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human reproduction update

Potential of human twin embryos generated by embryo splitting in assisted reproduction and research

Laila Noli[1,2], Caroline Ogilvie[3], Yacoub Khalaf[1,2], and Dusko Ilic[1,2,*]

[1]Division of Women's Health, Guy's Hospital, London SE1 9RT, UK [2]Assisted Conception Unit, Guy's Hospital, London SE1 9RT, UK [3]Genetics Laboratories, King's College London, Guy's Hospital, London SE1 9RT, UK

*Correspondence address. E-mail: dusko.ilic@kcl.ac.uk

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BACKGROUND: Embryo splitting or twinning has been widely used in veterinary medicine over 20 years to generate monozygotic twins with desirable genetic characteristics. The first human embryo splitting, reported in 1993, triggered fierce ethical debate on human embryo cloning. Since Dolly the sheep was born in 1997, the international community has acknowledged the complexity of the moral arguments related to this research and has expressed concerns about the potential for reproductive cloning in humans. A number of countries have formulated bans either through laws, decrees or official statements. However, in general, these laws specifically define cloning as an embryo that is generated *via* nuclear transfer (NT) and do not mention embryo splitting. Only the UK includes under cloning both embryo splitting and NT in the same legislation. On the contrary, the Ethics Committee of the American Society for Reproductive Medicine does not have a major ethical objection to transferring two or more artificially created embryos with the same genome with the aim of producing a single pregnancy, stating that 'since embryo splitting has the potential to improve the efficacy of IVF treatments for infertility, research to investigate the technique is ethically acceptable'.

OBJECTIVE AND RATIONALE: Embryo splitting has been introduced successfully to the veterinary medicine several decades ago and today is a part of standard practice. We present here an overview of embryo splitting experiments in humans and non-human primates and discuss the potential of this technology in assisted reproduction and research.

SEARCH METHODS: A comprehensive literature search was carried out using PUBMED and Google Scholar databases to identify studies on embryo splitting in humans and non-human primates. 'Embryo splitting' and 'embryo twinning' were used as the keywords, alone or in combination with other search phrases relevant to the topics of biology of preimplantation embryos.

OUTCOMES: A very limited number of studies have been conducted in humans and non-human primates. The published material, especially the studies with human embryos, is controversial. Some reports suggest that twinning technology will find clinical use in reproductive medicine in the future, whereas others conclude the opposite that human twin embryos created *in vitro* are unsuitable not only for clinical, but also for research, purposes.

WIDER IMPLICATIONS: The blastomere biopsy technique of embryo splitting seems to be unsuitable for either clinical or research purposes; however, embryo bisection, a preferable method of cloning in veterinary medicine, has not yet been tested on human embryos.

Key words: embryo splitting / embryo twinning / blastomere biopsy / lineage commitment / developmental clock

Introduction

The *in vitro* production of genetically identical copies of organisms can be done in two ways: somatic cell nuclear transfer (SCNT) and embryo twinning or splitting. Embryo splitting mimics the natural process that creates identical twins, whereas SCNT is completely artificial. Depending on the developmental stage of an embryo, splitting can be done using either blastomere biopsy (for cleavage-stage embryos) or bisection (for morula or blastocysts). Unlike cloning by SCNT that theoretically can produce multiple, genetically identical, copies, the number of clones that can be produced by embryo splitting is limited by the degree to which preimplantation embryos can be efficiently subdivided.

Research into embryo splitting dates back to the late 1800s, with early studies by Hans Dreisch on sea urchin embryos providing proof-of-concept evidence that individual blastomeres from 2- and 4-cell embryos could develop into larvae (Driesch, 1894). Subsequent studies on salamanders by Hans Spemann demonstrated that individual blastomeres of 2-cell stage embryos possess the potential to develop into full organisms. Hans Spemann also performed the first nuclear transfer (NT) experiments in 1914 (Spemann, 1921).

The obvious advantage of using genetically identical animals is in research, reducing the number of test animals needed for comparative studies (Biggers, 1986; Yang and Anderson, 1992). Nowadays, however, embryo splitting has also been extensively used in veterinary medicine and breeding to maintain high quality and healthy livestock with desirable genetic characteristics (Yang *et al.*, 2007). In most cases, the artificially generated twin embryos are transferred into different recipients to avoid the risks of multiple births (Norman *et al.*, 2004). The first calves generated by embryo splitting were registered with the Holstein Association USA in 1982 and by 2002, a total of 2319 such animals were registered. So far, studies have not detected any differences between products from cloned and non-cloned animals (Norman *et al.*, 2004; Yang *et al.*, 2007).

Until 1997, many thought that cloning from somatic cells would be impossible; however, the turning point came following the first successful cloning of a mammal, Dolly the sheep, 83 years after NT was first described by Spemann (1921). Much of the ethical debate surrounding embryo research since then has become focused on the future potential of cloning adult humans.

