Original Article

Expression Pattern of the Human ABC Transporters in Pluripotent Embryonic Stem Cells and in Their Derivatives

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Background: ATP-binding cassette (ABC) transporters have key roles in various physiological functions as well as providing chemical defense and stress tolerance in human tissues. In this study, we have examined the expression pattern of all ABC proteins in pluripotent human embryonic stem cells (hESCs) and in their differentiated progenies. We paid special attention to the cellular expression and localization of multidrug transporter ABC proteins.

Methods: Stem cell differentiation was carried out without chemical induction or cell sorting, and specialized cell types were separated mechanically. Cellular features regarding pluripotency and tissue identity, as well as ABC transporter expression were studied by flow cytomtery, immuno-microscopy, and qPCR-based low-density arrays.

Results: Pluripotent hESCs and differentiated cell types (cardiomyocytes, neuronal cells, and mesenchymal stem cells) were distinguished by morphology, immunostaining markers, and selected mRNA expression patterns. We found that the mRNA expression levels of the 48 human ABC proteins also clearly distinguished the pluripotent and the respective differentiated cell types. When multidrug and lipid transporter ABC protein expression was examined by using well characterized specific antibodies by flow cytometry and confocal microscopy, the protein expression data corresponded well to the mRNA expression results. Moreover, the cellular localization of these important human ABC transporter proteins could be established in the pluripotent and differentiated hESC derived samples.

Conclusions: These studies provide valuable information regarding ABC protein expression in human stem cells and their differentiated offspring. The results may also help to obtain further information concerning the specialized cellular functions of selected ABC transporters. © 2014 International Clinical Cytometry Society

Key words: human pluripotent stem cell; mesenchymal cell; cardiomyocyte; neural cells; ABC transporters; pluripotency; stem cells; flow cytometry; microscopy

Additional Supporting Information may be found in the online version of this article.

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INTRODUCTION

The ATP-binding cassette (ABC) protein family is present from bacteria to humans, and the 48 human ABC proteins include several transporters, channels, as well as nonmembrane proteins (1). During the past decades a large amount of information became available about the expression and function of these human proteins, while there are still numerous unresolved questions in this field. We have shortly summarized the recent knowledge about the human ABC proteins in Supporting Information Table 1 [see references (9,35-38) cited therein, and for more information see http://humanabc.4t.com/main.htm].

Certain ABC transporters form a special network of chemo-defense system, as these ATP-dependent active transporters extrude a wide variety of substrates from the cells, including endo- and xenobiotics. While these ABC transporter (with a key role of ABCB1/Pgp, ABCC1/MRP1, and ABCG2/BCRP) proteins play an important role in protecting our body, they are also involved in causing multidrug resistance in cancer cells (2,3). One member of these multidrug transporters, ABCG2, has been shown to play a major role in the protection of stem cells against toxic compounds. In addition, the ABCB6 protein has been indicated to play a major role in the defense against toxic heme derivatives, while the ABCA1 protein has a key role in the extrusion of excess cellular lipid derivatives, especially cholesterol (see Refs. 4–9).

Since the human embryonic stem cells (hESC) require special protection during development, and some of the differentiated tissues are also well protected against toxic agents, an important task is to follow the changes in the expression and localization of the ABC multidrug transporters during these early developmental processes.

In our previous studies we have shown that the HUES9 embryonic stem cells express the ABCG2 protein at the cell surface (10), and this transporter has an important role in defending the HUES9 cells during stress conditions (5). In the current work we have examined the full pattern of 48 human ABC protein mRNA expression levels by qPCR-based microarray, in order to provide information about changes in their tissue expression patterns in undifferentiated and selectively differentiated hES cells. We have also followed the expression of selected ABC transporter proteins by flow cytometry and immunostaining during early human cell differentiation. In addition to provide basic information about ABC protein expression, these studies also allowed to examine the cellular localization of these transporter proteins, which was not exactly known for embryonic stem cells and their derivates.

MATERIALS AND METHODS

Cell Culture and Differentiation

hES cell lines HUES9, HUES3, and HUES4 (originally provided by Dr. Douglas Melton, Harvard University) were maintained on mitotically inactivated mouse embryonic fibroblasts (MEF) and spontaneous differentiation were performed via embryoid body (EB) formation as described previously (11). After 6 days EB were placed onto gelatin coated 24 well plates, where they attached to the surface and underwent spontaneous differentiation. The desired cell types were separated as follows:

Some of the EB-outgrowths start spontaneously beating within a few days postplating. These rhythmically contracting areas were isolated mechanically and hES cell-derived cardiomyocytes were replated on gelatin coated 8 well confocal chambers or harvested to RNA isolation. Neuronal progenitor cells with rosette-like morphology were mechanically isolated for generation of hES cell-derived neural cells, between days 8 and 10. These rosettes were able to reattach onto gelatin coated surfaces and continue their further differentiation to mature neural cell types. The RNA isolation and immunostaining were performed at day 24.

The mesenchymal stem cell like (MSCl) cells were generated and maintained as described previously (12). For more details see the Supporting Information Methods.

Flow Cytometry

Single cell suspensions were prepared by gentle trypsinization, and the cells were labeled in PBS containing 0.5% bovine serum albumin with appropriate antibodies. In all hESC samples an anti mouse Sca-1 (Ly-6A/E) (FITC or PE, BD Pharmingen) antibody was employed for gating out the positively labeled mouse feeder cells.

We used the following directly labeled anti-human antibodies: SSEA4-APC and PODXL-PE (R&D Systems) for investigation of pluripotency. For indirect staining of ABC transporters the following monoclonal antibodies were applied: 5D3 (BD Pharmingen) for ABCG2, MRK16 (Alexis Biochemicals) for ABCB1, anti-Lan (OSK43 (generated and kindly provided by Hideo Takahashi Japanese Red Cross Osaka Blood Center, Japan) (13) for ABCB6, R1 (Abcam) for ABCC1 and AB H10 (Abcam) for ABCA1 labeling. The 5D3 labeling was performed in the presence of Ko143, a specific inhibitor of ABCG2, which maximizes 5D3 binding (14). Control staining with appropriate isotype-matched control mAbs was included. For fixation and permeabilization of the cells the Fix&Perm (Invitrogen) solution was used, according to the manufacturer's instruction. Samples were

analyzed by a BD FACSCantoII flow cytometer (Becton Dickinson Immunocytometry Systems [BDIS]). To ascertain the specificity of the antibodies we have performed various studies, including Western blotting in several cell lines (see Refs. 15–18 and data not shown). For more details see the Supporting Information Methods.

