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Initial differentiation of blastomeres in 4-cell human embryos and its significance for early embryogenesis and implantation

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Abstract This brief review is devoted to the nature of early blastomere differentiation in human 4-cell embryos and its consequences for embryonic development. Precursor cells of inner cell mass, germline, and trophectoderm may be formed at this stage, the clearest evidence being available for trophectoderm. The sites of these precursor cells in the embryo could be ascertained using markers for animal and vegetal poles, observing specific cleavage planes, and assessing gene and protein expression. This opens new opportunities for studying 4-cell embryos and removing or replacing specific cells. Knowledge of the properties of individual blastomeres should help in improving assisted human reproduction, performing preimplantation genetic diagnosis, and perhaps establishing specific stem cell lines. Special attention is paid to well-characterized trophectoderm, the trophectoderm stem cell, and possible new forms of clinical application. 

KEYWORDS: blastomere allocation, germline, inner cell mass, preimplantation human embryogenesis, stem cells, trophectoderm

Introduction

The nature of differentiation in early mammalian embryos attracts constant attention. Previous studies have revealed how single blastomeres in 4-cell human embryos may have entered their earliest forms of allocation (Edwards and Beard, 1997, 1999; Hansis and Edwards, 2003; Edwards, 2005). This stage is probably established as polarized ooplasm segregates under the control of meridional and transverse cleavage divisions. Maternal factors thus exert a primary role in early embryogenesis and much depends on the establishment of oocyte polarity, timing, and the strict control of cleavage planes. Recent data on the genes responsible for establishing oocyte polarities and on differentiation in the preimplantation human embryo were presented in a companion paper that drew attention to the fundamental properties of *Cdc42* (Edwards, 2005). This small GTPase of the Rho family has a unique sensitivity to changes in concentration gradients of numerous proteins and

factors and in external influences making it the 'centre of polarity' (Etienne-Manneville, 2004). Earlier studies on the fundamental roles of well-characterized genes, including the *par* family, *staufer*, *Bicaudal-C*, and others, in establishing and maintaining polarities throughout successive embryological stages in *Drosophila*, *C. elegans*, and *Xenopus laevis* were also described. Human homologues have been identified and some might have similar roles in early embryonic development (Edwards, 2001).

Fundamental differences emerging among individual blastomeres at the 4-cell stage provide the foundation for themes running throughout this brief review. The molecular and cellular bases of these events in human and murine embryos are described, and this knowledge is then applied to a better understanding of embryogenesis and implantation. It also leads to novel concepts designed to improve assisted reproductive technology, preimplantation genetic diagnosis (PGD), and the isolation of different types of stem cells.

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Basic embryology of 4-cell stages

Animal and vegetal ooplasm segregate in 4-cell embryos via successive cleavage planes

Human and murine oocytes are polarized from their earliest stages of formation (Antczak and Van Blerkom, 1997; Edwards and Beard, 1997; Edwards, 2001). After fertilization, animal and vegetal ooplasm are partitioned in the first three cell divisions by characteristic cleavage planes, as were reported in detail by Gulyas (1975). A typical meridional first cleavage produces two nearly identical daughters, each inheriting animal and vegetal cytoplasm. Frequently the first 2-cell stage daughter blastomere again divides meridionally to produce two identical cells inheriting full polarities. In contrast, the second 2-cell stage daughter divides equatorially, or nearly so, to produce one cell with mostly animal cytoplasm and another cell with mostly vegetal cytoplasm.

These specific cleavage planes lead to significant variations in the nature of apposition among blastomeres in the 4-cell mouse embryo as expressed by different numbers of contacts between them (Graham and Deussen, 1978). Furthermore, in many 4-cell human and mouse embryos, they lead to the tetrahedral arrangement of three blastomeres with animal cytoplasm associating with the polar body, while the fourth blastomere, inheriting only vegetal cytoplasm, is distant to the polar body (Figure 1).

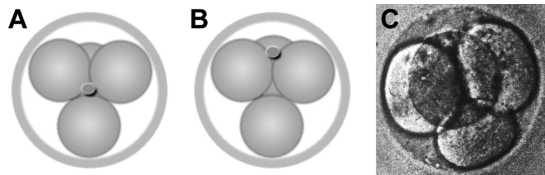


Fig. 1. The typical tetrahedral arrangement of individual blastomeres in relation to the polar body. (A, B) As seen from above and sideways, respectively. (C) A living 4-cell human embryo displaying the tetrahedral blastomere pattern (adapted from Edwards, 1980). The three cells located together might represent one trophectodermal precursor and two precursors of inner cell mass, while the remaining large separate blastomere might be the precursor of germline.

Detailed analyses of these and modified forms of cleavage led Edwards and Beard (1997) to propose that the two blastomeres inheriting a full animal/vegetal axis were precursors of inner cell mass (ICM) (Figure 2). The single 4-cell stage blastomere inheriting animal cytoplasm was proposed to be the precursor of trophectoderm and the blastomere inheriting vegetal cytoplasm the clonal originator of germline. Thus, the cleavage planes are thought to determine various aspects of later development. Based on these early studies and on considerable amounts of published data, fate maps were designed to forecast the developmental capacities of each blastomere in preimplantation stages of mammalian

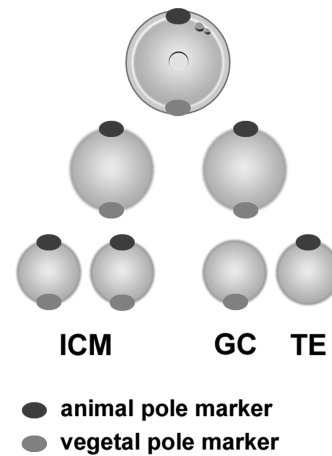


Fig. 2. Pole marking and fate map of human and murine preimplantation embryonic cells. ICM = inner cell mass; GC = germ cells; TE = trophectoderm.

development (Figure 2) (Edwards and Beard, 1997; Edwards, 2001, 2005; Hansis and Edwards, 2003).

Segregation of maternal proteins in 4-cell stages

These conclusions were supported when typical distributions of the maternally inherited proteins leptin and STAT3 were clarified in human and mouse embryogenesis (Antczak and Van Blerkom, 1997). Located in the cell cortex at the animal pole in oocytes, successive cleavage planes described above distributed these proteins differentially to daughter blastomeres in 4-cell embryos. Two of the 4-cell stage blastomeres retained intermediate levels of leptin and STAT3 indicative of their full animal-vegetal axes. The third 4-cell stage blastomere harboured large amounts of leptin and STAT3, presumably transmitted with the animal cytoplasm. The fourth blastomere possessed very small amounts of both proteins, presumably due to its inheritance of mostly vegetal cytoplasm. The location of leptin and STAT3 at the animal pole could be significant in order to organize preparations for implantation at the earliest stages of embryogenesis. Several other proteins also showed polarized distribution in human oocytes and cleavage stage embryos, including the growth factors TGF β 2 and VEGF, the apoptosis-associated proteins BCL-X and BAX, and the growth factor receptors c-KIT and EGF-R (Antczak and Van Blerkom, 1999).

The generation of ICM, trophectoderm, and germline by single stem cells should not be surprising. The power of single murine embryo stem cells was demonstrated some years ago by their capacity to contribute to most cell lines by full term after injection into the blastocoelic cavity of recipient mouse blastocysts (Gardner, 1978). Individual multipotent adult progenitor cells (MAPC) also colonized many tissues in similar experiments (Jiang *et al.*, 2002). Further evidence comes from the notion that single

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Table 1 Individual gene or protein expression and active molecular and cellular systems during preimplantation and post-implantation stages of human trophoctoderm differentiation.

