammalian tissues. Whereas AGR2 is currently not embedded in a known pathway, AGR2 can mediate metastasis in animal models. Indeed, when an expression vector for AGR2 cDNA was transfected into benign non-metastatic rat mammary tumour cells (Rama37), metastases occurred in the lungs of animals receiving the AGR2-transfected cells in 77–92% of animals with primary tumours compared with no metastases in the control groups. AGR2-transfected cells exhibited enhanced rates of adhesion to a plastic substratum.[48] This observation was recently substantiated by a study showing that AGR2 was a cell surface antigen-promoting tumour cell dissemination through the activation of cathepsins B and D.[41] The extracellular role of AGR2 has been clearly illustrated by nAG, a new member of the anterior gradient family of proteins that was shown to be important in the regeneration of amputated limbs in amphibians.[49] Artificial reintroduction of nAG by electroporation is sufficient to rescue regeneration in denervated salamander limbs. It is suggested that nAG is secreted by the nerve sheath cells and then acts directly on limb blasternal cells. In this study, a potential nAG-binding partner Prod1 was identified by yeast two hybrid, although the protein–protein interactions between these two proteins have not been validated in cell systems. This is a glycosylphosphatidylinisotol-anchored protein of the Ly6 superfamily as is the C4.4 protein identified in the yeast two-hybrid screen carried out against human AGR2 and that has been proposed to serve as AGR2 receptor,[49] although this has not been validated at the molecular level. The mechanisms whereby AGR2 can stimulate tissue regrowth might be due to its regulation of the adhesion and migration pathways required to remodel cells into a new environment. Altogether, evidences for AGR2 binding to cell surface proteins and for its presence in the extracellular milieu suggest a role for this protein outside the cell (Figure 5).

The molecular chaperone landscape of AGR2-emerging protein–protein interactions

The 'substrate-binding loop' of AGR2. Although AGR2 protein forms mixed disulphides with the mucin family and has a specific interaction with the AAA$^+$ protein Reptin, we are only beginning to learn about the functional motifs in the protein that regulate protein–protein interactions. The founder gene *AGR1* (ERP18 (PDB code 2K8V)) shows no evidence of being monomeric, while our unpublished data demonstrate that AGR2 forms homodimers in solution and in cells indicating that biochemical models of AGR2 function will likely incorporate a dimeric structure to protein–protein interactions. Yeast two-hybrid and peptide combinatorial libraries have been used to build on the AGR2 interactome. The yeast two-hybrid screen identified the listed proteins, as given in Table 1; only one of these has been well validated, namely the AAA$^+$ ATPase protein Reptin.[47] The AGR2:Reptin protein–protein interactions were validated using biochemical approaches and subsequently the interaction site for Reptin on AGR2 was fine-mapped to a peptide interface, comprising the proposed substrate-binding loop on AGR2, within amino acids F104 to Y111. Mutations in AGR2 at the junction codons of the loop at 104 and 111 attenuate Reptin binding to the protein.[47] Small peptides from the AGR2 substrate-binding loop are sufficient to reconstitute the ADP-responsive binding of Reptin (Maslon *et al.*[47] and unpublished data), thus identifying the first potential protein–protein interaction assay to develop drug leads for inhibiting the Reptin–AGR2 complex. Whether the substrate-binding loop is a dominant interaction site for the many AGR2-interacting proteins will be one key aim of future work on its mechanism of action and on developing possible strategies to therapeutically target this class of protein–protein interactions. Indeed, phosphosite plus annotated a phosphorylation site at Y111, suggesting that phosphorylation at this motif could regulate protein–protein interactions of AGR2.

Table 1. AGR2-interacting proteins identified in Y2H (acquired from *Hybrigenics*) or published co-immunoprecipitation studies

Gene name	*Function*	*Method*	*Reference*
ARHGAP29	GTPase activating protein (Rho)	Y2H	Unpublished data
CKAP2	Cytoskeletal-linked protein involved in mitosis	Y2H	Unpublished data
CHD6	Chromatin remodelling factor	Y2H	Unpublished data
DAG1	Links the cytoskeleton and the extracellular matrix	Y2H	Fletcher *et al.*[50]
GPSM2	Regulates G-protein activation	Y2H	Unpublished data
HECTD1	E3 ubiquitin ligase	Y2H	Unpublished data
HIVEP1	DNA binding protein	Y2H	Unpublished data
NRIP1	Binds hormone-dependent receptors	Y2H	Unpublished data
NRXN3	Controls adhesion and receptor signalling	Y2H	Unpublished data
RUVBL2	AAA$^+$ ATPase—DNA repair and transcription	Y2H	Maslon *et al.*[47]
LYPD3	Regulates cell migration	Y2H	Unpublished data
KDELR	KDEL receptors	CoIP	Raykhel *et al.*[42]
C4.4	Metastasis-associated GPI-anchored protein	Y2H	Fletcher *et al.*[50]
MUC2	Mucin 2	CoIP	Park *et al.*[33]
PROD1	Axotolt homolog for human CD59	Y2H	Kumar *et al.*[49]

Abbreviations: AGR2, anterior gradient 2; GPI, glycosylphosphatidylinisotol.

The 'consensus peptide-binding activity' of AGR2

Some molecular chaperones like heat-shock protein 70 can harbour canonical consensus binding motifs[51] that direct the class of interactions targeted by these proteins. There is no *a priori* reason as to why PDI/chaperones like AGR2 would have an intrinsic and specific peptide-binding function that directs its potential protein folding activity in the ER, other than its ability to interact with reduced and oxidized cysteines through its thioredoxin fold. However, when AGR2 was presented to a combinatorial peptide library, the synthetic AGR2 protein expressed in *Escherichia coli* exhibited an intrinsic affinity for peptides containing the pentapeptide consensus (T/S)–X–(I/V/M)–(Y/W/F)–(Y/W/F).[16] The minimal pentapeptide TXIYY was shown to bind specifically to the AGR2 in crude lysates and the site of interaction was later mapped to the same C-terminal region on AGR2 to which Reptin binds.[52] However, the structural basis for this specific protein–protein interaction remains unsolved and whether the peptide forms a 1:1 or 1:2 stoichiometry with the AGR2 dimer remains undefined.

An intrinsic peptide docking function of a target protein can also be exploited to develop synthetic 'peptide mimetics' to determine whether a protein is potentially 'drugable' *in vivo*. This approach has been most exploited with the MDM2 and MDM4 oncoproteins.[53–55] In the case of AGR2, synthetic EGFP–peptide fusion protein expression constructs transfected into cells can 'stabilize' AGR2 protein and stimulate the activity of the p53 tumour suppressor,[44] a pathway that is negatively regulated by AGR2.[45] Such peptides synergize with ultraviolet stress to induce the nuclear import of p53 and the synthesis of the consensus peptide-binding motif to the cell-membrane-permeable delivery sequence[45,56] similarly induced the nuclear translocation of p53. These data highlight the potential drugability of the AGR2 pathway with respect to stimulation of the p53 tumour suppressor.

Future research areas on protein structure and function in cells

Structure–function analyses of PDIs have largely focused on the role of the thioredoxin fold in mediating protein folding landscapes. However, whether these proteins have consensus docking sites on their cargo proteins and whether they might be drugable have not been addressed, except perhaps for the 2,4-dinitrochlorobenzene-mediated inhibition of TRX1.[57] Although various PDIs, like thioredoxins, have been implicated in radiation resistance or drug resistance, the interacting proteins are essentially undefined and the role of disulphide motifs not well understood in cell systems. This forms a compelling area of future work for AGR2: to exploit its functional domains (leader sequence, substrate-binding loop, dimeric nature, thioredoxin fold, ER retention site and TXIYY peptide consensus binding domain) to identify its global interactome using proteomic technologies and how these protein interactions towards specific motifs might direct AG2 to function in the ER, outwith the ER, and when shuttled extracellularly. In addition, the development of a panel of protein-interaction mutants in these same motifs would create a toolbox for this emerging field that can be used to develop goalposts for understanding AGR2 functions in a range of biological situations.

AGR2 EXPRESSION IN CANCERS

AGR2 exhibits the basic features of a pro-oncogenic protein

To evaluate the role of AGR2 in cancer (Figure 4), the over-expression and suppression of AGR2 in cancer cell lines has been used to determine whether it has growth-suppressive or pro-growth functions. Clonogenic assays were used to demonstrate that AGR2 can enhance cancer cell survival, rather than inhibit cell growth.[32] Deletion of the last 10 amino acids of AGR2, harbouring the ER retention site of AGR2, prevented clonogenic growth

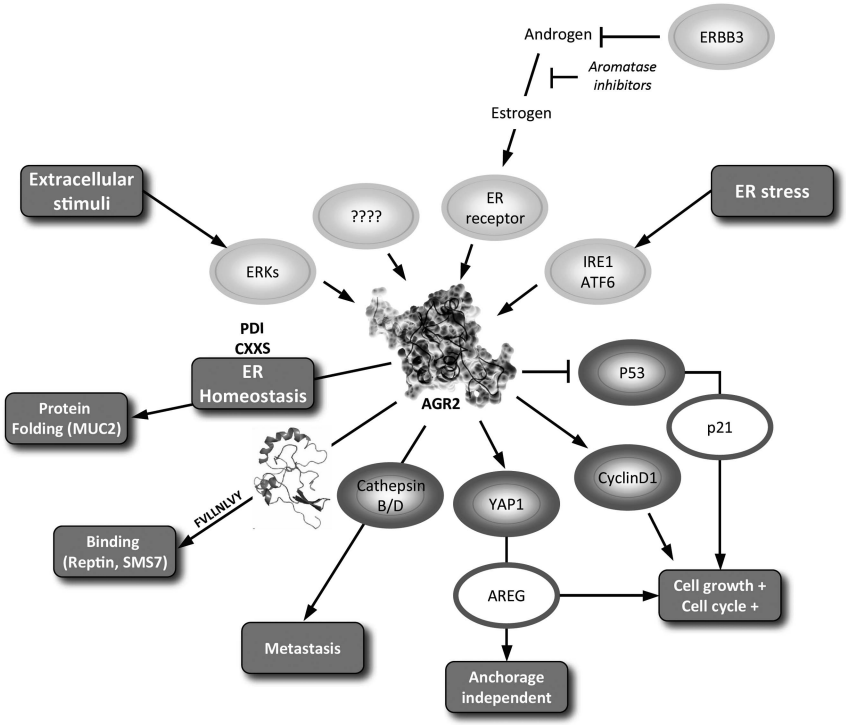

Figure 4. AGR2 biological pathways. AGR2 is presented as the central point of this picture (structure derived from ERP18[31]). Pathway intermediates known to regulate AGR2 expression are indicated in light grey ovals. AGR2-dependent functions are indicated in dark grey ovals. Potential (not experimentally demonstrated) intermediates are indicated as empty ovals. Physiological/pathophysiological inputs or outputs are represented as grey boxes.

stimulation, highlighting a role for this motif in AGR2 survival signalling. Further, AGR2 silencing was shown to inhibit proliferation, invasion and survival *in vitro* in pancreatic[43] and breast cancer cell lines.[58] AGR2 is also upstream of and stimulates key cancer-signalling pathways, such as cyclin D1, c-Myc, p-Src and survivin,[58] based on using both short interfering RNA and ectopic expression to manipulate AGR2 levels. Moreover, conditioned media from cells silenced in AGR2 have a reduced ability to stimulate proliferation of pancreatic cancer cells.[43] AGR2 has also been described as an oncogene[59] supporting a role in cellular transformation and adenocarcinoma growth. In the premalignant Barrett oesophagus and oesophageal cancer models, AGR2 overexpression induces colony formation and transformation.[45,59] Conversely, short interfering RNA- or short hairpin RNA-mediated AGR2 knockdown inhibits colony and subcutaneous growth in oesophageal and pancreatic cancer models.[43,59]

AGR2 as a mediator of Tamoxifen drug resistance in human breast cancers

As discussed above, the *AGR2* promoter responds to oestrogen in normal tissue as well as some cancer cell lines. Coincidentally, AGR2 protein is overexpressed in ER-positive human cancers. The anti-oestrogen Tamoxifen can be used successfully to treat human breast cancers; however, intrinsic resistance and/or acquired resistance remains a significant problem. It has been suggested that some genes paradoxically act as agonists of Tamoxifen and might mediate intrinsic resistance. As such, identification of oestrogen-responsive genes induced by Tamoxifen, yet play a survival role, has been one key aim in the field of drug resistance.[60] Three nearly simultaneous observations pointed towards AGR2 as being one of these elusive genes that might mediate the resistance of breast cancer to Tamoxifen. Firstly, was the observation that in cancer patients treated with the oestrogen suppressor Letrazole, the *AGR2* gene (not *AGR3*) was one of the top suppressed genes in biopsies from patients post-treatment that respond well to the drug, suggesting that resistance to anti-oestrogens might be due to failure to suppress *AGR2*.[32] Second, in a 'deep' proteomics screen using the label-free approach PaCIFIC to search for dominant proteins induced by Tamoxifen, AGR2 was the second most induced protein,[61] and xenograft studies demonstrated that the *AG2* gene can mediate cisplatin resistance.[61] Third, an analysis of *AGR2* gene expression in relation to prognostic markers in ER-positive breast cancers indicated that high *AGR2* expression was linked significantly to poor prognosis.[32]

At the molecular level, it was also demonstrated that this induction of AGR2 by Tamoxifen was a direct effect of ERα activation using chromatin immunoprecipitation. Elevated ERα was bound to the *AGR2* promoter in the presence of oestradiol or Tamoxifen and the *AGR2* promoter (-175 to $+35$) was activated in a luciferase reporter assay in the presence of oestradiol or Tamoxifen.[32] Understanding the molecular details of why Tamoxifen, selectively, induces AGR2 expression while acting as an anti-oestrogen for the majority of oestrogen-responsive genes might identify novel drug targets that sensitize Tamoxifen-resistant/AGR2-positive cancers.

A dominant role for AGR2 in oesophageal cancer progression

Oesophageal cancer progression is one of the minority of cancer types known to evolve through progressive changes in tissue architecture, from metaplasia to dysplasia, and adenocarcinoma. Oesophageal adenocarcinoma differs from other cancer types in that the environmental stress of bile acid reflux plays an apparently important role in disease progression and that p53 tumour suppressor gene mutation can occur very early in the carcinogenic sequence.[62] The early selection pressure for p53 mutation in oesophageal cancer occurs during the replacement of squamous epithelium with metaplastic epithelium, also called 'Barrett's oesophagus'. To identify potentially novel p53 inhibitors in this cancer 'intermediate', a clinical proteomics screen had been set up in a proliferative disease (Barrett's epithelium) and identified AGR2, which was validated as a potent inhibitor of p53-dependent transcription and a growth-promoting proto-oncogene.[45] Subsequently, it was shown that overexpression of AGR2 is maintained in oeosphageal cancer tissue, as the large majority of adenocarcinomas express AGR2 as defined using immunohistochemical methods.[46] *AGR2* mRNA expression is also a dominant feature of a recently identified murine model of Barrett's oesophageal epithelium induced through deletion of the squamous stem cell progenitor p63.[63] As oesophageal cancers are presumably oestrogen-independent, developing therapeutic strategies to inhibit the AGR2 pathway (if not the PDI machine) might prove to be different than those used to inhibit its Tamoxifen-resistance activity in breast cancers.

Uncoupling of AGR2 and AGR3 expression in human ovarian cancers

Despite the link between the oestrogen receptor and expression of AGR2 in breast cancers, oestrogen-independent expression can be observed in other human cancers.[46] It is notable that AGR2, and not AGR3, is the gene/protein that was found by various OMICs platforms to be expressed by oestrogen.[64] Follow-up studies confirmed that AGR3 is overexpressed in breast tumours and that AGR3 is co-expressed with AGR2 in breast cancer tissue with a strong positive correlation with ERα status.[50] However, AGR3 was not co-expressed to a high degree with AGR2 in prostate cancers, indicating that AGR2 and AGR3 expression can be uncoupled. The expression of both AGR2 and AGR3 was evaluated in complex hormone-independent human cancer to identify a suitable clinical model in which to begin to study AGR3/AGR2 function. Studies found that AGR2 and AGR3 are overexpressed in four different subtypes of primary human ovarian cancer, but although the expression of AGR3 and AGR2 can be co-incident in mucinous ovarian cancers, they are uncoupled in the other three types of primary ovarian cancers.[16] These later data suggest that distinct stresses can selectively induce either AGR2 or AGR3 *in vivo*.

Selective expression of AGR2 in liver cancers

AGR2 is mainly expressed in the normal biliary tree in both fetal and adult normal liver. More particularly, the tall epithelial cells covering the large bile ducts as well as gallbladder epithelial cells showed strong AGR2 staining.[65] Moreover, hepatocellular carcinoma tumours do not show any significant AGR2 staining, whereas fibrolamellar carcinoma did.[65,66] Both hilar and extrahepatic cholangiocarcinoma reveal positive AGR2 staining. In contrast, only 50% of the intrahepatic cholangiocarcinoma analysed display strong AGR2 staining.[65] This result was further investigated and led to the correlation of AGR2 staining with mucus production (Lepreux *et al*.[65] and Bioulac-Sage, Balabaud and Chevet, unpublished results). These results can be put in perspective of those obtained using $AGR2^{-/-}$ mice[33] and might provide a selective advantage to mucus-producing tumour cells. This observation might also provide some information on the cellular origin of intrahepatic cholangiocarcinoma, which still remains poorly defined.[67]

AGR2 and metastasis

Many cancer studies revealed that overexpression or suppression of AGR2, in different model systems, can affect cell proliferation, invasion and survival *in vitro*, metastasis and tumour growth

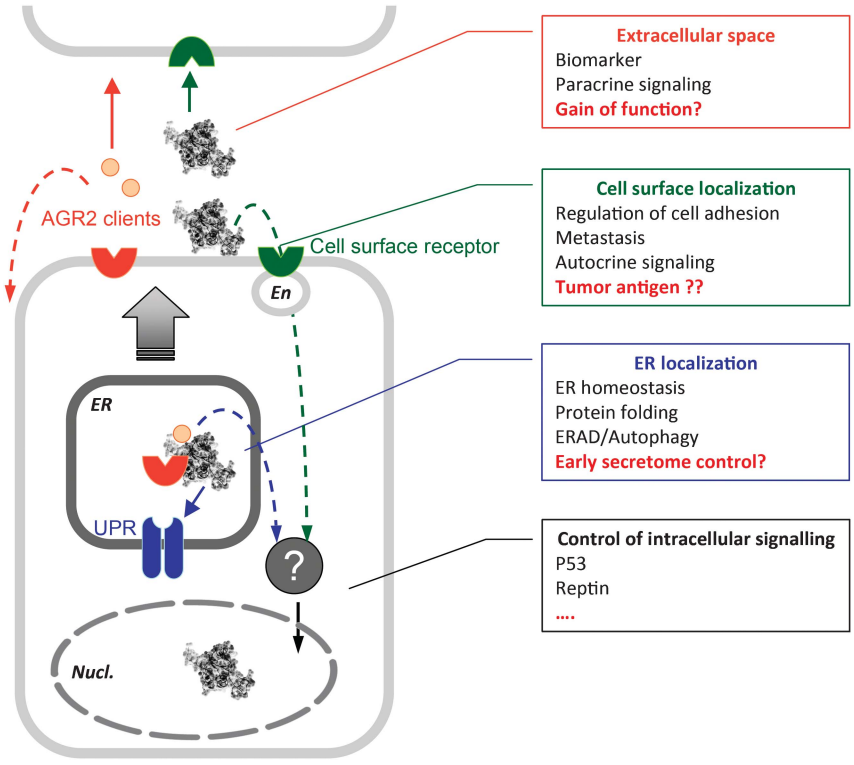

Figure 5. Localization and functions of AGR2. The different location of AGR2 are reported and correlated with its reported functions in these compartments.

in vitro.[43,59] However, the molecular mechanisms at the origin of these phenotypes are not yet clearly understood, although it was recently shown that AGR2 is present at the cell surface of pancreatic cancer cells,[41] a phenomenon similar to that previously reported in tumour immunity.[68,69] As demonstrated for other ER-resident proteins,[2] we have recently shown that the increased expression of AGR2 can enhance ER folding capacity, allowing cancer cells to cope with increased protein production and secretion.[31] Genomic analysis of AGR2-stable cells by cDNA microarray revealed that AGR2 overexpression upregulates the expression of genes involved in cell proliferation, invasion and angiogenesis,[70] which are very important for tumour progression and metastasis. Conversely, the genes involved in the negative regulation of cell proliferation, adhesion and death are downregulated.[70] Moreover, the overexpression of AGR2 in grafted cells results in greater propensity to form lung metastases when propagated as xenografts in nude mice,[48] showing that AGR2 has an influence on the *in vivo* tumour biology. In breast cancer models, overexpression of AGR2 failed to alter tumour formation *in vivo* or growth rate *in vitro*, but rather, reduced cell adhesion and increased the numbers of metastases.[48] A similar observation was obtained in head and neck squamous cell carcinoma cells, in which AGR2 silencing (also observed upon CD147 signalling) reduced cell proliferation migration and invasion,[71] thus CD147 could also represent an AGR2 client in this model. Thus, these results suggest that AGR2 does not only promote cell migration but can also control the cell adhesion rates of detached cells, which may correlate with the ability of metastatic cells to colonize distal sites. The differences in whether migration or adhesion dominates through an AGR2 signal are likely due to the fact that different cancer cell models were used in distinct experiments and that each cell type exploits distinct pathways that mediate cancer survival. AGR2 involvement in metastasis is also demonstrated in prostate adenocarcinoma cells in which an enhanced invasive behavior of cell-overexpressing AGR2 is shown.[72] AGR2 promotes migration and invasion of prostate cancer cells, consistent with previous results in other tumour types, but at the opposite, it attenuates cell growth.[73] In this study, overexpression of AGR2 led to a reduction of colony formation and cell proliferation of prostate cells, possibly brought by a cell cycle arrest.[73] Thus, AGR2 could regulate epithelial–mesenchymal transition (EMT) in development and cancer. EMT is a developmental programme used in cell differentiation during embryogenesis, but parts of this genetic programme are believed to be reactivated during metastasis to transform malignant epithelial cells into motile and invasive mesenchymal-like cells.[74,75] It is believed that EMT is a transient state and that the process is reversed once the cells form a metastatic lesion. Similar observations have also been reported for SIP, a transcription factor that control EMT. SIP1 induced an invasion phenotype while at the same time attenuating cell cycle progression.[76] Nevertheless, the involvement of AGR2 in EMT needs to be further investigated.

Cell signalling mechanisms of AGR2 in cancer

As yet, the cellular mechanism by which AGR2 promotes growth are poorly understood, but there have been a number of observations that are beginning to shed light on the role it plays in cell signalling networks. One pathway that AGR2 has been implicated in is the EGFR pathway. Recently, Dong *et al*.[46] have shown that AGR2 induced expression of AREG, a growth-promoting EGFR ligand. This study functionally linked AGR2 and AREG and supported a significant role for AGR2 in lung adenocarcinomas and the regulation of cell growth. As a result of AGR2 expression, AREG may stimulate the EGFR signalling pathway and may be responsible for the increased cell proliferation and anchorage-independent growth observed in transformed cells.[43,59] It was also shown that this was a specific induction of AREG, there was no induction of other known EGFR

ligands detected and that activation resulted in increased phosphorylation of both EGFR itself and AKT downstream of the receptor. AREG expression has also been shown to be regulated by the Hippo pathway, which serves to regulate cell proliferation and apoptosis, and functions in regulating organ size.[77] Repression of the Hippo pathway results in YAP dephosphorylation followed by transport to the nucleus, where it inhibits apoptosis and promotes cell division. Nuclear YAP protein is associated with neoplasia and has been observed in lung, colon, ovarian and breast adenocarcinoma.[46,78] Another study showed that the Erb3 binding protein 1 could inhibit the expression of AGR2 in prostate cancer cells. The mechanism by which this occurs is yet to be determined, but the Erb3 binding protein 1 can negatively regulate the androgen receptor and so this could be one possible mode.

A key clinical model used to evaluate cancer progression mechanisms is oesophageal adenocarcinoma where selection pressures are being placed on the survival of cells with either wild type-p53 or mutant p53, early in carcinogenesis. The discovery of AGR2 as a dominant overexpressed protein in oesophageal metaplasia and its validation as a p53 inhibitor suggests that AGR2 pathway might be a key mechanism to silence p53 signalling.[45] The introduction of the *AGR2* gene into cancer cell models can:[45] (i) enhance cell survival in a clonogenic assay, similar to the p53 mutant HIS175 allele; (ii) p53 transcriptional activity was reduced when co-transfected with AGR2; (iii) phosphorylation at p53-activating phosphorylation sites (Ser15 and Ser392) upon exposure to ultraviolet was attenuated in cells overexpressing AGR2, which suggest that AGR2's ability to act as a survival factor may be linked to its activity as a p53 kinase inhibitor; and (iv) the introduction of the AGR2 to HCT116 colon cancer cells resulted in the redistribution of p53 from the nucleus to the cytoplasm in ultraviolet-irradiated cells.[44] In agreement with this, targeting AGR2 with short interfering RNA or the use of the TXIYY peptide aptamers induced a redistribution of p53 protein from the cytosol to the nucleus,[44] suggesting that the AGR2 pathway might be targeted therapeutically to stimulate the p53 pathway. The role of AGR2 as an oncogenic wild type-p53 inhibitor cannot, at present, be reconciled with the role of AGR2 as a pro-oncogenic migration/invasion factor, as the cell models used in these latter studies have mutant p53.

Future research areas on challenging the function of AGR2 in cancers

Murine transgenic technologies have revolutionized our understanding of gene–gene interactions with proteins such as RAS, APC, p53, PTEN and SMAD4. The vast majority of this research is based on genes identified from human genetic studies. AGR2 represents a gene identified from OMIC approaches and it has not yet been evaluated in the many oncogenic murine transgenic systems that exist. The AGR2 field will need to integrate the function of AGR2 as an ER-resident chaperone into the genetic matrix identified from key oncogenic nodes to link protein folding/secretome functions of AGR2 to cancer cell survival. Further, the use of specific mutant alleles of AGR2 in such murine cancer models will also define the interactome of the protein that is linked to other oncogenic pathways. For example, we would expect AGR2-overproducing transgenes to put selective pressures on silencing p53 and we might therefore expect that tumours that evolve from $AGR2^{+}$ background might not have selected for p53 mutations to the same extent as AGR2-negative cancers.

QUESTIONS AND FUTURE PERSPECTIVES

In conclusion, AGR2 can stimulate cancer cell proliferation, invasion and survival *in vitro*, resistance to chemotherapy using murine xenografts, metastasis and tumour growth *in vitro*, is relatively unique as a PDI in that it is known to be overexpressed in a large range of human cancers types, and its expression levels can be used to predict patient prognosis in a number of cancers. In an attempt to better explain the function of AGR2 in cancer, we have proposed a fundamental, core role for AGR2 is in regulating the ER capacity to adapt. This ER buffering capacity of AGR2 can in turn have an impact on the nature of the cell secretome under physiological or pathological conditions, as well as affect intracellular transcription networks such as p53 that are known to sense the unfolded protein response. Consequently, shifts in this equilibrium could contribute to the development and progression of human cancers. Although the biological manifestations of AGR2 as a proto-oncogenic protein are now evident, the molecular mechanisms of how it functions and is regulated, nevertheless, remains poorly defined. On the basis of the unique properties of AGR2 we have reviewed here, a number of compelling basic and clinical research questions remain, including (i) why does AGR2 have a 'weak' or non-canonical ER-retention sequence and does this play a role in novel intracellular trafficking outwith the ER that drives cancer growth (Figure 5); (ii) what significance does the changes in the cellular redox balance of a cancer cell have on a PDI-like AGR2 with a single cysteine in its thioredoxin fold (CXXS); (iii) what is the role of the specific 'peptide-binding' function of AGR2 in cargo binding/trafficking and does this provide a peptido-mimetic, therapeutic intervention strategy; (iv) is the AGR2 pathway, including its upstream regulators, its cofactor-interacting proteins or its dimerization interface 'drugable'; (v) is the 'secretome' of AGR2 that mediates its core functions in cell migration, growth or adhesion 'drugable'; and (vi) will transgenic or genetic models that overproduce missense mutant alleles of AGR2 shed light on its ER signalling and pro-oncogenic functions? Answers to these fundamental questions would shed light on the role of the ER protein folding quality control in human cancer cell growth and provide an understanding of how the secretome provides adaptive advantages to the pro-metastatic cancer cell.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Příloha 3

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Mini-review

Anterior gradient 2: A novel player in tumor cell biology

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ABSTRACT

AGR2 has evolutionarily conserved roles in development and tissue regeneration and is linked with several human cancers. The exact functions and regulation of AGR2 are poorly understood, but current data identify AGR2 as a clinically relevant factor that modulates the behavior and response of hormone-dependent cancers (breast, prostate) and hormone-independent cancers (colorectal, pancreatic, esophageal and other common cancers). AGR2 protein expression induces metastasis, acts as a p53 tumor suppressor inhibitor and survival factor, participates directly in neoplastic transformation and is involved in drug resistance. Thus, AGR2 is an important tumor biomarker and negative prognostic factor potentially exploitable in clinical practice.

1. Introduction

Anterior gradient (AG) genes were first identified in *Xenopus laevis* and are named according to their specific expression patterns during early development [1]. Three AG transcripts were discovered by dissection of variously aged embryos of *X. laevis*. XAG-1, XAG-2 and XAG-3 are all approximately 2 kb in length and are first expressed in the anterior region of the dorsal ectoderm in late gastrula embryos [1,2]. The temporal and spatial pattern of XAG-2 expression during amphibian embryogenesis corresponds to the anterior region of the dorsal ectoderm at late gastrula and later the expression gradually becomes restricted to the anterior-most part of the dorsal ectoderm. At the onset of neurulation XAG-2 mRNA is located at the anterior border between the dorsal and ventral ectoderm. This region corresponds to the cement gland anlage, where XAG-2 transcripts are exclusively expressed in successive stages of the frog embryo [3]. The activation of XAG-2 expression is under the control of neuralizing signals including the noggin, chordin, follistatin and cerberus molecules emanating from the organizer region [3]. XAG-2 has been reported to be a secretory protein and a signal peptide was identified in the first 18 amino acid residues [3,4].

Strikingly, the newt AG homolog (nAG) was the first protein ever discovered with the ability to promote limb regeneration [5]. Newt limbs regenerate from de-differentiated fibroblast, muscle, skeletal and Schwann cells into their earlier stem cell forms. These stem cells then proliferate to produce a mass of undifferentiated cells in a process that requires factors for proliferation and overall regeneration. nAG protein is a mitogen for newt blastema cells and also a secreted ligand for Prod-1 protein. During early stages of de-differentiation, nAG was detected in Schwann cells of the distal nerve sheath, later on it was found to be expressed in gland cells of the wound epidermis [5].

Recently, another homologous protein to the anterior gradient secretory protein family was discovered in Xenopus embryo. This protein, named XAgr2, is strongly expressed in the cement gland [6] and other ectoderm-derived organs such as otic vesicles and notochord during neurula and tailbud stages of frog embryogenesis [7]. Interestingly, XAgr2 has greater sequence homology to the human AGR2 or mouse Agr2 (MAgr2) genes than to XAG-1 and XAG-2 genes of *X. laevis*. Anterior gradient proteins have been identified in zebrafish *Danio rerio*

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Table 1
AGR2 expression in normal tissues (derived from Human Protein Atlas, http://www.proteinatlas.org).

Normal tissues IHC[a]	Cell type	Protein expression
Appendix	Glandular cells	Strong
Breast	Glandular cells	Weak
Bronchus	Respiratory epithelial cells	Strong
Cerebral cortex	Neuronal cells	Moderate
Cervix, uterine	Glandular cells	Strong
Colon	Glandular cells	Strong
Corpus, uterine	Glandular cells	Strong
Duodenum	Glandular cells	Strong
Epididymis	Glandular cells	Strong
Fallopian tube	Glandular cells	Strong
Gall bladder	Glandular cells	Strong
Kidney	Cells in tubules	Moderate
Lung	Alveolar cells	Moderate
Nasopharynx	Respiratory epithelial cells	Strong
Pancreas	Exocrine glandular cells	Moderate
Placenta	Trophoblastic cells	Moderate
Prostate	Glandular cells	Strong
Rectum	Glandular cells	Strong
Seminal vesicle	Glandular cells	Strong
Small intestine	Glandular cells	Strong
Stomach	Glandular cells	Strong
Tonsil	Squamous epithelial cells	Moderate
Urinary bladder	Urothelial cells	Strong

[a] Immunohistochemical staining.

(Zagr2), pufferfish *Tetraodon nigroviridis* (Tagr2), Atlantic salmon Salmo salar (Sagr2) and in mammals including human AGR2 (AG2, GOB_4, HAG-2, PDIA17, XAG-2) and AGR3 or MAgr2 and mouse Agr3 (MAgr3) [6]. In contrast to *X. laevis*, where anterior gradient proteins are mainly expressed in ectoderm-derived organs, human and mouse anterior gradient proteins are mainly distributed in endoderm-derived organs [8].

Interestingly, phylogenetic studies have revealed several branches in the AG protein family tree. These studies suggest closer relationships between human AGR3, MAgr3 and Xenopus XAG/1/2 genes, and the similarity within the fish branch with human AGR2 and MAgr2 genes [6].

2. Human AGR2

AGR2 and AGR3 are the two human homologs of the originally discovered *X. laevis* secreted protein XAG-2. Human AGR2 is strongly expressed in the lung, stomach, colon, prostate and small intestine – tissues that contain mucus secreting cells and/or function as endocrine organs [6,9] (Table 1). From an evolutionary perspective, AGR2 may be involved in the epithelial barrier function, because AGR2 promoter was found to be regulated by transcriptional factors typical for epithelial goblet cells [10].

The gene for human AGR2 lies at chromosomal position 7p21.3 and shares 71% identity with its homolog AGR3 [11]. AGR2 gene consists of eight exons and nine transcripts in total have been described for this gene. However only six of them, AGR2 001, AGR2 005, AGR2 006, AGR2 007, AGR2 201 and AGR2 202 are protein coding. These splice variants of the AGR2 protein are within the range of 119 and 188 amino acids length (Fig. 1). The most abundant variant, AGR2 001, consists of 175 amino acids with a predicted molecular weight of 19,979.2 Da and a predicted pI value of 9.03. The alignment of the protein sequence of AGR2 revealed 54% identity and 71% similarity with XAG-2, and 91% identity and 96% similarity with MAgr2 [4].

The AGR2 protein contains a hydrophobic endoplasmic reticulum leader sequence with a signal peptidase cleavage site at Ala20/Lys21 and also has a retention sequence in the C-terminus that is likely to effect intracellular trafficking [12].

With regard to taxonomy, AGR2 is a member of the protein disulfide isomerase (PDI) family of endoplasmic reticulum-resident proteins [13]. These proteins contain one to four CXXC active domain motifs facilitating oxidative/reductive reactions, which are also found in thioredoxins (Tx) and thus referred as Tx-domain motifs. Several PDIs including AGR2 contain thioredoxin-like domain CXXS, which exhibits lower activity associated with disulfide bond reorganization compared to the CXXC domain, nevertheless they may contribute to isomerization of disulfide bridges and possibly perform other specialized functions in the endoplasmic reticulum [13].

The fundamental role of these motifs with active cysteine residues is to form mixed disulfide bonds with substrates. AGR2 itself has recently been shown to form mixed disulfides with intestinal mucin. AGR2 is present within the endoplasmic reticulum of intestinal secretory epithelial cells and interacts with Mucin 2 (Muc2), a major component of intestinal mucus, enabling the large number of Muc2 Cys residues to pair correctly as it is processed by mucus-producing cells. A structural relationship involving putative functional domains between AGR2 and proteins of the disulfide isomerase family of molecular chaperones suggests a role in protein folding [14].

In our recent work we identified an ATP binding protein, Reptin, as a protein that interacts with AGR2 in a yeast two-hybrid screen and validated as an AGR2 binding protein in human cells. We found that the PDI thioredoxin domain of AGR2 is not directly involved in binding to Reptin, but we detected a specific domain that can bind stably to Reptin, and this was localized by mutagenesis within amino acids 104–111 on the AGR2 primary sequence [15]. This region represents a divergent loop, which apart from AGR2 and AGR3 was also found in Erp18 (the smallest member of PDI family) and has been proposed to represent substrate-binding sites for the molecular chaperone function of the protein [16,17]. Accordingly, this loop insertion forms the minimal docking site for Reptin within AGR2 and in turn probably regulates Reptin's many functions such as ATPase activity, ATP binding, helicase functions, telomerase/Pontin binding, APPL1/2 binding, TIP60 interactions, and other related protein signaling functions.

3. AGR2 biological function

Although the role of AGR2 at the level of cells or organisms has not been fully described in mammals, some speculations about their possible functions may be derived from the literature referring to the Xenopus homologs XAG-1 and XAG-2 [9].

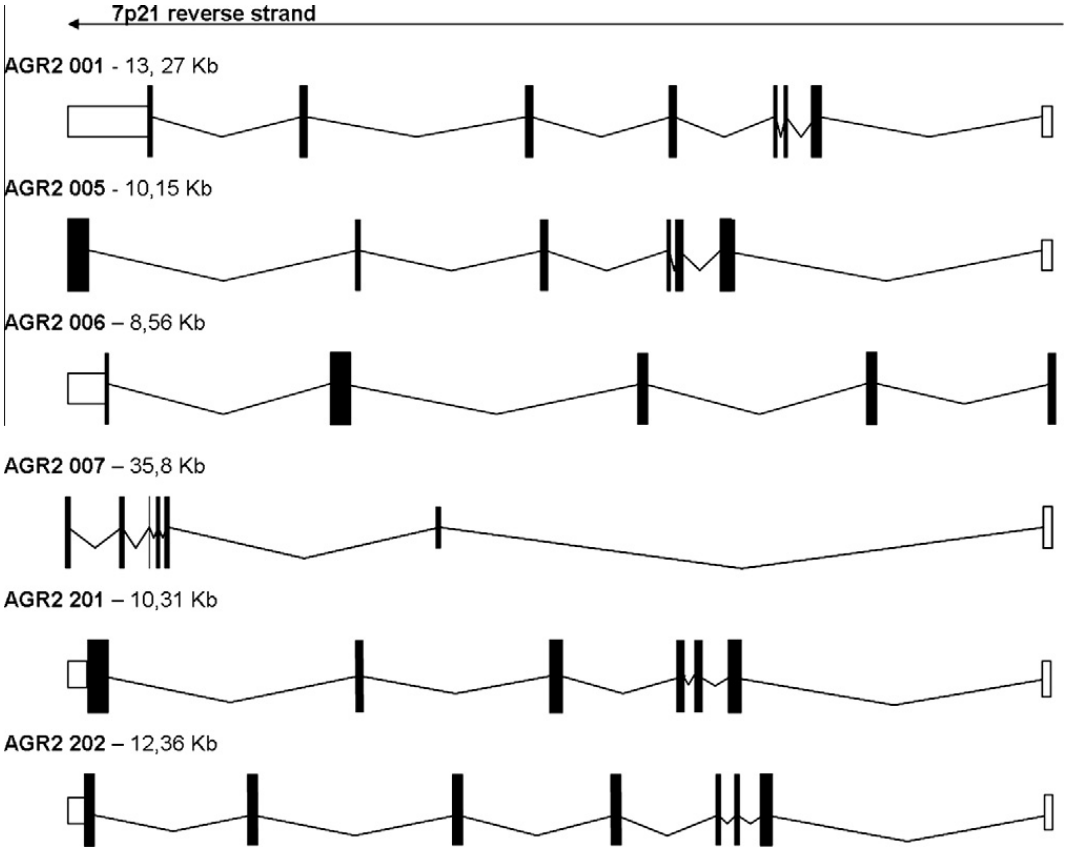

Fig. 1. Protein coding transcriptional variants of AGR2 gene according to Ensembl (Internet). Release 59 Aug 2010c (cited 2010 Oct 13), available from: http://www.ensembl.org.

With regard to the possible roles of AGR2 in the cell, several fragmentary pieces of information are available that provide us with some ideas about its function and regulation. There are also relationships between different human disorders and changes in AGR2 protein expressions. These changes comprise both down- and up-regulations in AGR2 expression in different human tissues.

Concerning downregulation, AGR2 represents a candidate gene for inflammatory bowel disease. Mice with mutant AGR2 have functional abnormalities of intestinal goblet cells and develop diarrhea. In molecular epidemiology studies, decreased AGR2 mRNA is associated with increased risk of both Crohn's disease and ulcerative colitis [10]. Expression of AGR2 protein in tissues that contain mucous-secreting cells and/or cells operating in endocrine organs supports its involvement in epithelial barrier function. This hypothesis is supported by positive regulation of the AGR2 promoter activity by Foxa1 and Foxa2 molecules that are transcription factors typical for epithelial goblet cells [10,18]. Based on the mouse model, phylogenetic background and activation of transcriptional factors, AGR2 may be functionally involved in the maintenance of epithelial integrity [10]. On the other hand, the expression changes linked with up-regulation of AGR2 were particularly observed in neoplasm development and elevated AGR2 levels have been described in various tumor tissues. Nevertheless, the conditions leading to up-regulation of AGR2 in tumor cells as well as the clinical effect of AGR2 expression in tumors are largely unclear and poorly understood.

4. AGR2 expression in human cancer cells

The evidence that AGR2 may be involved in tumor biology arises from a number of studies primarily concerning hormone-dependent tumors such as breast, prostate and ovarian cancers, and non-hormone cancers such as neoplasia of esophagus, gastro-intestinal tract and lungs.

Prometastatic characteristics of AGR2 protein are demonstrated by its interaction with C4.4A and alpha-dystroglycan (DAG-1) – two proteins participating in metastasis formation. Both are transmembrane proteins playing a role in either cell-cell or cell-matrix interactions between cancer and non-cancer cells [11]. After transfection of the AGR2 coding sequence, benign rat mammary cell line Rama 37 gains a metastatic phenotype *in vivo* in the mammary fad pads of syngeneic rats [19]. Moreover, reduced AGR2 expression in SEG-1, originally implied as an esophageal carcinoma cell line, but recently confirmed as non-small cell lung carcinoma cell line [20], decreases anchorage-independent colony growth and leads to a reduction in tumor xenograft size [21].

Pohler et al. [12] demonstrated another characteristic of AGR2 as a survival factor, with the ability of AGR2 to attenuate p53 activity by suppressing p53 phosphorylation

after DNA damage. Nevertheless the physiological conditions in which AGR2 and p53 pathways are coordinated have not been defined to date and further analyses are required to elucidate how the AGR2 pathway is regulated and regulates p53 in response to stresses that classically activate p53 dependent pathways.

In experiments where cells are cultured under serum depleted conditions and/or hypoxia, genes that promote survival, angiogenesis, motility or invasion are activated. The AGR2 gene emerged in a screen for such genes, implying that AGR2 contributes to the survival of cells undergoing physiological stress in the microenvironment of the tumor [22]. This sharp induction of AGR2 expression could be of potential interest, since a major characteristic of metastatic cells is the ability to adapt and survive under pathophysiological stress conditions. Successful metastases most likely require activation of genes that promote their survival during periods of pathophysiological stress such as hypoxia, loss of growth factors etc. Thus it is important to identify and characterize clinically relevant metastatic markers induced in response to pathophysiological stress.

4.1. Breast carcinoma

AGR2 has been found to be co-expressed with estrogen receptor alpha (ER) in breast cancer cell lines [9] and clinical specimens [11]. These findings are strengthened by a recent paper demonstrating that AGR2 is transcriptionally activated by estrogen via direct binding of ER to the AGR2 promoter [23]. Generally, ER-positive breast carcinomas are well-differentiated and less aggressive than ER-negative breast tumors. These data therefore provide a potential inconsistency, where ER is usually associated with a better outcome for breast cancer, while markers of metastasis, AGR2 in this case, associate with a worse outcome [9,23,24]. However, increased expression of AGR2 is associated with decreased survival specifically in ER-positive breast cancers [23,24], whereas in studies of patient cohorts with mixed ER status, the expression of AGR2 was associated with better outcome since AGR2 negative tumors were ER-negative as well [25]. Thus, in the evaluation of the clinical effects of AGR2 expression in breast cancers, it is important to take into account the presence of functional ER.

Importantly, AGR2 was shown as a mediator of prooncogenic signals in human breast cancer cells and as a significant player in the development of tamoxifen resistance in breast cancer. Despite its' proven benefits, tamoxifen surprisingly exhibits partial agonistic properties and side effects comprising induction of AGR2 mRNA and protein levels in tamoxifen treated breast cancer cells both *in vitro* and *in vivo* [23]. These findings are also supported by the fact that AGR2 over-expression strongly correlates with expression of cyclin D1 [23,26], which is well known as a negative predictive factor for tamoxifen response in postmenopausal breast cancer patients [27].

Finally, the role for AGR2 in breast cancer prognosis is not solely related to ER, since ER-positive status is itself associated with a better patient outcome. Other factors that influence expression of AGR2 probably exist, which is consistent with Innes et al. [24] showing that almost one third of ER-negative cases of breast carcinomas are positively stained for AGR2 and that not all ER-positive cases express AGR2. Nonetheless, AGR2 is a useful predictive biomarker for tamoxifen response and patient survival in ER-positive breast cancer [23].

4.2. Prostate cancer

The majority of prostate tumors are dependent on androgens for growth in the initial stages, and are effectively treated by androgen ablation therapy, however, in most cases the tumor eventually progresses to an androgen-independent phenotype. Androgen-independent prostate cancer tends to progress and metastasize and has a low survival rate [28]. AGR2 was shown as an androgen-inducible gene whose mRNA expression was detected in the normal prostate gland, but analysis of microdissected prostate adenocarcinoma samples showed striking elevation of AGR2 mRNA and protein levels in the majority of tumors in comparison to adjacent benign tissues. The androgen-regulated expression, secretory nature and tumor specific over-expression make AGR2 a promising prostate cancer biomarker, however its' use is limited due to expression in several normal human tissues including colon, pancreas, and lung [10]. Recent reports suggest that two proteins belonging to the family of forkhead transcriptional factors positively regulate the AGR2 promoter in prostate cancer cells. These two proteins, Foxa1 and Foxa2, both are important in prostate carcinogenesis. Another molecule likely to modulate the expression of AGR2 in prostate cancer is the ErbB3 binding protein 1 (EBP1) – an endogenous negative regulator of androgen receptor signaling that suppresses the invasive ability of cells by inhibiting the expression of AGR2. EBP1 inversely correlates with AGR2 and is a potent AGR2 inhibitor, but the mechanism remains unknown [18,29]. The data relating to AGR2 levels and patient prognosis are conflicting [30,31], but, as in breast cancer, there may be a role for AGR2 as a prognostic marker for prostate cancer in relation to endocrine status and response to treatment.

4.3. Pancreatic carcinoma

Ramachandran et al. found increased expression of AGR2 in precancerous lesions and in neoplastic cells of pancreatic tumors and cancer cell lines. These pancreatic cell lines secreted AGR2 into the culture media in variable levels, suggesting that AGR2 may act in an autocrine or paracrine manner. High expression of AGR2 in pancreatic carcinoma tissues was confirmed with cDNA microarrays, immunohistochemistry and tissue microarrays [32,33]. It was previously reported that AGR2 expression in breast and prostate tumors is induced by estrogen and androgen, [34,35] but it is not known whether AGR2 is hormone-inducible in pancreatic cancer cells. Kreiger et al. [36] found significantly higher AGR2 expression in female patients, which is of potential interest since epidemiological data have shown that pancreatic carcinomas are at least partly estrogen driven.

It was also shown that transient silencing of AGR2 increased the effectiveness of gemcitabine treatment in two pancreatic cancer cell lines *in vitro* [32,37]. These data indicate that AGR2 provides a survival benefit for pancreatic cancer cells and can protect tumor cells from chemotherapeutic treatments in pancreatic cancer, which is well recognized as being highly resistant to therapeutics.

4.4. Other carcinomas

Besides mentioned malignancies, AGR2 protein has also been studied in other tumors including liver, esophagus, gastrointestinal and lung cancers.

Vivekanandan et al. [38] characterized AGR2 expression in primary hepatocellular neoplasms including typical hepatocellular carcinomas, fibrolamelar carcinoma and hepatic adenomas. The study shows AGR2 protein to be strongly associated with fibrolamelar carcinomas (both primary and metastatic) but not typical hepatocellular carcinomas.

Interestingly, AGR2 was identified as a suitable candidate gene for the detection of circulating tumor cells (CTC) in patients with gastrointestinal cancer [39]. These data are in agreement with the findings of Smirnov et al. [40] who developed a novel approach for the identification of molecular markers to detect and characterize CTC's in peripheral blood. Using RNA extracted from the CTC-enriched portion of the sample and comparing it with the RNA extracted from the corresponding CTC-depleted portion, a list of cancer specific genes was obtained. AGR2 was identified as a novel CTC-associated gene that is expressed in the majority of metastatic samples and can be used for CTC monitoring in peripheral blood of advanced cancer patients.

A premalignant lesion characterized by intestinal metaplasia, Barrett esophagus significantly increases the risk of developing esophageal adenocarcinoma. Due to pathological changes evoked by gastroesophageal reflux a metaplastic change in esophageal mucosa occurs. Normal esophageal squamous epithelium is transformed into gastric-like mucosa, with the presence of intestinal metaplasia specific for Barrett esophagus. Such premalignant mucosa has an increased proliferation rate, decreased apoptosis, and an increased fraction of diploid and aneuploid cells compared to normal epithelium [41]. Importantly, AGR2 expression in Barrett esophagus is notably elevated compared to normal esophageal epithelia [21]. However, the suggestion that AGR2 may play a role in cell-cycle progression in this highly proliferative epithelium and manifests its' pro-oncogenic characteristics by this way is not conclusively supported and increased AGR2 expression is rather linked with mucous-secreting epithelium engaged in the metaplasia.

The potential oncogenic role of AGR2 in lung cancer was studied by Zhu et al. [42]. The genomic abberations of lung adenocarcinoma cell lines were established by metaphase and array comparative genomic hybridization with subsequent expression analysis. In this study, concordant genomic gain at 7p21 and almost 5-fold increased expression of AGR2 in adenocarcinomas compared to normal lung tissues indicates that AGR2 could mediate oncogenic roles in lung cancers. However genomic abberations or epigenetic changes are probably not the major mechanism responsible for the over-expression of the AGR2 gene, as its' expression is rather controlled as part of a larger signaling and metabolic pathway [18,38].

5. Perspectives

In a search to identify the differences between hormone-responsive and hormone-unresponsive breast cancers, AGR2 protein was found to be co-expressed with estrogen receptors in ER-positive breast cancer cell lines [9]. Furthermore, AGR2 expression was confirmed as hormone-responsive and elevated levels of AGR2 protein were found in a range of human malignancies, which were characteristic especially by either functional steroid receptors or mucous secretary epithelial cells.

On the other hand, a percentage of ER-negative breast cancer specimens are also AGR2 positive, supporting the view that the potential role of AGR2 in breast carcinogenesis can be independent of hormone activity as well [22,24]. Accordingly, elevated AGR2 expression was also found in other carcinomas, both hormone non-responsive and non-mucinous, indicating involvement of other signaling or metabolic pathways that are associated with AGR2 expression. In breast cancers, other growth factor signaling pathways, especially cross-talk with ER, could be involved in the regulation of AGR2 expression. For example, Her2/Her3, MAPK, AKT-2 and other components of cellular signaling may be considered as potential modulators of AGR2 expression. This cross-talk makes a breast cancer cell potentially more resistant to single forms of molecular therapy, such as ER-targeted therapy. Hence AGR2 overexpression may represent a clinically relevant factor, suggesting that simultaneous inhibition of the ER and other pathways may be necessary to overcome cancer growth. Consistent with this hypothesis, MAPK/ERK signaling was proven as a potent regulator of AGR2 expression in tumor cell lines in the context of physiological stress [22]. Furthermore AGR2 expression was effectively blocked by a synthetic inhibitor of ERK1/2, demonstrating that the activation signal for AGR2 induction during stress is mediated through an ERK1/2-dependent pathway [22].

The mechanism of AGR2 action remains largely unanswered. Apart from the CXXS motif characteristic for a PDI subclass of molecular chaperones that use this motif as a redox catalyst able to form a covalent complex with other target proteins, similar to how Mucin has been reported to with AGR2 [14], AGR2 also uses a divergent peptide substrate-binding loop to bind to several proteins including Reptin. Reptin in turn uses two allosterically interacting ATP binding motifs to control its binding activity toward AGR2 [15]. Considering the proven oncogenic potential of both AGR2 and Reptin proteins, it will be important to analyze the role of the AGR2-Reptin complex in tumor cells and to develop screening assays for testing potential therapeutic reagents that target the active sites of these proteins. The potential ability of AGR2 to influence such diverse pathways can account for its effects in normal development, regeneration and the diverse

functions attributed to AGR2 in malignancy, including enhanced survival, enhanced cell motility and invasiveness and altered prognosis in hormone-dependent and hormone-independent tumors.

Taken together, although AGR2 aberrant expression was described in a range of hormone-dependent tumors, other molecular mechanisms responsible for regulation of AGR2 expression probably participate in regulation of this protein. The present knowledge about AGR2 identifies this protein as a candidate biomarker to enhance the effectiveness of cancer therapy in selected tumors. Based on literature reports and our results, determination of AGR2 expression may help to predict response to therapy, at least in gemcitabine treated tumors of pancreas and tamoxifen treated breast cancers. However, further functional studies and evaluations of AGR2 role in cancer cells are necessary for the utilization of this protein as a potential predictive or prognostic biomarker of particular malignant diseases.

Conflict of interest statement

The authors declare no conflict of interest related to this work.

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Příloha 4

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ORIGINAL ARTICLE

The pro-metastatic protein anterior gradient-2 predicts poor prognosis in tamoxifen-treated breast cancers

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Transcriptomic screens in breast cancer cell lines have identified a protein named anterior gradient-2 (AGR2) as a potentially novel oncogene overexpressed in estrogen receptor (ER) positive tumours. As targeting the ER is responsible for major improvements in cure rates and prevention of breast cancers, we have evaluated the pro-oncogenic function of AGR2 in anti-hormone therapeutic responses. We show that AGR2 expression promotes cancer cell survival in clonogenic assays and increases cell proliferation and viability in a range of cancer cell lines. Chromatin immunoprecipitation and reporter assays indicate that AGR2 is transcriptionally activated by estrogen through ERα. However, we also found that AGR2 expression is elevated rather than inhibited in response to tamoxifen, thus identifying a novel mechanism to account for an agonistic effect of the drug on a specific pro-oncogenic pathway. Consistent with these data, clinical analysis indicates that AGR2 expression is related to treatment failure in ERα-positive breast cancers treated with tamoxifen. In contrast, AGR2 is one of the most highly suppressed genes in cancers of responding patients treated with the anti-hormonal drug letrozole. These data indicate that the AGR2 pathway represents a novel pro-oncogenic pathway for evaluation as anti-cancer drug developments, especially therapies that by-pass the agonist effects of tamoxifen.
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Keywords: AGR2; tamoxifen; letrozole; estrogen receptor; breast cancer

Introduction

Genomic and proteomic technologies provide the opportunity to discover clinically relevant and novel oncogenic pathways that would have been overlooked by genetic and model organism cancer gene screens of the past. In line with this, suppression subtractive hybridization has allowed the identification of anterior gradient-2 (AGR2) gene, the human homologue of the *Xenopus laevis* cement gland gene XAG-2, as being coexpressed with estrogen receptors (ERs) in breast cancer cell lines (Thompson and Weigel, 1998). More recently, gene expression profiling techniques identified AGR2 as highly expressed in human cancers (Iacobuzio-Donahue *et al*., 2003; Kondrakhin *et al*., 2008; Barraclough *et al*., 2010). A proteomic analysis to discover cell membrane-associated proteins with potential clinical relevance to breast cancer also identified AGR2 (Adam *et al*., 2003).

AGR2 was originally identified as a potential secretory protein that is highly expressed in frog eggs (Aberger *et al*., 1998). The human AGR2-coding sequence is located on chromosome 7p21, well known as a locus of frequent genetic alterations (Petek *et al*., 2000). Subsequent studies have shown a significant function for AGR2 in a range of biological pathways including regulation of p53 (Pohler *et al*., 2004), cell migration and cellular transformation (Wang *et al*., 2008). In a rat somatic model for breast carcinoma, although the overexpression of AGR2 in grafted cells did not enhance tumour initiation, the resulting tumours exhibited considerably greater propensity to form lung metastases compared with AGR2-negative counterparts (Liu *et al*., 2005). Importantly, the evidence suggested that AGR2 does not promote cell migration but instead controls the cell adhesion rates of detached cells, which may correlate with the ability of metastatic cells to colonize distal sites. Strikingly, AGR2 was identified as the first protein able to promote cell migration and regeneration in severed nerves, which normally provide signal to stimulate regeneration (Kumar *et al*., 2007). Clinical studies have also implicated the protein in inflammatory bowel disease (Zheng *et al*., 2006), hormone-dependent breast cancers (Thompson and Weigel, 1998; Fletcher *et al*., 2003; Innes *et al*., 2006), and a range of non-hormone cancers (Lee *et al*., 2006; Zhu *et al*., 2007). Furthermore, AGR2 predicts poor prognosis in prostate cancers (Zhang *et al*., 2007). Finally, expression of genes including AGR2 and others could detect circulating tumour cells in peripheral blood of advanced cancer patients (Smirnov *et al*., 2005).

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The clinical effects of AGR2 expression in breast cancers are unclear as AGR2 has been associated with either increased or decreased survival (Fritzsche *et al*., 2006; Innes *et al*., 2006), although the former study included ER-positive and ER-negative tumours, which may complicate the analysis due to the overwhelming impact of ER for prognosis after treatment. A more recent study using patients that have only undergone operative breast cancer surgery indicated that high AGR2 expression is linked to poor survival (Barraclough *et al*., 2009). Therefore, the precise function of these proteins and the mechanisms of their hormonal regulation in breast cancers remain to be elucidated.

As AGR2 expression is presumed to occur in hormone-responsive tumours, development of a breast cancer model to study the function of novel proteins such as AGR2 is required. Recent research has still failed to translate a broader clinical utilization of a range of breast cancer biomarkers because only two markers, ER and ErbB2 (Her2), are presently accepted worldwide for breast cancer diagnosis. These proteins have important functions in breast cancer development and provide effective treatments for many breast tumours. As breast cancer is a classical hormone-dependent tumour, estrogen is well known to have a major function in the development and progression of the disease. Therefore, the determination of the ER status is an essential part of the diagnostic procedure in all breast cancer patients. The presence of ER and progesterone receptors is indicative of response to endocrine therapy and improved disease-free survival (Robertson *et al*., 1996; Katzenellenbogen and Frasor, 2004). ER expression is generally found in only 6–10% of normal breast epithelial cells, whereas ~60–70% of primary breast cancers are ER-positive (Jacquemier *et al*., 1990; Normanno *et al*., 2005).

ER-positive/Her2-negative breast cancers typically respond well to endocrine therapy, which suppresses estrogen signalling in cancer cells and halts their proliferation and/or induces apoptosis. Although endocrine therapy is proven effective, its clinical benefit is seriously limited by drug resistance. Approximately 40% of ER-positive early stage breast cancers, which initially respond to anti-hormonal therapy, become resistant during the course of therapy (acquired resistance) and relapse with endocrine therapy resistant disease. Thus, endocrine therapy resistance is an important clinical challenge in breast cancer treatment (Coser *et al*., 2009).

Although there is growing clinical and biological data showing AGR2 has a function in cancer development, there are few studies of AGR2 signalling pathways. As AGR2 and its orthologue AGR3 are overexpressed in breast cancers and AGR2 was identified as an estrogen-inducible gene, we have analysed whether the AGR2 protein is indeed estrogen responsive, whether tamoxifen can control the AGR2 pathway, and what the effects of this might hold for tamoxifen-resistant breast cancers. Our data show that expression of AGR2 is at least partially under control of ERα and is tightly associated with the agonist effects of tamoxifen. The contrasting suppression of AGR2 by letrozole suggests a strategy for optimizing treatment of cancer based on the AGR2 pathway.

Results

AGR2 expression promotes cell survival

We evaluated whether transfection of the AGR2 gene into ER-negative and AGR2-negative cell lines H1299 (lung cancer, p53-null) and MDA-MB-231 (breast cancer, mutant p53) can alter cell survival in a clonogenic assay *in vitro*. Transient transfection of vectors encoding AGR2 gene indicated that AGR2 increases colony-forming ability in both cell lines (Figures 1a and b). To investigate whether AGR2 expression increases survival after chemotherapy in breast cancer, we used the MTT assay in MDA-MB-231 cells transfected with AGR2 and demonstrated an increase in cell viability after drug treatment (Figure 1c). Together, these results clearly show that AGR2 promotes cellular viability, indicating that AGR2 can mediate survival under conditions of stress. These effects are independent of p53 and ER status. In the light of these facts, we evaluated AGR2 expression in the more common breast cancer cell systems that naturally express both ER and AGR2. We tested four miRNAs, denoted as 261, 522, 575 and 654, for silencing AGR2 expression (Supplementary Figure 1). Two miRNAs, 261 and 575, the most potent AGR2 inhibitors, were chosen for further experiments. MCF-7 cells (breast cancer, ER-positive, wild-type p53) with silenced AGR2 expression showed twofold to fourfold reduced clonogenic efficiency in response to tamoxifen treatment compared with control cells (Figures 1d and e).

