

Markers that define stemness in ESC are unable to identify the totipotent cells in human preimplantation embryos

G. Cauffman^{1,4}, M. De Rycke^{1,2}, K. Sermon^{1,2}, I. Liebaers^{1,2}, and H. Van de Velde^{1,3}

¹Department of Embryology and Genetics, UZ Brussel and the Vrije Universiteit Brussel, Laarbeeklaan 101, Brussels 1090, Belgium ²Centre for Medical Genetics, UZ Brussel and the Vrije Universiteit Brussel, Laarbeeklaan 101, Brussels 1090, Belgium ³Centre for Reproductive Medicine, UZ Brussel and the Vrije Universiteit Brussel, Laarbeeklaan 101, Brussels 1090, Belgium

⁴Correspondence address. E-mail: greet.cauffman@uzbrussel.be

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BACKGROUND: During human preimplantation development, early blastomeres are believed to be totipotent. It is likely, however, that blastomeres are allocated to a specific lineage prior to any morphological differentiation. NANOG, SOX2 and SALL4 are transcription factors that play a key role in controlling stemness in embryonic stem cells (ESC) and are therefore candidate markers for developmental triggers in early embryos. *KRT18*, a trophoblast-determining gene, may mark early differentiation. Examining the expression pattern of these genes may inform us about when and in which cells totipotency is lost during early human development.

METHODS: Thirteen oocytes, 124 preimplantation embryos and 7 human embryonic stem cell (hESC) lines were examined for the presence of NANOG, SOX2, SALL4 or KRT18 proteins using immunostaining and confocal microscopy.

RESULTS: All stemness markers were expressed in the hESC, but none of them was specific for totipotent cells during human preimplantation development, and none of them seemed to mark cells allocated to the inner cell mass (ICM) or trophectoderm. After lineage specification, only the nuclear expression of NANOG and SOX2 became restricted to the ICM, at least to some cells because only a subpopulation expressed NANOG. *KRT18* expression was seen for the first time during compaction in some outer cells. *KRT18* was not expressed in hESC.

CONCLUSION: We conclude that the protein expression patterns of markers that define stemness in ESC do not identify the totipotent cells in human preimplantation embryos. Assessing the presence of KRT18 proteins implied that the outer cells of compacting embryos have probably lost their totipotent competence prior to any visible differentiation.

Key words: human preimplantation embryos / KRT18 / NANOG / SOX2 / totipotency

Introduction

Totipotency is the ability of a single cell to divide and differentiate into all cell types in an organism and produce fertile offspring (Edwards and Beard, 1997). Oocytes and sperm are the most differentiated cells in our body; yet, after fertilization, a zygote is formed—the ultimate totipotent cell giving rise to all cell types and live offspring. Development can be described as a progressive loss of totipotency, pluripotency and unipotency, finally resulting in differentiation into definite cell types. The initial loss of totipotency occurs during preimplantation development and becomes apparent for the first time when two distinct cell lineages in the blastocyst segregate forming the inner cell mass (ICM), which contains the founder cells of the embryonic and extra-embryonic layers, and the trophoblast (TE), an epithelial layer which contributes to the trophoblast portion of the placenta. The point at which preimplantation cells lose their totipotent competence is still unknown. Early blastomeres are believed to be totipotent in order to overcome aberrations in the development of the embryo, although data suggesting prepatterning have also been reported (Hansis et al., 2005). The ultimate proof of the totipotency of early embryonic cells is provided when an isolated blastomere is able to develop into normal fertile offspring (Edwards and Beard, 1997). In humans, totipotency is difficult to prove because of ethical objections and legal regulations. However, understanding totipotency during human preimplantation development will have a great impact on the current knowledge of development, cell loss by fragmentation, cryopreservation and preimplantation genetic diagnosis and the derivation of human embryonic stem cells (hESC) without destroying embryos.