| Preimplantation | Post-implantation | Reference |
|--|--|---|
| Individual genes or proteins | | |
| β -HCG (6 genes) | | Hansis <i>et al.</i> (2002, 2004b) |
| β -LH | | Hansis <i>et al.</i> (2002, 2004b) |
| Leptin | | Antczak and Van Blerkom (1997) |
| sHLA-G (3 isoforms) | | Fuzzi <i>et al.</i> (2002), Sher <i>et al.</i> (2004) |
| STAT3 | | Antczak and Van Blerkom (1997) |
| | <i>Aldose reductase</i> | Huch <i>et al.</i> (1998) |
| | <i>ATP synthetase U6</i> | Huch <i>et al.</i> (1998) |
| | <i>bFGF</i> | Shams and Ahmed (1994) |
| | Cyclin D1 | De Falco <i>et al.</i> (2004) |
| | <i>GCM1</i> | Janatpour <i>et al.</i> (1999), Nait-Oumesmar <i>et al.</i> (2000) |
| | <i>H19</i> | Lustig <i>et al.</i> (1994) |
| | <i>ID2</i> | Janatpour <i>et al.</i> (2000) |
| | <i>Integrin β1</i> | Huch <i>et al.</i> (1998) |
| | <i>LIF-R</i> | Sharkey <i>et al.</i> (1999) |
| | <i>MASH2</i> | Alders <i>et al.</i> (1997), Janatpour <i>et al.</i> (1999) |
| | <i>PBK1</i> | Huch <i>et al.</i> (1998) |
| | <i>Placental lactogen</i> | Hoshina <i>et al.</i> (1982) |
| | <i>STRA13</i> | Janatpour <i>et al.</i> (1999) |
| | <i>TCF5</i> | Jacquemin <i>et al.</i> (1998) |
| Molecular and cellular systems | | |
| Hatching, e.g. by 'zona breakers' | | Sathananthan <i>et al.</i> (2003) |
| Attachment to uterine epithelium, e.g. by integrins | | Lessey and Castelbaum (2002), Carson <i>et al.</i> (2000), Gonzalez <i>et al.</i> (2001) |
| | Uterine invasion, e.g. by metalloproteases | Carson <i>et al.</i> (2000), Castellucci <i>et al.</i> (2000), Gonzalez <i>et al.</i> (2001) |
| | Formation of placenta, e.g. cytotrophoblast cells | Janatpour <i>et al.</i> (1999) |
| | Suppression of immunological rejection e.g. by interaction with decidual NK cells | Hanna <i>et al.</i> (2003), Carosella <i>et al.</i> (2000) |

murine haematopoietic stem cells apparently retain the ability to colonize bone marrow in irradiated recipients and many other tissues (Krause *et al.*, 2001; Cao *et al.*, 2004; Edwards, 2004; Matsuzaki *et al.*, 2004). Finally, a single murine embryonic stem (ES) cell seems to be sufficient in generating a viable mouse when injected into blastocysts lacking an ICM (Wang and Jaenisch, 2004).

β -HCG and β -LH mRNA expression in individual cleavage stage blastomeres

A third and totally different analysis now corroborated the model proposed by Edwards and Beard (1997). Previous work using autoradiography or polymerase chain reaction (PCR) had identified specific mRNAs for the beta subunit of the trophoctoderm marker *human chorionic gonadotropin* (β -HCG) in intact cleaving human embryos, but did not assess single blastomeres

(Bonduelle *et al.*, 1988; Adjaye *et al.*, 1999; Jurisicova *et al.*, 1999). In human blastocysts, β -HCG mRNA is differentially expressed in trophoctoderm cells, with very little or none in ICM (Ohlsson *et al.*, 1989). After hatching, HCG protein was detected in culture media from human blastocysts from day 7 onwards, indicating that the trophoctoderm is now fully committed and functional (Fishel *et al.*, 1984; Hay and Lopata, 1988).

β -HCG mRNAs were then identified in a single blastomere in a 4-cell human embryo, but not in a 2-cell embryo (Hansis *et al.*, 2002). This evidence by marker gene expression indicated a single putative trophoctodermal precursor blastomere had emerged in the 4-cell stage (Table 1). The secretion of HCG protein by this and its sister blastomeres has not yet been studied. However, preliminary data have shown that secreted HCG protein can be detected in

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culture media of human preimplantation embryos from the 4-cell stage onwards, independently supporting the concept of early trophoctoderm allocation (C Hansis, unpublished data). The totipotency marker gene *Oct-4* was apparently silenced in this putative trophoctoderm precursor cell, but was expressed in its sister blastomeres with putative fates in ICM (Hansis *et al.*, 2001, 2004b; Hansis and Edwards, 2003). Single blastomeres were also characterized by β -HCG mRNA expression in 5-, 7-, and 8-cell stages (Hansis *et al.*, 2002). These data provided the first direct evidence of a single trophoctoderm stem cell in 4- and 8-cell human embryos.

In contrast to β -HCG, the beta subunit mRNA of the closely related gene and further putative trophoctoderm marker *luteinizing hormone* (β -LH) was found in 2-cell, but not in 4-cell embryos. β -LH mRNA might thus be produced before β -HCG mRNA in early trophoctodermal lineages and have a role in the very early embryo. This temporal divergence of trophoctoderm marker gene expression could be explained by mechanisms such as epigenetics, i.e. the differential methylation of CpG (cytosine-phosphate-guanine) islands or histone modifications at consensus DNA sequences, thereby influencing the synthesis and actions of promoters, enhancers, or inhibitors controlling mRNA synthesis. A recent analysis showed how the expression of the transcription factor *Oct-4* in trophoctoderm cells depended on the DNA methylation and histone acetylation status of the promoter region immediately upstream of the *Oct-4* gene (Hattori *et al.*, 2004). *Oct-4* in turn inhibits β -HCG mRNA expression by directly binding to regulatory regions in the β -HCG 5' gene promoter (Liu and Roberts, 1996). Enhanced survival or degradation of β -HCG and β -LH mRNAs could also be determined by temporarily bound regulatory proteins or antisense RNA molecules. Translationally competent or incompetent varieties could finally be generated by alternative splicing producing functionally active (full length) or inactive (truncated) forms.

HCG is widely produced in various forms and tissues in men and women. Among its many known functions is the induction of oocyte maturation. Qualitative and quantitative differences in N- and O-linked glycosylation of secreted HCG proteins allow the differentiation between ongoing pregnancies, early lost pregnancies, diabetic pregnancies, hydatidiform mole, and choriocarcinoma (Elliott *et al.*, 1997; O'Connor *et al.*, 1998). Early pregnancies in the first 5–6 weeks of gestation are characterized by hyperglycosylated HCG resembling trophoblastic malignancies and can be discriminated from later pregnancies (Kovalevskaya *et al.*, 1999, 2002). Hyperglycosylated HCG can be reasonably measured by some home pregnancy tests (Butler *et al.*, 2001). If HCG is given at mid-cycle to IVF patients with low endogenous LH concentrations due

to administration of a gonadotropin-releasing hormone (GnRH) agonist, endometrial thickness growth is induced and embryo implantation rates improved (Tesarik *et al.*, 2003). LH might play a similar role in gamete maturation and pregnancy. For example, LH enhances survival of male germ cells during spermatogenesis by the stimulation of *insulin-like 3* (*INSL3*) transcripts with the translated INSL3 protein then binding to LGR8, a G-protein coupled receptor rich in leucine (Kawamura *et al.*, 2004). Treatment with INSL3 suppresses male germ cell apoptosis *in vivo* and also initiates meiotic progression of arrested oocytes *in vivo* and *in vitro*.

HCG and other trophoctoderm markers of early embryogenesis can be functionally linked. For example, leptin stimulates HCG secretion in human cytotrophoblast cells *in vitro* and HCG then exerts negative feedback on leptin secretion (Chardonnes *et al.*, 1999; Cameo *et al.*, 2003; Islami *et al.*, 2003). Leptin also modulates the trophoblastic expression of integrins and metalloproteases facilitating embryo attachment and uterine invasion, respectively (Castellucci *et al.*, 2000; Gonzalez *et al.*, 2001). These findings support concepts of molecular systems that sustain specific cellular functions in trophoctoderm and its derivatives (Table 1). Assessing more members of these systems should further the specific identification of trophoctoderm precursors and clarify their cellular physiology. The most useful combinations of markers should be determined experimentally.

Secretion of sHLA-G by cleaving embryos

A non-classic HLA class Ib antigen in soluble form, sHLA-G, may also be a promising marker in characterizing trophoctoderm precursors. sHLA-G is released from human oocytes and early embryos into culture medium and serves as an *in-vitro* marker assessing the rate of cleavage at 48 h post-fertilization (Menicucci *et al.*, 1999). In post-implantation phases, sHLA-G is produced by cytotrophoblast cells at maternal–embryonic interfaces. HLA-G consists of at least seven isoforms: four are membrane-bound (HLA-G1, -G2, -G3 and -G4) and three are soluble proteins (HLA-G5, -G6 and -G7) (Carosella *et al.*, 2000). HLA-G can induce immune tolerance against paternal antigens of the fetus during pregnancy by causing apoptosis of cytotoxic alloreactive T cells and inhibiting natural killer (NK) cells. sHLA-G was identified in media samples used to culture grouped human embryos and those secreting it at cleavage stages implanted successfully (Fuzzi *et al.*, 2002). Individual cleavage stage embryos were also found to release sHLA-G into their culture media (Sher *et al.*, 2004). Similarly, implantation rates approached 40% in patients aged less than 39 years if one or more of their transferred embryos were secreting high concentrations of sHLA-G. In contrast, implantation

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rates were only 9% per embryo for those with low concentrations.

Molecules such as sHLA-G, HCG, and LH are likely to be good candidates for the quality assessment of embryos prior to their transfer since functioning peri-implantation trophoctoderm seems to be related to its ability to secrete those factors. This embryo quality testing is non-invasive and reduces rates of multiple births and costs of embryo culture. More objective standardized machine-based assays can replace personal observations on morphology when judging embryo quality. Data on β -HCG/ β -LH mRNA expression in cleaving embryos supports this approach, since high concentrations seem to be associated with younger-aged donors and embryonic euploidy (Hansis *et al.*, 2002, 2004b; Hansis and Edwards, 2003). Furthermore, there is preliminary evidence that high HCG concentrations in culture media of individual embryos 48 and 72 h after fertilization are predictive for high implantation chances, indicating that HCG is indeed a promising prospective quality marker (C Hansis, unpublished data).