AGR2 gene is expressed in response to tamoxifen in vitro and represents agonist responsive locus

These data validating the pro-survival activity of AGR2 directed us to analyse whether the expression of AGR2 is associated with response to tamoxifen, which is the endocrine treatment of choice for all stages of ER-positive breast cancer for the past 30 years. Treatment of MCF-7 cells with dialyzed serum resulted in a loss of AGR2 protein expression (Figure 2, lanes 5–8), whereas supplementing dialyzed serum with 17β-estradiol maintains AGR2 protein levels (Figure 2, lanes 9–12), indicating that the protein is estrogen responsive. Consistent with these data, AGR2 gene expression can be stimulated in MCF-7 cells exposed to estrogen (Figure 3a). As compared with MCF-7 cells, basal AGR2 mRNA expression in LCC1 and LCC9 cells especially (Figure 3a) was relatively higher. LCC9 cells were derived as estrogen non-responsive and tamoxifen-resistant derivatives of MCF-7 cells. As the LCC9 cells have been selected under fulvestrant exposure and acquired cross-resistance to both fulvestrant and tamoxifen (Brunner *et al*., 1997), we prepared other tamoxifen-resistant cells T47D-R, which have been selected under tamoxifen treatment. Interestingly, MCF-7 (tamoxifen-

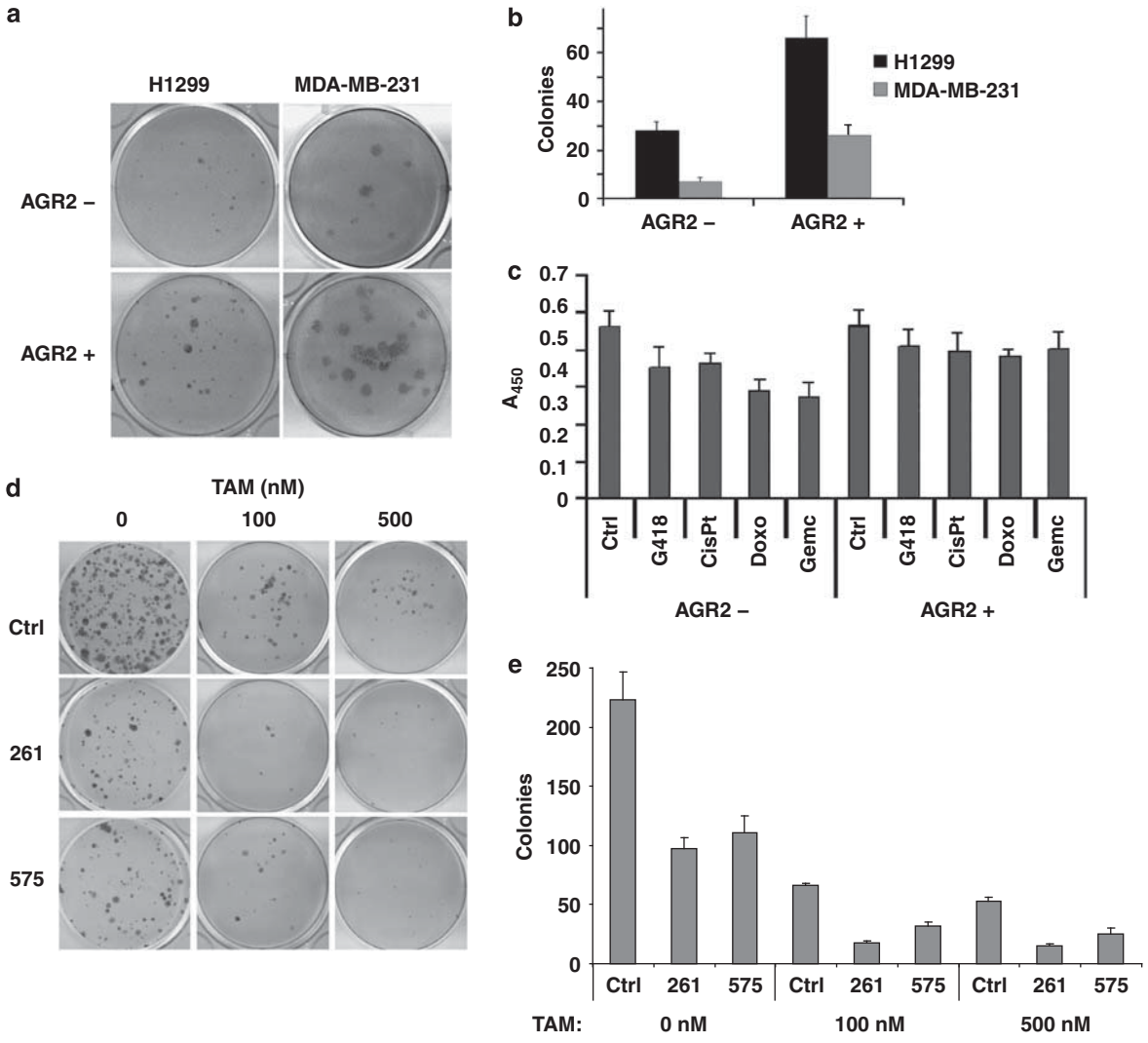

Figure 1 AGR2 expression enhances clonogenic cell survival. (**a**) H1299 and MDA-MB-231 cells were transiently transfected with vectors expressing AGR2, trypsinized 48 h later, and seeded in media containing G418 at concentration 0.5 mg/ml. After 3 weeks, the cells were washed with PBS and fixed for 30 min in methanol, then visualized with Giemsa stain and (**b**) quantified using TotalLab (Nonlinear dynamics, Newcastle upon Tyne, UK). The final colony number was determined as an average of three independent experiments. (**c**) Cell proliferation was determined using MTT assay in cells exposed to geneticin, cisplatin, doxorubicin and gemcitabine for a period of 72 h. MDA-MB-231 cells were transfected by empty pDEST12.2 as control and pDEST12.2 carrying AGR2-coding sequence, respectively. This experiment was performed once in quintuplicate for each treatment. (**d**) MCF-7 cells were transfected by 261 and 575 miRNAs silencing AGR2 expression and empty plasmid (Ctrl), trypsinized 48 h later, and seeded in media containing tamoxifen at 100 and 500 nM concentrations, respectively. After 3 weeks, the colonies were visualized and (**e**) quantified as indicated earlier. The final colony number was determined as an average of three independent experiments.

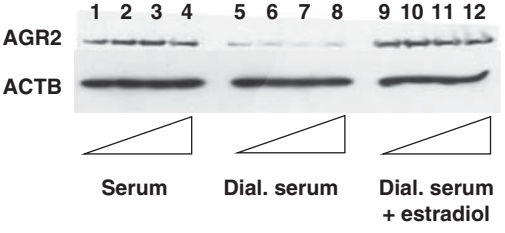

Figure 2 AGR2 protein expression is hormone dependent. MCF-7 cells were grown in media containing serum (10%) (lanes 1–4), media containing dialyzed serum (10%) (lanes 5–8), or media containing dialyzed serum (10%) supplemented with estradiol (lanes 9–12). Incubations were carried out for 1 day (lanes 1, 5, and 9), 2 days (lanes 2, 6, and 10), 3 days (lanes 3, 7, and 11), or 4 days (lanes 4, 6, and 12). Lysates were immunoblotted with antibody to AGR2.

sensitive) and both tamoxifen-resistant cell lines LCC9 and T47D-R showed induction of AGR2 mRNA level in response to tamoxifen treatment in contrast to T47D cells that did not show elevated AGR2 expression in response to tamoxifen exposure (Figures 3a and b).

Because of the estrogen responsiveness of the AGR2 pathway, we next evaluated whether AGR2 protein level could be modulated by tamoxifen treatment. Surprisingly, AGR2 mRNA and protein were induced by tamoxifen treatment of the MCF-7 cells (Figures 3 and 4). The combination of tamoxifen and estrogen synergized to elevate AGR2 expression in MCF-7 cells, LCC9 cells and T47D-R cells (Figure 3). AGR2 expression as well as induction of AGR2 by 17β-estradiol was inhibited by

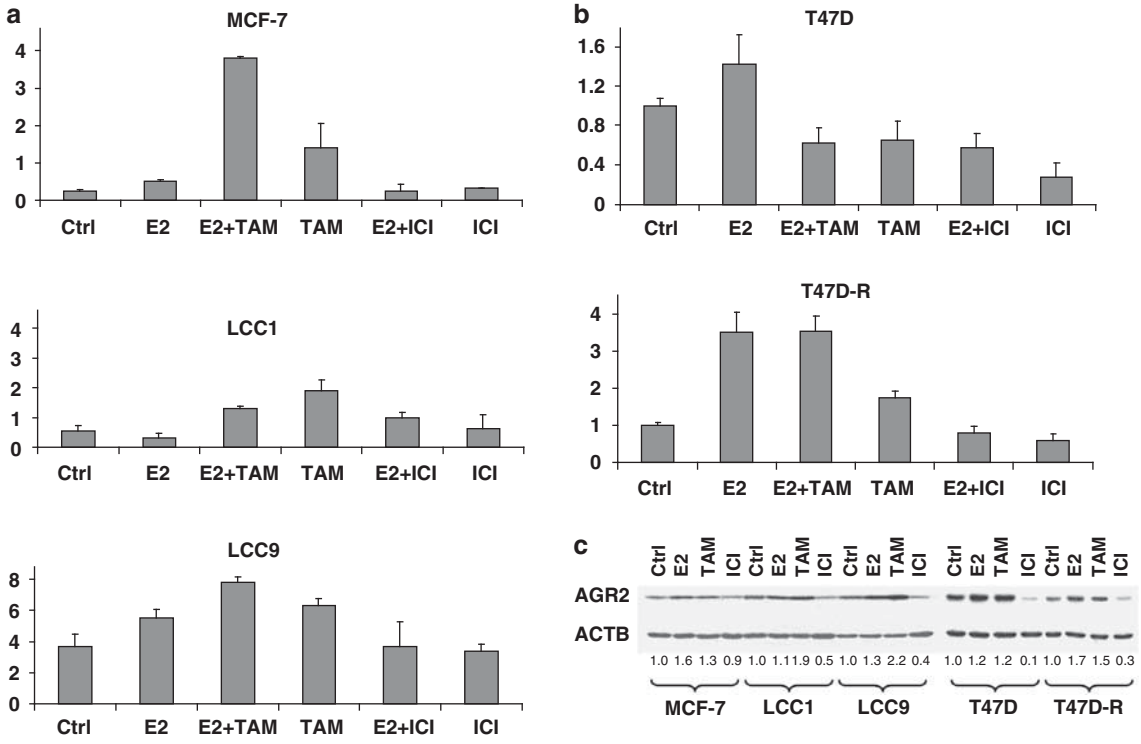

Figure 3 Stimulation of AGR2 expression. (**a**) The normalized AGR2 mRNA levels in MCF-7, LCC1 and LCC9 were quantified by the relative standard curve method. Graphs represent average of three independent experiments including s.d. of three independent analyses of AGR2 gene expression by qRT–PCR. Cells were exposed to 17β-estradiol (E2), tamoxifen (TAM) and fulvestrant (ICI) for 48 h and then total RNA was purified to analyse gene expression. (**b**) Determination of AGR2 expression in T47D (parental cell line) and T47D-R (tamoxifen-resistant) cells. Graphs represent average of three independent experiments including s.d. of three independent analyses of AGR2 gene expression by qRT–PCR using relative quantification method. The expression of untreated cells was used as a calibrator. (**c**) Determination of AGR2 protein levels. Cells were treated as indicated in the panel and immunoblotted for AGR2 protein and β-actin. Numbers correspond to fold changes in reference to respective controls.

fulvestrant (Figures 3 and 4), suggesting AGR2 induction by tamoxifen functions through the agonist effect on the ER.

ER transcriptionally regulates AGR2 expression

To confirm that the induction of AGR2 expression is a direct effect of activated ERα, we performed chromatin immunoprecipitation assay on the AGR2 promoter. The amount of AGR2 promoter that coimmunoprecipitated with ERα antibody was approximately twofold increased in response to 17β-estradiol or tamoxifen treatment (Figure 5a).

To mechanistically support the implication of ER in transcriptional regulation of AGR2 expression, we cloned the minimal AGR2 promoter sequence (Figure 5b) into pGL3 basic plasmid with luciferase reporter and cotransfected this construct with pSG5-ERα construct into H1299 cells (both AGR2 and ERα-negative). Our results clearly show that coexpression with ERα activates the AGR2-luc reporter; however, the treatment of cells with both 17β-estradiol and tamoxifen induced only slight increases in AGR2 promoter activity (Figure 5c).

AGR2 expression is elevated in tamoxifen-resistant cancers and predicts poor prognosis

To investigate links between tumour progression *in vivo* and AGR2 expression, 109 primary breast cancer tissue samples ranked to Cohort I were subjected to quantification of mRNA level by qRT–PCR. This cohort contained 64-paired specimens (tumour tissue plus healthy tissue dissected in proximity of the tumour). AGR2 mRNA expression was on average sevenfold higher in breast tumour tissue compared with normal breast tissue (Supplementary Figure 2). The relationships between AGR2 expression and other clinicopathological parameters are shown in Supplementary Table 1. The upregulation of AGR2 gene expression revealed strong association with expression of the ERα and progesterone receptors. Interestingly, AGR2 also correlated with cyclin D1, which is a direct transcriptional target of estrogen signalling mediated by ER/AP-1 and ER/Sp1 transcription complexes (Riggins *et al*., 2007).

AGR2 as potential predictive factor for disease-free survival was first analysed in Cohort I, which included both ER-positive and ER-negative cases and AGR2 expression was not a statistically valuable prognostic clinicopathological variable (data not shown). As our *in vitro* results showed increased AGR2 levels in ER-positive cells treated by tamoxifen, we next investigated AGR2 expression in correlation with the clinical response to tamoxifen treatment. In Cohort I, 78 patients were ER-positive cases (72%) and underwent adjuvant therapy with tamoxifen. This subgroup was subjected to disease-free survival analysis in respect to

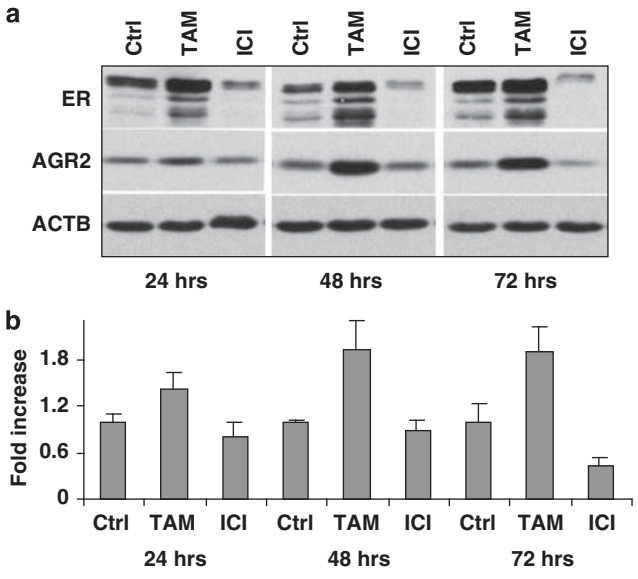

Figure 4 AGR2 protein expression is stimulated by tamoxifen. (**a**) MCF-7 cells were treated by tamoxifen (TAM) and fulvestrant (ICI). Three different time exposures were used (24, 48 and 72 h) and immunoblot analysis of ER, AGR2 and β-actin protein levels is depicted. (**b**) Densitometry of AGR2 protein level normalized by β-actin. Graph shows average of three independent experiments including s.d. of three independent experiments.

AGR2 expression. Tumours formed two distinct groups with respect to AGR2 expression levels. Those tumours with levels below the median expression value were classed as the low expression group and tumours with expression above the median were classified as high expression. We found that increased AGR2 expression is significantly associated with disease relapse or metastasis ($P = 0.008$, Log-Rank test; Figure 6).

To confirm the function of AGR2 in mechanisms responsible for tamoxifen treatment failure more exactly, we analysed AGR2 expression in Cohort II, which consists of 60 ERα-positive breast cancers who developed a locoregional and/or distant relapse during the 5-year period of adjuvant tamoxifen treatment. These specimens were divided into two groups: group A contained 25 biopsies resected from primary ER-positive breast cancers before tamoxifen adjuvant therapy and group B contained 35 samples taken from locoregional relapses or distant metastases, resected after adjuvant therapy. We found a statistically significant difference for AGR2 expression ($P = 0.036$, Mann–Whitney U test), indicating elevated expression of AGR2 in locoregional relapses or distant metastases evolving and growing during the period of adjuvant tamoxifen treatment of ERα-positive breast carcinomas (Figure 7a; Supplementary Table 2). We also analysed AGR2 expression in the subset of 16 patients who had a paired sample in both groups A and B and again found a significant difference between these paired samples ($P = 0.023$, Mann–Whitney U test).

Together, these data suggest that AGR2 expression contributes to cell survival and tamoxifen resistance in breast cancers. If so, then it would be of interest to

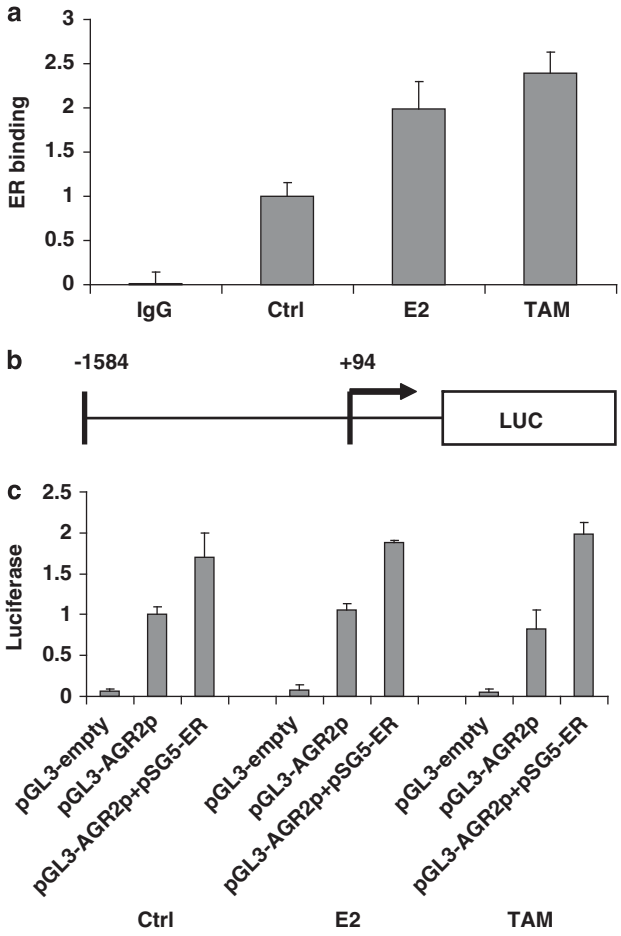

Figure 5 (**a**) Chromatin immunoprecipitation assay. The binding of ERα to the AGR2 promoter was estimated in MCF-7 cells either untreated or exposed to 17β-estradiol (E2) or tamoxifen (TAM) by three independent chromatin immunoprecipitation assays. Non-specific mouse IgG was used as negative control. Graph shows relative fold changes in binding affinity of ERα to AGR2 promoter sequence in reference to untreated cells. (**b**) Summary of AGR2 promoter construct pGL3-AGR2p. A DNA construct pGL3-AGR2p contains AGR2 gene sequence, −1584 to +94, was cloned upstream of the firefly luciferase gene. (**c**) Reporter assay. AGR2-luc promoter activity was determined in cells cotransfected (1) by empty pGL3 basic plasmid + pRL-TK as negative control, (2) by pGL3-AGR2p + pRL-TK and (3) by pGL3-AGR2p + pRL-TK + pSG5-Erα. H1299 cells were cotransfected in 24-well plate format with 100 ng of pGL3-AGR2p, 50 ng of pRL-TK and 100 ng of pSG5-ERα. The cells were subsequently treated with 17β-estradiol (E2) and tamoxifen (TAM). Activity of the AGR2 promoter was determined by luciferase assay. Promoter activity of a Renilla promoter construct was used for data normalization. Graph shows relative fold changes in reference to luciferase activity in H1299 cells cotransfected by pGL3-AGR2p + pRL-TK constructs. Data are average of three independent experiments including s.d.

determine whether AGR2 expression was downregulated after the use of other cancer therapies that do not exploit the anti-hormonal effects of tamoxifen. Fulvestrant or aromatase inhibitors like letrozole are a promising new therapy that can function upstream of the ER target by suppressing the hormonal stimulus. In cell culture experiments, we showed that administration of fulvestrant inhibited hormonal-induced AGR2

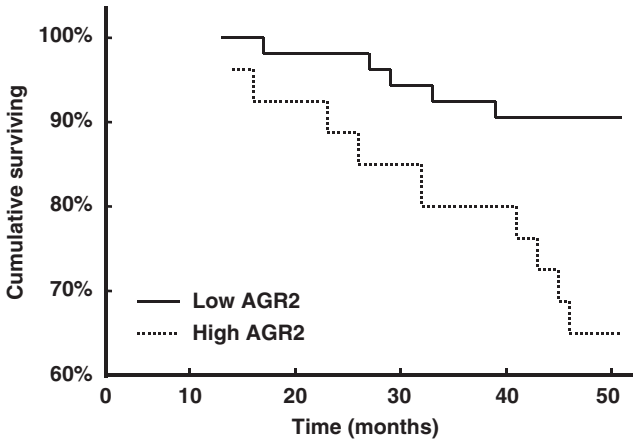

Figure 6 Association of the AGR2 expression with disease-free survival of 78 ER-positive breast cancer patients, who underwent tamoxifen adjuvant therapy (Log-Rank test, $P = 0.008$). There were censored observations at 48 months of follow-up. The continuous curve represents the group of breast tumours with very low or negative AGR2 expression. The dashed curve represents the breast tumours with high AGR2 expression.

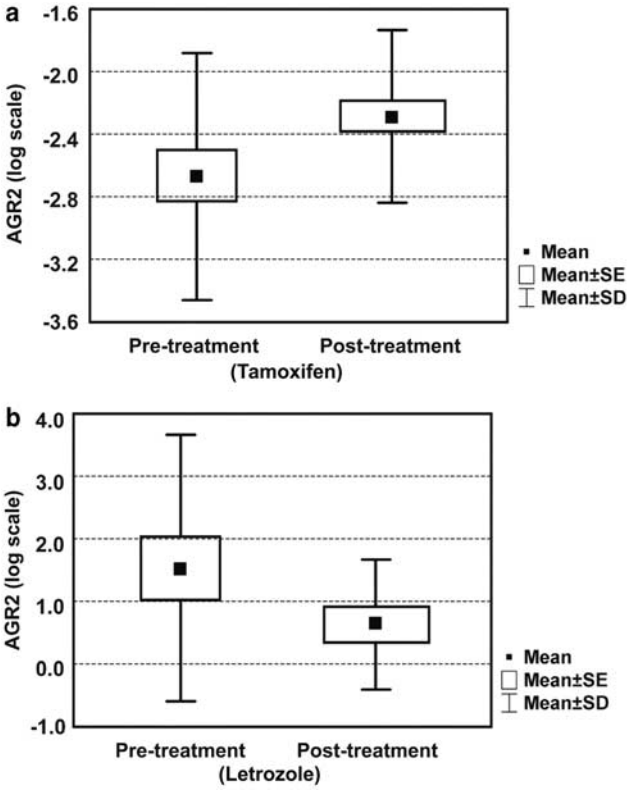

Figure 7 Determination of AGR2 expression in response to endocrine therapy in human breast cancers. (**a**) Box/Whisker plot analysis of AGR2 mRNA level in Cohort II of specimens before (group A) and after treatment by tamoxifen (group B). (**b**) A summary of Box/Whisker plot analysis of AGR2 expression in 19 pairs of breast cancer core biopsies (Cohort III) taken before and after 14-day treatment with letrozole. AGR2 expression was normalized by geometric mean of specifically validated reference genes ACTB and GAPDH.

expression (Figures 3 and 4). Moreover, our data of paired breast cancer samples before and after letrozole treatment shows that short-term estrogen deprivation by letrozole reduces AGR2 mRNA expression *in vivo* ($P = 0.016$, Mann–Whitney U test) (Figure 7b).

Discussion

ER is a member of a large superfamily of nuclear hormone receptors operating as transcription factors whose activity is under hormonal control. Binding of estrogen to ER induces activation of the receptor. In fact, ER dissociates from heat shock proteins, and undergoes conformational changes, dimerization and phosphorylation (Osborne and Schiff, 2005). The activated ER binds to estrogen response elements (EREs) that are located upstream of estrogen-regulated genes. A number of genes induced by the transactivation function of the ER have a coordinated function in mediating the pro-oncogenic functions of this signal transduction pathway. Of these many genes, ER-responsive gene named AGR2 was identified as potential target for breast cancer therapy and/or diagnostic markers of the disease (Fletcher *et al*., 2003).

Preliminary studies have shown that AGR2 and its orthologue AGR3 are expressed in primary breast cancers and that expression of AGR2 correlates with ERα expression in human breast cancer cell lines, suggesting that expression of AGR2 is regulated via ER. Computer analysis of the 3-kb sequence upstream of AGR2 gene revealed a consensus sequence for four potential EREs in chicken oviduct (Kim *et al*., 2007). However, our computer analysis of the human 3 kb segment upstream of AGR2 initiation codon identified only four half sites (½ ERE) and one AP-1 site, which is also a potential docking site for ERα (Gruber *et al*., 2004) (data not shown). On the other hand, it is well known that ERα can form complexes with ERE half-sites with lower affinity (Tyulmenkov and Klinge, 2001; Gruber *et al*., 2004). Interestingly, human sequence in positions −554 and −314 upstream of AGR2 initiation codon contains TGACC direct repeats, which can serve as docking site for ERα as a homodimer with one ERα monomer bound to each ½ ERE (Anderson and Gorski, 2000). Involvement of ERα in transcriptional activation of AGR2 is supported by our results from chromatin immunoprecipitation and luciferase reporter assays.

Transcriptional activity of ER is regulated through two separate transactivation domains termed AF-1 in the amino-terminal region and AF-2 in the carboxyl-terminal region (Kumar *et al*., 1987). Tamoxifen, like estrogen, allows ER dimerization and binding of the ER homodimer to the ERE; however, tamoxifen–ER complexes possess an altered conformational shape that results in block of gene transcription through the AF-2 domain, whereas AF-1 mediated gene transcription may still occur (Tzukerman *et al*., 1994). Thus, tamoxifen-based antagonist effect could be mediated by inhibiting ER's ligand-dependent AF-2 activity. The mechanisms responsible for the agonist properties of tamoxifen are not fully understood but appear to involve the

transactivation of responsive genes in a promoter- and cell-specific context through the AF-1 transactivation function of the ER (Metzger *et al*., 1995).

Preclinical studies as well as emerging data from breast tumour biopsies indicate that ER function is augmented by cross-talk with cell-cycle regulators including cyclin D1, cyclin E and/or growth factor receptors and downstream signalling molecules such as ErbB family, MAPK, PI3K/AKT and other and that this cross-talk can be associated with tamoxifen resistance (Riggins *et al*., 2007). Accordingly, it was showed that AGR2 contributes to the survival of metastatic and primary breast cancer cells in the pathophysiological environment of the growing tumour, revealing a novel mode of AGR2 regulation independent of the ER status (Zweitzig *et al*., 2007).

We examined whether there is a link between the clinical response to anti-hormonal treatment and AGR2 expression in ER-positive breast cancers. Our results show that AGR2 acts as a survival factor and is involved in the development of tamoxifen resistance in breast cancer. In fact, a recent study on the effects of the aromatase inhibitor letrozole on global gene expression identified AGR2 as one of the most significant suppressed post-treatment, suggesting that AGR2 expression can predict if not contribute to the extents of sensitivity to letrozole drug treatment (Mackay *et al*., 2007).

Targeting the ER using tamoxifen is responsible for major improvements in cure rates and prevention of breast cancers. Despite its proven benefit, tamoxifen also exhibits partial agonistic properties and side effects (Goetz and Loprinzi, 2003). Moreover, drug benefits are limited by tumour recurrence in a significant proportion of initially drug-responsive breast cancer patients because of acquired resistance (Johnston, 1997). As the molecular mechanisms leading to tamoxifen resistance are not fully understood, the identification of factors that have a function in the development of tamoxifen resistance is of great interest. Our data show induction of AGR2 at the mRNA and protein levels in tamoxifen-treated breast cancer cells but not in cells treated by fulvestrant and further support the ER responsiveness of the AGR2 gene. Our *in vitro* data predict an involvement of AGR2 in response to tamoxifen-based therapy and initiated our evaluation of AGR2 expression in clinical samples from patients with primary breast carcinoma and from secondary tumours developed during tamoxifen treatment, where we found significantly higher levels of AGR2 in tumours exposed to tamoxifen (Figure 7a). Our findings contradict speculations that the anti-estrogenic effects of tamoxifen would suppress the expression of AGR2 as a negative prognostic factor (Innes *et al*., 2006). This presumption was based only on the observation that expression of AGR2 is downstream of ER and is present at much higher levels in estrogen-responsive MCF-7 cells in the presence of estrogen (Liu *et al*., 2005). Further, when tamoxifen-treated ERα-positive breast cancers were subdivided into two separate classes according to AGR2 expression, those breast carcinomas with high AGR2 level showed poorer disease-free survival (Figure 6). Likewise in prostate cancer, AGR2 expression predicts poor prognosis (Zhang *et al*., 2007). Recent data have shown that AGR2 may have a prominent function in therapy resistance (Ramachandran *et al*., 2008). They found that AGR2 silencing in pancreatic cancers significantly inhibits cell proliferation, invasion and survival *in vitro*, inhibited tumour growth *in vivo* and sensitized cancer cells to gemcitabine both *in vitro* and *in vivo*. These findings are consistent with our *in vitro* data and our analyses of tumour material showing that AGR2 is linked to resistance to tamoxifen treatment, suggesting that elevated AGR2 expression contributes to the extents of tumour resistance or sensitivity to drug treatment. These statements are also supported by the fact that AGR2 overexpression correlates with expression of cyclin D1, which is well known as a negative predictive factor for tamoxifen response in post-menopausal breast cancer patients (Stendahl *et al*., 2004). Moreover, our *in vitro* and *in vivo* data suggest that AGR2 levels predict tamoxifen resistance.

In summary, we have shown that AGR2 mediates a pro-survival pathway in human breast cancer cells and is involved in pro-oncogenic signals of the ER. In addition to increasing colony growth, AGR2 also inhibits the effects of cytotoxic chemotherapeutic agents. Surprisingly, the estrogen antagonist, tamoxifen, induces AGR2 expression *in vitro* in ER-positive breast cancer cell lines and we also show that ER-positive breast cancers have increased levels of AGR2 after tamoxifen treatment. Most strikingly, pre-treatment levels of AGR2 act as a prognostic biomarker in ER-positive breast cancers treated with tamoxifen adjuvant therapy. Although the mode of action of AGR2 after tamoxifen treatment remains to be more precisely defined, our *in vitro* and clinical findings indicate that AGR2 mediates an agonist effect of this drug that may form the basis for novel anti-cancer drugs aimed at suppressing this function. In this regard, the ability of aromatase inhibitors to suppress AGR2 expression in patients suggests a first possible agent with which to begin to treat tamoxifen-resistant tumours with a high AGR2 status. It will be important to determine whether AGR2 expression is downregulated after the use of other cancer therapies, particularly as AGR2 expression can increase cancer cell growth and survival independently of both estrogen and p53.

Materials and methods

Cell culture and treatment

Cells referred as LCC1 and LCC9 were selected from human cancer cell line MCF-7 as described earlier (Brunner *et al*., 1997) (Source of LCC1 and LCC9 cells: Dr Robert Clarke, V T Lombardi Cancer Research Center, Georgetown University Medical School, Washington, DC, USA). In comparison to parental MCF-7 cell line (both estrogen and tamoxifen sensitive), LCC1 cells are tamoxifen sensitive but estrogen independent and LCC9 cells, which were selected under fulvestrant exposure also showed increased cross-resistance

towards tamoxifen. Tamoxifen-resistant T47D-R cells were derived from T47D cells as described earlier (Gunthert *et al*., 2005; Knowlden *et al*., 2005). All cells were cultivated in phenol-red free DMEM with 2 mM L-glutamine and dialyzed 10% foetal bovine serum. Cells were grown to 60–80% confluence before experimental treatments. Unless otherwise indicated, tamoxifen, (Z)-1-[4-[(dimethylamino)ethoxy]-phenyl]-1,1-diphenyl-1-butene, was added to a final concentration of 2 μM; fulvestrant, (ICI 182 780) (7a,17b)-7-[9-[(4,4,5,5,5pentafluoropentyl)Sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol, was applied in 2 nM concentration; 17β-estradiol (estrogen) 3,17-β-dihydroxyestra-1,3,5(10)-triene was applied in 1 nM concentration (all Sigma-Aldrich, St Louis, MO, USA). Culture medium was changed each 48 h.

Development of microRNAs silencing AGR2
Single-stranded DNA oligos were designed using RNAi Designer (Invitrogen, Carlsbad, CA, USA), double-stranded oligos denoted as 261, 522, 575 and 654 (Supplementary Table 3) were generated according to the manufacturer's instructions subsequently. The double-stranded oligos were cloned into the pcDNA 6.2-GW/EmGFP-miR vector (Invitrogen).

Reverse transcription and quantitative PCR
Total cellular RNA was extracted using TRI Reagent (MRC, Cincinnati, OH, USA). cDNA synthesis was carried out using the M-MLV reverse transcriptase (Invitrogen). Triplicate samples were subjected to quantitative PCR analysis using SYBR Green for AGR2 and TaqMan for 18S rRNA (Applied Biosystems, Foster City, CA, USA) that was used as an endogenous control. cDNA prepared from T47D cells that show strong expression of AGR2 was used as the calibrator. The primer pairs used for AGR2 PCR were as follows: AGR2 Forward: 5′-GGAGCTCTATATAAATCCAAGACAAGCA-3′ and AGR2 Reverse: 5′-GCCAATTTCTGGATTTCTTTATT TTC-3′. AGR2 gene expression was quantified in all clinical samples using the relative standard curve method according to User Bulletin #2 for ABI PRISM 7700 Sequence Detection System (Applied Biosystems).

SDS–PAGE and immunoblotting
Before lysis, the cells were washed twice and lysed in 1% NP-40, 150 mM NaCl, 20 mM Tris, pH 7.4 in the presence of complete protease inhibitor cocktail (Sigma-Aldrich). A measure of 20 μg of total protein was separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) in 10% gels and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). The blotted membranes were blocked in 5% milk and 0.1% Tween 20 in PBS for 2 h at room temperature and probed overnight with specific monoclonal antibodies or rabbit polyclonal sera. The antibodies used in this study are listed below: (1) AGR2 rabbit polyclonal antibody (Moravian Biotechnology, Brno, Czech Republic); (2) ER (Clone SP1) rabbit monoclonal antibody (Lab Vision, Fremont, CA, USA) and (3) AC-40 monoclonal antibody, which recognizes actin (Sigma-Aldrich). Peroxidase-conjugated rabbit anti-mouse immunoglobulin antiserum or swine anti-rabbit immunoglobulin antiserum (Dako, Glostrup, Denmark) were used as the secondary antibody. To visualize peroxidase activity, ECL reagents from Amersham Pharmacia Biotech (Little Chalfont, UK) were used according to the manufacturer's instructions.

Chromatin immunoprecipitation
Cells were cross-linked with 1% formaldehyde for 10 min at 37 °C. The reaction was stopped with 1 M glycine. After sonication, DNA bound to proteins was immunoprecipitated with anti-ERα mouse ascites (Millipore, Temecula, CA, USA) or non-specific IgG. The purified DNA was used for qPCR to amplify the region −1324 to −1063 upstream of initiation codon of the AGR2 gene using the following primers: forward 5′-GATATCAGCTAGGCGTGGTG-3′ and reverse 5′-TGG AGTACAGTGGCACAATC-3′. The results were standardized by measuring GAPDH in the input samples.

Luciferase reporter assay
The AGR2 promoter from −1584 to +96 was amplified from 100 ng of human genomic DNA by PCR under standard conditions and cloned into the pGL3-basic plasmid (Promega, Madison, WI, USA). This pGL3-AGR2p construct was sequence verified before using for transfections. To determine the regulation of AGR2 promoter activity by ERα, cells were cotransfected with pGL3-AGR2p (firefly luciferase), Renilla luciferase control reporter (pRL-TK) and expression vector pSG5 with cloned human ERα-coding sequence (pSG5-ERα), which was kindly provided by Professor M Marin (Seccion Bioquỳmica, Facultad de Ciencias, Montevideo, Uruguay). 17-β estradiol or tamoxifen were added 12 h post-transfection and luciferase activity was measured 24 h later. The dual luciferase assay was performed according to the manufacturer's instructions (Promega).

Clinical samples and processing
Three different cohorts of patients with developed breast cancer were included. Cohort I consisted of 109 patients referred to the hospital with newly diagnosed breast cancer over the 2-year period from 2004 to 2005. The second cohort (Cohort II) consisting of 60 breast cancer samples was divided into two groups, A and B. Group A consisted of primary tumour samples (resected before adjuvant therapy) from 25 patients with ERα-positive carcinomas, who developed a locoregional and/or distant relapse during the 5-year period of adjuvant tamoxifen treatment, 16 of them had a paired sample in following group B. Group B consisted of 35 samples of ERα-positive breast carcinomas taken from locoregional relapses or distant metastases, evolving and growing during the period of adjuvant tamoxifen treatment (resected after adjuvant therapy). All specimens were received within 20 min of surgical removal according to standardized hospital protocol and immediately evaluated by a pathologist. Tissue blocks were fixed in 4% neutral formaldehyde for ∼24 h before processing into paraffin wax. Total RNA was isolated from formalin-fixed, paraffin-embedded tissue sections using the paraffin block RNA isolation kit (Ambion, Austin, TX, USA). Ethical permission was granted after review at the Masaryk Memorial Cancer Institute and all patients gave written consent. The third cohort (Cohort III) consisted of 19 pairs of breast cancer core biopsies taken before and after 14-day treatment with letrozole where AGR2 expression was previously identified as being suppressed by letrozole using microarray analyses.

Statistical methods
All *in vitro* experiments were conducted in triplicate and carried out on three or more separate occasions. Data presented are means of the three or more independent experiments ± s.e. Statistical analysis was performed using STATISTICA 7.1 (StatSoft, Tulsa, OK, USA).

Conflict of interest

The authors declare no conflict of interest.

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Příloha 5

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ORIGINAL RESEARCH

Anterior gradient protein 3 is associated with less aggressive tumors and better outcome of breast cancer patients

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Abstract: Anterior gradient protein (AGR) 3 is a highly related homologue of pro-oncogenic AGR2 and belongs to the family of protein disulfide isomerases. Although AGR3 was found in breast, ovary, prostate, and liver cancer, it remains of yet poorly defined function in tumorigenesis. This study aimed to determine AGR3 expression in a cohort of 129 primary breast carcinomas and evaluate the clinical and prognostic significance of AGR3 in these tumors. The immunohistochemical analysis revealed the presence of AGR3 staining to varying degrees in 80% of analyzed specimens. The percentage of AGR3-positive cells significantly correlated with estrogen receptor, progesterone receptor (both $P<0.0001$) as well as low histological grade ($P=0.003$), and inversely correlated with the level of Ki-67 expression ($P<0.0001$). In the whole cohort, AGR3 expression was associated with longer progression-free survival (PFS), whereas AGR3-positive subgroup of low-histological grade tumors showed both significantly longer PFS and overall survival. In conclusion, AGR3 is associated with the level of differentiation, slowly proliferating tumors, and more favorable prognosis of breast cancer patients.

Keywords: AGR3, patient survival, protein disulfide isomerase, ER-positive breast cancer, immunohistochemistry

Introduction

Breast cancer is the most common female malignancy and a leading cause of deaths among women worldwide. Only in 2012, in Europe, roughly 464,000 new incidences were registered, and 131,000 women died from breast cancer.[1] Despite intensive research on various diagnostic and/or prognostic markers, thorough understanding of factors affecting breast cancer patients' outcome remains of great importance. In recent years, an increasing number of reports have linked anterior gradient protein (AGR) 2 with many aspects of breast tumor biology. AGR2 is a human homologue of *Xenopus laevis*-secreted protein XAG-2 and belongs to an evolutionary broad family with prominent role in developmental processes and regeneration of body appendages.[2,3] There are three subfamilies of AGRs: AGR1, AGR2, and AGR3, all showing the highest homology to non-secreted protein disulfide isomerase (PDI) of the TLP19 subfamily.[3] PDIs are involved in proper folding and maturation of newly synthesized proteins and the regulation of endoplasmic reticulum homeostasis.[4]

Following the first characterization of AGR2 in the estrogen receptor (ER)-positive breast cancer cell line MCF-7,[5] AGR2 has been frequently shown as an estrogen-responsive gene/protein. It was demonstrated that AGR2 is upregulated in response to estradiol treatment both in vitro[5] and in vivo,[6] and its high expression correlates with ER status[7] and predicts poor prognosis in ER-positive breast

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cancers[8,9] as well as resistance to tamoxifen.[10] Moreover, chromatin immunoprecipitation (ChIP) confirmed direct AGR2 regulation by ER.[10–12] In normal mammary gland, AGR2 induces cell proliferation and differentiation as shown in the mouse models,[13] whereas in breast tumors, it promotes cell progression and survival through, among others, ER, cyclin D1, c-Myc, and survivin signaling pathways.[14] Furthermore, when introduced into benign rat mammary epithelial cell line, AGR2 was found to contribute to metastasis development.[15]

Closely related AGR2 homologue, AGR3,[7] has also been identified in breast cancer cell lines using proteomics screen as one of the membrane-associated proteins.[16] Although both molecules share 71% sequence identity and lie adjacent to one another at chromosomal position 7p21,[7,17] AGR2, but not AGR3, is a dominant factor identified in many OMICS screens. Thus, to date, only few reports describing AGR3 expression in various tumors were published, and there are limiting amount of data depicting AGR3 prognostic relevance in these malignancies. It has been shown that AGR3 is strongly expressed in breast carcinomas when compared to healthy tissues[16] and that its expression correlates with ER status in breast tumors.[7] In another study, single ER-binding site on *AGR3* promoter has been found using ChIP-Seq approach.[12] Our group has recently demonstrated that intrahepatic cholangiocarcinomas (ICCs) express AGR3 protein, while hepatocellular carcinomas are predominantly AGR3 negative. Furthermore, we postulated that together with acid mucopolysaccharides, AGR3 could serve as a diagnostic marker of well-differentiated ICCs.[18] It has also been shown that AGR3 is overexpressed in different histological types of ovarian cancers. In non-mucinous types (including serous papillary, endometrioid, and clear cell), AGR3 expression was found to be ER independent and uncoupled with AGR2 expression, whereas in mucinous ovarian cancers, both AGR2 and AGR3 showed cognate expression patterns.[19] In serous type, AGR3 staining correlated with the level of differentiation and was associated with longer patient survival.[20] Additionally, *AGR3* was found to be androgen-regulated gene,[21,22] expression of which was highly elevated in human prostate cancer.[21] The aim of this study is to examine the significance between AGR3 expression, clinicopathological characteristics, and patient outcome in primary breast carcinomas.

Materials and methods

Study group and tissue specimens

The study group consisted of 129 patients undergoing surgical procedure for primary breast cancer at the Masaryk Memorial Cancer Institute (MMCI) between 2003 and 2006. Patient age at the time of diagnosis ranged from 29 years to 84 years (median 57 years). The clinical, histological, and molecular characteristics of the analyzed set of tumors are summarized in Table 1. Histological typing of tumors was carried out according to the criteria of World Health Organization.[23] Tumor stage was determined according to the guidelines of the Union for International Cancer Control (UICC).[24] Tumor grade was established according to Bloom and Richardson in the modification of Elston and Ellis.[25] ER, progesterone receptor (PR), human epidermal growth factor receptor 2 (Her2/neu), and Ki-67 statuses were extracted from pathological records obtained from the MMCI database. For the evaluation of AGR3 prognostic relevance without regard to ER status, additional ER-negative group of 90 breast

Table 1 Clinicopathological characteristics of primary breast carcinomas

Variable[a]	Group	N[b]	%[c]
Histology	Ductal	95	73.6
	Lobular	18	14
	Other	9	7
	NA	7	5.4
Histological grade	G1	27	20.9
	G2	43	33.4
	G3	56	43.4
	NA	3	2.3
Tumor size	pT_1	44	34.1
	pT_2	65	50.3
	pT_3	6	4.7
	pT_4	9	7
	NA	5	3.9
Nodal status	Negative	45	34.9
	Positive	73	56.6
	NA	11	8.5
ER status	Negative	29	22.5
	Positive	100	77.5
	NA	0	0
PR status	Negative	34	26.4
	Positive	94	72.8
	NA	1	0.8
Her2/neu status	Negative	92	71.3
	Positive	36	27.9
	NA	1	0.8
Ki-67[d]	$<15\%$	55	42.6
	$\geq 15\%$	61	47.3
	NA	13	10.1
AGR3 expression	1	25	19.4
	2	25	19.4
	3	79	61.2

Notes: [a]Defined in the "Materials and methods" section. [b]Number of patients. [c]Percentage of total patients, out of a total of 129. [d]Cut-off for Ki-67 was used according to St Gallen Consensus in 2009. AGR3 expression: 1 – negative/border, 2 – weakly/moderately positive, and 3 – strongly positive.

Abbreviations: NA, not available; ER, estrogen receptor; PR, progesterone receptor; Her2/neu, human epidermal growth factor receptor 2; AGR3, anterior gradient protein 3.

cancer patients treated at MMCI between 1995 and 2006 were included for survival analysis. Informed consent has been obtained from all patients involved in this study. The study was approved by ethical committee of MMCI, and the data used were anonymized and were handled according to Czech Republic existing legislation.

Immunohistochemistry

Tumor samples were fixed in 10% neutral buffered formalin for 24 hours and then embedded in paraffin wax. Immunohistochemical analysis was performed on 4 μm thick sections cut from formalin-fixed, paraffin-embedded archival tissue blocks, mounted on slides, deparaffinized in xylene, and rehydrated in phosphate-buffered saline through a graded ethanol series. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in phosphate-buffered saline for 15 minutes. Antigen retrieval was performed in citrate buffer pH 6 at 94°C for 20 minutes. For AGR3 immunodetection, the sections were incubated overnight at 4°C with mouse monoclonal antibody to AGR3 (clone 1, in house).[19] A streptavidin–biotin peroxidase detection system was used according to the manufacturer's protocol (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA). Signal was visualized by 3,3′-diaminobenzidine (Liquid DAB+ Substrate Chromogen System; Dako Denmark A/S, Glostrup, Denmark). Nuclear counterstaining was performed with Gill's hematoxylin. For immunohistochemical evaluation, three conventional categories according to the number of positive cells were assessed: 1 – negative/border (0%–5% of positive cells); 2 – weakly/moderately positive (5%–50% of positive cells); 3 – strongly positive (more than 50% of positive cells).[26]

Reverse transcription and quantitative PCR

Under the supervision of a pathologist, corresponding samples of tumor tissue were collected and used for extraction of total cellular RNA by TRI Reagent (MRC, Cincinnati, OH, USA). cDNA synthesis was carried out using the M-MLV reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Triplicate samples were subjected to quantitative polymerase chain reaction (PCR) analysis using SYBR Green (Sigma-Aldrich, St Louis, MO, USA) for AGR2 and AGR3. The primer pairs used were as follows: for AGR2 – forward: 5′-GGAGCTCTATAT AAATCCAAGACAAGCA-3′ and reverse: 5′-GCCAAT TTCTGGATTTCTTTATTTTC-3′; for AGR3 – forward: 5′-GCCTAGAATCATGTTTGTAGACC-3′ and reverse: 5′-GCTTTCTTCATGTTTTCTATCAAT-3′. PCR was performed using default conditions: initial denaturation at 95°C, and then 40 cycles at 95°C for 15 seconds and at 60°C for 1 minute. To obtain absolute quantification, dilution series of plasmids pDEST12.2 with cloned respective sequences were used in range from 20 to 2 millions of copies to generate standard curves. For data normalization, 18S rRNA levels were determined using TaqMan assay for 18S rRNA (Thermo Fisher Scientific, Waltham, MA, USA).

Statistical analysis

All statistical analyses were performed using STATISTICA Version 12 (StatSoft, Inc., Tulsa, OK, USA) and IBM SPSS Statistics 20.0. Fisher's exact test and Pearson's chi-squared test were applied to assess the associations of immunohistochemical staining for AGR3 with clinicopathological variables. Progression-free survival (PFS) was defined as the time from the date of surgery to the date of death or relapse of disease. Overall survival (OS) was defined as the time from surgery to death or last record. Patients who had not died or who were lost to follow-up were censored when they were last known to be alive. Differences between survival curves were assessed with the Breslow test. Unadjusted hazard ratios (HRs) ±95% confidence intervals (CIs) were obtained using Cox's multivariate analysis with backward selection. Differences at $P \leq 0.05$ were considered to be statistically significant.

Results

Association of AGR3 expression with other tumor variables

Due to the high homology between AGR2 and AGR3, protein specificity of the anti-AGR3 antibody was tested (Figure S1). The analyzed cohort composed of 95 (73.6%) tumors classified as ductal breast carcinomas, 18 (14%) as lobular type, and remaining 16 (12.4%) specimens were either of different or unknown origin. The remaining clinicopathological characteristics of the study group and their distributions are summarized in Table 1. Staining of primary breast carcinomas for AGR3 varied from tumor to tumor and was mainly cytoplasmic. Overall, of the 129 cases, 25 (19.4%) were classified as negative or borderline stained for AGR3 (<5% of positive cells), and the remaining 104 (80.6%) showed AGR3 positivity to different degrees (from weak to strong) (Figure 1). Immunohistochemical staining for AGR3 was then cross-tabulated with selected tumor features including histological type, tumor size, nodal status, histological grade, ER, PR, and Her2/neu status, and Ki-67 expression level. AGR3 positivity was significantly correlated with ductal type and slowly proliferating tumors as measured by expression level of Ki-67 marker ($P<0.0001$) as well as lower tumor grade ($P<0.0001$). Moreover, the degree of staining for

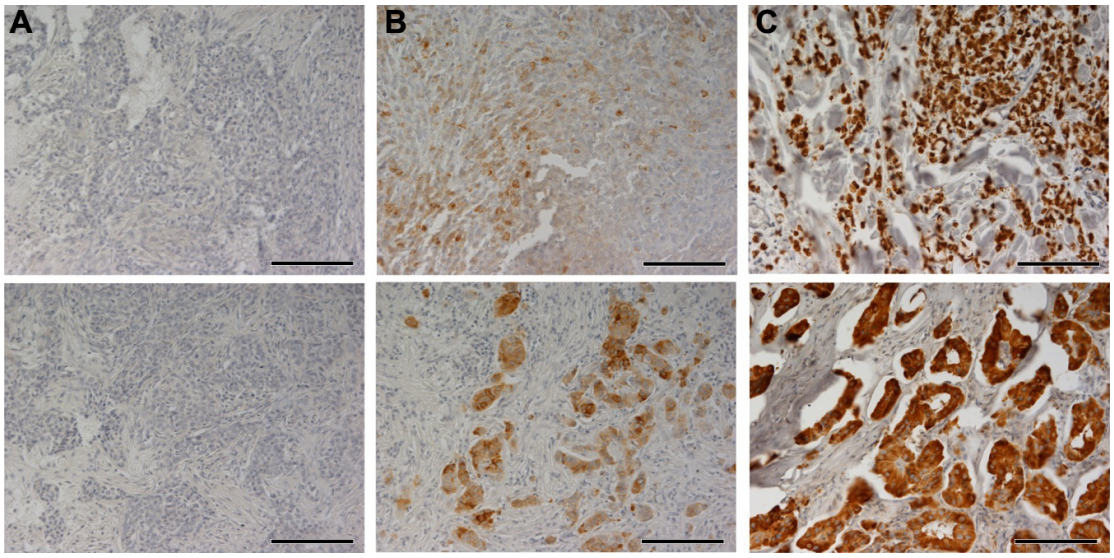

Figure 1 Immunohistochemical staining for AGR3.
Notes: The level of AGR3 expression in primary breast carcinomas was determined by immunostaining in 3-point scale: (**A**) negative or border; (**B**) weak to moderate; and (**C**) strong. Scale bars represent a length of 100 μm.
Abbreviation: AGR3, anterior gradient protein 3.

AGR3 was significantly associated with that for the ER ($P<0.0001$) and PR ($P<0.0001$). There was no significant correlation between AGR3 positivity and tumor size, nodal status, or Her2/neu status (Table 2).

AGR3 expression determined by immunohistochemistry was also compared with AGR3 mRNA levels and evaluated in relation to other clinicopathological variables. Interestingly, except Ki-67, whose elevated expression was

Table 2 Association of immunohistochemical staining for AGR3 with other tumor variables

Variable	N (%)[a]				Statistical significance
	Patients	AGR3 negative/border	AGR3 weak/moderate	AGR3 strong	
Histological grade					
G1	27	3 (11.1)	4 (14.8)	20 (74.1)	<0.0001[b]
G2	43	3 (7)	7 (16.3)	33 (76.7)	
G3	56	18 (32.1)	14 (25)	24 (42.9)	
Tumor size					
pT_1	44	8 (18.2)	9 (20.4)	27 (61.4)	0.664[b]
pT_2	65	11 (16.9)	11 (16.9)	43 (66.2)	
$pT_3 + pT_4$	6	5 (33.3)	2 (13.3)	8 (53.3)	
Nodal status					
Negative	45	10 (22.2)	10 (22.2)	25 (55.6)	0.332[c]
Positive	73	13 (17.8)	10 (13.7)	50 (68.5)	
ER status					
Negative	29	20 (69)	8 (27.6)	1 (3.4)	<0.0001[b]
Positive	100	5 (5)	17 (17)	78 (78)	
PR status					
Negative	34	19 (55.9)	9 (26.5)	6 (17.6)	<0.0001[c]
Positive	94	6 (6.4)	16 (17)	72 (76.6)	
Her2/neu status					
Negative	92	18 (19.6)	15 (16.3)	59 (64.1)	0.318[c]
Positive	36	7 (19.4)	10 (27.8)	19 (52.8)	
Ki-67					
<15%	55	6 (10.9)	9 (16.4)	40 (72.7)	<0.0001[c]
≥15%	61	14 (23.0)	14 (23.0)	33 (54.0)	

Notes: [a]Number (percentage) of patients with tumors characterized by negative/border, weak/moderate, or strong expression of AGR3. [b]Probability, *P*, from Fisher's exact test with the Freeman–Halton extension. [c]Probability, *P*, from Pearson's chi-squared test.
Abbreviations: AGR3, anterior gradient protein 3; ER, estrogen receptor; PR, progesterone receptor; Her2/neu, human epidermal growth factor receptor 2.

associated predominantly with negative or weak AGR3 expression (Table 2), we found similar trends for AGR3 on both protein and mRNA level in relation to other clinicopathological parameters (Tables 2 and S1).

We also examined AGR2 mRNA levels under the same parameters and found almost similar association between AGR2 gene expression and clinicopathological variables as seen for AGR3 (Table S1). In line with these observations, we also confirmed a strong correlation between AGR2 and AGR3 mRNA levels ($P<0.0001$, $R=0.6327$) according to Spearman Rank Order correlation. On the other hand, we also observed several statistically significant differences in the association between AGR2 expression and clinicopathological variables with respect to AGR3 indicating that the expression of these genes is similar but not identical. The evaluation of AGR2 and AGR3 mRNA levels revealed only marginal correlation of AGR2 mRNA levels with ER ($P=0.083$) in comparison with AGR3 and ER ($P<0.001$). In accordance with immunohistochemical staining ($P=0.003$), determination of AGR3 transcription levels showed significant association ($P=0.037$) with grade as well. Conversely, determination of AGR2 mRNA levels did not show this trend ($P=0.166$; Table S1).

Association of AGR3 with patient survival

For the survival analysis, follow-up was determined for 10 years since surgical removal. Median PFS was 92 months (range 1–120), and median OS was 103 months (range 1–120). As there was almost no difference in survival curves between negative/border and weak/moderate subgroups (data not shown), for further statistical analyses, the above subgroups were combined (further denoted as AGR3 "low") and were compared with patients whose tumors showed strong AGR3 positivity (more than 50% of stained cells, denoted as AGR3 "high"). While OS was not significantly affected by AGR3 expression, despite the fact that Kaplan–Meier curves indicated some trend in favor of increased AGR3 expression ($P=0.111$), these patients had significantly longer PFS ($P=0.037$) (Figure 2).

Association of AGR3 and other tumor variables with patient survival

As expected, patients with larger tumor size, higher histological grade, positive nodal status, and positive Her2/neu status had significantly poorer prognosis at 10 years of follow-up (Table S2). For multivariate survival analysis, the following clinicopathological parameters were included in Cox's model with backward selection: histological type, tumor grade, tumor size, nodal status, and ER, PR, Her2/neu, and AGR3 status. As a result, tumor size and Her2/neu status were found to be independent prognostic factors for PFS, whereas tumor size and grade reached statistical significance for OS time in the studied cohort (Table 3). The remaining clinical and histological characteristics, including AGR3, failed statistical significance and were removed from the analysis during the selection process. When further pairwised with other variables (Table S3), AGR3 positivity was associated with better outcome in the subgroup of patients with tumors defined by smaller histological grade (G≤2; OS: $P=0.005$; PFS: $P=0.024$) but not by higher histological grade (G>2;

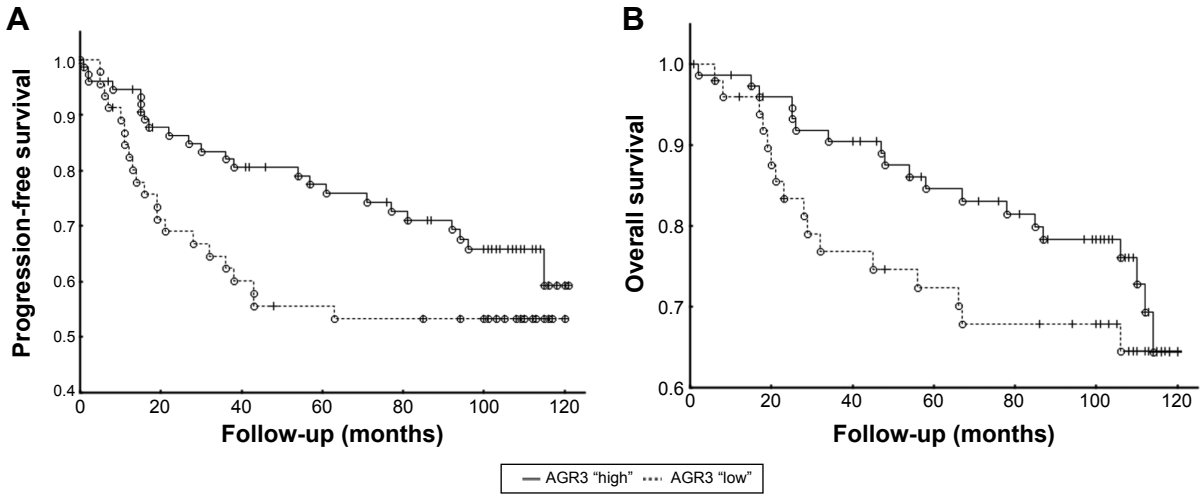

Figure 2 Association of immunohistochemical staining for AGR3 with patient survival.
Notes: (**A**) Determination of progression-free survival by Kaplan–Meier analysis in patients with "high" AGR3 expression (more than 50% of positive cells) and patients with "low" AGR3 expression (less than 50% of positive cells) using Breslow test ($P=0.037$). (**B**) Determination of overall survival by Kaplan–Meier analysis in patients with "high" AGR3 expression and patients with "low" AGR3 expression using Breslow test ($P=0.111$).
Abbreviation: AGR3, anterior gradient protein 3.

Table 3 Independent prognostic factors for the analyzed set of tumors according to Cox's multivariate survival analysis

Variable	HR	95% CI	Statistical significance
Progression-free survival			
pT_1	1.00		0.003
pT_2	1.99	0.83–4.73	0.121
pT_3	8.25	2.68–25.44	<0.0001
Her2/neu status	3.60	1.64–7.88	0.001
Overall survival			
pT_1	1.00		0.006
pT_2	1.38	0.51–3.76	0.531
pT_3	13.56	3.06–60.04	0.001
G1	1.0		0.015
G2	1.87	0.20–17.34	0.582
G3	6.38	0.82–49.42	0.076

Abbreviations: HR, hazard ratio; CI, confidence interval; Her2/neu, human epidermal growth factor receptor 2.

OS: P=0.583; PFS: P=0.945). In Her2/neu-negative set of tumors, AGR3 expression significantly correlated with longer PFS (P=0.019) as well as OS (P=0.009). On the other hand, when ER-positive cases were considered separately, AGR3 expression did not reach statistical significance for improved survival (for PFS: P=0.228; for OS: P=0.234). Therefore, the subgroup of ER- and PR-negative patients was extended to determine the impact of AGR3 on patients' outcome. However, within the additional ER-negative group of 90 patients, no significant association between AGR3 expression and patient outcome was observed as well with regard to both PFS and OS (P=0.282 and P=0.867, respectively; Figure S2). Statistical analysis of AGR3 IHC staining patterns with other clinicopathological parameters in cohort of ER- and PR-negative breast tumors revealed significant association between AGR3 expression and presence and Her2/neu status only (Table S4).

Discussion

AGR2 and AGR3 are conserved human homologues of *X. laevis* XAG-2 protein implicated in development and regeneration.[2] AGR2 and AGR3 share high-sequence homology, localize to the same chromosomal position 7p21,[7] and both respond to estrogen[12] and androgen stimulation,[21,22] which suggests their possible functional overlap. AGR2 is a well-studied pro-oncogene, promoting aggressive tumor phenotype and less favorable patient outcome in various malignancies.[27–29] On the other hand, AGR3 function in health and disease remains ambiguous, since data published so far are relatively contradictory. AGR3 expression was demonstrated in various cancers, including breast,[7] prostate,[21] ovary,[19,20] and liver.[18] Moreover, it was shown that AGR3 binds to metastasis-associated GPI-anchored C4.4a protein and extracellular alpha-dystroglycan (DAG-1)[7] and mediates resistance to cisplatin in mouse xenograft model,[19] providing clear evidence for its important involvement in tumor biology. In our descriptive study, we analyzed a cohort of 129 primary breast carcinomas in order to assess clinical and prognostic relevance of AGR3 expression. We have detected AGR3 in 104 (80%) out of 129 specimens, hence confirming previously reported predominant expression of AGR3 protein in breast tumors.[7,16] In the analyzed group, AGR3 was significantly associated with ER and PR positivity and tumor grades G≤2 but not with tumor size and nodal status, which is consistent with other studies.[7,8,30] Moreover, we observed that increase in AGR3 positivity negatively correlated with the proliferation rate defined by the level of Ki-67 expression. Notably, similar trends in relation to other clinicopathological parameters were also found for AGR3 mRNA level. Correlation with ER and PR positivity and slowly proliferating and well-differentiated tumors suggests that AGR3 expression is associated with less aggressive tumors that are more prone to effective treatment and therefore favorable outcome. Indeed, in our work, we demonstrated for the first time that the presence of immunohistochemical staining for AGR3 is associated with improved patient PFS. Although, in the whole cohort, AGR3 expression did not predict longer OS, patients whose tumors were characterized by strong AGR3 positivity showed better response to therapy. Moreover, AGR3 predicted better outcome in the subgroup of patients with well-differentiated tumors, which is consistent with previously demonstrated significance of AGR3 expression in ovarian cancers.[20] Quite the contrary, AGR2 is often described as an indicator of poor prognosis,[8,9] metastasis,[15,31] and resistance to commonly used treatments,[10,32] indicating divergent and/or context-dependent roles of AGR proteins in breast cancer. It is of note that similar antagonistic impact of AGR proteins on patient outcome is also observed in ovarian cancers where AGR3 promotes better outcome,[20] whereas AGR2 predicts shortened OS,[33] possibly due to the stimulation of cell growth and migration.[34] However, given that AGR3 was also shown to mediate cisplatin resistance, an explicit conclusion of AGR3-protective, antitumor role cannot be conclusively drawn. Moreover, in our recent work, we have compared AGRs distribution both in human healthy tissues and carcinomas using Genevestigator platform,[35] and we found that *AGR3* mirrors *AGR2* expression in many cases, such as stomach, colon, pancreas, breast, female reproductive system, or respiratory system.[36] In accordance, here, we have demonstrated strong correlation between AGR2 and AGR3 mRNA levels in breast carcinomas as well as similar

associations of both genes with clinicopathological variables, which suggests their cognate physiological function and role in pathological conditions.