hESC have the capacity to self-renew indefinitely in an undifferentiated state and to differentiate into cell types representing the three embryonic germ lineages, germ cells, extra-embryonic tissue and trophoblast, thus showing totipotent potential. However, as long as their contribution to all cell types of the human body is not proven, they are, by definition, pluripotent. *POU5F1* (formerly called *OCT-4*), *SOX2* and *NANOG* are the earliest expressed set of genes known to control stemness in hESC (Hyslop et al., 2005; Zaehres et al., 2005). Stemness refers to the stem cell properties of self-renewal and the generation of differentiated progeny (Melton and Cowan, 2004). *POU5F1*, *SOX2* and *NANOG* encode the transcription factors POU5F1, SOX2 and NANOG, respectively, which activate their own genes as well as genes that encode components of key signaling pathways and repress genes that are essential for developmental processes (Boyer et al., 2005). POU5F1, SOX2 and NANOG collaborate and form an intertwined network in the control of hESC fate through autoregulatory and feed-forward loops (Boyer et al., 2005; Chew et al., 2005; Okumura-Nakanishi et al., 2005; Rodda et al., 2005). POU5F1 and SOX2 regulate each other and a few thousand regulatory sites in the ESC genome including the *NANOG* promoter through the formation of a heterodimer (Boyer et al., 2005; Kuroda et al., 2005; Rodda et al., 2005). *SALL4*, another transcription factor, has been reported in mouse ESC as a new component of the transcription regulatory network. *SALL4* may form a regulatory circuit together with *NANOG* similar to that of *POU5F1* and *SOX2* (Wu et al., 2006). Recently, expression studies of *POU5F1* in hESC have shown that only one of the isoforms of *POU5F1*, namely *POU5F1_iA*, plays a role in stemness (Cauffman et al., 2006; Lee et al., 2006).

Because these factors play a key role in the regulatory network of stemness in ESC and because hESC lines have been derived from various preimplantation stages (Thomson et al., 1998; Strelchenko et al., 2004; Klimanskaya et al., 2006), they are candidate genes for developmental decisions in early human embryos. Expression patterns of *POU5F1* during human preimplantation development have recently been described, and it has been shown that *POU5F1* does not identify the totipotent cells, at least not on its own (Cauffman et al., 2006). The aim of this study is to examine whether the protein expression pattern of the other stemness genes *NANOG*, *SOX2* and *SALL4* can inform us about when and in which cells totipotency is lost during early human development. In addition, we have also examined the expression of cytokeratin 18 (*KRT18*), a trophoblast-determining gene that encodes epithelial-specific intermediate filaments. *KRT18* transcripts mark early differentiation in hESC (Bhattacharya et al., 2004, 2005) and are enriched in the TE samples of human blastocysts (Adjaye et al., 2005).

Materials and Methods

Human embryonic stem cells

Seven hESC lines were used, of which two are presumed to be genetically normal (VUB02 and VUB07) and five carry mutations for myotonic dystrophy type I (VUB03_DMI), cystic fibrosis (VUB04_CF), facioscapulohumeral muscular dystrophy (VUB09_FSHD), fragile X (VUB11_FRAXA) or adult polycystic kidney disease (VUB12_APKD) (Mateizel et al., 2006). Cell lines were cultured as described previously (Mateizel et al., 2006).

Human oocytes and preimplantation embryos

Human oocytes and preimplantation embryos were obtained for research at our Centre for Reproductive Medicine with the informed consent of the couples and the approval of the institutional ethical committee. Oocytes were denuded from surrounding cumulus and corona cells (Van de Velde et al., 1997). The oocytes used were immature, at the germinal vesicle stage or the metaphase I stage, or were *in vitro*-matured metaphase II oocytes. Embryos were obtained from normally fertilized oocytes after conventional IVF or ICSI (Devroey and Van Steirteghem, 2004). The embryos used were assessed as unsuitable for transfer or cryopreservation, or were obtained by applying ICSI on *in vitro*-matured oocytes donated for research. Preimplantation embryo development was evaluated daily (Van Landuyt et al., 2005). Blastocyst evaluation relied on the scoring system described by Gardner and Schoolcraft (1999). Early blastocysts have blastocoels that do not completely fill the embryo, and the TE and the ICM are morphologically not clearly defined. Full blastocysts have blastocoels that completely fill the embryo and a morphologically clearly defined TE and ICM. In an expanded blastocyst, the blastocoel volume is enlarged and the zona pellucida is thinning.