Oct-4 as a marker of inner cell mass precursors

Foundation cells of inner cell mass that will develop into embryonic disc and fetal tissues presumably inherit a full animal/vegetal axis and might be identified through their expression of the totipotency-associated transcription factor *Oct-4* (Okamoto *et al.*, 1990; Rosner *et al.*, 1990; Schöler *et al.*, 1990). *Oct-4* mRNA expression was measured in individual blastomeres of cleavage stage human embryos by Hansis *et al.* (2001). It was found that *Oct-4* mRNA is expressed in the majority of blastomeres by 4–5-cell stages, and then reduced to one-third by 7–10-cell stages. At the blastocyst stage, *Oct-4* mRNA was found to be down-regulated by 31-fold in trophoctoderm as compared with ICM (Hansis *et al.*, 2000). The variability of *Oct-4* mRNA expression between human cleavage stage blastomeres, the progressive decline of *Oct-4* mRNA positive cells towards the 10-cell stage, and the down-regulation of *Oct-4* mRNA in trophoctoderm were confirmed in recent studies (Huntriss *et al.*, 2004; Cauffman *et al.*, 2005). As mentioned above, *Oct-4* mRNA expression was negatively related to β -HCG mRNA expression at the 4–5-cell stage, signifying that individual blastomeres with potential ICM fate may then be formed (Hansis *et al.*, 2004b). However, caution is needed regarding the classification of *Oct-4* mRNA positive cells as ICM precursors, since this gene is also active in human primordial germ cells (PGC) (Goto *et al.*, 1999). Furthermore, *Oct-4* expression might be reactivated in previously negative cells at compacting stages (Cauffman *et al.*, 2005). Several other genes may be useful to characterize ICM precursors as well, including *Rex-1* and *Nanog*, which are known to be active in

totipotent cells (Hosler *et al.*, 1989; Chambers *et al.*, 2003; Mitsui *et al.*, 2003).

So far, there have been no published searches for the multidifferentiation potential of individual ICM cells and very little is known about their specific properties. However, in human and mouse ES cells, the canonical Wnt pathway is known to maintain self-renewal. Activated by 6-bromoindirubin-3'-oxime (BIO), it sustains the expression of *Oct-4*, *Rex-1*, and *Nanog* and specifically inhibits glycogen synthase kinase-3 (GSK-3) (Sato *et al.*, 2004). Removal of BIO reverses this situation and results in normal multidifferentiation in ES cells. Can such systems also operate in the inner cell mass? They may function to maintain pluripotency among ICM cells as well until one or more are selectively activated to initiate the formation of a specific tissue. Examples of such activation factors could be stromal cell-derived factor 1 (SDF1) and fibroblast growth factor 4 (FGF4), which promote the thrombopoietin-independent platelet production by megakaryocyte progenitors (Avecilla *et al.*, 2004). They could operate in the founding cells of the haematopoietic system that might develop soon after trophoctoderm, among the earliest tissues to differentiate. Indeed, cells in human blood islands start to synthesize fetal haemoglobin after embryonic day 12, indicating they were allocated several days before (Edwards, 2004).

Markers of putative germline precursors

The cell containing vegetal cytoplasm may be the precursor of germline. Confirming its identity will be more difficult than with the trophoctoderm or ICM stem cells, since the classic germline genes typical of amphibians are not expressed in mammalian embryos (Edwards and Beard, 1997, 1999). Nevertheless, several investigators have identified sex-specific genes including *Sry*, *Zfy*, *Zfx*, and *Sox-2* in mammalian embryos as early as the 1-cell stage (for references, see Edwards and Beard, 1999). The significance of these findings remains a matter for debate, although it would be well worth examining single blastomeres for their expression. The gene *PGC7* might well be a suitable germline marker since it is expressed in mouse preimplantation embryos and in germ cells and could thus be significant in early germline determination (Sato *et al.*, 2002). The germline marker genes *stella* and *fragilis* may not be expressed in early cleavage stages, since they were proposed to define developing murine germline cells in the proximal epiblast after activation by bone morphogenetic protein 4 (Bmp4) (Saitou *et al.*, 2002).

As mentioned above, *Oct-4* may also be expressed in germline precursor cells in early embryos, since it is present in human PGC isolated from male and female fetal gonads at 10 weeks gestation (Goto *et al.*, 1999).

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Its value as a marker of germline should be reinforced by evidence of other specific germline genes.

Stem cells of human 4-cell embryos

Identifying and locating stem cells

Genetic markers are expected to characterize individual stem cells on a molecular level. However, on a morphological level, it is equally important to identify and locate those cells for potential IVF applications. This could be achieved on the assumption of successive cleavage planes determining structure and shape in 4-cell human embryos (Gulyas, 1975). Tiny markers could be utilized to mark animal and vegetal poles, an approach that has been widely applied when assessing developmental fates of single cells in mammalian embryos (Gardner, 1978; Gardner and Davies, 2003; Zernicka-Goetz, 2003). One distinct marker could be placed on the surface of or just inside the animal pole in the zygote or each 2-cell stage blastomere (Figure 2). A second distinctive marker could be placed at the vegetal pole. Markers could be oil droplets, tiny particles, beads, DNA constructs, or colour-coded. If their position is reasonably stable, individual blastomeres could be identified in disaggregated embryos as well. Studies in mice could employ injections inside the membrane; human studies may best be initiated by surface markers to ensure the safe growth of the embryo. Ultimately, experience with this approach might lead to the identification of specific morphological characteristics in the putative stem cells.

Piotrowska-Nitsche and Zernicka-Goetz (2005) and Piotrowska-Nitsche *et al.* (2005) recently reported how marking animal and vegetal poles had been achieved in mice. This enables follow-up studies on the fate of the marked human cells: a blastomere with only an animal marker could be confirmed as trophoblast precursor by identifying its content of β -HCG or β -LH mRNA, high concentrations of maternally inherited proteins leptin and STAT3, and possibly secreted sHLA-G. As described earlier, the exact differentiation of inner cell mass versus germline precursors will be more difficult due to the lack of definite markers for either cell line. The introduction of transcriptomes should soon overcome this obstacle.

Excising and replacing stem cells

Once identified, single stem cells from 4-cell embryos could be specifically excised for fundamental studies on differentiation. Cell excision in embryos is already widely practised for preimplantation genetic diagnosis (PGD). The excised cell could be allowed to cleave, enabling one daughter to be assessed for its content of trophoblast, germline, or ICM markers and for genes enhancing or impairing implantation. The

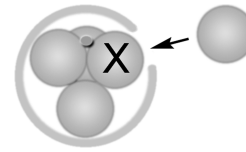


Fig. 3. Replacing a low-quality trophoblast stem cell with a high-quality one in a 4-cell embryo.

second daughter could be returned to the embryo either at its original or at a different location, thereby revealing positional effects. This approach might identify e.g. high- or low-quality trophoblast precursors and those of low quality could then be replaced with better cells to sustain implantation and avoid placental insufficiency (Figure 3).

Stem cell lines offer sources for replacement of a particular embryonic cell. A human ES cell line free of animal feeder cells or proteins has been derived from ICM cells (Richards *et al.*, 2002). Human embryonic germ (EG) stem cell lines are also available, albeit not xeno-free, and retain many of the properties of their precursors (Shamblott *et al.*, 1998; Turnpenny *et al.*, 2003). Clonal trophoblast stem cell lines ($n=58$) were derived from 91 mouse blastocysts at day 3.5, i.e. roughly akin to a day 5 human blastocyst (Tanaka *et al.*, 1998). They differentiated into tissues resembling extra-embryonic ectoderm as it occurs in murine trophoblastic lineages *in vivo*. Those stem cell lines differed from ES cell lines in being responsive to FGF4 and probably to other factors released from feeder cells. Even though these murine cell lines are obviously not suited for human replacement, their linear potential was promising, as indicated by their exclusive colonization of trophoblast in aggregation and blastocyst injection chimeras (Tanaka *et al.*, 1998). Currently, human studies would be best restricted to trophoblast for ethical reasons until experience has been gained on ICM or germline substitutions.

The results of cell replacement may differ according to the characteristics of the substitute cell. Fully potent replacement cells may divide immediately after reinsertion to restore a normal cell complement. Replacement cells with mostly animal or vegetal cytoplasm may be unable to respond as quickly. It would also be interesting to assess the growth of the remaining 3-cell embryos without any cells being replaced. This could help to clarify if and how their developmental competence is restored, for example, by mitotic divisions of the fully potent cells.