Immuno-Cytochemical Staining

Immunostaining of all cell types was performed as described previously (10), except the cell surface labeling of ABCG2, which was carried out as described by Ref. 5. For labeling of pluripotent and differentiation markers the following primary antibodies were applied: Oct4 (SantaCruz), Nanog, SSEA4, and PODXL (RnD Systems) cardiac Troponin-I (Sigma), Nestin (Abcam), β-III Tubulin (RnD Systems), and CD-44-FITC (BD Pharmigen). The ABC transporters were studied by the same antibodies used for flow cytometry. Hoechst33342 (Invitrogen) was used for nuclear staining. The stained samples were examined by an Olympus FV500-IX confocal laser scanning microscope. For more details see the Supporting Information Methods.

Gene Expression Analysis

Total RNA was isolated from the cells using the Trizol reagent (Life Technologies). RNA integrity was checked by standard gel electrophoresis, RNA concentration was determined by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Scientific). Gene expression profiles were determined by analyzing TaqMan® Low Density Arrays (TLDA cards, Life Technologies) designed for measuring pluripotency marker genes (TaqMan® Array Human Stem Cell Pluripotency Panel, cat. #: 4385344) or ABC transporters (TaqMan® Array Human ABC Transporter Panel, cat. #: 4378700). Briefly, 500 ng of total RNA was used to prepare cDNA samples using the Reverse Transcription System Kit (Promega), according to the manufacturer's instruction. cDNA samples corresponding to 100 ng of total RNA were combined with 2× TaqMan® Gene Expression Master Mix and loaded on one channel of the appropriate TLDA card; real-time PCR reactions were run on a 7900HT System according to the manufacturer's protocol (Life Technologies). For data analysis, the DataAssist™ Software v3.0 (Life Technologies) was used. Gene expression values were determined by the ΔΔCt method using multiple endogenous control genes on the TLDA cards (ACTB and GAPDH genes for the pluripotency array data and RPLP0 and PPIA genes for the ABC transporter array data). During further analysis we have selected and excluded several genes with very low expression levels (e.g., genes with $Ct > 38$, such as HBB, INS, and PAX4 on the pluripotency panel and ABCA6, ABCB5, ABCC11, and ABCC12 on the ABC transporter panel). The average linkage clustering method was applied to analyze the expression data and to calculate Pearson's correlation values to define distances of our samples sets.

RESULTS

Differentiation of hES Cells—Characterization by Differentiation Markers

For studying the expression of human ABC proteins in pluripotent cells and in their differentiated derivatives, we have generated cardiac cells, mesenchymal-like stem cells [MSCls (12)] and neural cells from the HUES9 cells, by using a method of spontaneous differentiation, via EB formation (Supporting Information Fig. 1). With this method the desired cell types could be generated from hESC without the addition of special chemicals, and the selection of the differentiated progenies was performed by enzymatic digestion and/or mechanical selection, without applying any drug selection or cell sorting. All the differentiated cell types were cultured in the same media and under similar conditions (see Materials and Methods section). These uniform conditions were used because the expression levels and localization of several ABC proteins have been shown to be influenced by chemical inducers, selection drugs or antibiotics (see Refs. 19,20).

When selecting various tissue types, we performed a detailed phenotypic characterization of the parental and differentiated cell types, in order to assure that any further analysis should be performed by using properly selected populations. This characterization included the investigation of several cell type specific markers by flow-cytometry and immunocytochemistry.

As shown in Figure 1A, by flow cytometry studies we found that the pluripotent hES cells (HUES9) highly expressed the SSEA-4 and PODXL pluripotency markers on the cell surface. Using immunostaining and confocal microscopy, we could clearly show the presence of the nuclear pluripotency markers, Oct4, and Nanog (Fig. 1B).

During differentiation studies, cell surface markers for hESC-s and hESC derived MSCls were analyzed (Supporting Information Fig. 2) as described in detail in our previous work (see Ref. 12). In all cases, immunostaining revealed proper separation of cells showing positive staining for representative markers of cardiac (cardiac troponin-cTNI), neural (neuron specific β-III tubulin and Nestin), and mesenchymal (CD44) cell types (Fig. 1C).

After this phenotypic selection and verification we have analyzed the mRNA expression patterns of the selected cell types derived from HUES9 cells by using a *TLDA pluripotency array*. Besides the differentiated cell types, the common progenies of the differentiated cells (mesenchymal, cardiac, and neural) from 6 days old EBs were included in the further investigation. The array included 36 pluripotency, 19 mesoderm, 13 ectoderm, 17 endoderm, and 4 trophectoderm markers, along with several housekeeping genes. An overview of these data (summarized in Supporting Information Table 2) is demonstrated in a heat-map (Fig. 2A), showing relative gene expression levels. The cluster analysis (see Methods section), documented in Figure 2B, shows close correlation of the mRNA expression pattern with the differentiation