Methods of embryo splitting

Using a range of different techniques, embryo splitting or *in vitro* twinning has been performed in several animal species. Early in the 20th century, experiments in fish showed that lowering the incubation temperature or reducing the oxygen concentration decreased the rate of development and thereby increased the incidence of monozygotic (MZ) twins (Stockard, 1921). Similarly, a large number of more recent studies have demonstrated that delayed fertilization in rabbits also led to MZ twinning (Hall, 2003; Aston *et al.*, 2008). It has been suggested that, in these cases, twinning may have been induced by disruptions in communication between blastomeres at various stages of development (Otsuki *et al.*, 2016). The latest improvements in microscopy and micromanipulation technologies have allowed the mechanical induction of MZ twinning *via* blastomere biopsy or blastocyst bisection.

Blastomere biopsy/separation

The blostomere biopsy technique involves the removal of one or more blastomeres from a cleavage-stage embryo and their insertion into a previously prepared empty zona pellucida (ZP) for further development (Fig. 1). To achieve this, the donor embryo is treated first with acidified Tyrode's solution, which produces an opening in the ZP. Blastomeres are then removed *via* an aspirating pipette that is inserted through the ZP hole. The free blastomere is subsequently transferred to a ZP that was previously emptied by removing its cellular content. Embryo splitting using the blastomere biopsy/separation technique has been favourable and the pregnancies have been achieved in most of the large animal species tested, including sheep (Williadsen, 1979), cattle (Williadsen and Polge, 1981), horses (Allen and Pashen, 1984) and pigs (Ash *et al.*, 1989). Similar success rates of the procedure in these species were confirmed in later studies from multiple groups. However, in non-human primates such as *Rhesus* monkeys, the studies with a similar strategy were not successful (Chan *et al.*, 2000; Mitalipov *et al.*, 2002), and the molecular basis for this difference has not been addressed.

Bisection

This method is used to mechanically divide post-compaction embryos into two equal halves which, in case of the blastocyst, include an even

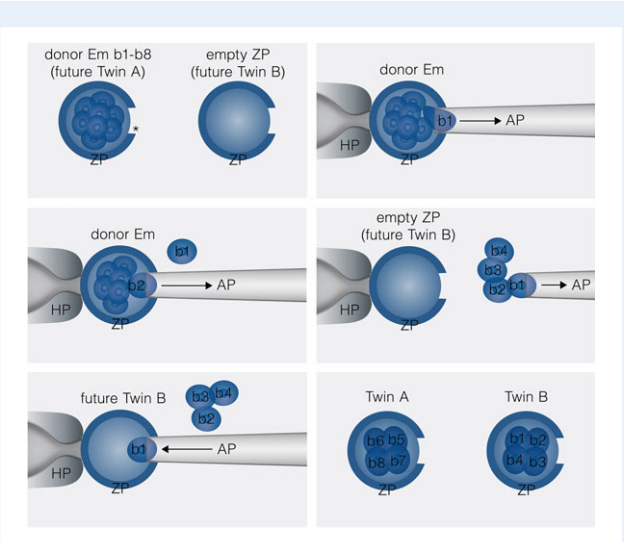

Figure 1 Embryo splitting of 8-cell embryo using blastomere biopsy approach. The donor embryo (future Twin A) is treated first with acidified Tyrode's solution or a laser beam to generate an opening in the zona pellucida (ZP). Blastomeres are then removed *via* an aspirating pipette (AP) that is inserted through the ZP hole. The free blastomeres are subsequently transferred to a ZP that was previously emptied by removing its cellular content (future Twin B). b1–b8, individual blastomeres of 8-cell cleavage-stage embryo; Em, embryo; HP, holding pipette.

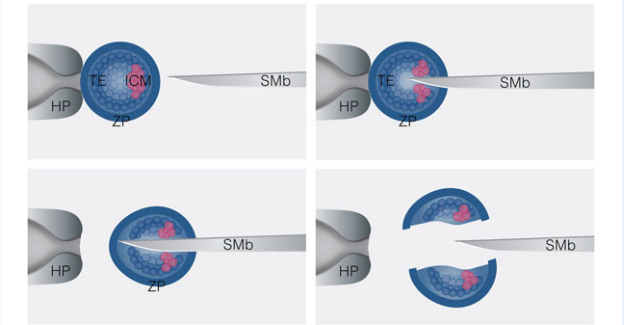

Figure 2 Embryo splitting using blastocyst dissection approach. Post-compaction embryos are divided mechanically into two equal halves which, in the case of the blastocyst, include an even distribution of inner cell mass (ICM) and trophectoderm (TE) between the resultant demi-embryos. SMb, surgical microblade; ZP, zona pellucida.

distribution of inner cell mass (ICM) and trophectoderm (TE) between the resultant demi-embryos (Fig. 2). Using this technique, The MZ twin embryos are then immediately cultured *in vitro* using a culture medium that encourages further development. Although this procedure has not been attempted in humans, blastocyst bisection has been effective in a number of large mammalian species, including goat (Udy, 1987), sheep (Széll and Hudson, 1991), cattle (Ozil *et al.*, 1982; Williams *et al.*, 1984) and pigs (Nagashima *et al.*, 1989). Numerous studies have followed, most of them in cattle.

In general, no difference has been found in the proportion of pregnancies or twins born from demi-embryos created by either of these two methods (Tagawa *et al.*, 2008).

Methods

A comprehensive literature search was carried out using PUBMED and Google Scholar databases to identify studies on embryo splitting in humans and non-human primates. 'Embryo splitting' and 'embryo twinning' were used as the keywords, alone or in combination with other search phrases relevant to the topic of biology of preimplantation embryos.