In the present work, we observed that better outcome in AGR3-positive group was independent of ER status (considered separately, neither ER-positive nor negative-subgroups had significantly longer survival time when pairwised with AGR3). These findings suggest more complex control of AGR3 expression in breast carcinomas, not solely dependent on ER, similarly to that of AGR2.[8] Thus, some clues regarding AGR3 regulation could be derived from the studies focusing on AGR2 homologue. For instance, in addition to ER, AGR2 was reported to be a component of, among others, EGFR, cyclin D1, survivin, AKT, and transforming growth factor-beta signaling pathway.[14,29,37,38] However, mechanisms triggering expression of AGR2 and AGR3 could be relatively unrelated as manifested by the uncoupled expression of both proteins in prostate and ovarian cancers,[7,19] and thus, further in vitro and in vivo studies are warranted to understand AGR3's function(s) in tumor biology. Relying on our in silico analyses, we have recently shown that AGR2 and AGR3 plausibly control similar aspects of tumor biology including cell cycle control, differentiation, migration, invasion, and metastasis.[36] Additionally, we performed promoter analysis and demonstrated that most of the transcription factors potentially binding to AGR2 or AGR3 promoters are exclusive for each protein,[36] which could partially elucidate their uncoupled expression. One possible explanation of observed AGR3 ambiguity is that dependent on the cellular context, it could support different phenotypes leading either to tumor progression or to regression.

In the light of what has been reported to date, it would be necessary not only to verify whether AGR3 plays tumor-suppressive or tumor-promoting role but also to evaluate the plausible relevance of AGR3 presence in patient's fluids. AGR3 was firstly characterized in breast cancer cell membranes and was found to localize in secretory or endosome-like vesicles in both T47-D and MDA-MB-468 cells,[16] suggesting more prominent role of secreted form of AGR3. Indeed, recent works have depicted emerging role of extracellular AGR2 in the control of tumor aggressiveness through both autocrine and paracrine effects,[39,40] indicating that similar mechanism can also be valid for AGR3. Lastly, taking into account cognate expression pattern of AGR proteins in different carcinomas,[7,19] it can be speculated that there is a functional cross talk between these proteins. However, whether they compete with each other, compensate for one's lost, or support one another requires further investigation.

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Disclosure

The authors declare that they have no competing interest.

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Supplementary materials

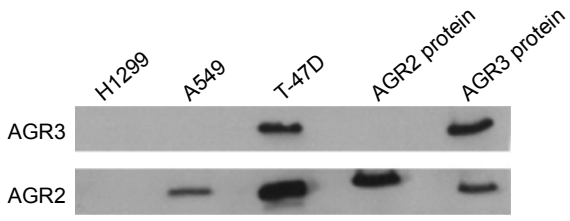

Figure S1 Determination of anti-AGR3 antibody cross-reactivity.
Notes: The specificity of our in-house anti-AGR3 antibody (AGR3.1) was confirmed by Western blot (upper panel). For comparison, we added testing of rabbit polyclonal sera raised against AGR2 protein, which recognizes AGR3 as well (bottom panel).
Abbreviation: AGR, anterior gradient protein.

Table S1 Association of AGR2 and AGR3 mRNA levels with other tumor variables

	Patients (n)	AGR2 mRNA	AGR3 mRNA
Histological grade			
G1	26	0.166[a]	0.037[a]
G2	33		
G3	34		
Tumor size			
pT_1	35	0.774[a]	0.990[a]
pT_2	50		
$pT_3 + pT_4$	8		
Nodal status			
Negative	37	0.822[b]	0.541[b]
Positive	52		
ER status			
Negative	17	0.081[b]	<0.001[b]
Positive	76		
PR status			
Negative	19	0.124[b]	<0.001[b]
Positive	73		
Her2/neu status			
Negative	57	0.364[b]	0.603[b]
Positive	36		
Ki-67			
<15%	44	0.169[b]	0.494[b]
≥15%	48		

Notes: [a]Determination of *P*-level using Kruskal–Wallis analysis of variance. [b]Determination of *P*-level using Mann–Whitney *U*-test.
Abbreviations: AGR, anterior gradient protein; ER, estrogen receptor; PR, progesterone receptor; Her2/neu, human epidermal growth factor receptor 2.

Table S2 Univariate survival analyses for the analyzed set of tumors

Variable[a]	Statistical significance[b]	
	PFS	OS
AGR3 expression	0.037	0.111
Histology		
DU vs LO	0.665	0.393
DU vs OTH	0.537	0.980
LO vs OTH	0.851	0.675
Grade		
G1 vs G2	0.159	0.137
G1 vs G3	0.002	0.009
G2 vs G3	0.020	0.055
Tumor size		
pT1 vs pT2	0.249	0.333
pT1 vs pT3	0.000	0.000
pT2 vs pT3	0.000	0.000
Nodal status	0.043	0.415
ER status	0.110	0.344
PR status	0.023	0.145
Her2/neu status	0.000	0.000
Ki-67 expression	0.004	0.018

Notes: [a]AGR3 expression, AGR3 "low" (less than 50% of stained cells) vs AGR3 "high" (more than 50% of stained cells); histology, ductal vs lobular vs others; nodal status, negative vs positive; estrogen receptor status, negative vs positive; progesterone receptor status, negative vs positive; Her2/neu status, negative vs positive; Ki-67 expression, <15% vs ≥15%. [b]Probability, *P*, from Breslow test.
Abbreviations: PFS, progression-free survival; OS, overall survival; AGR3, anterior gradient protein 3; DU, ductal; LO, lobular; OTH, others; ER, estrogen receptor; PR, progesterone receptor; Her2/neu, human epidermal growth factor receptor 2.

Table S3 Survival analysis of patients with AGR3-expressing tumors

Subgroup	Statistical significance[a]	
	PFS	OS
Histological grade		
G≤2	0.024	0.005
G>2	0.945	0.583
Her2/neu status		
Negative	0.019	0.009
Positive	0.781	0.278
PR status		
Negative	0.669	0.911
Positive	0.448	0.224
ER status		
Negative	0.431	0.507
Positive	0.228	0.234

Note: [a]Probability, *P*, from Breslow test.
Abbreviations: AGR3, anterior gradient protein 3; PFS, progression-free survival; OS, overall survival; Her2/neu, human epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor.

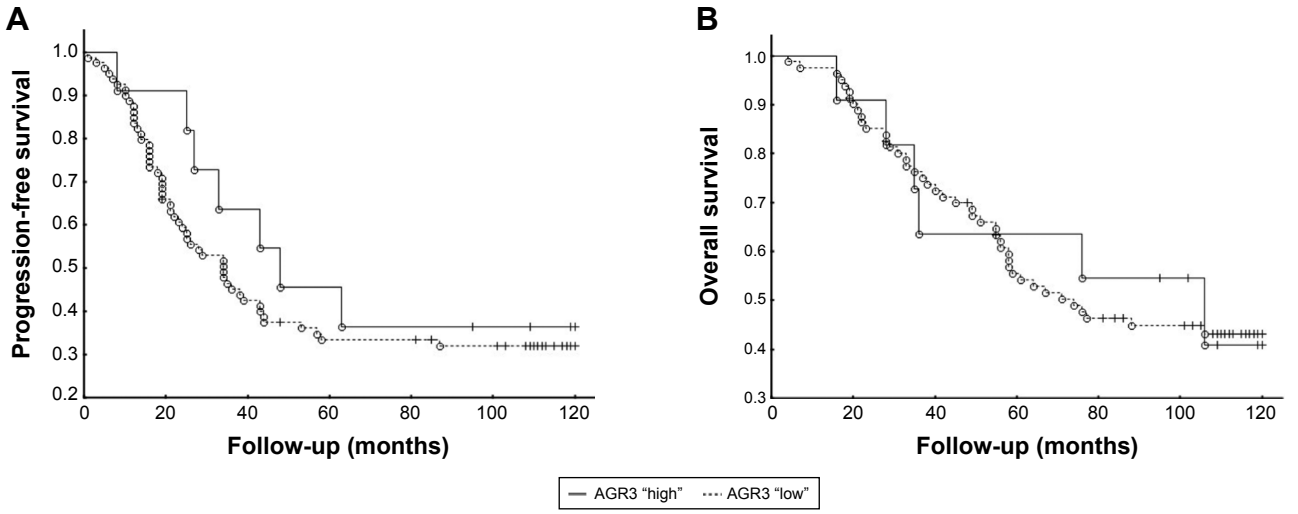

Figure S2 Survival analysis of cohort of ER- and PR-negative breast cancer patients.
Notes: Kaplan–Meier analysis of (**A**) progression-free survival in relation to AGR3 expression (P=0.282, Breslow test) and (**B**) overall survival in relation to AGR3 expression (P=0.867, Breslow test).
Abbreviations: ER, estrogen receptor; PR, progesterone receptor; AGR3, anterior gradient protein 3.

Table S4 Association of immunohistochemical staining for AGR3 with other tumor variables in a cohort of ER-negative breast cancer patients

Variable	N (%)[a]				Statistical significance[b]
	Patients	AGR3 negative/border	AGR3 weak/moderate	AGR3 strong	
Histological grade					
G1	1	1 (100.0)	0 (0.0)	0 (0.0)	0.917
G2	10	6 (60.0)	3 (30.0)	1 (10.0)	
G3	71	47 (66.2)	15 (21.1)	9 (12.7)	
Tumor size					
pT_1	32	24 (75.0)	5 (15.6)	3 (9.4)	0.729
pT_2	44	26 (59.1)	11 (25.0)	7 (15.9)	
$pT_3 + pT_4$	11	8 (72.8)	2 (18.2)	1 (9.0)	
Nodal status					
Negative	34	25 (73.5)	7 (20.6)	2 (5.9)	0.282
Positive	53	31 (58.5)	13 (24.5)	9 (17.0)	
Her2/neu status					
Negative	40	34 (85.0)	5 (12.5)	1 (2.5)	0.001
Positive	49	24 (49.0)	15 (30.6)	10 (20.4)	
Ki-67					
<15%	6	4 (66.7)	2 (33.3)	0 (0.0)	0.626
≥15%	42	30 (71.4)	7 (16.7)	5 (11.9)	

Notes: [a]Number (percentage) of patients with tumors characterized by negative/border, weak/moderate, or strong expression of AGR3. Probability, [b]P, was calculated using Fisher's exact test with the Freeman–Halton extension.
Abbreviations: AGR3, anterior gradient protein 3; ER, estrogen receptor; Her2/neu, human epidermal growth factor receptor 2.

Příloha 6

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Clinical Study

AGR2 Predicts Tamoxifen Resistance in Postmenopausal Breast Cancer Patients

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Endocrine resistance is a significant problem in breast cancer treatment. Thus identification and validation of novel resistance determinants is important to improve treatment efficacy and patient outcome. In our work, AGR2 expression was determined by qRT-PCR in Tru-Cut needle biopsies from tamoxifen-treated postmenopausal breast cancer patients. Our results showed inversed association of AGR2 mRNA levels with primary treatment response ($P = 0.0011$) and progression-free survival ($P = 0.0366$) in 61 ER-positive breast carcinomas. As shown by our experimental and clinical evaluations, elevated AGR2 expression predicts decreased efficacy of tamoxifen treatment. From this perspective, AGR2 is a potential predictive biomarker enabling selection of an optimal algorithm for adjuvant hormonal therapy in postmenopausal ER-positive breast cancer patients.

1. Introduction

Breast cancer is the most common women's malignancy, with growing incidence primarily in advanced countries. Despite improvements in treatment, 30–40% of women are diagnosed with metastatic cancer or develop metastases and die from their disease [1]. The most important group of breast cancers is hormone sensitive tumors, characterized by expression of estrogen and progesterone receptors (ER and PgR). These tumors encompass approximately 70% of all breast cancers and are significantly clinicopathologically different from ER-negative tumors. Thus, determination of ER status is an essential part of the diagnostic procedure in breast cancer patients. The presence of ER and PgR indicates response to endocrine therapy and improved disease-free survival [2, 3]. The treatment of choice represents tamoxifen, which has been used for systemic treatment for all stages of ER-positive breast cancer during the past 30 years. Despite the undeniable benefit, approximately one third of patients with ER-positive breast cancer either do not respond to tamoxifen or develop resistance, which constitutes a serious clinical problem. Thus, identification of novel, reliable, and easily identifiable biomarkers indicating resistance to this drug is of general interest.

Recent findings suggest that AGR2 plays a prominent role in mediating pro-oncogenic signals of ER, and there is a correlation between increased AGR2 expression and poor outcome of therapy in patients with ERα-positive breast cancers [4, 5]. Historically, AGR2 mRNA was discovered as selectively expressed in ER-positive breast cancer cell lines [6]. AGR2 has been functionally characterized and shown to act as an inhibitor of the tumor suppressor p53 [7] and a mediator of metastatic spread in rodent models [8]. Data from Wang et al. have shown that AGR2 can also transform cells and mediate cell migration [9]. In our previous work, we found that AGR2 mediates a prosurvival pathway in human breast cancer cells and is involved in pro-oncogenic signals of ER. More strikingly, AGR2 expression was elevated both *in vitro* and *in vivo* in response to tamoxifen adjuvant therapy, indicating that AGR2 mediates an agonist effect of this drug [4, 10]. Although the mode of action of AGR2 after tamoxifen treatment remains to be defined, we hypothesized that AGR2 may significantly affect the development and

progression of hormone sensitive breast tumors and response to anti-hormonal treatment.

2. Material and Methods

2.1. Clinical Samples and Processing. Our retrospective study includes 61 Tru-Cut needle biopsies from ER-positive invasive breast carcinomas of postmenopausal patients who received tamoxifen as primary treatment at the Masaryk Memorial Cancer Institute (MMCI) during the period 2000–2004. These patients due to advanced age (median 79 years) or comorbidities could not take any other primary treatment for their disease. More than half of the patients had locally advanced disease, which on one hand facilitated the assessment of treatment response; on the other hand, complications associated with advanced tumor resulted in the addition of local radiotherapy in 15 patients and a surgical solution in 1 patient. Detailed characteristics of the patients are given in Table 1. Biopsies were fixed in 10% formalin, embedded in paraffin wax, and stained with hematoxylin/eosin for histological examination. Clinical data including response to therapy were evaluated by oncologist from the hospital's patient records. The study was approved by the local Ethical Commission and informed consent was obtained from each patient.

2.2. Treatment Evaluation. The best response recorded during the primary treatment was used for response analyses. Patients who achieved complete or partial response (reduction of disease by 30% or more) or had long-lasting disease stabilization (stable disease for at least 33 months; median of PFS) were classified as responders. Patients who never responded to primary treatment or achieved stable disease for less than 12 months were classified as nonresponders. Tumor response to tamoxifen treatment was evaluated using mammography or ultrasound. Patients whose general health status or disease state (e.g., extensive T4 tumors) did not allow these tests were examined using caliper and palpation of regional lymph nodes. Progression-free survival (PFS) was measured from the first day of tamoxifen therapy until progression or death from any cause occurred. Patients who were alive and who had not experienced disease progression, or who were lost to follow up, were censored at the date that they were last known to be alive and progression-free. Overall survival (OS) was measured from the date of diagnosis until death from any cause. Patients who have not died or who were lost to follow-up were censored when they were last known to be alive.

2.3. Reverse Transcription and Quantitative PCR. Under the supervision of an experienced pathologist, corresponding samples of tumor tissue were collected and used for extraction of total cellular RNA by TRI Reagent (MRC, Cincinnati, OH, USA). cDNA synthesis was carried out using the M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Triplicate samples were subjected to quantitative PCR analysis using TaqMan for 18S rRNA (Applied Biosystems, Foster City, CA, USA) and SYBR Green (Sigma-Aldrich, St Louis, MO, USA) for AGR2 and GAPDH. The primer pairs used for AGR2 were as follows: forward: 5′-GGAGCTCTATATAAATCCAAGACAAGCA-3′ and reverse: 5′-GCCAATTTCTGGATTTCTTTATTTTC-3′, and the primer pairs for GAPDH were as follows: forward: 5′-GAAGGTGAAGGTCGGAGTC-3′ and reverse: 5′-GAAGATGGTGATGGGATTTC-3′. PCR reaction was performed using default conditions: initial denaturation 95°C, then 40 cycles 95°C 15 sec and 60°C 1 min. To obtain absolute quantitation, dilution series of plasmids pDEST12.2 with cloned respective sequences were used in range from 2 to 20 million of copies to receive standard curves. Two different housekeeping genes 18S rRNA and GAPDH were used, to confirm experimental setup and homogeneity of biopsy samples. The results obtained in both cases were fully comparable.

Table 1: Characteristics of patients.

	Total (n = 61)	%
Age (years)		
Median (range)	79 (62–93)	
Age >70	54	89
Performance status		
Karnofsky index ≤70%	32	52
Histology		
Invasive ductal	52	85
Invasive lobular	9	15
Clinical stage		
I	2	3
II	22	36
III	27	44
IV	10	17
T4 tumors	33	54
N+	48	79
Primary treatment		
Hormonal therapy	61	100
Tamoxifen only	54	89
Tamoxifen switched to AI*	7	11
Radiation therapy	15	25
Surgery	1	2
Initial response to primary treatment		
Responders**	48	79
Complete response (CR)	6	10
Partial response (PR)	39	64
Stable disease (SD)	3	5
Nonresponders***	13	21
Progressive disease (PD)	9	15
Stable disease (SD)	4	6

Annotations. *Switch of the tamoxifen to an aromatase inhibitor was carried out due to tamoxifen's side effects (mostly endometrial hyperplasia). All seven patients achieved partial remission in the course of tamoxifen therapy.
**Patients who achieved complete or partial (reduction of disease by 30% or more) remission or had long-lasting disease stabilization (stable disease for at least 33 months; median of PFS).
***Patients who never responded to primary treatment or achieved stable disease for less than 12 months during the period of primary treatment.

2.4. Immunohistochemical Staining. Immunohistochemical (IHC) staining was performed on 4 μm thick freshly cut tissue sections. Sections were deparaffinized in xylene and rehydrated into PBS through a graded ethanol series. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in PBS for 15 minutes. Antigen retrieval was performed in citrate buffer pH 6 in 94°C for 20 minutes. The sections were incubated overnight at 4°C with either anti-AGR2 antibody (HPA007912, Sigma-Aldrich, St. Louis, MO, USA) or SP1 antibody against ER or SP2 antibody against progesterone receptor (both Lab Vision and NeoMarkers, Fremont, CA, USA). A streptavidin-biotin peroxidase detection system was used according to the manufacturer's instructions (Vectastain Ellite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Signal was visualized by 3,3′-diaminobenzidine (Liquid DAB+ Substrate Chromogen System, Dako, Glostrup, Denmark). Tissue sections of small intestine and lymph node served as external positive and negative controls for AGR2.

2.5. Statistical Analysis. Fisher's exact test was used to derive P values from the 2×2 contingency tables. Survival analyses were performed by Kaplan-Meier method, and the differences between the survival curves were evaluated by the log-rank test to determine statistical significance levels. In all cases, a P value less than 0.05 was considered significant. The MedCalc Version 9.3.9.0 (MedCalc Software, Ostend, Belgium) was used for all calculations.

3. Results

The study included 61 postmenopausal ER-positive breast cancer patients receiving tamoxifen in first line of therapy. In particular, there were patients who could not undergo surgery due to their advanced age on date of diagnosis ranging from 62 to 93 years with median age 79 and average age 78.2 years or comorbidities (more than 52% of patients had the Karnofsky index 70% or less). More than half of the cases showed significantly locally advanced disease (54% T4 tumors, 79% had proven involvement of regional lymph nodes), which facilitated the evaluation of response to treatment. Systemic hormonal treatment by tamoxifen was accompanied by radiotherapy in 15 patients and mastectomy in 1 patient due to assessment of local tumor extent. Breast cancer was diagnosed in a fourth clinical stage in 10 patients; however, seven of them showed bone involvement only. In total, 48 patients (79%) were classified as "responders" who benefited from tamoxifen; most frequently partial regression of disease was detected. The remaining 13 patients (21%) did not respond to treatment and were classified as "nonresponders". Detailed characteristics of the patients are given in Table 1.

AGR2 mRNA expression showed an exponential distribution ($P < 0.01$; Kolmogorov-Smirnov test) within the studied group of patients. Thus AGR2 mRNA expression was defined according to the median, where tumors with levels below the median were classified as the low expression group, and tumors with expression above the median were classified as high expression. There is an inverse relationship between AGR2 mRNA expression and response to tamoxifen ($P = 0.0011$; Fisher's exact test) (Figure 1). In parallel, immunohistochemical staining was used to determine AGR2 expression on protein level (Figure 2). IHC staining confirmed AGR2 expression in all tested samples. IHC score was calculated as the sum of the percentage of cells with weak, moderate, and strong staining with a range from 0 to 300. This scoring system has been used as it takes into account both extent of reactivity and intensity [11, 12]. Due to IHC score data distribution, ROC analysis was used to determine cut-off level for "low" and "high" AGR2 levels. Nevertheless, no significant association with treatment response was found ($P = 0.3183$; Fisher's exact test).

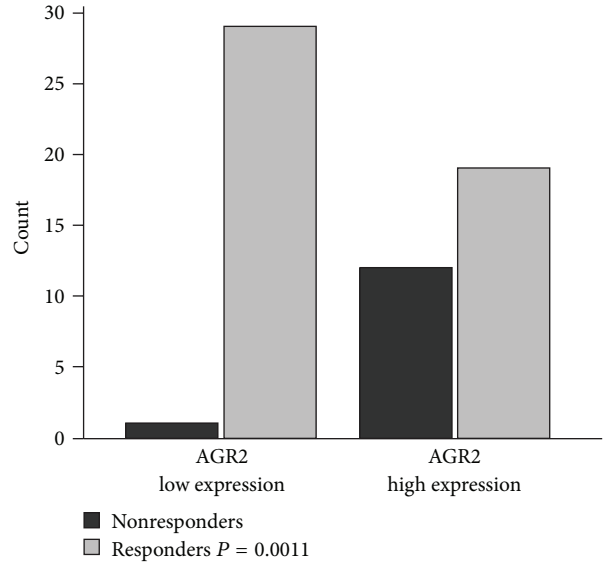

Figure 1: Numbers of postmenopausal breast cancer patients who responded or did not respond to primary tamoxifen treatment in relation to AGR2 expression.

Disease progression was observed in 36 patients during or after primary treatment. Second line of therapy with aromatase inhibitors was applied in 33 of these patients. Median progression-free survival (PFS) reached 33.5 months and overall survival (OS) 52.3 months in the whole cohort of patients. Patients with low AGR2 mRNA expression showed significantly longer PFS compared to cases with elevated AGR2 expression (HR 0.57; 95% CI: 0.31 to 0.96; $P = 0.0366$; Figure 3(a)). Although there was a similar trend between AGR2 mRNA expression and OS, the data were not statistically significant (HR 0.69; 95% CI: 0.38–1.18; $P = 0.1655$). As expected, neither PFS (HR 1.34; 95% CI: 0.69 to 2.75; $P = 0.3569$; Figure 3(b)) nor OS (HR 1.31; 95% CI: 0.68 to 2.66; $P = 0.3985$) did correlate significantly with the level of AGR2 determined by IHC.

4. Discussion

Tamoxifen resistance in breast cancer patients represents the main problem limiting treatment efficacy. We previously showed in consecutive group of ER-positive breast carcinomas that elevated levels of AGR2 mRNA predict significantly shorter disease free survival [4]. Nevertheless, the direct effect

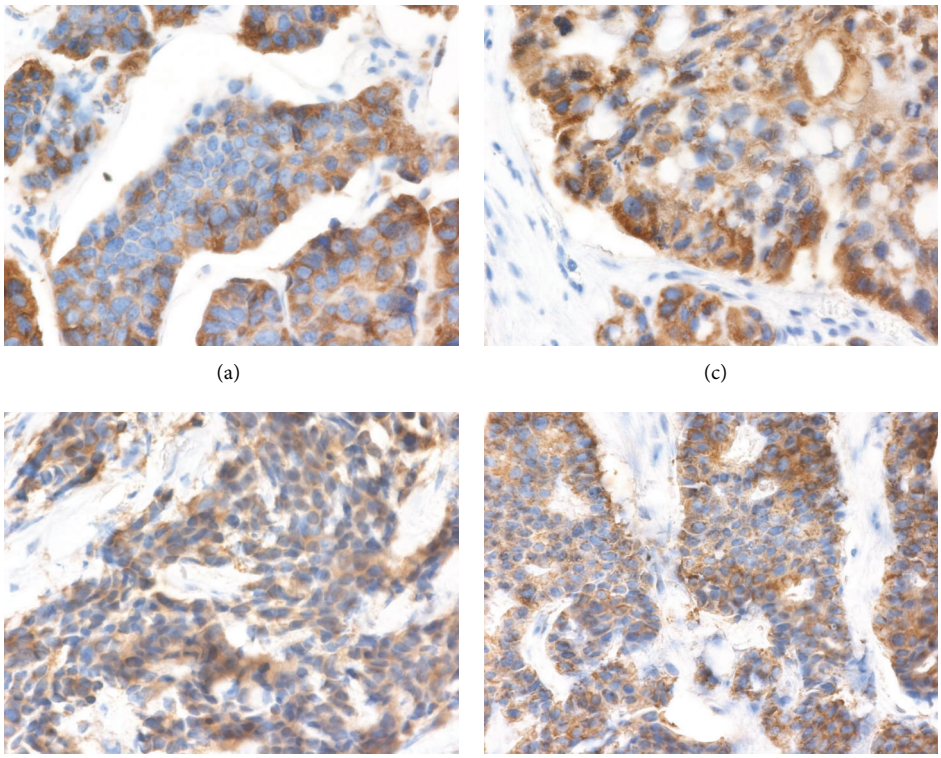

Figure 2: Illustration of immunohistochemical detection of AGR2 protein in Tru-Cut needle biopsies. (a) IHC score 80, (b) IHC score 100, (c) IHC score 180, and (d) IHC score 220. As cut-off level determined by ROC analysis was 110, samples (a) and (b) were classified in group denoted as "low" and AGR2 expression and samples (c) and (d) were included in group denoted as "high" AGR2 expression.

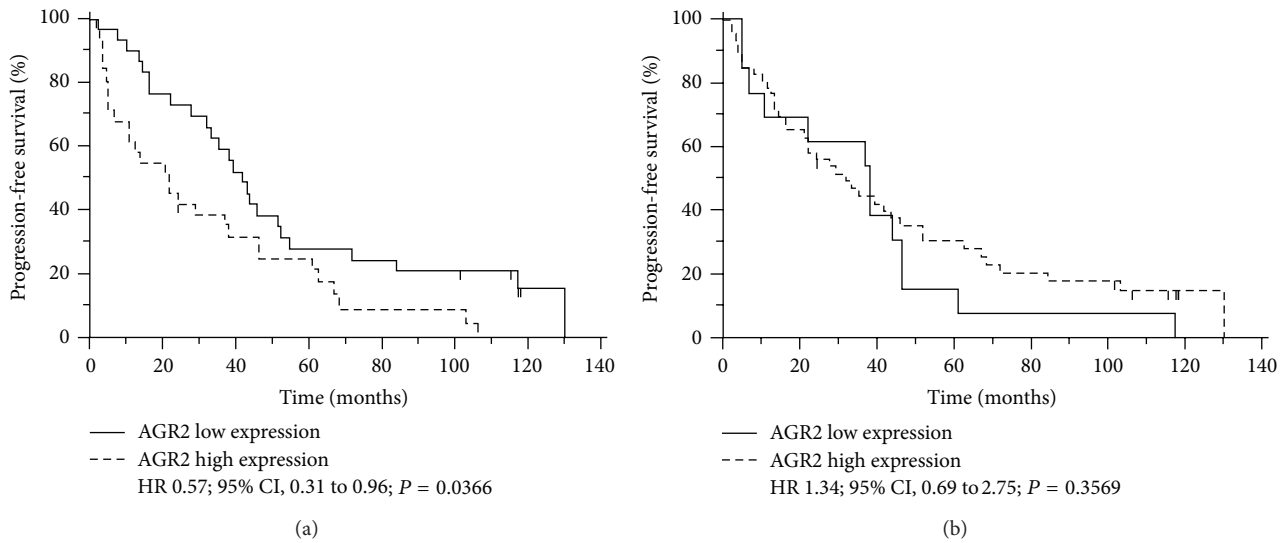

Figure 3: Progression-free survival of patients with low or high AGR2 tumor expression is determined using Kaplan-Meier curves. (a) Determination of PFS with respect to AGR2 mRNA levels. There were 4 censored observations in survival curve for low AGR2 mRNA expression and 2 censored observations in survival curve for high AGR2 mRNA level. (b) Determination of PFS with respect to AGR2 IHC staining. There were no censored observations in survival curve for low AGR2 IHC level and 6 censored observations in survival curve for high AGR2 IHC level. Hazard ratio (HR) with 95% CI as well as P value calculated using log-rank test is provided for both curves.

of AGR2 overexpression on the sensitivity to treatment with tamoxifen needs more precise characterization. Thus, in the present work, we have investigated the expression of AGR2 in a cohort of inoperable postmenopausal breast cancer patients with respect to response to tamoxifen treatment.

We confirmed that breast cancer patients with low AGR2 mRNA expression more readily respond to primary treatment by tamoxifen compared with tumors exhibiting AGR2 overexpression (Figure 1). These data are supported by determination of PFS showing more favorable outcome in patients with decreased AGR2 mRNA levels, which underscores the importance of AGR2 as a predictive biomarker. On the other hand, statistical analysis of OS proved to be insignificant, although survival curves showed similar tendencies as PFS, which may be due to (i) old age associated with increased mortality not only due to cancer and/or (ii) the effect of second or next line of endocrine therapy using aromatase inhibitors in progressing patients.

Although the correlation between AGR2 mRNA and protein levels has been demonstrated by previous studies [4, 5], IHC staining in our cohort of patients did not show statistical association between AGR2 protein level and response to tamoxifen treatment due to semiquantitative character of the IHC staining. This is also supported by the fact that in comparison with AGR2 mRNA levels, where cut-off levels determined by median and ROC analysis were more and less the same, ROC analysis for IHC staining calculated significantly different cut-off compared to median. It is important to take into account that our cohort of sample biopsies consists of ER-positive breast carcinomas only, which show elevated AGR2 levels. IHC staining sensitivity seems to be insufficient to distinguish modest differences in AGR2 levels in our group of tumor samples. This is in fact supported by examples of IHC staining in Figure 2 showing very similar levels of AGR2 in more than half of all samples (IHC score: 80–220) within both groups divided according to AGR2 at "low" and "high". On the other hand, we positively confirmed association of ER with AGR2 expression on both mRNA and protein levels *in vivo* since AGR2 was detected in all analyzed samples. In particular, we have shown that the increased expression of AGR2 detectable on mRNA level may reflect increased transcriptional activity of ER linked to tamoxifen agonistic effect predicting worse response to tamoxifen treatment [13].

Although ER expression itself is the main predictor of response to endocrine therapy, crosstalk between ER and other signaling pathways involved in regulation of cellular growth, survival, stress, and cytokine levels has been mechanistically described in resistance to endocrine agents. The clinical relevance of ER crosstalk with growth factor signaling pathways was confirmed by prospective trials in patients with metastatic disease, showing that tamoxifen resistance is associated with high expression of receptor tyrosine kinases HER2 and EGFR [14]. Interestingly, recent reports indicate that AGR2 is involved in the crosstalk between ER and EGFR [15] or PI3K/AKT [16] resulting in endocrine resistance, providing a potential mechanistic basis for our observations.

The identification of novel predictive biomarkers is essential for personalized endocrine therapies. Our data indicate that AGR2 may serve as one such biomarker with decreased AGR2 mRNA levels identifying a subset of postmenopausal breast cancer patients who respond and have clearly benefit from tamoxifen-based therapy. On the other hand, our data suggest that high AGR2 mRNA levels may predict a subset of postmenopausal breast cancer patients that are less likely to show adequate tumor growth control following tamoxifen therapy and for whom other options may therefore be more appropriate.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Identification of an AKT-dependent signalling pathway that mediates tamoxifen-dependent induction of the pro-metastatic protein anterior gradient-2

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ABSTRACT

The pro-metastatic protein anterior gradient-2 (AGR2) was previously demonstrated as a predictive factor of poor response to tamoxifen treatment. In this study we aimed to delineate the key signalling pathway that may contribute to regulation of AGR2 protein induction in order to identify novel targets to overcome tamoxifen resistance in tumour cells. Together, our data identify PDPK1-AKT as a pro-oncogenic signalling pathway that triggers AGR2 protein induction in response to tamoxifen and suggest that AKT inhibitors could be used as part of a therapeutic strategy to treat tamoxifen resistant, AGR2 over-expressing cancers.

1. Introduction

Cancer is often referred as a "disease of genes", which is manifested by two main characteristics: (i) uncontrolled growth of the cells and (ii) the ability of these cells to migrate from the original site and spread to distant sites. Importantly, cancer is not just one disease but a broad spectrum of diseases. There are more than 100 different types of cancer and within each cancer type we encounter high variability which can impede effective treatment. Thus it is becoming clear that there is a growing requirement to identify new biomarkers which enable the better characterisation of particular tumour types and more efficient targeted therapeutic strategies.

Comprehensive analysis of known oncogenic signalling pathways, deeper understanding of molecular mechanisms responsible for drug resistance and the associated identification of novel biomarkers represent one of the most important challenges of recent oncological research. In our previous work, we focused on anterior gradient-2 protein and its role in breast cancer signalling in response to tamoxifen treatment. AGR2 was originally identified as an oestrogen-receptor inducible gene and as a potential secretory protein that is highly expressed in *Xenopus* eggs [1] and subsequently as protein important for limb regeneration [2]. Clinical genomics and transgenic studies have also implicated AGR2 in inflammatory bowel disease [3,4] and asthma [5]. Recent studies in cancer cells demonstrated a role for AGR2 in cell migration, cellular transformation and drug resistance [6–8]. In addition it has been shown to function as a p53 inhibitor [9] and activator of EGFR [10]. Further clinical studies confirmed that AGR2 is over-expressed in hormone-dependent cancers such as breast and ovarian [11,12]; it is also over-expressed in a range of hormone independent cancers [13,14], and the over-expression of this protein usually predicts poor prognosis of the disease [15,16]. Xenograft studies have shown that *AGR2* or *AGR3* gene expression can mediate cisplatin resistance in p53-negative cancer cell lines [17–19] indicating that these protein disulphide-isomerases can reprogramme the proteome of the cell to regulate cancer cell survival. However, the molecular mechanisms underlying these wide-ranging cancer linked pathways, where AGR2 plays a role, are still not fully defined.

Although there is very little known about the physiological factors that regulate the AGR2 pathway in breast cancers, it has been shown that *AGR2* gene expression is induced by oestrogen [12] and in primary breast cancer tissue it is linked with oestrogen-receptor expression [6,20]. In analysing the effects of oestrogen on *AGR2*

Abbreviations: AGR2, anterior gradient-2; ER, oestrogen receptor; IGFR, insulin-like growth factor receptor; HER2, human epidermal receptor 2; EGFR, epidermal growth factor receptor; PDPK1, phosphoinositide-dependent protein kinase 1.

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expression in breast cancer derived cell lines, we found that AGR2 expression at both the mRNA and protein level is induced by the oestrogen antagonist tamoxifen [15,18]. These data suggest that the AGR2 protein represents an agonist effector of tamoxifen and that treatments that attenuate expression of the pro-survival functions of the AGR2 pathway, like the aromatase-inhibitor letrozole [15], might provide a more effective therapeutic strategy for sensitising breast cancer cells to anti-hormonal therapy.

A small molecule kinase inhibitory library coupled with high-throughput genetic screen, using an RNA interference library was carried in an attempt to identify the kinase signalling pathways implicated in resistance to tamoxifen [21]. In this study, Iorns et al. identified inhibitors of the phosphoinositide-dependent protein kinase 1-AKT (PDPK1-AKT) pathway triciribine and tetrandrine, as the most potent sensitisers to tamoxifen treatment. Having dissected the effects of tamoxifen on AGR2, we focused on analysis of the link between regulation of AGR2 and the PDPK1-AKT pathway. We found that AGR2 induction by tamoxifen is mediated by a PDPK1-AKT signalling pathway whose inhibition can sensitise cells to tamoxifen. To our knowledge this has not been previously observed and may have important implications for endocrine treatment with respect to the potential pro-oncogenic effect of tamoxifen in breast cancer patients showing elevated expression of AGR2.

2. Materials and methods

2.1. Cell biology and cellular manipulations

FBS (foetal bovine serum), DMEM (Dulbecco's modified Eagle's medium) were supplied by Gibco (Life Technologies, Carlsbad CA). Trypsin/EDTA solution and Lipofectamine™ 2000 were supplied by Invitrogen (Life Technologies, Carlsbad CA). HRP (horseradish peroxidase)-conjugated secondary antibodies were supplied by Dako (Dako, Glostrup, Denmark). Triciribine, tetrandrine, and Akti-1/2 were obtained from Enzo Life Sciences (EI-333), Enzo Life Sciences (CA-260) (Enzo Life Sciences Inc., Farmingdale, NY), and Calbiochem (124018) (Calbiochem, San Diego, CA), respectively. MCF-7 breast cancer cells were cultured in complete DMEM containing phenol red supplemented with 10% FBS in a humidified incubator in 5% CO_2 at 37 °C. Cells were plated at a density of 1.5×10^5 cells per well in 6-well tissue culture plates (Nunc, Roskilde, Denmark) 1 day prior to treatment. Tamoxifen was dissolved in DMSO to give a concentration of 1 mM. For treatment of cells this was diluted in DMEM with 10% FBS to give a final concentration as indicated in the figure legends. After this time cells were harvested into ice cold PBS and frozen in liquid nitrogen prior to lysis.

For siRNA treatment, cells were plated as described above and at the same time as the initial tamoxifen treatment cells were also transfected with siRNA. The on-target siRNAs from Dharmacon (Dharmacon, Inc., Chicago, IL) included: Control (D-001206-14-20), AGR2 (J-003626-05) or PDPK1 (M-003017-02). AKT siRNA was supplied by Cell signalling (#6211) (Cell Signaling Technology, Inc., Danvers, MA). siRNA was added to cells according to manufacturer's guidelines at a final concentration of 100 nM. Culture medium was changed after 48 h (without the addition of fresh siRNA) and cells were harvested into ice cold PBS after a total incubation period of up to 96 h.

2.2. Clinical samples and processing

In this study, tissue microarrays were analysed from a cohort of 94 breast cancer patients referred to the Masaryk Memorial Cancer Institute (Brno, Czech Republic) with newly diagnosed HER2/neu positive breast cancer during 2001–2009. All specimens were received within 20 min of surgical removal according to standardised hospital protocol and immediately evaluated by a pathologist. Tissue blocks were then fixed in 4% neutral formaldehyde for approximately 24 h before processing into paraffin wax. The tissue microarrays were constructed from routinely prepared formalin-fixed-paraffin-embedded tissue blocks. Two 1.8 mm cores from each sample were mapped into a tissue array blocks. Sections were cut at a thickness of 4 mm and collected onto positively charged slides (Superfrost plus, Thermo Scientific, Waltham, MA) for immunohistotochemical staining (IHC). To determine AKT and HER2/neu phosphorylation, we used phospho-AKT Ser473 and Phospho-HER2/neu Tyr1221 antibodies (both Cell Signaling Technology, Inc., Danvers, MA). Clinical data for each sample were reviewed retrospectively from medical records. Statistical analysis was performed using STATISTICA 9.1 (StatSoft, Inc., Tulsa, OK). Ethical permission was granted following review at the Masaryk Memorial Cancer Institute and all patients gave written consent.

2.3. Clonogenic cell assays

Colony formation assays were developed by transfecting the MCF-7 cells using Genejuice (Merck KGaA, Darmstadt, Germany) with 1 μg of the construct, which contains miRNA responsible for silencing of AGR2 expression [15]. The single-stranded DNA oligonucleotides targeting AGR2 were designed using RNAi Designer (Invitrogen, Life Technologies, Carlsbad, CA). The sequences are:

Forward 5′TGCTGATATGTCTGAGTCCAGATGAGGTTTTGGCCACTGACTGACCTCAT CTGCTCAGACATAT-3′ and Reverse 5′CCTGATATGTCTGAGCAGATGAGGTCAGTCA GTGGCCAAAACCTCATCTGGACTCAGACATATC-3′.

The double-stranded oligonucleotide was cloned into the pcDNA 6.2-GW/EmGFP-miR vector (Invitrogen, Life Technologies, Carlsbad CA). 48 h later the transfected cells were trypsinised, and seeded in media containing tamoxifen, triciribine, and tetrandrine either individually or in combination, at concentrations 10 nM or 100 nM. Two weeks later the cells were washed twice with PBS, fixed for 30 min in methanol and then visualised by Giemsa–Romanowski staining and quantified using TotalLab (Nonlinear dynamics, Newcastle upon Tyne, UK).

2.4. Immunochemical analyses

For immunoblotting analysis of protein expression, cells were lysed in NP-40 lysis buffer: 150 mM NaCl, 50 mM TrisHCl, pH 8.0, 50 mM NaF, 5 mM EDTA, 1% NP-40, 1:100 phosphatase inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, P5726) and 1:100 protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MO, P8340). AGR2 was detected using a polyclonal antibody to AGR2 raised in rabbit (in-house generation), β-actin was quantified using AC-15 antibody (Sigma–Aldrich, St. Louis, MO), AKT2 was detected using polyclonal antibody (Cell Signaling Technology, Inc., Danvers, MA, 2962) and to detect phospho-AKT Ser473 and Phospho-HER2/neu Tyr1221 we used the same antibodies as for IHC.

2.5. Quantitative RT-PCR

For qRT-PCR analysis, total RNA from cells was extracted and purified using RNeasy Mini kit (Qiagen, Hilden, Germany). cDNA was generated using Qiagen's Omniscript RT kit. *PDPK1*, *AGR2*, and *GAPDH* (endogenous control) expression was detected using SybrGreen with the following oligonucleotide primers; PDPK1 forward: CAACTCATTCGTGGGAACAG, PDPK1 reverse: GCCCAAAGGTCTGAACTCTT, AGR2 forward: GCTCCTTGTGGCCCTCTCCTACAC, AGR2 reverse: ATCCTGGGG ACATACTGGCCATCAG. GAPDH forward: GTCAGTGGTGGACCTGACCT, GAPDH reverse ACCTGGTGCTCAGTGTAGCC. Gene expression was determined by relative quantitation with data from the 7900HT Fast Real-Time PCR Detection System using comparative C_T method.

3. Results

3.1. Identification of the dominant kinase pathway that regulates AGR2 protein turnover in response to tamoxifen

Previous parallel RNAi and inhibitor screens identified the PDPK1-AKT signalling pathway as a strong determinant of sensitivity to tamoxifen [21]. In concordance with this work we attempted to determine the effect of specific inhibitors of PDPK1-AKT signalling pathway triciribine, tetrandrine and Akti-1/2 to define the kinase signalling pathway that triggers the expression of AGR2 in breast cancer cells. We found that all three AKT inhibitors were able to reduce basal AGR2 levels (Fig. 1A lanes 3, 5, 7) and attenuate the induction of AGR2 by tamoxifen (Fig. 1A lanes 4, 6, 8). To confirm the specific effect of the drugs, the total level of AKT as well as AKT phosphorylation was analysed in MCF-7 cells exposed to the AKT inhibitors. Our data show that in response to these drugs the total level of AKT remained almost unaffected with only a slight decrease after application of Akti-1/2. Conversely all three inhibitors attenuated AKT phosphorylation at Ser^{437} in both tamoxifen treated and untreated cells, albeit tetrandrine attenuated AKT phosphorylation in response to tamoxifen with lower efficiency (Fig. 1A, Supplementary Fig. 1).

In parallel *AGR2* expression at the mRNA level was determined in the same experimental setup (Fig. 1B). We found that application of AKT specific inhibitors caused decrease in *AGR2* mRNA levels indicating that AKT signalling pathway regulates AGR2

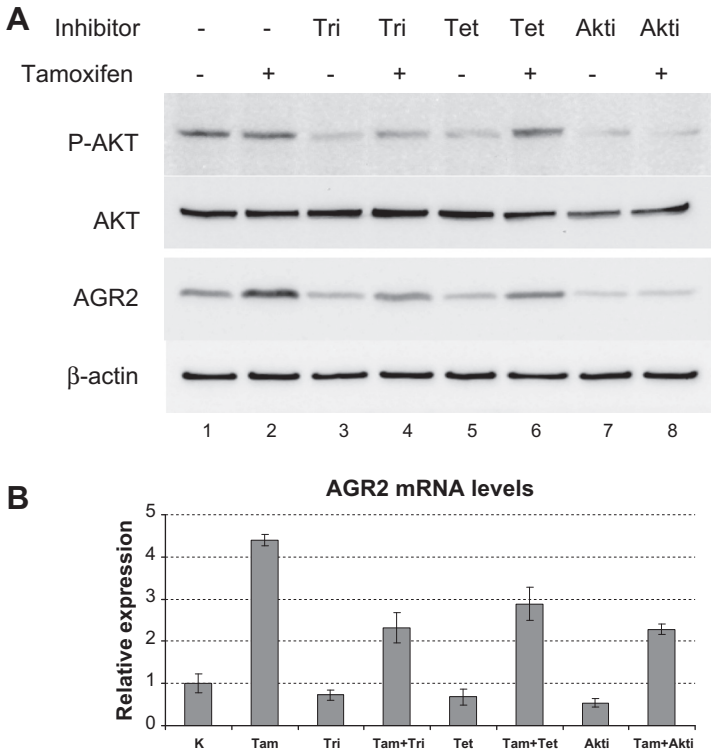

Fig. 1. AKT inhibitors block AGR2 protein induction in tamoxifen treated cells. MCF-7 cells grown in complete DMEM medium were incubated with tamoxifen (1 μM) and the indicated kinase inhibitors (triciribine, lanes 3 and 4; Tetrandrine, lanes 5 and 6 and Akti-1/2, lanes 7 and 8) for 48 h. (A) Lysates were processed for immunoblotting to quantify changes in phospho-AKT-Ser^{473}, AKT, AGR2 and β-actin protein expression using enhanced chemiluminescence. The data are representative of three independent experiments. (B) In parallel *AGR2* mRNA levels were determined in triplicates by qRT-PCR using comparative C_T method.

expression at the level of transcription. Interestingly, as was observed at the protein level, treatment with each AKT inhibitor individually resulted in a slight decrease in AGR2 expression, however, the decrease observed was more pronounced in cells that were treated with tamoxifen and the AKT inhibitors in combination.

Since AKT is activated by PDPK1, we further screened for changes in AGR2 expression using specific siRNA that reduces the steady-state levels of the AKT activator PDPK1. The siRNA mediated reduction in *PDPK1* mRNA levels (Fig. 2A) resulted in statistically significant ($p = 0.25$, One-Way ANOVA) decreased induction of AGR2 mRNA by tamoxifen (Fig. 2B). In accordance with these results the siRNA mediated attenuation of PDPK1 at the protein level led to decreased induction of AGR2 protein by tamoxifen (Fig. 2C). Taken together these data suggest that PDPK1-AKT signalling pathway is directly involved in the regulation of AGR2 protein turnover.

3.2. Combined depletion of AGR2 protein using miRNA and triciribine sensitises cells to tamoxifen

We further evaluated the effects of tamoxifen and AKT inhibitors triciribine and tetrandrine on cell survival using a clonogenic assay. First we confirmed that AGR2 protein level decreased by specific miRNA [15] results in reduced clonogenic survival (Fig. 3, panels 1). When the cells were treated with two different (10 nM and 100 nM) concentrations of tamoxifen (Fig. 3A and B, panel 2 vs 1) or triciribine (Fig. 3A and B, panel 3 vs 1), or tetrandrine independently (Fig. 3A and B, panel 4 vs 1), 10 nM treatment of all these drugs had little effect on cell survival. Conversely 100 nM dose of tamoxifen significantly decreased colony formation and exposure of the cells to 100 nM triciribine caused strongest reduction in colony formation. Interestingly, reduced clonogenic survival in response to these drugs was even more reduced by AGR2 silencing at both concentrations of administered individual drugs. The most efficient reduction in clonogenic cell survival was observed in cells with miRNA depleted AGR2 exposed to combined treatment with tamoxifen and triciribine indicating synergistic effect of oestrogen receptors signalling and AKT pathway on cell survival (Fig. 3A and B, panel 5).

To support our conclusions that PDPK1-AKT signalling pathway triggers AGR2 protein induction in response to tamoxifen we also examined whether PDPK1 and AKT siRNAs may sensitise cells to tamoxifen in colony forming assays. However, we did not find any significant change in the number of colonies in response to either PDPK1 or AKT siRNA treatment using similar time periods as in previous experiments (Fig. 3C). However, colonies developed from cells transfected with PDPK1 or AKT siRNA were significantly smaller in comparison with cells transfected with siRNA negative control; this trend was observed in both the control and tamoxifen treated cells (Supplementary Fig. 2). Thus, it would appear that specific siRNA mediated knock-down of PDPK1 or AKT does indeed result in attenuated cell proliferation. However, the use of siRNA mediated knock-down is not permanent, and during the course of this experiment it is likely that the cells were able to resume normal protein production; this probably explains why we only see a decrease in colony size rather than number.

3.3. Co-expression of phospho-Ser^{473} AKT protein and AGR2 protein in primary human breast cancers

Finally, we evaluated the possible clinical significance of AGR2 protein expression in relation to the level of AKT phosphorylation at Ser^{473} to determine whether or not a correlation exists between these two events *in vivo*. We quantified AGR2 protein (Fig. 4A), phospho-Ser^{473} AKT (Fig. 4B), and phospho-Tyr^{1221} HER2 protein

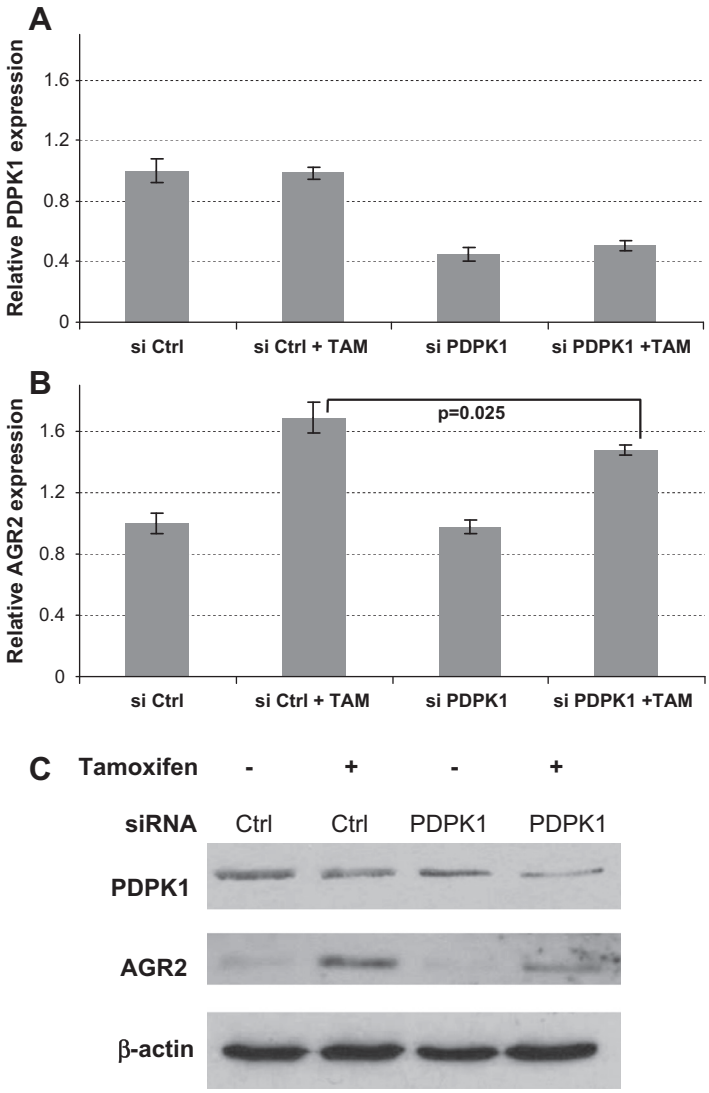

Fig. 2. Effects of siRNA targeting PDPK1 on AGR2 expression in tamoxifen treated cells. MCF-7 cells grown in complete DMEM medium were transfected with control siRNA (si Ctrl) or siRNA targeting *PDPK1* (si PDPK1), incubated with 1 μM tamoxifen for 48 h, as indicated. Then (A) *PDPK1* and (B) *AGR2* mRNA levels were determined in triplicates by qRT-PCR using comparative C_T method. (C) Protein lysates were processed for immunoblotting to quantify changes in PDPK1, AGR2, and β-actin protein expression using enhanced chemiluminescence. The data are representative of three independent experiments.

expression (Fig. 4C) by immunohistochemical staining of formalin fixed sections from primary breast cancer tissue (94 samples). Activation of AKT was shown to predict poor prognosis in hormone-treated breast cancer patients [22,23]. Therefore, we examined whether AGR2 expression is linked to expression of these activated signalling pathways. The samples were scored as follows: no/low (0 or 1), intermediate (2), or high expression (3). To validate our IHC staining and scoring system respectively, the patients' overall survival was evaluated with respect to AKT Ser^{473} phosporylation and showed the expected association of reduced survival with enhanced phospho-Ser^{473} AKT protein expression (Fig. 4D). Importantly, we found significant correlation between high AGR2 and phospho-Ser^{473} AKT protein expression (Fig. 4E) and marginal association for AGR2 and phospho-Tyr^{1221} HER2 protein expression (Fig. 4F). These data suggest that *in vivo*, activation of the AKT pathway may play a role in driving the pro-metastatic and survival functions of AGR2 protein. Given our prior demonstration that AGR2 expression predicts poor responses to tamoxifen treatment [15], these data suggest that AKT pathway would be an attractive target for inhibiting the growth of tamoxifen resistant cancer cells.

4. Discussion

The link between hormones and breast cancer growth and development has been recognised for more than a century. Subsequent identification of the oestrogen receptors (ER) provided a mechanism to describe the target site specificity of oestrogen action in many tumours including breast carcinomas. Oestrogen signalling occurs through multiple pathways in which oestrogen receptors directly or indirectly regulate transcription of many target genes. The clinical relevance of many of these signalling pathways still remains to be fully defined including physiological system controlling AGR2 protein expression. At a mechanistic level, very little is known about the function and the regulation of the AGR2 pathway. However, a key observation published recently demonstrates that the pro-survival function of AGR2 in oestrogen receptor positive breast carcinomas can be linked to its induction by tamoxifen, thus potentially identifying a novel gene that could mediate resistance to tamoxifen [15,18].

Tamoxifen was extensively studied for breast cancer treatment since 1970s and soon became the drug of choice for the endocrine

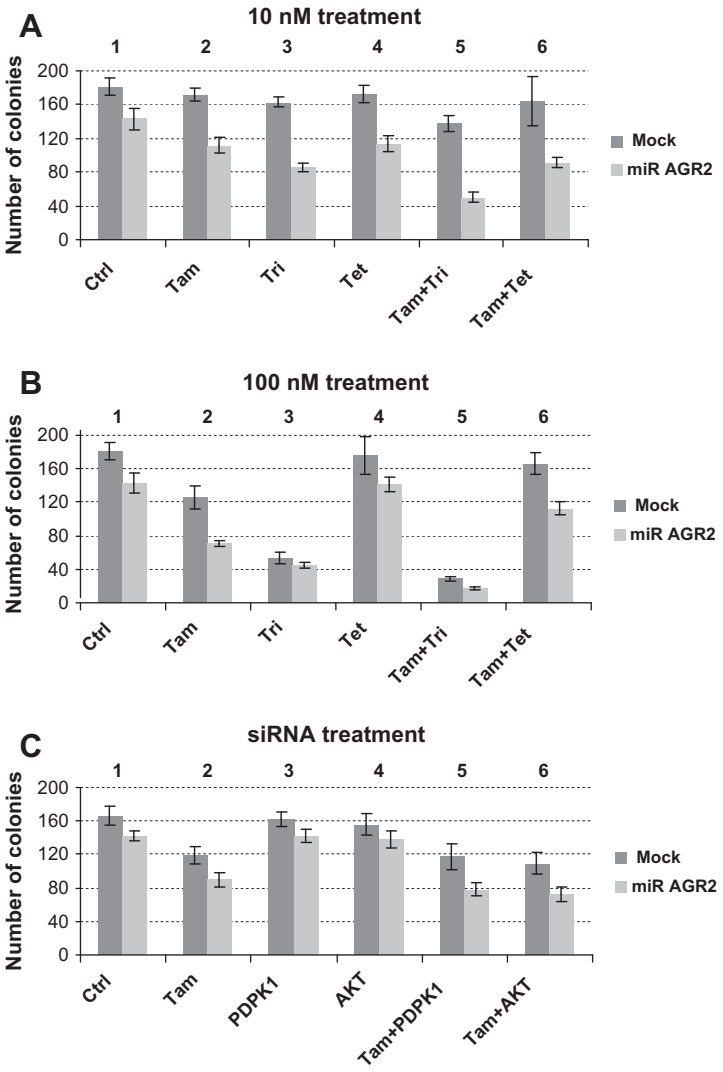

Fig. 3. Depletion of AGR2 sensitises cells to tamoxifen in clonogenic assays. MCF-7 cells grown in complete medium were transfected with control shRNA vector (black bar) or shRNA vector targeting AGR2 (grey bar; as previously validated [15]) and then incubated alone or with (A) 10 nM or (B) 100 nM of the indicated small molecules: (1) DMSO; (2) tamoxifen; (3) triciribine; (4) tetrandrine; (5) tamoxifen and tricribine; and (6) tamoxifen and tetrandrine. (C) Untreated or tamoxifen treated MCF-7 cells were transfected with either siRNA negative control (panels 1 and 2) or PDPK1 (panels 3 and 5) and AKT (panels 4 and 6) specific siRNAs, respectively. After 2 weeks, the colonies were fixed with methanol and visualised using Giemsa–Romanowski staining. The final colony number was determined as an average of three independent experiments.

treatment of advanced breast cancer in postmenopausal women who were considered likely to respond to endocrine treatment [24]. Nevertheless, although 5 years of adjuvant tamoxifen treatment significantly reduces the risk of breast cancer mortality in patients with ER-positive tumours, there is a portion of patients who immediately fail to respond to tamoxifen treatment and in many responding patients the disease ultimately progresses to a tamoxifen-resistant phenotype [25]. Since the definition of the specific genetic alterations, pharmacology of tamoxifen and molecular processes that determine clinical endocrine resistance is incomplete, it is a reasonable goal to identify the determinants of tamoxifen resistance and sensitivity to develop a reliable predictive test for tamoxifen resistance.

Small molecule and siRNA library screens were carried out to investigate enzymes that mediate tamoxifen resistance in breast cancer cells and independently identified: (i) AKT as a potential target for the sensitisation of cells to tamoxifen using the kinase inhibitors triciribine and tetrandrine; and (ii) PDPK1 as a potential target for the sensitisation of cells to tamoxifen. The consistency of this conclusion is based on the knowledge that PDPK1 and AKT form a signalling cascade known to be a general pro-oncogenic signalling pathway in many human cancers. This type of hypothesis-based screen is an example of how to develop more rational strategies for selecting patients harbouring drug-resistant cancers based on over-expression of pro-oncogenic drugable enzymes. In our current study, the identification of AKT and PDPK1 as potential regulators of AGR2 protein induction is consistent with the known ability of PDPK1 and AKT inhibition to sensitise cells to tamoxifen [21].

In other words, our data suggest a link between AGR2 expression and ER non-genomic activity, which generally involves ER interaction with and activation via phosphorylation of a number of key signalling molecules such as insulin-like growth factor receptor (IGFR), human epidermal receptor 2 (HER2), epidermal growth factor receptor (EGFR), etc. [26]. Many of these interactions lead to the activation of key secondary signalling messengers and downstream kinase pathways, such as the p21Ras/p42/44 MAPK and AKT pathways, leading to the activation of various cellular processes such as proliferation, growth, and survival. Clinical relevance of ER crosstalk with growth factor signalling pathways was confirmed by prospective trials in patients with metastatic disease showing that tamoxifen resistance is associated with high levels of

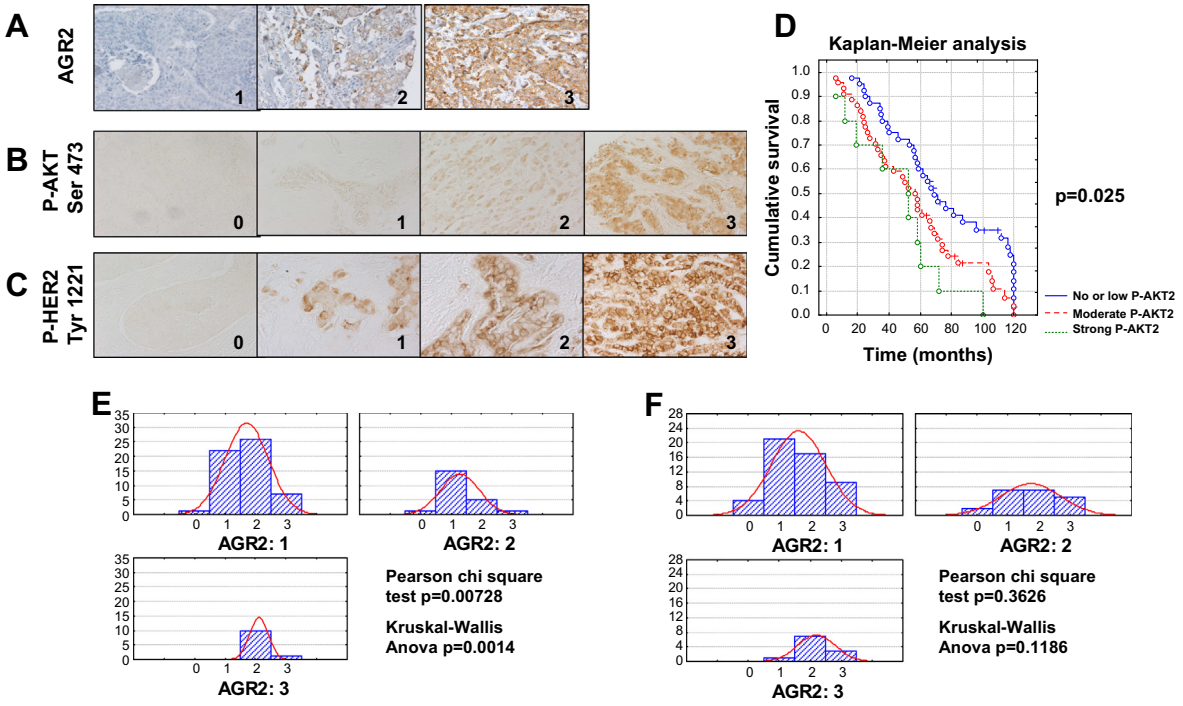

Fig. 4. AGR2 and phospho-AKT are co-expressed *in vivo* in primary breast cancers. Representative immunohistochemical staining of formalin fixed sections from a primary breast cancer cohort (94 samples) that have been graded with respect to levels of (A) AGR2 protein, (B) phospho-Ser^{473} AKT, or (C) phospho-Tyr^{1221} HER2 protein expression. The samples have been scored from no/low (0 or 1), intermediate (2), or high protein expression (3). Using this immunohistochemical scoring system, we evaluated expression of AGR2 protein in relation to the activation of the HER2 and AKT proteins in primary breast cancers. (D) Cumulative overall survival of patients as a function of the degree of phosphorylation of AKT2 at Ser^{473} using a Kaplan–Meier analysis. The *p* value using a Chi-square test is 0.025. (E and F) Statistical analysis of AGR2 protein expression as a function of expression of the HER2-AKT pathway activation *in vivo*. Expression of phospho-AKT (E) and phospho-HER2 (F) that were quantified according to criteria in Fig. 4 (A–C) were plotted as a function of (1) negative/low, (2) intermediate, or (3) high levels of AGR2 protein expression.