Characteristics and clinical relevance of trophoblast

Trophoblast development and genetics

Most immediate attention will likely be devoted to trophoblast in preimplantation human and

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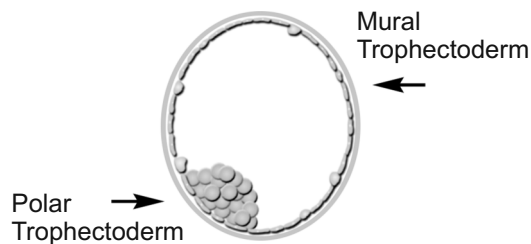


Fig. 4. Structure of the human and mouse blastocyst showing the location of polar and mural trophoblast (modified after Hansis *et al.*, 2004b).

mouse embryos due to its relevance for implantation. Trophoblast becomes morphologically distinct from ICM by the development of outer cells in compacting morulae. In mice, trophoblast emerges either simultaneously or earlier than ICM, as shown by the failure of ICM-derived ES cell lines to colonize trophoblast in blastocyst chimera experiments (Niwa *et al.*, 2000; Rossant, 2001). Further evidence of rapid murine trophoblastic differentiation emerges from its early production of apical tight junctions (Fleming *et al.*, 1989) and sodium pumps responsible for drawing fluid into the blastocoelic cavity (Watson and Kidder, 1988). Trophoblast later separates into actively dividing cells overlying the inner cell mass (polar trophoblast) and the remainder forming a thin epithelium enclosing the cavity (mural trophoblast) which, in mice, endoreplicates DNA to ultimately form polyploid trophoblast giant cells (Figure 4). Polar trophoblast regulates embryonic development by producing factors which activate differentiating cell lines in inner cell mass and its derivatives.

Human trophoblast differs somewhat in the nature of its development from murine by developing cytotrophoblast and then syncytiotrophoblast as many cells fuse and form multiple-nucleated cells. 'Zona breaker' cells in mural trophoblast control the hatching of the human blastocyst from its zona pellucida, thus enabling trophoblast to attach to and invade uterine epithelium (Sathananthan *et al.*, 2003). Interestingly, in contrast to mice, human trophoblast-like cells can develop from ES cells

in vitro (Thomson *et al.*, 1998). In mouse embryos, continued division of polar trophoblast leads to extra-embryonic ectoderm, which forms a pool of trophoblast stem cells and has inducing activity. Bone morphogenetic proteins provide an example for the latter as *Bmp4* was proposed to stimulate gene activity in differentiating germline stem cells (Saitou *et al.*, 2002). Trophoblastic descendants also direct the attraction of a defined subset of CD16⁻ NK cells with decreased killing activity towards the decidua via the CXCR4/CXCL12 receptor/ligand system (Hanna *et al.*, 2003). This establishes maternal immune tolerance in addition to the above-described sHLA-G system. Other well-known functions of trophoblastic descendants include nourishment of the growing fetus and hormone production.

Genetically, members of the FGF family, especially *Fgf4* released from inner cell mass or inherited maternally, may assist in the differentiation and proliferation of murine trophoblast from cleavage stages onwards (Niswander and Martin, 1992). This process involves the trophoblastic expression of four FGF tyrosine kinase receptors (*Fgfr1-4*), especially *Fgfr2*, which is expressed from oocyte to blastocyst stages (Rappolee *et al.*, 1998; Haffner-Krausz *et al.*, 1999). Knowledge about the significance of *Fgf4* in trophoblast proliferation and differentiation also enabled the *in-vitro* establishment of murine stem cell lines with trophoblastic characteristics (Tanaka *et al.*, 1998). These trophoblast stem cells are metabolically active, requiring support from human FGF4, heparin, and mouse embryonic fibroblasts (MEF). With withdrawal of any one, the stem cells subsequently differentiated to trophoblast derivatives *in vitro* and were restricted to the trophoblast lineage in chimeras *in vivo*.

Numerous genes are known to be active in murine trophoblast. Some of them are expressed in trophoblast stem cells and others in differentiating trophoblast cells as described in Table 2 (Cross *et al.*, 2003; Kunath *et al.*, 2004). Note that this list does not include classic ICM genes such as *Oct-4*. Even so, those genes are also essential for

Table 2 Comparison of genes expressed in mouse trophoblast stem cells and differentiating trophoblast (based on Cross *et al.*, 2003, and Kunath *et al.*, 2004).

| Trophoblast stem cell maintenance and proliferation | Trophoblast differentiation |
|--|---|
| <i>Cdx2</i> (caudal-related homeobox transcription factor) | <i>Flt1</i> (vascular endothelial growth factor tyrosine kinase receptor) |
| <i>Eomes</i> (T-box transcription factor) | <i>Gcm1</i> (novel transcription factor) |
| <i>Esrrb</i> (<i>Err2/Errβ</i> , orphan nuclear receptor) | <i>Hand1</i> (bHLH transcription factor) |
| <i>Fgfr2 IIIc</i> (receptor tyrosine kinase isoform) | <i>Id2</i> (dominant-negative HLH factor) |
| <i>Tcfap2c</i> (AP-2γ, transcription factor) | <i>Mash2</i> (bHLH transcription factor) |
| | <i>Stra13</i> (bHLH transcription factor) |
| | <i>Tcf1/Lef1</i> (Wnt-activated transcription factors) |
| | <i>Tpp1/4311</i> (novel) |

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maintaining and developing trophoblast if ICM is, for example, to produce Fgf4. Hundreds of genes have been identified via transcriptomes as being active in morulae and blastocysts, some deduced as being trophectodermal (Sharov *et al.*, 2003; Hamatani *et al.*, 2004). Not yet applied to isolated trophectoderm, this approach has revealed specific stages of massive gene activity, for example, at 4- to 8-cell stages. It seems that some trophectodermal genes will be included in this group while others are activated later (Hamatani *et al.*, 2004). Furthermore, some genes are expressed both in early and later stages of trophectodermal growth, such as the SAPK/JNK subgroup of mitogen-activated protein kinases (MAPK) present in murine pre- and post-implantation embryos, human and mouse trophectodermal cell lines, and human placenta. These proteins are intracellular signal transduction enzymes that may mediate the effect of various types of stress in trophoblast cells (Mirkes *et al.*, 2000; Zhong *et al.*, 2004).

Low implantation rates of human embryos

Low rates of implantation still characterize clinical IVF programmes, a fact constituting a major drawback to this technique. In almost every published study, implantation rates per starter embryo without any application of embryo selection have seldom raised much above 25%. Indeed, each transferred fresh embryo from non-donor eggs by women under the age of 35 years has a mere 15.9% chance of resulting in a live birth according to the 2002 US National Report on Assisted Reproductive Technology Success Rates (US Department of Health and Human Services, 2002). This value does not differ greatly from estimates of embryo implantation rates *in vivo*, which are often quoted at less than 20%.

Modern methods utilize selection in order to identify and transfer those embryos capable of implantation and so attain much higher pregnancy rates. Several of these involve timing and polarities. For example, growing embryos to blastocysts *in vitro* is a natural means of ensuring they are developing on time and with the correct structure. Timers are also significant in selecting those embryos that grow fastest through their first two cleavage divisions and have high implantation chances (Edwards *et al.*, 1984). Polarization provides markers when it is measured in patterns formed by nucleoli in fertilized eggs (Balaban *et al.*, 2004). Scoring blastomere fragmentation (Alikani *et al.*, 1999; Antczak and Van Blerkom, 1999; Van Blerkom *et al.*, 2001) could involve both systems. All these approaches permit implantation rates of 45% or more per embryo. Yet high-quality embryos are scarce, and infertile couples returning time and time again for treatments may never produce any.

Many embryos fail to develop and this perhaps frequently includes the 30–50% that are thought to

contain one or more aneuploid blastomeres (Harper *et al.*, 1995; Munné *et al.*, 1995; Delhanty *et al.*, 1997). Other embryos, seemingly diploid, develop to blastocysts, but fail to implant. They may be impaired by ineffective ICM/trophectoderm interactions or an incompetent trophectoderm that fails to attach to and invade endometrial epithelium. Some embryos may succumb at early post-implantation stages, as indicated by the occurrence of biochemical pregnancies. However, maternal conditions such as submucous uterine fibroids, uterine endometriosis, endometrial polyps and cancer, scar tissue in the uterine cavity, or congenital anomalies (e.g. septate uterus) may prevent successful implantation even for the most competent embryos.

The trophoblastic vesicle: will its use raise implantation rates in assisted human reproduction?

Trophoblastic vesicles have been widely used in animal research. They lack an ICM and hence provide an opportunity to replace an inadequate trophectoderm of an embryo with a fully capable alternative. In mice, they can be prepared by excising ICM manually. Alternatively, the two blastomeres of a 2-cell embryo can be electrically fused, thereby creating tetraploid embryos that fail to develop ICM (Nagy *et al.*, 1990, 1993). Individual ICM prepared by mechanical excision or by lysing trophectoderm with antisera and complement (Solter and Knowles, 1975) can be surgically placed into those empty vesicles or sandwiched between two of them, thus allowing for establishment of successful pregnancies. The resulting mouse chimeras are a mixture of trophectoderm-descendant cells from the vesicle and fetal tissues from the donated ICM (Nagy *et al.*, 1990).