Results

Potential benefits of embryo splitting

Embryo splitting has potential benefits in both assisted reproduction programmes and research.

For patients with low response to hormonal stimulation, the technology, if proven to be safe, may provide additional embryos for intrauterine transfer and in such a way as to increase the likelihood of pregnancy (Illmensee *et al.*, 2010).

Currently, there is a severe shortage of human embryos that have been donated for research purposes, and there is an associated potential for future restrictions on human developmental research. Since the policy to transfer embryos at the blastocyst stage of development was introduced, there has been a reduction in the number of cleavage-stage embryos that are available for research. Therefore, splitting embryos at the cleavage stage offers the potential to increase the number of viable embryos, and it may therefore be a suitable means of addressing the current shortage in the availability of research embryos. In addition, embryo splitting provides the opportunity to obtain genetically identical embryos, a feature that is ideal for comparative research. Having genetically identical embryos in control (non-treated) and experimental (treated) groups would eliminate the bias of genetic background and fewer embryos would be needed to reach conclusions .

Embryo splitting in farm animals

The most common outcome of producing MZ offspring is twins or singletons, but triplets and quadruplets have also been reported in cattle following the transfer of quartered embryos (Willadsen and Polge, 1981; Johnson *et al.*, 1995). An increase in the production of cattle from the transfer of split embryos produced using these approaches has been reported (Leibo and Rall, 1987). In addition to fresh transfer, the use of frozen-thawed demi-embryos has also been attempted (Seike *et al.*, 1991). Remarkably, in the case of cattle, the normal pregnancy rate from a whole embryo transfer is ~70%. The equivalent rate for a demi-embryo is ~50–55%, and this method, therefore, provides a 30–40% increase in the chance of conception (Seike *et al.*, 1989; Wood and Trounson, 2000). In addition, no developmental or physiological defects have been reported in the offspring resulting from these split embryos, which develop into healthy

animals. However, a study in horses (Allen and Pashen, 1984) has shown that the size of twins born from embryos of unequal allocations of cells (one versus two blastomeres) is different and that the disparity persists into adult life.

Embryo splitting in non-human primates

Paving the way towards the splitting of human embryos, the technology was investigated first in rhesus macaques (*Macaca mulatta*). Rhesus macaques are a non-human primate model that is highly related to humans in evolutionary, genetic and physiological terms. Therefore, they can be used to gain crucial information for human research (Van de Berg and Williams-Blangero, 1996). Specifically, the successful development of methods for producing MZ twins in monkeys could lead to significant advancements in the scientific understanding of human disease, MZ twinning and the effects of the maternal environment on the epigenetic profile of a developing embryo. In addition, these studies could also lead to the development of better animal models for vaccine trials and tissue transplantation studies (Schramm and Paprocki, 2004a, b). However, current strategies aimed at producing MZ twins in rhesus monkeys have met with only limited success (Schramm and Paprocki, 2004a,b). Blastomere separation studies performed in rhesus monkeys gave rise to blastocysts with significantly different total cell numbers within a given demi-embryo pair (Mitalipov *et al.*, 2002). This may have resulted from the asymmetric distribution of cytoplasm between the blastomeres during separation or a difference in the polarity of cells within the embryo. There were 22 pairs of demi-embryos created using blastomere separation and then transferred, resulting in a pregnancy rate of 33% (7 out of 22). Among these pregnancies, two twin pregnancies (9%) were initiated, but neither of the twin pairs developed to term (Mitalipov *et al.*, 2002). In another study, a total of 368 embryos were created by splitting 107 rhesus embryos at the 8-cell stage. The compaction rate was not affected by number of identical clones produced from one embryo. However, the blastocyst formation was reduced by each identical clone produced and no blastocyst was formed when splitting beyond sextuplets was attempted. In an attempt to produce sets of identical quadruplets, each originally consisting of two blastomeres, a pair of the quadruplet embryos was transferred to each of two fertile surrogates. However, only one monkey was born: a healthy female named Tetra (Chan *et al.*, 2000).

Data from Chan *et al.* showed a reduction in the developmental potential of the blastocysts when blastomere separation was performed at later cleavage stages between the 8- and 16-cell stages (Chan *et al.*, 2000). Mitalipov demonstrated that blastomere separation at the 2- or 4- cell stage can produce demi-embryos that develop into blastocysts comparable to non-manipulated control embryos (Mitalipov *et al.*, 2002). The ratio of ICM to TE and the ratio of ICM to total cells in these split blastocysts were similar to the ratios in non-manipulated control blastocysts. However, the total number of cells in the split blastocysts was almost 50% lower than the number in the controls, similar to results recorded in other species (Willadsen and Polge, 1981; Willadsen *et al.*, 1981; Willadsen, 1981; Willadsen, 1989).

In the case of the demi-embryos that were developed using blastocyst bisection methods, a pregnancy rate of 33% (4 out of 12) was achieved. However, no twin pregnancies were established, and all of the pregnancies were singletons (Mitalipov *et al.*, 2002). While blastocyst bisection led to the formation of higher numbers of demi-embryos, the number of clinical pregnancies per oocyte was higher for embryos produced by blastomere separation (Mitalipov *et al.*, 2002). However, in spite of the fact that pregnancies have been established using both methods of embryo splitting in rhesus monkeys, they have both resulted in only singleton offspring, whether they were implanted in different or the same recipients (Chan *et al.*, 2000; Mitalipov *et al.*, 2002).

