

Human trophectoderm cells are not yet committed

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STUDY QUESTION: Are human trophectoderm (TE) cells committed or still able to develop into inner cell mass (ICM) cells?

SUMMARY ANSWER: Human full blastocyst TE cells still have the capacity to develop into ICM cells expressing the pluripotency marker NANOG, thus they are not yet committed.

WHAT IS KNOWN ALREADY: Human Day 5 full blastocyst TE cells express the pluripotency markers POU5F1, SOX2 and SALL4 as well as the TE markers HLA-G and KRT18 but not yet CDX2, therefore their developmental direction may not yet be definite.

STUDY DESIGN, SIZE, DURATION: The potency of human blastocyst TE cells was investigated by determining their *in vitro* capacity to develop into a blastocyst with ICM cells expressing NANOG; TE cells were isolated either by aspiration under visual control or after labeling with fluorescent 594-wheat germ agglutinin. Further on, aspirated TE cells were also labeled with fluorescent PKH67 and repositioned in the center of the original embryo.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human preimplantation embryos were used for research after obtaining informed consent from IVF patients. The experiments were approved by the Local Ethical Committee and the 'Belgian Federal Committee on medical and scientific research on embryos *in vitro*'. Outer cells were isolated and reaggregated by micromanipulation. Reconstituted embryos were analyzed by immunocytochemistry.

MAIN RESULTS AND THE ROLE OF CHANCE: Isolated and reaggregated TE cells from full human blastocysts are able to develop into blastocysts with ICM cells expressing the pluripotency marker NANOG. Moreover, the majority of the isolated TE cells which were repositioned in the center of the embryo do not sort back to their original position but integrate within the ICM and start to express NANOG.

LIMITATIONS, REASONS FOR CAUTION: Owing to legal and ethical restrictions, manipulated human embryos cannot be transferred into the uterus to determine their totipotent capacity. The definitive demonstration that embryos reconstructed with TE cells are a source of pluripotent cells is to obtain human embryonic stem cell 'like' line(s), which will allow full characterization of the cells.

WIDER IMPLICATIONS OF THE FINDINGS: Our finding has important implications in reproductive medicine and stem cell biology because TE cells have a greater developmental potential than assumed previously.

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Introduction

Preimplantation development starts with the fusion of two highly differentiated cells—the oocyte and the spermatozoon—resulting in a totipotent zygote. Totipotency of a single cell refers to its ability to generate offspring or to contribute to all embryonic and extraembryonic tissues (Adjaye *et al.*, 2008; Tarkowski *et al.*, 2010). The differentiation capacity of early embryonic cells (blastomeres) is subject to continuous investigation. In animal models, the totipotency of blastomeres can be demonstrated by transferring embryos derived from a single blastomere into a pseudo-pregnant foster mother in order to obtain offspring (Tarkowski *et al.*, 1967; Johnson *et al.*, 1995). Experiments have shown that the potential of blastomeres depends on the cell stage of the embryo from which they are derived, the position of the blastomeres (inner versus outer) and their cellular mass (Tarkowski *et al.*, 2001; Suwinska *et al.*, 2008).

Owing to both ethical and legal restrictions, early human development can only be studied *in vitro*. The capacity of a single blastomere to develop into a blastocyst with an inner cell mass (ICM) and trophectoderm (TE) cells or its ability to contribute to both the ICM and the TE demonstrates at least pluripotency. Only limited data exist on the potency of human blastomeres and it remains unknown at which point of time their totipotent capacity is lost. Recently, we showed that all four blastomeres of a 4-cell stage embryo are able to develop individually into blastocysts with an ICM and TE cells (Van de Velde *et al.*, 2008). The birth of a child after transferring a Day 2 embryo of which only one out of four cells had survived the freezing-thawing procedure confirmed that at least one of the 4-cell stage blastomeres is totipotent (Veiga *et al.*, 1987). Furthermore, it was shown that human embryonic stem cell (hESC) lines can be derived from both single 4-cell stage (Feki *et al.*, 2008; Geens *et al.*, 2009) and 8-cell stage (Klimanskaya *et al.*, 2006; Klimanskaya *et al.*, 2007) blastomeres and blastomeres from early arrested embryos (Zhang *et al.*, 2006; Lerou *et al.*, 2008), indicating that some of the early blastomeres are at least pluripotent.

The aim of this study was to investigate the potency of outer cells from the compaction stage and the blastocyst stage in the human. We hypothesized that the outer cells from compacted embryos, early and full blastocysts (blastocyst scoring according to Gardner and Schoolcraft, 1999) still have the capacity to develop into ICM cells because they express the pluripotency markers POU5F1, SOX2 and SALL4 (Cauffman *et al.*, 2006; Cauffman *et al.*, 2009; Chen *et al.*, 2009) as well as the TE markers HLA-G (Verloes *et al.*, 2011) and KRT18 (Cauffman *et al.*, 2009) but not yet CDX2 (Chen *et al.*, 2009).

Materials and Methods

The experiments were approved by the Local Ethical Committee and the 'Belgian Federal Committee on medical and scientific research on embryos *in vitro*'. Compacted embryos (14–28 cells, no fragmentation) and top quality full and expanding blastocysts (≥ 32 cells, BI3AA and BI4AA, respectively, blastocyst scoring according to Gardner and Schoolcraft (1999)), which were diagnosed to carry a genetic disease after removal of one blastomere on Day 3, were used after obtaining informed consent. For some experiments, cryopreserved 8-cell stage embryos were thawed and cultured to the blastocyst stage, after becoming available

for research after the legally determined 5-year period of storage, and if consent was previously obtained.