Trophoblastic vesicles proved useful as a diagnostic tool to assess the characteristics of embryonic stem cells *in vivo*. For example, two genes normally expressed maternally, *Igf2r* and *H19*, and two paternally expressed genes, *Igf2* and *U2af1-rs1*, displayed altered methylation patterns in ES cells upon derivation and culturing which persisted in chimeric offspring when the cells were placed in tetraploid vesicles (Dean *et al.*, 1998). *Igf2r* showed novel biallelic methylation and expression, while a reduced expression of *H19* was thought to be due to biallelic methylation of an upstream region. Loss of methylation and biallelic expression characterized *U2af1-rs1* expression and maternal methylation and expression was imposed on *Igf2*. All chimeras grew poorly, with polyhydramnios, poor mandibles, and interstitial bleeding. Interestingly, these chimeras are apparently most often derived from only one or two founder ES cells independently of the total number of injected cells, thus indirectly supporting the theory of two totipotent ICM-destined cells at the 4-cell stage

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(Wang and Jaenisch, 2004). Similar conclusions can be drawn from aggregation chimeras probably derived from two 4-cell stage blastomeres (Tarkowski *et al.*, 2001).

Mice cloned from ES cells and raised via tetraploid vesicles showed likewise variations in expression of imprinted genes (Humpherys *et al.*, 2001). In a further study using tetraploid vesicles, mouse embryos were derived from cloned PGC. Again, evidence of improper imprinting was found, including abnormal biallelic expression of *H19*, *Nnat*, and possibly *Peg1/Mest*, *Peg3*, and *Snrpn*, as well as abnormal biallelic repression of *Igf2*, *Mash2*, *p57kip2*, and *Igf2r* (Kato *et al.*, 1999). Embryos displayed preimplantation failure, weak post-implantation growth, and abnormal placentae resulting in early embryonic death (Kato *et al.*, 1999). In addition, chorionic growth was retarded, diploid trophoblast undeveloped, spongiotrophoblast absent, labyrinthine trophoblast defective, and numbers of giant cells elevated. The failure of recipient tetraploid trophoblastic vesicles to substantially rescue ICM of those cloned embryos indicated that the ICM cells were almost certainly impaired before this stage of growth.

Some investigators have demonstrated the therapeutic potential of trophoblastic vesicles in mice. For example, inherited deficiencies of the *retinoblastoma* (*Rb*) gene were shown to induce fetal and placental anomalies in newborn pups such as neurological and erythroid abnormalities, trophoblastic overgrowth, anomalous development of the labyrinthine layer, and occlusion of placental vessels (Wu *et al.*, 2003). When, in a variation of the above described methods, *Rb*^{-/-} morula-stage embryos were aggregated with tetraploid embryos of the same stage, the degree of placental insufficiency was reduced and anomalies in fetuses were virtually eliminated (Wu *et al.*, 2003). Fetal defects were thus secondary to placental insufficiency rather than to direct actions of the mutant gene. Interestingly, in The Netherlands the incidence of retinoblastoma tumours occurs with a 4.9–7.2 times greater frequency in children conceived by assisted reproduction than by natural conception (Moll *et al.*, 2003). Retinoblastoma tumours can be caused by germline or somatic mutations, or the epigenetic silencing of the *Rb* gene. So far, no attempts have been made to correct this defect in human embryos using donor trophoblastic vesicles. The therapeutic value of trophoblastic vesicles was also illustrated in mice when the feasibility of combining gene therapy with therapeutic cloning to repair a genetic defect, in this case the *Rag2*^{-/-} phenotype, was demonstrated (Rideout *et al.*, 2002).

Trophoblastic vesicles might provide an efficient host trophoblast for transplanted inner cell masses in patients with repeated IVF failures (Figure 5).

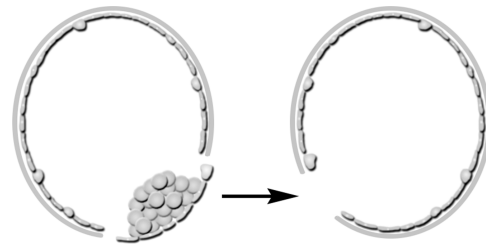


Fig. 5. Excising the inner cell mass from a blastocyst with defective trophoblast and transferring it to a recipient trophoblastic vesicle with fully functional trophoblast.

Physiological placental function and fetal development could thus be ensured. If the trophoblastic vesicle is prepared by excising the ICM of the host blastocyst, it will be essential to remove all the ICM cells in order to avoid contaminating the transplanted ICM. Incorporation of defective carry-over trophoblast attached to the transplanted ICM is a possibility if the ICM is mechanically removed. However, the host trophoblast cells would outnumber the transplanted ones and might have a growth advantage due to their healthier status. It should also be helpful to know that contamination of fetal tissues by occasional multipotent cells from the vesicle trophoblast seems to be rare or absent. Trophoblastic vesicles might further be applied for future cloned human offspring (Zavos, 2003) to overcome putative placental insufficiencies leading to fetal anomalies or demise. This concept has already been proven useful in mouse (Eggan *et al.*, 2001).

Discussion

New opportunities to advance basic understanding of embryonic and fetal differentiation could arise from the proposed model. Cell movements, cell fates, and polarities in embryos could be determined. Transcriptomes established for each blastomere could identify additional specific genetic markers for cell fates and animal and vegetal poles. The replacement of deficient trophoblast or trophoblast precursors by better quality cells might help patients with repeated IVF failures. Furthermore, gene therapy in trophoblast stem cells might overcome metabolic or other disturbances in the early embryo. Finally, embryo surgery could be applied to potential ICM and germline precursors. Clearly, conclusions of this brief review on the presence of trophoblastic, ICM, and germline progenitor cells in 4-cell human embryos must be confirmed by follow-up analyses of the work of Antczak and Van Blerkom (1997) and Hansis *et al.* (2001, 2002, 2004b). It is encouraging that new evidence in mouse studies provides first steps in this direction: individually labelled 4-cell stage blastomeres contributed either exclusively to trophoblast derivatives, ICM derivatives, or to a

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combination of the two (Fujimori *et al.*, 2003). By contrast, blastomeres labelled at the 2-cell stage almost always developed into both trophectoderm and ICM derivatives.

Indications of the formation of stem cells for trophectoderm, inner cell mass, and possibly germline in human cleavage stage embryos raise many implications for assisted reproduction, PGD, and stem cell research. For example, excising specific blastomeres may interfere with normal embryonic development and produce characteristic developmental syndromes. This issue should clearly be considered when practising PGD and indeed some reports have been made about impairment of embryo implantation after applying this technique (Van de Velde *et al.*, 2000; Vandervorst *et al.*, 2000). Embryos may face more stress from the removal of an allocated unique 4- or 8-cell stage blastomere than from the excision of a non-allocated pluripotent blastomere, since the latter might be more easily replaceable by division of a remaining pluripotent sister cell.

Initial studies from Zernicka-Goetz (1998) concluded that animal and vegetal poles did not regulate murine embryogenesis, since fertile offspring could infrequently be derived from fertilized eggs in which a substantial amount of cytoplasm from either region was removed. However, other evidence later showed how such operative methods might have failed to remove all regions of ooplasm likely to contribute to the polar axes, suggesting that the experiment was inadequate (Antczak and Van Blerkom, personal communication; see Edwards, 2001). Supporting the proposed model, recent data also showed that 4-cell stage mouse blastomeres, which presumably inherited largely animal or vegetal cytoplasm from a secondary equatorial division, most often gave rise to mural trophectoderm or superficial ICM cells and their overlying trophectoderm (Piotrowska-Nitsche and Zernicka-Goetz, 2005). In contrast, cells that presumably inherited full polarity usually developed into deeper ICM cells and polar trophectoderm. Furthermore, mouse chimeras lacking the animal- or vegetal-inheriting blastomere at the 4-cell stage often displayed developmental abnormalities at both late preimplantation and early post-implantation stages (Piotrowska-Nitsche *et al.*, 2005). Morulae were arrested, empty trophoblastic vesicles formed, the ICM was reduced in size, and embryos were often retarded in growth or disorganized, with inadequate extra-embryonic ectoderm. Growth defects of chimeras constructed from vegetal-inheriting blastomeres only were the most severe and embryos never developed to term. By contrast, chimeras made from 4-cell stage blastomeres inheriting full polarity developed normally in most cases. Apparently, those cells were able to remedy the loss of animal- and vegetal-inheriting blastomeres in subsequent cell divisions.