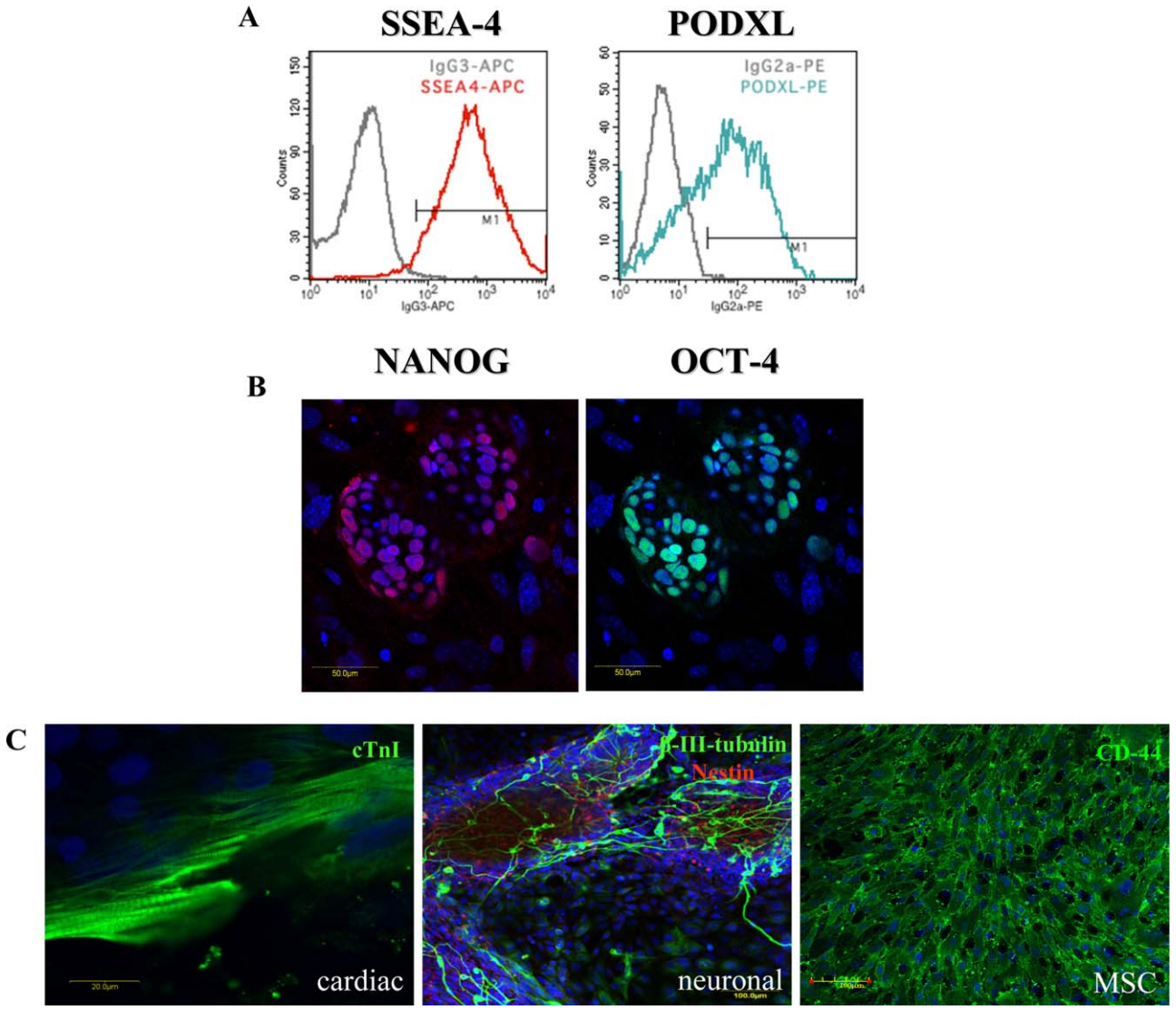
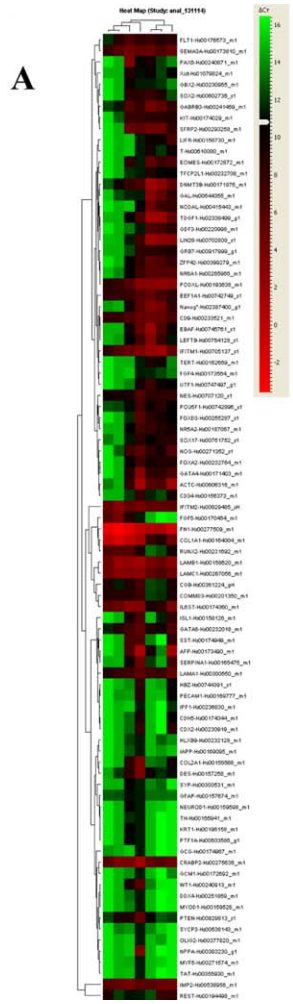


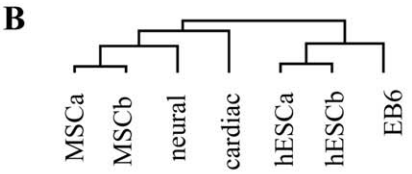
FIG. 1. Characterization of HUES9 cells and their derivates by following the expression of selected proteins. (A) Investigation of the pluripotent state of HUES9 cells by flow cytometry. More than 90% of the cells show cell-surface SSEA4 and PODXL expression. M1: cell population with marker positivity was gated based on the relevant isotype-matched control mAbs. (B) Investigation of the pluripotent state of HUES 9 cells by immunomicroscopy. HUES 9 cells were grown on MEF feeder cells for two days in eight-well chambers for confocal microscopy. Coculture of HUES9 and feeder cells were fixed, permeabilized, and stained with the antibodies recognizing the Nanog (red) and Oct4 (green), markers. Nuclei were counterstained with Hoechst33342 (blue). Nanog and Oct4 transcription factors showed nuclear localization. (C) Investigation of the differentiated forms of HUES9 cells by immunomicroscopy. HUES9-derived cell types were differentiated as described in Materials and Methods section and were transferred mechanically into eight-well chambers for confocal microscopy. The samples were fixed, permeabilized, and stained with antibodies recognizing specific proteins for each cell type; cTNI (green) for cardiac, β-III tubulin (green), and Nestin (red) for neural and CD44 (green) for mesenchymal cell type. Nuclei were counterstained with Hoechst33342 (blue).

status of the cell samples. As shown, the biological parallels (for hESC and MSCl) clustered together, while the undifferentiated hESC samples are clearly separated from the partially differentiated sample (6 days EB). The mRNA clusters of the differentiated cell types are noticeably different from that of the hESCs and from one another and the biological replicates of the most differentiated cell types (the MSCl cells, after 80 days of differentiation) are located away from the undifferentiated cells with the longest distance in the dendrogram. As shown by the highest expression levels found in the pluripotency array data, the hES cells showed high levels of the pluripotency mRNA markers, while the derivative cells showed upregulated levels of the proper lineage-specific markers (Fig. 2C).

ABC Transporter Expression in Pluripotent and Differentiated hES Cells—Flow Cytometry Studies

In these experiments we investigated the ABC transporter expression in the pluripotent and differentiated





C

HUES9		HUES9-EB6		Neural		Cardiac		MSC	
FN1	0.874	**FN1**	-1.320	**FN1**	- 2.309	**FN1**	- 0.123	**FN1**	- 3.080
TDGF1	1.888	**AFP**	1.301	**COL1A1**	- 0.120	**AFP**	0.915	**COL1A1**	- 0.979
IFITM1	2.193	**ACTC**	1.617	**LAMB1**	2.444	**COL1A1**	1.117	**LAMB1**	3.397
DNMT3B	2.361	**LAMB1**	2.970	**IL6ST**	3.033	**ACTC**	1.332	**LAMC1**	3.901
LIN28r	2.485	**PODXL**	2.990	**LAMC1**	3.402	**CRABP2**	1.637	**IFITM2**	4.210
PODXL	3.042	**LIN28r**	3.392	**PAX6**	3.619	**LAMB1**	1.784	**CRABP2**	4.737
Nanog	3.963	**IL6ST**	3.979	**CRABP2**	3.816	**LAMC1**	3.016	**FGF5**	5.047
GAL	4.010	**CRABP2**	4.340	**IMP2**	4.170	**GATA4**	3.160	**IL6ST**	5.279