There could be multiple reasons why twinning approaches in rhesus monkeys have not met with similar success to that seen in mice or large domestic animals. Normally, rhesus monkeys do not carry twins; only ~0.25% of naturally occurring pregnancies are twin pregnancies. Even then, the offspring rarely survive due to various complications (Hendrickx and Binderd, 1980; Schramm *et al.*, 2002). Pregnancy rates following transfer of two embryos generated *in vitro* are in range of 25–40%, with <15% resulting in twin gestations (Lanzendorf *et al.*, 1990; Mitalipov *et al.*, 2002; Schramm *et al.*, 2002). In both seminal primate studies (Chan *et al.*, 2000; Mitalipov *et al.*, 2002), more than one twin embryo was transferred into each recipient. Single twin embryo transfers into separate recipients might improve outcomes greatly. In addition, rhesus monkeys cannot be synchronized and optimal timing for transfer requires cryopreservation, which has not been very successful for split embryos regardless of species (Weston *et al.*, 1996).

Embryo splitting in humans

The first human embryo splitting procedure was reported by a team of researchers including Robert Stillman and Jerry Hall from George Washington University in Washington, DC, in October 1993, at a joint meeting of the American Fertility Society and the Canadian Fertility and Andrology Society (Hall *et al.*, 1993). Researchers used polyspermic embryos that would not survive and would have been routinely discarded. They separated blastomeres from 17 2- to 8-cell embryos, covered them in an artificial ZP and cultured them for up to 32-cell divisions. The researchers claimed that their results pointed a way for enhanced infertility treatment in humans. However, it was later found that the study did not possess the valid Institutional Review Board approval, and the authors were reprimanded and instructed to destroy their data (Fackelmann, 1994; Macklin, 1995). The case led to fierce ethical debate on embryo cloning (Cohen and Tomkin 1994; Cohen, 1994; National Advisory Board on Ethics in Reproduction, 1994; Verhey, 1994; Macklin, 1995; Burke 1996). In the wake of protests from the scientific community and media, the American Society for Regenerative Medicine's (ASRM's) Ethics Committee formulated a statement concerning embryo splitting and its use in infertility treatment, which was subsequently accepted by the Board of Directors in December 1995 (The Ethics Committee of the American Society for Reproductive Medicine, 2004).

Ethical considerations

Despite the fact that spontaneous MZ twinning is a natural form of cloning, twinning of human embryos *in vitro* continues to be a matter

of ethical debate. The ethical considerations have given rise to a regulatory framework to restrict research and development in human cloning, which in the UK, includes the particular methodology of embryo splitting. The process of embryo splitting falls under the generic heading of human cloning, which is an emotive and controversial topic and the ethical debate regarding embryo splitting is, therefore, more likely to attract public attention and scrutiny.

Biological barriers are likely to prevent human reproductive cloning by NT in the foreseeable future and, if the cloning ever happens, it is more likely to be achieved by embryo splitting. There is an argument that embryos produced from splitting are not artificial clones and that they should be available for use in therapy. First, embryo splitting is distinct from the process of NT, which transfers nuclear content from somatic cells for the purposes of creation of a child or therapeutic application. NT effectively duplicates a fully formed human being, whereas embryo splitting replicates the natural process that forms MZ twins during embryogenesis. NT circumvents normal gametogenesis and fertilization and prevents the normal programming of an embryo's genome. Second, the split embryos are dichorionic and diamniotic, with a separate placenta and amnion, which reduces the risk of common complications in twin pregnancies, such as cord entanglement or twin–twin transfusion. Third, since MZ twinning is also a natural phenomenon, significant information can be obtained from analysing the behaviour of twins. Finally, the embryo splitting procedure familiarizes parents with the possibility of twin pregnancies and their risk, which better prepares them for these events.

Most mammalian reproductive cloning that is performed using SCNT gives rise to offspring that either die during gestation or suffer from large offspring syndrome, which is typified by respiratory and metabolic abnormalities and an enlarged, dysfunctional placenta (Jaenisch, 2004). Clones that do survive usually have a normal phenotype and are physiologically able to produce healthy offspring (French *et al.*, 2006). In addition, no significant behavioural or psychological problems related to MZ twining have so far ever been reported and the technology therefore seems to be safe. Hence, the ethical debate centres on whether human reproductive cloning by embryo splitting, if possible in the foreseeable future without increasing the risk of abnormalities in the child, is ethically justifiable.

In order to avoid complications of the twin pregnancies, only one of the split embryos might be transferred, whereas the other one could be cryopreserved for future use. However, this would likely give rise to an ethical debate on MZ twins of different age.

There are various issues to consider in this ethical debate, including the right to life of the embryo and the interests of the child, the societal consequences and teleological perspectives (Strong, 2005). For example, one controversial and highly discussed aspect of embryo splitting is whether artificial twinning violates the right of an unborn child to be unique. However, given that embryo splitting replicates a natural process, none of these arguments carry sufficient ethical justification to warrant a total ban on human reproductive cloning using this methodology. It is widely accepted that embryo splitting must not be used for unethical purposes, such as the generation of histocompatible embryos with the intention of organ transplantation. Therefore, the main consideration, from both a scientific and a clinical perspective, is whether this methodology can be used without an increased risk of abnormalities.

