The four blastomeres of a 4-cell stage human embryo are able to develop individually into blastocysts with inner cell mass and trophectoderm

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BACKGROUND: Early mammalian blastomeres are thought to be flexible and totipotent allowing the embryo to overcome perturbations in its organization during preimplantation development. In the past, experiments using single blastomeres from 2-, 4- and 8-cell stage mammalian embryos have provided evidence that at least some of the isolated cells can develop into healthy fertile animals and therefore are totipotent. We investigated whether isolated blastomeres of human 4-cell stage embryos could develop in vitro into blastocysts with trophectoderm (TE) and inner cell mass (ICM). METHODS: Six 4-cell stage human embryos were split and the four blastomeres were cultured individually. The expression of NANOG, a marker for ICM cells, was analysed by immunocytochemistry. RESULTS: The majority of the blastomere-derived embryos followed the normal pattern of development with compaction on Day 4 and cavitation on Day 5 and developed into small blastocysts with TE and ICM on Day 6 (n = 12). The four cells of one embryo were individually capable of developing into blastocysts with TE and ICM, and NANOG was expressed in the ICM. CONCLUSIONS: Although based on a small number of embryos, we conclude that the blastomeres of a 4-cell stage human embryo are flexible and able to develop into blastocysts with ICM and TE.

Keywords: blastomere; totipotent; inner cell mass; NANOG; blastocyst

Introduction

Early blastomeres are assumed to be totipotent allowing the embryo to regulate its development in order to overcome perturbations in its organization such as cell loss. Direct evidence for totipotency is provided when an isolated blastomere is able to develop into a normal fertile offspring (reviewed in Edwards and Beard, 1997). In mice, splitting experiments have shown that a single blastomere of a 2-cell stage embryo is totipotent, but a single blastomere of a 4-cell stage embryo is not. A blastomere of a 4-cell stage mouse embryo can develop into a blastocyst and implant, but will die soon because of its small size and the insufficient cell number of its inner cell mass (ICM). Indirect evidence that at least some of the blastomeres of a 4-cell stage mouse embryo are totipotent was provided in chimeric models using carrier blastomeres of a different genotype to keep the size and cell number of the embryos normal (Tarkowski et al., 2001). Some isolated cells of an 8-cell stage mouse embryo form only small trophoblasts (Edwards and Beard, 1997). Both blastomeres of 2-cell stage avine embryos can develop into lambs (Willadsen and Godke, 1984), and all the cells of a 4-cell stage bovine embryo are totipotent (Johnson et al., 1995).

The aim of our study was to investigate whether isolated blastomeres of human 4-cell stage embryos could develop in vitro into blastocysts with trophectoderm (TE) and ICM. To our knowledge, this is the first time such an experiment has been carried out.

Materials and Methods

Oocytes and embryos

According to Belgian legislation, embryos can be generated for research purposes as long as the study is filed to the Federal Ethical Committee on Embryos. The Ethical Committee of UZ Brussel gave its approval for this project because the embryos would not be transferred into a uterus and would therefore be destroyed by the experimental procedure. Therefore, reproductive cloning, which is not allowed in Belgium, was not an issue here. Mature oocytes, empty zona pellucida (ZP) from immature oocytes and blastocysts were donated for research by couples treated at our IVF centre, who had given their informed consent. Embryos were obtained after micro-injection with the spermatozoa of a consenting donor.

In vitro culture, micromanipulation and inverted microscopy

Oocytes were denuded and injected with spermatozoa and the resulting zygotes were cultured (Medicult series, Lucron Bioproducts,
Belgium) as described previously (Van de Velde et al., 2000). Fertilization was assessed 16–22 h after microinjection, embryo quality was evaluated on Day 2 prior to biopsy, embryos with <10% fragmentation and regular sized blastomeres were split. A hole was made in the ZP using laser pulses (Fertilase, MTM Medical Technologies Montreux, Switzerland) as described before (Van de Velde et al., 2000). Blastomeres were removed by aspiration using a pipette with an inner diameter of 50 μm. All blastomeres were put one by one into an empty ZP (ZP of immature oocytes had been emptied by aspiration). Culturing in a ZP enabled size comparison between the small blastomere-derived blastocysts and regular human embryos in the IVF laboratory. They were cultured individually for 6 days in sequential medium, as used in the IVF programme (Van Landuyt et al., 2005). In vitro development was followed by an experienced clinical embryologist using an inverted microscope (×100 or ×200 magnification, Diaphot 300, Nikon, Tokyo, Japan) and photographs were taken daily (OCTAX Eyeware MX).