This model suggests that trophectoderm, ICM, and germline differentiate in a linear, clonal fashion from their founder stem cells. Furthermore, it stresses the significance of maternal factors in establishing polarities, regulating cleavage planes, and allocating specific blastomeres to particular fates. However, this view has to be complemented by evidence showing that stem cells retain considerable plasticity and abilities to revert to more primitive stages or switch to different lineages (Hansis *et al.*, 2004b). As with mesodermal haematopoietic stem cells giving rise to endodermal differentiated liver cells, two mechanisms could sustain this phenomenon: microenvironmental cues may convert cells directly (Jang *et al.*, 2004; Hansis *et al.*, 2004a) and cell fusion may increase their potency (Tada *et al.*, 2001; Vassilopoulos *et al.*, 2003; Wang *et al.*, 2003). In a recent study on murine embryos, it was shown that down-regulation of the atypical protein kinase C (aPKC) and its binding protein Par3 in randomly located blastomeres at the 4-cell stage directed their clonal descendants towards ICM irrespective of their original presumed fate (Plusa *et al.*, 2005). This effect might have been caused by disturbance of the naturally polarized localization of aPKC and Par3 from the 8-cell stage onwards. Furthermore, in murine adult melanocyte stem cells, the transcription factor Pax3 seems to have the ability to determine their fate by initiating a melanogenic cascade while simultaneously maintaining their undifferentiated state (Lang *et al.*, 2005). This is thought to be the result of Pax3 promoting the expression of the transcription factor Mitf and at the same time competing with it for binding at an enhancer region required for the Mitf-driven expression of the melanin synthesis enzyme dopachrome tautomerase. Only after activated β -catenin displaces Pax3 from this enhancer are melanoblasts free to fully differentiate.

This model might also have to be extended to incorporate the views of many embryologists who followed Spemann's work in the 1920s, which stressed the importance of gradients along an axis, especially in amphibian development (Spemann and Mangold, 1924). In essence, cells exposed to high concentrations of signalling molecules are induced to a different fate than those exposed to low concentrations. It is possible that similar interactions occur as early as in 4-cell human embryos and the two blastomeres inheriting full polarity may be transmitting inducers to the other two unipolar cells in order to initiate their allocation. Clearly, more information of this kind is needed on the establishment, differentiation, and properties of individual blastomeres in 4-cell stages of mammalian development.

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References

- Adjaye J, Bolton V, Monk M, 1999, Developmental expression of specific genes detected in high-quality cDNA libraries from single human preimplantation embryos. *Gene* **237**, 373–383.
- Alders M, Hodges M, Hadjantonakis AK *et al.*, 1997, The human Achaete-Scute homologue 2 (ASCL2, HASH2) maps to chromosome 11p15.5, close to IGF2 and is expressed in extravillous trophoblasts. *Human Molecular Genetics* **6**, 859–867.
- Alikani M, Cohen J, Tomkin G *et al.*, 1999, Human embryo fragmentation in vitro and its implications for pregnancy and implantation. *Fertility and Sterility* **71**, 836–842.
- Antczak M, Van Blerkom J, 1999, Temporal and spatial aspects of fragmentation in early human embryos: possible effects on developmental competence and association with the differential elimination of regulatory proteins from polarized domains. *Human Reproduction* **14**, 429–447.
- Antczak M, Van Blerkom J, 1997, Oocyte influences on early development: the regulatory proteins leptin and STAT3 are polarized in mouse and human oocytes and differentially distributed within the cells of the preimplantation stage embryo. *Molecular Human Reproduction* **3**, 1067–1086.
- Avecilla ST, Hattori K, Heissig B *et al.*, 2004, Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nature Medicine* **10**, 64–71.
- Balaban B, Yakin K, Urman B *et al.*, 2004, Pronuclear morphology predicts embryo development and chromosome constitution. *Reproductive BioMedicine Online* **8**, 695–700.
- Bonduelle ML, Dodd R, Liebaers I *et al.*, 1988, Chorionic gonadotrophin-beta mRNA, a trophoblast marker, is expressed in human 8-cell embryos derived from triprounucleate zygotes. *Human Reproduction* **3**, 909–914.
- Butler SA, Khanlian SA, Cole LA, 2001, Detection of early pregnancy forms of human chorionic gonadotropin by home pregnancy test devices. *Clinical Chemistry* **47**, 2131–2136.
- Cameo P, Bischof P, Calvo JC, 2003, Effect of leptin on progesterone, human chorionic gonadotropin, and interleukin-6 secretion by human term trophoblast cells in culture. *Biology of Reproduction* **68**, 472–477.
- Cao YA, Wagers AJ, Beilhack A *et al.*, 2004, Shifting foci of hematopoiesis during reconstitution from single stem cells. *Proceedings of the National Academy of Sciences of the USA* **101**, 221–226.
- Carosella ED, Paul P, Moreau P *et al.*, 2000, HLA-G and HLA-E: fundamental and pathophysiological aspects. *Immunology Today* **21**, 532–534.
- Carson DD, Bagchi I, Dey SK *et al.*, 2000, Embryo implantation. *Developmental Biology* **223**, 217–237.
- Castellucci M, De Matteis R, Meisser A *et al.*, 2000, Leptin modulates extracellular matrix molecules and metalloproteinases: possible implications for trophoblast invasion. *Molecular Human Reproduction* **6**, 951–958.
- Cauffman G, Van de Velde H, Liebaers I *et al.*, 2005, Oct-4 mRNA and protein expression during human preimplantation development. *Molecular Human Reproduction* **11**, 173–181.
- Chambers I, Colby D, Robertson M, 2003, Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. *Cell* **113**, 643–655.
- Chardonens D, Cameo P, Aubert ML *et al.*, 1999, Modulation of human cytotrophoblastic leptin secretion by interleukin-1alpha and 17beta-oestradiol and its effect on HCG secretion. *Molecular Human Reproduction* **5**, 1077–1082.
- Cross JC, Baczyk D, Dobric N *et al.*, 2003, Genes, development and evolution of the placenta. *Placenta* **24**, 123–130.
- De Falco M, Fedele V, Cobellis L *et al.*, 2004, Pattern of expression of cyclin D1/CDK4 complex in human placenta during gestation. *Cell and Tissue Research* **317**, 187–194.
- Dean W, Bowden L, Aitchison A *et al.*, 1998, Altered imprinted gene methylation and expression in completely ES cell-derived mouse fetuses: association with aberrant phenotypes. *Development* **125**, 2273–2282.
- Delhanty JD, Harper JC, Ao A *et al.*, 1997, Multicolour FISH detects frequent chromosomal mosaicism and chaotic division in normal preimplantation embryos from fertile patients. *Human Genetics* **99**, 755–760.
- Edwards RG, 2005, Genetics of polarity in mammalian embryos. *Reproductive BioMedicine Online* **11**, 104–114.
- Edwards RG, 2004, Stem cells today: B1. Bone marrow stem cells. *Reproductive BioMedicine Online* **9**, 541–583.
- Edwards RG, 2001, Ovarian differentiation and human embryo quality. 1. Molecular and morphogenetic homologies between oocytes and embryos in *Drosophila*, *C. elegans*, *Xenopus* and mammals. *Reproductive BioMedicine Online* **3**, 138–160.
- Edwards RG, 1980, *Conception in the Human Female*. Academic Press, London.
- Edwards RG, Beard HK, 1999, Hypothesis: sex determination and germline formation are committed at the pronucleate stage in mammalian embryos. *Molecular Human Reproduction* **5**, 595–606.
- Edwards RG, Beard HK, 1997, Oocyte polarity and cell determination in early mammalian embryos. *Molecular Human Reproduction* **3**, 863–905.
- Edwards RG, Fishel SB, Cohen J *et al.*, 1984, Factors influencing the success of in vitro fertilization for alleviating human infertility. *Journal of In Vitro Fertilization and Embryo Transfer* **1**, 3–23.
- Eggan K, Akutsu H, Loring J *et al.*, 2001, Hybrid vigor, fetal overgrowth, and viability of mice derived by nuclear cloning and tetraploid embryo complementation. *Proceedings of the National Academy of Sciences of the USA* **98**, 6209–6214.
- Elliott MM, Kardana A, Lustbader JW *et al.*, 1997, Carbohydrate and peptide structure of the alpha- and beta-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. *Endocrine* **7**, 15–32.
- Etienne-Manneville S, 2004, Cdc42 – the centre of polarity. *Journal of Cell Science* **117**, 1291–1300.
- Fishel SB, Edwards RG, Evans CJ, 1984, Human chorionic gonadotropin secreted by preimplantation embryos cultured in vitro. *Science* **223**, 816–818.
- Fleming TP, McConnell J, Johnson MH *et al.*, 1989, Development of tight junctions de novo in the mouse early embryo: control of assembly of the tight junction-specific protein, ZO-1. *Journal of Cell Biology* **108**, 1407–1418.
- Fujimori T, Kurotaki Y, Miyazaki J *et al.*, 2003, Analysis of cell lineage in two- and four-cell mouse embryos. *Development* **130**, 5113–5122.
- Fuzzi B, Rizzo R, Criscuoli L *et al.*, 2002, HLA-G expression in early embryos is a fundamental prerequisite for the attainment of pregnancy. *European Journal of Immunology* **32**, 311–315.
- Gardner RL, 1978, The relationship between cell lineage and differentiation in the early mouse embryo. *Results and Problems in Cell Differentiation* **9**, 205–241.
- Gardner RL, Davies TJ, 2003, Is the plane of first cleavage related to the point of sperm entry in the mouse? *Reproductive BioMedicine Online* **6**, 157–160.
- Gonzalez RR, Devoto L, Campana A *et al.*, 2001, Effects of leptin, interleukin-1alpha, interleukin-6, and transforming growth factor-beta on markers of trophoblast invasive phenotype: integrins and metalloproteinases. *Endocrine* **15**, 157–164.
- Goto T, Adjaye J, Rodeck CH *et al.*, 1999, Identification of genes expressed in human primordial germ cells at the time of entry of the female germ line into meiosis. *Molecular Human Reproduction* **5**, 851–860.
- Graham CF, Deussen ZA, 1978, Features of cell lineage in preimplantation mouse development. *Journal of Embryology and Experimental Morphology* **48**, 53–72.
- Gulyas BJ, 1975, A reexamination of cleavage patterns in eutherian mammalian eggs: rotation of blastomere pairs during second cleavage in the rabbit. *Journal of Experimental Zoology* **193**, 235–248.