FIG. 2. TLDA analysis of differentiation status of HUES9 cells and their differentiated offspring. (A) Heat map representation of human pluripotency marker gene mRNA expressions in 7 samples of 4 cell types (hESCs a and b, cardiac, neural, and MSCs a and b) and the 6 days old EB culture (the common progenitors of differentiated cell types). The pluripotency genes are shown on the *y*-axis, the 7 samples are ordered on the *x*-axis with biological replicates ("a" and "b"). The color code ranges from high (dCt = −2, light red) through medium (black), to low (dCt = 16, light green) levels of gene expression relative to selected housekeeping genes. (B) Hierarchical clustering of 7 samples allowing a separation based on differentiation status of the samples. (C) Genes with the highest expressions values for each cell type show the proper characteristics for a given differentiation status. The table shows dCt data relative to the geometric mean of housekeeping control genes (ACTB and GAPDH). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells at the protein level. The application of specific and properly reactive antibodies is a crucial question in these experiments. Especially, in the case of the flow cytometry studies, we used antibodies against extracellular epitopes to examine the expression and cell surface localization of ABCG2 (mAb 5D3), ABCB1 (mAb MRK16), and ABCB6 (mAb OSK43). However, most of the antibodies recognizing human ABC transporters have been generated against intracellular epitopes, and in several experiments we have used such antibodies (see Methods section). In each case, the specific protein recognition was assured by examining various cell types overexpressing these ABC transporters (Supporting Information Fig. 3).

The two major cell types in which ABC transporter expression could be studied without major cellular damage were the undifferentiated hES cells and the MSCs. In these cell types we focused on the expression of ABC multidrug or lipid transporters with key roles in cellular chemodefense and stress response. As shown in Figure 3, in the case of ABCB1, ABCB6, and ABCG2 we used the respective antibodies recognizing extracellular epitopes, while in the case of ABCC1 and ABCA1 we employed antibodies generated against intracellular protein domains.

Figure 3A shows flow cytometry detection of five ABC transporters (ABCB1, ABCB6, ABCG2, ABCC1, and ABCA1) in HUES9 cells under nonpermeabilized conditions using antibodies recognizing external epitopes, while panel B of Figure 3 shows similar measurements after permeabilization of hESCs. As documented, in all cases we found a very low level expression in the

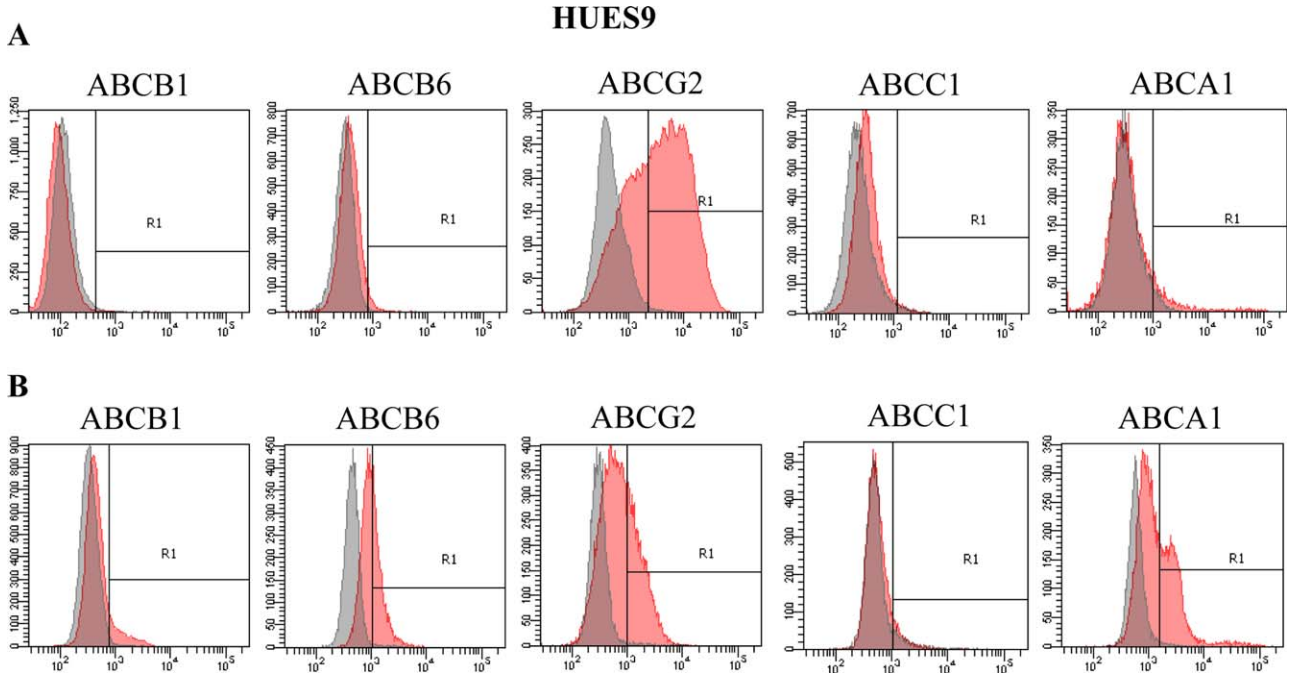


FIG. 3. Flow cytometry analysis of ABC transporter expression in hES cells. Single cell suspensions from HUES9 cells were obtained as described in Materials and Methods section. Nonviable cells were gated out by 7AAD staining. Monoclonal antibodies specific for ABCB1, ABCB6, ABCG2, ABCC1, and ABCA1 were used to detect transporter expression (A) in intact cells (without fixation and permeabilization) and (B) in fixed and permeabilized cells (for details see Materials and Methods section). R1: cell population with marker positivity was gated based on the relevant isotype-matched control mAbs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

nonpermeabilized cells except ABCG2, which showed elevated level of cell surface expression, while a slight increase in the apparent expression of all these transporters, especially ABCA1 could be found in the permeabilized hESCs. Only ABCG2 expression level showed an apparent reduction under these conditions, showing that the 5D3 antibody binding was reduced by the applied fixation-permeabilization. Similar results were obtained when analyzing HUES3 and HUES4 cells as documented in Supporting Information Figures 4 and 5.

Figure 4 documents similar studies carried out with hES-derived MSCls by flow cytometry. In this case the expression levels of all studied transporters were negligible without permeabilization (Fig. 4A), but were well detectable in the permeabilized cells in the case of ABCB6, ABCC1, and especially of ABCA1. It is important to note that ABCG2 expression was not detectable in these cells, with or without permeabilization.

In the case of ABCB1 and ABCB6 (antibodies reacting with external membrane epitopes) the potential expression of these proteins in intracellular membrane compartments may be suggested by these data. Since the ABCC1 and ABCA1 antibodies recognize intracellular epitopes, the increased protein levels found in the permeabilized cells most probably correspond to increased general expression levels, and in this case the plasma membrane or intracellular membrane expression levels cannot be distinguished. Similar results were obtained when analyzing HUES3 and HUES4 cells as documented in Supporting Material Figures 6 and 7.