Micromanipulation and culture

Embryos were biopsied in $\text{Ca}^{2+}\text{Mg}^{2+}$ -free medium (Vitrolife, Stockholm, Sweden) using laser biopsy. Blastocysts were decavitated by aspirating the fluid out of the blastocoele using an ICSI pipette. Decavitated blastocysts and compacted embryos were incubated in $\text{Ca}^{2+}\text{Mg}^{2+}$ -free medium until full decompaction. Blastomeres were manipulated using a self-made pipette with an inner diameter of 40 μm . Outer blastomeres were aspirated under visual control. Several steps were undertaken to exclude contamination with inner cells: the hole for biopsy was larger than usual in order to be able to intrude the pipette into the perivitelline space without disturbing the position of the blastomeres; by orientating and firmly fixing the blastocyst at the embryonic pole, exclusively mural TE blastomeres were removed; the pipette was never inserted inside the embryo and blastomeres were only taken when they were immediately accessible at the laser hole; blastomeres were taken in one move in order not to re-intrude the pipette and disturb the position of the blastomeres; aspiration was stopped whenever there was the smallest doubt on the original position of the blastomere. All steps were carried out under visual control of two persons. Embryos were reconstituted by putting the blastomeres in an empty zona pellucida. Reconstituted 'outer-cells-only' embryos and the founder (remaining outer + all inner cells) embryos were cultured individually in blastocyst medium (Vitrolife, Stockholm, Sweden; Sage, Pasadena, CA, USA) in 25 μl droplets under oil in 5% O_2 6% CO_2 conditions up to Days 6 and 7 of preimplantation development. For each experiment, unmanipulated embryos were cultured as well. The newly formed blastocysts were morphologically scored before fixation according to Gardner and Schoolcraft (1999): a good-quality blastocyst was considered to be at least BI3BB. For each experiment, non-manipulated embryos were cultured, serving as controls (data not shown).

Immunocytochemistry

Embryos were analyzed by immunocytochemistry and confocal microscopy for the expression of NANOG (ICM) and HLA-G (TE) as described (Verloes *et al.*, 2011). Embryos were fixed for 10 min with 3.7% formaldehyde (Merck, VWR International), permeabilized for 20 min with 0.1% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) at room temperature and incubated overnight at 4°C with the mouse monoclonal immunoglobulin (Ig) G1 anti-HLA-G antibody MEM-G/9 (8 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology, CA, USA), a mouse monoclonal IgG1 anti-NANOG antibody (5 $\mu\text{g}/\text{ml}$; Abcam, Cambridge, UK) and/or rabbit polyclonal anti-NANOG antibodies (3 $\mu\text{g}/\text{ml}$; Abcam). Control reactions for non-specific binding were included in each experiment by replacing the antibodies with a mouse monoclonal IgG1 antibody (BD Biosciences, Erembodegem, Belgium) or rabbit IgGs (Abcam) at the same concentration as the primary antibodies. Alexa Fluor 647-conjugated goat anti-mouse F(ab')₂ fragments (Molecular Probes, Invitrogen, Gent, Belgium), Alexa Fluor 488-conjugated donkey anti-mouse IgG (Molecular Probes, Invitrogen) and Alexa Fluor 488-conjugated donkey anti-mouse IgGs (Molecular Probes, Invitrogen) were used as secondary antibodies at a concentration of 10 $\mu\text{g}/\text{ml}$ for 2 h at 4°C in the dark. All primary and secondary antibody solutions were prepared in phosphate-buffered saline supplemented with 2% bovine serum albumin (Sigma-Aldrich). After staining, embryos were mounted between two glass cover slips (24 × 50 mm) in the Slow-Fade® Gold antifade reagent and round glass cover slips (diameter of 10 mm) were put between the cover slips to prevent squeezing. Confocal scanning microscopy with an Ar-HeNe laser (488/633) (IX71 Fluoview 300; Olympus, Aartselaar, Belgium) was performed to record the fluorescent images.

Blastomere labeling and tracing

To separate outer from inner cells, blastocysts were labeled with Alexa Fluor 594-wheat germ agglutinin (594-WGA) (Molecular Probes, Invitrogen) (Wright, 1984; Basbaum and Menetrey, 1987; Hoshino et al., 2010). Blastocysts were incubated for 10 min in 1 mg/ml pronase (Sigma-Aldrich) to remove the zona pellucida, rinsed in the HEPES medium (Gynotec, Malden, The Netherlands) and cultured for 30 min in blastocyst medium to recover before labeling. Subsequently, they were incubated for 10 min in 5 µg/ml 594-WGA and finally rinsed in the HEPES medium; blastocysts that collapsed during the procedure were not used further. Blastocysts were decapitated as described above and dissociated in $\text{Ca}^{2+}\text{Mg}^{2+}$ -free medium by pipetting. Blastomeres were put individually in 2 µl droplets of HEPES medium and labeling was evaluated using an Axioplan 2 Imaging HBO100 microscope (Zeiss, Zaventem, Belgium). Labeled and unlabeled blastomeres were separated, collected in one droplet of HEPES medium and embryos were reconstituted by putting separated blastomeres in an empty zona pellucida using a 40 µm biopsy pipette.

For tracing experiments, outer blastomeres were isolated from blastocysts, as described above by micromanipulation under visual control of two persons and labeled using PKH67 Fluorescent Cell Linker kit

(Sigma-Aldrich) (Tarkowski et al., 2010). Blastomeres were individually incubated for 30 s in 1/200 dye solution while pipetting. They were rinsed in $\text{Ca}^{2+}\text{Mg}^{2+}$ -free medium and placed in the center of the founder embryo using a 40 µm biopsy pipette.