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- Haffner-Krausz R, Gorivodsky M, Chen Y *et al.*, 1999, Expression of Fgfr2 in the early mouse embryo indicates its involvement in preimplantation development. *Mechanisms of Development* **85**, 167–172.
- Hamatani T, Carter MG, Sharov AA *et al.*, 2004, Dynamics of global gene expression changes during mouse preimplantation development. *Developmental Cell* **6**, 117–131.
- Hanna J, Wald O, Goldman-Wohl D *et al.*, 2003, CXCL12 expression by invasive trophoblasts induces the specific migration of CD16-human natural killer cells. *Blood* **102**, 1569–1577.
- Hansis C, Edwards RG, 2003, Cell differentiation in the preimplantation human embryo. *Reproductive BioMedicine Online* **6**, 215–220.
- Hansis C, Barreto G, Maltry N *et al.*, 2004a, Nuclear reprogramming of human somatic cells by *Xenopus* egg extract requires BRG1. *Current Biology* **14**, 1475–1480.
- Hansis C, Grifo JA, Krey LC, 2004b, Candidate lineage marker genes in human preimplantation embryos. *Reproductive BioMedicine Online* **8**, 577–583.
- Hansis C, Grifo JA, Tang Y *et al.*, 2002, Assessment of beta-HCG, beta-LH mRNA and ploidy in individual human blastomeres. *Reproductive BioMedicine Online* **5**, 156–161.
- Hansis C, Tang YX, Grifo JA *et al.*, 2001, Analysis of Oct-4 expression and ploidy in individual human blastomeres. *Molecular Human Reproduction* **7**, 155–161.
- Hansis C, Grifo JA, Krey LC, 2000, Oct-4 expression in inner cell mass and trophoblast of human blastocysts. *Molecular Human Reproduction* **6**, 999–1004.
- Harper JC, Coonen E, Handyside AH *et al.*, 1995, Mosaicism of autosomes and sex chromosomes in morphologically normal, monospermic preimplantation human embryos. *Prenatal Diagnosis* **15**, 41–49.
- Hattori N, Nishino K, Ko YG *et al.*, 2004, Epigenetic control of mouse Oct-4 gene expression in embryonic stem cells and trophoblast stem cells. *Journal of Biological Chemistry* **279**, 17063–17069.
- Hay DL, Lopata A, 1988, Chorionic gonadotropin secretion by human embryos in vitro. *Journal of Clinical Endocrinology and Metabolism* **67**, 1322–1324.
- Hoshina M, Boothby M, Boime I, 1982, Cytological localization of chorionic gonadotropin alpha and placental lactogen mRNAs during development of the human placenta. *Journal of Cell Biology* **93**, 190–198.
- Hosler BA, LaRosa GJ, Grippo JF *et al.*, 1989, Expression of REX-1, a gene containing zinc finger motifs, is rapidly reduced by retinoic acid in F9 teratocarcinoma cells. *Molecular and Cellular Biology* **9**, 5623–5629.
- Huch G, Hohn HP, Denker HW, 1998, Identification of differentially expressed genes in human trophoblast cells by differential-display RT-PCR. *Placenta* **19**, 557–567.
- Humpherys D, Eggan K, Akutsu H *et al.*, 2001, Epigenetic instability in ES cells and cloned mice. *Science* **293**, 95–97.
- Huntriss J, Hinkins M, Oliver B *et al.*, 2004, Expression of mRNAs for DNA methyltransferases and methyl-CpG-binding proteins in the human female germ line, preimplantation embryos, and embryonic stem cells. *Molecular Reproduction and Development* **67**, 323–336.
- Islami D, Bischof P, Chardonnens D, 2003, Modulation of placental vascular endothelial growth factor by leptin and hCG. *Molecular Human Reproduction* **9**, 395–398.
- Jacquemin P, Sapin V, Alsat E *et al.*, 1998, Differential expression of the TEF family of transcription factors in the murine placenta and during differentiation of primary human trophoblasts in vitro. *Developmental Dynamics* **212**, 423–436.
- Janatpour MJ, McMaster MT, Genbacev O *et al.*, 2000, Id-2 regulates critical aspects of human cytotrophoblast differentiation, invasion and migration. *Development* **127**, 549–558.
- Janatpour MJ, Utset MF, Cross JC *et al.*, 1999, A repertoire of differentially expressed transcription factors that offers insight into mechanisms of human cytotrophoblast differentiation. *Developmental Genetics* **25**, 146–157.
- Jang YY, Collector MI, Baylin SB *et al.*, 2004, Hematopoietic stem cells convert into liver cells within days without fusion. *Nature Cell Biology* **6**, 532–539.
- Jiang Y, Jahagirdar BN, Reinhardt RL *et al.*, 2002, Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41–49.
- Juriscicova A, Antenos M, Kapasi K *et al.*, 1999, Variability in the expression of trophoblastic markers beta-human chorionic gonadotropin, human leukocyte antigen-G and pregnancy specific beta-1 glycoprotein by the human blastocyst. *Human Reproduction* **14**, 1852–1858.
- Kato Y, Rideout 3rd WM, Hilton K *et al.*, 1999, Developmental potential of mouse primordial germ cells. *Development* **126**, 1823–1832.
- Kawamura K, Kumagai J, Sudo S *et al.*, 2004, Paracrine regulation of mammalian oocyte maturation and male germ cell survival. *Proceedings of the National Academy of Sciences of the USA* **101**, 7323–7328.
- Kovalevskaya G, Birken S, Kakuma T *et al.*, 2002, Differential expression of human chorionic gonadotropin (hCG) glycosylation isoforms in failing and continuing pregnancies: preliminary characterization of the hyperglycosylated hCG epitope. *Journal of Endocrinology* **172**, 497–506.
- Kovalevskaya G, Birken S, Kakuma T *et al.*, 1999, Early pregnancy human chorionic gonadotropin (hCG) isoforms measured by an immunometric assay for choriocarcinoma-like hCG. *Journal of Endocrinology* **161**, 99–106.
- Krause DS, Theise ND, Collector MI *et al.*, 2001, Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105**, 369–377.
- Kunath T, Strumpf D, Rossant J, 2004, Early trophoblast determination and stem cell maintenance in the mouse – a review. *Placenta* **25** (Suppl. A), S32–38.
- Lang D, Lu MM, Huang L *et al.*, 2005, Pax3 functions at a nodal point in melanocyte stem cell differentiation. *Nature* **433**, 884–887.
- Lessey BA, Castelbaum AJ, 2002, Integrins and implantation in the human. *Reviews in Endocrine and Metabolic Disorders* **3**, 107–117.
- Liu L, Roberts RM, 1996, Silencing of the gene for the beta subunit of human chorionic gonadotropin by the embryonic transcription factor Oct-3/4. *Journal of Biological Chemistry* **271**, 16683–16689.
- Lustig O, Ariel I, Ilan J *et al.*, 1994, Expression of the imprinted gene H19 in the human fetus. *Molecular Reproduction and Development* **38**, 239–246.
- Matsuzaki Y, Kinjo K, Multigan RC *et al.*, 2004, Unexpectedly efficient homing capacity of purified murine hematopoietic stem cells. *Immunity* **20**, 87–93.
- Menicucci A, Noci I, Fuzzi B *et al.*, 1999, Non-classic sHLA class I in human oocyte culture medium. *Human Immunology* **60**, 1054–1057.
- Mirkes PE, Wilson KL, Cornel LM, 2000, Teratogen-induced activation of ERK, JNK, and p38 MAP kinases in early postimplantation murine embryos. *Teratology* **62**, 14–25.
- Mitsui K, Tokuzawa Y, Itoh H *et al.*, 2003, The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell* **113**, 631–642.
- Moll AC, Imhof SM, Cruysberg JR *et al.*, 2003, Incidence of retinoblastoma in children born after in-vitro fertilisation. *Lancet* **361**, 309–310.
- Munné S, Alikani M, Tomkin G *et al.*, 1995, Embryo morphology, developmental rates, and maternal age are correlated with chromosome abnormalities. *Fertility and Sterility* **64**, 382–391.
- Nagy A, Rossant J, Nagy R *et al.*, 1993, Derivation of completely cell culture-derived mice from early-passage embryonic stem cells. *Proceedings of the National Academy of Sciences of the USA* **90**, 8424–8428.