Since Dolly the sheep was born in 1997, the international community acknowledged the complexity of the moral arguments that are related to this research and has expressed concern about the potential for reproductive cloning in humans. Numerous countries have formulated bans either through laws, decrees or official statements (UNESCO, 2004).

Regulatory framework

In terms of regulation at the international level, the General Conference of UNESCO unanimously acclaimed the Universal Declaration on the Human Genome and Human Rights in 1997. This international instrument was subsequently endorsed by the General Assembly of the United Nations in 1998, which declared that human reproductive cloning is a practice against human dignity (UNESCO, 2004).

At the European level, the Additional Protocol to the Convention of the Council of Europe for the Protection of Human Rights and Dignity of the Human Being with regard to the Application of Biology and Medicine on the Prohibition of Cloning Human Beings was developed in 1998 and took effect in 2001. It states that 'any intervention seeking to create a human being genetically identical to another human being, whether living or dead, is prohibited' (Council of Europe, 1997).

In terms of the UK and the US regulations, following controversy over the original research in the USA, the ASRM published a statement concerning embryo splitting in 1995 stating that 'splitting one embryo into two or more embryos could serve the needs of infertile couples in several ways' and that they did not recognize a significant ethical objection to the placement of two or more embryos with the same genome in the recipient uterus with the aim of producing a single pregnancy, as long as the parents undergoing the fertility treatment were duly apprised of the outcome of this procedure (The Ethics Committee of the American Society for Reproductive Medicine, 2004). A bill was subsequently passed by the House of Representatives in 2003 that banned reproductive and therapeutic cloning. The bill paved the way for legislation to be passed in different states that outlawed either reproductive cloning or both therapeutic and reproductive cloning. Fifteen states have laws on human cloning. These laws specifically define cloning as an embryo that is achieved *via* NT (National Conference of State Legislatures, 2003; 2008) and do not include embryo splitting. Thus, in the USA, legislation on reproductive cloning relates specifically and exclusively to NT methodologies. Furthermore, since the 1995 statement by the ASRM, legislation has allowed embryo splitting as an infertility treatment. The UK includes cloning both by embryo splitting and NT in the same legislation. There is, therefore, a major difference in laws between these two countries.

The Human Fertilisation and Embryology Authority (HFEA) have not supported the views of the ASRM. The original HFEA Act 1990 (The Human Fertilisation and Embryology Authority, 1990), which regulates the medical and scientific manipulation of embryos, defined an embryo as a 'live human embryo where fertilization is complete', and therefore the Human Reproductive Cloning Act was brought into force in 2001 to cover embryos created by reproductive cloning techniques. It prohibits reproductive cloning and states in Chapter 23 that 'a person who places in a woman a human embryo which has

been created otherwise than by fertilization is guilty of an offence and this offence carries up to 10 years and/or an unlimited fine' (The Human Reproductive Cloning Act, 2001). In 2002, a ruling came into force that allowed for clones produced by nuclear replacement to be classified as embryos, and reproductive cloning therefore subsequently fell under the HFEA Act (The Human Fertilisation and Embryology Authority, 1990). Furthermore, the 6th HFEA Code of Practice (paragraph 8.9 ii) specifies that the embryo splitting procedure must not be used by fertility clinics to produce embryos for treatment purposes (The Human Fertilisation and Embryology Authority, 2003). The HFEA now stipulates that a license must be granted for therapeutic cloning research. The first license was awarded by the HFEA in 2004 to scientists from the University of Newcastle to create human embryonic stem cells (hESC) *via* cell NT (The Human Fertilisation and Embryology Authority, 2004).

In Australia, under a license issued by the National Health and Medical Research Council (NHMRC) Embryo Research Licensing Committee, cloning by SCNT for therapeutic purposes is permitted, whereas reproductive cloning is banned (The Prohibition of Human Cloning for Reproduction Act, 2002). Other countries are even more restrictive. Hong Kong prohibits the 'replacing of the nucleus of a cell of an embryo with a nucleus taken from any other cell' as well as the 'cloning of any embryo' (Human Reproductive Technology Ordinance, 2000). The scope of the latter, therefore, is arguably the widest prohibition, as it rules out all cloning techniques, such as cell nucleus replacement, embryo splitting, parthenogenesis and cloning using stem cell lines. At the present, there is no country which permits reproductive cloning of humans by legislation or guidelines (National Legislation Concerning Human Reproductive and Therapeutic Cloning. UNESCO. 2004). However, only a few, such as the UK (The Human Fertilisation and Embryology Authority, 2003), Australia (The Prohibition of Human Cloning for Reproduction Act, 2002), Singapore (Human Cloning and Other Prohibited Practices Act, 2004) and India (Ethical Guidelines for Biomedical Research on Human Participants, 2006), define embryo splitting. Even when they do, the embryo splitting technology always ends up under the umbrella of forbidden activity of human cloning.