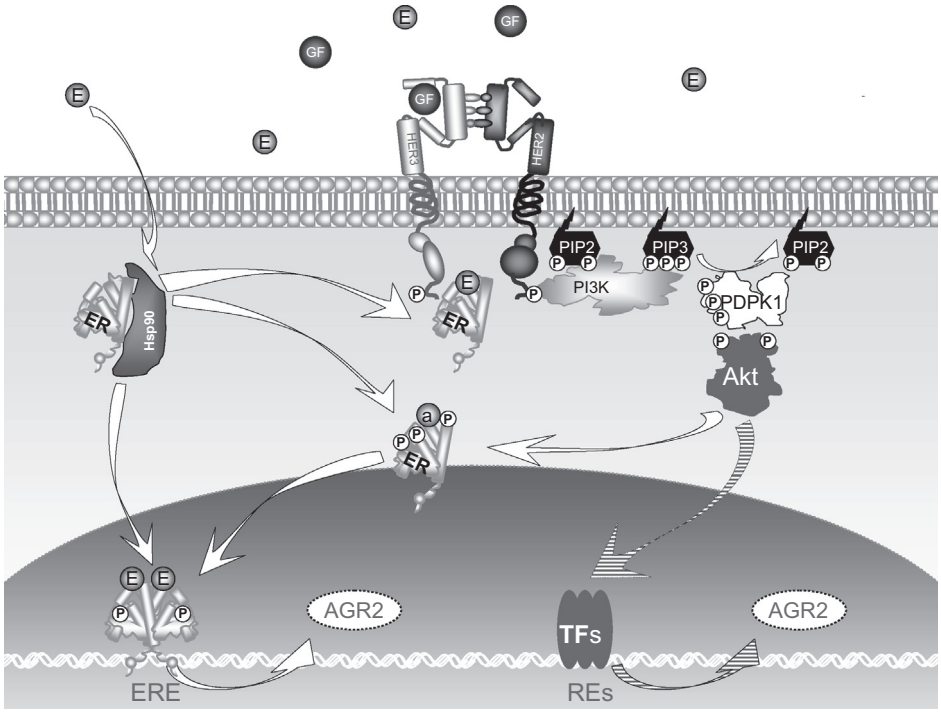

Fig. 5. The scheme of modulation of AGR2 expression through ER and PDPK1/AKT signalling. AGR2 expression is affected by direct genomic mode of ER involving estradiol (E) diffusion through cell membrane and binding to ER to form ER-E active complex. The complex acts as a transcription factor and triggers transcription of target genes including AGR2. Recently described additional molecular mechanism of ER action includes interaction of active ER-E complex with ErbB2/PI3K/Akt signalling pathway. ER-E mediated activation of ErbB2 heterodimer results in phosphorylation of AKT kinase and launches downstream signals possibly including AGR2 transcription regulation (striped arrows). ER-E can in turn be phosphorylated by AKT kinase at the same time leading to formation of transcriptionally active ER-ER homodimer.

expression of receptor tyrosine kinases HER2 and EGFR [27]. These concepts might be confined to breast cancer, since AGR2 expression occurs independent of the oestrogen receptor in ovarian cancer [17].

These findings together with our results are consistent with recently published work by Dong et al. identifying that AGR2 induces expression of amphiregulin, a growth promoting EGFR ligand, through the Hippo signalling pathway co-activator, YAP1 [10]. They also show that induced amphiregulin expression in adenocarcinoma cells is able to rescue the transformed phenotype that is lost when AGR2 expression is reduced. Establishment of a link between AGR2, Hippo and EGFR signalling pathways would appear to support the concept that AGR2 takes part in ER signalling as a potential determinant of resistance to tamoxifen. Taken together, AGR2 would appear to be actively involved in molecular crosstalk between ER and other pro-oncogenic signalling pathways such as PDPK1/AKT or EGFR resulting in endocrine resistance (Fig. 5). Therefore, development of new therapeutic strategies to overcome resistance to endocrine therapy in breast cancer patients consisting in the simultaneous inhibition of the ER and other associated pathways is an important goal of cancer research.

5. Conclusions

Since tamoxifen is the most widely used drug to be able to perturb the ER-transcriptome, we evaluated whether the AGR2 pathway responds to the presence of this drug and whether other key regulatory pathways could be identified that signal from tamoxifen to AGR2. We report that a PDPK1-AKT signalling pathway substantially contributes to the regulation of AGR2 turnover in response to tamoxifen, thus identification of a signalling cascade whose inhibition has the effect on depletion of AGR2 protein, indicates a potential therapeutic strategy for treating tamoxifen resistance in AGR2 positive breast cancers. Additionally, since the oestrogen receptors have been a target of anti-cancer therapeutic programmes for many years, the characterisation or description of specific AGR2-inhibitory pathways may greatly facilitate anti-cancer drug development programmes.

Conflict of Interest

The authors declare no conflict of interest related to this work.

Acknowledgement

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2013.01.034.

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A Divergent Substrate-Binding Loop within the Pro-oncogenic Protein Anterior Gradient-2 Forms a Docking Site for Reptin

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cancer;
ATP

Anterior gradient-2 (AGR2) functions in a range of biological systems, including goblet cell formation, limb regeneration, inhibition of p53, and metastasis. There are no well-validated binding proteins for AGR2 protein despite the wealth of data implicating an important cellular function in vertebrates. The yeast two-hybrid system was used to isolate the ATP binding protein Reptin as an AGR2-interacting protein. AGR2 formed a stable complex in human cell lysates with Reptin, thus validating Reptin as an AGR2 binding protein in cells. Reptin was also shown to be overproduced in a panel of primary breast cancer biopsy specimens, relative to normal adjacent tissue from the same patient, suggesting a role in cancer growth *in vivo*. Mutations were made at the two ATP binding motifs in Reptin to evaluate the effects of ATP on Reptin–AGR2 complex stability. Loss-of-ATP binding mutations at the Walker A motif (K83A) or gain-of-ATP binding mutations at the Walker B motif (D299N) resulted in Reptin mutants with altered oligomerization, thermostability, and AGR2 binding properties. These data indicate that the two ATP binding motifs of Reptin play a role in regulating the stability of the AGR2–Reptin complex. The minimal region of AGR2 interacting with Reptin was localized using overlapping peptide libraries derived from the AGR2 protein sequence. The Reptin docking site was mapped to a divergent octapeptide loop in the AGR2 superfamily between amino acids 104 and 111. Mutations at codon Y104 or F111 in full-length AGR2 destabilized the binding of Reptin. These data highlight the existence of a protein docking motif on AGR2 and an ATP-regulated peptide-binding activity for Reptin. This knowledge has implications for isolating other AGR2-interacting proteins, for developing assays to isolate small molecules that target the Reptin ATP binding site, and for measuring the effects of the Reptin–AGR2 complex in cancer cell growth.

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Abbreviations used: AGR2, anterior gradient-2; PDI, protein disulfide isomerase; ER, endoplasmic reticulum; HA, hemagglutinin; GST, glutathione *S*-transferase; DLS, dynamic light scattering; WT, wild type; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; HUBMA/B/C, His-ubiquitin modification buffer A/B/C.

Introduction

Anterior gradient-2 (AGR2) is a protein whose function is proving to play an increasingly critical role in a diverse range of biological systems, including vertebrate tissue development, inflammatory tissue injury responses, and cancer progression. AGR2 was identified initially as a secretory factor expressed in the anterior region of the dorsal ectoderm in *Xenopus laevis* embryos, where it was postulated to mediate the specification of dorsoanterior ectodermal fate, particularly in the formation of the cement gland.[1,2] AGR2 was subsequently cloned as a gene whose expression is induced by the estrogen receptor α,[3] and subsequent studies in primary breast carcinomas have also shown significant associations between AGR2 expression and estrogen receptor-α positivity or tamoxifen resistance.[4,5] Clinical studies have shown that the AGR2 protein is overexpressed in a wide range of human cancers, including carcinomas of the esophagus, pancreas, breast, prostate, and lung.[4,6–9] More biological studies in cell lines have shown a significant role for AGR2 in tumor-associated pathways, including tumor growth, cellular transformation, cell migration, limb regeneration, and metastasis.[8,10–12]

AGR2 protein was also identified as part of a clinical proteomics screen aimed at discovering novel inhibitors of the tumor suppressor p53, and it was subsequently validated as a potent inhibitor of p53 activity and of the p53-dependent response to DNA damage.[11] The latter data provide a specific oncogenic pathway into which AGR2 integrates; however, the signaling mechanisms that drive AGR2 to inhibit p53 are not defined. Although there are no well-validated binding proteins in human cells that can explain how AGR2 can act as a pro-oncogenic protein, peptide aptamer screens have identified a specific peptide-binding activity for the AGR2 protein for peptides containing an (S/T)xIΦΦ consensus motif, suggesting that the AGR2 protein might prove to have a peptide groove able to interact with cellular proteins containing such a consensus motif.[13] Furthermore, penetratin peptides linked to this AGR2-binding (S/T)xIΦΦ motif or EGFP fusions to the (S/T) xIΦΦ motif can stabilize AGR2 in cells and stimulate p53 activity, indicating that the AGR2 protein can interact with this peptide consensus motif *in vivo*.[14] A yeast two-hybrid screen has also been used to previously identify the prometastatic proteins C4.4 and DYS1 as interactors of AGR2[15]; however, there was no biological validation of C4.4 and DYS1 as a bona fide protein–protein interaction in human cells. However, potential extracellular receptor functions for AGR2 in human cells remain possible, because an interaction between the newt extracellular receptor PROD1 and newt AGR2 was identified using a yeast two-hybrid screen and validated to demonstrate a direct signaling role for AGR2 in amphibian limb regeneration.[10]

Although the general biochemical functions of AGR2 in human cells remain undefined, *AGR2* is part of the protein disulfide isomerase (PDI) superfamily that contains core thioredoxin folds (CxxC or CxxS motif), which have the potential to act as molecular chaperones that regulate protein folding via regulation of disulfide-bond formation.[16] There are five protein members of this family: TRX1 (thought to be predominantly the nuclear thioredoxin), TRX2 (thought to be predominantly the mitochondrial thioredoxin), endoplasmic reticulum (ER) protein 18 (ERP18; the ancestral protein in the AGR2/AGR3 group that has potent reducing potential),[17] AGR2, and the AGR2 ortholog AGR3. AGR2 and AGR3 are confined to vertebrates, and both have the CxxS core motif instead of the CxxC motif of TRX1, TRX2, and ERP18.[18] The majority of PDIs/ERPs harbor a typical H/KDEL ER retrieval signal. A putative ER retention sequence that has been shown to regulate the intracellular localization of AGR2 in human cells has been identified at the C-terminus of AGR2.[14] It is therefore possible that at least one function of the AGR2 is to act as a PDI and hence as a protein molecular chaperone. A recent study has confirmed that AGR2 is essential for the production of the intestinal mucin MUC2, a cysteine-rich glycoprotein that forms the protective mucus gel lining the intestine. The cysteine residue within the AGR2 thioredoxin-like domain was shown to form a mixed disulfide bond with a cysteine in the N-terminus or C-terminus of MUC2 as it is being processed.[19] However, there are currently no established biochemical mechanisms to explain the function or the regulation of AGR2 protein.

In this report, a yeast two-hybrid screen was used to identify a potentially novel interacting protein for the AGR2 protein from a human breast cancer library. A protein named Reptin was identified by the yeast two-hybrid screen and validated as an interacting protein of AGR2 in human cells. Reptin is a highly conserved member of the AAA+ family that can be found in numerous multiprotein complexes linked to transcription, DNA damage response, and nonsense-mediated RNA decay.[20–25] This protein is a member of the highly conserved RuvBl1/2 superfamily containing ATP binding motifs and DNA binding and helicase functions and an ability to form biologically relevant protein–protein interactions with proteins implicated in cancer, including Myc, Tip60, APPL1, Pontin, and telomerase holoenzyme complexes.[20,23,26–29] The validation of Reptin as an AGR2 binding protein gives rise to a potentially novel signaling complex involved in prometastatic cancer development. Our mapping of the determinants that mediate a specific

Reptin–AGR2 protein–protein complex *in vitro* provides biochemical insights for understanding how the Reptin–AGR2 complex can be regulated in cells. These data also provide ideas for development of *in vitro* enzyme assays for the screening of small molecules that might be used to disrupt the AGR2–Reptin complex in cancer cells as potential therapeutic leads.

Results

Reptin is overexpressed in primary human cancers and forms a stable protein–protein complex with AGR2 protein in cancer cells

In our search for proteins interacting with human AGR2 protein, we used a yeast two-hybrid assay with LexA fused to AGR2 as bait screened against a cDNA library derived from breast cancer cells (Fig. 1a). These hits appear relatively specific for the AGR2 bait because a parallel yeast two-hybrid screen performed on the AGR2 ortholog AGR3 (sharing approximately 75% homology) yielded a completely distinct set of interacting proteins (data not shown).

Two extracellular prometastatic receptors identified (Fig. 1a; C4.4A and DAG1) were previously published to be AGR2-interacting proteins based on a yeast two-hybrid screen,[15] but these interactors were not validated as AGR2 binding proteins. Yeast two-hybrid approaches for identifying interacting proteins can be prone to the generation of false-positives[30]; for example, we have been unable to validate despite numerous approaches in human cell systems or *in vitro* using purified proteins a direct interaction between the HECTD1 ubiquitin ligase (Fig. 1a) and AGR2 protein (unpublished data). Furthermore, as the interactions proposed between AGR2 and C4.4A or DAG1 based on the yeast two-hybrid screen require an extracellular localization of AGR2, we did not further validate C4.4A and DAG1 as potential interactors of AGR2. The reason for excluding these potential interactors is that our laboratory cannot observe AGR2 secreted and/or localized at the plasma membrane, as has been suggested by others.[15] Rather, we have published data showing the localization of endogenous or red fluorescent protein-tagged AGR2 to the ER, perinucleus, and nucleus.[14] Peptide aptamers generated to AGR2[13] also shift AGR2 out of nuclear fraction into cytosolic compartments.[14] As such, we focused our efforts at validating nuclear proteins from the yeast two-hybrid screen as potential AGR2 interactors—particularly one protein of specific relevance within the AAA+++ superfamily named Reptin.

Reptin can be a nuclear protein involved in range of functions, including DNA repair, transcription regulation, and chromatin structural control.[24] Similar to AGR2, Reptin is also involved in prometastatic signaling using cell lines *in vitro*, but this involves, in part, interactions of Reptin with the transcription regulator Myc or Tip60.[31] Furthermore, there is some evidence that Reptin is

(a)

Name	Accession	Function
Reptin	CAG38538	Interacts with Tip60 and Myc transcription factors, contains intrinsic ATPase and helicase functions
Rip140	NP_003480	Nuclear de-acetylase that interacts with hormone receptor activation domains
LGN	AAN01266	Modulation of G protein activation
HECTD1	AAW65983	HECT-homology domain containing Ubiquitin ligase superfamily
CKAP2	AAH10901	Regulates cyclin- kinase functions
TMEM123	AL050161	Cell-membrane mediated cell death
C4.4A	AF082889	Metastasis linked activity, previously published, unvalidated AGR2-Y2H interactor
DAG1	L19711	Metastasis linked activity, previously published, unvalidated AGR2-Y2H interactor

(b)

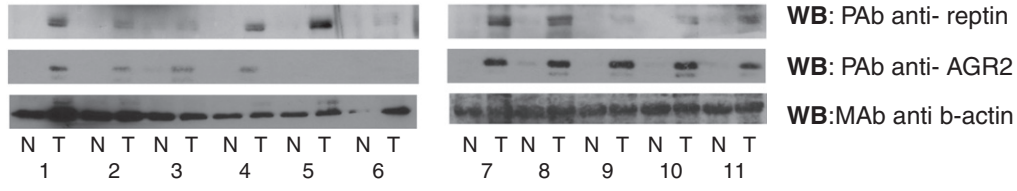


Fig. 1. Reptin and AGR2 are both overproduced in primary human cancers. (a) Summary of AGR2 yeast two-hybrid interactors. Yeast two-hybrid analysis was performed by Hybrigenics using the bait vector consisting of pB27 containing a LexA C-terminal fusion to human AGR2 (21–175) and screened against human breast tumor epithelial cell RP1. Only highly significant overlapping hits are depicted including name, accession number, and functions. (b) Immunoblotting of lysates derived from breast cancers and normal adjacent tissue from the same patient. Sample biopsy specimens [from T (tumor) and N (normal)] were prepared in lysis buffer as described previously,[5,44] separated by electrophoresis, and immunoblotted using antibodies to Reptin, AGR2, and actin, as indicated.

overproduced in primary cancers, hepatocellular carcinoma,[32] and gastric cancer.[33] However, there are no data on whether Reptin and AGR2 can be coexpressed in primary human cancers. If there were, then this would form an important rationale for continued evaluation of Reptin–AGR2 interactions. A panel of primary human breast cancers and normal adjacent tissue from the same patient were lysed to examine whether Reptin is in fact overexpressed in primary cancers. These human cancers were previously used to demonstrate that AGR2 overproduction predicts poor prognosis in tamoxifen-resistant breast cancers.[5] The immunoblotting of lysates from this panel of breast cancer biopsy specimens or normal adjacent tissue from the same patient demonstrated the overproduction of AGR2 in the cancers in the majority of patients (Fig. 1b, samples 1, 2, 3, 4, and 7–11), as expected from previous immunohistochemical evaluations.[5] Similarly, Reptin was also overproduced in the

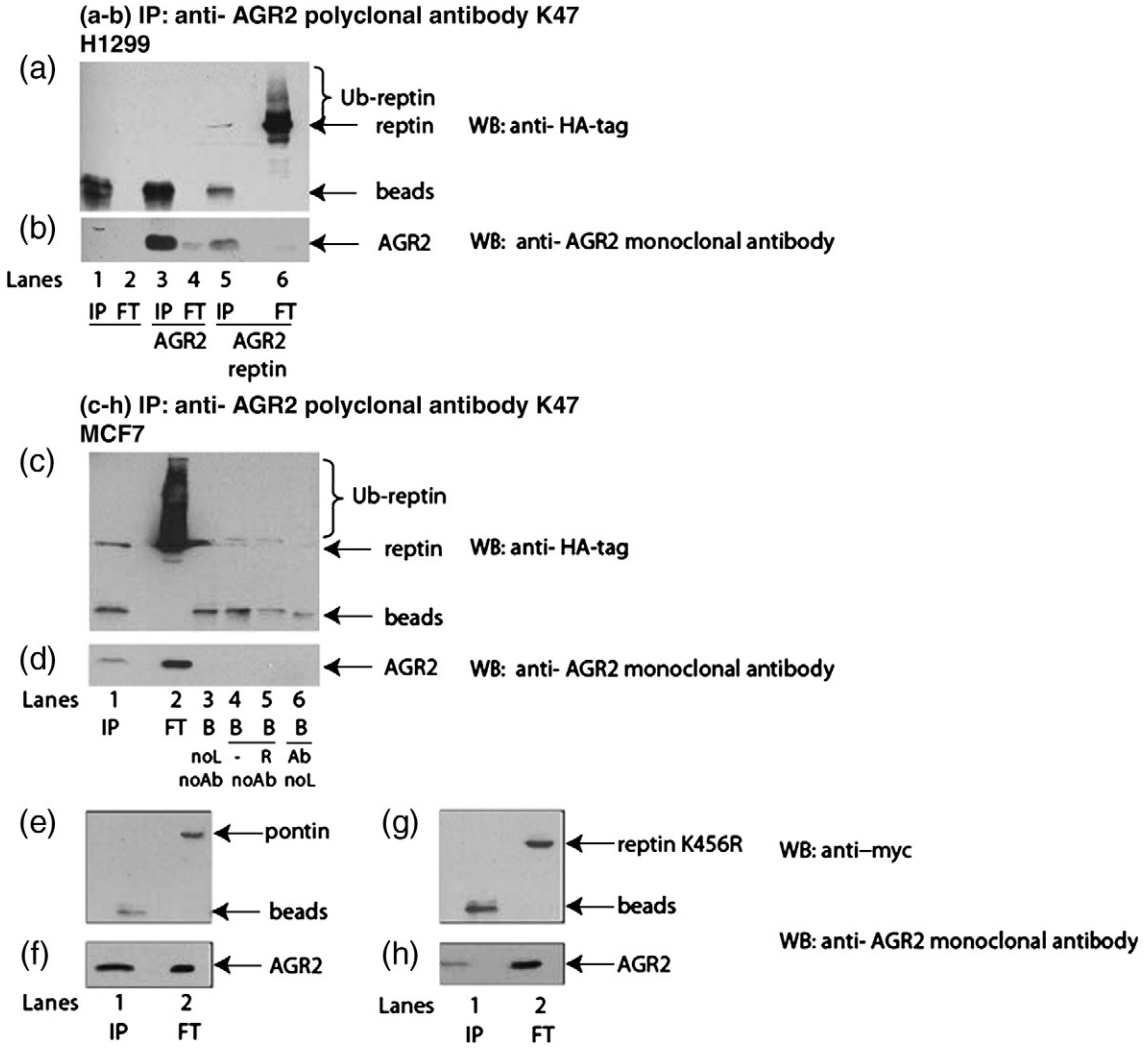


Fig. 2. Co-immunoprecipitation of human Reptin and AGR2 protein in human cancer cells. (a and b) Reptin and AGR2 form a complex in cells. Cell lysates from H1299 human breast cancer cells (no AGR2) transfected with vector control (lanes 1 and 2), with the AGR2 vector (lanes 3 and 4), or with AGR2 and HA-tagged Reptin (lanes 5 and 6) were incubated with the anti-AGR2 rabbit polyclonal antibody (K47) and protein G beads. The AGR2 immunoprecipitate [IP] (lanes 1, 3, and 5) or the unbound, flow-through fraction [FT] (lanes 2, 4, and 6) was immunoblotted to quantify the extent of (a) HA-tagged Reptin or (b) AGR2 in each fraction. Reptin, ubiquitinated Reptin, and AGR2 are highlighted. (c–h) Neither mutant Reptin nor Pontin forms a complex with AGR2 in cells. Cell lysates from MCF7 cells expressing endogenous AGR2 transfected with (c and d) WT Reptin, (e and f) Myc-tagged Pontin, and (g and h) Myc-tagged Reptin^{K456R} were immunoprecipitated with the anti-AGR2 polyclonal antibody K47. The bound [IP] and unbound [FT] fractions were immunoblotted to quantify the extent of Reptin, Pontin, or Reptin^{K456} proteins bound to AGR2. The immunoblots were exposed for the same times, and Reptin, ubiquitinated Reptin, protein G beads, Pontin, and AGR2 are highlighted.

majority of these cancer biopsy specimens (Fig. 1b, samples 1–11). Thus, the combined overexpression of AGR2 and Reptin in primary human breast cancer makes Reptin a more compelling and physiologically relevant AGR2-interacting protein.

To evaluate whether the association between Reptin and AGR2 is physiological in human cells, we performed co-immunoprecipitation experiments using lysates of H1299 lung carcinoma cells transiently expressing hemagglutinin (HA)-tagged Reptin and AGR2. The transfection of AGR2 alone into cells demonstrated that the transfected AGR2 protein could be immunoprecipitated with a polyclonal antibody (K47) specific for AGR2 (Fig. 2b, lane 3), with a small proportion in the unbound, flow-through fraction (Fig. 2b, lane 4). As a control, AGR2 is not detectable in cells that were not transfected with AGR2 (Fig. 2b, lanes 1 and 2). The transfection of HA-tagged Reptin into cells resulted in the immunoprecipitation of the Reptin protein with AGR2 (Fig. 2a, lane 5).

A pool of Reptin protein in the unbound fraction was composed of high-molecular-mass adducts (Fig. 2a, lane 6), presumably due to ubiquitin-like modification. A control was performed to determine whether the high-molecular-mass ladder of ubiquitin-like adducts was ubiquitin, NEDD8, or SUMO-1. His-tagged versions of the latter ubiquitin/ubiquitin-like genes were co-transfected into cells with HA-tagged Reptin, and following purification of the *in vivo* His-tagged proteins on a nickel affinity column, the pellets were analyzed by immunoblotting for Reptin. The data demonstrate that the dominant adduct on Reptin is ubiquitin, not NEDD8 or SUMO-1 (Fig. 3b, lane 2). This is distinct from a prior report that suggested a dominant modification on Reptin is SUMO-1.[21] This might reflect a cell-specific difference in the type of ubiquitin-like modification that is catalyzed on Reptin. As an additional control, basal p53 modification by ubiquitin and SUMO-1 could be detected (Fig. 3b, lanes 2 and 4), whereas MDM2 could drive enhanced ubiquitination or NEDDylation of p53 (Fig. 3b, lanes 6 and 7). These latter data indicate that integrity of the His-tagged NEDD8 and SUMO-1 genes is adequate and that the low amount of NEDDylation or SUMOylation of Reptin presumably highlights the specificity of ubiquitination on Reptin.

In order to define determinants that mediate the specificity of the co-immunoprecipitation of Reptin with AGR2, we also evaluated the ability of the Reptin ortholog and partner protein Pontin/RuvBl1 to interact with endogenous AGR2 in MCF7 cells. Pontin is thought to form a hetero-oligomer with Reptin,[34] although it has also been published that Reptin has biochemical functions that are Pontin independent.[21] Although transfected Reptin was able to form an immune complex with endogenous

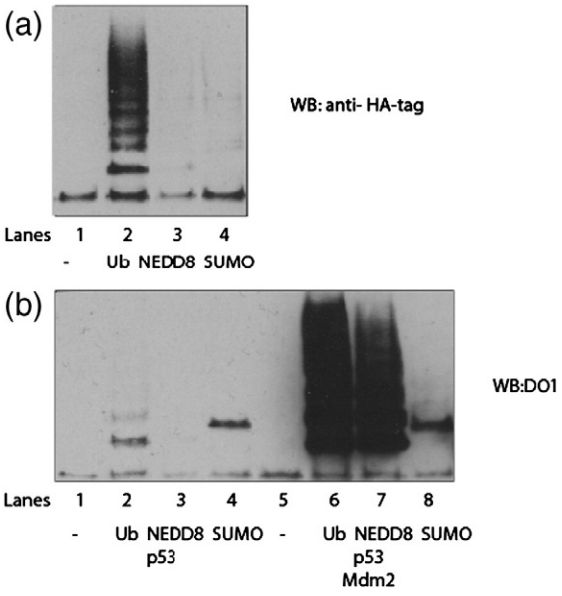


Fig. 3. Reptin is selectively ubiquitinated in cells. (a) Evaluation of Reptin ubiquitin-like modification in cells. The HA-tagged vector expressing Reptin was co-transfected with His-ubiquitin, His-NEDD8, and His-SUMO-1 expression vectors. After the nickel affinity purification stage, the expressed Reptin was examined for changes in the amount of posttranslational modification by immunoblotting with an anti-HA-tag antibody. (b) Evaluation of p53 ubiquitin-like modification in cells. A vector expressing WT p53 was co-transfected with vectors expressing His-ubiquitin, His-NEDD8, and His-SUMO-1 without (lanes 1–4) or with (lanes 5–8) the co-transfection of MDM2. The expressed p53 was examined for changes in the amount of posttranslational modification by immunoblotting with an anti-p53 antibody (DO-1) after the nickel affinity chromatography stage.

AGR2 (Fig. 2c and d, lane 1), Pontin did not form any detectable complex with Reptin (Fig. 2e and f, lane 1 *versus* lane 2). Furthermore, the Reptin mutant K456R, which cannot form ubiquitin-like adducts,[21] was not able to form a stable complex with AGR2 (Fig. 2g and h, lane 1 *versus* lane 2). Together, these results confirm that the yeast two-hybrid system did in fact reveal a specific protein–protein interaction between AGR2 and Reptin that can be detected in human cell lines. As such, we developed biochemical approaches in order to validate the determinants that define the specific binding between Reptin and AGR2.

ATP regulates the stability of the Reptin–AGR2 protein complex

In order to determine whether Reptin binding to AGR2 is direct and not due to a bridging factor *in vivo* (in the yeast or human cell systems), we

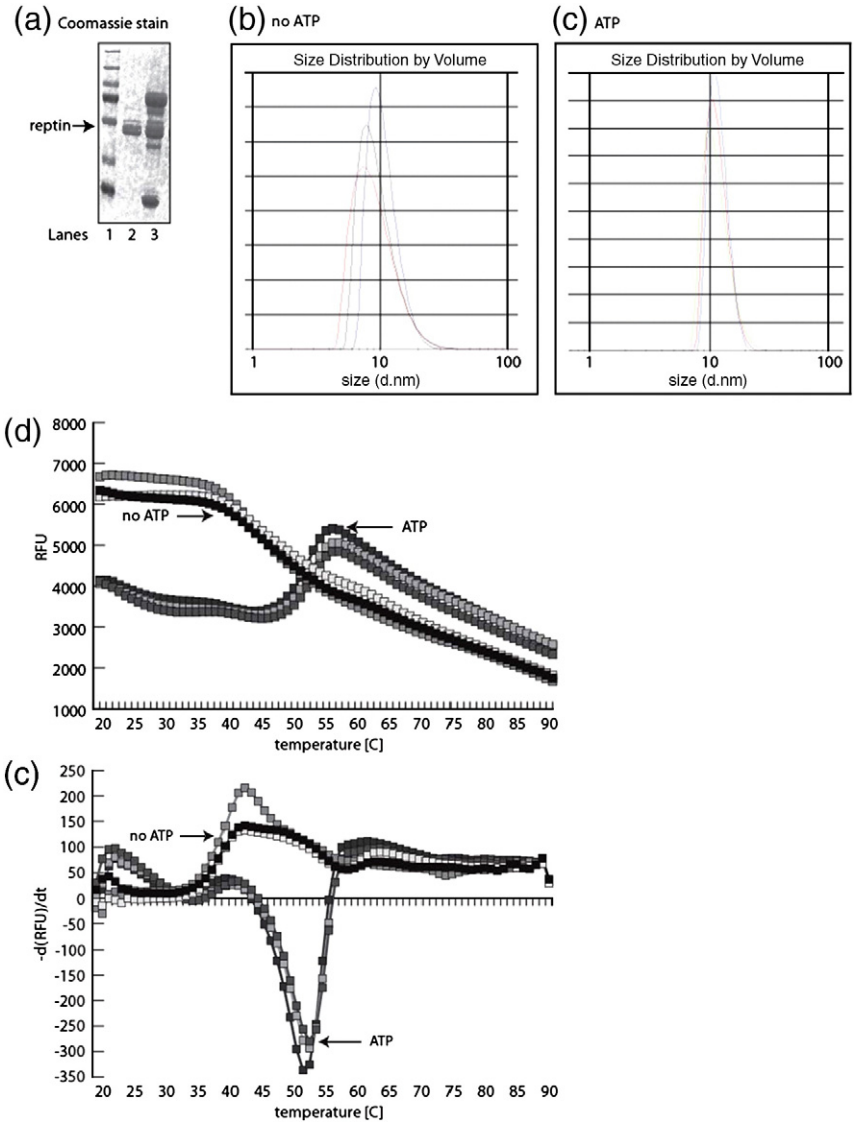


Fig. 4. Purification and ATP binding properties of untagged human Reptin protein. (a) Purification of recombinant Reptin. Reptin was cloned into a vector producing a GST fusion protein containing a PreScission protease cleavage site after adsorption of GST-Reptin onto the glutathione beads, followed by rigorous washing and elution of Reptin protein in purified form and then by dialysis into buffer as indicated in Methods. Lane 1, markers; lane 2, full-length untagged Reptin; lane 3, GST-tagged Reptin. (b and c) Reptin (50 μM) was subjected to analysis by light scattering in the absence and in the presence of ligand ATP, as indicated in Materials and Methods. (d) Reptin unfolding as a function of temperature change in the absence and in the presence of ligand. SYPRO Orange fluorescence was used to measure the thermal unfolding events between 20 °C and 89 °C, and experiments were done in triplicate in the absence or presence of ATP. (e) Phase transitions in the Reptin thermal melt profile. The gradient of protein unfolding was plotted against the temperature gradient to obtain the midpoint temperature of transition (T_m) in the absence and in the presence of ATP.

first purified and characterized Reptin protein biochemically to ensure that the integrity of the protein was adequate for biochemical analyses. Recombinant human Reptin protein was over-produced as a recombinant glutathione *S*-transferase (GST)-tagged fusion protein expressed in *Escherichia coli* and purified after protease cleavage from the GST tag (Fig. 4a, lane 3 *versus* lane 2). The use of dynamic light scattering (DLS) to characterize the oligomeric state of highly purified Reptin revealed a protein fraction with a relatively homogenous nature (Fig. 4b) that was shifted in apparent mass as defined by changes in DLS after the inclusion of ATP (Fig. 4c). The latter data are consistent with previous reports that Reptin can bind ATP, although one previous report indicated that His-tagged Reptin does not increase its oligomeric nature in the presence of ATP.[34]

Lastly, we analyzed the ATP binding potential of Reptin using thermal-shift assay. Increase in protein denaturation or unfolding as a function of temperature can be measured by the interaction with SYPRO Orange, a dye that binds to hydrophobic regions exposed or unfolded regions of proteins and results in fluorescence.[35] Ligand binding can alter the thermostability of a target protein and cause a change in the rate of unfolding as a function of increasing temperature. In the absence of ligand, there is a high basal fluorescence of Reptin (~6400 RFU) that reduces gradually as a function of increasing temperature (Fig. 4d). When the data are plotted as rate of change in fluorescence at each temperature, Reptin does not undergo a classic unfolding transition (Fig. 4e). These data suggest that hydrophobic regions on ATP-free Reptin are being concealed rather than exposed upon heating. However, in the presence of ATP, a classic ligand-dependent thermal unfolding transition was observed as defined by increases in the rate of SYPRO Orange dye binding as a function of increasing temperature (Fig. 4d and e). Also, in the presence of ATP, there is a reduction in the basal fluorescence at 20 °C (~4100 RFU; Fig. 4d). These data suggest that the higher fluorescence seen at 20 °C in the absence of ATP is due to the SYPRO Orange dye binding to the hydrophobic ATP binding motifs and ATP conceals the pocket from dye binding. Together, these data suggest that the biochemical integrity of the Reptin protein we have purified by this method is sufficient and that the protein is active as an ATP binding protein. This form of Reptin was used to determine if it indeed binds directly to AGR2 and if so to define the determinants in Reptin that drive specific binding to AGR2 protein.

Nitrocellulose filter-based ATP binding assays were utilized to define the apparent stoichiometry of the Reptin–ATP complex. A titration of Reptin protein (from 0.25–1.0 pmol) using α-^{32}P-labeled ATP as a ligand revealed that approximately 0.5 pmol of ATP can be bound per picomole of Reptin (Fig. 5a). With the use of the nonhydrolyzable ATP analog γ-S-labeled ATP, up to approximately 0.18 pmol of ATP binding activity could be detected per picomole of Reptin (Fig. 5a). Because Reptin has two ATP binding sites and can become more oligomeric in the presence of ATP (Fig. 4), these data suggest that the hydrolysis of ATP might stimulate or stabilize ATP binding allosterically or that the nonhydrolyzable ATP analog does not functionally mimic ATP in these ATP binding pockets. In addition, as we were unable to detect 2 mol ATP bound per mole of Reptin, these data suggest a negative cooperativity between the two ATP binding pockets on the protein (see Discussion). Using this ATP-binding active form of Reptin, we determined whether Reptin bound to AGR2 protein directly using an ELISA to measure protein–protein interactions. When AGR2 was adsorbed onto the solid phase (96-well microtiter plate) and subjected to a Reptin titration in the mobile phase, a

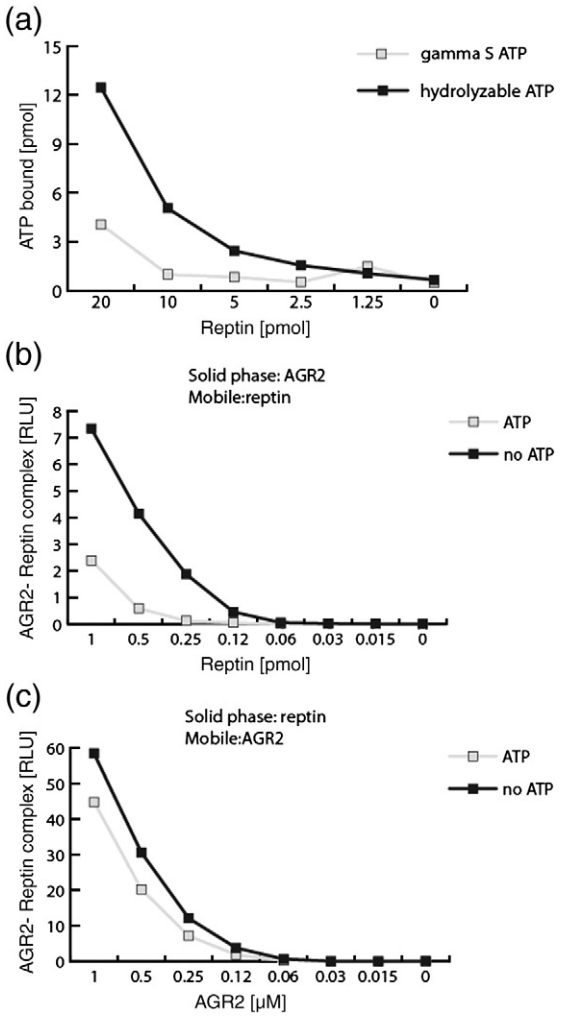


Fig. 5. The effects of ATP on the stability of the Reptin–AGR2 protein complex. (a) ATP binding activity of Reptin. ATP binding was measured by quantifying the amount of radioactive ATP bound to a nitrocellulose filter as a function of increasing Reptin protein levels. The black line represents binding to ATP, and the light line represents binding to nonhydrolyzable ATP. The data are plotted as picomoles of ATP bound as a function of increasing Reptin protein levels (in picomoles). (b and c) ATP destabilizes the Reptin–AGR2 protein complex. Either AGR2 (b) or Reptin (c) was immobilized in the solid phase, and in the mobile phase either Reptin (b) or AGR2 (c) was titrated in the absence or presence of ATP. The amount of Reptin or AGR2 bound was quantified with antibodies specific for either protein using chemiluminescence. The data are plotted as the extent of protein–protein complex formation (in relative luminescence units) as a function of increasing protein in the mobile phase (in micromolar concentrations).

stable complex can be detected between the two proteins (Fig. 5b). This confirms that the immunoprecipitation and yeast two-hybrid observed between AGR2 and Reptin *in vivo* (Fig. 2) can be due to a direct protein–protein interaction between the two proteins. A titration of Reptin in the presence of ATP reveals that ATP can reduce the stability of the Reptin–AGR2 complex by approximately threefold (Fig. 5b). Corroborating this assay, when Reptin is adsorbed to the solid phase, with AGR2 titrated in the mobile phase, a stable complex can be detected between AGR2 and Reptin that is also attenuated by the inclusion of ATP, but by approximately 20% (Fig. 5c). The difference in the extent of ATP-dependent reductions in AGR2 binding to Reptin might be due to the conformation of the respective protein (AGR2 in Fig. 5b and Reptin in Fig. 5c) when adsorbed to the solid phase. These data confirm that Reptin and AGR2 can form a direct complex and that the conformation of Reptin in its ATP-bound state can affect the stability of the complex.

Mutation of either of the two ATP binding sites on Reptin destabilizes the AGR2–Reptin complex

Reptin has a number of conserved sequence motifs. Notably, it has two motifs critical for nucleotide binding and hydrolysis, namely the P-loop NTP binding motif (also known as the Walker A box) and the DEAD motif (also known as the Walker B box),[31] respectively. In order to further evaluate the effects of the two ATP binding domains of Reptin (Fig. 6ai) on the stability of the AGR2–

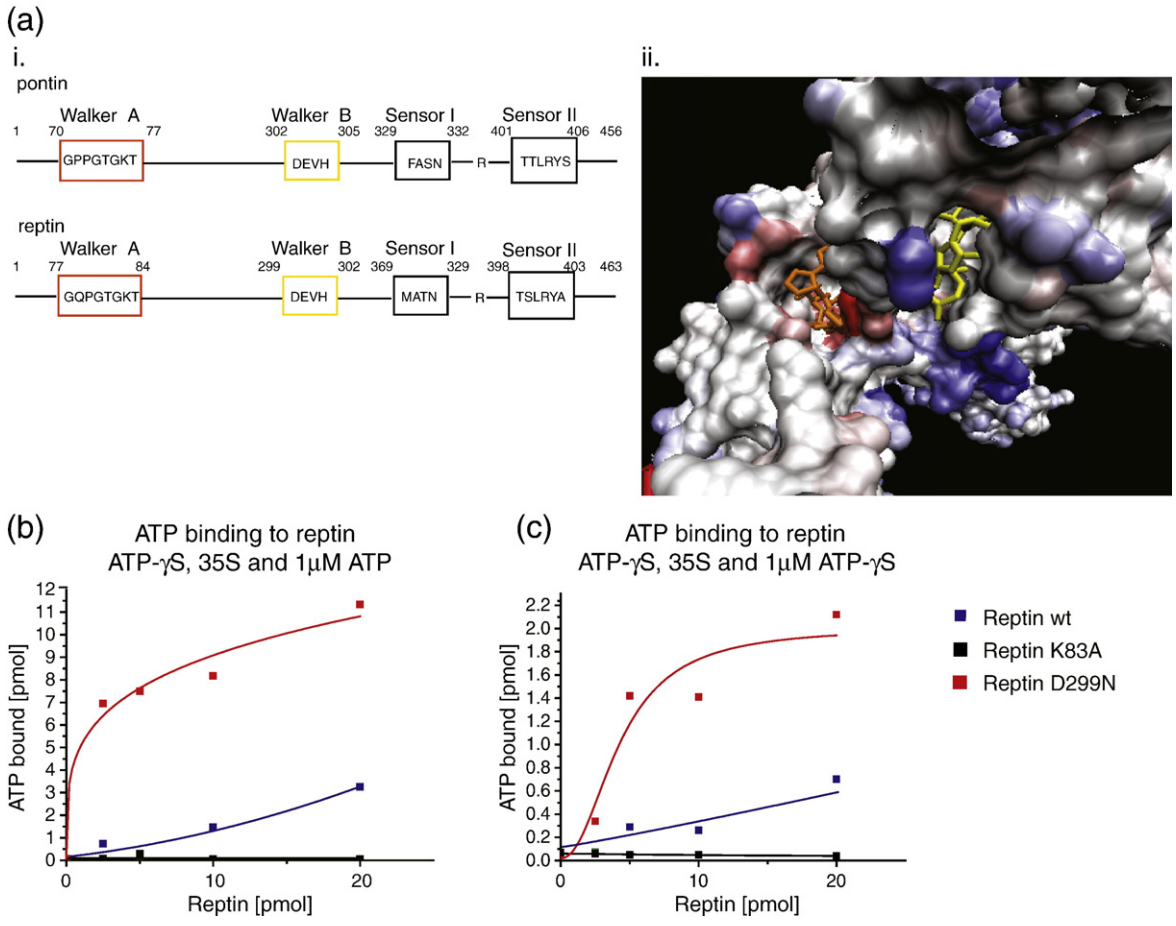


Fig. 6. The effects of Reptin ATP binding site mutations on binding to ATP. (a) (Left panel i) The key functional domains of Reptin and its ortholog Pontin are shown, including the Walker A and Walker B ATP binding motifs and the sensor motifs. (Right panel ii) Diagram of the structure of human Pontin with the Walker A and B ATP binding sites highlighted in blue and red, respectively; the Protein Data Bank code for Pontin is 2C9O. (b and c) ATP binding activity of Reptin ATP site mutants. Radioactive ATP binding was measured by quantifying the amount of (b) ATP (spiked with radioactive γ-^{35}S-labeled ATP or (c) γ-S-labeled ATP (spiked with radioactive γ-^{35}S-labeled ATP) bound to a nitrocellulose filter as a function of increasing WT and mutant Reptin protein levels. The data are plotted as picomoles of ATP bound as a function of increasing Reptin protein levels (in picomoles).

Reptin complex, we generated the single point mutants in the ATP binding sites, K83A and D299N. The Walker A mutation (K to A) is thought to induce a loss-of-function mutation, whereas the Walker B mutation (D to N) is thought to reduce ATP hydrolysis, but it actually increases ATP binding.[36] As expected, Reptin^{D299N} exhibited a higher affinity for the radioactive nonhydrolyzable ATP analog γ-^{35}S-labeled ATP (in the presence of nonradioactive ATP) than the wild-type (WT) Reptin, whereas the mutant Reptin^{K83A} exhibited a reduced binding affinity for γ-^{35}S-labeled ATP (Fig. 6b). Similar results were observed using radioactive γ-S-labeled ATP in the presence of nonradioactive γ-S-labeled ATP (Fig. 6c). The data also suggest that mutation of codon 83 Walker A motif attenuates ATP binding allosterically by the remaining WT Walker B motif. However, the inactivity of Reptin^{K83A} in this assay is complicated by the observation that this mutant appears to interact with ATP using three assays (described below). This suggests that the nitrocellulose binding assay underestimates the specific activity of Reptin^{K83A} as an ATP binding protein, possibly due to the remaining WT Walker B motif.

Chemical cross-linking was performed to determine if changes in the ATP-dependent oligomeric structure of Reptin (Fig. 4c) correlate with differences in the ATP binding functions of the Reptin^{K83A} and Reptin^{D299N} mutant proteins. Increasing the concentration of cross-linker into reactions with WT Reptin yielded an oligomeric ladder (Fig. 7a, lane 6 *versus* lanes 1–5) that was partially resistant to cross-linking as defined by the resilience of a pool of monomeric protein to cross-linking at the highest concentration of 0.2% (Fig. 7a, lane 1 *versus* lane 6). In the presence of ATP, there was a marginal difference in the oligomerization of Reptin (Fig. 7a, lanes 7–12), indicating that the cross-linking assay is not revealing dramatic changes in the conformation of the WT Reptin. Nevertheless, the Reptin^{K83A} and Reptin^{D299N} mutant proteins exhibited striking and opposing behavior in the cross-linking assay to each other and to WT Reptin. The Reptin^{K83A} mutant protein was sensitive to loss of monomeric subunit as a function of increasing concentration of cross-linker in the presence of ATP (Fig. 7b, lane 12 *versus* lanes 7–11), whereas this mutant appeared similar to WT Reptin in the absence of ATP (Fig. 7b, lanes 1–6, *versus* Fig. 7a, lanes 1–6). These data indicate that the Reptin^{K83A} mutant protein can in fact interact with ATP in this assay format, contrasting with its inactivity in the nitrocellulose filter ATP binding assay. By contrast, Reptin^{D299N} exhibited a loss in monomeric Reptin as a function of increasing cross-linking in the absence of ATP (Fig. 7c, lanes 1–6) but was resistant to cross-linking of its monomer in the presence of ATP (Fig. 7c, lanes 7–12). These data indicate that the Reptin^{K83A} and Reptin^{D299N} have different conformational responses to ATP binding between each other (summarized in Fig. 7d and e) and compared with WT Reptin. The oscillating response of WT Reptin might be due to a dynamic equilibrium between monomeric and oligomeric states at the temperature used in these assays (21 °C), as we have data (unpublished results) showing that the oligomeric nature of Reptin can be dramatically influenced by temperatures ranging from the extreme of 0 °C to 37 °C.

In conclusion, characterization of Reptin^{K83A} and Reptin^{D299N} ATP binding site mutant proteins indicated that they both respond differently from WT Reptin and opposing each other in terms of ATP-dependent changes in oligomeric cross-linking and in ATP binding. The proximity of the ATP binding motifs containing the K83 and D299 codons based on the homology of Reptin to the structure of its ortholog Pontin (Fig. 6ai) suggests a potential allosteric shift of one ATP binding motif might locally affect the affinity of the other ATP binding motif for its ligand. The data also suggest that mutating the individual Walker B ATP binding site in Reptin creates a gain-of-function activity due to reduction in ATP hydrolysis and thus increases in binding affinity for ATP.

Using the WT Reptin and these Reptin ATP binding site mutants, we evaluated whether the mutant proteins exhibited differences in stable binding to AGR2 protein. When AGR2 was incubated in the solid phase with WT Reptin in the mobile phase, the typical ATP-dependent reduction in AGR2 binding (as in Fig. 4c) could be observed (Fig. 8a). With the use of the loss-of-ATP binding function mutant (Walker A motif, K83A), significant reduction in AGR2 binding could be observed (Fig. 8a), highlighting an important role for the Walker A motif in regulating the AGR2 binding activity of Reptin.

The gain-of-function mutant in the Walker B motif (D299N) was analyzed alone and in combination with the double mutation D299N and K83A to also evaluate how these mutations affected Reptin binding to AGR2. As with WT Reptin, we first evaluated whether the D299N mutant protein had an altered thermostability using the thermal-shift assay. Interestingly, Reptin^{D299N} exhibited an intrinsically more thermoresistant property as a function of increasing temperature (Fig. 8b) that was further stabilized approximately 15 °C by the inclusion of ATP (Fig. 8b). These data are further consistent with a gain-of-function conformational effect on the Reptin structure, with respect to ATP binding. Although the Reptin^{D299N} mutant protein bound well to AGR2 (Fig. 8c) and was partially destabilized from AGR2 binding by ATP (Fig. 8c), the double Reptin mutant was significantly reduced in AGR2 binding activity in the absence or presence of ATP (Fig. 8c). These data indicate that the loss-of-ATP

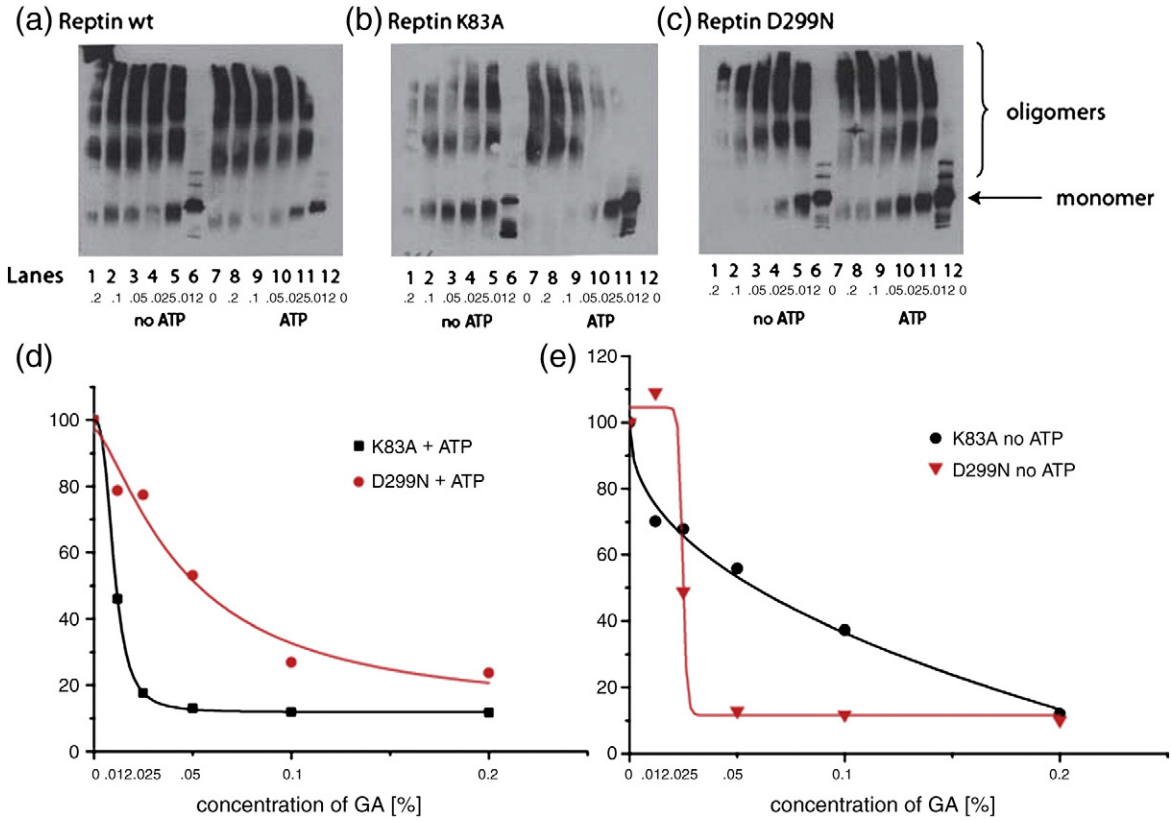


Fig. 7. Reptin mutants display distinct changes in ATP-dependent oligomerization as defined using a cross-linking assay. (a–c) Oligomerization of Reptin. Reactions were set up using (a) WT Reptin, (b) Reptin^{K83A}, and (c) Reptin^{D299N} mutant proteins. A titration of glutaraldehyde (from 0.012% to 0.2%) in the absence or presence of ATP was followed by immunoblotting for changes in the extent of oligomerization of Reptin from the monomeric state (arrow). (d and e) Quantitation in the changes in the extent of monomeric oligomerization of Reptin^{K83A} and Reptin^{D299N} mutant proteins in the absence or presence of ATP.

binding K83A mutation is dominant over the gain-of-ATP binding D299N mutation, with respect to AGR2 binding. Together, these data establish that Reptin can bind directly to AGR2 and that this interaction is likely to be specific since the ATP binding domains and in turn the conformation of Reptin play a role in driving the stability of the AGR–Reptin complex. Having validated one regulatory determinant in Reptin that regulates its stable binding to AGR2, we next evaluated whether we could identify determinants in AGR2 that mediate its stable binding to Reptin.

The divergent substrate-binding loop in the AGR2 superfamily forms the dominant Reptin binding interface

A significant proportion of the human proteome is composed of intrinsically disordered peptides and linear domains that form docking sites for protein–protein interactions.[37] As such, we used an overlapping peptide library derived from the AGR2 open reading frame to determine whether any linear domain/peptide docking sites exist for Reptin within the AGR2 protein sequence. The overlapping peptide library was composed of 15 amino acids with 10 amino acid overlaps, and each peptide contained an N-terminal biotin-SGSG spacer (Fig. 9a). The incubation of the biotinylated peptides derived from AGR2 with human cell lysates containing endogenous Reptin resulted in the specific binding of Reptin to one peptide motif, named peptide 10 (Fig. 9b). Intriguingly, this peptide motif overlaps with the previously identified unique surface loop in the AGR2 gene family (Fig. 9cii). This family is composed of the founder gene Erp18 and the AGR2 ortholog AGR3, which have both appeared in the vertebrate lineage and have not undergone gene expansion since their appearance in vertebrates.[18] The position in the three-dimensional structure of the divergent loop extension containing the sequence from AGR2 was modeled into the Erp18 crystal structure[38] (Fig. 9ci, red), and it has been proposed that this divergent surface loop could form a substrate docking site for distinct interacting proteins in this Erp18/AGR2/AGR3 family.[39] The sequence

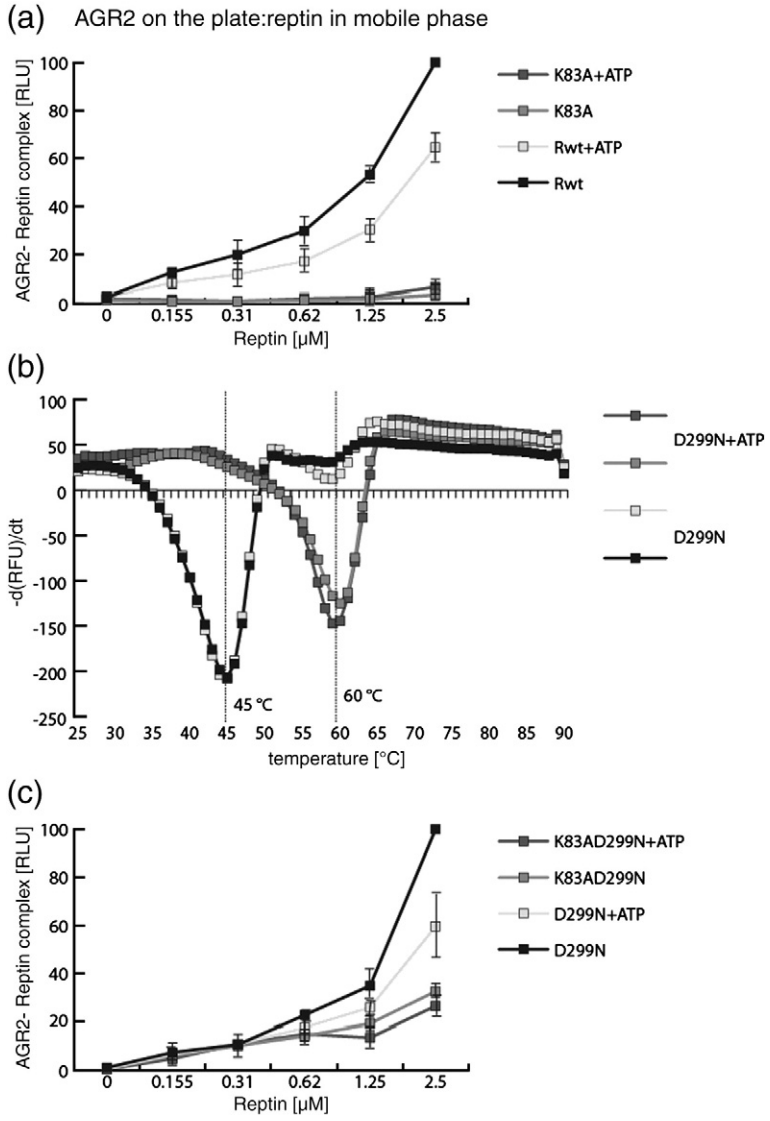


Fig. 8. The effects of ATP binding site mutations in Reptin on binding to AGR2. (a). The effects of the Walker A site mutation on the Reptin–AGR2 protein complex. Reactions were set up using (i) WT Reptin or (ii) Reptin^{K83A} in which AGR2 is in the solid phase and Reptin is in the mobile phase. The amount of Reptin bound was quantified with antibodies specific for Reptin protein titrated in the mobile phase using chemiluminescence. The data are plotted as the extent of protein–protein complex formation (in relative luminescence units) as a function of increasing protein in the mobile phase (in micromolar concentrations). (b). Reptin^{D299N} unfolding as a function of temperature change in the absence and in the presence of ligand. SYPRO Orange fluorescence was used to measure the thermal unfolding events between 20 °C and 89 °C, and experiments were done in triplicate in the absence or presence of ATP. The raw data were plotted as the gradient of protein unfolding was plotted against the temperature gradient to obtain the midpoint temperature of transition (T_m) in the absence and in the presence of ATP. (c) The effects of the Walker B site mutation and double ATP site mutations on the Reptin–AGR2 protein complex. Reactions were set up using (i) Reptin^{D299N} or (ii) Reptin$^{D299N/K83A}$ mutant proteins in which AGR2 is in the solid phase and the amount of Reptin or bound was quantified with antibodies specific for Reptin titrated in the mobile phase using chemiluminescence. The data are plotted as the extent of protein–protein complex formation (in relative luminescence units) as a function of increasing protein in the mobile phase (in micromolar concentrations).

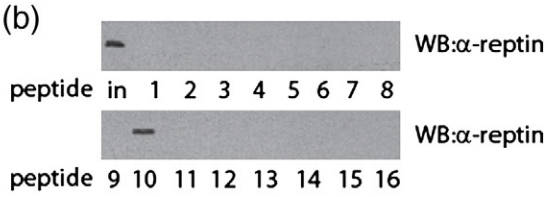
of amino acids in this divergent loop in the Erp18/AGR2/AGR3 protein family is shown in Fig. 9cii.

Alanine scan and truncation mutagenesis were performed on peptides containing the surface loop (Fig. 10a). The data reveal that most amino acid residues from 104-FVLLNLVY-111 play a rate-limiting role in binding to Reptin, as their mutation to alanine attenuates Reptin binding to AGR2 (Fig.