Indirect immunocytochemistry

Embryos were washed in phosphate-buffered saline (PBS; Sigma-Aldrich, Bornem, Belgium) supplemented with 2% bovine serum albumin (BSA; Sigma-Aldrich), fixed with 3.7% formaldehyde (Merck; VWR International, Leuven, Belgium) in PBS for 10 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Serum albumin (BSA; Sigma-Aldrich), fixed with 3.7% formaldehyde (Merck; VWR International, Leuven, Belgium) in PBS for 20 min at room temperature. They were then incubated with 2 μg/ml anti-NANOG rabbit polyclonal antibody (ab21624; Abcam, Cambridge, UK) in PBS with 2% BSA at 4°C overnight. Next, samples were incubated with 10 μg/ml Alexa Fluor 647 donkey anti-rabbit immunoglobulin (Ig) G (A-31573, Invitrogen, Merelbeke, Belgium) in PBS with 2% BSA for 2 h at 4°C in the dark. Between every step, samples were washed three times for 5 min in PBS with 2% BSA on a shaking plate at room temperature. Positive and negative control experiments on regular preimplantation embryos, which were donated for research after informed consent, were included in each experiment (data not shown). In positive controls, regular IVF embryos were stained for NANOG. In negative controls, the NANOG antibody was replaced with rabbit IgGs (R-9133, Sigma-Aldrich) at a concentration of 2 μg/ml. After staining, embryos were put between two glass cover slips (24 × 60 mm) in 2 μl SlowFade® Light Antifade reagent (Invitrogen). To prevent squeezing of the embryos, round glass cover slips (10 mm diameter) fixed with acrytol mounting medium (Surgipath, Peterborough, UK) were used as spacers. Confocal scanning microscopy with a HeNe laser (633 nm) (IX71 Fluoview 300; Olympus, Aartselaar, Belgium) was performed to record the fluorescent images.

Results

Twenty-four blastomeres of six human embryos (Table I) were, one by one, aspirated after ZP drilling as in the case of embryo biopsy for preimplantation genetic diagnosis (PGD) (Van de Velde et al., 2000) and put into an empty ZP (Fig. 1). Their size could thus be compared with the size of regular in vitro developing human embryos. One blastomere did not survive the aspiration; 23 single blastomeres inside a ZP were cultured individually in vitro. They were evaluated daily until Day 6 of preimplantation development. Four blastomeres degenerated during the in vitro culture; the majority of the blastomeres followed the normal pattern of preimplantation development in accordance with the biological clock of compaction on Day 4 and cavitation on Day 5. At the moment of evaluation on Day 3 (72 h after injection), 19 blastomeres divided into two (Table I). One day later, 19 embryos were compacting/compacted (made up of 2–4–8 cells). On Day 5, these 19 blastomere-derived embryos developed into blastocysts, with a cavity, consisting of a small number of TE cells. Finally, 16 blastomeres developed into full-expanded blastocysts on Day 6, of which 12 had an ICM. Some blastocysts were artificially hatching through the hole previously made in the ZP. In order to support the microscopical observation of ICM cells, we looked for the presence of the stemness marker NANOG, a transcription factor exclusively expressed by ICM cells in human embryos (Hyslop et al. 2005). After immunocytochemistry and confocal microscopy, we found that in 9 of 11 blastocysts (one was lost during fixation) with ICM and TE tested, some ICM cells expressed NANOG. In five out of six embryos, three of four blastomeres developed into blastocysts on Day 5 (in total 15). In 12 of these blastocysts, an ICM was visible. In the sixth embryo, the four blastomeres were each capable of developing individually into a good quality expanded blastocyst with cohesive TE and tightly packed ICM (two type B4AA with many ICM cells and two type B4BA with fewer cells, according to the scoring system of Gardner and Schoolcraft, 1999) (Fig. 2a). In the ICM of the two B4AA and two B4BA blastocysts, 6, 6, 3 and 2 cells, respectively, were found to express NANOG (Fig. 2b). We therefore conclude that the cells of a 4-cell stage human embryo can individually develop into small blastocysts with ICM and TE cells.

Table I. Result of splitting six 4-cell stage human embryos.

<table>
<thead>
<tr>
<th>Embryo</th>
<th>Day 2 Survived</th>
<th>Day 3 2 cell</th>
<th>Day 4 Compaction</th>
<th>Day 5 Cavity</th>
<th>Day 6 Full-expanded blastocyst (ICM/NANOG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2 (1/ND)</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3 (2/2)</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3 (1/1)</td>
<td>3 (3/2)</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4 (4/4)</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3 (3/2)</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1 (1/0)</td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>16 (12/9)</td>
</tr>
</tbody>
</table>

The number of blastomeres that survived biopsy on Day 2, divided on Day 3, showed signs of compaction on Day 4, had a cavity on Day 5 and developed into full/expanded blastocysts on Day 6 are represented in the respective columns. In the last column, the number of blastocysts with inner cell mass (ICM)/the number of ICM expressing NANOG is also presented.