ABC Transporter Expression in Pluripotent and Differentiated hES Cells—Immunomicroscopy Studies

Flow cytometry examinations could not be performed with vulnerable cell types such as cardiomyocytes and neural cells, without injuring the cells. Comparative examination of the hESC and hESC derived cell types by immunohistochemistry is thus an additional method to characterize protein expression and yield more informative cellular localization data than flow cytometry.

In Figure 5 we summarize the results of the immunostaining experiments. This figure compiles representative data for each cell type and for the above described antibodies recognizing selected ABC transporters. As shown in Supporting Information Figure 8, we have examined several selected cell types, overexpressing these ABC transporters, as positive controls (see also Materials and Methods section).

As documented in Figure 5, in the case of *ABCA1* all hESC-derived cell types showed well measurable expression, and in the undifferentiated hESCs and the MSCls, the expression was homogenously high showing both plasma membrane and cytosolic localization. In contrast, only a small number of cardiac and neural cells showed ABCA1 expression, and this expression was mostly localized in the cytosol. In the case of *ABCB1*, we found measurable expression only in the differentiated cardiac and neural cell types, which was in agreement with the flow cytometry data indicating no ABCB1 expression in the undifferentiated hESCs and the MSCls. The *ABCB6*

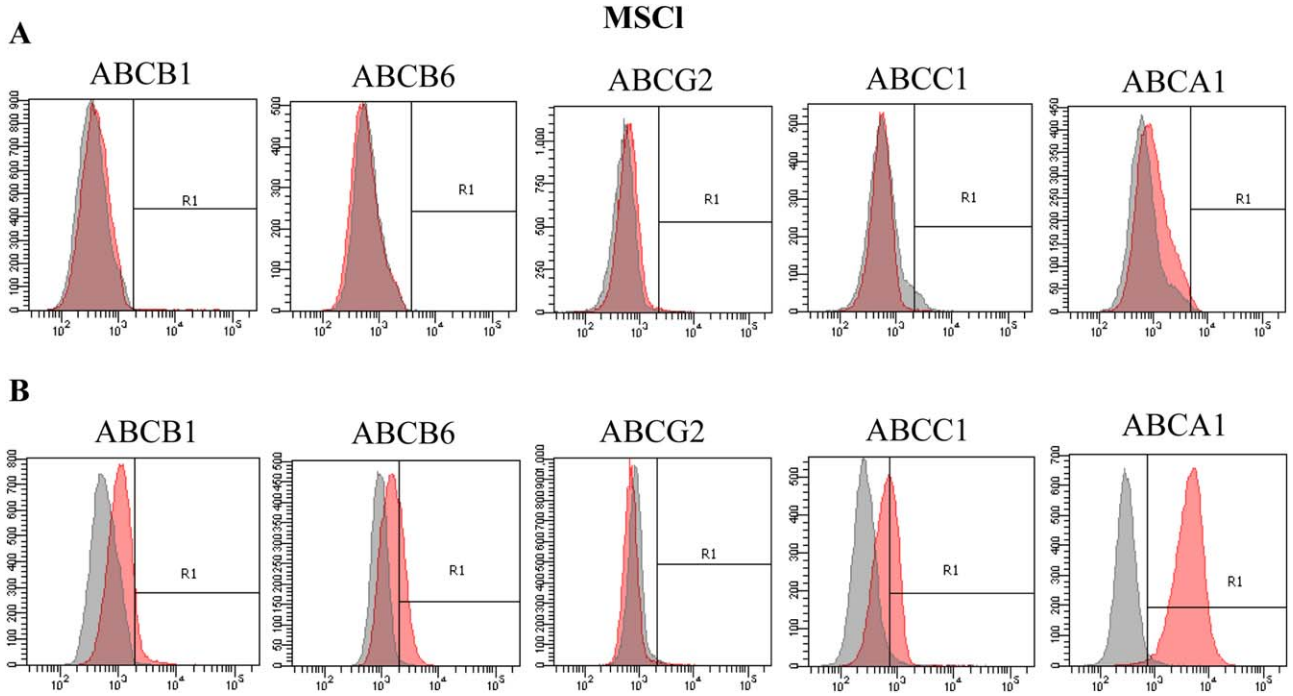


FIG. 4. Flow cytometry analysis of ABC transporter expression on MSCl cells. Single cell suspensions from MSCl cells were obtained by gentle trypsinization. Nonviable cells were gated out by 7AAD staining. Monoclonal antibodies specific for ABCB1, ABCB6, ABCG2, ABCC1, and ABCA1 were used to detect transporter expression (A) on intact cells (without fixation and permeabilization) and (B) in fixed and permeabilized cells (for details see Materials and Methods section). R1: cell population with marker positivity was gated based on the relevant isotype-matched control mAbs. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

protein was expressed ubiquitously in all of the examined cell types, and the expression was found in intracellular compartments. These data are again in line with the flow cytometry results, which also indicated intracellular expression of this protein. For *ABCC1*, a well measurable expression could be observed for most of the cell types, except for the cardiac cells, showing a much lower protein level. In all cases, ABCC1 expression was detected in the plasma membrane compartment.

As shown earlier in Figure 3 and Supporting Information Figures 4 and 5, in the undifferentiated hES cells high level of ABCG2 protein expression was detected on the cell surface by flow cytometry and the immunostaining experiments shown in Figure 5 verified this phenomenon. In contrast, we could not detect a significant ABCG2 expression in the differentiated MSCl cell types and some expression could be detected on cardiac and neural cultures however this expression was confined to the border of tissues. In Figure 5, we have also included immunostaining data for the ABCC6 protein, as this transporter has been indicated to play an important role in cardiac tissues (21,22). However, we could not detect ABCC6 expression in any of the examined hESC-derived cell types.

ABC Protein mRNA Expression in Pluripotent and Differentiated hES Cells

Because of the lack of reliable antibodies and staining protocols for numerous human ABC proteins, a systematic analysis can only be performed for the respective mRNA expression levels. For this purpose we used a quantitative RT-PCR technology and ABC protein specific microarray, and examined the same mRNA samples as used in the pluripotency array analysis (see Fig. 2), obtained from pluripotent hESCs and the differentiated cell types. A visual overview of these gene expression data is demonstrated in a heat-map (Fig. 6A), while the dCt data are presented in the Supporting Information Table 3.

