The roles of FGF and MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine and human embryos

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SUMMARY
At the blastocyst stage of mammalian pre-implantation development, three distinct cell lineages have formed: trophectoderm, hypoblast (primitive endoderm) and epiblast. The inability to derive embryonic stem (ES) cell lines in a variety of species suggests divergence between species in the cell signaling pathways involved in early lineage specification. In mouse, segregation of the primitive endoderm lineage from the pluripotent epiblast lineage depends on FGF/MAP kinase signaling, but it is unknown whether this is conserved between species. Here we examined segregation of the hypoblast and epiblast lineages in bovine and human embryos through modulation of FGF/MAP kinase signaling pathways in cultured embryos. Bovine embryos stimulated with FGF4 and heparin form inner cell masses (ICMs) composed entirely of hypoblast cells and no epiblast cells. Inhibition of MEK in bovine embryos results in ICMs with increased epiblast precursors and decreased hypoblast precursors. The hypoblast precursor population was not fully ablated upon MEK inhibition, indicating that other factors are involved in hypoblast differentiation. Surprisingly, inhibition of FGF signaling upstream of MEK had no effects on epiblast and hypoblast precursor numbers in bovine development, suggesting that GATA6 expression is not dependent on FGF signaling. By contrast, in human embryos, inhibition of MEK did not significantly alter epiblast or hypoblast precursor numbers despite the ability of the MEK inhibitor to potentially inhibit ERK phosphorylation in human ES cells. These findings demonstrate intrinsic differences in early mammalian development in the role of the FGF/MAP kinase signaling pathways in governing hypoblast versus epiblast lineage choices.

KEY WORDS: FGF, Lineage segregation, MAP kinase, Epiblast, Hypoblast, Bovine, Human

INTRODUCTION
Mammalian pre-implantation development is characterized by the development of the trophectoderm (TE), the hypoblast (primitive endoderm) and the epiblast cell lineages. The three different cell types in peri-implantation embryos are the result of two consecutive lineage segregation events. The first lineage specification occurs at the morula stage, when outer cells segregate from the inner cells and differentiate into the TE. The second lineage specification takes place in the inner cell mass (ICM) of blastocyst stage embryos, where the precursors of the hypoblast segregate from precursors of the epiblast. In the mouse (Mus musculus), the TE gives rise to parts of the trophectoderm/placenta and the chorion, whereas the primitive endoderm (PE) develops primarily to parietal endoderm and visceral endoderm, and the epiblast gives rise to the embryo proper, the umbilical cord, the amnion and part of the chorion (Ralston and Rossant, 2005). In bovine (Bos taurus) and human (Homo sapiens) embryos, the hypoblast gives rise to the primitive yolk sac and the secondary yolk sac (Docherty et al., 1996; Maddox-Hyttel et al., 2003).

In the ICM of embryonic day (E) 3.5 mouse blastocysts, the epiblast and PE progenitors can be recognized by the mosaic expression of the transcription factors NANOG and GATA6, respectively (Chazaud et al., 2006). Mouse embryos that lack Nanog do not develop epiblast cells (Mitsui et al., 2003; Silva et al., 2009), whereas embryos mutant for Gata6 initiate the formation of the PE but fail to develop functional visceral endoderm (Morrisey et al., 1998; Koutsourakis et al., 1999).

Various lines of evidence indicate that the formation of the murine PE is initiated upon FGF-mediated activation of GRB2 and subsequent activation of the mitogen-activated protein kinase (MAP kinase) signal transduction pathway. Indeed, mouse embryos that lack Fgf4, Fgfr2 or Grb2 fail to develop PE cells and all ICM cells of E3.5 Grb2 mutant blastocysts have epiblast characteristics (Feldman et al., 1995; Arman et al., 1998; Cheng et al., 1998; Chazaud et al., 2006). Moreover, recent studies have demonstrated that it is possible to shift the balance between epiblast and PE precursors through modulation of the levels of FGF/MAP kinase signaling in pre-implantation mouse embryos (Nichols et al., 2009b; Lanner et al., 2010; Yamanaka et al., 2010).

MAP kinase signaling is also involved in the differentiation of mouse embryonic stem (ES) cells. It is possible to prevent mouse ES cells from differentiating through inhibition of MEK (Hamazaki et al., 2006; Ying et al., 2008). Moreover, by simultaneous inhibition of FGF/MAP kinase signaling and glycogen synthase kinase 3β (GSK3β), mouse ES cells can be captured in a ground
state of self-renewal (Ying et al., 2008). Similar conditions have allowed the generation of ES cell lines from hitherto impermissive mouse strains and from rat embryos (Buehr et al., 2008; Li et al., 2008; Nichols et al., 2009a).