ND, not done because lost during fixation.
The results of the experiments performed in this study provide evidence that the isolated blastomeres of a human 4-cell stage embryo can individually develop in vitro into blastocysts with TE and ICM. We conclude that the cells are flexible and may not yet be irreversibly allocated to either cell lineage.

Polarity and pre-patterning in the mammalian embryo are currently under debate (Vogel, 2005; Hiiragi et al., 2006). Having an up–down or left–right axis seems to be clearly defined in frog and fly oocytes where morphogens in varying concentrations regulate differentiation after fertilization. In non-mammals, gradients of mRNA and morphogens are established by the cytoskeleton; they cross the zygote and early embryo and dictate the neighbouring cells to differentiate. In mammals, blastomeres are thought to be totipotent at least up to the 8-cell stage, although data showing some pre-patterning have been reported. The formation of outer and inner cells during the fourth cleavage is the result of differential division into polar/outer cells and apolar/inner cells (inside–outside hypothesis) (Tarkowski and Wroblewska, 1967). The first morphological sign of polarity appears in the blastocyst when at least two cell types can be distinguished: the inner cells (ICM), which will form the fetus as well as extraembryonic tissue and the outer cells (TE), which will develop into part of the placenta. In this regulative or cleavage-driven model, cells are allocated based on order and/or orientation of cleavage divisions. Lately, however, several research groups found evidence in mice that the fertilized oocyte is already polarized (morphological and/or molecular asymmetry) and that the early blastomeres are predestined to become either ICM or TE (pre-patterning model). Gardner’s group initially found that the embryonic (ICM)–abembryonic (cavity) axis in the blastocyst is approximately orthogonal to the plane of the first cleavage, indicating that polarity in the mouse blastocyst might find its origin already in the fertilized egg (Gardner, 1997; 2001). This idea is supported by Zernicka-Goetz’ group, who reported that it is already possible at the 2-cell stage to predict the fate of the sister cells (Piotrowska et al., 2001; Gray et al., 2004): the early-dividing cell tends to form the embryonic region, whereas the late-dividing cell tends to form the abembryonic region. By creating chimeric embryos from the individual cells from a 4-cell stage mouse embryo, they showed that the cells have distinct fates (Piotrowska-Nitsche et al., 2004; Torres-Padilla et al., 2007). Others argue that this pre-patterning model has only been established in particular mouse strains under certain experimental conditions (manipulation, invasive techniques and mechanical pressure) that might have induced tendencies/patterns that are biologically irrelevant (Vogel, 2005; Hiiragi et al., 2006). Nevertheless, in mice an isolated blastomere of a 2-cell stage embryo is totipotent (Edwards and Beard, 1997).

Edwards, who supports the pre-patterning model, has introduced a model—extrapolated from the non-mammalian situation—where in the 4-cell stage embryo, two cells are determined to become ICM; one cell will become TE and one cell will contribute to the germline (Edwards and Beard, 1997; Edwards, 2005). Data in support of this model were provided by Hansis et al. (2004) after expression analysis of β-HCG (potential marker of TE), POU5F1 (potential marker of totipotent cells, formerly called OCT-4) and β-ACTIN (positive control) in single blastomeres of five human 2- to 5-cell stage embryos. The expression of β-HCG and POU5F1 was negatively correlated in these embryos; however, caution should be taken when interpreting these data since the result in a number of blastomeres was not considered because no transcripts of β-actin were found. The human embryonic genome is switched on at the 4- to 8-cell stage, but embryonic expression is not necessarily synchronized and therefore variability in expression should be taken into account when drawing conclusions at the early cleavage-stage (Braude et al., 1988; Monk and Holding, 2001; Cauffman et al., 2005). Moreover, experiments with POU5F1 should be reinterpreted because the isoforms POU5F1_iA and POU5F1_iB are often disregarded, and only the former plays a role in totipotency (Cauffman et al., 2006; Lee et al., 2006). Expression analysis of POU5F1_iA throughout human preimplantation development showed that POU5F1_iA is expressed in TE and ICM cells of the human blastocyst and does not induce a specific allocation of the dividing blastomeres towards a certain lineage (Cauffman et al., 2006). Our splitting results contradict Edwards’ human pre-patterning model, because at the 4-cell stage all cells still have the individual capacity to develop into the two lineages ICM and TE. We cannot exclude that within the structure of the embryo, the blastomeres communicate and behave differently, but individually they are equivalent and may not yet be irreversibly allocated. The timing of human embryonic genome activation between the 4- and 8-cell stage (Braude et al., 1988) explains the flexibility of the cells at the 4-cell stage. We postulate that allocation to ICM or TE occurs after the maternal to embryonic genome switch on Day 3. The embryonic genome becomes activated earlier in the mouse
(Flach et al., 1982; Bolton et al., 1984), which could explain the differences between the two species.