Immunostaining and confocal microscopy

Human foreskin fibroblasts, which were used as a negative control for the antibodies, and hESC colonies were plated 1 or 2 days before staining in a four-well Multidish Nunclon™Δ (NUNC A/S, Roskilde, Denmark). Oocytes and embryos were individually stained in 50 µl droplets in a 96-well plate (Cellstar, Greiner Bio-one, Frickenhausen, Germany). Fixation was performed with 3.7% formaldehyde (Merck, Darmstadt, Germany) for 10 min at room temperature and was followed by permeabilization with 0.1% Triton X-100 (Sigma-Aldrich, St Louis, MO, USA) for 20 min at room temperature. Both solutions were made in phosphate-buffered saline (PBS). Samples were incubated overnight at 4°C with either

a rabbit polyclonal IgG antibody against NANOG (2 µg/ml; ab21624, Abcam, Cambridge, UK) or SOX2 (3 µg/ml; ab15830, Abcam), or a mouse monoclonal IgG1 antibody against SALL4 (2 µg/ml; H00057167-M03, Abnova, Taipei, Taiwan, Republic of China) or KRT18 (1 µg/ml; ab668, Abcam). Control reactions for the non-specific binding of the primary antibodies were included in each experiment and carried out by replacing the rabbit antibodies with rabbit serum (R9133, Sigma-Aldrich) and the mouse antibodies with mouse IgG1s (349040, Becton Dickinson, Franklin Lakes, NJ, USA) under the same conditions as the primary antibodies. Alexa Fluor 647-conjugated donkey anti-rabbit IgGs (A-31573, Molecular Probes, Invitrogen, Stockholm, Sweden) and Alexa Fluor 488-conjugated goat anti-rabbit F(ab')₂ fragments (A-11070, Molecular Probes) and donkey anti-mouse IgGs (A-21202, Molecular Probes) were used as secondary antibodies. Samples were incubated at a concentration of 10 µg/ml for 2 h at 4°C in the dark. All antibody solutions were prepared in PBS supplemented with 2% bovine serum albumin (BSA) (Sigma-Aldrich). Extensive washing with PBS (supplemented with 2% BSA in the case of oocytes and embryos) was performed between all steps. After staining, wells containing hESC were covered with ProLong[®] Gold antifade reagent (P36930, Invitrogen) or with SlowFade[®] Gold antifade reagent with DAPI (S36939, Invitrogen). Oocytes and embryos were put between two glass cover slips (24 × 50 mm) in 2 µl ProLong[®] Gold antifade reagent or SlowFade[®] Gold antifade reagent with DAPI. To prevent squeezing, round glass cover slips (Ø 10 mm) were put between the cover slips using nail polish. Before examination, samples were put at 4°C in the dark for at least 30 min.

Confocal scanning microscopy with an Ar–HeNe laser (488/633) (IX71 Fluoview 300; Olympus, Aartselaar, Belgium) was performed to record the fluorescent images. Fluorescent images were also taken using the FviewII camera (Olympus). Control and test images were captured using identical settings.

Results

Human embryonic stem cells

NANOG, SOX2 and SALL4 proteins were detected in the nuclei of hESC, but not in the nucleoli. SOX2 was also occasionally detected

in the cytoplasm, in particular in the middle of a colony. KRT18 proteins were not detected in undifferentiated colonies. At early differentiation, KRT18 staining was shown in the cytoskeleton of some cells, especially at the periphery of the colony. Representative images are shown in Fig. 1 and Supplementary Fig. S1.

Human oocytes and preimplantation embryos

NANOG expression was examined in 5 oocytes, 10 cleavage-stage embryos, 13 compacted embryos and 26 blastocysts. No expression of NANOG could be detected before the blastocyst stage. Of the 9 early blastocysts tested, only 1 showed a clear nuclear expression in 10 inner cells. Of the eight full blastocysts tested, three blastocysts were negative and four blastocysts showed a clear expression in some nuclei of the ICM (one, four, five and eight nuclei, respectively), and once in one nucleus of the polar and mural TE. The remaining full blastocyst displayed a strong nuclear staining in four cells of the ICM and in two cells of the mural TE. All nine expanded blastocysts showed a strong nuclear staining in a subpopulation of the ICM (ranging from 3 to 12 cells), and in four of these blastocysts, there was also staining in a few mural or polar TE cells. The intensity of the TE staining was similar or less than in the ICM. The NANOG-positive mural cells were positioned at the same height as the ICM.