(a) AGR2 spanning peptides

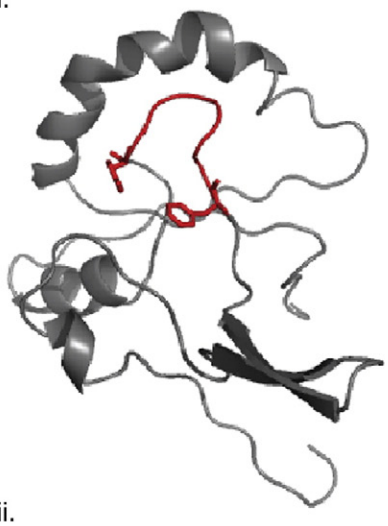
1. llvalsytlardttv
2. rdttvkpgakkdtkd
3. kdtkdsrpklpqtls
4. pqtlsrgwgdqliwt
5. qliwtqtyeealyks
6. alyksktsnkplmii
7. plmiihhldecphsq
8. cphsqalkkvfaenk
9. faenkeiqklaeqfv
10. aeqfvllnlvyettd
11. yettdkhlspdgqyv
12. dgqyvprimfvdpsl
13. vdpsltvraditgry
14. itgrysnrlyayepa
15. ayepadtallldnmk
16. ldnmkkalkllktel

(b)



(c)

i.