Notwithstanding our mechanistic insight into the development of the pluripotent cell population and the differentiation thereof in mouse embryos, this has yielded limited benefit for the establishment of ES cell lines in species that were hitherto recalcitrant to ES cell derivation (Kuijk et al., 2008). This lack of success probably reflects mechanistic differences between species in early lineage specification. Moreover, it is well understood that mouse and human ES cells are rather different from each other, but it is not well understood why mouse ES cell culture conditions fail to support human ES cell self-renewal (Buecker and Geijsen, 2010; Kuijk et al., 2011). In other words, there is an urgent need for comparative studies on pre-implantation development, including that of human embryos (Rossant, 2011a; Rossant, 2011b).

Studies on the development of the epiblast and hypoblast cell lineages in species other than mouse have mainly focused on morphological changes and the expression of marker genes (Kuijk et al., 2008; Cauffman et al., 2009; Hall et al., 2009). Functional studies are more cumbersome in non-mouse mammals, and consequently little is known about the degree of conservation in the developmental mechanism that establishes the PE and epiblast lineages.

Here we performed functional studies on the development of the hypoblast and epiblast cell lineages through interference with signaling pathways in bovine and human embryo cultures. In bovine embryos we could alter the balance of epiblast and hypoblast precursors by modulating FGF and MAP kinase signaling. Stimulation of bovine embryos with FGF4 resulted in ICMs composed entirely of hypoblast precursors. However, contrary to our expectations, inhibition of the FGF pathway did not alter the balance toward more epiblast precursors, even though inhibition of MEK resulted in a significant shift toward more epiblast cells and fewer hypoblast cells in bovine blastocysts. Despite potent inhibition of MAP kinase signaling in human cells, manipulating this signal transduction cascade in developing human embryos did not have an effect on the formation of hypoblast or epiblast cells. Our study provides evidence for species divergence in the mechanisms of early mammalian development.

**MATERIALS AND METHODS**

**Growth factors and inhibitors**

The following inhibitors/growth factors were used: 25 μM PD98059 (MEK inhibitor; Sigma-Aldrich), 3 μM CHIR99021 (GSK3B inhibitor; Stemgent), 0.5 μM A-83-01 (ALK5 inhibitor; Stemgent) and 1 μg/ml human recombinant FGF4 (R&D Systems, Minneapolis, MN, USA). Within experiments, all embryos were cultured in equal concentrations of DMSO.

**Bovine in vitro fertilization (IVF) and embryo culture**

Bovine oocyte retrieval, in vitro oocyte maturation, IVF and subsequent in vitro bovine embryo culture were as previously described (Kuijk et al., 2008). In short, cumulus oocyte complexes (COCs) were retrieved from 3- to 8-mm follicles of cow ovaries, which were collected at a local slaughterhouse. Oocytes with an intact cumulus oophorus were matured in M199 (Gibco, Paisley, UK) supplemented with 0.25 IU/ml recombinant human FSH (Organon, Oss, The Netherlands) and 1% (v/v) penicillin-streptomycin ( Gibco). Matured oocytes were transferred to fertilization medium (Fert-Talp) supplemented with 1.8 IU/ml heparin, 20 μM di-penicillamine, 10 μM hypotaurine and 1 μM epinephrine (all Sigma-Aldrich). Subsequently, frozen-thawed semen from a fertile bull was centrifuged over a Percoll gradient and sperm cells were added to the COCs at a final concentration of 1×10^6 spermatozoa/ml. After 20 hours of incubation, presumptive zygotes were denuded and placed in synthetic oviduct fluid (SOF) medium. Incubation took place at 39°C in a humidified atmosphere with 7% O2 and 5% CO2. On day 5 of embryo culture, cleaved embryos were transferred to fresh SOF medium (containing growth factors or inhibitors as appropriate) and cultured to day 8, when blastocysts were collected and fixed for immunofluorescence.