Results

We performed three types of manipulations to determine at which stage the decision of a blastomere to become either an ICM or a TE cell is irreversibly taken (Fig. 1).

First (manipulation 1), we isolated the outer cells by aspiration under visual control (Fig. 1A). We investigated the potency of outer cells from embryos at the compaction stage (Day 4) and at the blastocyst stage (Days 5 and 6) to develop *in vitro* into blastocysts with both ICM and TE cells. (Table 1 and Fig. 2). The vitality of the *de novo* formed blastocysts was determined by both morphological scoring (Gardner and Schoolcraft, 1999) and immunocytochemistry determining the integrity of the TE epithelium (HLA-G expression) (Verloes et al., 2011) and the potency of the ICM cells (NANOG expression) (Mitsui et al., 2003; Hyslop et al., 2005; Cauffman et al., 2009) (Fig. 3).

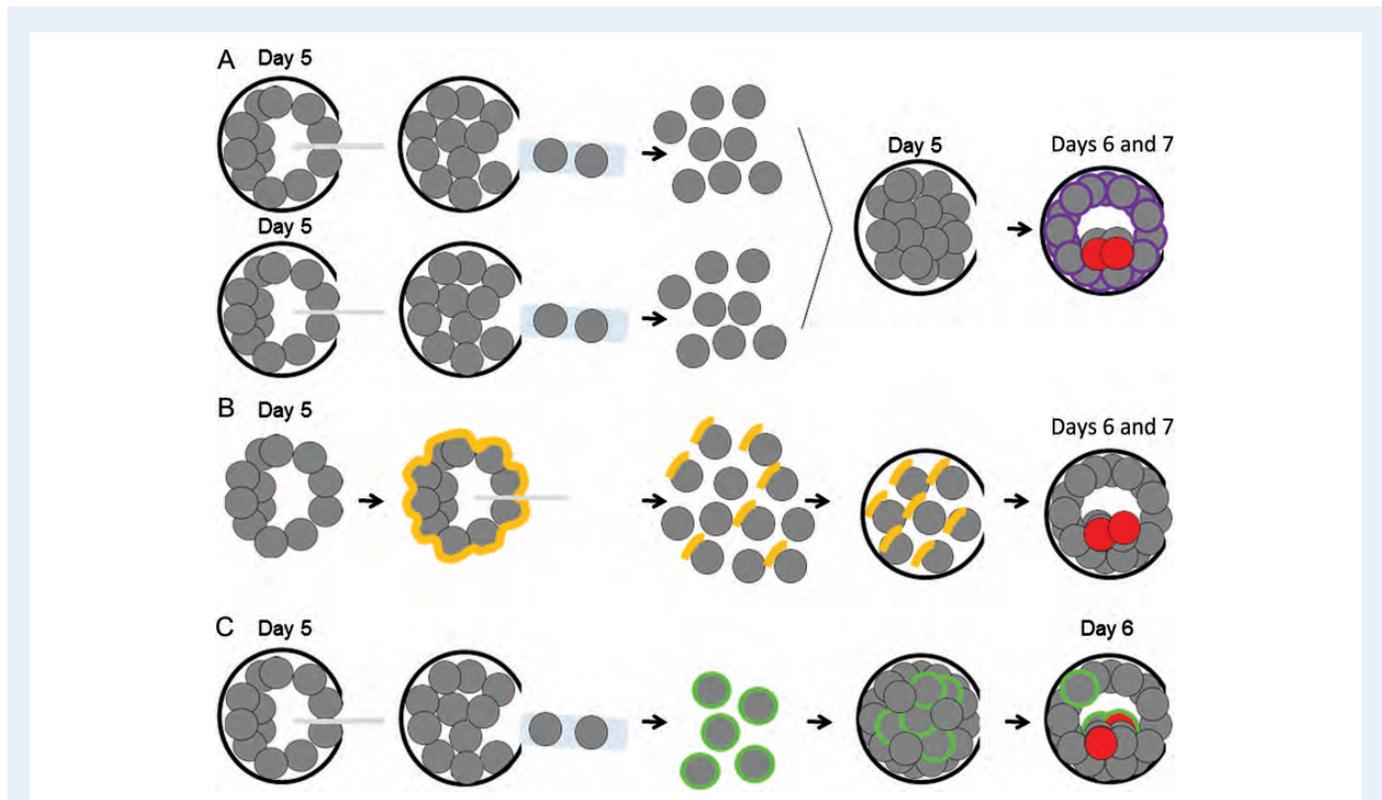


Figure 1 Schematic representation of the manipulations. **(A)** Human blastocysts were decapitated and decompacted, outer blastomeres of two blastocysts were aspirated under visual control, embryos were reconstituted by putting the outer blastomeres in an empty zona pellucida, reconstituted embryos were cultured up to Days 6 and 7 of preimplantation development and immunostained for NANOG (red) and HLA-G (purple); **(B)** the zona pellucida of the blastocysts was removed, blastocysts were labeled with 594-wheat germ agglutinin (WGA) (yellow), decapitated and decompacted, labeled and unlabeled blastomeres were separated by immunofluorescence microscopy, labeled outer cells were collected and put in an empty zona pellucida, reconstituted embryos were cultured up to Days 6 and 7 of preimplantation development and immunostained for NANOG (red); **(C)** blastocysts were decapitated and decompacted, outer blastomeres were aspirated under visual control, labeled with PKH67 (green) and placed in the center of the founder embryo, the position of the labeled cells was determined on Day 6 of preimplantation development, the embryos were immunostained for NANOG (red). The number of blastomeres drawn and the colors used are not representative of the manipulations performed.