Controversies of the initial studies

More recent studies from two groups has suggested that the use of these types of embryo splitting techniques may result in the formation of viable and morphologically adequate blastocysts in humans (Van de Velde *et al.*, 2008; Illmensee *et al.*, 2010, 2011). However, neither of these three studies presented comprehensive qualitative analyses of the embryos that were created using splitting techniques. In addition, the results have been somewhat contradictory. For example, Van de Velde *et al.* (2008) reported that blastomeres derived from 4-cell embryos possessed sufficient plasticity to form a proper blastocyst. Illmensee *et al* (2010) could not reproduce that result; they reported that the blastomeres from 8-cell embryos, rather than the blastomeres derived from embryos at earlier stages, led to the development of the blastocysts. The most recent study by Noli *et al.* (2015a) suggested that human twin embryos created *in vitro* using a blastomere biopsy technique were unsuitable for not only for clinical but also research purposes, regardless of the stage of development of the parental embryos.

Split embryos were evaluated in terms of their size, biological behaviour, morphology and expression of an ICM marker, NANOG, using immunocytochemistry (Van de Velde *et al.*, 2008). Blastocysts that were derived from individually cultured blastomeres resulted in embryos that were one-quarter the size of regular human embryos that were cultured *in vitro*. It was also shown that in spite of their smaller size, the blastocysts underwent compaction on Day 4 and cavitation on Day 5, similar to the control human embryos. On Day 6, the majority of these split embryos were able to form complete blastocysts that possessed a distinct ICM and TE, even though the yield of cells per embryo was very low. The presence of NANOG-positive cells suggested that the ICM cells in the split embryos are pluripotent. In one embryo, all four blastomeres developed into viable blastocysts, each with a cohesive TE and a tightly packed ICM, with some cells expressing NANOG. Although the sample size was small, the authors successfully demonstrated that the single cells isolated from a 4-cell stage human embryo could individually develop into mini-blastocysts with delineated ICM and TE cells (Van de Velde *et al.*, 2008).

Illmensee *et al.* (2010) demonstrated that the ideal developmental stage for splitting human embryos is the 6–8 cells stage, in terms of both splitting and developmental efficiency. The authors claimed that the number of blastocyst-stage embryos that formed in the study significantly exceeded the original number of embryos that were split at this stage. The split embryos appeared to hatch earlier, however, possibly because of compromised ZP integrity from the blastomere biopsy. Earlier hatching may enhance the implantation capacity of embryos, especially in patients who may have experienced multiple implantation failures (Primi *et al.*, 2004; Petersen *et al.*, 2005). In a second study by the same group, the authors showed that the MZ characteristics of triploid embryos were not altered by embryo splitting, with the resulting twin embryos containing the same allelic short tandem repeats (STR) sequences as expected (Illmensee *et al.*, 2011). Six selected polymorphic STR markers in the HLA locus on Chromosome 6 were selected and subjected to nested multiplex PCR analysis. Fluorograms from five pairs of twin blastocysts showed that peak positions for the detected STR profiles were identical between twin embryos. This was the first study to demonstrate the monozyogocity of twinned human embryos at the DNA level (Illmensee *et al.*, 2011).

Our group has analysed the largest number of twin embryos ($n = 176$) reported to date. Twin embryos created by splitting either early (2–5 blastomeres, $n = 43$) or late (6–10 blastomeres, $n = 45$) cleavage-stage embryos were compared with IVF embryos that later resulted in pregnancy and live birth upon single blastocyst transfer ($n = 42$) (Noli *et al.*, 2015a). The comparative methods used include morphokinetics and immunodetection of lineage markers. In addition, we attempted a derivation of hESC lines following our standard protocols (Ilic *et al.*, 2012; Stephenson *et al.*, 2012). We found that twin embryos were smaller and that the size of the twin blastocyst was proportional to the number of blastomeres used for creation of the embryo. In addition, the ICM was generally relatively poorly developed, if distinguishable at all. Immunostaining revealed that the majority of the cells expressed markers of both ICM and TE, which raises a question about their developmental competence. Indeed, none of the 10 twin embryos with a morphologically distinguishable ICM-like structure gave rise to hESC lines, even though the success rate of the protocol is 30–50% when using IVF embryos.

Molecular mechanisms

Human development is under strict temporal control

Embryogenesis follows a precise and specific programme shared by all individuals of the same species including human. The programme is strictly regulated by developmental timers that are set at fertilization and inherited in every daughter cell. Timers are not cell–cell contact dependent; they are intrinsic to each blastomere. For example, the transition from rapid and symmetrical cell divisions to slow and asymmetrical divisions in *Xenopus laevis* embryos always occurs at the 12th cleavage after fertilization (Masui and Wang, 1998; Wang *et al.*, 2000). After the 12th cleavage, the cells become contact dependent and cell cycle durations become variable. The molecular mechanisms governing such strict schedules are unclear. It could be simply due to the physical time needed for successive gene activations and biochemical reactions to take place or to the nuclear/cytoplasmic ratio (Masui and Wang, 1998; Wang *et al.*, 2000). Research on the spatial and temporal monitoring of mouse embryonic development has suggested that a developmental clock also exists in mammals (Morris *et al.*, 2012). The landmark developmental events of cell compaction, lineage commitment and cavitation took place at the same time in the embryos split at the 2-cell stage as in the intact non-manipulated controls. However, their developmental potential was not the same. Unless the ICM contained a minimum of four NANOG-positive pluripotent stem cells at the time of implantation, development did not proceed. Modulation of Fgf and Wnt signalling increased the number of pluripotent cells in demi-embryos and rescued the developmental failure phenotype. Interestingly, the same treatment did not work if the blastomeres were separated at the 4-cell stage indicating that at that stage, at least in mouse, all blastomeres do not have equal developmental potential. Indeed, cell fate in 4-cell mouse embryos is biased through heterogeneity in Oct4 and Sox2 targets (Goolam *et al.*, 2016).