SOX2 expression was examined in 2 oocytes, 7 cleavage-stage embryos, 5 compacted embryos and 18 blastocysts. In oocytes and in Day 2 cleavage-stage embryos, SOX2 expression was detected in the cytoplasm but not in the nuclei. Day 3 cleavage-stage embryos displayed a cytoplasmic staining in all cells and a nuclear staining in some with an intensity similar to the cytoplasm. Compacted embryos and early blastocysts expressed SOX2 in the cytoplasm and in all nuclei. The nuclear staining was similar or more intense compared with the cytoplasm. In full blastocysts, all cells of the ICM showed a stronger nuclear expression. Some nuclei of the TE showed no expression, whereas others showed a stronger or similar expression compared

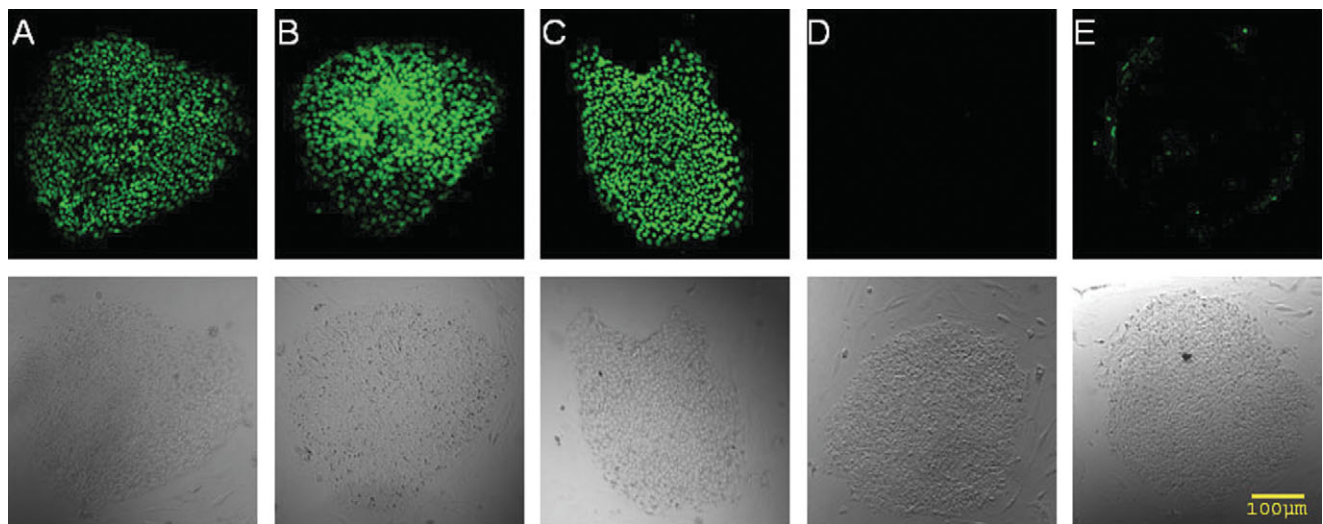


Figure 1 NANOG (A), SOX2 (B), SALL4 (C) and KRT18 (D) expression in hESC colonies and KRT18 expression in an early differentiated colony (E). Each immunofluorescent image is accompanied by the differential interference contrast image of the colony.

with the cytoplasm. Expanded blastocysts displayed a much stronger SOX2 expression in the nuclei of the ICM compared with the cytoplasm, and no expression in the nuclei of the TE, except for in a few blastocysts in which a nuclear staining was still found in some TE cells, located at the same height as the ICM.

SALL4 expression was examined in 4 oocytes, 1 zygote, 8 cleavage-stage embryos, 3 compacted embryos and 15 blastocysts. Oocytes, a zygote and Day 2 cleavage-stage embryos displayed a weak *SALL4* expression in the nuclei and cytoplasm. In Day 3 cleavage-stage embryos, the nuclear expression remained but the intensity of the staining in the cytoplasm decreased. In Day 4 cleavage-stage embryos, the nuclear staining increased and the cytoplasmic staining almost vanished and completely disappeared in compacted embryos. All blastocysts displayed a very strong nuclear staining and no staining in the cytoplasm. A similar intensity of staining was observed in the ICM and the TE at all stages of blastocyst expansion.