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- Nagy A, Gocza E, Diaz EM, 1990, Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110**, 815–821.
- Nait-Oumesmar B, Copperman AB, Lazzarini RA, 2000, Placental expression and chromosomal localization of the human Gcm 1 gene. *Journal of Histochemistry and Cytochemistry* **48**, 915–922.
- Niswander L, Martin GR, 1992, Fgf-4 expression during gastrulation, myogenesis, limb and tooth development in the mouse. *Development* **114**, 755–768.
- Niwa H, Miyazaki J, Smith AG, 2000, Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genetics* **24**, 372–376.
- O'Connor JF, Elish N, Kakuma T *et al.*, 1998, Differential urinary gonadotrophin profiles in early pregnancy and early pregnancy loss. *Prenatal Diagnosis* **18**, 1232–1240.
- Ohlsson R, Larsson E, Nilsson O *et al.*, 1989, Blastocyst implantation precedes induction of insulin-like growth factor II gene expression in human trophoblasts. *Development* **106**, 555–559.
- Okamoto K, Okazawa H, Okuda A *et al.*, 1990, A novel octamer binding transcription factor is differentially expressed in mouse embryonic cells. *Cell* **60**, 461–472.
- Piotrowska-Nitsche K, Zernicka-Goetz M, 2005, Spatial arrangement of individual 4-cell stage blastomeres and the order in which they are generated correlate with blastocyst pattern in the mouse embryo. *Mechanisms of Development* **122**, 487–500.
- Piotrowska-Nitsche K, Perea-Gomez A, Haraguchi S *et al.*, 2005, Four-cell stage mouse blastomeres have different developmental properties. *Development* **132**, 479–490.
- Plusa B, Frankenberg S, Chalmers A *et al.*, 2005, Downregulation of Par3 and aPKC function directs cells towards the ICM in the preimplantation mouse embryo. *Journal of Cell Science* **118**, 505–515.
- Rappolee DA, Patel Y, Jacobson K, 1998, Expression of fibroblast growth factor receptors in peri-implantation mouse embryos. *Molecular Reproduction and Development* **51**, 254–264.
- Richards M, Fong CY, Chan WK *et al.*, 2002, Human feeders support prolonged undifferentiated growth of human inner cell masses and embryonic stem cells. *Nature Biotechnology* **20**, 933–936.
- Rideout 3rd WM, Hochedlinger K, Kyba M *et al.*, 2002, Correction of a genetic defect by nuclear transplantation and combined cell and gene therapy. *Cell* **109**, 17–27.
- Rosner MH, Vigano MA, Ozato K *et al.*, 1990, A POU-domain transcription factor in early stem cells and germ cells of the mammalian embryo. *Nature* **345**, 686–692.
- Rossant J, 2001, Stem cells from the mammalian blastocyst. *Stem Cells* **19**, 477–482.
- Saitou M, Barton SC, Surani MA, 2002, A molecular programme for the specification of germ cell fate in mice. *Nature* **418**, 293–300.
- Sathananthan H, Menezes J, Gunasheela S, 2003, Mechanics of human blastocyst hatching in vitro. *Reproductive BioMedicine Online* **7**, 228–234.
- Sato M, Kimura T, Kurokawa K *et al.*, 2002, Identification of PGC7, a new gene expressed specifically in preimplantation embryos and germ cells. *Mechanisms of Development* **113**, 91–94.
- Sato N, Meijer L, Skaltsounis L *et al.*, 2004, Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nature Medicine* **10**, 55–63.
- Schöler HR, Dressler GR, Balling R *et al.*, 1990, Oct-4: a germline-specific transcription factor mapping to the mouse t-complex. *EMBO Journal* **9**, 2185–2195.
- Shamblott MJ, Axelman J, Wang S *et al.*, 1998, Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proceedings of the National Academy of Sciences of the USA* **95**, 13726–13731.
- Shams M, Ahmed A, 1994, Localization of mRNA for basic fibroblast growth factor in human placenta. *Growth Factors* **11**, 105–111.
- Sharkey AM, King A, Clark DE *et al.*, 1999, Localization of leukemia inhibitory factor and its receptor in human placenta throughout pregnancy. *Biology of Reproduction* **60**, 355–364.
- Sharov AA, Piao Y, Matoba R *et al.*, 2003, Transcriptome analysis of mouse stem cells and early embryos. *Public Library of Science Biology* **1**, E74.
- Sher G, Keskinetepe L, Nouriani M *et al.*, 2004, Expression of sHLA-G in supernatants of individually cultured 46-h embryos: a potentially valuable indicator of 'embryo competency' and IVF outcome. *Reproductive BioMedicine Online* **9**, 74–78.
- Solter D, Knowles BB, 1975, Immunology of mouse blastocyst. *Proceedings of the National Academy of Sciences of the USA* **72**, 5099–5102.
- Spemann H, Mangold H, 1924, Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. *Archiv für Mikroskopische Anatomie und Entwicklungsmechanik* **100**, 599–638.
- Tada M, Takahama Y, Abe K *et al.*, 2001, Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Current Biology* **11**, 1553–1558.
- Tanaka S, Kunath T, Hadjantonakis AK *et al.*, 1998, Promotion of trophoblast stem cell proliferation by FGF4. *Science* **282**, 2072–2075.
- Tarkowski AK, Ozdzinski W, Czolowska R, 2001, How many blastomeres of the 4-cell embryo contribute cells to the mouse body? *International Journal of Developmental Biology* **45**, 811–816.
- Tesarik J, Hazout A, Mendoza C, 2003, Luteinizing hormone affects uterine receptivity independently of ovarian function. *Reproductive BioMedicine Online* **7**, 59–64.
- Thomson JA, Itskovitz-Eldor J, Shapiro SS *et al.*, 1998, Embryonic stem cell lines derived from human blastocysts. *Science* **282**, 1145–1147.
- Turnpenny L, Brickwood S, Spalluto *et al.*, 2003, Derivation of human embryonic germ cells: an alternative source of pluripotent stem cells. *Stem Cells* **21**, 598–609.
- US Department of Health and Human Services, Centers for Disease Control and Prevention, National Center for Chronic Disease Prevention and Health Promotion, Division of Reproductive Health, 2002, Assisted Reproductive Technology Success Rates. National Summary and Fertility Clinic Reports.
- Van Blerkom J, Davis P, Alexander S, 2001, A microscopic and biochemical study of fragmentation phenotypes in stage-appropriate human embryos. *Human Reproduction* **16**, 719–729.
- Van de Velde H, De Vos A, Sermon K *et al.*, 2000, Embryo implantation after biopsy of one or two cells from cleavage-stage embryos with a view to preimplantation genetic diagnosis. *Prenatal Diagnosis* **20**, 1030–1037.
- Vandervorst M, Staessen C, Sermon K *et al.*, 2000, The Brussels' experience of more than 5 years of clinical preimplantation genetic diagnosis. *Human Reproduction Update* **6**, 364–373.
- Vassilopoulos G, Wang PR, Russell DW, 2003, Transplanted bone marrow regenerates liver by cell fusion. *Nature* **422**, 901–904.
- Wang X, Willenbring H, Akkari Y *et al.*, 2003, Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* **422**, 897–901.
- Wang Z, Jaenisch R, 2004, At most three ES cells contribute to the somatic lineages of chimeric mice and of mice produced by ES-tetraploid complementation. *Developmental Biology* **275**, 192–201.
- Watson AJ, Kidder GM, 1988, Immunofluorescence assessment of the timing of appearance and cellular distribution of Na/K-ATPase during mouse embryogenesis. *Developmental Biology* **126**, 80–90.

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- Wu L, de Bruin A, Saavedra HI *et al.*, 2003, Extra-embryonic function of Rb is essential for embryonic development and viability. *Nature* **421**, 942–947.
- Zavos PM, 2003, Human reproductive cloning: the time is near. *Reproductive BioMedicine Online* **6**, 397–398.
- Zernicka-Goetz M, 2003, Determining the first cleavage of the mouse zygote. *Reproductive BioMedicine Online* **6**, 160–163.
- Zernicka-Goetz M, 1998, Fertile offspring derived from mammalian eggs lacking either animal or vegetal poles. *Development* **125**, 4803–4808.
- Zhong W, Sun T, Wang QT *et al.*, 2004, SAPKgamma/JNK1 and SAPKalpha/JNK2 mRNA transcripts are expressed in early gestation human placenta and mouse eggs, preimplantation embryos, and trophoblast stem cells. *Fertility and Sterility* **82** (Suppl. 3), 1140–1148.

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