Table 1 Human TE cells are not yet committed.

Experiment	Stage	Embryos manipulated (n)	Blastocysts developed (n)	Blastocysts which developed an ICM (n)
1	Compaction Day 4 1 founder	11	10	8
	Outer-cells-only (5–9 cells)	11	8	0
2	Compaction Day 4 2 founder	22	22	17
	Outer-cells-only (12–14 cells)	11	11	10
3	B11–B12 Day 5 2 founder	10	9	8
	Outer-cells-only (10–23 cells)	5	3	2
4	B13 Day 5 2 founder	6	5	5
	Outer-cells-only (19–30 cells)	3	2	1
5	B13 Day 5 3 founder	6	6	6
	Outer-cells-only (21–45 cells)	2	2	2

Outer cells were extracted from one (experiment 1), two (experiments 2, 3 and 4) or three (experiment 5) embryos at different stages and days of preimplantation development: compaction on Day 4 (experiments 1 and 2); early blastocysts B11 and B12 (experiment 3) on Day 5 and full blastocyst B13 on Day 5 (experiments 4 and 5). The founder embryos and the 'outer-cells-only' embryos were cultured until Days 6 and 7, developing into blastocysts with a clearly visible ICM.

First we investigated the potency of outer cells from compacted embryos. A limited number of outer cells (5–9) was aspirated and reagggregated in an empty zona pellucida. The 'outer-cells-only' embryos recompact and developed into blastocysts but none of them appeared to have an ICM (Table 1, Figs 2A and 3A). This could be due to loss of capacity at compaction or to insufficient cell mass to generate an inner population. To compensate for the presumed insufficient cell mass, we reagggregated outer cells from two distinct embryos (12–14 cells in total). These 'outer-cells-only' embryos recompact and developed into good-quality blastocysts with an ICM in which NANOG was found (Table 1, Figs 2B and 3B). Next we investigated the potency of outer cells from early blastocysts. Outer cells from two blastocysts (10–23 cells in total) were reagggregated. They recompact, recavitated and some developed into blastocysts with ICM cells expressing NANOG (Table 1). In case of full blastocysts, we reconstituted embryos with outer cells from two blastocysts (Table 1, Figs 2C and 3C) and three blastocysts (Table 1 and Fig. 3D) to have sufficient cellular mass (19–45 cells in total). The 'outer-cells-only' embryos recompact, recavitated and developed into blastocysts with an ICM expressing NANOG. The majority of the founder embryos developed into good-quality blastocysts with an ICM in which NANOG was found (Table 1). Similar experiments with Days 5 and 6 expanded blastocysts failed (data not shown). Day 5 expanding blastocysts could be decavitated and decompact; however, isolated TE cells used to reconstitute embryos were not able to recompact but vacuolated. Day 6 expanded blastocysts could not be completely decavitated and decompaction did not occur. Additional attempts at Days 5 and 6 to isolate TE cells by laser cutting (Schoolcraft *et al.*, 2010) and incubation in $\text{Ca}^{2+}\text{Mg}^{2+}$ -free medium to obtain single TE cells resulted in significant cell death. Our results show that outer cells from human full blastocysts can still change their developmental direction, whereas outer cells from expanding blastocysts no longer have this capacity.

To confirm our results (manipulation 2), we performed additional labeling experiments in which we could use nearly all TE cells from the original blastocyst and thus we did not need to pool outer cells from distinct embryos (Fig. 1B). Outer cells were labeled with 594-WGA. To test the specificity of 594-WGA, we labeled one compacted embryo and four full blastocysts; 594-WGA was shown to stick to the membrane of the outer cells and not to enter the cavity; labeled inner cells were not detected (Fig. 4A). We separated labeled outer and unlabeled inner cells from six full blastocysts by fluorescence microscopy and reconstituted 'outer-cells-only' embryos with labeled cells. The *in vitro* development of the reconstituted embryos was delayed because recompaction after 594-WGA labeling seemed to be impaired. Three out of six 'outer-cells-only' embryos developed into good-quality blastocysts with an ICM on Days 6 and 7 (Table II and Fig. 4B). From one of the six full blastocysts we also reconstituted an embryo with unlabeled 'inner-cells-only', it developed into a good-quality blastocyst with an ICM on Day 7. NANOG was found in ICM cells of both the 'inner-cells-only' and 'outer-cells-only' embryo on Day 7, supporting our hypothesis that outer cells of a full human blastocyst have the capacity to change their developmental direction as well as inner cells.

Next we hypothesized that the outer cells can change their developmental direction when they are put in an inner position. To investigate this (manipulation 3), we repositioned labeled TE cells from a full human blastocyst in an inner position (Fig. 1C). However, 594-WGA could not be used to trace outer cells because it disappeared from the membrane after 16 h *in vitro* culture. Therefore a limited number of outer cells (maximum eight to avoid contamination with inner cells) were extracted and labeled with PKH67 Fluorescent Cell Linker kit (Tarkowski *et al.*, 2010). This lipid dye could be used for cell tracing after 24 h *in vitro* culture but not after 48 h. Labeled cells were placed in the center of the decavitated and decompact founder embryo and their position was determined after 20–24 h *in vitro* culture using confocal microscopy. The manipulated embryos