KRT18 expression was examined in 2 oocytes, 3 cleavage-stage embryos, 5 compacted embryos and 13 blastocysts. Expression was seen for the first time during compaction in the cytoskeleton of some outer cells. At all stages of blastocyst expansion, a strong expression was found in the cytoskeleton of the TE and the outer cells of the ICM, which will form the hypoblast at implantation.

Representative images of the staining are shown in Fig. 2, Supplementary Figs S2 and S3 and Supplementary videos/serial sections S6–S10.

Negative controls

The staining of the human foreskin fibroblasts and the control reactions for the non-specific binding of the primary antibodies were negative (Supplementary Figs S4–S5).

Discussion

NANOG, SOX2 and POU5F1 are the key transcription factors in regulating stemness. In hESC, NANOG, SOX2 and POU5F1 work in concert, and knockdown of one of them induces differentiation (Hay et al., 2004; Matin et al., 2004; Chew et al., 2005; Zaehres et al., 2005; Babaie et al., 2007). None of these factors is dispensable or capable of compensating the role of the other. Recently, it has been shown that NANOG, SOX2 and POU5F1 in combination with LIN28, and SOX2 and POU5F1 in combination with KLF4 and c-MYC are capable of reprogramming human somatic cells into pluripotent stem cells (Takahashi et al., 2007; Yu et al., 2007). In mice, knockdown of *Sall4* leads to a reduced level of ESC-specific gene expression and an increased expression of differentiation-associated marker genes, implying a role in maintaining the undifferentiated state (Wu et al., 2006; Zhang et al., 2006). Hence, it seems that the signature of stemness can be identified by a set of genes expressed by stem cells. In order to identify the totipotent cells during human preimplantation development and determine when and in which cells totipotency is lost and developmental decisions are taken, the expression of the stemness genes NANOG, SOX2 and *SALL4* was examined at the protein level. Expression studies of *POU5F1* in human embryos have already shown that *POU5F1* is not a marker for totipotent cells during human preimplantation development, at least not on its own (Cauffman et al., 2006).

We report that none of the key stemness factors was able to identify the totipotent cells during human preimplantation development, and that these factors did not direct cells towards the ICM or TE lineage (Fig. 3). Until now, only zygotes have been proven to be totipotent, and blastomeres from early cleavage-stage embryos before Day 3 of development are all, or at least some of them, assumed to be totipotent. However, only *SALL4* was weakly expressed in the nucleus of a zygote and early blastomeres. The lack of expression of the other stemness markers before Day 3 of development could be explained, because global human embryonic expression starts at Day 3 (Braude et al., 1988; Dobson et al., 2004). Until this point, maternal RNA and proteins direct early development. After the embryonic genome is activated, we found the expression of stemness markers in cleavage-stage embryos either in the nuclei of all cells (SOX2 and *SALL4*) or in none of the cells (NANOG). Lineage specification in the early embryo becomes morphologically distinguishable at the early blastocyst stage. As has already been reported for *POU5F1* (Cauffman et al., 2006), SOX2 and *SALL4* were expressed in the nuclei of all cells of early blastocysts and of compacted embryos, i.e. the stage just before the first signs of differentiation become apparent. NANOG proteins could at the earliest be detected at the blastocyst stage and were consistently present in expanded blastocysts. Therefore, none of these proteins seems to be involved in the decision that determines which cells will become ICM and which become TE. After the ICM and the TE could clearly be distinguished, only the nuclear expression of NANOG and SOX2 became as good as restricted to the ICM, at least to some of the cells because only a subpopulation of the ICM expressed NANOG. NANOG expression has also been reported in another study using a different antibody from ours (Hyslop et al., 2005). The authors found only the expression of NANOG in the ICM of expanded blastocysts; however, it is not clear whether earlier stages of blastulation were examined and whether all the cells of the ICM of expanded blastocysts expressed NANOG. Possible explanations for NANOG not being expressed in all the ICM cells might be that NANOG marks (i) the quality of the ICM cells, (ii) the ICM cells from which hESC lines can be derived, (iii) cells committed to the epiblast (Darr et al., 2006), (iv) future primordial germ cells (Zwaka and Thomson, 2005) or (v) precursors of the primitive streak (Rossant, 2008). hESC lines have been derived from the ICM, compacted embryos and blastomeres from 8-cell-stage embryos (Thomson et al., 1998; Strelchenko et al., 2004; Klimanskaya et al., 2006), though during human preimplantation development, the stemness genes NANOG, SOX2, *SALL4* and *POU5F1* are only commonly expressed in a subpopulation of the ICM. Because these stemness factors have been shown to work together to maintain the properties of hESC, it might be possible that these properties are only maintained in some ICM cells. A sporadic NANOG expression was also detected in a minority of TE cells located near the ICM. An explanation cannot readily be given, but it might be possible that TE cells that were in contact for longer with the ICM during blastocyst formation are still shifting their expression pattern before adopting their final one. However, our conclusions are based on the results of one technique and should be confirmed by other techniques.