ii.

```
AGR2            -MEKIPVSAFLLLVALSYTLARDTTVKPGAKKDTKDSRPKLPQTLSRGWGDQLIWTQTYE 59
AGR3            MMLHSALGLCLLLVTVSSNLA--IAIK-------KEKRP--PQTLSRGWGDDITWVQTYE 49
ERP18           -METRPRLGATCLLGFSFLLLVISSDG--------------HNGLGKGFGDHIHWR-TLE 44
                 *   .      *: .*  *    :                 : *.:*:**.: *  * *

AGR2            EALYKSKTSNKPLMIIHHLDECPHSQALKKVFAENKEIQKLAE-QFVLLNLVYET--TDK 116
AGR3            EGLFYAQKSKKPLMVIHHLEDCQYSQALKKVFAQNEEIQEMAQNKFIMLNLMHET--TDK 107
ERP18           DGKKEAAASGLPLMVIIHKSWCGACKALKPKFAESTEISELSH-NFVMVNLEDEEEPKDE 103
                :.   :  *  ***:* * . *  .:***  **:. **.:::. :*:::**  *   .*:

AGR2            HLSPDGQYVPRIMFVDPSLTVRADITGRYSNRLYAYEPADTALLLDNMKKALKLLKTEL- 175
AGR3            NLSPDGQYVPRIMFVDPSLTVRADIAGRYSNRLYTYEPRDLPLLIENMKKALRLIQSEL- 166
ERP18           DFSPDGGYIPRILFLDPSGKVHPEIINENGNPSYKYFYVSAEQVVQGMKEAQERLTGDAF 163
                .:**** *:***:*:*** .*:.:* .. .*  * *   .   :::.**:* . :  :

AGR2            ---------
AGR3            ---------
ERP18           RKKHLEDEL 172
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Fig. 9. Identification of a specific binding site for Reptin on AGR2. (a) Human cell lysates expressing Reptin were incubated in a buffer containing the indicated biotinylated peptides (peptides 1–16 of human AGR2) coupled to streptavidin beads. The amount of Reptin bound was evaluated by immunoblotting the bound fractions as indicated in panel (b): "in" indicates input fraction, and the numbers 1–16 represent the peptide sequences in part (a). (c) [i] Homology model of the position of the divergent peptide loop from AGR2 that has the Reptin binding site based on the structure of the AGR2 ortholog Erp18 (Protein Data Bank code 2K8V). [ii]. Sequences in the divergent loop between AGR2, AGR3, and ERP18 proteins.

10b, lanes 4–7 and 9–11); the exceptional amino acid is N108 (lane 8). Alanine substitution of residues outwith this region does not attenuate Reptin binding to the peptides (Fig. 10b, lanes 1–3 and 12–15). Truncation mutagenesis also confirmed that this loop motif forms the specific interface for Reptin. C-terminal truncations from E111 to D114 do not reduce Reptin binding (Fig. 10b, lanes 16–19). However, the removal of Y111 reduces Reptin binding (Fig. 10b, lanes 20–22). This defines the C-terminal end of the Reptin binding site on the AGR2 peptide. Similarly, the N-terminal regions in the loop peptide were also truncated to define the N-terminal residues in the peptide that are important for binding to Reptin. The deletion of A101 to Q103 did not reduce Reptin binding (Fig. 10b, lanes 23–25). However, deletion of F104 reduced Reptin binding (Fig. 10b, lanes 26–39), highlighting the N-terminal residue required for binding to Reptin. The minimal peptide that exhibited binding to Reptin was 8 amino acids long (104-FVLLNLVY-111; Fig. 10b, lane 34), and the peptide displayed reduced binding to Reptin when the F104A or Y111A mutation was introduced (Fig. 10b, lanes 35 and 36 *versus* lane 34).

This divergent loop in AGR2 from 104–111 (Fig. 9c) might mediate specificity in its interactions with potential partner proteins. We examined this by evaluating whether the peptide derived from this loop in AGR3 (Fig. 9cii) bound with higher or lower affinity to Reptin. The peptide used from AGR3 had the sequence QNKFIMLNLMHETTD, and it did not bind Reptin (Fig. 10b, lane 37), compared with the AGR2 sequence AEQFVLLNLVYETTD, which did bind Reptin (Fig. 10b, lane 1 or lane 41). Mutating the KF in AGR3 to the QF present in AGR2 did not restore AGR3 peptide binding to Reptin (Fig. 10b, lane 38). However, introducing the H-to-Y mutation in the C-terminal region of the AGR3 peptide restored its binding to Reptin (Fig. 10b, lane 39 *versus* lane 37).

We finally evaluated whether inactivating mutation of full-length AGR2 protein in this divergent loop (based on the peptide screens in Fig. 10) reduced the interaction between Reptin and AGR2. We did not delete this loop as this might make gross mutations that alter the structure of the AGR2 protein. The peptide mapping data indicated that mutation of seven of eight residues could attenuate the binding of Reptin to AGR2 peptides, suggesting that the entire peptide contains side chains making important contacts with a potential peptide-binding groove in Reptin protein. As such, it might not be possible to make single point mutations on the full-length AGR2 protein if the structure of the constrained loop would require multiple mutations in the loop to attenuate the stability of the full-length Reptin–AGR2 protein complex. Nevertheless, we first focused on making two single point mutations in residues F104 and Y111, because these two residues form the N-terminal and C-terminal ends of the peptide binding between Reptin and the AGR2 peptide and these are bulky hydrophobic residues that might contribute significantly to the stability of the full-length AGR2–Reptin protein complex.

With the use of ELISA binding assays where AGR2 is immobilized in the solid phase, mutation of full-length AGR2 at codon 111 (Y to A) did not reduce the stability of the Reptin–AGR2 complex (Fig. 11b) and the $AGR2^{Y111A}$ mutant protein still retained destabilization in the presence of ATP similar to WT AGR2 (Fig. 11b). However, the $AGR2^{F104A}$ mutant protein was substantially destabilized in its binding to Reptin (Fig. 11b), indicating that the F104 residue forms an important contact point for Reptin in the context of the full-length protein complex. When Reptin was first immobilized in the solid phase, the $AGR2^{Y111A}$ mutant protein was reduced in Reptin binding and the $AGR2^{F104A}$ mutant protein was substantially destabilized in its binding to Reptin (Fig. 11a).

Discussion

Defining regulatory motifs in Reptin that regulate binding to AGR2

AGR2 is a prometastatic and p53 inhibitory protein involved in a range of oncogenic pathways, such as tamoxifen resistance and cell migration, as well as additional biological functions in limb regeneration and inflammatory responses.[5,10,12,19] Despite the wealth of data accumulating on AGR2, there are no validated interacting proteins for the AGR2 protein in human cells, with only the newt receptor PROD1 identified as an AGR2-interacting protein in yeast two-hybrid that functions in newt limb regeneration.[10] In this current work, we report on yeast two-hybrid interactors for AGR2 from a human cDNA library, one of which (Reptin) was evaluated using biochemical approaches for whether it formed a bona fide interaction with the AGR2 protein. In order to validate the interaction between the two proteins, we set out to determine whether we could identify limiting determinants in both AGR2 and Reptin proteins, whose characteristics would allow us to measure the validity of the potential protein–protein interaction. In the case of Reptin, we exploited its major functional motif; its two ATP binding sites. In the case of AGR2, because there is no identified ligand binding domain, we exploited the likelihood that small linear domains/unstructured motifs might mediate a specific interaction between AGR2 and Reptin. Our data indeed demonstrate that the ATP binding motifs of Reptin and the proposed substrate-binding loop of AGR2

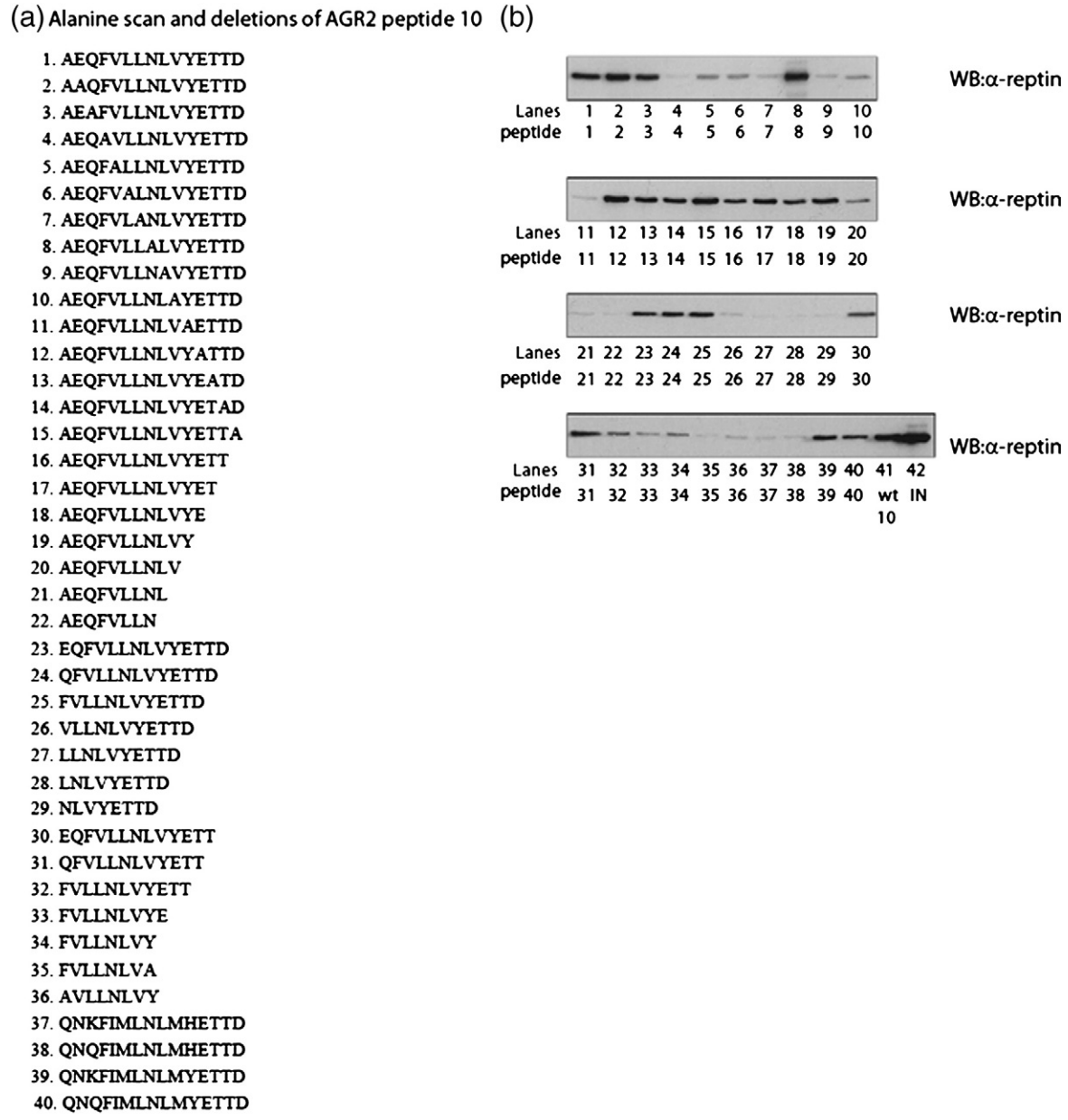

Fig. 10. Identification of key residues that stabilize the AGR2 peptide–Reptin complex. (a) Peptides 1–36 represent modifications in peptide 10 (Fig. 9), including C-terminal and N-terminal truncations and alanine-substituted mutants. Peptides 37–40 represent the divergent loop in the AGR2 ortholog AGR3 (peptide 37 is the AGR3 sequence and 38–40 mutations in this sequence). (b) Human cell lysates expressing Reptin were incubated in a buffer containing the indicated biotinylated peptides [as in panel (a); peptides 1–40 of human AGR2 or AGR3] coupled to streptavidin beads. The amount of Reptin bound was evaluated by immunoblotting the bound fractions as indicated in lanes 1–40. Lane 41 contains the positive control peptide 41, and lane 42 is the input lysate [IN].

are determinants that drive a specific complex between AGR2 and Reptin proteins.

The biochemical integrity of Reptin was measured with respect to its ATP binding activity using WT and ATP binding site mutants of the protein and included the following: (i) increases in mass as defined by light scattering in the presence of ATP; (ii) classic thermal unfolding transition in the presence of ATP; (iii) ATP binding activity using nitrocellulose filter binding assays; (iv) ATP binding quantified as defined by changes in oligomerization potential using chemical cross-linkers; and (v) ATP-dependent changes in binding to AGR2. In these cases, there was only one experimental discrepancy: the Reptin^{K83A} mutant protein was completely defective in ATP binding as defined using the filter binding assay. However, this

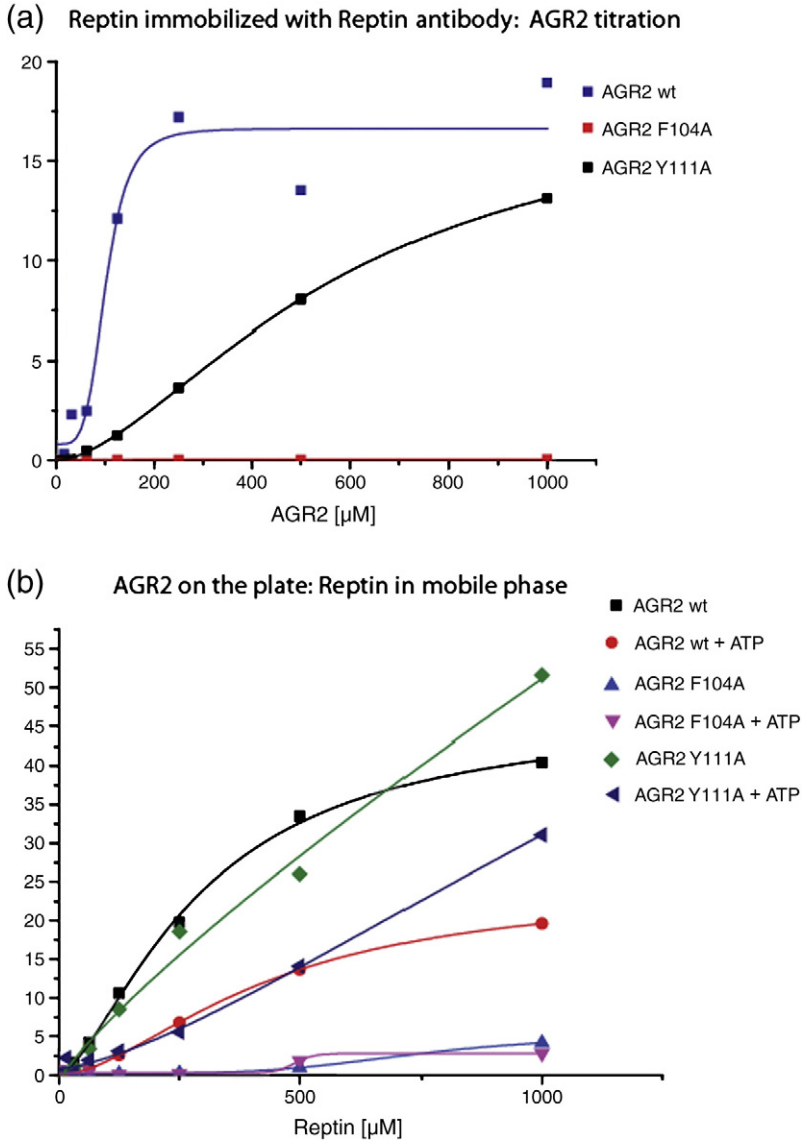

Fig. 11. The effects of AGR2 loop mutations on the stability of the Reptin–AGR2 complex. (a and b) Reactions were set up using WT AGR2, AGR2^{Y111A}, and theAGR2^{F104A} mutant proteins in which (a) Reptin is in the solid phase or (b) AGR2 is in the solid phase in the absence and in the presence of ATP. The amount of Reptin or AGR2 bound was quantified with antibodies specific for either protein titrated in the mobile phase using chemiluminescence. The data are plotted as the extent of protein–protein complex formation (in relative luminescence units) as a function of increasing protein in the mobile phase (in micromolar concentrations).

Reptin^{K83A} mutant protein was more responsive in ATP-mediated oligomerization than WT Reptin using the chemical cross-linking assay. This discrepancy in the ATP nitrocellulose filter binding assay might involve a potential inactivation of Reptin^{K83A} mutant protein ATP binding activity when it is adsorbed to the nitrocellulose filter and washed at 0 °C. The protein binding assays, thermal shifts, and the oligomerization assay are evaluated in solution at room temperature or higher. The ATP filter binding assay is very sensitive to temperature as this assay involves exploiting the difference between ATP and target protein in their hydrophobic interactions with the nitrocellulose filter at low temperatures.[40] Thus, the lower temperatures required for wash buffer in this assay might alter the affinity of ATP for the Reptin^{K83A} mutant protein. Indeed, our continuing characterization indicates that the oligomerization of WT Reptin is dependent on temperature, especially at very low temperatures, and this might also underestimate the ATP binding function of the Reptin^{K83A} mutant protein.

In contrast to the Walker A K83A mutant that essentially acts as a loss-of-function mutation with

respect to ATP binding, the Walker B mutant Reptin^{D299N} mutant protein was more active than WT Reptin in ATP binding using the nitrocellulose filter binding assay, more responsive to ATP in the oligomerization cross-linking assay, and more intrinsically thermoresistant in the thermal-shift assays even without ATP inclusion. Because there are two ATP binding sites for Reptin, it can be implied that the ATP binding function for both Reptin^{K83A} and Reptin^{D299N} mutant proteins could be regulated through an allosteric effect of one site on the other remaining active motif. Indeed, with the use of the double ATP binding site mutant, it is established that the K83A mutation is dominant over the D299N mutation with respect to AGR2 binding (Fig. 8c), suggesting that an allosteric effect between the two sites does exist. Although there is no high-resolution structure available for Reptin, a fragment of its ortholog Pontin has been crystallized[41] and is summarized in Fig. 6a. The ATP binding motifs are proximal, being poised by two structural lobes, and the proximity of the ATP binding motifs to each other might alter allosterically the ATP binding function of Reptin.

Prior to the current work, there have been minimal studies on potential allostery in the ATP binding properties of Reptin. One study reported that mutating the Reptin ATP binding site (D299N) did not inhibit the ATP binding function of the mixed Pontin–Reptin oligomer[34] and that ATP did not change the oligomeric state of WT Reptin.[34] This are in contrast with our data showing that the WT Reptin increases in apparent mass defined using light scattering and that our preparations of Reptin^{D299N} mutant protein display elevated intrinsic thermostability, elevated ATP binding, and a different response to ATP-dependent oligomerization as defined by cross-linking. These might be due to differences in the protein concentration or temperatures we have used in light scattering or cross-linking experiments compared with the gel-filtration method used in the prior report. In addition, our purified recombinant Reptin preparations are untagged, whereas the prior report utilized His-tagged protein.[34] Although a tag is often thought to be neutral, it can sometimes have an effect on a protein; indeed, this is specifically the case for Reptin. When we initially used a GST-tagged Reptin protein in our biochemical characterizations, it displayed an activated rather than an attenuated binding affinity for AGR2 protein in the presence of ATP (unpublished data). As a result, we only used untagged recombinant Reptin in this current work. Together, these data provide a proof of concept that a protein–protein interaction between Reptin and a target protein can be modified allosterically by the ATP binding motifs of the protein. How these affect other Reptin–protein interactions, including Pontin, APPL1/2, telomerase, or Tip60, as well as helicase activities, remains to be determined.

Defining amino acid motifs in AGR2 that form a docking site for Reptin

Having identified determinants in Reptin that contribute to its stable binding of AGR2, we next determined whether determinants in AGR2 that mediate its binding to Reptin could be identified. AGR2 is a member of the PDI superfamily that contains thioredoxin/PDI homology folds (CxxC or CxxS motif). These proteins can function similar to molecular chaperones to regulate protein folding via regulation of disulfide-bond formation.[18] Proteins with a CxxC motif, such as Erp57, have been shown to have their interactome trapped by using the CxxS artificial mutant that apparently traps normal *in vivo* interactors with the mutant Erp57 protein.[39] This PDI motif within AGR2 is the CxxS motif characteristic of a PDI subclass of molecular chaperones that use this motif as a redox catalyst able to form a covalent complex with other target proteins, similar to how Mucin has been reported to with AGR2.[19] In our evaluations, the AGR2^{C81A} mutant protein is able to form a Reptin complex and WT AGR2 (data not shown), indicating that the PDI thioredoxin fold of AGR2 is not directly involved as a rate-limiting determinant in binding stably to Reptin. As such, we set out to evaluate how other determinants in AGR2 protein sequence might be exploited to determine whether Reptin has a specific docking site on the protein.

One of the recent paradigm shifts in the field of protein science is the realization that a large percentage of the protein sequence information is not only in stable globular domains but also in small linear motifs, linear domains, or intrinsically unstructured domains that form small docking sites for a protein with a peptide-binding groove.[37] Proteins that have a large percentage of the protein sequence information in an unstructured landscape include regulatory proteins, such as p53, that sit in signaling hubs that form a scaffold for the formation of large numbers of diverse, low-affinity, and transient but specific protein–protein interactions.[42] These linear motifs can also be embedded within structural domains themselves. In the case of AGR2, we utilized an overlapping peptide library that represents the primary linear amino acid sequence information of the protein with the aim of determining whether or not there are any linear motifs that can form a stable complex with full-length Reptin. Using this approach, we were in fact able to detect a linear domain that can bind stably to Reptin, and this was localized by mutagenesis with amino acids 104–111 on the AGR2 primary sequence. This region is notable in that it represents a divergent loop in the Erp18/AGR2/AGR3 family of proteins that has been proposed to represent substrate binding sites for the molecular chaperone function of the protein.[39] That amino acids 104–111 from AGR2 form the minimal docking site

peptide that can bind to Reptin and that the AGR2 mutant protein Y104A completely loses the ability to bind stably to Reptin are consistent with this substrate docking model. What this model proposes, however, is that perhaps AGR2 can bind to Reptin at this loop and in turn regulate Reptin's many functions by chaperoning: ATPase activity, ATP binding, helicase functions, telomerase/Pontin binding, APPL1/2 binding, TIP60 interactions, and other related protein signaling functions. As we can convert the AGR3 peptide loop to Reptin binding protein by generating an H111–Y111 conversion (Fig. 10), we are currently generating an AGR3 protein with a WT AGR2 loop to determine if we can switch AGR3 to a Reptin binding protein *in vitro* and in cells. As AGR3 has a distinct subcellular localization from AGR2 (unpublished data; with AGR3 being more mitochondrial and of the plasma membrane), we are also evaluating whether a change in this loop on AGR3 changes its intracellular localization.

In summary, we report on the first well-validated protein–protein interaction for the pro-oncogenic protein AGR2. Reptin was identified as an AGR2 binding protein in a yeast two-hybrid screen and validated as an AGR2 binding protein in human cells. Limiting determinants for both AGR2 and Reptin were identified using *in vitro* protein enzymological approaches; AGR2 uses a divergent peptide substrate-binding loop to bind to Reptin, and Reptin in turn uses two allosterically interacting ATP binding motifs to control its binding activity toward AGR2. Because Reptin can also function as a prometastatic transcription protein and we show for the first time that Reptin can be overproduced in human breast cancers (Fig. 1b), future research in cell-based studies will inform whether AGR2 uses its substrate-binding loop to chaperone Reptin into inactive and activated transcriptional states and/or whether the allosteric ATP binding motifs of Reptin regulate AGR2 function as a prometastatic factor in cancer. The sets of AGR2 and Reptin mutants that were generated will be useful for such cellular assays. In addition, as both Reptin and AGR2 can be thought of as potential anticancer drug targets due to their prometastatic functions, biochemically based screening assays that utilize the substrate-binding loop of AGR2 or ATP binding motifs of Reptin might form biochemical assays for the development of small molecules that regulate this protein–protein complex *in vivo*.

Materials and Methods

Reagents

Fetal bovine serum was from Autogen Bioclear. Dulbecco's modified Eagle's medium and RPMI were provided by Gibco. Trypsin/EDTA solution and penicillin–streptomycin were supplied by Invitrogen. Attractene was from Qiagen. Hybond-C nylon membrane for immunoblotting was supplied by Amersham Pharmacia Biotech. ATP-γS [adenosine 5′-*O*-(3-thiotriphosphate)] and ATP (adenosine 5′ thiotriphosphate) were from Calbiochem. The following antibodies were used: anti-HA tag monoclonal antibody and anti-Myc tag rabbit polyclonal antibody (Sigma), anti-His tag monoclonal antibody (Novagen), anti-AGR2 monoclonal antibody (Abnova), anti-AGR2 polyclonal antibody (Moravian Biotechnologies), and anti-Reptin rabbit polyclonal antibody (Abnova). Secondary antibodies were from Dako Cytomation. All peptides were synthesized with a biotin tag and an SGSG spacer at the N-terminus and were from Mimotopes.

Plasmids

The human HA-tagged Reptin and AGR2 for mammalian and bacterial expression were cloned into Gateway Entry clones (Invitrogen) for subsequent use. The human Reptin sequence for cloning into a new *E. coli* expression system was amplified using the following primers: forward primer 5′-GGGGACAAGTTTGTACAAAAAAG-CAGGCTTCCTGGAAGTTCTGTTCCAGGGGCCC ATGGCAACCGTTACAGCCACAACC-3′ and reverse primer 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTC-CAGGAGGTGTCCATGGTCTCG-3′. The forward primer had a PreScission protease cleavage site inserted. Following amplification, the polymerase chain reaction (PCR) product was first inserted into pDONR201 and then into pDEST-15 using Gateway technology (Invitrogen) to generate GST-tagged Reptin. Myc-tagged Reptin and Pontin for mammalian expression were a kind gift from Dr Marta Miaczynska (International Institute of Molecular and Cell Biology, Warsaw, Poland). Point mutations in the abovementioned plasmids were introduced using the following primers: for the Reptin D299N mutant, forward primer 5′-GAGTGCTGTTCATCAACGAGGTCCA-CATGC-3′ and reverse primer 5′-GCATGTGGACCTCGTT-GATGAACAGCACTC-3′; for the Reptin K83A mutant, forward primer 5′-GCACGGGGGCGACGGCCATCG-3′ and reverse primer 5′-CGATGGCCGTCGCCCCCGTGC-3′; for generating the AGR2 F104A mutant, forward primer 5′-TGGCAGAGCAGGCTGTCCTCCTC-3′ and reverse primer 5′-GAGGAGGACAGCCTGCTCTGCCA-3′; and for generating the AGR2 Y111A mutant, forward primer 5′-CCTCAATCTGGTTGCTGAAACAACTGAC-3′ and reverse primer 5′-GTCAGTTGTTTCAGCAACCAGATT-GAGG-3′.

Cell culture and transfection

H1299 and MCF7 cell lines were grown in RPMI or Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) penicillin–streptomycin mix. Both cell lines were maintained at 5% CO_2. Cells were seeded 24 h prior to transfection, and DNA was transfected using Attractene, following standard protocol. Cells were lysed in immunoprecipitation

lysis buffer as described below. Clarified cell lysates were normalized for the protein concentration using the Bradford method.[43]

Western blotting

Samples were resolved by SDS-PAGE through 12% (w/v) Tris-glycine gels. Gels were transferred onto nitrocellulose membranes in transfer buffer [0.192 M glycine, 25 mM Tris, and 20% (v/v) methanol] at 300 mA for 90 min. Following transfer, the membrane was stained with black Indian ink to confirm even protein transfer and loading. Membranes were blocked for 1 h in 5% milk-PBST [5% (w/v) dried skimmed milk and 0.1% (v/v) Tween 20 in phosphate-buffered saline]. Membranes were then incubated with primary antibodies overnight, followed by secondary antibodies conjugated to horseradish peroxidase for 1 h. Bound antibody was detected by enhanced chemiluminescence, and immunoblots were quantified using ImageJ. When indicated, gels were stained with Coomassie blue R-450 (Sigma; 5% Coomassie blue in 40% methanol and 10% acetic acid). Lysates and preparation from primary breast cancer and normal tissue samples have been previously described.[5,44]

Expression and purification of WT and mutant Reptin

GST-Reptin WT and mutants were expressed in BL21-AI (Invitrogen) and purified from soluble lysates using glutathione beads (GE Healthcare) according to the manufacturer's instructions. In detail, cells were lysed with 10% sucrose, 50 mM Tris, pH 8.0, 400 mM NaCl, 0.5% Triton X-100, 1 mM DTT, 1 mM benzamidine, 0.5 mg/ml lysozyme, and protease inhibitors for 30 min on ice and then sonicated. Lysate was incubated with glutathione beads for 90 min at 4 °C with rotation, followed by thorough washes with 20 mM Hepes, pH 7.5, 1 M NaCl, 1 mM DTT, and 1 mM benzamidine; thorough washes with 20 mM Hepes, pH 7.5, 0.05 mM NaCl, 1 mM DTT, and 1 mM benzamidine; and a final wash with 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, and 1 mM DTT. The rigorous washes were required to remove nonspecifically bound proteins. Reptin protein was cleaved off the column using PreScission protease (GE Healthcare) into 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, and 1 mM DTT and stored frozen in liquid nitrogen at 6–9 mg/ml.

Expression and purification of WT and mutant AGR2

His-tagged AGR2 was expressed in BL21-AI and purified using Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) according to the manufacturer's instructions. In detail, cells were lysed in a buffer containing 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 10 mM $MgCl_2$, 0.05% Tween 20, 10% glycerol, 20 mM imidazole, pH 8.0, and 0.1 mg/ml on ice for 30 min and then sonicated. Lysate was incubated with Ni-agarose (on rotary shaker at 4 °C for 1 h) and washed two times in lysis buffer and three times in lysis buffer with 40 mM imidazole, and then protein was eluted with lysis buffer containing 150 mM imidazole.

Thermal protein unfolding assay and light scattering

Thermal shifts

Extent of protein unfolding was measured using fluorescent SYPRO Orange dye (Invitrogen). Purified Reptin (5 μM) with or that without ATP was added in buffer containing 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, and 1 mM DTT before the addition of SYPRO Orange. Samples were aliquoted onto a 96-well PCR plate and sealed with optical-quality sealing film (Bio-Rad). The rate of protein unfolding was measured using an iCycler iQ Real-Time PCR system (Bio-Rad) by heating samples from 20 °C to 90 °C at increments of 1 °C and with a 30-s incubation at each increment. Fluorescence intensity was measured using excitation/emission wavelengths of 485 nm/575 nm in relative light units, and thermal denaturation graphs were plotted as a function of the gradient of protein unfolding against the temperature gradient [d(RFU)/dT].

Light scattering

Light scattering was measured in a temperature-controlled Zetasizer Auto Plate Sampler. Buffer (50 mM Tris–HCL, pH 8.0, 1 mM EDTA, 120 mM NaCl, and 1 mM DTT) was filtered using 0.2 μM as a background control. The mean hydrodynamic radius, R_h, of Reptin was measured by DLS with a Zetasizer APS (Malvern Instruments, UK) equipped with a 50-mW laser light source of wavelength 830 nm. Scattering data were collected at a scattering angle of 90 ° for 10 s, repeated at least 12 times, and averaged. The experiments were repeated in triplicate. Autocorrelation data were fit to a model of a multiple-exponential form suitable for polydisperse solutions using the protein-specific software supplied with the instrument. This generated a distribution of particles by size. DLS is very sensitive to aggregation as scattering is a function of R_h to the sixth power. Size analysis was carried out on 50 μM Reptin in 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 120 mM NaCl, and 1 mM DTT at 10 °C. Samples were passed through a 0. 22-μm filter (Ultrafree-MC, Millipore, UK), centrifuged at 4 °C, 12*g*, prior to analysis.

Protein–protein interaction assays (ELISA)

Purified recombinant His-AGR2 (100 ng), Reptin (100 ng), anti-AGR2 monoclonal antibody (100 ng), anti-His monoclonal antibody (100 ng), and anti-Reptin antibody (100 ng) were immobilized on a microtiter plate 0.1 M $NaHCO_3$ buffer, pH 8.6, at 4 °C overnight. Excess protein was washed away in PBS containing 0.1% (v/v) Tween 20. Nonreactive sites were blocked using PBS containing 3% bovine serum albumin. A titration of the protein of interest (in the solid phase or mobile phase, as highlighted in each figure) with or that without ATP or ATP-γ-S as indicated in the legend to the figures was added in 1× reaction buffer [25 mM Hepes, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 5% (v/v) glycerol, 0.1% (v/v) Tween 20, and 2 mg/ml bovine serum albumin] for 1 h at room temperature. After washing in PBS containing 0.1% (v/v) Tween 20, anti-His tag monoclonal, anti-Reptin polyclonal, and anti-AGR2 polyclonal antibodies were

added. The unbound primary antibody was washed away, and then the appropriate secondary antibody was added. After washing, electrochemical luminescence was quantified using a luminometer (Fluoroskan Ascent FL, Labsystems).

ATP filter binding assay

Reptin was incubated with 0.57 μCi γ-^{35}S-labeled ATP at 4 °C for 15 min in 20 μl of ATP binding assay buffer (20 mM Tris–HCl, pH 7.5, 70 mm KCl, and 1 mM $MgCl_2$). In some experiments, Reptin was further incubated with DNA fragments at 30 °C for 5 min in the same buffer. Samples were passed through nitrocellulose membranes (Millipore HA, 0.45 μm) at room temperature and washed rapidly with 20 volumes of ice-cold buffer T using a vacuum suction. The radioactivity remaining on the filter was monitored with a liquid scintillation counter (Perkin Elmer).

Peptide binding assays

H1299 cells were lysed in 0.1% Triton X-100 lysis buffer (50 mM Hepes, 0.1 mM EDTA, 150 mM NaCl, 10 mM NaF, 2 mM DTT, 0.1% Triton X-100, and 1× protease inhibitor mixture). Lysates were incubated with 40 μg/ml avidin for 30 min on ice. Then, lysates were precleared by incubation with streptavidin-agarose (Sigma) beads for 1 h. In the meantime, 0.4 μl of peptide was incubated with streptavidin-agarose (Sigma) beads in buffer W (100 mM Tris, pH 8.0, 150 mM NaCl, and 1 mM EDTA) for 1 h at room temperature with gentle rotation and then washed three times with the same buffer. Cleared lysate (0.2 mg) was then added to the peptide-coated beads in a final volume of 200 μl and rotated at room temperature. After 1 h, beads were washed once with buffer W, four times with PBS + 0.2% Triton X-100, and once more with buffer W. Sample buffer was then added to the beads, and bound protein was eluted by boiling for 3×5 min and immunoblotted to quantitate the amount of Reptin bound.

Immunoprecipitation of protein complexes from cell lysates

The cells were harvested and lysed in co-immunoprecipitation buffer (25 mM Tris, pH 7.2, 0.4 M KCl, 1% NP-40, and 1 × protease inhibitor mixture). The lysates were precleared by incubation with Sepharose CL 4B (Sigma-Aldrich) and protein G-Sepharose™ 4 FastFlow (GE Healthcare) at 4 °C with rotation for 40 min. Subsequently, 1 μg of primary antibody (K47 polyclonal specific for AGR2) was incubated with 600 μg of protein in the precleared lysate in a final volume of 200 μl for 2 h at 4 °C with gentle rotation. A total of 15 μl of protein G-Sepharose was then added to the abovementioned samples and incubated for 1 h at 4 °C with gentle rotation. Supernatant (flow-through) was collected, and the beads were washed four times with 500 μl of co-immunoprecipitation buffer. Samples were eluted by adding 50 μl of 4 × SDS sample buffer containing 0.2 M DTT and incubating at 95 °C for 5 min. The eluate was then collected and analyzed by Western blotting.

His-ubiquitin conjugate pull-down assay

Cells co-transfected with HA-tagged Reptin or p53 and/or human MDM2 along with pCMV-His-ubiquitin, NEDD8, or SUMO plasmids for 24 h were incubated with 10 μM MG132 for 4 h and then harvested and washed in PBS before the addition of 6 ml of HUBMA (His-ubiquitin modification buffer A; 6 M guanidinium chloride, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0,10 mM Tris–HCl, and 10 mM 2-mercaptoethanol) and 5 mM imidazole. The lysate was homogenized using a 24-G syringe needle before adding 75 μl of Ni^{2+}-nitrilotriacetic acid-agarose beads and rotating at room temperature (21 °C) for 4 h. The beads were centrifuged at 2000 *g* for 5 min, and the supernatant was discarded before washing sequentially with 750 μl of HUBMA, HUBMB (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 8.0, 10 mM Tris–HCl, and 10 mM 2-mercaptoethanol), HUBMC (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, pH 6.3, 10 mM Tris–HCl, and 10 mM 2-mercaptoethanol), HUBMC with 0.2% (v/v) Triton X-100, and finally HUBMC and 0.1% (v/v) Triton X-100. To the washed beads, 75 μl of His-ubiquitin elution buffer [0.2 M imidazole, 5% (w/v) SDS, 0.15 M Tris–HCl, pH 6.7, 10% (v/v) glycerol, and 0.72 M 2-mercaptoethanol] was added and incubated for 20 min at room temperature. A total of 75 μl of 2XSDS sample buffer was added to the eluted ubiquitin conjugates and subjected to Western blot analysis as indicated in the legend to Fig. 3.

Oligomerization assay using cross-linker

Recombinant WT or mutant Reptin protein (2 μg) with or without 1 mM ATP was mixed with serial twofold dilutions of glutaraldehyde, from 0.2% to 0%, in a total volume of 20 μl and incubated for 1 h at room temperature. The reaction was stopped with 10 μl of 1 M Tris, pH 8.0, sample buffer was added, and samples were boiled, separated using 8% SDS-polyacrylamide gel, and immunoblotted to detect monomeric and oligomeric Reptin.

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AGR2 oncoprotein inhibits p38 MAPK and p53 activation through a DUSP10-mediated regulatory pathway

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ABSTRACT

The tumor suppressor p53 plays a key role in malignant transformation and tumor development. However, the frequency of p53 mutations within individual types of cancer is different, suggesting the existence of other mechanisms attenuating p53 tumor suppressor activity. Changes in upstream regulators of p53 such as MDM2 amplification and overexpression, expression of viral oncoproteins, estrogen receptor signaling, or changes in p53 transcriptional target genes were previously described in wild-type p53 tumors. We identified a novel pathway responsible for attenuation of p53 activity in human cancers. We demonstrate that AGR2, which is overexpressed in a variety of human cancers and provides a poor prognosis, up-regulates DUSP10 which subsequently inhibits p38 MAPK and prevents p53 activation by phosphorylation. Analysis of human breast cancers reveals that AGR2 specifically provides a poor prognosis in ER+ breast cancers with wild-type p53 but not ER- or mutant p53 breast cancers, and analysis of independent data sets show that DUSP10 levels also have prognostic significance in this specific sub-group of patients. These data not only reveal a novel pro-oncogenic signaling pathway mediating resistance to DNA damaging agents in human tumors, but also has implications for designing alternative strategies for modulation of wild-type p53 activity in cancer therapy.

1. Introduction

Wild-type (wt) p53 is a sequence-specific DNA-binding protein and stress-activated transcription factor. Depending on cell type and stress stimuli, p53 plays multiple roles in many cellular processes, including cell cycle arrest, apoptosis, differentiation and senescence (Lane, 1992). The main role of p53 is the maintenance of genome stability and integrity, therefore p53 inactivation represents a crucial step in tumor development and progression. The role of p53 as a tumor suppressor is reflected by its high rate of mutation in human tumors. Mutations in p53 act as useful markers for the follow-up of minimal residual disease, for comparison between primary and recurrent tumors, for tracing the origin of distant metastases and to determine tumor clonality (Olivier et al., 2010). Moreover, mutations in p53 have been consistently associated with poor prognosis in many cancers including

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breast, colorectal, head and neck, leukemia and others (Petitjean et al., 2007).

In cancers where wt p53 is present, the p53 network is usually altered by other genetic or epigenetic events that compromise the p53 response. The most studied is p53-inducible E3 ubiquitin ligase MDM2 that promotes p53 proteasomal degradation through an ubiquitin-dependent pathway (Haupt et al., 1997). Although MDM2 protein overexpression has also been found in the absence of gene amplification, it generally results from gene amplification observed in ~7% of all human cancers (Forslund et al., 2008). Another RING finger ubiquitin ligase involved in regulation of p53 is MDMX (MDM4), which regulates MDM2 activity (Wade et al., 2013). The causative viruses of human cancer possess several distinct mechanisms to inactivate p53 functions and signaling by the alterations of post-transcriptional modifications, localization, binding partners, turnover, and transcriptional activity (Sato and Tsurumi, 2013). Additional mechanisms resulting in attenuation of wt p53 activity are mutations of its upstream activators *e.g.* ATM or CHK2 (Banin et al., 1998; Hublarova et al., 2010). A strong relationship of estrogen signaling with p53 has also been documented. Activated estrogen receptors (ER) were shown to antagonize p53 function either directly through the recruitment of co-repressors (Konduri et al., 2010), or *via* independent inhibition of genes responsible for execution of the p53-triggered apoptotic program in response to DNA damage (Bailey et al., 2012).

In 1998, co-expression of the *AGR2* (*anterior gradient-2*) gene and ER was detected in mammary gland cancer cells (Thompson and Weigel, 1998). Later, it was shown that AGR2 expression is directly stimulated by ER signaling (Salmans et al., 2013) and correlates with poor outcome of patients with ER-positive breast cancer (Hrstka et al., 2010; Innes et al., 2006), which emphasizes the importance of estrogen receptors in the regulation of AGR2 expression. From a functional perspective, AGR2 was shown to promote tumor metastasis, cell survival, cell proliferation and resistance to therapy (Hrstka et al., 2013; Ramachandran et al., 2008; Wang et al., 2008). In addition to breast cancer, AGR2 was also detected in many other adenocarcinomas (Brychtova et al., 2011). Proteomic analysis of Barrett's epithelium, where alterations in *TP53* gene function are observed at high frequency, identified AGR2 protein as over-produced in pre-neoplastic cells. Subsequent data revealed that AGR2 functions as a pro-oncogenic survival factor that inhibits the p53 response to UV radiation (Pohler et al., 2004). However the mechanism by which AGR2 attenuates p53 activity is unknown.

In our work, we identify a novel signaling pathway where AGR2 exerts its inhibitory effect on p53 through attenuation of p38 mitogen-activated protein kinase (p38 MAPK) activity. The anti-cancer activity of many chemotherapy drugs relies on the induction of DNA damage and followed cellular response triggered by p53. Thus, the discovery of novel p53-inhibitory targets may significantly contribute to drug development programs. Our data demonstrate an important role for AGR2 as a pro-survival factor responsible for enhanced resistance to DNA damaging agents, proposing AGR2 as a target for the development of future treatments to improve anti-cancer therapy.

2. Experimental procedures

2.1. Cell cultures and treatments

The following AGR2-positive cell lines were used: MCF-7 (breast cancer, ER-positive, wt p53) and A549 (lung cancer, ER-negative, wt p53). AGR2-negative cell lines included in the study were H1299 (lung cancer, ER-negative, p53 null) and ARN8 cells (clone derived from A375 cells, malignant melanoma, ER-negative, wt p53, stably transfected with a p53 dependent β-galactosidase reporter construct) (Blaydes and Hupp, 1998). All cell lines were cultured in glucose-rich D-MEM supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. The Flp-In™ System (Invitrogen) was used to generate H1299-LZ4 cells containing a single integrated Flp Recombination Target (FRT) site. The coding sequence of the human *AGR2* gene was stably inserted into this site using Flp recombinase mediated site-specific DNA recombination to give H1299-LZ4-AGR2 cell line. To establish the knockout of all 4 copies of *AGR2* in A549 cells (A549-AGR2-KO), TALEN (transcription activator-like effector nucleases) pair was constructed to target *AGR2* coding sequence at position 12–63 from start codon as described previously (Kim et al., 2013). TALEN expression plasmids bearing *AGR2* targeting sequences were further delivered into A549 cells. Transfected cells were diluted and plated at an average density of 0.5 cells per well into a 96-well plate. Colonies were immunochemically tested for AGR2 expression. The AGR2 non-expressing clone denoted as A549-AGR2-KO was used for further experiments.

25 pmol *AGR2* specific silencing RNA and 25 pmol untargeted siRNA as a control (Dharmacon, Thermo Fisher Scientific, Waltham, MA, USA) per million cells were used for transfection by AMAXA system (Lonza Group Ltd., Basel, Switzerland). Unless stated otherwise, 32 h post-transfection the cells were exposed to 10 μM cisplatin or 0.5 μM doxorubicin (both Adriablastica, Ebewe, Unterach, Austria). After 16 h cells were harvested and lysed in NP-40 lysis buffer [150 mM NaCl, 1% NP-40, 50 mM TrisCl pH 8.0, 50 mM NaF, 5 mM EDTA pH 8.0 supplemented with 100× diluted protease and phosphatase inhibitors (Sigma–Aldrich Inc., St. Louis, MO, USA)].

2.2. Determination of protein expression

Immunoblotting was carried out as described previously (Hublarova et al., 2010). The membranes were probed overnight with in-house specific anti-p53 (DO-1), (Vojtesek et al., 1992) anti-p53-S392 (FP392) (Blaydes and Hupp, 1998), anti-MDM2 (Ab2A9) (Chen et al., 1993), anti-p53-S15 and anti-p38 MAPK antibody (both Cell Signaling Technology Inc., Danvers, USA), anti-actin AC-40 (Sigma–Aldrich Inc., St. Louis, MO, USA), anti-DUSP10 (Abcam, Cambridge, UK), anti-$p21^{WAF-1}$ (Ab-1) and anti-PARP-1 (Ab-2) (both Merck Millipore, Darmstadt, Germany) monoclonal antibodies, AGR2 was detected by our in-house rabbit polyclonal antibody K47 (Hrstka et al., 2010). p38 MAPK activity was measured using p38 MAP Kinase Assay Kit (Cell Signaling Technology Inc., Danvers, USA) according to the manufacturer's instructions. Briefly, cells were transiently transfected with siRNA and subsequently

exposed to cisplatin or doxorubicin. Phosphorylated (active) p38 MAP kinase was immunoprecipitated from whole cell lysates. The phosphorylation of ATF-2 fusion protein on Thr71 caused by selectively immunoprecipitated phosphorylated p38 MAP kinase in the presence of ATP was determined. In parallel, β-actin and AGR2 protein levels were determined to check correct protein loading and *AGR2* silencing efficiency respectively. Relative fold changes in protein levels were determined using TotalLab software (Nonlinear dynamics, Newcastle upon Tyne, UK).

2.3. β-galactosidase colorimetric assay

ARN8 cells were transfected and treated as described above and harvested using 200 μl 0.25 M TrisCl pH 7.5, cell suspensions were sonicated 3× for 5 s on ice and then centrifuged 30 min/14,000 RPM/4 °C. 150 μg of total protein was added to substrate mixture (0.001 M $MgCl_2$, 0.045 M β-mercaptoethanol, 0.264 mg o-nitrophenyl-β-D-galactopyranoside, 0.1 M sodium phosphate pH 7.5) in total volume of 300 μl per reaction. The reactions were incubated 30 min at 37 °C and then were stopped by 500 μl of 1 M Na_2CO_3, absorbance was measured at 420 nm. All samples were measured in triplicate.

2.4. Reverse transcription and quantitative PCR

Total cellular RNA was extracted by TRI-Reagent (MRC, Cincinnati, OH, USA). cDNA synthesis was carried out using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Triplicate samples were subjected to quantitative PCR analysis using TaqMan Array Human MAPK pathways plates containing 92 assays to MAPK associated genes and 4 endogenous control genes (Applied Biosystems, Foster City, CA, USA). Data were analyzed using StatMiner (Integromics, Madrid, Spain). Quantitative PCR analysis using TaqMan for 18S rRNA (Applied Biosystems, Foster City, CA, USA) and SYBR Green (Sigma–Aldrich, St Louis, MO, USA) for *AGR2* was performed as described previously (Hrstka et al., 2013).

2.5. Cellular viability and proliferation

MCF-7 and A549 cells transfected with siRNA and H1299 cells transfected with corresponding plasmid were seeded into 96-well plates using 10,000 cells per well. After 24 h incubation, doxorubicin and cisplatin were applied for an additional 48 h. 20 μl MTT reagent (2.5 mg/ml in PBS) was added after incubation with drugs and cells were incubated 3 h at 37 °C, followed by addition of 100 μl 10% SDS at room temperature overnight to dissolve violet formazan. Finally, absorbance at 595 nm was measured. Each sample was measured in five technical replicates.

In colony formation assays, one million H1299 cells were transfected with 1 μg of the construct bearing either AGR2 or p53 coding sequence. Co-transfection by both plasmids was conducted with 0.5 μg of each construct. In total, 500 transfected cells were seeded per well of six-well plates, 24 h later the media was changed with fresh media or media containing cisplatin or doxorubicin. Two weeks later, the cells were washed twice with PBS, fixed for 30 min in methanol and then visualized by Giemsa–Romanowski staining and quantified using TotalLab (Nonlinear dynamics, Newcastle upon Tyne, UK).

Cell proliferation and cytotoxicity of H1299-LZ4 or H1299-LZ4-AGR2 cells (mock or p53 transfected) were performed on E-plates using RTCA-DP instrument (Roche Diagnostics GmbH, Mannheim, Germany). After transfection cells were seeded into proliferation 16-well plates at 5,000 cells per well. After 12 h post-seeding low doses of cisplatin and doxorubicin were applied in quadruplicate within the same experiment and proliferation was measured. Cell index was monitored every 15 min during the experiment.

2.6. Clinical samples and processing

Our hospital-based study included 115 primary breast cancer tissue samples obtained from female patients diagnosed at Masaryk Memorial Cancer Institute (MMCI) in 2002–2005 (age 29–79 years). The study was approved by the local ethics committee of the MMCI and informed consent was obtained from each patient. All selected patients received anthracycline-containing adjuvant chemotherapy regimens. Samples were collected within 20 min of surgical removal and immediately evaluated by a pathologist according to standardized hospital protocol. Portions of tissues were used for preparation of formalin fixed paraffin embedded tissue blocks and separate tumor pieces of ~3 × 3 × 8 mm were stored in RNA later for 3–5 days at 4 °C and then frozen at −80 °C. RNA purification as well as determination of p53 status was carried out as described previously (Dobes et al., 2014). The main clinicopathological variables including ER and progesterone receptor (PR) status were extracted from pathological records obtained from the MMCI database. AGR2 immunohistochemical staining was performed on 4 μm thick freshly cut tissue sections. Sections were deparaffinized in xylene and rehydrated into PBS through a graded ethanol series. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in PBS for 15 min. Antigen retrieval was performed in citrate buffer pH 6.0 at 94 °C for 20 min. The sections were incubated overnight at 4 °C with anti-AGR2 antibody (HPA007912, Sigma–Aldrich, St. Louis, MO, USA). We used the semiquantitative H-score with a dynamic range of 0 (no staining) to 300 (diffuse intense staining), where intensity of staining is recorded on a scale of 0–3 (0 = none, 1 = weak, 2 = moderate, 3 = strong). The H-score is calculated as the percentages of cells stained at each intensity level multiplied by that intensity level and then summed. A score <50 was considered as a negative (AGR2-).

2.7. Ex vivo *chemosensitivity assay*

A portion of a patient's solid tumor, about the size of a core biopsy, was mechanically disaggregated and established in primary culture. Cellular viability was determined by Trypan blue dye exclusion test. The cell suspension was than diluted to 0.8–1.0 × 10^6/ml in culture medium: RPMI-1640 (Sigma–Aldrich Inc., St. Louis, MO, USA) supplemented with 15% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 300 mg/ml glutamine, 0.3 U/ml R-Humulin, 10 μg/ml holo-Transferrin and 5.6 μg/ml Amphotericin-B. *Ex vivo* sensitivity of isolated tumor cells to anti-cancer drugs was

determined according to Mosmann's protocol (Mosmann, 1983) with several modifications. Briefly, six final concentrations of each tested agent (including doxorubicin which was tested preferentially in case of a lower number of cells) were tested in triplicate, negative control (wells with culture medium only) and positive controls (wells with cell suspension without drug) were used as well. Tumor cells were incubated for 72 h at 37 °C/5% CO_2 and analyzed by MTT assay. The measured values of the absorbance were converted into the program Chemorezist 1.0 (Regner et al., 2000) and the dose response curves exported for each agent tested. The EC_{50} value was derived from the average response curve and the samples evaluated as resistant: $EC_{50} > 1/16 \times$ maximal tested concentration of the drug (c_{max}); partially sensitive: $EC_{50} = 1/16–1/32 \times c_{max}$; sensitive: $EC_{50} < 1/32 \times c_{max}$.

3. Results

3.1. AGR2-dependent inhibition of p53 activity

The tumor suppressor wt p53 is critically important for the cellular response to DNA damage, including that caused by chemotherapy. We investigated the potential links between AGR2, chemotherapy, and p53 activation, based on the previous observations linking AGR2 with resistance to therapy. First, we analyzed the effect of cisplatin and doxorubicin treatment on AGR2 protein level in A549 and MCF-7 cell lines (Figure 1). Although induction of AGR2 expression was observed predominantly in MCF-7, probably due to a different cellular content, these results encouraged us to assess the effect of AGR2 on p53 in tumor cells exposed to DNA damaging drugs. To generalize the effects of AGR2, two different cancer cell lines A549 and MCF-7 (both expressing AGR2 and wt p53) were studied. Both cell lines were pretreated with siRNA against *AGR2* for 32 h and then exposed for 16 h to cisplatin or doxorubicin. Treatment with these drugs induced p53 levels, along with Ser15 and Ser392 phosphorylation, especially in response to doxorubicin treatment. Simultaneously, the level of p53 downstream regulated genes *MDM2* and *CDKN1A* (encoding $p21^{WAF-1}$) were elevated, indicating the induction of p53 transcriptional activity (Figure 1, Figure S1). However, AGR2-depleted cells showed clearly increased p53 level in comparison with cells pretreated by control siRNA (in A549: 1.5× for untreated cells, 1.2× in response to cisplatin, 2.3× in response to doxorubicin; in MCF-7: 2× for untreated cells, 2.1× in response to cisplatin and 1.7× in response to doxorubicin). Similar effects were observed also for phosphorylation at Ser15 and Ser392, as well as in the levels of p53 target genes, which were higher in cells pretreated with *AGR2* specific siRNA, indicating enhanced transcriptional activity of p53 in response to AGR2 depletion (Figure 1). More than two-fold increase in the level of MDM2 has been observed in AGR2 silenced A549 cells exposed to both cisplatin and doxorubicin in comparison to cells pretreated with control siRNA, while $p21^{WAF-1}$ was induced by doxorubicin only, but again 1.2× more after AGR2 silencing. In MCF-7 cells the effect was clear predominantly in response to cisplatin treatment, where 3.3× for MDM2 and 1.6× stronger induction for $p21^{WAF-1}$ was observed after AGR2 depletion (Figure S1).

To demonstrate that AGR2 attenuates the transcriptional activity of p53, ARN8 cells carrying p53-responsive β-galactosidase reporter were used. These cells were transiently transfected with *AGR2* expressing plasmid and subsequently exposed to doxorubicin and cisplatin. *AGR2* transfection clearly decreased transactivation of the p53-responsive β-galactosidase reporter in comparison with mock transfected cells (Figure 2). Taken together, our data show that AGR2

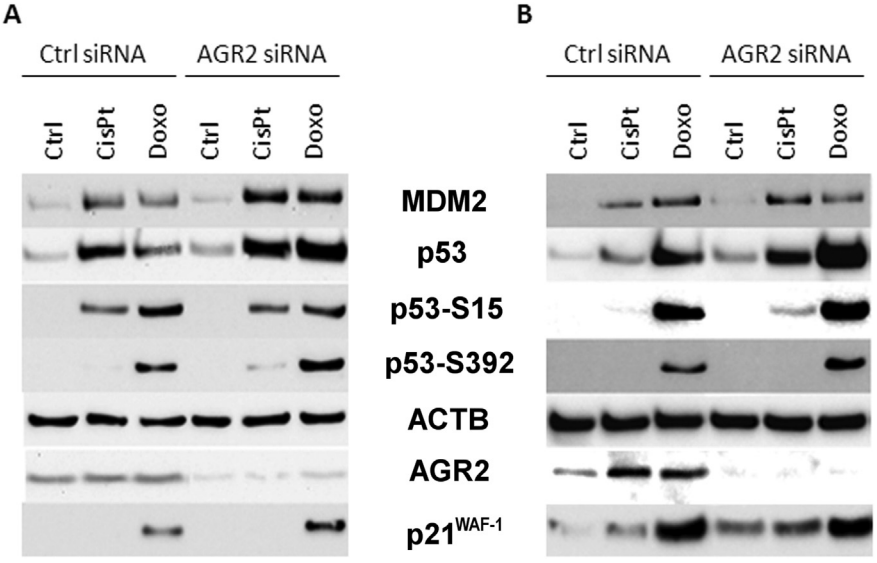

Figure 1 – The effect of AGR2 level on p53 and its downstream regulated proteins. The figure shows representative Western blots of A549 cells (A) and MCF-7 cells (B) transfected either with control or AGR2 specific siRNA and treated with cisplatin or doxorubicin. The levels of p53, p53 phosphorylation at Ser15 and Ser392, MDM2 and $p21^{WAF-1}$ protein were determined. Beta-actin (ACTB) was used as a loading control, AGR2 expression was determined to confirm siRNA efficacy. Biologically independent experiments were conducted at least 3 times with very similar results. The changes in the protein levels corresponding to this figure are graphically quantified in Figure S1.

reduces endogenous p53 levels, post-translational modification at Ser15 and Ser392 and transcriptional activity in response to both cisplatin and doxorubicin.

3.2. *Identification of AGR2-dependent mechanism responsible for inhibition of p53*

To identify downstream effectors of AGR2 signaling pathway, we screened mRNA levels of 92 key genes known to be involved in MAPK signaling in both A549 and MCF-7 cells pretreated either with control siRNA or AGR2 specific siRNA and then exposed to cisplatin or doxorubicin (Table S1). Based on the criteria that changes in gene expression must be of the same nature in response to both cisplatin and doxorubicin and that the extent of these changes should be at least 20%, we found only one gene, *Dual Specificity Phosphatase 10* (*DUSP10*) also known as *MAP Kinase Phosphatase 5* (*MKP5*). *DUSP10* mRNA levels were reduced in both MCF-7 and A549 in response to AGR2 depletion (Figure 3A and B). The involvement of AGR2 in maintaining *DUSP10* levels was then confirmed in biologically independent experiments using RT-qPCR (data not shown) and at the protein level. Decreased DUSP10 protein levels were seen predominantly in control MCF-7 cells exposed only to *AGR2* siRNA in comparison with A549 cells showing only negligible reduction of DUSP10. However, the effect was more pronounced in response to both cisplatin and doxorubicin and a clear decrease in DUSP10 expression was observed in both cell lines (Figure 3C and D).

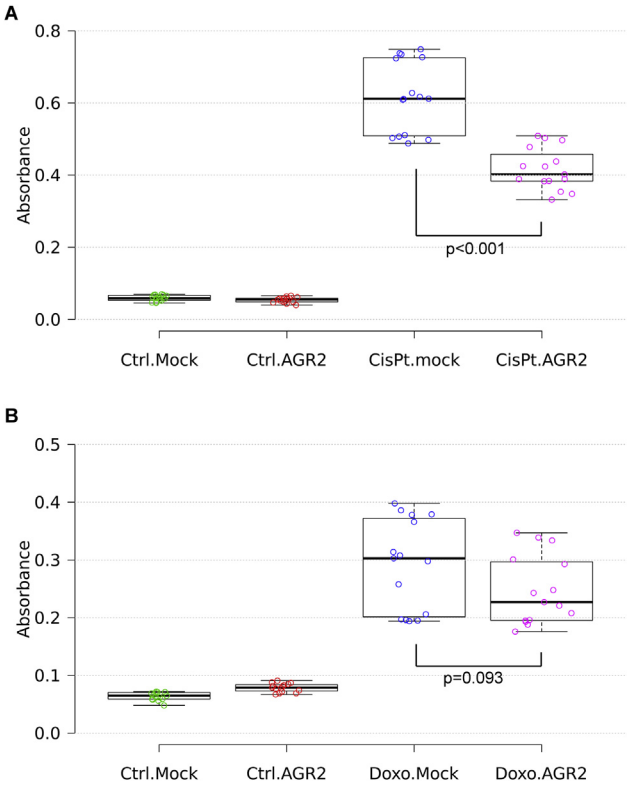

Figure 2 – AGR2 blocks p53 transactivation. ARN8 cells bearing β-galactosidase reporter construct were transiently transfected with either empty pcDNA3-GW plasmid or carrying the coding sequence for AGR2 (2 μg of plasmid per million cells using Amaxa nucleofector). Mock or AGR2 transfected cells were treated with either cisplatin (A) or doxorubicin (B). Determination of p53 transcriptional activity of p53 was conducted using β-galactosidase colorimetric assay in three independent biological replicates, each performed in quintuplicate. Statistical significance of differences in β-galactosidase activity was calculated using One-way Anova test.

To investigate the link between DUSP10 and AGR2 in relation to p53 activity, A549, A549-AGR2-KO (*AGR2* homozygous knockout) and MCF-7 cells were transfected with *DUSP10* expression plasmid and exposed to either cisplatin or doxorubicin (Figure 4). In response to these drugs two to three times lower induction of p53 was observed in *DUSP10* transfected A549 and MCF-7 cells compared to their mock transfected counterparts (Figure 4, Figure S2). A similar effect was observed for p53 phosphorylation, as well as expression of $p21^{WAF-1}$ and MDM2. Interestingly, the introduction of *DUSP10* into A549-AGR2-KO cells also attenuated p53 activity, when compared with mock transfected A549-AGR2-KO cells, but to a lesser extent (e.g. decrease in total p53 level 1.2× for cisplatin and 1.4× for doxorubicin treatment) than in parental A549 cells with endogenous AGR2 expression (Figure 4, Figure S3 – highlighted in blue), indicating potential role of AGR2 in DUSP10 mediated attenuation of p53. A synergistic effect of AGR2 and DUSP10 is also supported by the finding that the level of p53 is higher in A549-AGR2-KO cells than in parental AGR2-positive cells, in both mock and *DUSP10* transfected cells. Similar results were obtained for posttranslational modifications and expression of the p53 downstream targets $p21^{WAF-1}$ and MDM2. The results from this experiment correspond with the findings shown in Figure 1 and similar trends are observed for all three cellular models used, providing internal validation of the data.

Dual specificity protein phosphatases inactivate their target kinases by dephosphorylating both phosphoserine/threonine and phosphotyrosine residues. DUSP10 shows substrate selectivity towards the stress activated MAP kinases, predominately p38 MAPK and c-Jun amino-terminal kinase (JNK) (Owens and Keyse, 2007). Given that p38 MAPK phosphorylates p53 at Ser15 and Ser392 (Lim et al., 2007), we studied p38 MAPK as a potential effector of the AGR2-dependent inhibitory pathway towards p53. p38 MAPK protein levels were immunochemically determined in both AGR2 expressing and AGR2-siRNA treated cells (Figure 5). Since p38 MAPK protein levels do not necessarily reflect catalytic activity, we determined p38 MAPK activity using a nonradioactive IP-kinase assay in the same lysates. This assay clearly demonstrated increased phosphorylation of ATF-2 fusion protein by immobilized p38MAPK in response to both cisplatin and doxorubicin treatment. This effect was significantly enhanced when AGR2 was silenced (Figure 5). Increased catalytic activity even in the absence of increased p38MAPK protein levels after AGR2 silencing supports a role of AGR2 on DUSP10 expression, which in turn leads to inhibition of p38 MAPK activity. Taken together these data indicate a novel AGR2-triggered signaling pathway that attenuates p53 activity in response to DNA damage (Figure 6).

3.3. *AGR2 mediates drug resistance* in vitro *and* in vivo

The identification of an AGR2-dependent signaling pathway responsible for attenuation of wt p53 transcriptional activity

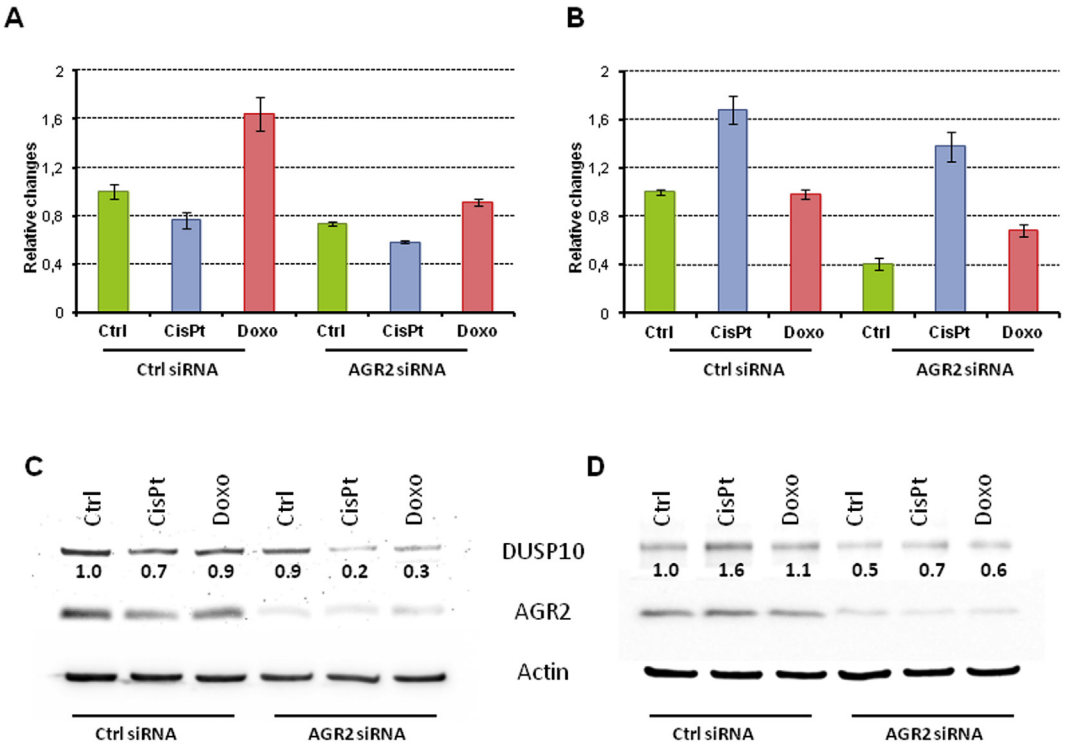

Figure 3 – AGR2 influences DUSP10 expression. Inhibition of AGR2 decreases *DUSP10* mRNA in both A549 (A) and MCF-7 (B) cells. Protein levels show similar trends in both A549 (C) and MCF-7 (D) cells. For this representative figure, the relative fold changes in DUSP10 expression in relation to untreated cells with endogenous AGR2 expression are indicated under corresponding bands.

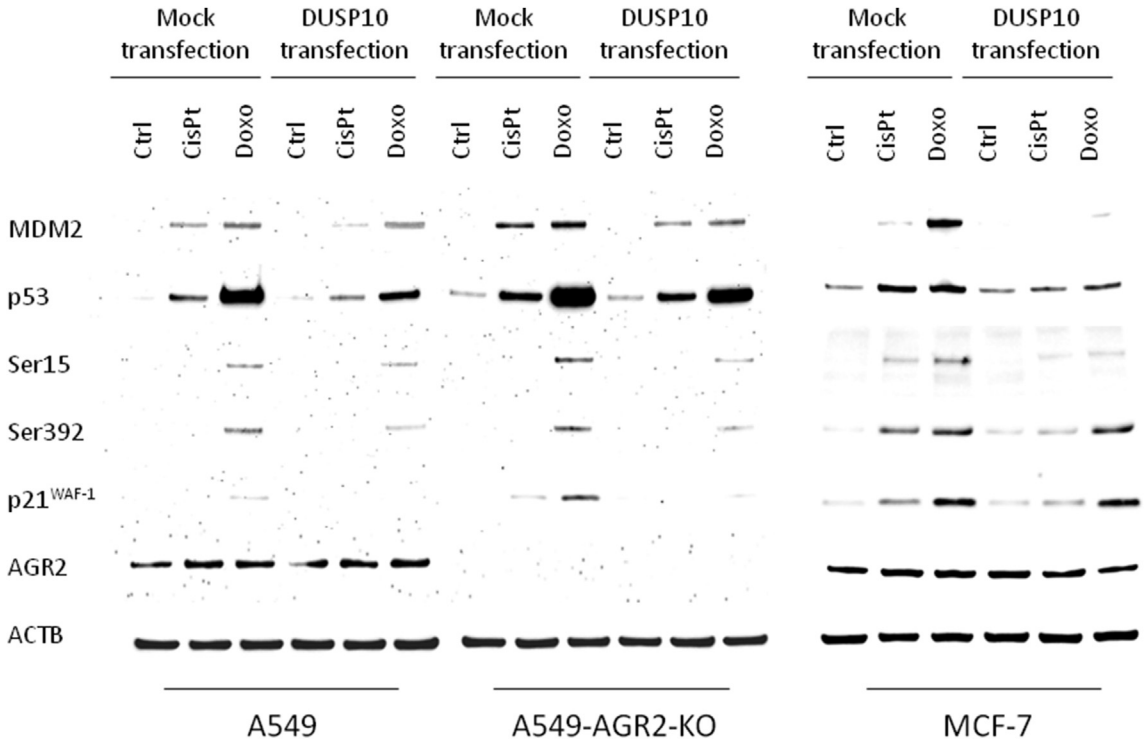

Figure 4 – DUSP10 expression directly influences p53. A549, A549-AGR2-KO and MCF-7 cells were either mock or pcDNA3-GW- DUSP10 transfected to determine the total level of p53, phosphorylation at Ser15 and Ser392, as well as the expression of p53 downstream regulated proteins $p21^{WAF-1}$ and MDM2. ACTB normalized protein levels are shown graphically in Figures S2 and S3.

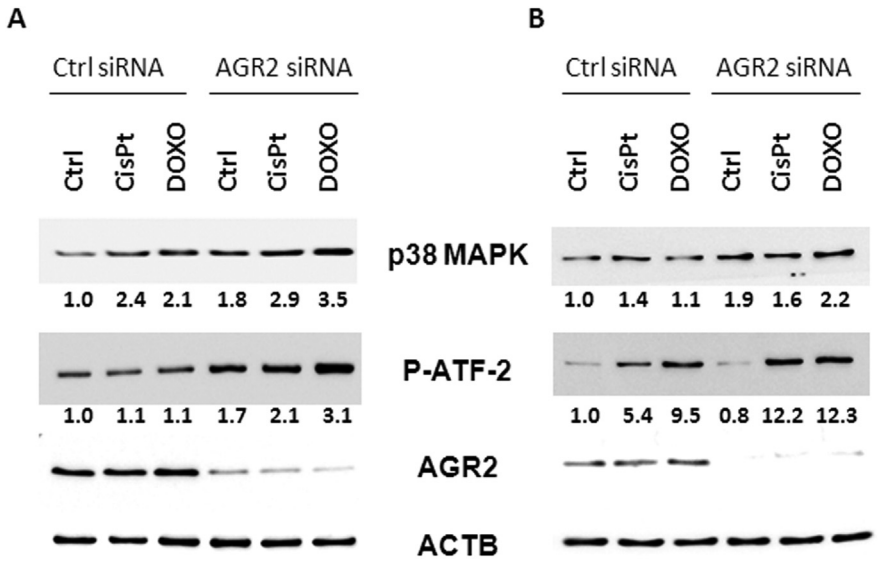

Figure 5 – Inhibition of AGR2 increases p38 MAPK activity. A549 (A) and MCF-7 cells (B) were transfected either with control or *AGR2* specific siRNA and then exposed to cisplatin or doxorubicin. Changes in p38 MAPK activity directly correspond to fold changes in degree of phosphorylation of ATF-2 fusion protein. Relative fold changes in amounts of phosphorylated ATF-2 fusion protein as well as p38MAPK are indicated under corresponding bands. These numbers were determined after normalization to ACTB in relation to untreated cells with unaffected endogenous AGR2 expression. This is a representative figure of two independent determinations of p38 MAPK expression and activity.

led us to investigate whether AGR2 expression may contribute to increased cell survival in response to chemotherapy, whose efficiency can greatly depend on the activity of p53. We used the MTT assay in A549 and MCF-7 cells either transfected with siRNA against *AGR2* or control siRNA and then exposed to either cisplatin or doxorubicin. Increased drug sensitivity was observed in AGR2-depleted cells (Figure S4). A similar trend was observed for the ability of AGR2 to block p53 functions and to rescue development of colonies (Figure S5). Briefly, clonogenic assays with H1299 cells (AGR2 and p53 negative) transfected either with *TP53* or co-transfected with *TP53* and *AGR2* together demonstrated increased colony development in cells with both *TP53* and *AGR2* in comparison with H1299 cells expressing p53 only.

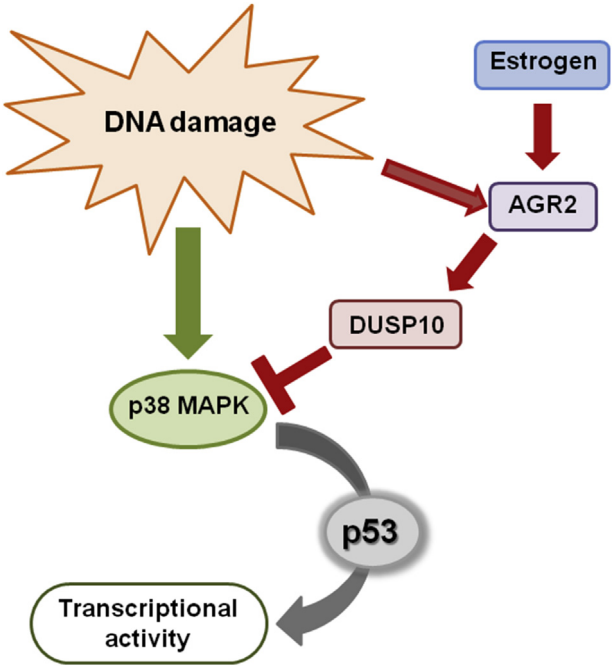

Figure 6 – Schematic representation of AGR2 signaling pathway. In response to DNA damage and other stimuli, AGR2 expression is elevated and induces DUSP10 expression. This process results in p38 MAPK inhibition and impaired p53 activity.

The pro-survival potential of AGR2 was also determined using real time monitoring of cell viability and proliferation kinetics using xCELLigence system. The proliferation of H1299-LZ4 cells (AGR2 negative) and H1299-LZ4-AGR2 (stably expressing AGR2) after transient transfection by expression vector coding p53 was monitored. The rate of proliferation in untreated H1299 cells was very similar for both AGR2-negative and -positive H1299 cells. Dramatic changes in proliferation were observed after introduction of wt p53, since H1299-LZ4-AGR2 cells were able to partially attenuate p53 activity and keep cellular proliferation, albeit with lower intensity (Figure S6A – black curve). When the cells were exposed to cisplatin and doxorubicin, we observed similar trends for both drugs (Figure S6B, C). AGR2 expressing cells showed increased proliferation rates compared to H1299-LZ4 cells, highlighting AGR2's pro-proliferative and pro-survival potential. In the presence of drugs, AGR2 rescued cells transfected by wt p53 even with increased efficiency compared to untreated cells, demonstrating that AGR2's inhibitory effect towards p53 is enhanced in response to genotoxic stress.

AGR2 expression in *ex vivo* cultured cells from breast cancer patients was determined to analyze the ability of AGR2

to mediate resistance to cytotoxic agents in primary cancers. This clinical study focused on chemosensitivity prediction of *ex vivo* cultivated malignant breast cancer cells was conducted at MMCI from August 2003 to September 2005 (Michalova et al., 2008; Poprach et al., 2008). In total 31 successfully evaluated breast cancer samples enabled us to determine AGR2 expression in relation to doxorubicin sensitivity. We found significantly higher *AGR2* mRNA level in tumor samples evaluated as doxorubicin insensitive ($p = 0.022$, Mann–Whitney U Test), supporting the role of AGR2 in resistance to DNA damaging drugs (Figure S7).

The effect of AGR2 expression determined by immunohistochemical staining on outcome of breast cancer patients treated by anthracyclines was evaluated in a cohort of 115 consecutive patients. No significant effect of AGR2 level on patients' outcome was found ($p = 0.313$, Log Rank test) (Figure S8A). Taking into account that AGR2 exerts its oncogenic functions via wt p53 inhibition, we focused only on tumors with wt p53 (80 cases) from the cohort of 115 patients, but again did not find a significant difference in overall survival with respect to AGR2 ($p = 0.159$, Log Rank test) (Figure S8B). Since ER status represents an important positive prognostic factor and was shown to regulate AGR2 expression, only wt p53 and concurrently ER-positive cases were selected. In this group of 67 patients, we found significantly worse overall survival of patients with AGR2 over-expressing tumors ($p = 0.043$, Log Rank test) (Figure 7).

To support our clinical results, the patients' outcome was analyzed in relation to expression of *AGR2* and *DUSP10* using the online survival analysis software Kaplan–Meier Plotter (http://kmplot.com/analysis/index.php?p=service) to assess the prognostic value of selected biomarkers using transcriptomic data in breast cancer patients (Gyorffy et al., 2010). Following our selection criteria involving wt p53 and ER-positivity (n = 234) we found both *AGR2* ($p = 0.034$) and *DUSP10* ($p = 0.002$) to have significant impact on patients' relapse free survival (Figure S9). On the other hand and similarly to our clinical data (Figure S8), the evaluation of *AGR2* and *DUSP10* expression in the whole cohort of 3554 breast tumors showed even a reverse trend for *AGR2* and an order of magnitude less significance for *DUSP10* (Figure S10).

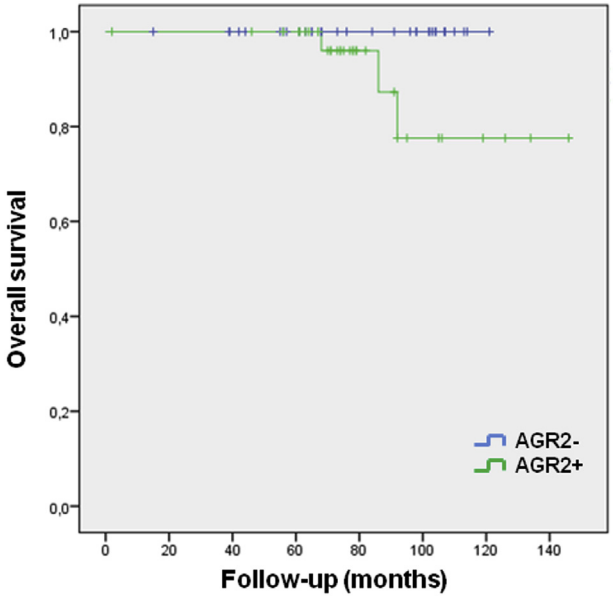

Figure 7 – Kaplan–Meier plots in relation to AGR2 expression. Overall survival of 67 patients with both positive ER and wt p53 selected from group of 115 consecutive breast cancer patients who received anthracyclines in adjuvant treatment.

4. Discussion

Unremitting efforts in p53 research over the past 35 years clearly illustrate that pathways driven by p53 or controlling p53 represent promising targets of newly developed anticancer therapeutics. The regulation of wt p53 is predominantly accomplished through two main mechanisms, comprising regulation of p53 stability by a series of distinct E3 ligases (Lee and Gu, 2010) and modulation of transcriptional activity *via* a myriad of posttranslational modifications including, but probably not limited to, phosphorylation, acetylation, methylation, glycosylation, ubiquitination, neddylation, sumoylation and poly-ribosylation (Kruse and Gu, 2009). This implies that p53 is under tight regulation by a large number of both negative and positive signals. Interestingly, many p53 negative regulators are in parallel p53 target genes forming auto-regulatory negative feedback loops with p53 (Harris and Levine, 2005). Overexpression and/or amplification of these negative regulators is proposed to attenuate p53 functions and promote tumorigenesis and indeed have been observed frequently in tumors (Jiang et al., 2015; Yu et al., 2014).

Recent comprehensive proteomic technologies provide the opportunity to discover novel, clinically relevant oncogenic pathways that would have been over-looked by cancer gene screens of the past. One of these recently discovered oncogenes related to p53 is AGR2. AGR2 induces metastasis, acts as a survival factor mediating drug resistance and has a direct involvement in malignant transformation (Brychtova et al., 2011). The ability of AGR2 to inhibit p53 represents another key role of AGR2 in human malignancies.

AGR2 expression has been described in a range of human malignancies including breast tumors, where the regulation of AGR2 expression is best described. Breast cancer encompasses a group of heterogeneous diseases where chemotherapy represents a standard treatment option predominantly for high risk patients, who benefit significantly from the addition of chemotherapy to endocrine treatment (Albain et al., 2010; Paik et al., 2006). p53 plays an important role in sensitivity to chemotherapy, as illustrated by many studies linking *TP53* mutations to drug resistance (Dobes et al., 2014; Chrisanthar et al., 2011). *TP53* mutations have especially been linked to resistance towards DNA damaging agents such as anthracyclines and mitomycin in breast cancer. Although alterations in *TP53* are observed at a relatively high frequency, it is reasoned that many other pathways, mainly in tumors with wt p53, may contribute to inhibition of this tumor suppressor. This is consistent with the fact that 70–80% of breast carcinomas are ER-positive and most of these express wt p53 (Olivier et al., 2006). One explanation could be that ER-dependent signaling, including AGR2

upregulation, actively participates in attenuation of p53 activity and in this way contributes to the malignant phenotype in the early stages of breast cancer development. Conversely, in advanced breast tumors where endocrine resistance is usually developed, p53 is almost always mutated (Bouchalova et al., 2014; Yamashita et al., 2006). This is consistent with numerous studies showing crosstalk between p53 and ER resulting in repression of p53 activity (Bailey et al., 2012; Konduri et al., 2010; Liu et al., 2009; Sayeed et al., 2007). We and others have demonstrated that AGR2 expression is directly regulated by estrogens and is associated with poor outcomes for ER-positive breast cancer patients (Hrstka et al., 2010; Innes et al., 2006; Thompson and Weigel, 1998). These findings indicate that ER-dependent induction of AGR2 may contribute to attenuation of p53 activity. Interestingly, tamoxifen induces AGR2 both *in vivo* and *in vitro* (Hengel et al., 2011; Hrstka et al., 2010), which is in accordance with observations that not only estrogen signaling via ER but also tamoxifen treatment may inhibit p53 activity (Bailey et al., 2012). On the other hand, to generalize inhibitory effect of AGR2 towards p53 in response to DNA damage, we used A549 cells to confirm that AGR2 blocks p53 transcriptional activity also independently of ER signaling, albeit with varying involvement of other corresponding signaling pathways with respect to cellular context.

Based on the critical role of p53 in carcinogenesis and response to genotoxic therapies, clarification and detailed description of the mechanism by which AGR2 exerts its p53 inhibitory activity may have impact on therapeutic strategies for the treatment of cancer with respect to AGR2 and p53 status. We found that AGR2 up-regulates *DUSP10* mRNA, whose product belongs to a subset of protein tyrosine phosphatases responsible for dephosphorylation of threonine and tyrosine residues on MAPKs (Jeffrey et al., 2007), while DUSP10 exhibits increased substrate selectivity for p38 MAPK (Theodosiou et al., 1999). With this in mind, it is reasonable to assume that AGR2 induces DUSP10 expression that subsequently inhibits p38 MAPK, well known to be involved in regulation of p53 *via* post-translational modifications enabling stabilization and activation of p53. Taken together, we have discovered a novel mechanism that contributes to AGR2-mediated oncogenic and anti-therapeutic effects through attenuating p53 transcriptional activity in response to DNA damage.