**Human IVF and embryo culture**

Human embryos were obtained at the Centre for Reproductive Medicine of the University Hospital in Brussels, with the informed consent of the couples and the approval of the Institutional Ethical Committee and the Federal Ethical Committee For Research On Human Embryos In Vitro. Ovarian hyperstimulation, oocyte maturation triggering and intracytoplasmic sperm injection (ICSI) were performed according to standard clinical protocols as described (Devroey and Van Steirteghem, 2004) and embryos were cultured in sequential medium (Sage, Irvine, CA, USA). Incubation took place at 37°C in a dry atmosphere with 6% O2 and 5% CO2. The embryos were biopsied for pre-implantation genetic diagnosis (PGD) on day 3 and diagnosed to carry harmful genetic mutations at day 4 of development, which rendered them unsuitable for transfer. The diagnosed mutations do not interfere with pre-implantation development and therefore should not have any effects on the outcome of the current study. Embryos were transferred to culture medium containing 0.5 μM PD0325901 or DMSO (negative control) immediately after the outcome of the PGD on day 4. Embryos were subsequently cultured in these conditions until embryonic day 6 and then fixed for 10 minutes in 4% paraformaldehyde (PFA) before they were subjected to whole-mount immunofluorescence.

**Whole-mount immunofluorescence**

Blastocysts were washed briefly in PBS and fixed for 10 minutes at room temperature (RT) in 4% PFA. After a brief wash in PBS with 10% fetal calf serum (FCS) and 0.1% Triton X-100 (PBST), embryos were permeabilized in PBS with 0.5% FCS and 0.5% Triton X-100 for 30 minutes at RT. Next, embryos were incubated in PBST for 1 hour at RT to block non-specific binding sites. This blocking step was followed by overnight incubation at 4°C in primary antibodies diluted in PBST. For the CDX2 staining, the blocking step was preceded by a 30-minute incubation in 2M HCl at RT and pH neutralization for 10 minutes with 100 mM Tris-HCl buffer (pH 8.5).

The primary antibodies used were: mouse anti-CDX2 (Biogenex, Freemont, CA, USA), mouse anti-GATA4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-GATA6 (Santa Cruz), mouse anti-NANOG (eBioscience, San Diego, CA, USA) and goat anti-NANOG (R&D Systems). The embryos were then washed in PBST and transferred to PBST containing Alexa Fluor-conjugated secondary antibodies (Invitrogen, Venlo, The Netherlands). After 1 hour in secondary antibody solution, embryos were counterstained with TO-PRO-3 (Invitrogen) and mounted in Vectashield mounting medium (Brunschwic Chemie, Amsterdam, The Netherlands). Fluorescent signals were visualized using an SPE confocal laser-scanning microscope (Leica, Rijswijk, The Netherlands). Images were further analyzed with ImageJ software (Abramoff et al., 2004). Image acquisition, analysis and processing were standardized within each experiment.

For all embryos identical image acquisition (laser power, light path, objective, gains, offsets) and z-stack settings were used to minimize potential differences in signal strength from embryo to embryo. Moreover, to minimize bleaching, embryos were not exposed to light emitted from mercury lamps and they were only briefly exposed to laser light to set the exposure window. Whole-mount immunofluorescence.

**Statistical analysis**

The experimenter responsible for image analysis and quantification was blinded to the treatments. Cell numbers were manually counted, aided by the Cell Counter plug-in in ImageJ (Abramoff et al., 2004). Day 8 bovine embryos with fewer than ten ICM cells were considered of poor quality and excluded from the final analysis. Two-tailed Student’s t-tests were used.
to evaluate statistical differences in absolute cell numbers between groups. Two-sided Mann-Whitney U tests were used to evaluate statistical differences in relative cell numbers between groups. \( P<0.05 \) was considered to denote significant differences between groups.

**Immunoblot analysis**

HUES1 human ES cells were grown in mouse embryonic fibroblast (MEF)-conditioned medium on gelatin until 80% confluency. They were starved for 30 minutes in 0.1% FCS followed by the addition of different concentrations (0, 5, 1, 0.2, 0.04, 0.008 \( \mu \)M) of the MEK inhibitor PD0325901 for 15 minutes. Protein extracts were isolated using RIPA buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich) and orthovanadate (Sigma-Aldrich) as phosphatase inhibitor. Equal amounts of protein were loaded onto precast polyacrylamide gels (Bio-Rad, Veenendaal, The Netherlands) and separated by electrophoresis. Immunoblots were performed with primary antibodies against ERK (Santa Cruz) and phospho-ERK (Cell Signaling, Beverly, MA, USA) and HRP-conjugated secondary antibodies. Antibodies were detected using the chemiluminescent substrate ECL (Millipore, Amsterdam, The Netherlands).