Data from our study with human embryos suggested that the human preimplantation development is also subject to strict temporal control (Noli *et al.*, 2015a). The size of twin embryos was proportionate to the number of cells used for their creation: more cells used as a starting material gave bigger blastocysts (Fig. 3). The average diameter of blastocysts generated from a single blastomere of 2- or 3-cell stage embryos was 86.93 µm, and from four blastomeres of 8- or 9-cell embryo, it was 102.25 µm, whereas an average diameter of blastocysts formed from intact embryos was 120.87 µm. The estimated time of development from the pronuclear (2PN) stage to expanding blastocyst for participating blastomeres is quite similar between control intact embryos (116.22 hours) and the twin embryos, regardless of the time of splitting (112.70–115.92 hours). Similar to the ICM in mouse (Morris *et al.*, 2012), sheep (Willadsen, 1981) and rhesus monkey demi-embryos (Schramm *et al.*, 2002), the ICM in human twin embryos had fewer cells in comparison with the ICM in intact controls (Noli *et al.*, 2015a). Whether the number of pluripotent cells in human twin embryos could be increased by manipulating fibroblast growth factor (FGF) and WNT signalling or some other pathways, such as IGF1 (Kimber *et al.*, 2008; Noli *et al.*, 2015b) remains to be investigated.

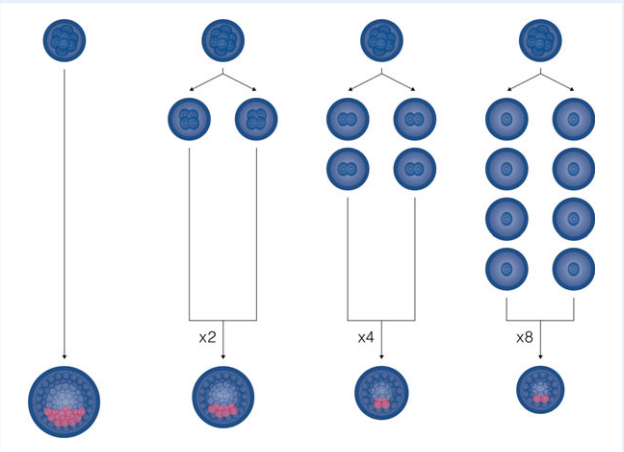

Figure 3 The size of twin embryos is proportionate to the number of cells used for their creation: more cells used as the starting material produce bigger blastocysts.

The role of cell–cell interactions in fate specification and Hippo signalling

Previous research in the murine system has supported the importance of continuous cell–cell interaction in the regulation of blastomere fate, as biopsied blastomeres tend to re-establish cell–cell interactions subsequent to their transfer into recipient ZP (Johnson and Ziomek, 1981; Lorthongpanich *et al.*, 2012). A pre-patterning model (Piotrowska and Zernicka-Goetz, 2001; Piotrowska *et al.*, 2001) posits that the ICM and TE lineages undergo predetermination due to the asymmetrical localization of molecular determinants in the oocyte; conversely, the inside–out (Tarkowski and Wroblewska, 1967) and cell polarity models (Johnson and Ziomek, 1981) hypothesize that a decision-making process is dependent on cell position within the embryo.

Our results have suggested that lineage determination in human embryos takes place through the cell position-dependent inside–out or cell polarity models (Noli *et al.*, 2015a). In the pre-patterning model, the number of blastomeres used for the creation of twin embryos is not a governing factor; therefore, the probability of forming twin embryos with better quality ICMs would have been higher than what we observed.

Recently completed studies in a mouse model have implied that the Hippo pathway is responsible for the translation of positional information to lineage specification, acting primarily through the downstream mediator proteins YAP1 and TEAD1-4 (reviewed in Lorthongpanich and Issaragrisil, 2015). The immunostaining studies of YAP1 expression in twin embryos suggested that this mechanism may also be conserved in human embryonic development (Noli *et al.*, 2015a).

Lineage commitment and reproductive competence

The first embryonic cell fate commitment, ICM and TE lineage segregation, begins at the compaction/morula stage, when asymmetric cell division pushes one cell inwards and the other daughter cell remains outside (Bruce and Zernicka-Goetz, 2010; Lorthongpanich *et al.*, 2012).