ESC may be an accessible model for studying early development; care should be taken however when assuming equivalence between ESC and early embryonic cells, because the former exist only *in vitro*, whereas the latter are a transient *in vivo* population.

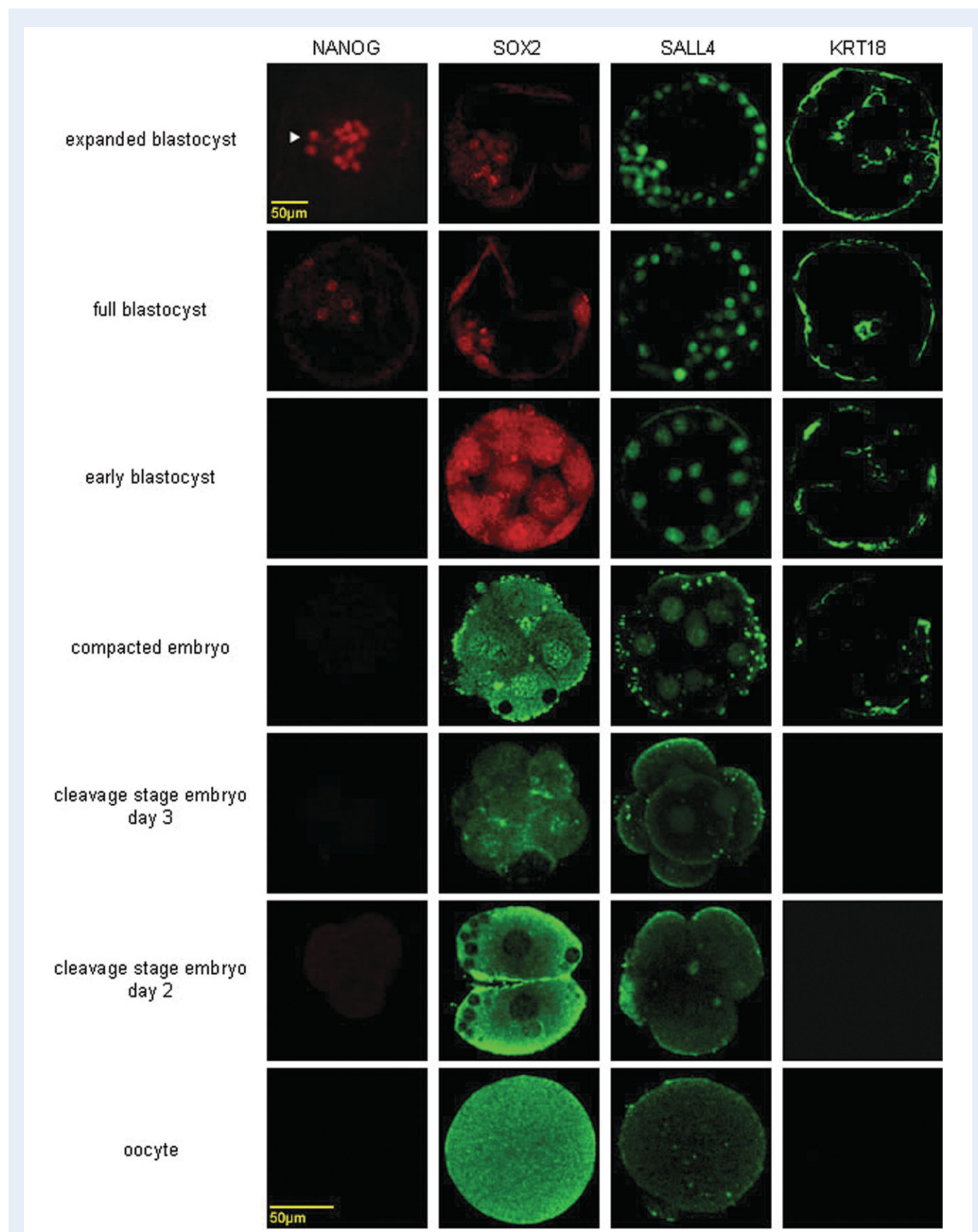
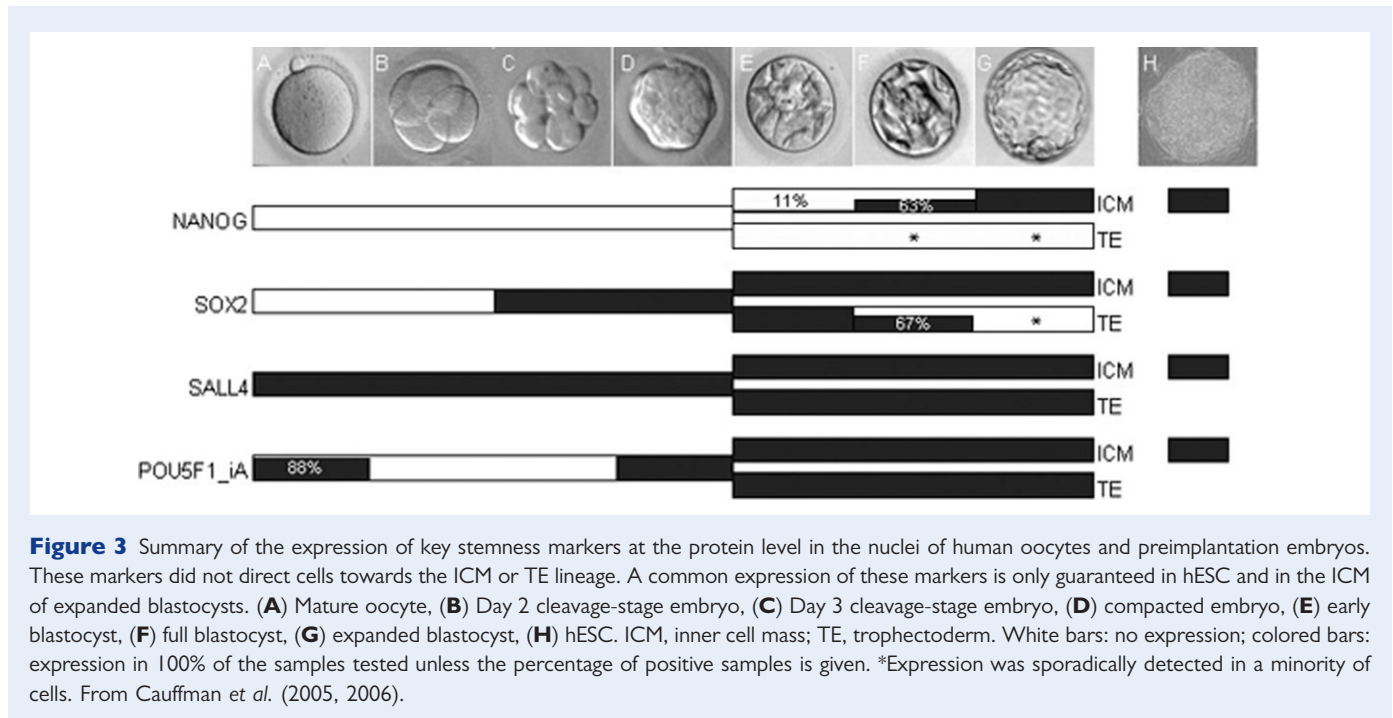


Figure 2 NANOG, SOX2, SALL4 and KRT18 expression throughout human preimplantation development and in a mature oocyte. Immunostaining was performed using either a rabbit polyclonal IgG antibody against NANOG or SOX2, or a mouse monoclonal IgG1 antibody against SALL4 or KRT18. As secondary antibodies, Alexa Fluor 647-conjugated donkey anti-rabbit IgGs, Alexa Fluor 488-conjugated goat anti-rabbit F(ab')₂ fragments and donkey anti-mouse IgGs were used. Each image represents a section throughout the examined material. The arrowhead indicates staining in two nuclei of the polar TE.