In summary, DNA damaging drugs are frequently used for the treatment of many human cancers, including breast, however resistance is often observed. Identification of this novel mechanism, through which AGR2 inhibits p53, may lead to development of useful diagnostic and therapeutic approaches focused on activation of p53 tumor suppressor. In accordance, AGR2 was shown to contribute to cell death resistance induced by the commonly used DNA damaging agents both *in vitro* and in mouse xenografts (Hengel et al., 2011; Ramachandran et al., 2008; Simpson et al., 2010). Our study extends these findings by showing a significant association of elevated *AGR2* mRNA levels with resistance to doxorubicin treatment in *ex vivo* cultured human breast cancer primocultures, as well as significant association of induced AGR2 protein levels with worse overall survival of wt p53, ER-positive breast cancer patients. Although it remains important to comprehensively determine whether AGR2 could be a potential drug target for reactivation of the p53 pathway in cancer cells, our findings clearly indicate that AGR2 could be useful as a prognostic marker in ER-positive wt p53 breast tumors and potentially also as a druggable molecular target (Arumugam et al., 2015).

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molonc.2015.12.003.

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Příloha 10

Anterior Gradient 2 and Mucin 4 Expression Mirrors Tumor Cell Differentiation in Pancreatic Adenocarcinomas, But Aberrant Anterior Gradient 2 Expression Predicts Worse Patient Outcome in Poorly Differentiated Tumors

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Objectives: This study aimed to determine anterior gradient 2 (AGR2) expression in biopsies from pancreatic ductal adenocarcinomas (PDACs) and to evaluate AGR2 as a potential independent prognostic factor.

Methods: Tissue sample sections from a cohort of 135 consecutive surgically resectable PDACs were subjected to semiquantitative immunohistochemical analysis of AGR2 and mucin 4 (MUC4) expression.

Results: Anterior gradient 2 was over-expressed in PDAC compared with normal ductal cells. Since tumor lesions of PDAC are heterogeneous and constitute structures with various differentiation states, expression of both AGR2 and MUC4 was evaluated in each separate component. Expression levels of both proteins reflected the degree of tumor differentiation. Generally, well differentiated regions of tumor lesions expressed high levels of both proteins, moderately differentiated regions showed less AGR2 and MUC4, and poorly differentiated structures showed only weak positivity or were entirely negative. Of particular interest were occasional cases of strong AGR2 expression in high-grade tumors, where elevated protein levels were associated with shorter patient survival.

Conclusions: Anterior gradient 2 and MUC4 reflect the level of differentiation of PDACs. However, in less differentiated tumors, aberrantly elevated AGR2 expression predicts poor patient outcome.

Key Words: pancreatic ductal adenocarcinoma, AGR2, MUC4, immunohistochemistry

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Despite advances in anticancer therapeutics, pancreatic cancer is a lethal disease with mortality almost reflecting the incidence.[1] According to the American Cancer Society, survival rates have improved only very slightly during last 5 years. The mortality rate is still too high because pancreatic cancer symptoms appear typically at late stage, usually when it has already metastasized, and currently, there is no reliable specific tool to detect the disease in its earlier stages. The research in past decades has shown that pancreatic cancer is fundamentally a genetic disease because multiple alterations in genes such as *KRAS*, *CDKN2A*, *TP53*, *SMAD4*/*DPC4*, and so on were confirmed to be involved in pancreatic cancer progression.[2] In addition, the description of early and late genetic alterations led to the formulation of a multistep progression model of pancreatic cancer.[3,4] The poor prognosis and late disease manifestation promote efforts to understand exact molecular processes responsible for the development of invasive carcinoma of the pancreas in more detail and to identify specific and reliable biomarkers.

One of the potential biomarkers appears to be anterior gradient 2 (AGR2) protein. Developmentally, AGR2 expression is linked to the cell population covering the biliary tract.[5] The pancreas along with the liver arises from the shared multipotent population in the foregut endoderm, which explains the presence of AGR2 protein in pancreatic ductal cells. In the context of pancreatic cancer, *AGR2* has been noted as an over-expressed gene in pancreatic cancer samples in 2 independent microarray studies.[6,7] Consequently, it has been shown that AGR2 expression may be involved in the malignant progression of pancreatic cancer because of its expression in both pancreatic cancer cell lines and clinical samples.[8] In addition, protein expression analyses showed high AGR2 expression in pancreatic intraepithelial lesions, invasive pancreatic carcinomas, and circulating tumor cells.[9]

Taxonomically, AGR2 is a member of the protein disulfide-isomerase family of endoplasmic reticulum (ER)-resident proteins possessing a thioredoxin-like CXXS domain.[10] A structural relationship to molecular chaperones suggests its role in protein folding.[11] Interestingly, AGR2 has an evolutionarily related homologue in newts, newt anterior gradient (nAG), whose function is to trigger mitogenic activity during limb regeneration, suggesting involvement of AGR2 in cell migration and proliferation.[12] At the molecular level in pancreatic ductal adenocarcinomas (PDACs), AGR2 was shown to stimulate growth, invasiveness, and survival of pancreatic cancer cells in both in vitro and in a xenograft mouse model.[6]

Anterior gradient 2 is presumed to be a secretory protein and indeed has also been detected in extracellular fluids, indicating its potential use in clinical biochemistry.[8,13] Its protumorigenic functions have also been demonstrated in a diverse range of human cancers both hormone dependent and independent,[14] and over-expression of this protein usually predicts poor prognosis.[15–19] However, the mechanism by which AGR2 contributes to tumor phenotype is not fully elucidated and still can be only estimated from its proven biochemical properties and cellular localization.

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Therefore, because of limited response to PDAC chemotherapy treatment and AGR2 potential involvement in cell proliferation, we focused on and discuss the role of AGR2 in pancreatic carcinogenesis. We immunohistochemically determined AGR2 protein expression in PDAC and in normal pancreas and evaluated its expression with respect to clinicopathologic parameters including patients' overall survival.

MATERIALS AND METHODS

Study Group and Tissue Specimens

The consecutive study group consisted of 135 patients with resectable pancreatic cancer who had undergone pancreatectomy at the Faculty Hospital Brno, at the Masaryk Memorial Cancer Institute, or at the St Anne's University Hospital between 2000 and 2011. The cohort consisted of 68 males (50.3%) and 67 females (49.7%). Because of limited tissue availability, AGR2 and mucin 4 (MUC4) were evaluated in 132 patients (Table 1). The patients' age at the time of diagnosis was within the range of 39 to 83 years (median 60 ± 8.9 years). Tissues were fixed in 10% neutral-buffered formalin for 24 hours and then embedded in paraffin wax. Hematoxylin-eosin staining was used to identify representative samples with structures of pancreatic invasive ductal adenocarcinoma for immunohistochemical analysis. Tumors were histologically confirmed to be invasive ductal adenocarcinomas of the pancreas, and the grading of tumor differentiation was performed according to the criteria of the World Health Organization, combining glandular differentiation including mucin production with mitotic count and nuclear features. Tumor staging was performed according to the *International Union Against Cancer TNM System* (seventh edition). Anonymized clinicopathologic data were gathered from the archival pathology reports. Survival data were obtained from the National Oncologic Register of the Czech Republic. Ultimately, the follow-up was available for 113 patients. Patients who were hospitalized in Masaryk Memorial Cancer Institute, in Faculty Hospital Brno, and in St Anne's University Hospital give standardized informed consent when undergoing surgery. All the patients involved in this work signed statements that the redundant biologic material, which was obtained from his/her body within the standard diagnostic and therapeutic procedures, can be used for research purposes and that the data from her/his medical documentation could be used for research studies. The study was approved by an ethical review board.

Immunohistochemistry

Immunohistochemical staining was performed on 4-μm thick freshly cut tissue sections, and the optimal antibody concentration and retrieval was set separately for each antibody used. Sections were deparaffinized in xylene and rehydrated into phosphate buffered saline through a graded ethanol series. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in phosphate buffered saline for 15 minutes. Antigen retrieval was performed in citrate buffer (pH 6.0) at 94°C for 20 minutes. For AGR2 immunodetection, the sections were incubated overnight at 4°C with rabbit polyclonal anti-AGR2 antibody (HPA007912l; Sigma-Aldrich, St Louis, Mo), and for MUC4 immunodetection, sections were incubated overnight at 4°C with mouse monoclonal antibody MUC4 (Clone 1G8; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). A streptavidin–biotin peroxidase detection system was used according to the manufacturer's instructions (VECTASTAIN Elite ABC Kit; Vector Laboratories, Burlingame, Calif). Signal was visualized by 3,3′-diaminobenzidine (Liquid DAB+ Substrate Chromogen System; Dako, Glostrup, Denmark). Nuclear counterstaining

TABLE 1. Clinicopathologic Parameters in Association With AGR2 and MUC4 Expression in the Tumor Set

	AGR2 Positivity Score							MUC4 Positivity Score							
	0	1	2	3	4	*P*	Total	0	1	2	3	4	*P*	Total	Patient Cohort
Age	3	10	24	82	12	0.29840	131	22	29	35	43	2	0.90370	131	134
N/A				1			1			1				1	1
Sex						0.95393							0.86058		
Male	2	5	12	42	5		66	11	17	16	22	1		67	68
Female	1	5	12	41	7		66	11	12	20	21	1		65	67
Grade						<0.00001							<0.00001		
G1				3	7		10	1	2		5	2		10	10
G2			5	66	5		76	4	11	24	35			74	76
G3	3	10	19	14			46	17	16	12	3			48	49
T status						0.44090							0.16790		
T1		1	1	3	1		6	2	1	1	1			5	6
T2		2	7	15	7		31	3	7	8	12	2		32	33
T3	3	7	16	65	4		95	17	21	27	30			95	96
N status						0.89904							0.61685		
N0	1	2	7	22	4		36	4	6	11	14	1		36	37
N1	1	8	17	61	8		95	17	23	25	29	1		95	97
N/A	1						1	1						1	1
R status						0.72990							0.61636		
R0	2	7	19	65	8		101	17	20	25	36	1		99	102
R1	1	3	4	16	4		28	5	9	9	7			30	30
N/A			1	2			3			2		1		3	3

N/A, not applicable.

was performed with Gill's hematoxylin. Tissue sections of small intestine and lymph node served as external positive and negative controls for anti-AGR2. Mucin 4 staining of goblet cells in duodenal mucosa served as internal control.

Evaluation of Immunostaining

In both AGR2 and MUC4 immunohistochemistry, the immunostaining in each component of differentiation within the individual tumor was evaluated separately, because a significant proportion of G3 tumors also contain moderately and well differentiated components, and G2 tumors may contain a well differentiated component. At least 3 representative (×400) high-power fields of each area of particular differentiation grade were examined. Cases with an area of less than 3 high-power fields of the representative tumor tissues were excluded. A semiquantitative scoring system was used for evaluation of both AGR2 and MUC4 expression levels, which were classified into 5 categories: numeric score 0 (component of the particular differentiation with completely negative immunostaining); numeric score 1 (weak to moderate intensity of immunostaining in some tumor cells of the particular differentiation component, ie nondiffuse immunopositivity); numeric score 2 (diffuse weak intensity of immunostaining of the particular differentiation component); numeric score 3 (diffuse moderate intensity of immunostaining of the particular differentiation component); and numeric score 4 (diffuse strong intensity of immunostaining of the particular differentiation component). All tissue samples were independently examined by 2 histopathologists, and discrepancies were resolved by consensus.

Statistical Analysis

All statistical analyses were performed using STATISTICA Version 10 (StatSoft, Inc, Tulsa, Okla). Differences at $P \leq 0.05$ were considered to be statistically significant. The association of AGR2 and MUC4 expression with various clinicopathologic features was analyzed using the Kruskal-Wallis and Pearson χ^2 tests. Survival curves were plotted using the Kaplan-Meier method, and statistical significance was assessed using the log-rank test. Multivariate Cox proportional hazards regression analysis was used to evaluate potential prognostic factors. The Cox proportional hazards regression model was undertaken by using a stepwise procedure with a significance level of 0.05 for entering and 0.15 for removing variables.

RESULTS

The consecutive cohort of PDACs comprised 10 (7.4%) well differentiated G1 cases, 76 (56.3%) moderately differentiated G2 cases, and 49 (36.3%) poorly differentiated G3 cases. According to the tumor size and extent, the study group encompassed 6 (4.4%) cases falling within T1 group, 33 (24.5%) cases of T2 group, and 96 (71.1%) cases of T3 group. At the time of diagnosis, 97 (71.9%) patients had lymph node metastases, whereas the nodal involvement data were absent in 1 patient (0.7%; Table 1). No distant metastases were revealed at initial diagnosis, only M0 patients with resectable ductal adenocarcinoma were included into the study.

Statistical analysis of demographic factors such as age and sex in relation to AGR2 and MUC4 levels did not show any significant association. Similarly, comparison of both AGR2 and MUC4 expression with clinicopathologic parameters such as tumor size, nodal involvement, and residual status did not reveal any statistical association. Strikingly, a significant association of AGR2 and MUC4 expression with histologic grade was found (Table 1).

The immunohistochemical analysis revealed weak AGR2 expression in normal ductal cells of pancreas, whereas high levels were detected in tumor cells of predominantly well and moderately differentiated PDAC samples (Fig. 1A vs 1B, C). The expression of AGR2 in these adenocarcinomas was restricted to tumor cells with homogenous diffuse cytoplasmic expression pattern and was not detectable in tumor stromal cells. Conversely, poorly differentiated cases of PDAC exhibited mostly weak heterogeneous staining for AGR2 (Fig. 1D).

Mucin 4 was negative in normal pancreatic ducts, and mucin granules were localized predominantly in the apical cytoplasm in tumor cells (Fig. 1). The expression level of MUC4

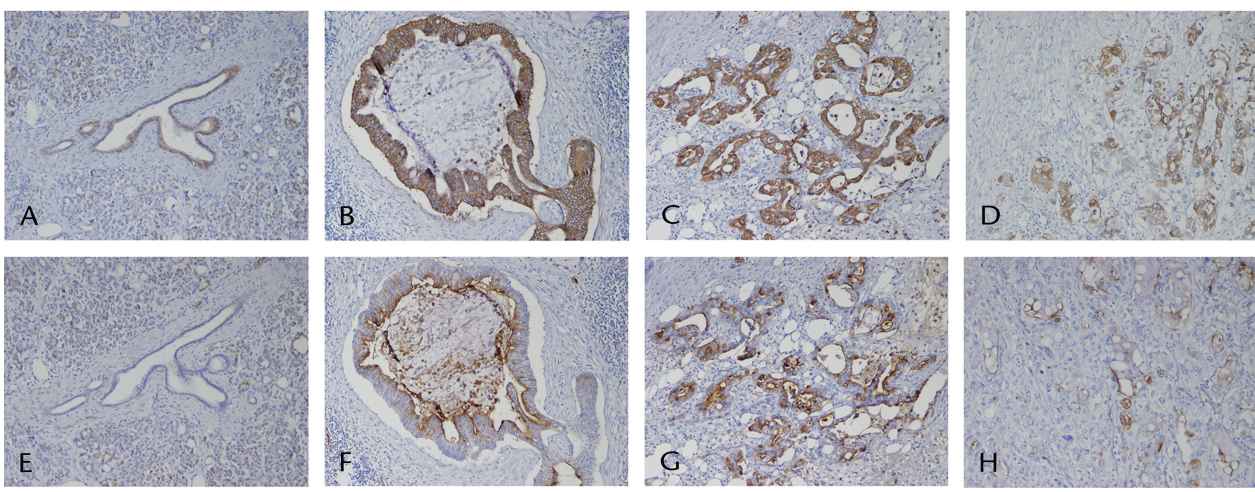

FIGURE 1. Immunohistochemical staining shows weak to moderate AGR2 expression in ductal cells of normal pancreas (A). Pancreatic tumor samples show significant diversity in AGR2 expression intensity, reflecting the level of cancer tissue differentiation. Strong cytoplasmic positivity was detected in well-differentiated PDAC (B), moderate expression in moderately differentiated PDAC (C), and nonhomogeneous weak expression in poorly differentiated PDAC (D). In contrast to AGR2, MUC4 is not expressed in normal pancreatic duct (E); however, MUC4 expression pattern in PDACs reflects the level of differentiation similarly as AGR2. Well-differentiated PDAC has strong apical luminal expression (F), similarly moderately differentiated PDAC has moderate expression (G), and the expression in poorly differentiated cancer tissue is weak (H). Immunohistochemistry AGR2 (A–D) and MUC4 (E–H); original magnification ×100.

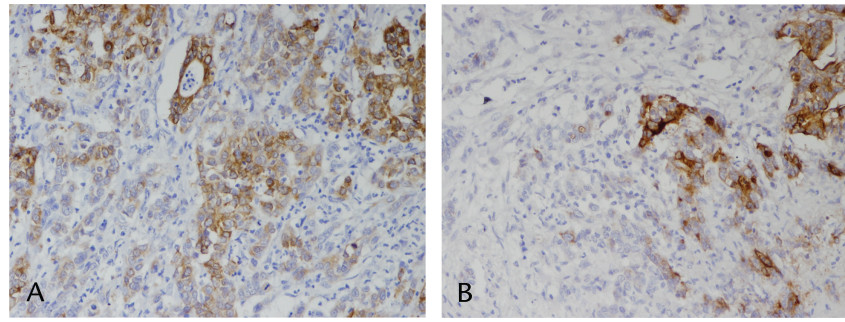

FIGURE 2. Immunohistochemical detection of AGR2 and MUC4 in PDACs shows variable expression in individual components of divergent differentiation within a single tumor. Anterior gradient 2 expression in PDAC tissue section of moderately and poorly differentiated component of pancreatic cancer tissue (A). Mucin 4 expression in PDAC tissue cut of moderately and poorly differentiated component of pancreatic cancer tissue (B). Immunohistochemistry AGR2 (A) and MUC4 (B) (E–H); original magnification ×200.

was increased, consistent with a more differentiated phenotype (Fig. 1F–H).

Distinct heterogeneity in the amount of AGR2 and MUC4 positive cells was found between and also within individual PDAC cases that corresponded to diverse differentiation of tumor tissue (Fig. 2). In the course of immunohistochemical sample evaluation, we observed variation in the degree of differentiation within the same neoplasm in approximately half of all samples analyzed. The expression of AGR2 and MUC4 was analyzed in 132 patients but encompassed 181 cases of individual components with various degrees of differentiation in which we assessed the expression separately. Determination of AGR2 expression revealed mainly score 3 in 121 (66.8%) of 181 components, and for MUC4 expression, we found mostly score 2 in 43 (23.7%) components and score 3 in 78 (43.1%) of 181 components studied (Table 2).

With respect to the level of differentiation of particular components, AGR2 and MUC4 exhibited strong and moderate expression (scores 3 and 4) of both proteins in well-differentiated structures. Components classified as moderately differentiated showed weaker expression largely classified as moderate staining (score 3). The largest diversity in AGR2 and MUC4 expression was observed among poorly differentiated components, but the trend was shifted toward lower expression levels (scores 0, 1, and 2; Fig. 3A, B; Table 2). Related statistical evaluation confirmed statistically significant correlation between the level of tumor cell differentiation and AGR2 and MUC4 expression (both, $P < 0.00001$).

Survival Analysis

The impact of all clinicopathologic variables on the outcome of patients with PDAC was assessed by Kaplan-Meier analysis. We found significant differences in survival rates with respect to sex, grade, node involvement, and AGR2 expression. Univariate analysis indicated that patients with PDAC with low AGR2 expression show significantly shorter survival rates than those patients with high expression (Fig. 4). However, taking into account that well-differentiated PDACs have generally more favorable prognosis compared with less differentiated counterparts and that increased AGR2 expression strongly correlates with well-differentiated PDACs; standard multivariate Cox proportional hazards regression analysis to evaluate real statistical significance of potential prognostic factors was used. Multivariate analysis of overall survival showed that only metastatic status of nodes at time of diagnosis and tumor grade affected overall survival. Node involvement was associated with a reduced survival ($P = 0.000821$), and low-grade carcinomas were associated with a better prognosis than high-grade tumors ($P < 0.000001$). In contrast, multivariate analysis showed neither sex nor AGR2 expression as independent prognostic factors in our cohort of PDACs.

We also assessed the prognostic value of AGR2 expression in univariate survival analysis in subgroups stratified to tumor grade. There is a statistically significant impact of AGR2 expression on patient survival in high-grade tumors, with patient samples possessing diffuse weak to moderate expression (scores 2 and 3) showing shorter survival than those patients with weak nondiffuse expression (score 1) or no AGR2 expression (score 0; $P = 0.01085$; Fig. 5).

DISCUSSION

The identification of genes differentially expressed in pancreatic cancer is critical for the development of strategies to detect and treat this lethal disease. Anterior gradient 2 has been linked to various types of cancer in promoting cell growth, survival, cell migration, and cellular transformation.[6,14,18,20] Although the actual mechanism of action is not fully known, a possible explanation may consist in its biochemical properties and structure. From this perspective, AGR2 belongs to a protein disulfide-isomerase family of proteins residing in the ER and

TABLE 2. AGR2 and MUC4 Expression According to the Level of Differentiation of Pancreatic Cancer Tissue

	AGR2 Positivity Score					*P*	MUC4 Positivity Score					*P*
	0	1	2	3	4		0	1	2	3	4	
Cancer tissue differentiation						<0.00001						<0.00001
Well				6	13		2	2		9	5	
Moderately			4	107	6		6	11	33	68		
Poorly	1	13	23	8			17	17	10	1		
Total	1	13	27	121	19		25	30	43	78	5	

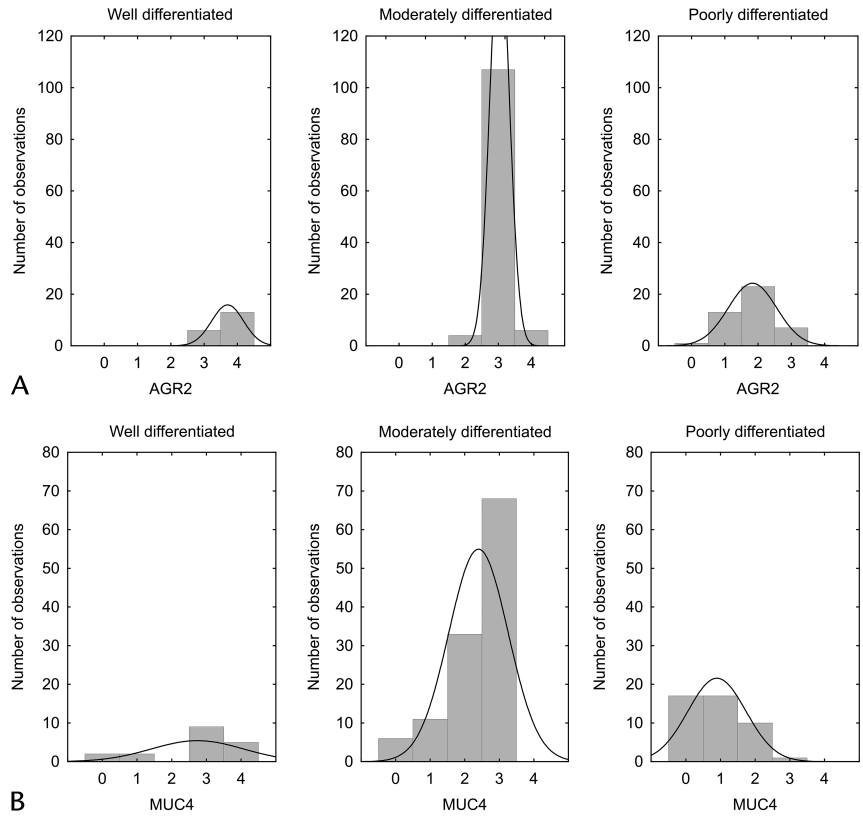

FIGURE 3. The distribution of AGR2 expression in PDAC tumor samples correlates to the categories of tumor differentiation (A). The distribution of MUC4 expression in PDAC tumor samples within the categories of tumor differentiation indicates the correlation between MUC4 expression and the level of tumor differentiation (B).

participating in disulfide bond formation and breakage during protein-folding processes.[21] Detrimental conditions such as hypoxia, abnormal calcium levels, low nutrients, or accumulation of misfolded proteins lead to ER stress response, where ER stress proteins are activated to support cellular survival and to equalize the imbalances in cellular homeostasis. In tumor cells, cellular protective features of ER stress are chronically activated and thus provide support for continuous proliferation and survival.[22] Although AGR2 belongs to ER stress–associated gene, the possible role in supporting cancer development seems plausible.

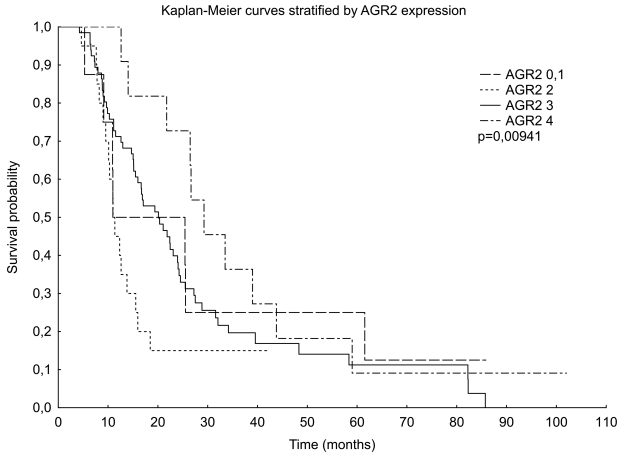

FIGURE 4. Kaplan-Meier survival curves for patients with AGR2 expression from 0 to 4 (0, negative; 1, weak/moderate nondiffuse staining; 2, weak diffuse staining; 3, moderate diffuse staining; and 4, strong diffuse staining) show significantly longer survival times for AGR2 expressing tumors ($P = 0.00941$).

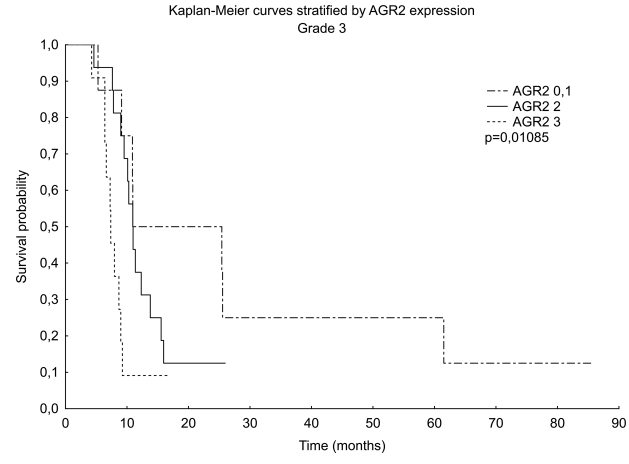

FIGURE 5. Kaplan-Meier survival curves for the subgroup of grade 3 patients. Lower AGR2 expression (0, negative; and 1, weak/moderate nondiffuse staining) in tumors is significantly associated to longer survival times in contrast to higher AGR2 expression (2, weak diffuse staining; and 3, moderate diffuse staining; $P = 0.01085$). Strong AGR2 expression (4) was absent in this subgroup.

A potential role for AGR2 in pancreatic cancer was initially demonstrated almost a decade ago in differential gene expression studies that identified *AGR2* as a significantly overexpressed gene in pancreatic cell lines and pancreatic adenocarcinomas vs human pancreatic duct epithelial cell lines and normal pancreatic tissues, respectively.[23,24] Subsequently, the expression of AGR2 has been analyzed analogously also in premalignant lesions. Anterior gradient 2 messenger RNA and protein levels are reported to be elevated in all grades of pancreatic intraepithelial neoplastic lesions, showing its involvement in early pancreatic cancer progression.[25] Since pancreatic cancer is a multistep phenomenon characterized by the progressive accumulation of multiple genetic alterations occurring in an ordered sequence, elevation of AGR2 in the early phases of carcinogenesis implies that it has a fundamental role in the neoplastic process and that it may serve as an early marker of disease.

In our study, we evaluated AGR2 expression in formalin-fixed paraffin embedded samples of PDAC. Immunohistochemical staining has shown elevated AGR2 predominantly in adenocarcinomas, in concordance with previously published works.[6,7,20] In line with our expectations, weak expression was also detected in normal pancreatic duct epithelium because AGR2 expression naturally occurs in epithelial cells.[26] Anterior gradient 2 staining in tissue sections exhibited heterogeneous staining corresponding to the level of differentiation in particular tumor tissue components. The variability of AGR2 levels in PDAC samples and AGR2 expression in pancreatic cancer cell lines has been already described.[7] These findings were prerequisite to evaluate AGR2 expression in each of these structures separately. We believe that with respect to nonhomogeneity of analyzed samples, the evaluation in whole sample may improve the tumor scoring and provide much more objective view to AGR2 expression in pancreatic cancer in contrast to studies based on tissue arrays, which may be responsible for distortion and inconsistency of obtained data.

Statistical analysis showed significant correlation between AGR2 expression and the level of tumor tissue differentiation. These findings could be of potential interest, because current predictive model for pancreatic cancer based on traditional TNM staging has been extended by additional factors, one of which is the degree of differentiation or tumor grade. Although the survival within particular stage is highly variable, the likelihood of disease-free survival after resection of pancreatic tumors can be predicted better, if the classic scoring system is accompanied with the level of tissue differentiation evaluation. Tumor grade is now widely accepted as an independent predictor of survival across all stages of pancreatic cancer, which is also supported by our data and other recent studies.[27]

In our study, we also found a strong correlation between MUC4 expression and the degree of PDAC differentiation. Current research has shown that MUC4 is aberrantly expressed in pancreatic adenocarcinoma cells and that expression correlates positively with the degree of differentiation. Mucin 4 is a specific tumor marker for pancreatic malignancy, given its neoexpression in early precancerous intraepithelial neoplastic lesions with progressive increase with disease advancement. Although in some cancers, MUC4 expression is related to better prognosis, whereas in differentiated pancreatobiliary tumors, it is associated with a poor clinical outcome and may identify patients with particularly poor prognosis among morphologically determined adenocarcinomas of pancreas.[28,29]

In the case of AGR2, Kaplan-Meier plot suggested potential association between longer survival and elevated expression of AGR2 in patients with PDAC compared with cases with low AGR2 expression. However, multivariate Cox proportional hazards regression analysis elucidated that the survival rate is primarily influenced by histologic grade to which AGR2 expression is directly related. Although AGR2 expression clearly mirrors the degree of differentiation in PDACs, several poorly differentiated tumors retained strong AGR2 expression. These samples were associated with shorter patient survival in Kaplan-Meier survival analysis. Our findings support in vitro data demonstrating AGR2 as a promoter of pancreas carcinogenesis that influences cancer cell phenotype by enhancing proliferation, invasion, and survival of cancer cells.[6] Taken together with respect to presence of larger quantities of variously differentiated structures within a pancreatic tumor mass, our results clearly show significant AGR2 contribution to worse prognosis in poorly differentiated high-grade tumors.

In summary, the data published so far demonstrate the potential role of AGR2 in PDACs as a cancer cell proliferation facilitating factor participating in tumor progression from its very early onset. Our observations contribute to existing knowledge by finding that AGR2 increased expression is tightly associated with well differentiated PDACs and is predominantly lost during tumor progression to high grade. Nevertheless, in high-grade pancreatic adenocarcinomas, where cancer cells retain the AGR2 expression, our data indicate the role of AGR2 in worse prognosis of these tumors.

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Mini Review

The role of AGR2 and AGR3 in cancer: Similar but not identical

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ABSTRACT

In the past decades, highly related members of the protein disulphide isomerase family, anterior gradient protein AGR2 and AGR3, attracted researchers' attention due to their putative involvement in developmental processes and carcinogenesis. While AGR2 has been widely demonstrated as a metastasis-related protein whose elevated expression predicts worse patient outcome, little is known about AGR3's role in tumour biology. Thus, we aim to confront the issue of AGR3 function in physiology and pathology in the following review by comparing this protein with the better-described homologue AGR2. Relying on available data and *in silico* analyses, we show that AGR proteins are co-expressed or uncoupled in context-dependent manners in diverse carcinomas and healthy tissues. Further, we discuss plausible roles of both proteins in tumour-associated processes such as differentiation, proliferation, migration, invasion and metastasis. This work brings new hints and stimulates further thoughts on hitherto unresolved conundrum of anterior gradient protein function.

Introduction

Anterior gradient (AGR) proteins form an evolutionarily broad family with prominent, however, poorly understood physiological functions in vertebrates. The first identified was a secreted protein XAG-2, originally discovered in *Xenopus laevis* and implicated in the formation of the anteroposterior axis during embryogenesis (Sive et al., 1989). AGR2 extracellular role was also documented during limb regeneration in salamanders by stimulation of blastemal growth (Kumar et al., 2007). In addition, it was recently demonstrated that AGR proteins in *X. laevis* promote regeneration of hindlimb buds and tails of tadpoles (Ivanova et al., 2013). During the last decades, intense research has commenced in order to elucidate biological function of human homologues, namely AGR2 and AGR3 both in health and disease. Strikingly, although both molecules share 71% sequence identity and lie adjacent to one another at chromosomal position 7p21 (Fletcher et al., 2003; Petek et al., 2000), AGR2, but not AGR3, is a dominant factor identified in many OMICS screens, and therefore, many more reports have been published in relation to its characterization. Thus, relying also on our recent observation of uncoupled AGR2 and AGR3 expression in various tumour tissues (unpublished data), we sought to compare both proteins by analysing their expression pattern and regulatory mechanisms in this review. For this reason, we have reviewed all the published data and used the Genevestigator tool (Hruz et al., 2008), which enables not only prediction of AGRs tissue distributions but also their co-expressed partners. Moreover, we performed *in silico* promoter analysis to find upstream regulatory pathways potentially triggering AGR2 and AGR3 expression. All the analyses were conducted on the data available online in May 2014.

Abbreviations: AA, amino acids; ALS, familial amyotrophic lateral sclerosis; AGR, anterior gradient; AhR, aryl hydrocarbon receptor; AR, androgen receptor; CAPN8, calpain 8; CAPN9, calpain 9; CDDP, cisplatin; CFTR, cystic fibrosis transmembrane conductance regulator; CLDN3, claudin 3; DAG-1, alpha-dystroglycan; EGFR, epidermal growth factor receptor; ELK1, ETS-domain containing protein; EMT, epithelial-to-mesenchymal transition; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation; EsR, oestrogen receptor; FOX, forkhead box; GC-SBE, glycine-rich SMAD binding elements; HCC, hepatocellular carcinomas; HGSC, high-grade serous ovarian carcinomas; ICC, intrahepatic cholangiocarcinomas; JAK-STAT, Janus kinase-signal transducer and activator of transcription; LEF-1, lymphoid enhancer factor-1; LGSC, low-grade serous ovarian carcinomas; MAPK, mitogen-activated protein kinases; MICA, MHC I-related chain A; MUCs, mucins; NK, natural killer; PDB, Protein Data Bank; PDIs, protein disulphide isomerases; SLC44A4, protein member 4 of solute carrier family 44; TGF-β, transforming growth factor-beta; TGIF1, TG-interacting factor 1; TM4SF, transmembrane 4 superfamily; TMC4, transmembrane channel-like protein 4; TMC5, transmembrane channel-like protein 5; TMPRSS2, transmembrane protease serine 2; TRX, thioredoxin; TSPAN1, tetraspanin 1; TSS, transcription start site; UPR, unfolded protein response; ZEB1, zinc-finger enhancer binding-1.

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AGR2 and AGR3 as PDI family members

Structural features of the PDI family

The protein disulphide isomerase (PDI) family is part of the thioredoxin (TRX) superfamily, which also includes the glutaredoxins, thioredoxins, ferroredoxins and peroxiredoxins (Jacquot et al., 2002). Recent work has revealed that there are three subfamilies of AGRs: AG1, AGR2 and AGR3, all showing the highest homology to non-secreted PDI of the TLP19 subfamily. Remarkably, members of AGR2 and AGR3 subfamilies are present in amniotes, while members of AG1 subfamily (to which family founder, XAG-2 belongs) are restricted to lower vertebrates (Ivanova et al., 2013). Human AGR2 and AGR3's affiliation to the PDI family was based on the phylogenetic analysis performed by Persson et al. (2005), where it was shown that both proteins have high homology to ERp18/19 protein, also denoted as AGR1. To date, 21 members varying in size, structure, tissue distribution and enzymatic activity were identified (Galligan and Petersen, 2012). The common feature of all the PDI members is the presence of at least one domain with structural similarity to TRX, which can be either active or enzymatically inactive (Kozlov et al., 2010). Protein activity depends on the presence of CXXC motif, which determines the reaction with thiols of newly synthesized proteins. However, some of the proteins vary in their active site composition, including AGR2 and AGR3 proteins with CXXS motif (Galligan and Petersen, 2012); therefore, it is possible that in order to act as PDIs AGR proteins cooperate with other redox-active molecules. Within the family, ERp18, TMX, TMX2, TMX4, TMX5, AGR2 and AGR3 proteins possess a single active domain, while ERp27, ERp29, CASQ1 and CASQ2 proteins contain only inactive domains (Benham, 2012; Galligan and Petersen, 2012). Moreover, each protein of the family is characterized by the presence of a short NH_2-terminal signal peptide and the COOH-terminal endoplasmic reticulum (ER) retention sequence (with the exceptions of TMX1, TMX4, CASQ1 and CASQ2 that lack the ER retention sequence) (Appenzeller-Herzog and Ellgaard, 2008; Galligan and Petersen, 2012). An ample insight into the sequence characterization as well as structural overview of the PDI family can be found in the following works (Appenzeller-Herzog and Ellgaard, 2008; Galligan and Petersen, 2012; Kozlov et al., 2010).

The structure of the mature AGR2 protein, characterized by the lack of the first 20-amino acid signal peptide, was recently characterized. It was shown that unfolded N-terminal 21–40 amino acid region determines adhesion properties of AGR2, whereas the folded domain forms a dimer through specific intermolecular salt bridges. Moreover, in that work, the authors demonstrated that the proper topography of the dimeric structure relies on interaction between amino acids E60 and K64 (Patel et al., 2013). Relying on available dimeric structures of AGR proteins in Protein Data Bank [PDB accession numbers—AGR2: 2LNS, AGR3: 3PH9], it can be concluded that AGR2 secondary structure consists of α-β-α-α-β-α structural motifs, whereas AGR3 consists of α-β-α-α-β-α-β-α-α-α motifs. AGR2 and AGR3 thioredoxin domain with active site CPHS and CQYS, respectively, is situated on α2 helix. The main difference between AGR2 and AGR3 structure lies in the dimerization region (Fig. 1). The dimerization of AGR2 arises from interaction between random coils (residues 45–54) and α1 helices (between residues 60–67), whereas AGR3 is predicted to form dimer through random coils corresponding to 32–36, 42–46 and 100–105 residues as well as part of α1 helix (residues 47–53). Moreover, based on PDBsum database, we suggest that AGR3 dimerization occurs through specific hydrogen bonds between amino acids Q32 and Q46. However, to verify whether AGR homologues are able to dimerize with each other through random coils and/or α1 helices warrants further investigation.

Role of PDI family in cell homeostasis

The PDI family members are implicated in a variety of disorders, including neurodegenerative syndromes such as Parkinson's, Alzheimer's, Huntington's diseases, familial amyotrophic lateral sclerosis (ALS) as well as infertility and a diverse range of malignancies (as reviewed by Benham, 2012). Of note is also a report showing decrease of AGR2 expression in ulcerative colitis, where it was postulated to play a crucial role in the maintenance of epithelial integrity (Zheng et al., 2006).

The main function of the PDI family members is to form/disrupt, oxidize/reduce and isomerize the disulphide bonds between the cysteine residues of nascent proteins in the lumen of the endoplasmic reticulum (ER) in order to provide their proper folding and maturation prior to the release for cellular transport (Hatahet and Ruddock, 2009). Thus, they play a pivotal role in the maintenance of cellular homeostasis. They can also serve as molecular chaperones involved in the ER-associated degradation (ERAD) mechanisms that lead to protein removal (Ni and Lee, 2007). Apart from their ubiquitous expression in ER lumen, PDIs can be found in other cellular compartments, where they are shown to regulate among others cell adhesion, platelets activation, viral infections and protein–DNA interactions (Turano et al., 2002).

Although oxidative properties of AGR2 protein have not yet been validated *in vitro* or *in vivo*, some AGR2 client proteins have been identified, supporting its role in the protein folding machinery. For instance, AGR2 was demonstrated to regulate production of mucins (MUCs), including intestinal MUC2 (Park et al., 2009), the airway epithelial MUC5AC and MUC5B (Schroeder et al., 2012) as well as pancreatic MUC1 (Norris et al., 2013). Additionally, by forming a substrate loop between amino acids 104 and 111, it interacts with ATP-binding protein Reptin and consequently regulates many of its functions such as ATPase activity, ATP binding, helicase functions, telomerase/Pontin binding and others (Maslon et al., 2010). Further, AGR2-interacting proteins were identified in yeast two-hybrid screen, including neurexin 3, cytoskeleton-associated protein 2 or Ly6/PLAUR domain-containing protein 3, linking AGR2 with cell adhesion, division and migration (see review by Chevet et al., 2013).

Perturbation of ER homeostasis leads to the accumulation of unfolded or mis-folded proteins, ER stress and consequently activation of the unfolded protein response (UPR). UPR signalling results either in the degradation of mis-folded proteins by upregulating PDIs and molecular chaperones or in the attenuation of protein synthesis (Ron and Walter, 2007). However, if ER homeostasis cannot be restored, apoptotic pathways are induced (Tabas and Ron, 2011). Using both proteomic and biochemical approaches, Higa et al. identified AGR2 as one of the ER proteins that associates with membrane-bound ribosomes through nascent protein chains. They found that AGR2 is induced upon ER stress and that its basal expression is controlled by the IRE1α- and ATF6α-triggered arms of the UPR. They also showed that AGR2 silencing altered the expression of ERAD components, resulting in cell survival under stress condition (Higa et al., 2011). Additionally, an independent study showed that AGR2 homo-dimerization is important for the association with BiP/GRP78, a well-established chaperone involved in the cellular response to many stresses (Ryu et al., 2013).

AGR2 and AGR3 expression in tumour cells and tissues

Uncoupled expression of AGR proteins in carcinomas

Both AGR2 and AGR3 were originally found in breast cancer specimens. *AGR2* gene was first described in the oestrogen receptor (EsR)-positive MCF-7 cell line (Thompson and Weigel, 1998), and AGR3 protein was identified in the membrane of breast cancer

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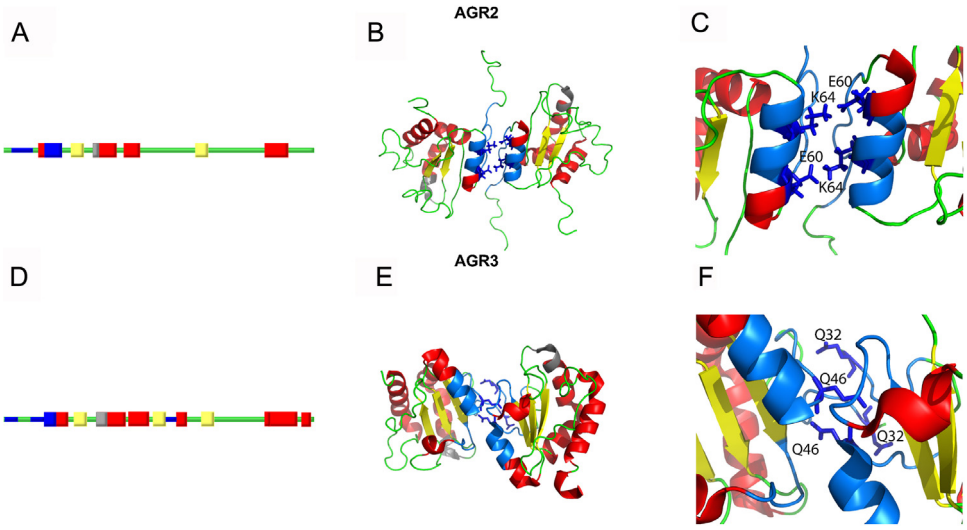

Fig. 1. Structure of AGR2 and AGR3 proteins (derived from Protein Data Bank, http://www.rcsb.org, accession numbers 2LNS and 3PH9, respectively). (a) AGR2 secondary structure with alpha helices (red), beta strands (yellow), CPHS active site (grey) and dimerization domain (blue) shown; (b) ribbon representation of $AGR2_{41-175}$ homodimer with secondary structures, dimerization domain and active site marked correspondingly to 1a; (c) AGR2 dimeric interface, interactions between E60 and K64, responsible for dimer stability are delineated in blue; (d) AGR3 secondary structure with alpha helices (red), beta strands (yellow), CQYS active site (grey) and dimerization domain (blue) shown; (e) ribbon representation of $AGR3_{31-165}$ homodimer with secondary structures, dimerization domain and active site marked correspondingly to 1d and (f) AGR3 dimeric interface, interactions between Q32 and Q46, with putative role in dimer stabilization are delineated in blue.

cell lines using proteomic screening (Adam et al., 2003). The latter study showed that AGR3 was present in the majority of analysed breast tumours, while in normal tissue, it was found only in the epithelial lining of the colonic mucosa (Adam et al., 2003). The same group reported concordant expression of both AGR2 and AGR3 in breast tumours, which positively correlated with EsR status and negatively with epidermal growth factor receptor (EGFR) expression (Fletcher et al., 2003). Regulation of AGR2 protein expression by oestrogen was later confirmed by both *in vivo* (Wilson et al., 2006) and *in vitro* experiments (Hrstka et al., 2010). Furthermore, based on ChIP-Seq experiments, it was validated that both *AGR2* and *AGR3* possess a single EsR-binding site in their regulatory regions (Welboren et al., 2009).

AGR proteins are also involved in response to other sex hormones. Using a suppression subtractive hybridization-based technique, Zhang et al. (2005) demonstrated that *AGR2* is an androgen-inducible gene. On the other hand, comparing benign, malignant prostate tissue and samples obtained from prostate cancer patients after surgical castration, Vaarala et al. (2012) found *AGR3* among other androgen-regulated genes, expression of which was highly elevated in human prostate cancer. This report is in agreement with recent work by Bu and co-workers in which they depicted androgen receptor (AR)-dependent up-regulation of both *AGR2* and *AGR3* genes by androgen and oestrogen (specifically 17β-oestrogen) in prostate cancer cell lines. Moreover, they characterized four AR-binding sites in the distal promoter region of *AGR2*, located 5.5, 8 and 11 and 17 kb upstream of the transcription start site (TSS), and one strong AR-binding site in *AGR3*, localized to the first intron approximately 1 kb downstream of TSS (Bu et al., 2013).

Furthermore, uncoupled expression of both AGR proteins was described in ovarian cancers. Gray et al. found that AGR3 is overexpressed in four divergent types of ovarian cancers, including serous papillary, endometrioid, clear cell (non-mucinous types) and mucinous carcinomas. In non-mucinous types, AGR3 expression was found to be heterogeneous, EsR-independent and not related to AGR2 expression. However, mucinous ovarian cancers showed corresponding positive staining of both AGR2 and AGR3 (Gray et al., 2012). In another study, AGR3 staining correlated with the level of differentiation in serous borderline ovarian tumours and low-grade serous ovarian carcinomas (LGSCs) and was associated with longer patient survival. On the other hand, high-grade serous ovarian carcinomas (HGSCs) showed AGR3 positivity to a much lesser extent (King et al., 2011). Conversely, strong AGR2 expression was reported in HGSCs and was statistically significantly linked to shorter overall survival and progression-free survival (Darb-Esfahani et al., 2012).

Liver cancer represents another example of malignancy in which AGR proteins exhibit a cognate expression pattern. Recently, our group has shown that intrahepatic cholangiocarcinomas (ICCs) express AGR3 protein, whereas hepatocellular carcinomas (HCCs) are predominantly AGR3-negative. Due to the statistically significant association between AGR3 expression and the presence of acid mucopolysaccharide, it was postulated that together they could serve as diagnostic markers of well-differentiated ICCs (Brychtova et al., 2014). In agreement with this report is work demonstrating AGR2 overexpression in fibrolamellar carcinomas compared to other HCCs (Vivekanandan et al., 2009). AGR2 expression also mirrors the level of differentiation of biliary tract cholangiocytes, similar to AGR3 (Lepreux et al., 2011). AGR2 expression in other carcinomas is out of scope of this review and can be found elsewhere (Brychtova et al., 2011; Chevet et al., 2013; Salmans et al., 2013).

The above data indicate that the mechanism of either coincident or separate *in vivo* expression of AGR proteins is a context-dependent event. Further investigation is required to verify whether similar phenomena can be observed in different types of tumours. Indeed, recently, our group observed different expression patterns of both proteins in various tumours (complete data not shown) including breast carcinomas, in some of which AGR proteins correlated while in others were uncoupled (Fig. 2).

AGRs expression based on Genevestigator data

In order to expand current knowledge regarding expression of AGRs in different types of cancer, we aimed to compare their distribution both in human healthy tissues and carcinomas (Fig. 3) using Genevestigator platform, a tool that integrates high-quality public microarray data (Hruz et al., 2008).

It is noteworthy that *AGR3* mirrors *AGR2* expression in many cases, suggesting their cognate physiological function and role in

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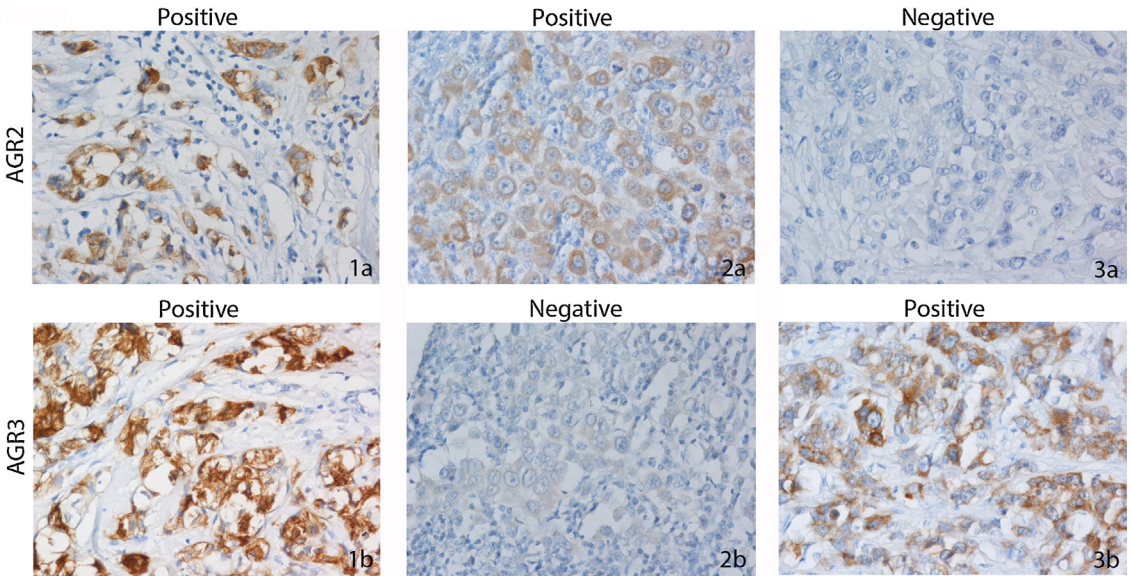

Fig. 2. Expression patterns of AGR proteins in breast cancer. Immunohistochemical detection of AGR2 and AGR3 proteins showed discordant expression pattern in triple-negative breast cancer. In some tumour samples, AGR2 and AGR3 proteins showed similar cytoplasmic positivity (1a, 1b) in identical tumour tissue, while in other samples, AGR2 and AGR3 expression was uncoupled in the corresponding tissue sections (2a, 2b; 3a, 3b) (AGR2 protein was detected using HPA007912 antibody, Sigma-Aldrich, St Louis, MO, USA; AGR3 protein was detected using in-house anti-AGR3.1 antibody (Gray et al., 2012) magnification 400×).

pathological conditions. However, they can also be expressed in a tissue-specific manner, such as normal skin or urinary bladder, where *AGR3* is not present or expressed to a lesser extent, respectively, when compared to *AGR2* that is expressed in both tissues. In early stages of low vertebrates development, AGR proteins are mainly expressed in ectoderm-derived organs, while in adults they can also be found in endoderm-derived organs such as intestine (Chen et al., 2012; Xia et al., 2009). Similarly, human AGRs are predominantly distributed in endoderm-derived organs (Xia et al., 2009). Hence, as expected, we found that both *AGR2* and *AGR3* are highly expressed in normal stomach, colon, female reproductive system and respiratory system tissues as well as the corresponding carcinomas.

In most of the cases, our findings are in concordance with the literature regarding AGRs expression in ovarian, gastric and lung carcinomas (Armes et al., 2013; Bai et al., 2011; Darb-Esfahani et al., 2012; Gray et al., 2012; King et al., 2011; Park et al., 2011; Pizzi et al., 2012), with the exception of *AGR2* in colon cancers, where it was found among down-regulated genes when compared with adenoma (Lee et al., 2006). Moreover, according to our analyses, expression of both *AGR2* and *AGR3* genes was elevated in pancreatic and breast carcinomas in comparison to healthy tissues, which is in line with published reports (Adam et al., 2003; Brychtova et al., 2014).

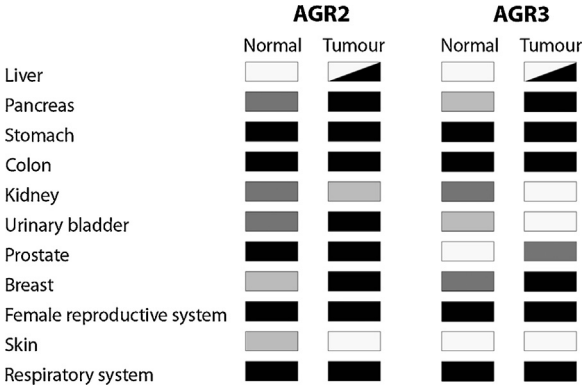

Fig. 3. Overview of AGR proteins distribution in normal and cancer tissues. Four-step grey scale indicates expression levels of AGR2 and AGR3 in normal and corresponding tumour tissue according to data from Genevestigator (lighter shades, lower AGR expression; darker shades, higher AGR expression). Liver tumour is characterized by heterogeneous expression of AGR proteins (shown by light/dark boxes).

Strikingly, unlike the increased levels of *AGR2* in urinary bladder carcinoma compared to normal tissues, we found *AGR3* to be downregulated in this type of tumour. To date, only one report describing AGR2 expression in bladder urothelial carcinoma was released, showing downregulation of this gene in tumours when compared to healthy controls (Izquierdo et al., 2010), which is in disagreement with our findings based on Genevestigator. However, further investigation is required to elucidate AGRs function in bladder tumours. According to Genevestigator data, *AGRs* expression in liver carcinomas is either elevated or downregulated, which could be explained by the fact that AGRs are differentially expressed in different histological types of these tumours (Brychtova et al., 2014; Vivekanandan et al., 2009). Although *AGR3* levels are increased in various carcinomas, as shown above, there are still a limited number of reports regarding its role in tumours, as well as its clinicopathological significance.

In normal tissues, AGR proteins could participate in the regulation of the total protein load in the cell. There are some lines of evidence to support this hypothesis. For instance, AGR2 is required for the production of airway epithelial MUC5AC and MUC5B in respiratory system tissues (Schroeder et al., 2012), whereas AGR2 was found to regulate cell proliferation and differentiation during normal mammary gland development, and its maximum expression appeared during late pregnancy and lactation, where there is the highest requirement for milk protein production (Verma et al., 2012). Further work is warranted to identify AGRs role in the physiology of other tissues, including those found in our *in silico* analysis, such as prostate, stomach, colon or urinary tract.

AGR2 and AGR3 *in silico* promoter analysis

Uncoupled expression of AGR2 and AGR3 in different carcinomas has already been reported (Bu et al., 2013; Gray et al., 2012), which indicates that their functions are required in divergent situations. Therefore, in order to elucidate the mechanisms

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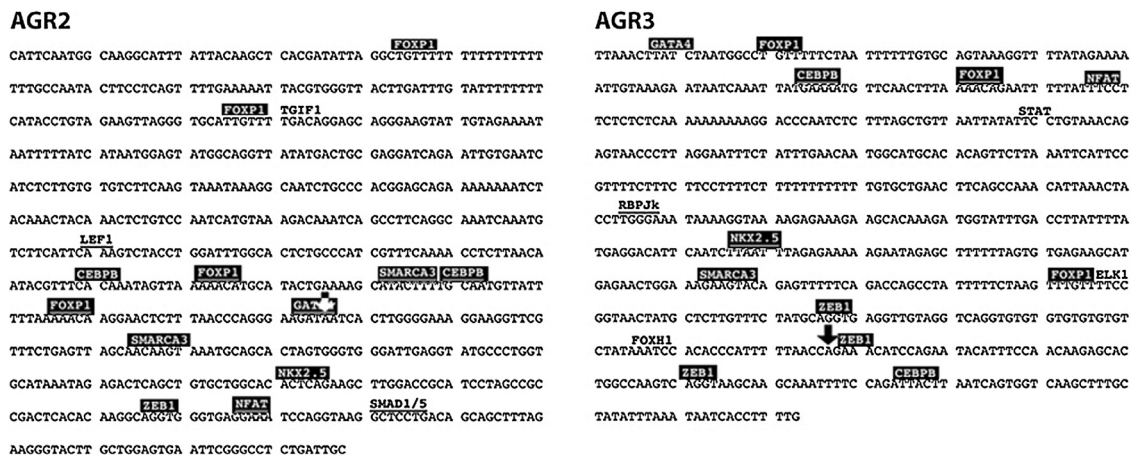

Fig. 4. Nucleotide sequence of AGR2 and AGR3 promoter with representative potential binding sites for transcription factors. Arrows indicate position of the transcription start site determined according to MatInspector. Underlined molecules represent transcription factors which bind to the (+) DNA strand, whereas molecules without highlights correspond to the transcription factors which bind to the (−) DNA strand. Transcription factors common for AGR2 and AGR3 are shown in black boxes.

triggering differential expression of these proteins, we aimed to analyse the promoter region of both AGR2 and AGR3 using MatInspector (Cartharius et al., 2005; Quandt et al., 1995) in search for transcription factor (TF) binding sites. TFs were selected according to the matrix similarity, and only TFs with score higher than 0.95 were taken into account. Many of the TFs found in the *in silico* analysis play significant roles in developmental processes and differentiation of tissues and/or organs of diverse origins (Fig. 4 and Table S1). This is consistent with the report demonstrating that anterior gradient proteins in lower vertebrates (particularly XAG-2) play a putative role in ectodermal patterning during amphibian embryogenesis, triggering cement gland differentiation as well as the expression of anterior neuronal marker genes (Aberger et al., 1998). Moreover, AGRs correlation with differentiation lineages has been shown in liver carcinomas (Brychtova et al., 2014; Lepreux et al., 2011).

Interestingly, most of the TFs potentially binding to AGR2 or AGR3 promoters are exclusive for each protein, with the exception of common FOXP1, ZEB1 (AREB6), SMARCA3, GATA4, NFAT, CEBPB and NKX2.5. Forkhead box (FOX) family members, namely FOXA1 and FOXA2, have been implicated in the regulation of AGR2 expression both in prostate cancer and in human embryonic kidney-derived HEK293 cell lines (Zhang et al., 2010; Zheng et al., 2006). Recently, it has also been shown that FOXA1 drives expression of AGR2 in both tamoxifen-sensitive and -resistant breast cancer cells (Wright et al., 2014). On the other hand, FOXP1 is considered to act as a tumour suppressor due to the fact that it is often downregulated in cancers and its loss correlates with worse patient outcome (Koon et al., 2007). Furthermore, elevated nuclear FOXP1 activity is associated with more favourable prognosis of breast cancer patients treated with tamoxifen (Shigekawa et al., 2011). Following this, it is plausible that FOXP1 inhibits AGR2 activation, which in consequence leads to prolonged patient survival by reduction of tumour aggressiveness. It is therefore necessary to verify whether FOXP1 is indeed able to bind the AGR2 promoter and control its activity. Whether FOXP1 has similar effects on AGR3, which is also expressed in EsR-positive breast cancers, remains to be established as well (Fletcher et al., 2003).

Another noteworthy transcription factor that potentially triggers expression of AGRs is zinc-finger enhancer binding-1 (ZEB1). ZEB1 is a master regulator of epithelial-to-mesenchymal transition (EMT), a fundamental cellular programme controlling embryonic development and homeostasis, as well as tumour progression and metastasis (Thiery et al., 2009). ZEB1 suppresses the epithelial cell–cell adhesion molecule E-cadherin (downregulation of which is a hallmark of EMT) and influences other key cellular processes such as cell cycle control, senescence and apoptosis (Brabletz and Brabletz, 2010; Browne et al., 2010). AGR proteins could act as downstream effectors of ZEB1-induced metastasis and promote tumour cell dissemination through modulation of their adhesion properties and interaction with the extracellular matrix (Dumartin et al., 2011; Fletcher et al., 2003).

Several pieces of evidence suggest that AGR2 is a mediator of various cancer signalling pathways including Hippo, EGFR, EsR, cyclin D1, Src, c-Myc, survivin, aryl hydrocarbon receptor (AhR) and transforming growth factor-beta (TGF-β) (see review by Salmans et al., 2013). Further evidence for AGR2 involvement in TGF-β signalling is provided by the presence of a binding site for TG-interacting factor 1 (TGIF1) within the *AGR2* promoter. TGIF1 is a well-documented co-repressor of this pathway (Wotton et al., 1999) and therefore can be expected to inhibit AGR2. Moreover, in pancreatic carcinomas, AGR2 expression is regulated by SMAD4, a downstream target of TGF-β signalling (Norris et al., 2013), which is in agreement with our finding of glycine-rich SMAD binding elements (GC-SBE) in the *AGR2* promoter. Additionally, we identified a lymphoid enhancer factor-1 (LEF-1)-binding site in the *AGR2* promoter region, indicating a possible functional involvement of AGR2 in Wnt/β-catenin/LEF-1 signalling. This pathway is activated in many types of cancers (Reya and Clevers, 2005) and regulates among others expression of c-Myc and cyclin D1 (He et al., 1998; Shtutman et al., 1999). Interestingly, AGR2 has been also demonstrated to modulate cyclin D and c-Myc expression and in consequence influence growth and survival of breast cancer cells (Vanderlaag et al., 2010).

Although AGR3's role as a component of tumour signalling is poorly understood, *in silico* promoter analysis revealed a binding site for the ETS-domain containing protein ELK1, which is a direct target for the mitogen-activated protein kinase (MAPK) signalling pathway (Janknecht et al., 1993), implicated in many aspects of tumour biology including proliferation, apoptosis, EMT, invasion and migration (Koul et al., 2013; Sui et al., 2014). Particularly, activation of ELK1 leads to the enhanced cell proliferation and survival (Huynh, 2002; Koul et al., 2013). Moreover, relying on our *in silico* analysis, we hypothesize that AGR3 may work as a downstream effector of other signal transduction pathways such as Notch (due to the presence of RBPjκ binding site (Jarriault et al., 1995)), TGF-β/activin/Nodal (presence of FOXH1 binding site (Schier and Shen, 2000)) and Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling (presence of STAT binding site). These pathways coordinate signalling cascades controlling

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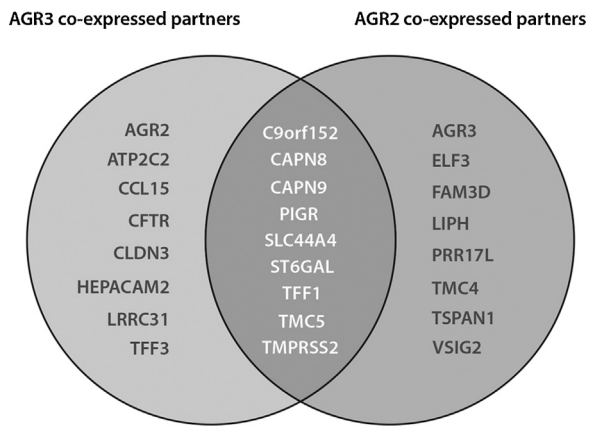

Fig. 5. AGR2 and AGR3 co-expressed genes. Co-expression tool from Genevestigator was used to find genes having an expression profile most similar to AGR genes.

cell proliferation, differentiation, migration, apoptosis, cell-to-cell communication as well as developmental processes (Kitisin et al., 2007; Lai, 2004; Rawlings et al., 2004).

In addition, given that during brain development in *X. laevis* amphibian's AGRs are induced in the FGF-dependent manner (Tereshina et al., 2014), it is plausible that expression of human AGRs is also driven through this signalling pathway.

Interestingly, although regulation of AGR2 and AGR3 expression by EsR has been demonstrated several times (Hrstka et al., 2010; Welboren et al., 2009; Wilson et al., 2006), we have not identified EsR-binding sites in the analysed promoter region of either of these two genes. It is thus possible that (i) EsR-binding sites are located in the distant promoter regions or in introns, (ii) EsR acts through interaction with other transcription factors, (iii) EsR is an enhancer or (iv) EsR signalling pathways activate AGR transcription or remove inhibition of their induction.

Although the number of *in silico* identified binding sites for specific transcription factors is in substance consistent with previously published data, it is clear that real understanding of involvement of these identified putative transcription factors and hierarchy of their interplay in regulating transcription of AGR genes requires further experimental evidence.

AGR2 and AGR3 co-expressed partners

In order to provide deeper insight into potential AGR functions, with a focus on the poorly characterized AGR3 protein, we performed co-expression analysis using Genevestigator (Hruz et al., 2008). We found several shared and unique genes, which could exemplify AGRs interacting molecules, downstream partners and/or cross-talking pathways (Fig. 5 and Table S2).

There are several lines of evidence that AGRs are secreted proteins. Although both AGR2 and AGR3 harbour ER-retention sequences (KTEL and QSEL. respectively) in their C-terminal parts, they can escape ER retrieval machinery and can be found in the cytoplasm as well as in the extracellular environment (Fletcher et al., 2003). In both T47-D and MDA-MB-468 cells, AGR3 is localized in secretory or endosome-like vesicles (Adam et al., 2003), while AGR2 was found to be secreted during the development of pancreatic cancer (Ramachandran et al., 2008) and was detected in the urine of prostate cancer patients (Bu et al., 2011) as well as in the blood of ovarian cancer patients (Edgell et al., 2010). Remarkably, Bergstrom et al. (2014) have recently demonstrated that in addition to the KTEL ER-retention signal, the presence of a single cysteine within the AGR2 TRX-like domain is required for the control of protein secretion.

Some of the transporter molecules identified in gene expression database (Table S2) could participate in AGRs export from the cell, including transmembrane channel-like protein 4 (*TMC4*), transmembrane channel-like protein 5 (*TMC5*), protein member 4 of solute carrier family 44 (*SLC44A4*) or cystic fibrosis transmembrane conductance regulator (*CFTR*).

Presence of AGR proteins outside the cells suggests their possible role in an autocrine and paracrine signalling. We also found tetraspanin 1 (*TSPAN1*) as a potential AGR2 co-expressed partner. Tetraspanins, also known as transmembrane 4 superfamily (TM4SF) members, mediate outside-in signal transduction events and consequently regulate cell differentiation, migration and proliferation (Richardson et al., 2011).

Yeast two-hybrid screen identified metastasis-associated GPI-anchored C4.4a protein and extracellular alpha-dystroglycan (DAG-1) as binding proteins for both AGR2 and AGR3. It was further discussed that through these interactions, AGR proteins can be involved in many pivotal processes promoting tumour progression including metastasis, migration and invasion (Fletcher et al., 2003). AGR2 role as a metastasis-related protein is intensively studied in many cancer types including breast carcinomas (Liu et al., 2005), prostate adenocarcinomas (Zhang et al., 2010), head and neck squamous cell carcinomas (Sweeny et al., 2012) or gastrointestinal tumours (Valladares-Ayerbes et al., 2008).

AGR3 function in cancer development remains ambiguous as yet; nevertheless, it was shown that AGR3 mediates resistance to cisplatin (CDDP) in a mouse xenograft model (Gray et al., 2012) and can bind to metastasis-related proteins C4.4a and DAG-1 (Fletcher et al., 2003). Co-expression analysis using Genevestigator depicted other possible AGR3 partners linking AGR3 with cell adhesion, motility, metastasis and regulation of cell cycle. We found that *AGR3* is co-expressed with a gene coding for tight junction protein, claudin 3 (CLDN3), responsible for control of tumour growth and metastasis (Shang et al., 2012) as well as suppression of epithelial-to-mesenchymal transition (Lin et al., 2013). On the other hand, overexpression of claudin 3 promotes the malignant potential of colorectal cancer cells, which is regulated by ERK1/2 and PI3K-Akt pathways (de Souza et al., 2013). Interestingly, it was also demonstrated that CLDN3 affects sensitivity of ovarian cancer cells to CDDP through regulation of copper influx transporter CTR1 function (Shang et al., 2013). Therefore, it is tempting to speculate that insensitivity of AGR3-positive tumours to the growth-inhibiting effect of CDDP (Gray et al., 2012) would be due to cooperation with claudin 3.

Another noteworthy *AGR3* co-expression partner is the gene coding for immunoglobulin-like hepatocyte cell adhesion molecule (hepaCAM), which is frequently downregulated in diverse human cancers (Chung Moh et al., 2005; Moh et al., 2005). There is mounting evidence for hepaCAM role as a tumour suppressor as it inhibits proliferation of human bladder cancer cells (Wang et al., 2013), causes G1 phase arrest and promotes c-Myc degradation in human renal cell carcinomas (Zhang et al., 2011), whereas in MCF7 breast cancer cells, it induces cellular senescence (Moh et al., 2008). It would be interesting to evaluate whether AGR3 may suppress hepaCAM activity and, in consequence, promote tumour aggressiveness.

In our analysis, both *AGR2* and *AGR3* were found to co-express with genes coding two gastrointestinal (GI) track-specific members of the calpain family, calpain 8 (CAPN8) and calpain 9 (CAPN9) (Lee et al., 1998; Sorimachi et al., 1993). Most calpains are ubiquitous cytosolic proteases involved in a plethora of cellular processes such as signal transduction, cell cycle progression, apoptosis or cytoskeletal remodelling (Storr et al., 2011). CAPN8 and CAPN9 were demonstrated to play a protective role in the gastric mucosa by forming an active protease complex (Hata et al., 2010). In addition, downregulation of CAPN9 has been reported in a subset of

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gastric cancer patients and gastric cancer cell lines (Yoshikawa et al., 2000). Therefore, it is possible that AGRs are essential for the maintenance of calpains physiological properties, similar to that of intestinal MUC2 (Park et al., 2009).

Conclusions and perspectives

In conclusion, AGR2 and AGR3 proteins due to their high sequence homology are similar but not identical and therefore can be expressed in cognate or uncoupled manner in divergent carcinomas and normal tissues.

Mechanisms triggering context-dependent expression of AGR proteins remain poorly defined and thus need deeper investigation. As a part of the PDI family, AGRs are predicted to control proper protein load in the endoplasmic reticulum, and indeed, there are reports showing AGR2's essential role, for instance, in mucin production. The information depicted in this review indicates that AGR2 and AGR3 plausibly control similar aspects of tumour biology including cell cycle control, differentiation, migration, invasion and metastasis. Moreover, relying on available data and on our *in silico* analysis, it can be speculated that, in addition to oestrogen receptor signalling pathway, they are components of additional signal transduction cascades. Following this, it would be interesting to verify whether a functional cross-talk between AGR proteins exists in order to regulate cell fate processes in health and disease. Taking into account their high expression and emerging roles, it is highly possible that they can act as either tumour suppressors or oncogenes, similar to TGF-β (Imamura et al., 2012). However, mechanisms that enable switch between these two contradictory actions require further investigation. In the light of recent developments in cancer research, proper understanding of AGR functions would be inevitable for new drugs development aiming both at these proteins and pathways driving their expression. Moreover, as secreted proteins they could serve as markers for cancer detection and/or prediction of patient outcome. Lastly, as suggested in this work, AGRs could cooperate with other druggable targets, which makes them even more attractive for thorough characterization.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejcb.2015.01.002.

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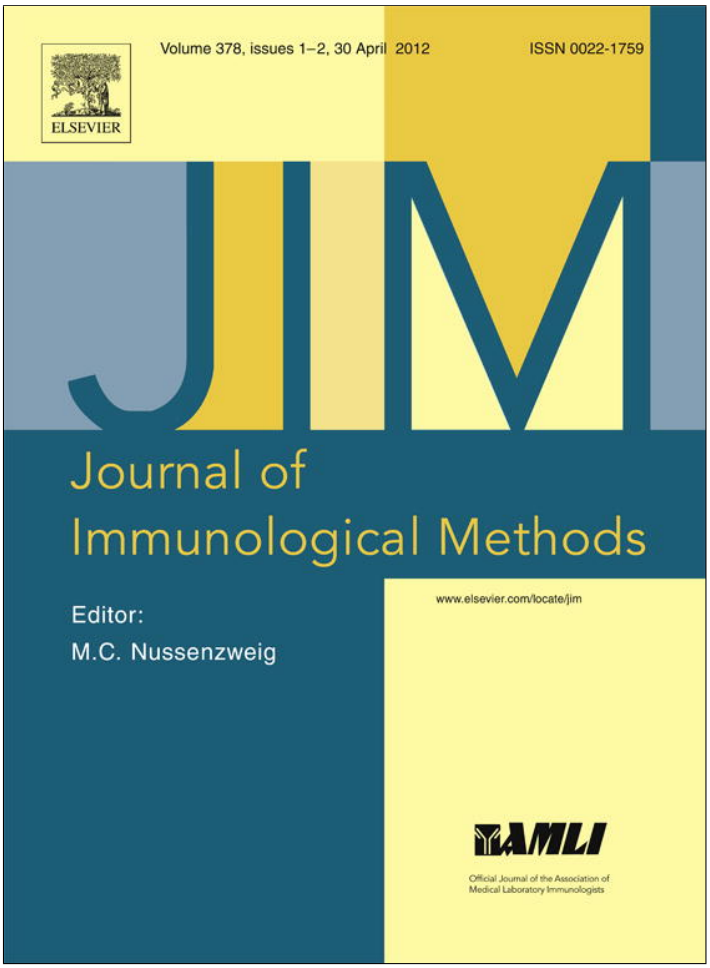
Příloha 12



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Research paper

Anterior Gradient-3: A novel biomarker for ovarian cancer that mediates cisplatin resistance in xenograft models

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ABSTRACT

The Anterior Gradient (AGR) genes *AGR2* and *AGR3* are part of the Protein Disulfide Isomerase (PDI) family and harbour core thioredoxin folds (CxxS motifs) that have the potential to regulate protein folding and maturation. A number of proteomics and transcriptomics screens in the fields of limb regeneration, cancer cell metastasis, pro-oncogenic oestrogen-signalling, and p53 regulation have identified AGR2 as a novel component of these signalling pathways. Curiously, despite the fact that the *AGR2* and *AGR3* genes are contiguous on chromosome 7p21.1-3, the AGR3 protein has rarely been identified in such OMICs screens along with AGR2 protein. Therefore there is little information on how AGR3 protein is expressed in normal and diseased states. A panel of three monoclonal antibodies was generated towards AGR3 protein for identifying novel clinical models that can be used to define whether AGR3 protein could play a positive or negative role in human cancer development. One monoclonal antibody was AGR3-specific and bound a linear epitope that could be defined using both pep-scan and phage-peptide library screening. Using this monoclonal antibody, endogenous AGR3 protein expression was shown to be cytosolic in four human ovarian cancer subtypes; serous, endometrioid, clear cell, and mucinous. Mucinous ovarian cancers produced the highest number of AGR3 positive cells. AGR3 expression is coupled to AGR2 expression only in mucinous ovarian cancers, whereas AGR3 and AGR2 expressions are uncoupled in the other three types of ovarian cancer. AGR3 expression in ovarian cancer is independent of oestrogen-receptor expression, which is distinct from the oestrogen-receptor dependent expression of AGR3 in breast cancers. Isogenic cancer cell models were created that over-express AGR3 and these demonstrated that AGR3 mediates cisplatin-resistance in mouse xenografts. These data indicate that AGR3 is over-expressed by a hormone (oestrogen-receptor α)-independent mechanism and identify a novel protein-folding associated pathway that could mediate resistance to DNA-damaging agents in human cancers.