The size of the blastocysts derived from the individually cultured blastomeres was smaller when compared with the size of regular in vitro cultured human embryos (actually four times smaller from Day 3 until Day 5). Nevertheless, they followed the biological clock of compaction on Day 4 and cavitation on Day 5. On Day 6, the majority developed into full/expanded blastocysts with clearly visible ICM and TE. The critical step seemed to be the production of cellular mass (expansion) between Day 5 and Day 6. For ethical and legal reasons, we could not transfer the blastocysts into a uterus to determine their implantation capacity and provide evidence that they are totipotent. The presence of trophoblast cells was obvious under the inverted microscope. In order to confirm the presence of ICM cells within the blastomere-derived blastocysts, we investigated the expression of \textit{NANOG}. \textit{NANOG} was chosen because, to our knowledge, it is the only marker that is specifically expressed in the ICM cells of expanded human blastocysts (Hyslop et al., 2005). Moreover, \textit{NANOG} is considered to be one of the key markers of totipotency/stemness (Hyslop et al., 2005). The expression of POU5F1_iA was not investigated because it is not restricted to ICM cells in human blastocysts (Cauffman et al., 2006). \textit{NANOG} null murine embryos have been shown to have normal ICM, but they fail to produce epiblast and die shortly after implantation (Mitsui et al., 2003). Therefore, we conclude that the blastomere-derived blastocysts are potentially totipotent.

Totipotency or rather pluripotency could have been proven by deriving embryonic stem cells (ESCs) from the ICM, but the chance to succeed was \textit{a priori} low. ESC lines have been derived from single blastomeres of 8-cell stage mouse (Chung et al., 2006) and human embryos (Klimanskaya et al., 2006) with a low success rate (respectively, 4 and 2%), indicating that some of these blastomeres are pluripotent. However, to provide evidence that the four cells are pluripotent, an ESC line should have been derived from each of the four blastomeres, which is practically almost impossible.

The finding that in one embryo the four cells can equally develop into blastocysts has implications on the current knowledge of preimplantation development and cell loss by fragmentation, biopsy for PGD or cryodamage. From the six split embryos, the four sister cells did not always develop \textit{in vitro} into blastocysts. This could be due to: (i) damage after manipulation; (ii) lack of developmental potential or (iii) normal \textit{in vitro} preimplantation development. Although

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{\textit{In vitro} development of four single blastomeres derived from one 4-cell stage human embryo. (a) Single blastomeres divided on Day 3, compacted on Day 4, cavitated on Day 5 and developed into expanded blastocysts on Day 6 (the same scale applies to all photographs); (b) immunocytochemistry and confocal microscopy showed \textit{NANOG} expression in cells of the inner cell mass on Day 6.}
\end{figure}
the lack of totipotency cannot be excluded, we believe that our observations reflect the daily observations in the IVF laboratory: not all embryos are of excellent quality and develop into blastocysts. Only 15% of the embryos are top quality on Day 5 (Van Landuyt et al., 2005), but the non-top quality blastocysts (retarded and/or fragmented) have capacity to implant. Fragmentation on Day 3 is associated with a lower blastocyst development rate on Day 5 and a lower implantation rate (Stone et al., 2005); in particular, large fragments resembling whole blastomeres are associated with a low pregnancy outcome. Interestingly, small fragments can be reabsorbed by the blastomeres during preimplantation development (Hardarson et al., 2002). Removal of small fragments (localized or distributed) might improve the implantation capacity of the embryo (Alikani et al., 1999), though this remains to be proven in a prospective randomized controlled study. Pregnancy rates were reported from partially intact frozen–thawed cleavage-stage embryos (Veiga et al., 1987; Van den Abbeel et al., 1997). Removal of the lysed cells might improve the implantation capacity of the frozen–thawed embryo (Nagy et al., 2005; Rienzi et al., 2005), but this needs to be proven in an appropriate trial. Cell loss after biopsy for PGD seems to be less detrimental for the embryo since the implantation rate of biopsied embryos is acceptable (Van de Velde et al., 2007). Obviously, the cleavage-stage cells are flexible and the embryo has intrinsic capacities to overcome cell loss. Cell loss at the cleavage-stage is unlikely to result in an altered ICM/TE ratio on Day 5 (Hardy et al., 1990); however, the loss of a large volume seems to be detrimental for the vitality of the embryo. Biopsied embryos seem to implant better than fragmented or cryodamaged embryos; therefore, the possibility that fragments or lysed cells may obstruct normal preimplantation development (compaction, cavitation and allocation) needs to be investigated.

For the first time, we provide evidence that, at the 4-cell stage, the individual cells of a human embryo are highly flexible so as to overcome cell loss during preimplantation development.

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