Cluster analysis based on ABC protein expression patterns is shown in Figure 6B. Remarkably, clustering based on ABC transporter profiles was found to be similar to the hierarchy defined by pluripotency markers (Fig. 2), indicating that all the examined cell types have characteristic expression patterns (fingerprints) for the ABC proteins, depending on the cell maturation status. As shown in Figure 6C, some of the ABC proteins showed similar (medium) level of expression in all cell types (Fig. 6C, left panel), although most of these ABC proteins are not well described transporters, and some are not even membrane proteins (Supporting Information Table 1). These data suggest a role of these ABC proteins in the general maintenance of cellular homeostasis.

In the following analysis, we focused on the recognized ABC membrane transporters, and excluded those proteins, which showed high Ct values, indicating extremely low or no expression (Fig. 6C, right panel).

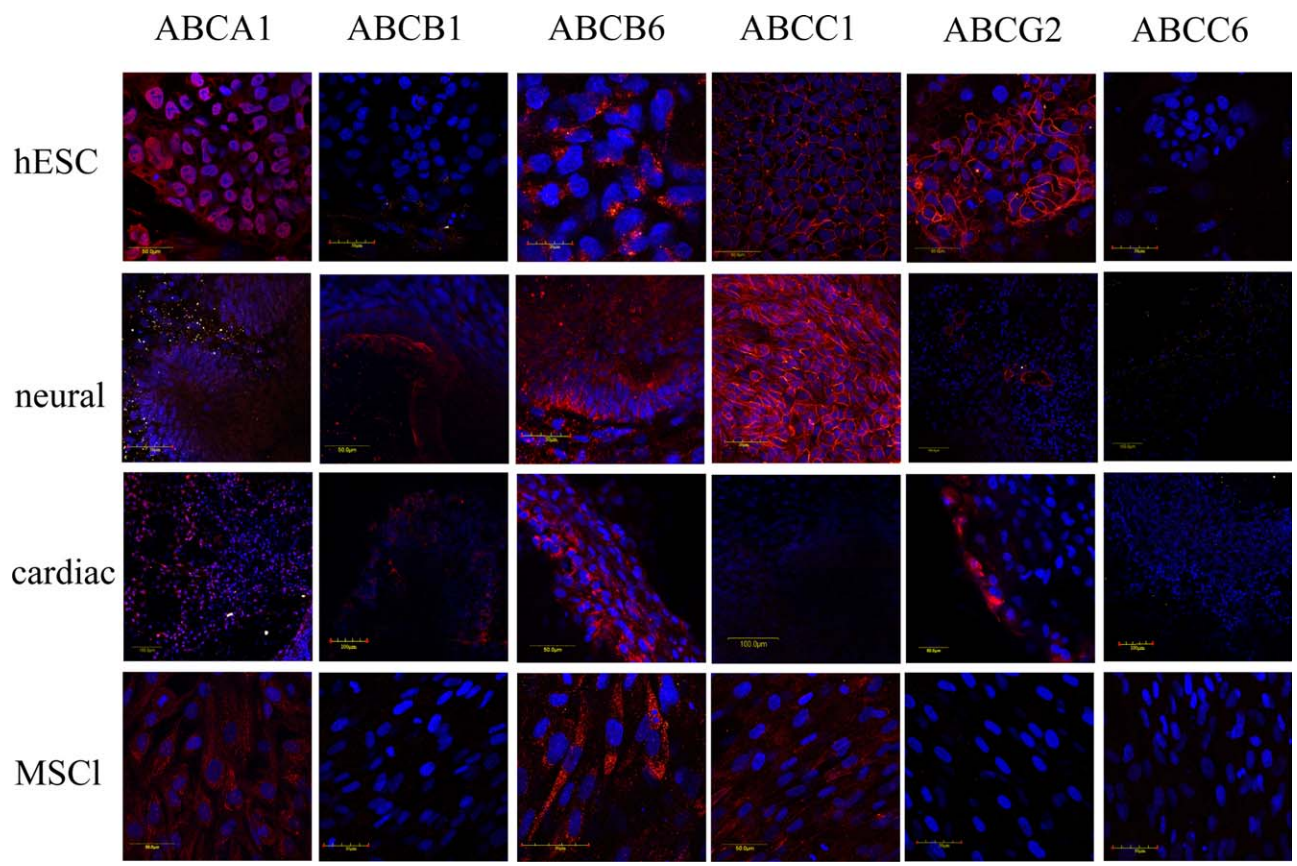


FIG. 5. Immuncytochemical detection of ABC transporters in HUES9 cells and their derivatives. HUES 9 cells were grown on MEF feeder cells for two days in eight-well chambers and HUES9-derived cell types were differentiated as described in Materials and Methods section and were transferred mechanically into eight-well chambers for confocal microscopy. The samples were stained with specific antibodies to visualize ABCA1, ABCB1, ABCB6, ABCC1, ABCG2, and ABCC6 proteins (red) as described in the "Materials and Methods" section. Nuclei were counterstained with Hoechst33342 (blue). Stained samples were examined by an Olympus FV500-IX confocal laser scanning microscope. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

We compared the relative expression of ABC transporter mRNAs in differentiated cells to those in undifferentiated hES cells (Supporting Information Table 4).

As shown in Figure 7 and presented in Supporting Information Table 4, we found that most of the ABC transporters were expressed at significantly higher level (although in different magnitudes, see Fig. 7A and B) in the differentiated cell types than in the pluripotent hESCs. When analyzing the cell-type dependent expression, we found that several ABC transporter mRNA levels showed large increase in differentiated progenies. These included the expression of ABCA8, ABCC3, ABCC9, and ABCG1. In contrast, there were several transporter mRNA expression levels characteristic for certain cell types: changes of ABCB1 and ABCC6 expressions were higher in cardiac cells and ABCA4 was higher in neural cells as compared to hESC (Fig. 7A). Note, that the only ABC transporter, which showed a decreased expression level in all differentiated cell types was ABCG2 (Fig. 7B).

This cell type-specific expression indicates a possible role of these proteins in the differentiated tissues and allows a further characterization of their physiological roles.

DISCUSSION

ABC transporters play an important role in various physiological functions, maintain cellular homeostasis, and provide defense mechanism against toxic endo- and xenobiotics. These ABC transporters work in a general defense network system, having overlapping functions and substrate specificity. On the basis of their promiscuous recognition of toxic agents, they can functionally substitute each other. While many of the ABC multidrug transporters also have broad tissue distribution, their expression levels may provide a tissue-specific "fingerprint," corresponding to special metabolic or other cellular functions, as well as tolerance against stress and drugs.