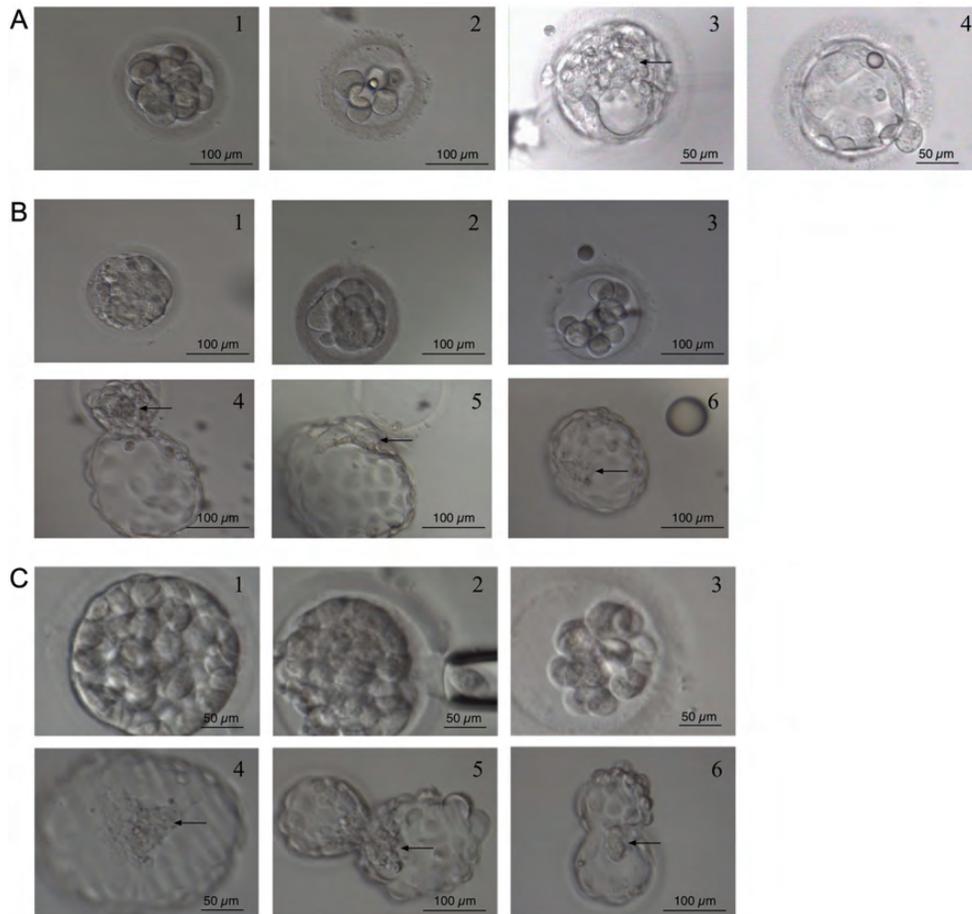


Figure 2 'Outer-cells-only' human embryos can develop into a blastocyst with ICM cells. **(A)** Decompacted embryo (A1) on Day 4, 'outer-cells-only' (A2) developed further into blastocysts without an ICM (A4), whereas the founder embryo developed into a blastocyst with an ICM (A3) on Day 6; **(B)** two decompacted embryos (B1 and B2), 'outer-cells-only' (B3) developed into blastocysts with an ICM (B6) as well as the founder embryos (B4 and B5) on Day 6; **(C)** two full blastocysts (C1 and C2) on Day 5, 'outer-cells-only' (C3) developed into blastocysts with an ICM (C6) as well as the founder embryos (C4 and C5) on Day 6. The arrow indicates the ICM. Images taken using an inverted microscope: Nikon Eclipse TE300.

developed into blastocysts with an ICM (Table II). We detected the majority of the PKH67-labeled cells in the ICM of the newly formed blastocysts (88.7%) (Fig. 5), indicating that the outer cells integrated within the developing ICM of the founder embryo. In these ICM, NANOG was found in PKH67-labeled cells (54.7%) as well as in PKH67-unlabeled founder cells. We conclude that the TE cells from a full human blastocyst have the capacity to change their developmental direction when put in an inner position, rather than sorting back to their original position.

Discussion

We found that TE cells from a full human blastocyst as well as ICM cells have the capacity to develop *in vitro* into a blastocyst with ICM cells expressing NANOG and we conclude that these cells can still change lineage direction. We suggest that human full blastocyst TE cells as well as ICM cells are potentially totipotent. Their totipotent capacity will never be proven because they cannot be transferred

into a uterus. When put in an inner position, full blastocyst TE cells can integrate within the ICM and start to express NANOG, indicating that their lineage direction is determined by position. Since full blastocyst TE cells still express essential master genes of pluripotency (Cauffman et al., 2006; Cauffman et al., 2009; Chen et al., 2009) as well as epithelium markers (Cauffman et al., 2009; Verloes et al., 2011), they probably do not need to completely alter their expression profile. The lineage direction of TE cells is irreversible at the expanding blastocyst stage and corresponds with the onset of nuclear CDX2 expression (Chen et al., 2009) and the down-regulation of nuclear expression of the pluripotency key players SOX2 (Cauffman et al., 2009) and POU5F1 (Chen et al., 2009).