1. Introduction

Anterior Gradient-2 (AGR2) was originally identified as a gene expressed in *Xenopus* development that specifies dorso-anterior ectodermal fate during the formation of the

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cement gland, a mucus-secreting organ involved in the attachment of the embryo to a solid support prior to swimming and feeding (Sive et al., 1989; Sive and Bradley, 1996; Aberger et al., 1998). *AGR2* is now known to have an orthologue named *AGR3* which together are part of the Protein Disulfide Isomerase (PDI) family (Persson et al., 2005). There are at least 18 human PDI family members (Hatahet and Ruddock, 2009) (Appenzeller-Herzog and Ellgaard, 2008). Protein Disulfide Isomerases (PDIs) contain core thioredoxin folds (CxxC or CxxS motifs), which regulate protein folding via regulation of disulfide bond formation (Persson et al., 2005). There are five highly conserved members within the AGR2 grouping that include: TRX1 (thought to be predominantly the nuclear thioredoxin), TRX2 (thought to be predominantly the mitochondrial thioredoxin), endoplasmic reticulum (ER) protein 18 (ERP18 (Alanen et al., 2003) which is the ancestral founder in the AGR2/3 group), AGR2, and the AGR2 orthologue AGR3. AGR2 and AGR3 are confined to vertebrates and both have the CxxS core motif instead of the classic thioredoxin fold/CxxC motif of TRX1, TRX2, and ERP18.

AGR2 is now known to be involved in normal intestinal mucus production (Park et al., 2009) and in normal limb regeneration in the newt (Kumar et al., 2007). AGR2 expression is also perturbed in human disease; it is part of a stress protein group that can be used as a diagnostic tool for oesophageal disease (Barrett's) (Groome et al., 2008) and in the aetiology of asthma and inflammatory bowel disease (Zheng et al., 2006; Di Valentin et al., 2009). Transcriptomic and proteomic approaches in the past ten years using human tissue biopsies have identified novel, clinically-relevant protein families involved in cancer that have not been identified from cancer genetic screens. AGR2 forms a prototype case as a gene not identified from powerful cancer genetics and oncogene discovery models of the 1980s and 1990s, but from more latterly developed OMICS expression screens that reflect dynamic transcriptional and translational functions of a cell that drive oncogenesis. AGR2 is involved in tumour-associated pathways, including tumour growth, cellular transformation, cell migration, metastasis and chemoresistance (Liu et al., 2005; Ramachandran et al., 2008; Wang et al., 2008), and may represent a novel prognostic factor in some cancer types (Smirnov et al., 2005; Barraclough et al., 2009). Human AGR2 can be induced by the oestrogen receptor α (ERα) (Thompson and Weigel, 1998) and clinical studies suggested a positive correlation between AGR2 levels and ERα expression (Fletcher et al., 2003). Surprisingly however, AGR2 expression is induced rather than suppressed by the anti-oestrogen cancer drug Tamoxifen thus identifying AGR2 as an important pro-oncogenic agonist of Tamoxifen (Hrstka et al., 2010). A label-free data-independent proteomics screen (PACIFIC) of Tamoxifen-treated MCF7 cancer cells identified AGR2 protein as one the top proteins elevated in Tamoxifen treated cells (Hengel et al., accepted for publication). Clinical studies have also shown that AGR2 expression can also predict poor responses to Tamoxifen in breast cancer patients identifying a potentially novel pathway to target for treating drug resistant cancers (Hrstka et al., 2010).

Oestrogen-independent expression of AGR2 can be observed in other human cancers including prostate (Fletcher et al., 2003; Kristiansen et al., 2005; Zhang et al., 2005, 2007), lung (Fritzsche et al., 2007; Zhu et al., 2007), pancreas (Missiaglia et al., 2004; Lowe et al., 2007; Ramachandran et al., 2008), liver (Lepreux et al., 2011), and oesophagus (Iacobuzio-Donahue et al., 2003; Hao et al., 2006). An oesophageal cancer progression model has also identified a function for AGR2. Oesophageal adenocarcinoma similarly proceeds by a "stepwise" process including mutation of p53 and p16 that drives the development of metaplasia, dysplasia, and adenocarcinoma (Leedham et al., 2008). Oesophageal adenocarcinoma differs from other cancer types in that the environmental stress of bile acid reflux plays an apparently important role in disease progression and that p53 tumour suppressor gene mutation can occur very early in the carcinogenic sequence (Leedham et al., 2008). The early selection pressure for p53 mutation in oesophageal cancer occurs during the replacement of squamous epithelium with metaplastic epithelium also called "Barrett's oesophagus". In order to identify novel pro-oncogenic proteins that function as proto-oncogenes in Barrett's epithelium, a proteomics screen was initiated to identify candidate proteins over-produced in Barrett's compared to normal squamous epithelium. This screen identified a highly over-expressed protein named Anterior Gradient-2 (AGR2) that was shown to inhibit the p53 response to DNA damage thus identifying a clinically relevant, novel p53 silencing pathway in metaplasia (Hopwood et al., 1997; Yagui-Beltran et al., 2001; Pohler et al., 2004). AGR2 mRNA expression is also a dominant feature of a recently identified murine model of Barrett's oesophageal epithelium induced through deletion of the squamous stem cell progenitor p63 (Wang et al., 2011). AGR2 is now known to be over-produced in the majority of oesophageal squamous cancers suggesting that once it is elevated in Barrett's metaplasia, selection pressures are placed on maintaining its expression (Dong et al., 2011).

It is notable that AGR2, and not AGR3, is the gene/protein that was found to be expressed by oestrogen (Thompson and Weigel, 1998), as a dominant protein induced in Tamoxifen treated cells (Hengel et al., accepted for publication), as an abundantly expressed p53-inhibitor in a Barrett's oesophagus proteomic screens (Pohler et al., 2004) (Murray et al., 2007), and as a predictive marker for Letrozole responses in patients (Mackay et al., 2007). As such, there are no molecular studies to indicate whether AGR3, like AGR2, is pro-oncogenic, anti-oncogenic, or perhaps functions in a redundant manner with AGR2. AGR3, also known as HAG-3, AG3 or BCMP11, was first identified using a proteomics screen as a protein present in breast cancer cell membrane fractions (Adam et al., 2003). Follow-up studies confirmed that AGR3 is over-expressed in breast tumours and that AGR3 is co-expressed with AGR2 in breast cancer tissue with a strong positive correlation with ERα status (Fletcher et al., 2003). However, AGR3 was not shown to be co-expressed to a high degree with AGR2 in prostate cancers, indicating that AGR2 and AGR3 expressions can be uncoupled. As there has been limited data published on AGR3 and considering the growing amount information on AGR2 functions in health and disease, we investigated the expression of AGR3 in human cancer in order to identify a suitable clinical model in which to begin to study AGR3 function. We therefore set out to generate immunochemical tools and to identify models in which to study the putative role of AGR3 in human cancer development. We have developed a panel of

well-characterised monoclonal antibodies to the AGR3 protein and have used biotinylated peptide libraries derived from the open reading frames of AGR2/AGR3, as well as peptide-phage display, to identify two monoclonal antibodies that do not cross-react with AGR2. Further characterization of the monoclonal antibodies to evaluate AGR3 protein expression has shown that (i) AGR3 does not co-localise with an endoplasmic reticulum marker to the same the extent as AGR2 protein; (ii) AGR3 is over-expressed in four different types of primary human ovarian cancer; (iii) AGR3 and AGR2 expressions are coupled in mucinous ovarian cancers but are uncoupled in the other three types of primary ovarian cancers; and (iv) that AGR3 can mediate drug resistant growth in an isogenic cancer cell panel. These data suggest that AGR3 is pro-oncogenic, has non-redundant functions that are independent of AGR2, and is a component of a potentially novel pathway to target for the improved treatment of drug-resistant cancers.

2. Materials and methods

2.1. Reagents and AGR3 protein production

All reagents were purchased from Sigma-Aldrich unless otherwise stated. Overlapping synthetic 15-mer biotinylated peptides from the coding sequence of AGR2 and AGR3 were obtained from Mimotopes (see Table 1). Monoclonal antibodies to AGR2 and ERα were purchased from Abnova and Thermo Fisher Scientific, respectively. Recombinant human AGR3 protein was used to generate a panel of hybridomas expressing monoclonal antibodies to AGR3 (Moravian Biotechnology). The *AGR3* gene was cloned by recombination from a human cDNA library into a Gateway vector pDONR221 (using primer sequences with the Gateway recombination sites; forward-5′GGGGACAAGTTTGTACAAAAAAGCTTCGAAGGAGA-TAGAACCATGATAAAAAAGGAAAAGAGGCC-3′ and reverse-5′ GGGGACCACTTTGTACAAGAAAGCTGGGTCTTATAGCTCTGACTGA ATAAGTC-3′) and then recombined into the pDEST17 destination vector for the expression of HIS-tagged protein in BL21AI. Following induction by Arabinose (Fig. 1) and purification on a nickel affinity column (Fig. 1), the protein was used for immunisation to generate antibodies by the fusing B cells from immunised animals with sp2 myeloma cells (Harlow and Lane, 1988). A phage-peptide library (12-mer library from New England Biolabs) was used to define a consensus site for the three AGR3 monoclonal antibodies generated. Briefly, purified monoclonal antibody (1 μg) was adsorbed onto an ELISA well and phage library screening was performed through three rounds of selection according to the buffers in the manufacturer's protocol. The enriched phage-peptide pools were plated and individual plaques were grown, DNA isolated, and sequences of the peptide insert were identified by DNA sequencing. Representative sequences are summarised in Fig. 3C. Horseradish peroxidase (HRP)-conjugated and biotinylated streptavidin-HRP conjugated secondary antibodies were obtained from Dako.

Table 1
AGR2 and AGR3 peptide amino acid sequences. Overlapping synthetic 15-mer biotinylated peptides derived from the coding sequence of AGR2 (peptides 1–16) and AGR3 (peptides 17–31).

Peptide	Sequence
1	LLVALSYTLARDTTV
2	RDTTVKPGAKKDTKD
3	KDTKDSRPKLPQTLS
4	PQTLSRGWGDQLIWT
5	QLIWTQTYEEALYKS
6	ALYKSKTSNKPLMII
7	PLMIIHHLDECPHSQ
8	CPHSQALKKVFAENK
9	FAENKEIQKLAEQFV
10	AEQFVLLNLVYETTD
11	YETTDKHLSPDGQYV
12	DGQYVPRIMFVDPSL
13	VDPSLTVRADITGRY
14	ITGRYSNRLYAYEPA
15	AYEPADTALLLDNMK
16	LDNMKKALKLLKTEL
17	LLVTVSSNLAIAIKK
18	IAIKKEKRPPQTLSR
19	QTLSRGWGDDITWVQ
20	ITWVQTYEEGLFYAQ
21	LFYAQKSKKPLMVIH
22	LMVIHHLEDCQYSQA
23	QYSQALKKVFAQNEE
24	AQNEEIQEMAQNKFI
25	QNKFIMLNLMHETTD
26	HETTDKNLSPDGQYV
27	DGQYVPRIMFVDPSL
28	VDPSLTVRADIAGRY
29	IAGRYSNRLYTYEPR
30	TYEPRDLPLLIENMK
31	IENMKKALRLIQSEL

2.2. Tissue specimens, cell culture, and xenograft

Primary ovarian tumour material was obtained from patients undergoing gynaecological surgery in Lothian Hospitals NHS Trust, Scotland, UK. Institutional ethical approval was granted by the Lothian University National Health Service Trust Medicine/Clinical Oncology Research Ethics Subcommittee. All the patient samples were anonymised during laboratory investigation. For evaluating the effects of AGR3 on cell viability after drug treatment, a H1299 cell line stably expressing AGR3 was generated by transfecting an AGR3 expressing plasmid cloned from pDONR into pDEST12.2 and selecting for stable AGR3 expressing clones for growth analysis. For xenograft studies, H1299 cell lines, either AGR3-negative or transfected to acquire AGR3, were processed in a xenograft system in the absence and presence of cisplatin, as previously indicated (Macleod et al., 2005). Transfected H1299 cells (5×10^6 cells/implant) were initially injected into xenograft lines. Animals were at least 8 weeks old at the time of experimentation and were maintained in negative pressure isolators (La Calhene, UK). For experiments, tumour fragments were implanted subcutaneously into both flanks of nude mice and allowed to grow to 4–6 mm in diameter (over a period of approximately 1 month). Animals were then allocated to cisplatin treatment (5 mg/kg; i.p.) or control (5 mice/group) groups and treatment was commenced (defined as day 0). Cisplatin was administered on days 0 and 7. Groups contained 8–10 tumours. Tumour size was measured twice weekly using callipers and the volume calculated according to the formula $\pi/6\times\text{length}\times\text{width}^2$. Relative tumour volumes (%) were then

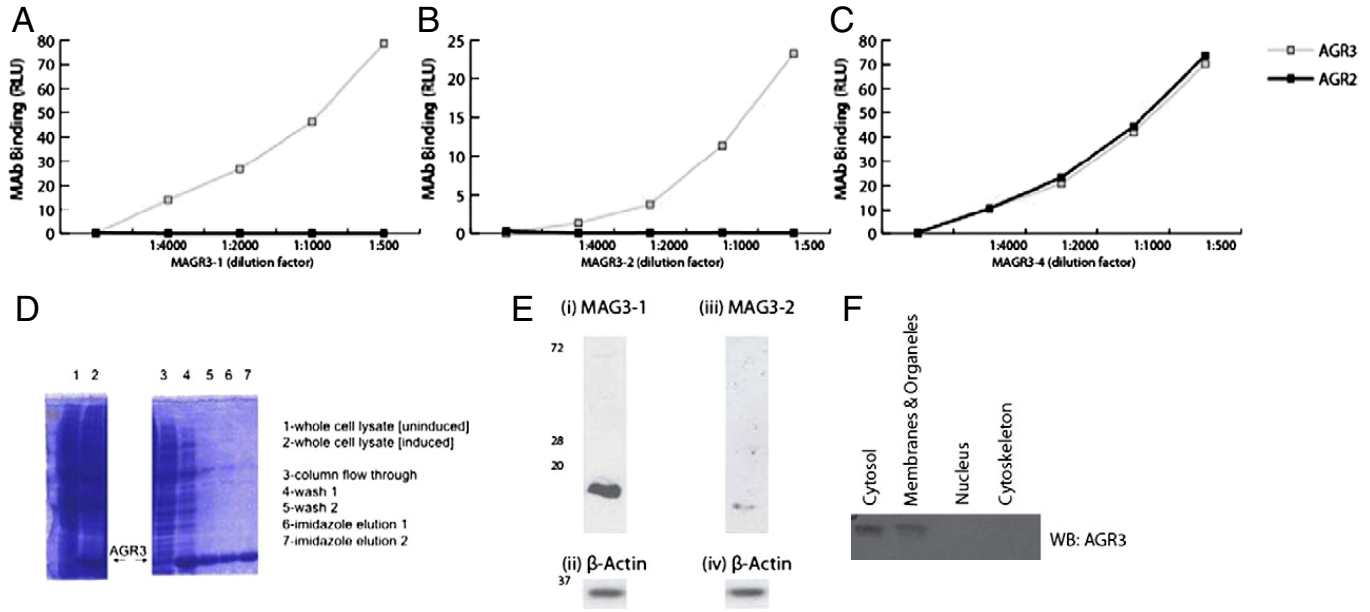

Fig. 1. Generation of monoclonal antibodies to AGR3 protein. (A–C) Recombinant human AGR3 or AGR2 proteins expressed in *E. coli* were used to define the specificity of the monoclonal antibodies MAGR3-1 (A), MAGR3-2 (B), and MAGR3-4 (C) by ELISA. Recombinant full-length AGR2 or AGR3 protein was absorbed on ELISA wells and the indicated monoclonal antibodies were titrated (by dilution) and assayed for specificity towards the full-length proteins. Data is plotted as relative binding of each monoclonal antibody (in relative light units) as a function of ascites fluid dilution. (D) Coomassie blue gel showing the relative purity of various fractions of recombinant human AGR3 protein before and after affinity purification, as indicated-cell lysates, washes, and elutions. Purified protein expressed in bacteria was used for the immunizations to generate the monoclonal antibodies. (E) MCF7 cell lysates that express both AGR2 and AGR3 proteins (data not shown) were examined for expression of AGR3 protein by Western blotting with antibodies specific for the indicated proteins using; (i) the MAGR3-1 monoclonal antibody; and (iii) the MAGR3-2 monoclonal antibody. β-Actin protein expression as a loading control is shown in ii and iv. (F) Subcellular chemical fractionation of MCF7 cell pellets (as in Pang et al. (2011)) into F1 (cytosol); F2 (membrane/organelles); F3 (nuclear); and F4 (cytoskeleton) demonstrates the MAGR3-1 can be used to localise AGR3 protein in cytosolic and organellar/membrane fractions.

calculated for each individual tumour by dividing the tumour volume on day t (V_t) by the tumour volume on day 0 (V_0) and multiplying by 100. Mean tumour volumes were then obtained. Ethical approval was granted by the University of Edinburgh ethical review committee and experiments were conducted according to UKCCCR guidelines (Workman et al., 2010).

2.3. ELISA

ELISA 96-well plates were coated with recombinant human AGR2 or AGR3 proteins in 0.1 M $NaHCO_3$ (pH 8.6). Alternatively, biotinylated peptides from the coding sequence of AGR2 and AGR3 (see Table 1) were added to ELISA 96-well dishes precoated with streptavidin. Dishes were incubated with monoclonal antibodies followed by secondary antibodies conjugated to HRP. Bound antibody was detected by enhanced chemiluminescence (ECL) using a Fluoroskan Ascent FL luminometer (Labsystems) and Ascent software version 2.4.1.

2.4. Western blotting

Cells were lysed in urea buffer (7 M urea, 0.1 M DTT, 0.05% Triton X-100, 25 mM NaCl, 20 mM HEPES–KOH pH 7.6, 5 mM NaF, 2 mM Na_3VO_4, 2.5 mM $Na_4P_2O_7$, 1× protease inhibitor cocktail). Proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) through 15% tris–glycine gels and transferred onto nitrocellulose membranes (Hybond ECL, GE Healthcare). Membranes were probed with primary antibodies, followed by secondary antibodies conjugated to HRP. Bound antibody was detected by ECL.

2.5. Immunofluorescence

MCF7 breast cancer cells were grown in DMEM (10%FCS) express both endogenous AGR2 and AGR3 due to the presence of oestrogen in serum. The cells were seeded onto glass coverslips at 40% confluency and incubated at 37 °C overnight. Coverslips were incubated with 4% formaldehyde for 10 min to fix the cells and then permeabilised in 2% BSA in 1× TBS containing 1% Triton X-100. Fixed cells were probed with primary antibodies at the appropriate dilution. Primary antibodies used were mouse monoclonal antibodies AGR3.1 or anti-AGR3.2 (this study), anti-AGR2 (Abnova), the endoplasmic reticulum marker anti-PDI antibody (Enzo Life Sciences), and Golgi apparatus biomarker Golgin 97 (Abcam). The cells were then incubated with Alexa Fluor conjugated secondary antibodies goat anti-mouse (488 nm) and donkey anti-rabbit (594 nm) at 1/200 dilutions. TO-PRO-3 nuclear stain (Invitrogen) diluted in 1× TBS was applied to coverslips, and incubated at 37 °C for 20 min providing a nuclear counterstain. Coverslips were mounted in Fluorescent Mounting Medium (DakoCytomation), and viewed using an Olympus FV1000 confocal microscope with a 60× oil-immersion objective and FV10-ASW software (Olympus).

2.6. Immunohistochemistry

25 formalin-fixed paraffin-embedded ovarian carcinoma samples were examined (see Table 2A for the distribution of the samples according to histological subtype). Sections were cut, antigens were retrieved, and samples were probed

Table 2
(A) Subtypes of ovarian carcinoma analysed by immunohistochemistry. (B). AGR3 and ERα expression patterns. Twenty five formalin-fixed paraffin-embedded sections were probed with the AGR3 monoclonal antibody MAGR3-2 and an antibody to ERα. Staining for ERα was scored as previously described (Hrstka et al., 2010).

Histological type of ovarian carcinoma	Count
Serous papillary	12
Endometrioid	2
Mucinous	4
Clear cell	7
Total	25

Histological type	AGR3 expression	AGR3 expression levels	ERα expression
Mucinous	Positive	100%	Negative
Mucinous	Positive	100%	Negative
Mucinous	Positive	100%	Negative
Mucinous	Positive	100%	Negative
Mucinous	Positive	100%	Negative
Mucinous	Positive	100%	Negative
Mucinous	Positive	100%	Negative
Serous papillary	Positive	50%	2+ Focal
Serous papillary	Positive	50%	Negative
Serous papillary	Positive	20%	2+ Diffuse
Endometrioid	Positive	20%	1+ Focal
Clear cell	Positive	10%	2+ Focal
Serous papillary	Positive	10%	Positive
Serous papillary	Positive	10%	1+ Focal
Clear cell	Positive	5%	Negative
Serous papillary	Positive	5%	Negative
Serous papillary	Positive	3%	Positive
Serous papillary	Positive	1%	Positive
Clear cell	Positive	1%	Negative
Serous papillary	Positive	1%	Negative
Serous papillary	Positive	1%	1+ Focal
Serous papillary	Negative	1%	1+ Focal
Serous papillary	Negative	0%	Negative
Clear cell	Negative	0%	Negative
Endometrioid	Negative	0%	2+ Focal
Total	21 Positive		11 Positive

with primary antibodies followed by biotinylated secondary antibodies conjugated to streptavidin–HRP. Sections were reacted with DAB (Dako), counterstained with haematoxylin and mounted. Samples were viewed using a 200× objective as previously described (Nenutil et al., 2005).

2.7. Quantitative RT-PCR

A panel of 18 ovarian carcinoma samples was examined (see Table 3 for the distribution of the ovarian cancer samples according to histological subtype). Tissue was retrieved from liquid nitrogen, dismembranated and RNA extracted using the Absolutely RNA RT-PCR Miniprep kit (Stratagene). Reverse transcription was performed using the Invitrogen Cloned AMV First-strand cDNA synthesis kit (1 μg RNA per reaction). Quantitative PCR was then performed using the Qiagen QuantiTect Sybr Green PCR kit (SYBR Green I dye, ROX passive reference dye, HotStarTaq DNA polymerase, and a dNTP mix in an optimised buffer), along with template cDNA (2 μl in a 10 μl reaction) and primers for AGR3 (forward 5′-CATCACCTGGAGGATTGTCAATAC-3′ and reverse 5′-TGAACT TATTCTGAGCCATTTCTTGT-3′) and for β-actin housekeeping control (forward 5′-CTACGTCGCCCTGGACTTCGAGC-3′ and reverse 5′-GATGGAGCCGCCGATCCACACGG-3′). Samples were run on a Rotorgene RG3000 machine and analysed with Rotorgene 6 software (Qiagen).

Table 3
Subtypes of ovarian carcinoma analysed by quantitative RT-PCR.

Histological type of ovarian carcinoma	Count
Serous papillary	5
Endometrioid	5
Mucinous	4
Clear cell	4
Total	18

3. Results

3.1. Generation of a panel of novel monoclonal antibodies to AGR3 that do not cross-react with AGR2

We set out to develop assays to define the expression patterns of AGR3 protein in clinical archival samples. Current polyclonal antibodies generated to AGR3 cross react with AGR2 (data not shown) and a polyclonal antibody generated to AGR2 cross-reacts with AGR3 (data now shown; see Fig. 2E). As such, monoclonal antibodies were generated that are AGR3-specific. Recombinant human AGR3 protein was overproduced in *Escherichia coli* using the pDEST17 *Gateway* expression systems (Fig. 1D) and the purified his-tagged protein was used to immunise mice for the generation of a panel of monoclonal antibodies. A panel of three distinct hybridoma cell lines were generated that express antibody (IgG, data not shown) recognising AGR3 protein in preliminary screens (data not shown). Further characterisation revealed that two of the three monoclonal antibodies were AGR3-specific using full-length protein in an ELISA (Fig. 1A and B, the monoclonal antibodies were named MAGR3-1 and MAGR3-2) and one of the monoclonal antibodies cross-reacted with full-length AGR2 (Fig. 1C, named MAGR3-4).

The monoclonal antibodies MAGR3-1 and MAGR3-2 could not be used to immunoprecipitate AGR3 protein from soluble cell lysate (data not shown), suggesting the epitopes require denaturation or require dissociation from an AGR3-binding protein prior to detection. However, one of the monoclonals (MAGR3-1) could be used to detect endogenous AGR3 protein by Western blotting of MCF7 cells lysates using a denaturing SDS polyacrylamide gel (Fig. 1Ei; exposure time using enhanced chemiluminescence 5 s). MAGR3-2 also detected AGR3 protein in an immunoblot but to a much weaker extent (Fig. 1Eiii; exposure time using enhanced chemiluminescence 2 min). Thus, MAGR3-1 has an apparently higher affinity than MAGR3-2 for endogenous AGR3 (Fig. 1E). MAGR3-1 antibody could also be used to detect endogenous AGR3 protein in the cytosolic and membrane fractions (F1 and F2) after subcellular fraction of MCF7 cells into cytosol (F1), membrane and organelles (F2), nucleus (F3), and insoluble cytoskeleton (F4) (Fig. 1F).

3.2. Epitope mapping of the monoclonal antibodies

We next set out to define the epitopes of these monoclonal antibodies in order to define the mechanism of specificity

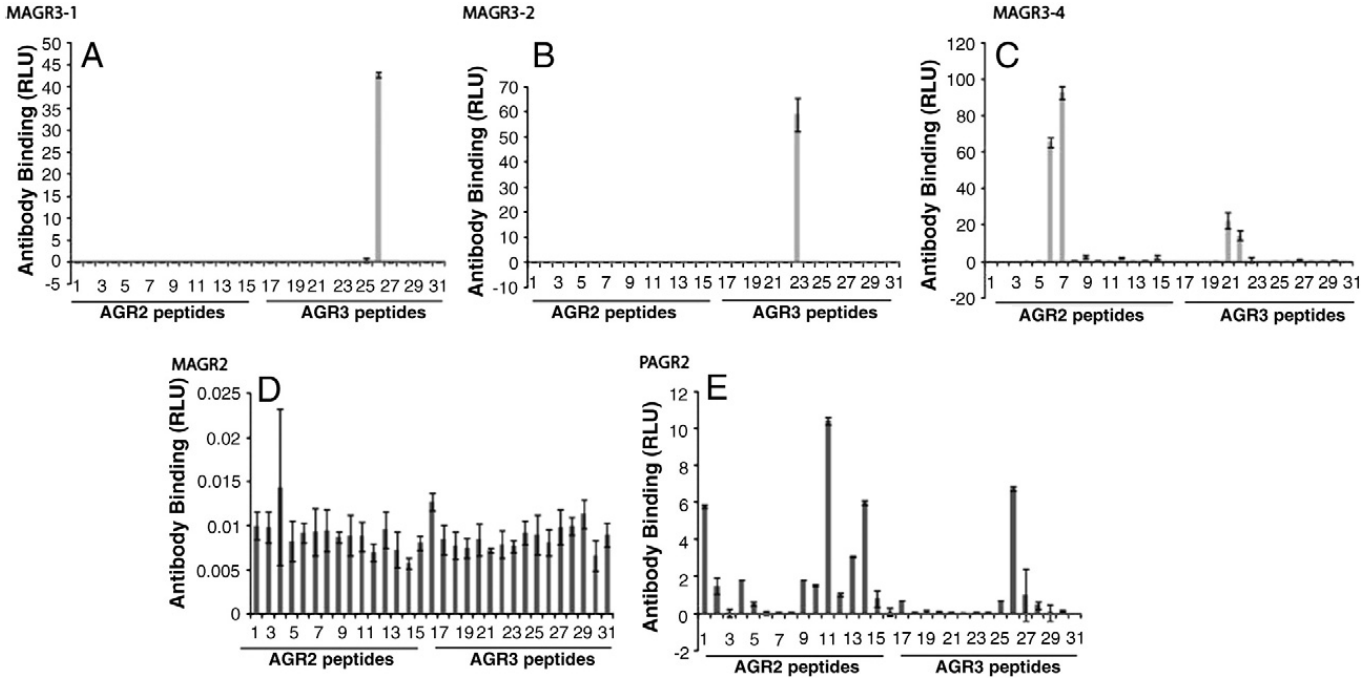

Fig. 2. Epitope mapping of monoclonal antibodies using a pepscan. Pepscans comprised of overlapping biotinylated 15-amino acid peptides derived from the sequence of AGR2 (peptides 1–16) and AGR3 (peptides 17–31) (see Table 1) were used to define the epitope of the monoclonal antibodies (A) MAGR3-1; (B) MAGR3-2; (C) MAGR3-4; (D) a commercially available AGR2-specific monoclonal antibody; and (E) a polyclonal antibody generated in rabbits to recombinant AGR2 protein. The biotinylated peptides were captured onto streptavidin microtitre wells and the indicated monoclonal antibody were incubated to define epitope specificity. Data is plotted as relative binding of each monoclonal antibody (in relative light units) as a function of peptide motif.

of MAGR3-1 and MAGR3-2, as well as cross-binding of MAGR3-4 to both AGR2 and AGR3 (Fig. 1). Pepscans comprised of overlapping biotinylated 15-amino acid peptides derived from the open reading frames of the AGR2 (peptides 1–16) and AGR3 (peptides 17–31) protein sequence (see Table 1) were used to define the epitope of the three monoclonal antibodies. The biotinylated peptides were captured onto streptavidin coated microtitre plates and the indicated monoclonal antibodies were incubated to measure relative binding to the overlapping peptides. Such a method will not necessarily work well if the epitope is discontinuous. The two AGR3-specific monoclonal antibodies (Fig. 2A and B) had their epitopes confined to a single linear peptide motif (peptide 26 for MAGR3-1 and peptide 23 for MAGR3-2), which is summarised in Fig. 3A. As expected, MAGR-3-4 cross-reacted with AGR2 peptides (peptides 6 and 7; Fig. 2C and summarised in Fig. 3A) and this latter monoclonal antibody could also immunoprecipitate AGR2 protein from cell lysates (data not shown).

Phage peptide library screening was next used to determine whether peptide consensus motifs could be defined for the monoclonal antibodies to determine whether independent evidence could be acquired to support epitope mapping using pepscans. MAGR3-1 gave rise to the generalised consensus phage peptide motif N(L/V)(S/T)PD (Fig. 3C), which has significant homology to the C-terminal location of the MAGR3-1 epitope defined using pep-scans (Fig. 3A). MAGR3-4 gave rise to the generalised consensus phage peptide motif P(L/M) I I (Fig. 3C), which has significant homology the location of the MAGR3-4 epitope defined using pepscans (Fig. 3A). MAGR3-2 did not yield a linear homology epitope (data not shown) that suggests it may have a larger discontinuous epitope that is not captured using a 12-mer peptide library. These phage-peptide screening data indicate that the pepscan accurately defined specificity of the antibodies MAGR3-1 and MAGR3-4 (summarised in Fig. 3). In addition, we do not think MAGR3-1 and MAGR3-2 cross-react with AGR2, ERP18, TRX1, and TRX2, since the epitopes are quite divergent within these orthologues (Fig. 3B).

3.3. Intracellular expression of AGR3 evaluated using confocal microscopy

As a key aim of this approach was to generate monoclonal antibodies that could be used to access AGR3 protein expression in immunohistochemistry of clinical cancer samples, we first evaluated whether AGR3 protein could be detected in cell lines using immunofluorescence. MAGR3-1 could detect using confocal microscopy AGR3 protein expression in a cytosolic location (Fig. 4Bi and ii; in green). However, only localised islands or foci of AGR3 protein co-localised with the endoplasmic reticulum marker protein PDI (Fig. 4Bi, see white arrows and D) and co-localization with Golgi biomarker protein Golgin (Fig. 4Bii) or the mitochondria biomarker Mitotracker (data not shown) were not observed. This is in contrast to AGR2 protein expression that shows a large majority of AGR2 protein co-localising with the PDI endoplasmic reticulum marker (Fig. 4Ai; yellow/orange fluorescent signal). Similar to MAGR3-1, the MAGR3-2 antibody could detect AGR3 protein in a cytosolic location (Fig. 4Ci and ii). However, co-localization was not as evident with the endoplasmic reticulum (Fig. 4Ci), nor with Golgi (Fig. 4Cii), nor with mitochondria (data not shown).

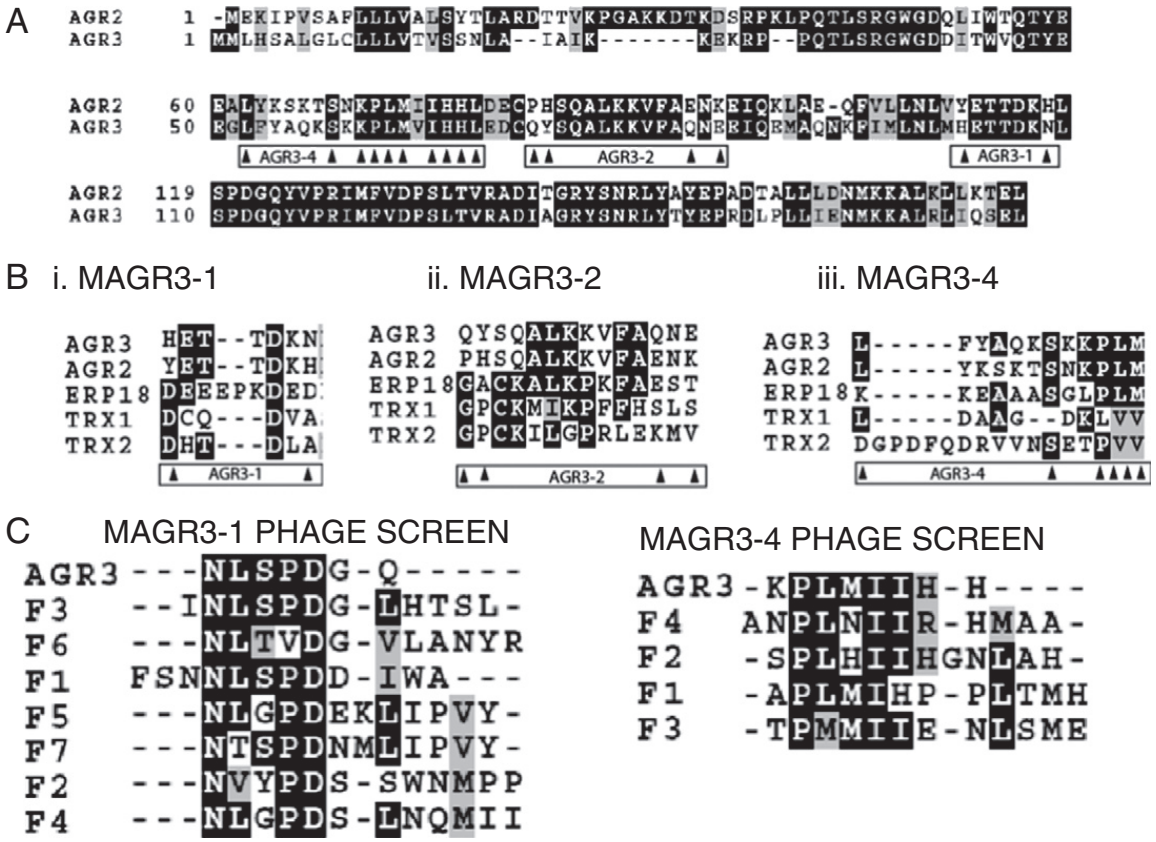

Fig. 3. (A) Epitope map of the AGR3-specific antibodies. (A) Based on the peptide binding specificity of each monoclonal antibody (in Fig. 2) and the divergence in amino acid sequence between AGR2 and AGR3 proteins (as indicated), the amino acid contacts that drive binding were defined. The arrows highlight amino acids that are presumably required to define specificity towards AGR3 protein for MAGR3-1 and MAGR3-2, whilst the arrows for MAGR3-4 highlight the amino acids shared between AGR2 and AGR3 that explain the cross-reaction of this monoclonal antibody for AGR2 and AGR3. (B) AGR3 monoclonal antibody epitopes do not appear to mimic those of closely related orthologues. The specific residues identified in the interaction of MAGR3-1 and MAGR3-2 with the substrate show little similarity amongst the five orthologues in this protein family. However, the monoclonal antibody AGR3-4 that recognises both AGR2 and AGR3 proteins recognises several amino acid residues conserved within the related proteins, ERP18, TRX1, and TRX2. (C) Summary of phage-peptides selected using MAGR3-1 and MAGR3-4. Linear epitopes could be defined that give rise to the consensus sequences in (i) MAGR3-1 and (ii) MAGR3-4. The consensus peptide motifs localise to the C-terminal region of the predicted epitope for the AGR3-specific monoclonal antibody MAGR3-1 and to the central region of the epitope defined for AGR3/AGR2 reacting monoclonal antibody AGR3.4. The inability to select peptide consensus motifs for MAGR3-2 might be due to the possibility that it harbours a discontinuous epitope that is longer than the 12-mer peptides used for the library screen.

AGR2 harbours a classical N-terminal endoplasmic reticulum signal peptide (Zhang and Henzel, 2004) and an atypical endoplasmic reticulum-retention motif (KTEL) normally required for endoplasmic reticulum localization (Raykhel et al., 2007a). Further, deletion of the KTEL motif on AGR2 prevents endoplasmic reticulum co-localization and mutagenesis of the KTEL to the optimal KDEL increases the extent of endoplasmic reticulum localization of AGR2 protein (Fourtouna et al., 2009). Thus, although endogenous or ectopically expressed AGR2 protein localises to a large extent with the endoplasmic reticulum, consistent with predictions, endogenous AGR3 protein appears to be largely diverted away from this compartment, at least with the endoplasmic reticulum biomarker protein PDI (Fig. 4). AGR3 also has a non-optimal endoplasmic reticulum retention sequence at the C-terminus; QSEL (Persson et al., 2005) and a N-terminal endoplasmic reticulum signal peptide (Zhang and Henzel, 2004). When the signal peptide of AGR3 is fused to a heterologous fusion carrier, then the fusion can be found secreted (Zhang and Henzel, 2004). Further, an AGR3-GFP fusion can be found localised to the endoplasmic reticulum (Raykhel et al., 2007b). These methods suggest that AGR3 should be predominantly endoplasmic reticulum localised and we did see some (although not predominant) co-localization of AGR3 with the PDI biomarker (Fig. 4). As endogenous AGR3 protein was identified in the membranes of breast cancer cells (Adam et al., 2003). It is therefore likely that at least one function of the AGR2 and AGR3 proteins is to act as endoplasmic reticulum-resident or membrane bound PDIs that regulate protein folding. Further, these data (Fig. 4) affirm that AGR3-specific reagents could be developed and that MAGR3-1 is the most robust tool for examining AGR3 protein production in cell lines and clinical models (see below).

3.4. AGR3 protein is expressed in four distinct ovarian cancer subtypes and is not linked to oestrogen receptor expression

After screening a range of cancer types (data not shown) for AGR3 protein expression using the panel of monoclonal antibodies developed above, we also found considerable expression of AGR3 in various sub-types of ovarian cancer.

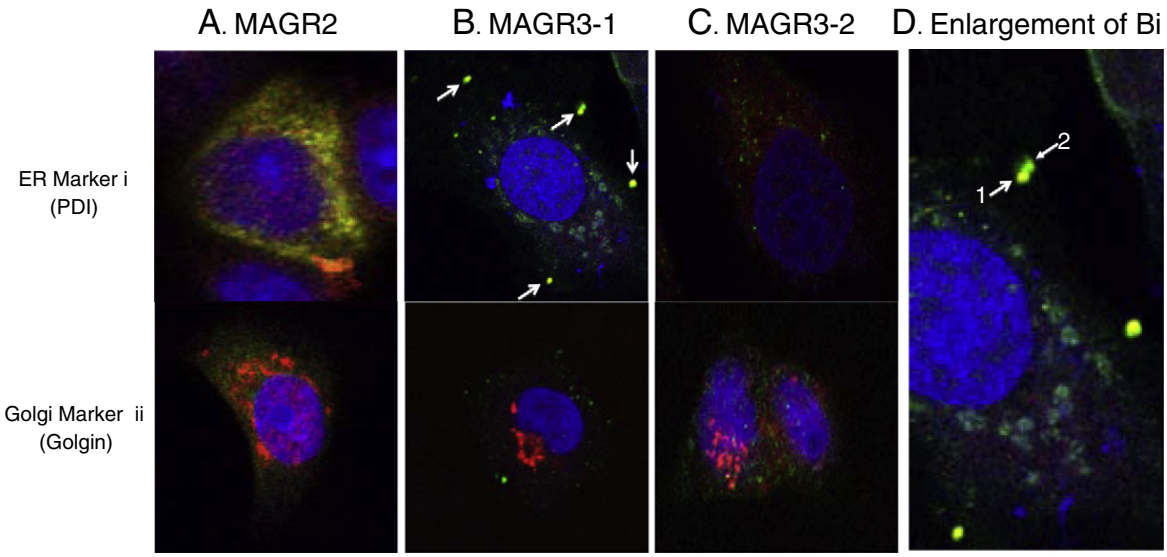

Fig. 4. Examination of AGR2 and AGR3 co-localization with endoplasmic reticulum and Golgi biomarker proteins. MCF7 cell lysates that express both AGR2 and AGR3 (data not shown) were examined for the subcellular expression of the indicated proteins. Cells were fixed, permeabilised and probed with the AGR3 monoclonal antibodies (in green); (A) AGR2; (B) MAGR3-1; and (C) MAGR3-2 along with a subcellular location markers (in red) highlighting endoplasmic reticulum (PDI) and Golgi apparatus (Golgin). TO-PRO-3 (shown in blue) was used as a nuclear counter stain. (D) Expansion of Bi; arrow 1 highlights co-localization of AGR3 protein with PDI and arrow 2 highlights AGR3 protein not co-localised with PDI.

Four subtypes of ovarian carcinoma were analysed (serous papillary, endometrioid, mucinous, and clear cell), which produced varying expressions of AGR3 protein from 0 to 100% (Table 2A and B). Each of these cancer types can differ in aetiology, incidence, responsiveness, and aggressiveness. AGR3 protein expression was generally cytosolic and granular, but two basic staining patterns were observed: (i) either "focal and patchy" with expression detectable in 0 to 50% of the tumour cells (Fig. 5A and Table 2B) or (ii) "diffuse" with expression detectable in 100% of the tumour cells in a field (Fig. 5B and Table 2B). These data suggest the existence of signalling pathways that differentially regulate AGR3 protein induction and/or intracellular localization *in vivo*.

During the course of our studies, a paper was published showing that AGR3 mRNA is expressed in serous ovarian cancers (King et al., 2011) and represented the first transcriptomics screen to identify aberrant regulation of the *AGR3* gene.

However, our current study differs first in that AGR3 mRNA and protein expression (and AGR2 expression — data not shown) was observed in 100% of mucinous ovarian cancer cells relative to adjacent tissue (Table 2B). Quantitative RT-PCR affirmed that *AGR3* mRNA levels are high in mucinous-type ovarian cancer (Fig. 5C and Table 3), suggesting that *AGR3* gene expression is the rate-limiting stage in AGR3 protein production. In addition, in the three other ovarian carcinoma subtypes, including serous cancer, AGR3 expression was heterogeneous, with expression detectable in 0 to 50% of the cancer cells (Table 2B).

Although AGR3 was previously shown to be linked to oestrogen receptor expression in breast cancers (Fletcher et al., 2003), the recent study showing *AGR3* mRNA is elevated in serous ovarian cancer (King et al., 2011) did not evaluate linkages to oestrogen receptor-α expression. In our studies using control immunostaining with antibodies to oestrogen receptor-α, we observed that all 7 mucinous carcinomas were oestrogen receptor-α negative, 8 of 12 serous carcinomas were oestrogen receptor-α positive, both endometrioid carcinomas were oestrogen receptor-α positive, and 1 of 4 clear cell carcinomas were also oestrogen receptor-α positive (Table 2B). Thus, there was no relationship between oestrogen receptor-α and AGR3 expression (Table 2B). This indicates that signalling pathways driving AGR3 protein expression in ovarian cancers can be distinct from those mediating AGR3 protein expression in oestrogen-receptor positive breast cancers.

In conclusion, there are two distinct staining patterns of AGR3 protein in ovarian carcinomas: a heterogeneous focal/patchy staining, not related to oestrogen receptor-α and/or AGR2 protein expression. Future *in vitro* cell-based studies will be required to determine whether AGR3 protein can be in fact induced independent of AGR2 protein. This staining pattern in tissue is present in the normal epithelium of the uterine tube and in non-mucinous ovarian carcinomas (serous, endometrioid and clear cell). The second pattern of AGR3 protein expression is a homogeneous diffuse staining independent of oestrogen receptor-α expression, but parallel to AGR2 staining, present in mucinous ovarian carcinomas. Our data indicates that AGR3 may act as a novel biomarker for ovarian cancer, in particular the endometrioid, clear cell, and mucinous subtype. This complements a recent study showing that *AGR3* mRNA is elevated in serous ovarian cancers (King et al., 2011). In addition, this study identifies four biologically distinct ovarian cancer subtypes as a physiological model for evaluating the functions of the AGR3 signalling pathway.

3.5. AGR3 can mediate drug resistance in xenografts

The AGR2 protein can play diverse roles in biology including limb regeneration, mucin secretion, cell migration and

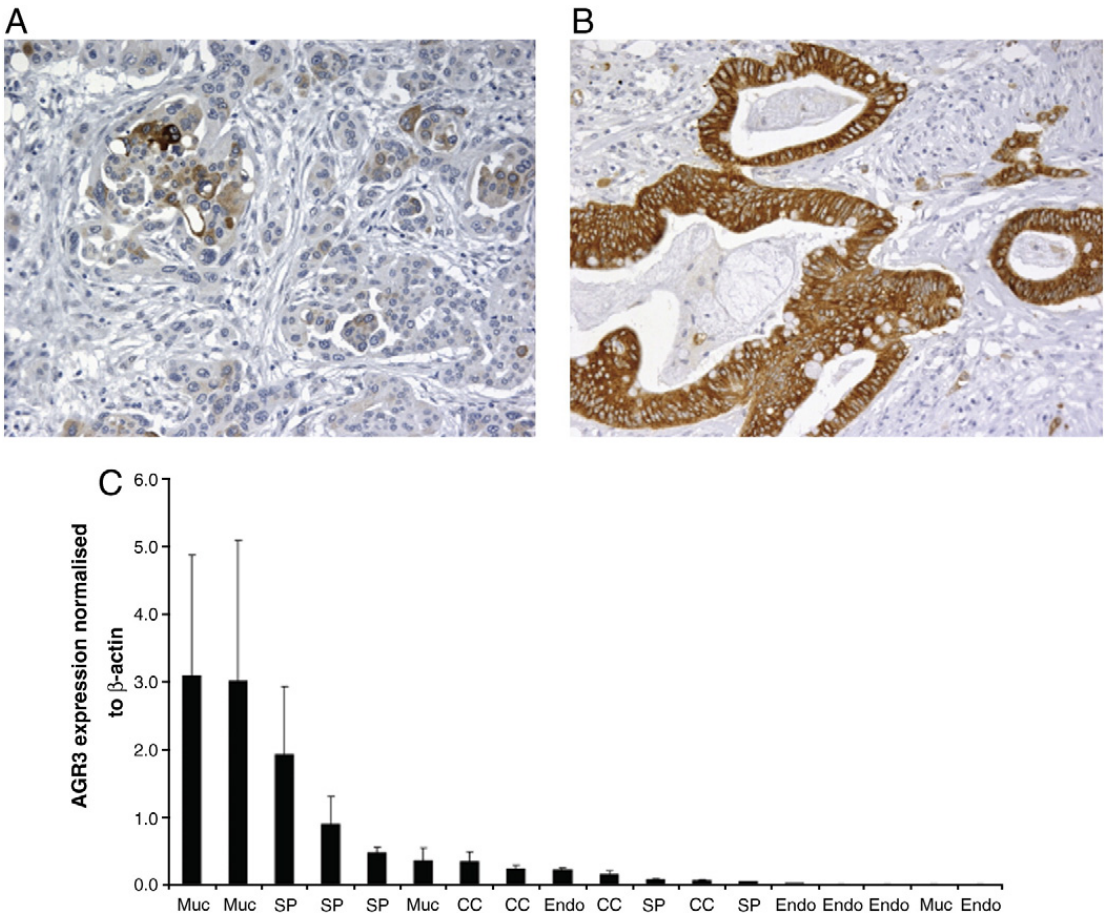

Fig. 5. AGR3 protein expression in ovarian cancer. (A and B) Formalin-fixed paraffin-embedded sections were probed with the AGR3 monoclonal antibody MAGR3-1. Representative heterogeneous focal/patchy cytoplasmic staining present in the normal epithelium of the uterine tube and in non-mucinous ovarian carcinomas (A), and homogeneous diffuse staining present in mucinous ovarian carcinomas (B). See Table 2 for summary. (C) AGR3 mRNA levels were determined in a panel of 18 ovarian carcinomas by quantitative RT-PCR relative to a β-actin control. Abbreviations: Mucinous (Muc), serous papillary (SP), clear cell (CC) and endometrioid (Endo).

transformation, and inhibition of the p53 tumour suppressor, but there is no known biological function for AGR3. As platinum analogues are often used for the treatment of mucinous ovarian cancer, where cisplatin resistance is often observed (Sato et al., 2009), we evaluated whether the *AGR3* gene could pay a role in cisplatin resistance in a model experimental system. The *AGR3* gene and vector controls were transfected in H1299 (AGR3-negative) cells to generate an AGR3+ and AGR3− isogenic cell panel. The cells were grown in mouse xenograft systems and then the cancers were treated with CDDP as previously published (Macleod et al., 2005). Although reduction in the rate of tumour growth was observed by standard treatments in control cells (Fig. 6A), cells that over-produce AGR3 were resistant and continued to grow in the presence of the drug (Fig. 6B). These are the first studies showing that the *AGR3* gene can mediate drug resistance in an experimental cancer model and suggest that AGR3 is pro-oncogenic.

4. Discussion

Transcriptomic and proteomic approaches in the past ten years using human tissue biopsies have identified novel and clinically-relevant protein families involved in cancer that have not been identified from cancer genetic screens. AGR2 is a prototype case as it was identified using various transcriptomic and proteomic screens in cancer models. For example, proteomic screens identified AGR2 in an oesophageal cancer/p53 inhibition screen using clinically derived metaplastic oesophageal biopsies (Pohler et al., 2004); data independent shotgun mass spectrometric screens identified AGR2 protein as a dominant Tamoxifen-agonist in MCF7 breast cancer cell lines (Hengel et al., accepted for publication); and the first paper using shotgun proteomics on Tamoxifen-resistant and sensitive human cancer biopsies using accurate mass and time (AMT) tag approach identified AGR2 as a dominantly expressed protein in resistance (Umar et al., 2009). By comparison, transcriptomic screens using cell models aimed at identifying pro-migratory/metastatic genes implicated in cancer resistance identified *AGR2* as a dominant outlier (Missiaglia et al., 2004). AGR2 is now known to mediate cell migration in pancreatic and breast cancer models (Liu et al., 2005; Ramachandran et al., 2008), thus demonstrating a novel biological function for AGR2. Cell biology studies have supported these conclusions as transfection of the *AGR2* gene into cells can promote clonogenic cell survival, cell transformation, drug-resistance, and cell migration in a p53-independent manner (Pohler et al., 2004; Ramachandran et al., 2008; Wang et al., 2008; Hengel et al., accepted for publication). Clinical studies have also been initiated to determine whether there is any clinical significance in AGR2 expression. One study

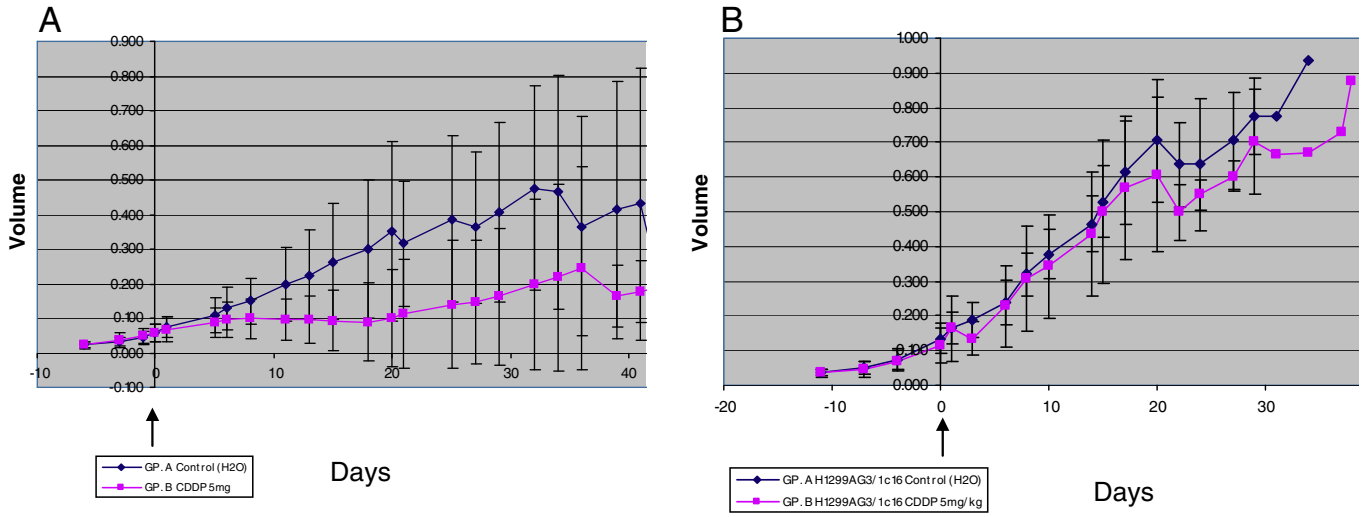

Fig. 6. The *AGR3* gene mediates resistance of Xenograft H1299 cells to cisplatin administration. Transfected isogenic H1299 cells (AGR3-negative or positive; (5×10^6 cells/implant)) were injected subcutaneously into nude mice to establish the xenograft lines. Tumour fragments were implanted subcutaneously into both flanks of nude mice and allowed to grow to 4–6 mm in diameter and then animals were allocated to cisplatin treatment (5 mg/kg; i.p.) or control (5 mice/group) groups and treatment was commenced (defined as day 0). Cisplatin (CDDP) was administered on days 0 and 7. Groups contained 8–10 tumours. Tumour size was measured twice weekly using callipers and the volume calculated according to the formula $\pi/6\times\text{length}\times\text{width}^2$. Relative tumour volumes (%) were then calculated for each individual tumour by dividing the tumour volume on day t (V_t) by the tumour volume on day 0 (V_0) and multiplying by 100. The raw data are plotted as growth rate (tumour volume) as a function of days of treatment.

demonstrated that AGR2 expression shows no relation to prognosis in prostate cancer (Kristiansen et al., 2005) in contrast to another study showing the opposite (Zhang et al., 2005). Similarly in breast cancers, AGR2 expression may be linked to decreased survival (Innes et al., 2006; Barraclough et al., 2009; Hrstka et al., 2010) and AGR2 expression predicts poor responses to Tamoxifen in breast cancer patients (Hrstka et al., 2010). These data together indicate that AGR2 can function as a pro-oncogenic effector protein with implications for cancer resistance.

Protein interaction screens have also shed potentially new light on the role of AGR2 linked to a cell migratory property. A yeast two hybrid screen was initiated using an receptor (named PROD1) involved in limb regeneration in the newt and this screen identified the newt homologue of AGR2, nAG2, as a PROD1-interacting protein that can promote limb regeneration after nerve severage (Kumar et al., 2007). These data have been interpreted to suggest that AGR2 protein is rate-limiting for limb regeneration in this model and suggests a reason, in part, why the *AGR2* gene may have evolved to function in vertebrates. However, the PROD1 protein has no significant homologue in mammals so the interaction between AGR2 and PROD1 remains unclear in human disease. A yeast two-hybrid screen using human AGR2 as a bait has also identified metastasis-associated GPI-anchored C4.4a protein and extracellular alpha-dystroglycan (DAG-1) as putative binding partners of both AGR2 and AGR3 (Fletcher et al., 2003). Another yeast two-hybrid screen using human AGR2 identified the pro-metastatic protein Reptin as an interacting protein at a divergent "substrate binding" loop in the AGR2 protein (Maslon et al., 2010). Together, these data highlight a series of protein–protein interactions that could mediate the cell invasion properties of the protein.

In addition to these yeast two hybrid screens, protein maturation functions for AGR2 protein were identified by characterising AGR2 knock-out mice. Mice defective in AGR2 exhibit reduced production of mucin-2 and enhanced sensitivity to stress-induced intestinal colitis (Park et al., 2009). Validation experiments demonstrated that transfected AGR2 could form a covalent cysteine-adduct with the transfected N-terminal domain of Mucin-2 (Park et al., 2009), thus suggesting that AGR2 can mediate directly the maturation of Mucin-2 in cells. Phage-peptide libraries have also been used to evaluate whether novel protein–protein consensus binding motifs can be identified for AGR2, as occurs for some molecular chaperones (Murray et al., 2007). Indeed, AGR2 exhibits a selective binding activity for pentapeptides containing the core (S/T)xI(Y/F)(Y/F) motif (Murray et al., 2007), suggesting that proteins harbouring the pentapeptide consensus site would be AGR2 binding proteins and could perturb AGR2 functions in cells (Fourtouna et al., 2009). Validated AGR3 protein interactors have not been published to our knowledge and these various protein–protein interactions of AGR2 also remain to be well validated in cell, tissue, and disease systems.

It is curious that the many OMICS screens identified predominantly AGR2, and not AGR3, as the predominant factor in disease progression. For example, a transcriptomic screen searching for genes implicated in the response of breast cancer patients to Letrozole: AGR2 was one of the most significantly down-regulated genes in patients with good responses, but AGR3 was not detected as an outlier in this cohort (Mackay et al., 2007). Related to this, AGR2 protein and not AGR3 protein was the 2nd most highly induced protein in Tamoxifen treated breast cancer cells (Hengel et al., accepted for publication). There are however two OMICs studies identifying AGR3, and not AGR2, in disease. One OMICs screen that identified AGR3 was a proteomics screen, searching for novel membrane proteins potentially involved in breast cancer, starting from insoluble cancer cell membrane fractions (Adam et al., 2003). Follow-up studies confirmed that AGR3

expression is enhanced in breast cancer in association with ERα positivity (Fletcher et al., 2003). A recent study also identified the first time using transcriptomics that *AGR3* mRNA is elevated in serous ovarian cancers (King et al., 2011).

In our current report, we have developed monoclonal antibodies that can discriminate AGR3 from AGR2 and used them to screen cancer archives for tumours that express AGR3 protein. The most striking and novel clinical expression was in ovarian cancer subtypes where two distinct expression patterns were observed. This includes: heterogeneous expression in non-mucinous subsets of ovarian carcinoma, and homogeneous expression in mucinous types of ovarian cancer that are oestrogen-receptor α-negative. At this superficial level, these data indicate that although breast cancers may exploit oestrogen-receptor-α to drive AGR3 production, ovarian cancers have an oestrogen-receptor-α-independent mechanism to drive AGR3 protein expression. As AGR2 was included as a control and was found co-expressed with AGR3 in 100% of mucinous ovarian carcinomas (Table 2B), these data further indicate a cancer type where oestrogen-receptor-α-independent expression of AGR2 and AGR3 proteins can both occur. The AGR3 expression noted within the mucinous carcinomas may reflect an independent role for the protein in the production of secreted proteins, similar to the recently documented function for AGR2 as a PDI essential for processing MUC2 in the mouse intestine (Park et al., 2009).

It would be interesting to investigate whether the different expression patterns of AGR3 and AGR2 in mucinous and non-mucinous (serous, endometrioid and clear cell) ovarian carcinoma play a role in patient prognosis. Indeed, distinct pathogenetic pathways are believed to be involved in the differentiation of the subsets of ovarian carcinoma from ovarian epithelial cells. For example, mutations in *KRAS* or *BRAF* are often found in low-grade serous carcinoma; mutations in β-catenin (*CTNNB1*), phosphatase and tensin homolog (*PTEN*) and phosphatidyl inositol 3-kinase catalytic subunit-α (*PIK3CA*) are frequently detected in low-grade endometrioid carcinoma; mutations in *KRAS* are most common in mucinous carcinoma, with some cases of *p53* mutation; and mutations in *PTEN* and/or *PIK3CA* are frequent in clear cell carcinoma (Bast et al., 2009). *p53* mutation is common in high-grade serous or endometrioid carcinoma, along with marked genomic instability, and some *BRCA1* dysfunction.

Despite their heterogeneity, clinical management of all ovarian carcinoma subtypes is standardised and consists of a combination of radical surgery and chemotherapy with the cisplatin analogue carboplatin, either alone or in combination with paclitaxel. Further knowledge of the chain of molecular events involved in the differentiation of the ovarian cancer subsets should ultimately enable the development of targeted therapies. Patients with advanced mucinous ovarian cancer have a lower response to first-line chemotherapy compared to patients with other histologic subtypes, and drug resistance is therefore thought to be one of the main causes for the poorer prognosis of patients with mucinous ovarian cancer (Hess et al., 2004; Pignata et al., 2008). As such, it is interesting to note that we have identified a function for AGR3 in mediating resistance to the inhibitory effects of the classic DNA-damaging agent cisplatin (Fig. 6). This indicates that AGR3 could represent a novel pathway with which to target drug resistance ovarian cancers. If this is confirmed by future studies, then the AGR3 pathway might represent an attractive target for sensitising certain cancer cell types to conventional chemotherapeutic agents.

In conclusion, the expression of AGR3 in four distinct types of ovarian cancer implicates *AGR3* as a novel clinically relevant gene that could be used for diagnostic and/or therapeutic purposes. In this study we describe the generation of monoclonal antibodies to AGR3 that could be utilised as tools for such diagnostic analyses. In addition, our results indicate that ovarian cancers represent a useful clinical model for evaluating the role of AGR3 in human cancer and in drug resistance.

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Differential expression of anterior gradient protein 3 in intrahepatic cholangiocarcinoma and hepatocellular carcinoma

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ABSTRACT

Intrahepatic cholangiocarcinoma (ICC) is the second most common primary liver cancer next to hepatocellular carcinoma (HCC). Despite the significant difference of the therapeutic strategy for both diseases, their histological appearance may be very similar. Thus the correct diagnosis is crucial for treatment choice but is often difficult to achieve. The aim of our study was to evaluate anterior gradient 3 (AGR3) as a new diagnostic marker helping to distinguish between ICC and HCC. AGR3 is a putative transmembrane protein implicated in breast, prostate and ovary tumorigenesis and belongs to the family of protein disulfide isomerases.

Since there is little information on how AGR3 is expressed in normal and diseased tissues and what its exact function is, we analyzed its expression pattern in normal liver and tumor tissue of ICC and HCC. The immunohistochemical analysis in normal tissue revealed specific AGR3 expression in intrahepatic bile duct cholangiocytes which was not present in liver hepatocytes. Consequently we analyzed AGR3 expression in 74 representative samples of puncture biopsies, tissue excisions and resection specimens from which 48 samples were diagnosed as HCC and 26 as ICC. Our results showed AGR3 expression negative and weakly positive respectively in hepatocellular carcinomas compared to stronger AGR3 positivity in cholangiocellular carcinomas. AGR3 expression statistically significantly correlated to acid mucopolysaccharide expression and negatively correlated to glypican-3 expression. We conclude that according to receiver operating characteristics (ROC) analysis AGR3 expression is relatively specific for ICC and is potentially linked to mucosecretion, which may indicate potential implication in treatment resistance.

Introduction

Malignant liver tumors are frequent malignancies worldwide. In Europe and North America, viral hepatitis C and in Asia and Africa viral hepatitis B are major risk factors. Both infectious illnesses result in their chronic stage in cirrhosis which is present in more than half of hepatocellular carcinoma patients (El-Serag and Rudolph, 2007; Jemal et al., 2011). The majority of primary adult liver tumors are classified as either hepatocellular carcinoma (HCC) or intrahepatic cholangiocarcinoma (ICC). There also exists a rare subtype of primary liver cancer designated as combined hepatocellular and cholangiocarcinoma (cHCC-CC) (Bosman et al., 2010).

The therapeutic strategy and the prognosis for HCC and ICC are significantly different and therefore the distinction between these two diagnoses is essential. Common immunohistochemical markers used for differential diagnostics favoring HCC include hepatocyte paraffin antigen-1 (Hep Par 1), carcinoembryogenic antigen (CEA), CD10, alpha-fetoprotein (AFP), β-catenin, Hsp70, glutamine synthetase and glypican-3 (GPC-3) (Fatima et al., 2011; Kakar et al., 2007; Tremosini et al., 2012). However, each of these markers has limitations given by suboptimal sensitivity or difficulty of interpretation at certain conditions. Particularly nonstandard conditions arise in poorly differentiated tissue, atypical cases or in needle core biopsies where available tissue is limited and the positive expression of markers is commonly only focal.

During our work in analyzing the expression of anterior gradient 3 (AGR3), we found that it shows a restricted expression pattern in the liver, being expressed by intrahepatic bile duct cholangiocytes but not hepatocytes. We thus tested AGR3 as a potential new marker of ICC applicable in differential diagnostics.

Abbreviations: ICC, intrahepatic cholangiocarcinoma; HCC, hepatocellular carcinoma; AGR3, anterior gradient 3; AGR2, anterior gradient 2; GPC-3, glypican-3; ROC, receiver operating characteristics; AUC, area under curve.

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AGR3 is also known as HAG-3 or BCMP11 and it is a homologue of anterior gradient 2 (AGR2) protein. AGR2 is an endoplasmic reticulum chaperone that participates in mucin production in intestine and asthmatic lung (Park et al., 2009; Schroeder et al., 2012) and has been associated to different cancers. Elevated AGR2 expression has been detected especially in cancers derived from epithelial cells including breast, prostate, ovarian, lung, esophageal and pancreatic cancer and in some cases was shown to be hormone dependent (Brychtova et al., 2011; Hrstka et al., 2010; Zhang et al., 2005). AGR2 is specifically expressed in tall epithelial cells of normal liver and liver cancer where it potentially promotes secretory functions of mucins (Lepreux et al., 2011). AGR2 overexpression was found in majority of fibrolamellar carcinomas but only exceptionally in conventional hepatocellular carcinomas (Vivekanandan et al., 2009).

There are only limited data on AGR3 expression in normal and cancer tissue. Originally it has been identified as a membrane bound protein in breast cancer cell lines (Adam et al., 2003) and together with AGR2 the expression has been associated with estrogen receptor positive breast cancer (Fletcher et al., 2003). Recent data on ovarian cancer has shown that AGR3 expression can be also independent of estrogen signaling and is coupled to mucinous type of ovarian cancer (Gray et al., 2012).

In this study we aimed to examine AGR3 expression in healthy liver and hepatic cancer. We focused on the analysis of AGR3 expression pattern in ICC and HCC as well as mucosecretion and verified the possibility to use AGR3 as a diagnostic marker for distinction between liver tumor types compared to GPC-3.

Materials and methods

Study group and tissue specimens

The study group consisted of 74 patients: 26 were diagnosed with ICC and 48 patients with HCC. All patients underwent surgical procedure at the Faculty Hospital Brno, at the Masaryk Memorial Cancer Institute, or at St. Anne's University Hospital from 2005 to 2011. The cohort consisted of 52 males (70.3%) and 22 females (29.7%). Patient age at the time of diagnosis was within the range of 17–81 years (median 65 years). Tissue material included both diagnostic biopsies and surgical specimens. Tissues were fixed in 10% neutral buffered formalin for 24 h, routinely processed and then embedded in paraffin wax. Informed consent has been obtained from all patients involved in this study. The data used were anonymized and they were handled according to Czech Republic existing legislation.

Immunohistochemistry

Immunohistochemical staining was performed on 4 μm thick freshly cut tissue sections and the optimal antibody concentration and retrieval were set separately for each antibody used. Sections were deparaffinized in xylene and rehydrated into PBS through a graded ethanol series. Endogenous peroxidase activity was quenched in 3% hydrogen peroxide in PBS for 15 min. Antigen retrieval was performed in citrate buffer pH 6 at 94 °C for 20 min. For AGR3 immunodetection, the sections were incubated overnight at 4 °C with mouse monoclonal antibody to AGR3 (clone 1.2, in house; Gray et al., 2012) and for GPC-3 immunodetection, the sections were incubated overnight at 4 °C with mouse monoclonal antibody to GPC-3 (clone 1G12, Cell Marque, CA, USA). A streptavidin-biotin peroxidase detection system was used according to the manufacturers' instructions (Vectastain Ellite ABC Kit, Vector Laboratories, Burlingame, CA, USA). Signal was visualized by 3,3′-diaminobenzidine (Liquid DAB + Substrate Chromogen System, Dako, Glostrup, Denmark). Nuclear counterstaining was performed with Gill's hematoxylin.

Histochemistry

Neutral and acid polysaccharides were detected by PAS-Alcian Blue. Briefly, histochemical staining was performed on 4 μm thick freshly cut tissue sections that were deparaffinized in xylene and rehydrated into distilled water through a graded ethanol series. The sections were then stained with Alcian blue in acetic acid at pH 2.5, washed with distilled water, treated with 0.6% periodic acid, washed with distilled water and stained with Shiff's reagent. Nuclear counterstaining was performed with Gill's hematoxylin.

Evaluation of immunostaining and PAS-Alcian Blue staining

For immunohistochemical evaluation, 5 conventional categories according to the number of positive cells were assessed: 0 — negative (less than 1% of positive cells); 1 — border (1–5% of positive cells); 2 — weakly positive (5–25% of positive cells); 3 — moderately positive (25–50% of positive cells) and 4 — strongly positive (more than 50% of positive cells). For PAS-Alcian Blue staining evaluation, two categories were considered: 0 — negative (less than 1% of positive cells) and 1 — positive (more than 1% of positive cells).

Statistical analysis

All statistical analyses were performed using STATISTICA Version 10 (StatSoft, Inc., Tulsa, OK, USA). The associations of AGR3 and acid mucopolysaccharides expression with selected clinico-pathological features were analyzed using Pearson's chi-squared test. Differences at $p \leq 0.05$ were considered to be statistically significant.

Results

The study group comprised 26 patients diagnosed with ICC and 48 with HCC. Regarding histologic grade, the cohort encompassed 5 (6.7%) G1 tumors, 39 (52.0%) G2 tumors, 15 (20.0%) G3 tumors and 16 cases (21.3%) with unknown grade (mostly comprising the needle biopsies due to their limited size). According to the tumor size, the study group included 7 (9.3%) cases falling into T1 group, 13 (17.3%) cases of T2 group, 21 (28.0%) cases of T3 group, 4 (5.4%) cases of T4 group and the rest 30 (40.0%) cases were of unknown T status (encompassing some of the diagnostic biopsies without further available pathological or clinical data). Considering nodal involvement, 31 (41.3%) patients did not have any affected lymph nodes, 11 (14.7%) had lymph node metastases and in 33 (44.0%) patients the data were absent. Distant metastases were not detected in 37 (49.3%) patients while 16 (21.4%) patients presented distant metastases and in 22 (29.3%) patients the data describing distant metastasis involvement were unavailable.

From demographical factors, only sex showed significant role in HCC incidence ($p = 0.00152$; Pearson's chi-squared test). The distribution of hepatocellular carcinoma between males and females was uneven with males suffering more often from hepatocellular carcinoma than females. There was no similar association in ICC. Neither tumor extent nor nodal and distant metastasis involvement showed any significant association with clinicopathological characteristics.

Immunohistochemical analysis showed the expression of AGR3 protein in tissue samples of healthy liver and liver neoplasm. In normal healthy liver tissue AGR3 was expressed in cholangiocytes, while hepatocytes were AGR3 negative (Fig. 1).

During evaluation of AGR3 expression in ICC and HCC distinct disparity between the two diagnoses was found. Immunohistochemical analysis showed that high AGR3 expression is a characteristic feature of ICC ($p < 0.00001$; Pearson's chi-squared test) (Fig. 2; Table 1). AGR3 expression was more homogenous in cholangiocellular rather than hepatocellular carcinoma, but rarely there were observed regions with different intensity. There were two negative samples which constituted

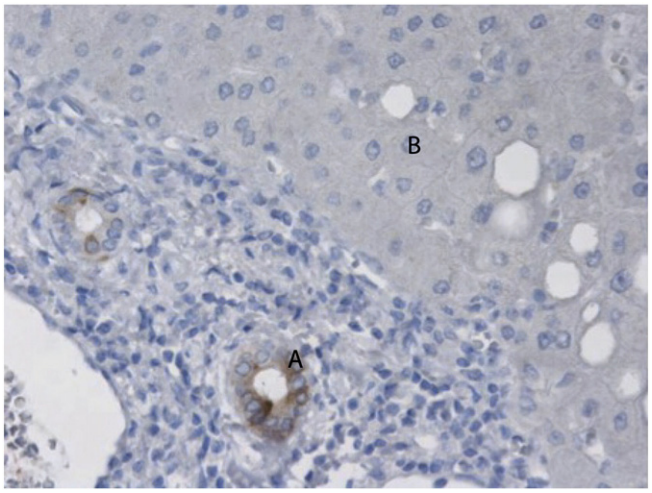

Fig. 1. AGR3 expression in normal liver. AGR3 is expressed in normal cholangiocytes of biliary duct (a) while hepatocytes are AGR3 negative (b).

a case of less differentiated clear cell ICC and a needle sample with limited representativeness, in which hepatocellular element could not have been excluded. GPC-3 expression was negative in ICC with the exception of one sample with border positivity (Fig. 3; Table 1).

In samples with the character of HCC, AGR3 expression was predominantly negative or heterogeneously weakly positive. In several resection specimens with weak positivity, AGR3 expression exhibited considerable heterogeneous character without any apparent correlation with the morphology of cancer tissue. Moderate and strong AGR3 expression was absent in hepatocellular carcinoma samples (Fig. 4; Table 1).

Immunohistochemical analysis of membranous GPC-3, a typical marker of hepatocellular carcinoma, exhibited significant correlation with hepatocellular carcinoma ($p = 0.00013$; Pearson's chi-squared test) (Fig. 5; Table 1).

Table 1
The distribution of AGR3 and GPC-3 expression in hepatocellular carcinoma and intrahepatic cholangiocarcinoma.
The table shows the number of cases for each intensity of sample AGR3 and GPC-3 positivity (0 — negative, 1 — border, 2 — weak, 3 — moderate and 4 — strong expression) in hepatocellular carcinoma and intrahepatic cholangiocarcinoma.

Protein	Type	Expression level					p value
		0	1	2	3	4	
AGR3	HCC	30	9	9	0	0	
	CC	6	2	4	6	8	$p < 0.00001$
GPC-3	HCC	24	7	4	7	2	$p = 0.00013$
	CC	25	1	0	0	0	

PAS-Alcian Blue differential staining for the presence of glycoproteins showed significantly higher expression of acid mucopolysaccharides in cholangiocellular carcinomas. Neutral polysaccharides were expressed with comparable intensity in both samples of cholangiocellular and hepatocellular carcinomas. There was a statistically significant association between AGR3 expression and acid mucopolysaccharides in ICC ($p = 0.00025$; Pearson's chi-squared test) (Fig. 6).

To evaluate the diagnostic relevance of AGR3 and GPC-3 expression in ICC and HCC we performed ROC curve analyses. In our cohort, the area under curve (AUC) for GPC-3, which is a frequently used marker for detection of HCC, reached 0.701 (95% CI 0.579–0.822; $p = 0.006$). In the analysis of AGR3 potential diagnostic application, AUC reached 0.801 (95% CI 0.681–0.922; $p < 0.001$). The combination of both markers increased the AUC value to 0.859 (95% CI 0.769–0.950; $p < 0.001$) (Fig. 7).

Discussion

The objective of this work was to evaluate AGR3 protein expression in liver cancer in order to utilize AGR3 determination as a differential marker discriminating ICC and HCC. In a screen for AGR3 expression in normal tissue, we have found that AGR3 is predominantly expressed

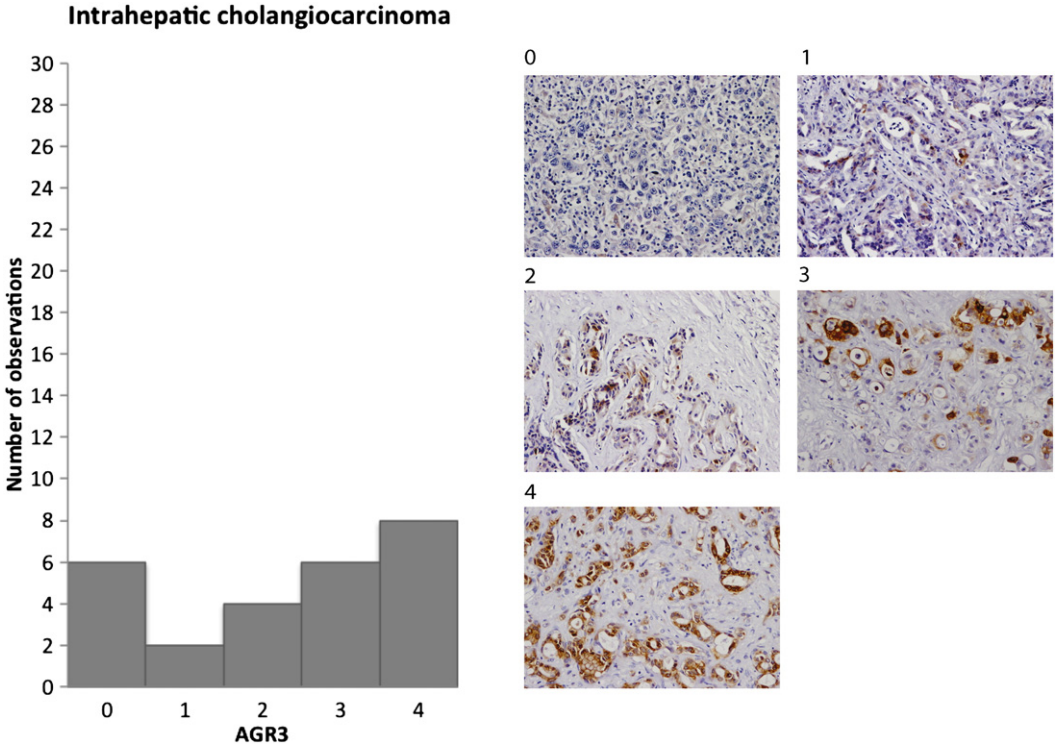

Fig. 2. AGR3 in intrahepatic cholangiocarcinoma. The immunohistochemical distribution of AGR3 expression in intrahepatic cholangiocarcinoma. The pictures show examples of negative (0), border (1), weak (2), moderate (3) and strong (4) expression of AGR3.

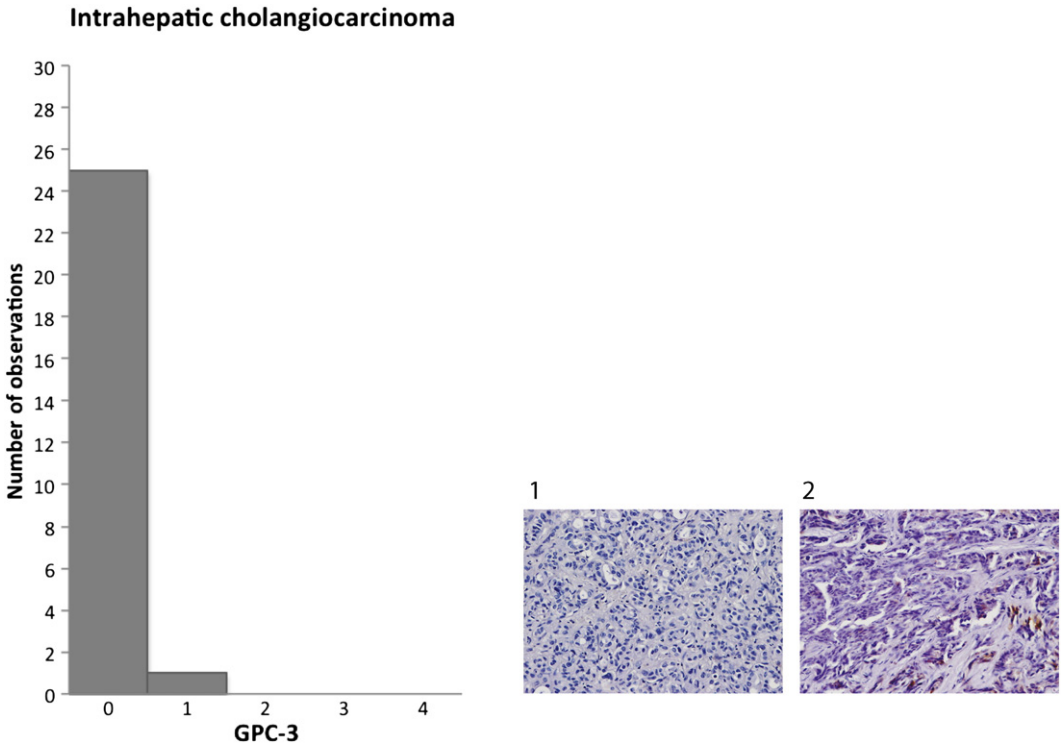

Fig. 3. GPC-3 in intrahepatic cholangiocarcinoma. The immunohistochemical distribution of GPC-3 expression in intrahepatic cholangiocarcinoma. The level of GPC-3 positivity of immunostaining is expressed by 0–4 scale (0 — negative; 1 — border; 2 — weak; 3 — moderate; 4 — strong). The pictures show examples of negative (0) and border (1) expression of GPC-3.

in liver cholangiocytes representing epithelial cells of bile ducts. Conversely liver hepatocytes express AGR3 protein only weakly and rarely (Fig. 1). These data are similar to work by Vivekanandan et al., who demonstrated expression of the AGR3 homologue, AGR2, in cholangiocytes located in septal-sized bile ducts and weakly in zone 3 hepatocytes (Vivekanandan et al., 2009).

Similarly to normal tissue, AGR3 expression in HCC was predominantly negative, whereas high expression was found for GPC-3 membrane

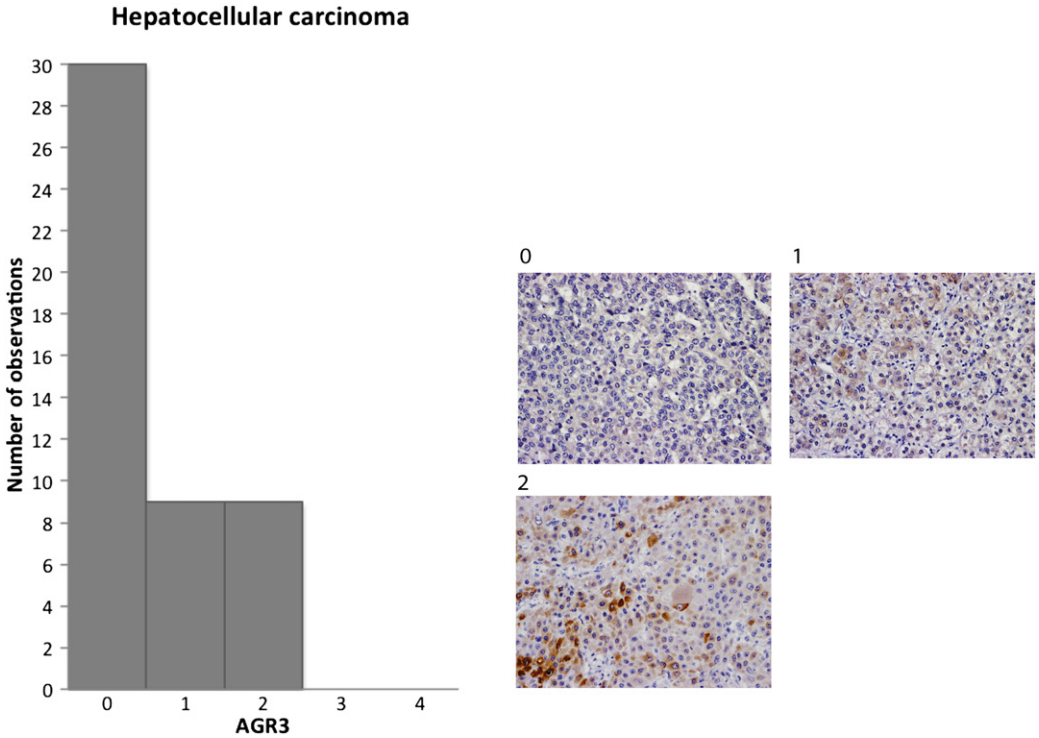

Fig. 4. AGR3 in hepatocellular carcinoma. The distribution of AGR3 expression in hepatocellular carcinoma. The level of AGR3 positivity OD immunostaining is expressed by 0–4 scale (0 — negative; 1 — border; 2 — weak; 3 — moderate; 4 — strong). The pictures show examples of negative (0), border (1) and weak (2) expression of AGR3.

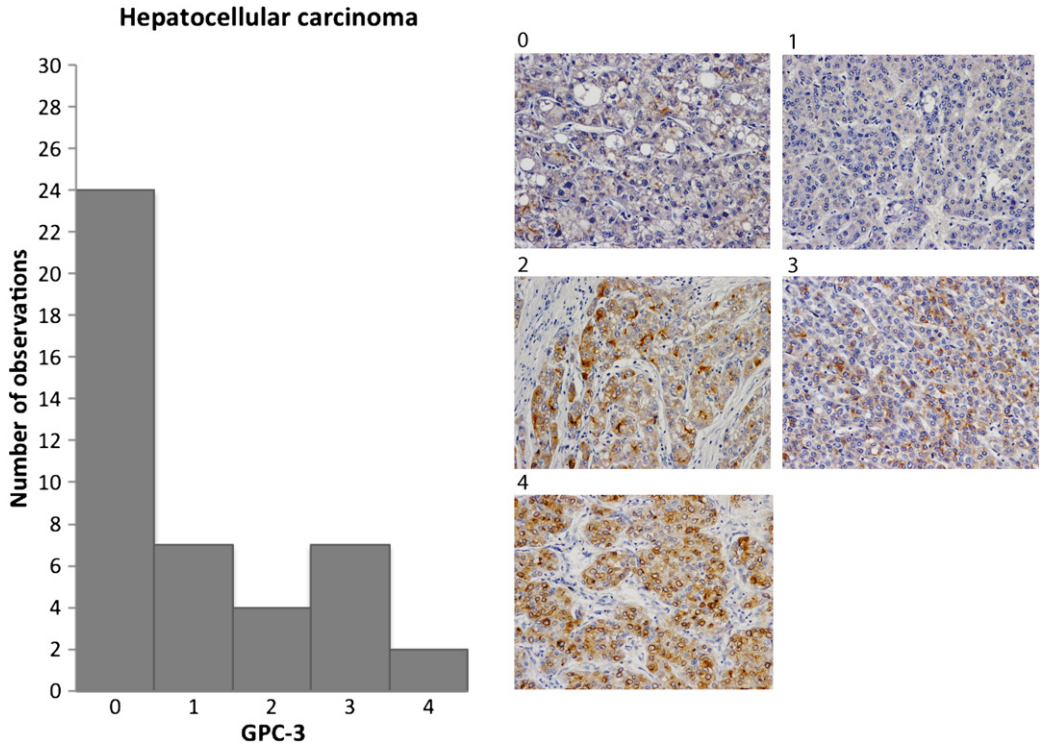

Fig. 5. GPC-3 in hepatocellular carcinoma. The distribution of GPC-3 expression in hepatocellular carcinoma. The pictures show examples of negative (0), border (1), weak (2), moderate (3) and strong (4) expression of GPC-3.

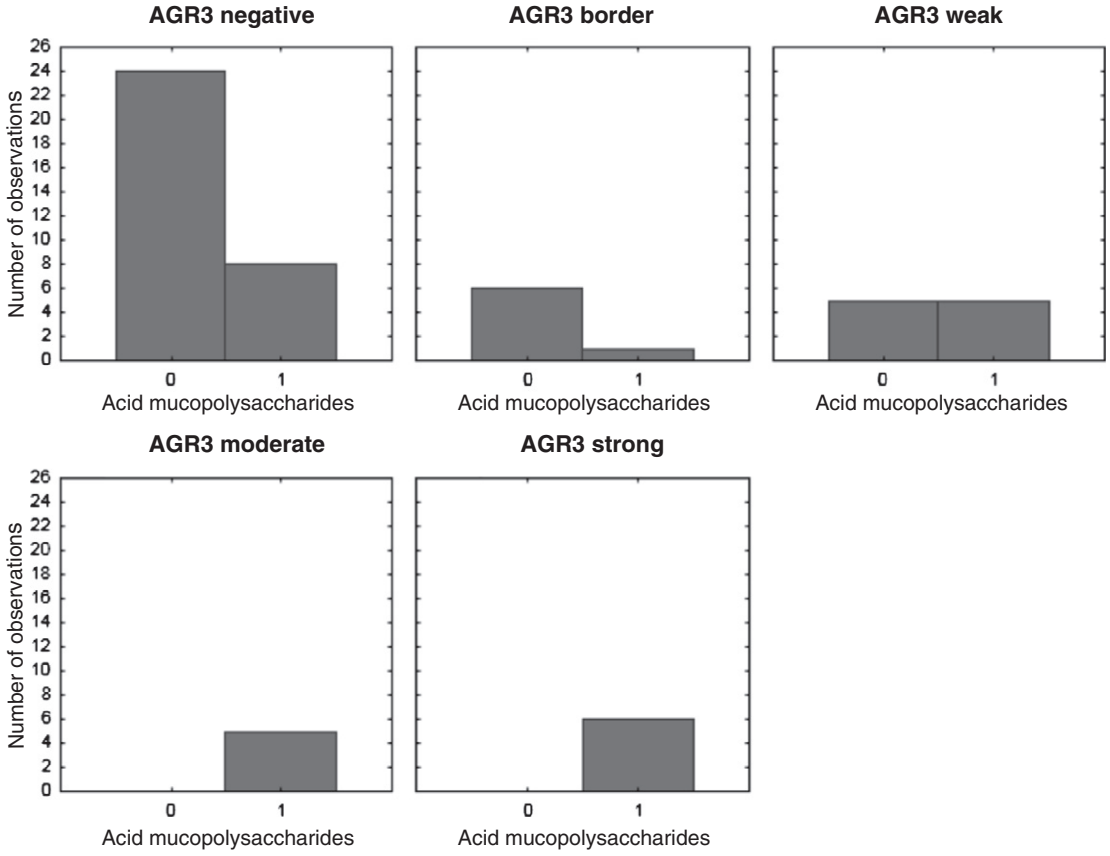

Fig. 6. Acid mucopolysaccharides distribution according to AGR3 protein levels. The distribution of acid mucopolysaccharides in samples of intrahepatic cholangiocarcinoma and hepatocellular carcinoma categorized according to the level of AGR3 expression (0 — negative; 1 — positive expression). The expression of acid mucopolysaccharides is significantly correlated to AGR3 expression.

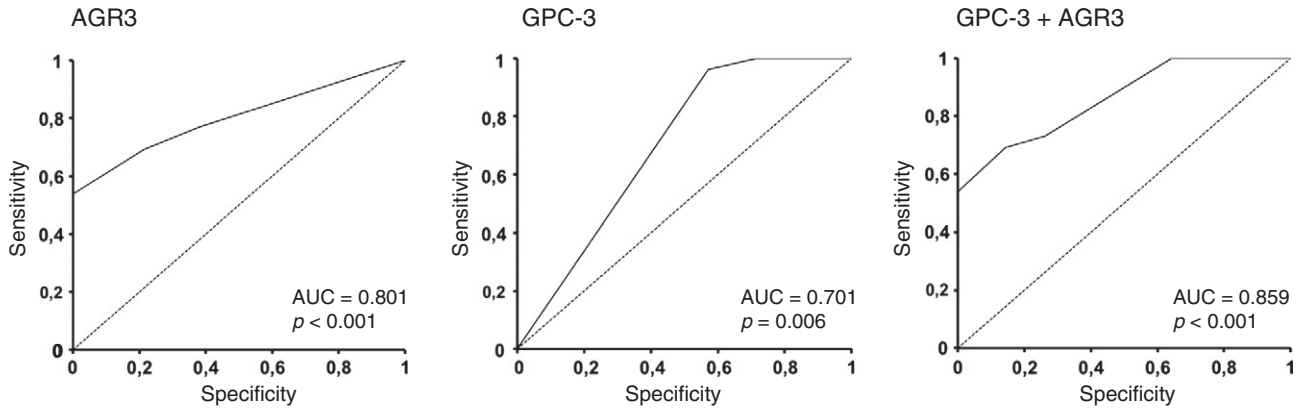

Fig. 7. ROC analysis. ROC curves for AGR3, GPC-3 and the combination AGR3/GPC-3 show that AGR3 has a higher AUC value than GPC-3 (0.801 versus 0.701). The best AUC value is provided by the combination AGR3/GPC-3 (0.859).

protein. Although GPC-3 expression in HCC was specific, the number of negative cases was higher than we expected, which we attribute to false negativity caused by focal expression of GPC-3 and also the quality of material processing, in particular, fixation. In ICC, AGR3 expression was typically moderate or strong and was linked to acid mucopolysaccharides.

ICC is a primary liver tumor derived from cholangiocytes which line intrahepatic bile ducts. The intrahepatic biliary epithelium lines the biliary tree from the small ductules to large extrahepatic bile ducts and possesses topographic heterogeneity within the different levels of the biliary tree. Large bile ducts are covered with mucin-producing cylindrical cells while the ductules are covered with mucin-negative cubic and bipotential hepatic progenitor cells. The cell population in large bile ducts comprises more differentiated cells that vary in nucleus to cytoplasm ratio and are functionally diverse from small cuboidal cells of bile ductules (Marzioni et al., 2002). These two different cell populations give rise to different classes of intrahepatic cholangiocarcinomas with mostly different symptoms, histopathological characteristics and protein expression profiles. Those tumors may develop either from the large hepatic bile ducts near the hilus (perihilar) or from the more peripheral small bile ducts and ductules (Nakanuma et al., 2010a, 2010b).

Large cholangiocytes have more abundant cytoplasm where RNA translation takes place (Marzioni et al., 2002) and since AGR3 protein belongs to protein disulfide isomerases of endoplasmic reticulum, AGR3 could participate in the formation and breakage of disulfide bonds and help protein folding in these cylindric cholangiocytes. We assume that the immunohistochemical assessment of the expression of AGR3 protein together with acid mucopolysaccharide expression could serve as a marker of well differentiated tumors developed from large biliary ducts and may contribute to improve differential diagnostics of HCC and ICC carcinomas.

Lepreux et al. (2011) has already published differential expression of AGR2 corresponding to the localization within biliary tree and suggested AGR2 protein involvement in the differentiation of the biliary tree cells. AGR2 protein was also shown to be involved in mucin 2 secretion in goblet cells and to be associated with mucosecretion in pancreatic cancer (Norris et al., 2013). Recently, AGR2 expression and MUC4 expression were correlated with tumor cell differentiation in pancreatic ductal adenocarcinomas (Brychtova et al., 2014). Based on our results and results published, the common feature for anterior gradient proteins in normal and tumor tissue seems to be the participation in mucosecretion and differentiation of cells.

Conclusion

This study has shown that although AGR3 protein expression has some limitation as to non-homogenity of AGR3 immunoprofile in hepatocellular carcinoma, it is highly specific and sensitive marker of intrahepatic cholangiocarcinoma that can be used as complementary marker to GPC-3 in distinguishing between intrahepatic cholangiocarcinoma and hepatocellular carcinoma in differential diagnostics.

Conflict of interest

The authors declare no conflict of interest related to this work.

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