In this study, we have examined the protein level and cellular localization of the major multidrug transporters, as well as the mRNA expression pattern of all 48 ABC proteins in pluripotent hESC and in their selected, differentiated progenies. To investigate the human ABC transporter expression "fingerprint" in differentiating cells, we have used and characterized an experimental system involving four human cell types of isogenic origin. We have cultured human pluripotent hESCs, and in a

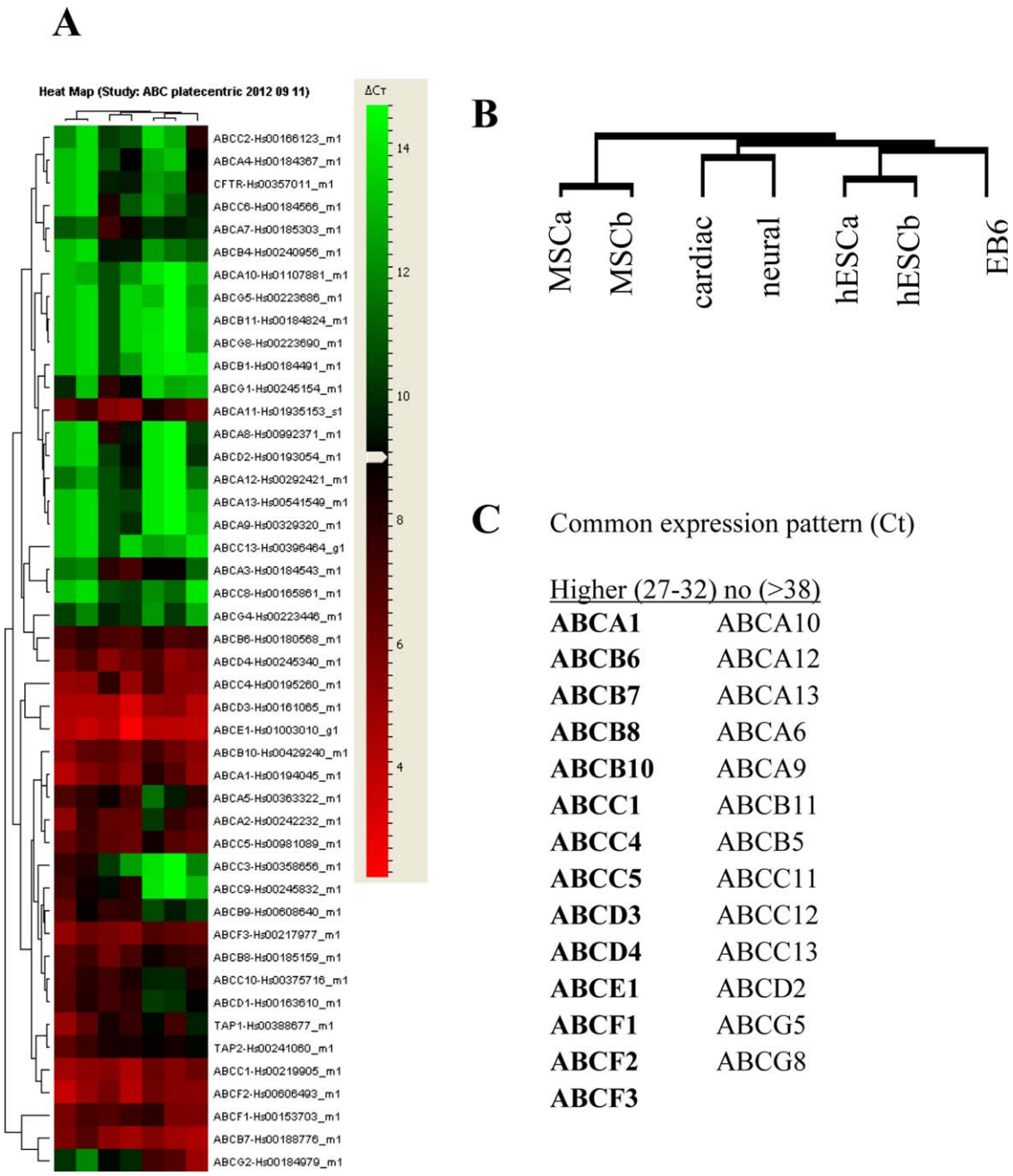


FIG. 6. TLDA analysis of ABC protein gene expression in HUES9 cells and their differentiated offspring. (A) Heat map representation of human ABC protein gene expressions in 7 samples of 4 cell types (hESCs a and b, cardiac, neural, and MSCIs a and b) and the 6 days old EB culture (the common progenitors of differentiated cell types). The ABC genes are shown on the *y*-axis, the 7 samples are ordered on the *x*-axis with biological replicates ("a" and "b"). The color code ranges from high (dCt = 4, light red) through medium (black), to low (dCt = 14, light green) gene expression relative to selected housekeeping genes. (B) Hierarchical clustering of 7 samples allowing a separation based on differentiation status of samples. (C) The listed highest ABC gene expressions ($27 < Ct > 32$, bold) and lowest gene expressions ($Ct > 38$, normal) were common for each cell type showing universal expression pattern for certain ABC proteins. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

spontaneous differentiation model generated well separable differentiated cell types, including cardiac cells, neural cells, and MSC-like cells. The major advantage of this system is that the hES cells and the differentiated progenies have the same genetic background and cell differentiation did not involve major changes in the culturing conditions.

In these experiments first we examined pluripotency and tissue-specific marker protein expression in the selected cell types by flow cytometry and