Our primary aim was to investigate the potency of outer cells from full blastocysts because they still express pluripotency markers. Therefore we decavitated and decompacted the blastocysts and aspirated single TE cells as for cleavage-stage biopsy. The cells of early and full blastocysts did not degenerate after decavitation, decompaction and aspiration, and they recompact

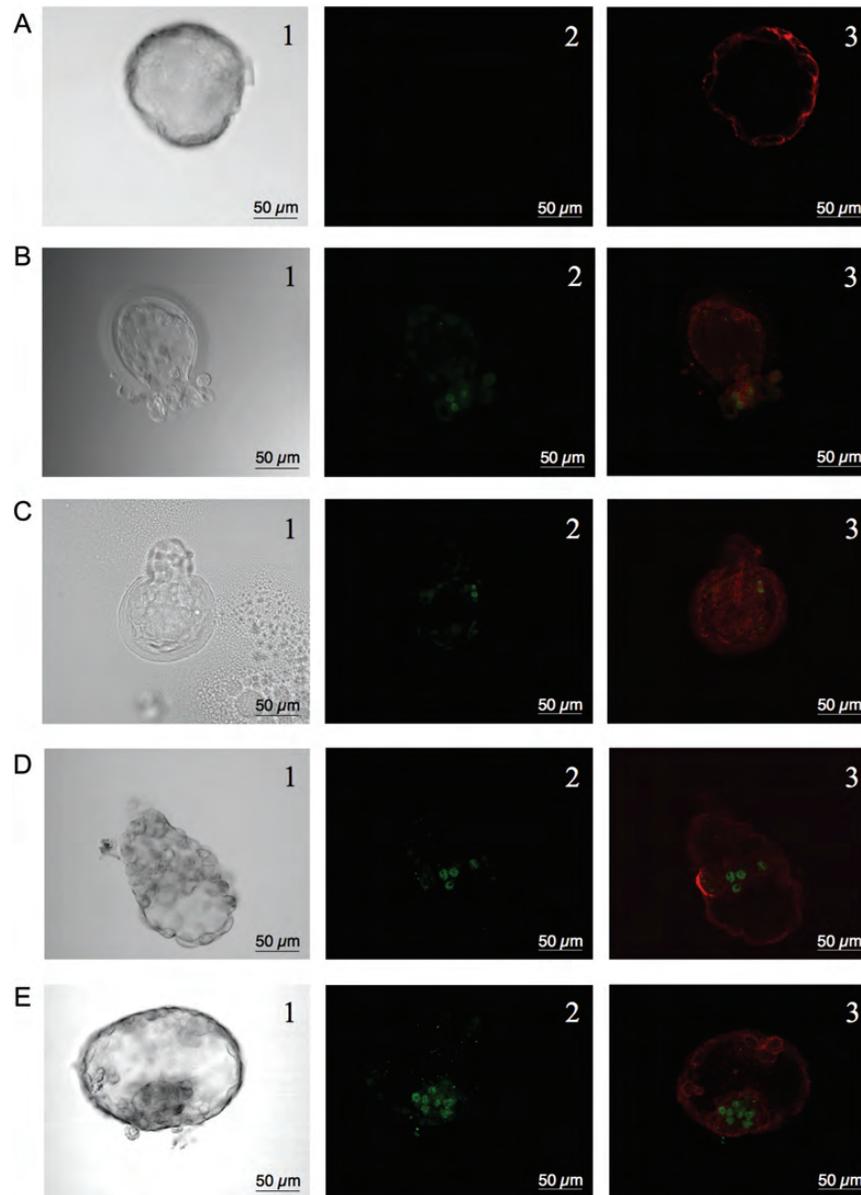


Figure 3 'Outer-cells-only' human embryos can develop into a blastocyst with ICM cells expressing the pluripotency marker NANOG. NANOG (green in 2 and 3) and HLA-G (red in 3) immunocytochemistry in Day 6 blastocysts reconstituted with outer-cells-only from (A) one Day 4 compacted embryo; (B) two Day 4 compacted embryos; (C) two Day 5 full blastocysts; (D) three Day 5 full blastocysts; and (E) a founder full blastocyst. The phase contrasts of the embryos are shown in (1).

easily. They developed into blastocysts with a cohesive TE expressing HLA-G (Verloes *et al.*, 2011) and an ICM. NANOG was found in some but not all ICM cells, as described (Mitsui *et al.*, 2003; Hyslop *et al.*, 2005; Cauffman *et al.*, 2009). We only took a limited number of outer cells from two to three blastocysts in order not to have contamination with inner cells on the one hand and to have enough cells to have an inner population in the newly formed embryos on the other hand. It was not possible to reconstitute embryos with the original number of blastomeres because of the scarcity of top quality embryos at the same developmental stage available for research.

To confirm our findings, we did additional experiments in which outer cells were labeled prior to biopsy. Experiments performed to specifically label outer cells with PKH67 Fluorescent Cell Linker, as described in the mouse (Suwinska *et al.*, 2008), failed. In all experimental conditions tested (incubation time and dye concentration), outer and inner cells from compacted embryos and blastocysts were labeled. The distinct labeling results obtained in the mouse and the human are most likely related to differences in the membrane of the blastomeres between the two species. We were able to label outer cells with 594-WGA which sticks to the membrane of the outer cells and could be used to separate them from the inner cells by