The process of ESC derivation may create a cell type that is not present in the normal embryo, and ESC differentiation occurs in response to an environment that does not reflect the developmental context of embryogenesis. ESC may therefore have different molecular and biological characteristics from the cells in the embryo (Hansson et al., 2007).

During human preimplantation development, it is most likely that cells are allocated to a specific lineage prior to any visible morphological differentiation. How and when these cells lose their totipotent competence still needs to be solved, and is currently being debated (Hiiragi et al., 2005; Vogel, 2005). Edwards hypothesizes that the cells of the early preimplantation embryo are already prepatterned at the four-cell stage: two cells are determined to become ICM, one cell to become TE and one cell to contribute to the germ line (Edwards and Beard, 1997; Edwards, 2005). Edwards' pre patterning model has recently been challenged by Van de Velde et al. (2008). The authors have shown that the blastomeres of a four-cell human embryo are individually capable of developing into blastocysts consisting of TE and ICM, hereby supporting the idea that the cells of early cleavage-stage embryos are flexible and potentially totipotent (Van de Velde et al., 2008). The examination of *KRT18* expression, a trophoblast-determining gene encoding epithelial-specific intermediate filaments, showed the presence of *KRT18* proteins as early as compaction in some outer cells. This implies that the outer cells of compacted embryos have probably lost their totipotent competence prior to any visible morphological differentiation. During blastocyst expansion, *KRT18* expression was found in the cytoskeleton of the TE and the outer cells of the ICM adjacent to the blastocoel cavity. At implantation, the latter cells form the hypoblast that will contribute to the yolk sac. So, *KRT18* may be used as a very early marker of differentiation during human preimplantation development. Our finding suggests that totipotency is lost in the outer cells of compacted embryos, supports the inside–outside hypothesis of Tarkowski and

Wroblewska (1967) and has narrowed the time frame in which the first developmental decisions are taken in the human preimplantation embryo, i.e. after the four-cell stage (Van de Velde et al., 2008) and before compaction.

The identification of *NANOG*, *SOX2* and *POU5F1* as master regulators in hESC and as a part of four genes that can reprogram human somatic cells into pluripotent stem cells has been a breakthrough in stem cell biology. Although their expression is crucial in regulating stemness, the presence of their proteins does not in itself define totipotency in the early embryo. Therefore, revealing the upstream mechanisms that control the stemness genes is indispensable in understanding potency. Oocytes and ESC share the capacity to reprogram a somatic nucleus into an undifferentiated one, and they have a high abundance of chromatin and DNA-modifying genes in common (Kocabas et al., 2006), which suggests that an epigenetic network is involved in the initiation and maintenance of the undifferentiated state in ESC and in normal development. Furthermore, epigenetic reprogramming occurs during mammalian development at stages when the developmental potency of cells changes, which suggests a role in early lineage development in the embryo (Morgan et al., 2005).

Conclusion

NANOG, *SOX2*, *POU5F1* and *SALL4* are key regulators in ESC. Although their expression is crucial in regulating stemness properties of ESC that are derived from preimplantation embryos, their protein expression pattern in embryos could not answer the question of when early embryonic cells lose their totipotent competence. None of these stemness factors could identify the totipotent cells during human preimplantation development or induce a specific allocation of the dividing blastomeres towards the ICM or TE lineage. All this suggests the involvement of additional regulators in establishing the totipotent state. Therefore, to elucidate the upstream mechanisms,

in particular the epigenetic mechanisms that control the stemness genes, might be of great importance. Assessing the presence of KRT18 proteins has enabled us to narrow the time window in which the first developmental decisions are taken. Further research on this topic is vital, not only because it will help us understand basic embryology and stem cell biology but also because it will have an impact on current practice in the IVF laboratory with respect to cell loss during preimplantation development and on the derivation of hESC lines without destroying embryos.

Supplementary Data

Supplementary data are available at <http://humrep.oxfordjournals.org/>.

Funding

Fonds Wetenschappelijk Onderzoek—Vlaanderen.

Acknowledgements

We wish to thank the colleagues at the Embryonic Stem Cell Laboratory and the Centre for Reproductive Medicine of the UZ Brussels. We also thank M. Whitburn of the Language Education Centre for proofreading the manuscript.

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Submitted on December 17, 2007; resubmitted on July 1, 2008; accepted on September 1, 2008