This decision, however, is not ultimate. The outer cells from the morula and the early blastocyst tend to retain their plasticity for a short period of time. These cells express the pluripotency markers POU5F1 (OCT4), SOX2 and SALL4 and the TE markers HLA-G and KRT18 but not CDX2 (Cauffman *et al.*, 2009; Chen *et al.*, 2009; Verloes *et al.*, 2011). NANOG expression has also been reported in the polar TE cells of the early blastocyst (Cauffman *et al.*, 2009). Upon isolation from fully developed human blastocysts and their subsequent reaggregation, TE cells were able to develop into blastocysts expressing the pluripotency marker NANOG (De Paepe *et al.*, 2013). Furthermore, most of the isolated TE cells did not regain their original position when placed in the centre of the embryo; instead, they integrated into the ICM with subsequent expression of NANOG, indicating that the TE cells at that stage of embryonic development were not yet fully committed (De Paepe *et al.*, 2013).

We found that Day 5 twin embryos expressed NANOG almost universally, with NANOG-positive cells co-localizing with the TE markers CDX2 and GATA2 (Noli *et al.*, 2015a). Although TE cells co-expressing NANOG, CDX2 and GATA2 were reduced in number by Day 6, they still constituted a significant fraction of the TE. However, at Day 6, we could find only 1–4 NANOG-positive cells that lost the expression of CDX2 and GATA2, indicating the initial formation of the ICM. SOX17, a marker of primitive endoderm, was also detected in twins with larger ICMs on Day 6. The data indicated that the molecular events responsible for first and second fate commitments of the embryos were taking place in the split embryos; however, they lagged behind the control intact blastocysts obtained by IVF. Irrespective of these findings, in almost all twins with distinguishable ICM-like structure on Day 6, the ICM was small and of poor quality. This may suggest that, similar to the experimental results with mouse split embryos, the epiblast possessed an insufficient number of cells to continue the post-implantation development of the conceptus, rendering the twin embryos reproductively incompetent (Balbach *et al.*, 2010; Morris *et al.*, 2012).

miRNAs are known regulators (mostly repressors) of target gene expression; they are secreted (Valadi *et al.*, 2007) from embryos *in vitro*, and attempts have been made to link specific miRNAs detected in spent blastocyst medium (SBM) with embryo ploidy status and reproductive competence (McCallie *et al.*, 2010, Kropp *et al.*, 2014, Rosenbluth *et al.*, 2014, Capalbo *et al.*, 2016). Analyses of spent culture media collected at different stages of preimplantation embryo development showed a marked increase in the number of miRNA detected at the blastocyst stage (Capalbo *et al.*, 2016). These authors compared miRNAs in SBM from 25 implanted euploid embryos which had later resulted in the birth of a healthy baby following a single embryo transfer, with 28 non-implanted embryos and identified miR-20a and miR-30c as statistically significantly more abundant. Analysis of miRNA detected in SBM of twin blastocysts revealed a unique profile (Noli *et al.*, 2016). Nearly a quarter (11 out of 48) of the miRNA found in SBM of twin embryos were not detected in SBM or TE samples of normal blastocysts. Furthermore, levels of those detected in spent culture media of both twin and normal blastocysts were consistently different, including miR-30c, which was significantly lower in media of twin embryos. Although the exact role of miRNAs secreted from developing embryos is unclear, it likely reflects development-related specific gene activity. Since the majority of cells in twin embryos express both TE and ICM markers, the unique miRNA profile in spent media of twin blastocysts might be a result of differential lineage commitment in these embryos.

Conclusion and prospectives

Data from the most recent studies on split human embryos have suggested that embryo splitting using the blastomere biopsy or separation techniques may be neither a suitable source of genetically identical embryos for research purposes, nor a novel-assisted reproduction treatment (Noli *et al.*, 2015a). Aberrant lineage commitment and a strict developmental clock demonstrated in human split embryos may also explain the poor results reported from twinning experiments in non-human primates (Chan *et al.*, 2000; Mitalipov *et al.*, 2002). However, data provided by the UK HFEA showed that in 1104 live births from cycles involving single embryo transfer, the rate of multiple pregnancies was 2.3%, which is higher than the rate of 0.4–0.45% reported in *in vivo* conceptions (Blickstein *et al.* 2003; Aston *et al.*, 2008). This suggests that IVF may lead to embryo splitting *in vivo* and that further exploration of *in vitro* techniques is warranted.

Embryo bisection, a preferable method of cloning in veterinary medicine, has not yet been used on human embryos. If the parental embryo is of high quality, especially with a large ICM, a careful bisection might yield two developmentally competent embryos. Although such a technical approach might benefit fertility patients, the approach is limited by lack of quality control, and there is as yet no country in which regulations permit embryo splitting for clinical purposes. The possibilities of maintaining human embryos in culture beyond Day 7 are still very limited and there would be little room for comparative studies. However, the most recent advances in extending the culture of human embryos beyond Day 7 may allow some progress (Deglincerti *et al.*, 2016; Shahbazi *et al.*, 2016); this extended culture will allow more detailed research into the early development of the embryo, leading to a better understanding of the limitations of embryo manipulation.

As IVF techniques improve, the need to provide additional embryos for transfer should become less pressing, and this, together with the ethical problems associated with this type of embryo manipulation, may mean that this approach may never reach the clinic.

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Authors' roles

L.N. conducted the review of literature and wrote the first draft of the paper. D.I. and Y.K. supervised the work, helped in performing the review of literature and corrected the paper. C.O. critically reviewed the manuscript. All authors approved the final version of the manuscript.

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Conflict of interest

All authors declare that they have no conflict of interest.

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