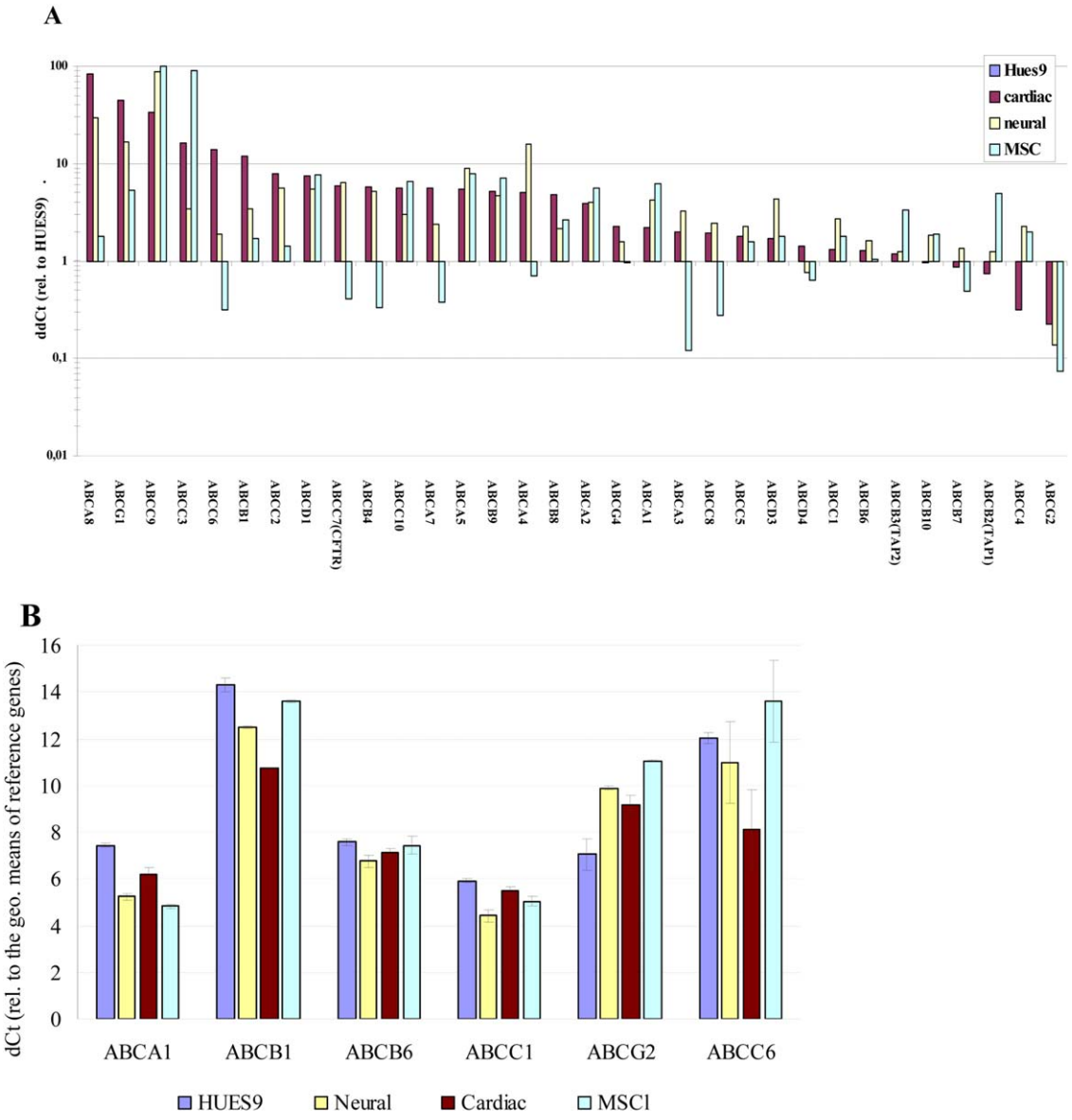


FIG. 7. Relative gene expressions of ABC transporters in HUES9 derived cell types as compared to undifferentiated cells. (A) The relative gene expression level values (dCt) of each transporter in HUES9 are set arbitrarily at 1, and the fold differences in expression (ddCt) in other cell types are presented on the graph. (B) The relative mRNA expression (dCt) of selected transporters in HUES9 cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

immunostaining. We have also carried out the investigation of the mRNA expression pattern of a wide range of pluripotency and differentiation markers by using RT-PCR based quantitative arrays. These studies established a proper characterization of the respective cell types. Surprisingly, fibronectin (FN1) showed the highest relative expression level in all samples; however this is likely caused by the similar cell culturing conditions.

When looking for ABC protein expression, we found that the mRNA expression levels of all the 48 human ABC proteins also clearly distinguished the pluripotent and the respective differentiated cell types. According to recent studies, distinct ABC protein patterns were observed when hESCs (HES2 and HES3) were compared to hematopoietic stem cells, unrestricted somatic stem cells and mesenchymal stem cells (23). It has also been shown that mRNA expression levels are different in hESCs and hESC-derived hMSCs (24). However, none of these studies have investigated the ABC transporter expression at protein level. To perform a detailed characterization, we have used three hES cell lines (HUES9, HUES3, and HUES4) and selected several key ABC transporters involved in multidrug resistance, providing defense against stress conditions, and modulating cellular lipid metabolism. These transporters were examined by using well characterized specific antibodies both in

flow cytometry and confocal microscopy experiments. This way the cellular expression pattern and also the subcellular localization of these important human ABC transporters could be established in the pluripotent and differentiated hES cell derived samples. It is important to note that the obtained ABC transporter protein expression data closely corresponded to the respective mRNA expression results (Fig. 7B). An exception was ABCC6 expression, which was under the detection limit of immunostaining, while the cardiac samples showed relatively high mRNA levels.

During our investigations, we found that the most prevalent multidrug transporter in the undifferentiated hES cells, in accordance with previous data, was the ABCG2/BCRP protein. Several other articles demonstrated ABCG2 expression at mRNA level in various human embryonic stem cell lines (23,25–29), while only one study indicated that human ES cells do not express ABCG2 (30). Recently, ABCG2 mRNA expression was confirmed in 10 different embryonic stem cell lines by Padmanabhan et al., while the ABCG2 protein was under the detection limit in this work. These discrepancies may be caused by the different hESC lines studied, the different culturing conditions, or the protein detection techniques. This latter can be significantly enhanced by using the 5D3 monoclonal antibody together with a specific inhibitor of ABCG2, as described earlier (10,14,31). According to our data, the ABCG2 protein is expressed on the cell surface and, although this expression is heterogeneous, may significantly contribute to the defense mechanisms in pluripotent stem cells (see Refs. 5,10,23,24,32,33).

Another multidrug transporter found at low levels in the undifferentiated cells was ABCC1 (on the cell surface), while we could not detect ABCB1/MDR1 in this cell type. Two other ABC transporters found to be expressed in the hES cells were ABCA1, an important player in lipid/cholesterol extrusion, and ABCB6, a potential player in protecting against toxic heme derivatives. Interestingly, this transporter, noted to be also in the plasma membranes of various cells (16,34), showed only intracellular expression in all cell types examined here.

In the human ES-derived neural cells we observed high level expression for ABCB6 and ABCC1, and in certain cell regions for that of ABCA1 and ABCB1. In the cardiomyocytes high level expression of ABCB6 and lower levels for ABCB1 and ABCA1 were observed. Interestingly, certain cells in the external regions of cardiac and neural tissue samples also showed ABCG2 expression. In the MSCs a predominant, but mostly intracellular expression was observed for ABCA1, ABCB6, and ABCC1, while there was no measurable expression for ABCB1 or ABCG2. As of note, we did not find any significant ABCC6 expression at protein level in any of the cell types examined here.

As a summary, these studies may provide important information for a selective ABC protein expression pattern in human stem cells and in their differentiated offsprings. However, further studies are needed for the establishment of specialized intertissue and interorgan distribution, as well as for the cellular functions of the key human ABC transporters during early human development.

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