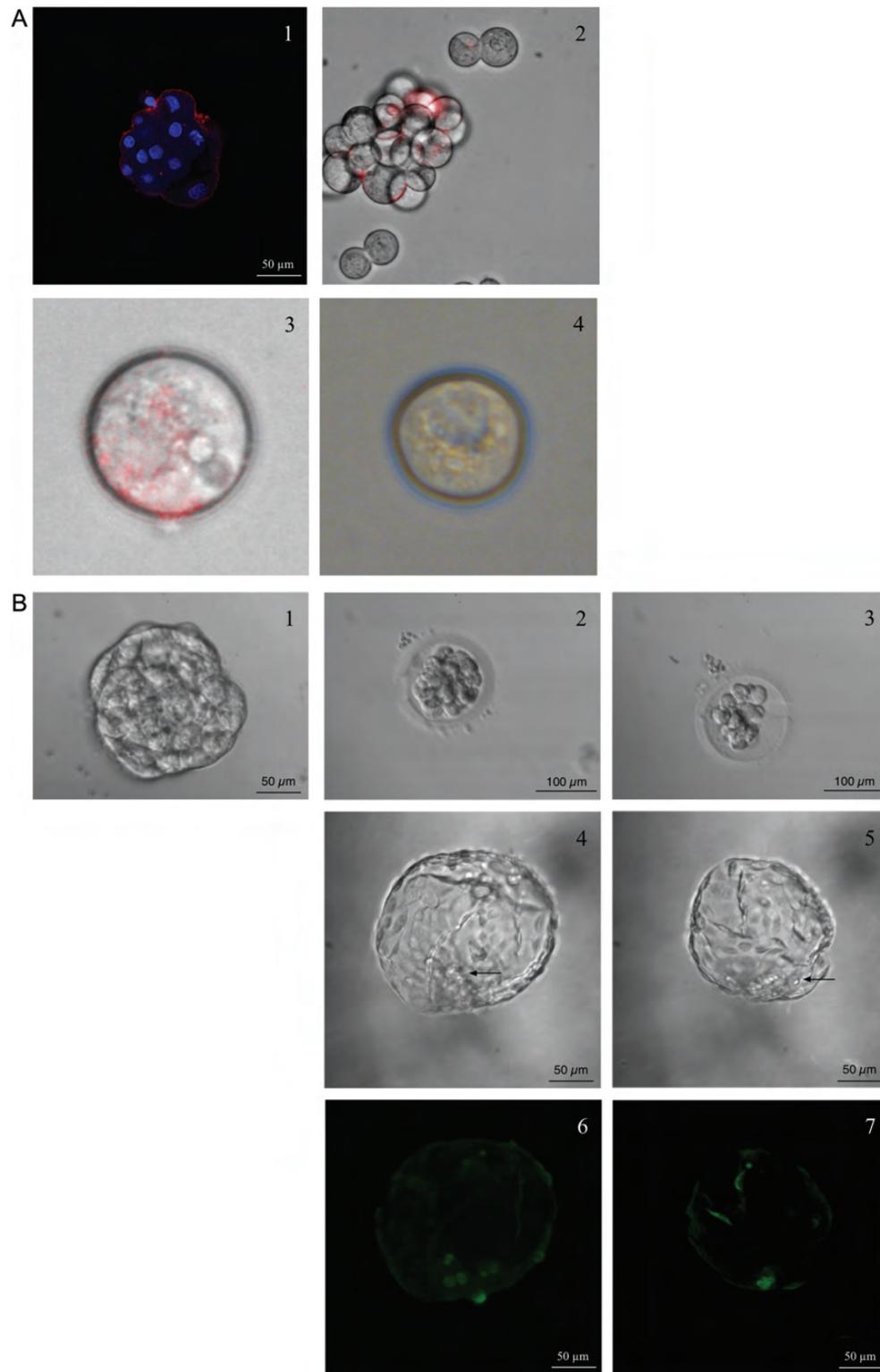


Figure 4 TE cells as well as ICM cells from a human full blastocyst are not yet committed. A Day 5 blastocyst was labeled with 594-WGA (A1); labeled outer cells and unlabeled inner cells could be distinguished by immunofluorescence microscopy (A2) and separated one-by-one into labeled (A3) and unlabeled (A4) cells; an embryo reconstituted with outer-cells-only (B2) developed into a blastocyst with ICM cells (phase contrast in B4) expressing NANOG (green in B6); an embryo reconstituted with inner-cells-only (B3) developed into a blastocyst with ICM cells (B3; phase contrast in B5) expressing NANOG (green in B7). The arrows indicate the ICM. The original embryo is shown in (B1).

Table II Human full blastocyst TE cells are not yet committed.

Experiment	Stage	Embryos reconstituted (n)	Blastocysts developed (n)	Blastocysts which developed an ICM (n)
1	BI3 Day 5			
	Outer-cells-only (20–36 cells)	6	3	3
	Inner-cells-only (25 cells)	1	1	1
2	BI3 Day 5	6	5	5

(Experiment 1) outer cells were labeled with 594-wheat germ agglutinin to separate outer from inner cells, embryos were reconstituted with labeled 'outer-cells-only' or unlabeled 'inner-cells-only' and cultured further until Days 6 and 7; (Experiment 2) four to eight outer cells extracted from Day 5 full blastocysts BI3 were labeled with PKH67, repositioned in the center of the founder decavitated and decompacted embryos, and the embryos were cultured further until Day 6.

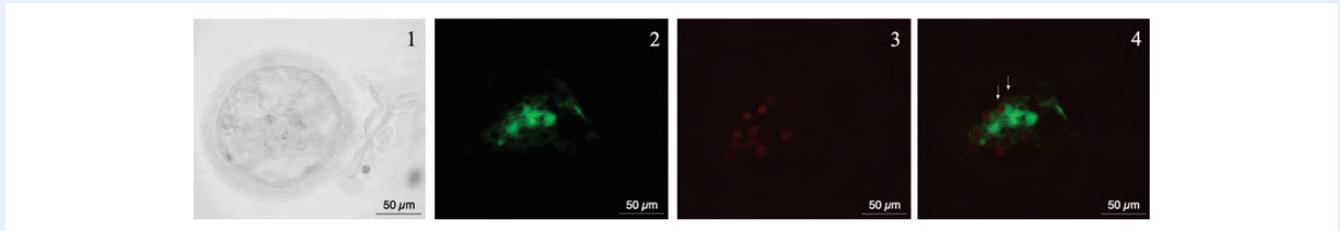


Figure 5 Human TE cells put in an inner position do not sort back to their original position and start to express NANOG. Isolated outer cells from a Day 5 full blastocyst were labeled with the PKH67 green fluorescent dye and reintroduced in the center of the founder decavitated and decompacted embryo. The labeled outer cells integrated within the founder ICM (green in 2) and expressed the pluripotency marker NANOG (red in 3) on Day 6. The green/red combination is shown in (4), the arrows indicate PKH67-labeled cells expressing NANOG. The phase contrast of the reconstituted embryo is shown in (1).

fluorescence microscopy. We confirmed that 'outer-cells-only' of a full human blastocyst can develop into blastocysts with an ICM, as well as 'inner-cells-only'. However, 594-WGA labeling interfered with recompaction and resulted in a developmental delay. Therefore, NANOG was not found in Day 6 ICM cells as described (Mitsui *et al.*, 2003; Hyslop *et al.*, 2005; Cauffman *et al.*, 2009) but only in some Day 7 ICM cells.

We also found that human full blastocyst TE cells are capable of changing lineage direction if they are put in an inner position, indicating that position determines fate (Tarkowski, 1967). These TE cells could integrate within the ICM and some cells started to express NANOG, confirming that they can become precursor epiblast cells (Mitsui *et al.*, 2003; Hyslop *et al.*, 2005; Cauffman *et al.*, 2009). Tracing outer cells with 594-WGA could not be performed because it disappeared after 16 h *in vitro* culture (Day 6) and NANOG could only be detected on Day 7. Although PKH67 could not be used to specifically label outer cells in the blastocyst, we could use PKH67 to label the outer cells after isolation. Also, only a limited number of outer cells were extracted in order to exclude contamination with inner cells. The majority of the labeled and repositioned outer cells did not sort back to an outer position but integrated within the ICM and started to express NANOG, confirming that they can change lineage direction.

The outer cells of expanding Day 5 human blastocysts could be isolated but the single cells were unable to recompact, and vacuolated; the outer cells of an expanded Day 6 blastocyst formed a strong epithelium and the cells could no longer be disaggregated into single cells, suggesting that at these stages the TE cells have lost their plasticity to change developmental direction. This stage of development (i.e.

expansion) coincides with the presence of CDX2 and the down-regulation of SOX2 and POU5F1 transcription factors in the nuclei of TE cells (Cauffman *et al.*, 2009; Chen *et al.*, 2009).

Similar experiments have been performed in the mouse and cattle, however, with a different outcome. Embryos reconstituted with inner or outer cells from compacted mouse embryos (16 cells) to the original cell number can develop into offspring, indicating that inner cells as well as outer cells are totipotent (Suwinska *et al.*, 2008). The inner cells of early blastocysts (32 cells) are able to develop into new blastocysts with an ICM but they fail to implant, indicating that they are pluripotent but not totipotent (Suwinska *et al.*, 2008). On the other hand, the outer cells of early blastocysts recavitate but fail to develop an ICM, indicating loss of totipotency and pluripotency. We believe this is related to their inability to recompact properly and generate an inner cell population after manipulation. Recently, using tetraploid carrier blastomeres able to form only TE cells, it was shown that single outer cells from a mouse 32-cell stage embryo can sporadically develop into a fetus (1/42 embryos transferred) (Tarkowski *et al.*, 2010). The data from Tarkowski's group indicate that TE cells from an early mouse blastocyst are rarely pluripotent (Suwinska *et al.* 2008; Tarkowski *et al.*, 2010). TE cells from bovine embryos are pluripotent at least until the expanding blastocyst stage on Day 8 (Berg *et al.*, 2011). We found that human outer cells were able to recompact and develop into blastocysts with an ICM up to the 64-cell stage, suggesting that their plasticity remains until a later cell stage in the human as compared with the mouse but not as long as in the bovine. Moreover, when the position of blastomeres of early mouse blastocysts is randomly changed, these embryos

overcome the perturbation by sorting the cells back to their original inside or outside position rather than by changing their developmental direction (Suwinska et al., 2008). We found that human TE cells from a full blastocyst do not sort themselves back to their outside position when placed in an inside position. They change their developmental direction, integrate within the ICM cells and some cells start to express the pluripotency marker NANOG.

Our finding has important implications in reproductive medicine because plasticity allows the early embryo to overcome perturbations in its organization until the very late stages of preimplantation development. This implies that the impaired implantation capacity of embryos after a 2-cell biopsy for PGD is linked to cell loss (De Vos et al., 2009). It would also mean that in the clinic, blastocysts lacking an ICM may benefit from manipulation (decavitation and decompaction) in order to generate an inner cell population and, subsequently, an embryo which is capable of implanting and developing further. Moreover, this finding may have important implications in regenerative medicine because full blastocyst TE cells could be a source of hESC lines, avoiding the destruction of the embryo which continues to develop normally *in vitro* and may be transferred into a uterus to obtain offspring (Geens et al., 2009).

We conclude that full human blastocyst TE cells are not yet committed towards TE epithelium. Therefore, as they are a potential source of hESC, the future development of hESC lines will provide sufficient material for the analysis of additional markers and demonstration of the ability of cells to differentiate into ectoderm, mesoderm and endoderm: these data would definitively confirm our conclusion that embryos reconstructed with TE cells are a source of pluripotent cells.

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Authors' roles

H.V.d.V. contributed to conception and design, provision of study material, experimental work, data analysis and interpretation and writing of the manuscript; C.P. and G.C. contributed to experimental work, data analysis and interpretation, revision and final approval of the manuscript; A.V. contributed to experimental work and final approval of the manuscript; J.S. contributed to experimental work and final approval of the manuscript; I.L. and H.T. contributed to revision and final approval of the manuscript; P.D. contributed to final approval of the manuscript.

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Conflict of interest

None declared.

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