**RESULTS**

**The formation of the epiblast and hypoblast cell lineages in bovine development**

In mouse development, NANOG is initially expressed in all blastomeres of compacting embryos, but its expression becomes restricted to the epiblast precursors at the blastocyst stage (Chambers et al., 2003; Chazaud et al., 2006; Dietrich and Hiiragi, 2007; Plusa et al., 2008). GATA6 expression has been reported from the 4-cell stage onwards, reaching ubiquitous expression at the morula stage, after which it is downregulated in the TE and becomes restricted to the hypoblast precursor cells (Koutsourakis et al., 1999; Chazaud et al., 2006; Plusa et al., 2008). Consequently, NANOG and GATA6 show a mosaic expression pattern in the ICM of late blastocyst stage mouse embryos (Chazaud et al., 2006; Plusa et al., 2008).

Using in vitro derived bovine embryos we performed whole-mount immunofluorescence for NANOG and GATA6 on day 5-8 embryos to examine the expression dynamics of these transcription factors in bovine development. NANOG expression was not observed in bovine morula stage embryos at day 5 of development (Fig. 1A; \( n=6 \); total number of cells: 19.7±6.9), but weak expression could be observed in non-expanded blastocysts at day 6 of development (in three out of seven embryos; total number of cells/embryo: 39.0±18.9). At days 7 and 8 of development, strong NANOG expression was observed in a subset of cells of the ICM. GATA6 expression was observed in some nuclei of day 5 embryos and in most nuclei of day 6 and day 7 embryos. Quantification revealed a logarithmic correlation between the number of blastomeres and the number of GATA6-positive cells in bovine embryos at days 5-7 of development (Fig. 1B). At day 8 of development, when the expanded blastocyst stage, strong GATA6 expression became restricted to cells of the ICM (Fig. 1A), although in some day 8 embryos weak GATA6 expression was observed in the TE cells, probably because the expression in these cells had not yet been fully downregulated. We also examined the expression of GATA4 in bovine day 8 blastocysts, but in contrast to the mouse where GATA4 is expressed in the PE (Plusa et al., 2008), GATA4 expression in bovine embryos was detected in both TE cells and ICM cells (Fig. 1C). We therefore considered GATA6 the better marker to study bovine hypoblast development.

GATA6 expression appeared mutually exclusive with the NANOG-positive cell population in the ICM (\( n=6 \)). However, on careful examination, a few cells of day 8 embryos were found to be double positive for GATA6 and NANOG (6.7% of all the NANOG-positive cells). By contrast, embryos at day 7 of development contained on average 39.5% double-positive cells. These results indicate that NANOG and GATA6 become progressively restricted to distinct cell lineages. In addition to a
‘pepper-and-salt’ distribution of NANOG and GATA6 in the ICM of day 8 embryos, we observed ICMs in which NANOG-positive cells were partially enclosed by GATA6-positive cells, indicating that the physical segregation of the epiblast and hypoblast cell lineages had already initiated in these embryos (Fig. 1A, bottom panels). We conclude from these experiments that at the bovine blastocyst stage the transcription factors NANOG and GATA6 are expressed in the epiblast and hypoblast precursors, respectively.

A role for MAP kinase signaling in the segregation of the epiblast and hypoblast cell lineages in bovine development

The MEK inhibitor PD98059 can successfully block PE differentiation in mouse ES cells (Hamazaki et al., 2006). To examine the function of MEK in bovine pre-implantation development, embryos were cultured from the zygote (day 1) to the blastocyst stage (day 8) in the presence of 25 μM PD98059. Whole-mount immunofluorescence revealed the mottled distribution of NANOG-positive cells and GATA6-positive cells in the ICM, indicating that hypoblast formation was not fully blocked (Fig. 2A). However, quantification of the number of NANOG-positive and GATA6-positive cells revealed that there were proportionally more NANOG-positive cells in the ICMs of the embryos that were cultured in the presence of the MEK inhibitor than in the DMSO negative control (Fig. 2B). These results suggest that MEK signaling plays a role in the segregation of the epiblast and hypoblast cell lineages in bovine embryos.

To confirm the role of MEK signaling in bovine lineage segregation, we subsequently cultured embryos from the zygote to the blastocyst stage in the presence of another MEK inhibitor, PD0325901 (Bain et al., 2007). In this condition we also observed NANOG-positive cells and GATA6-positive cells (Fig. 2C). In agreement with the above findings, embryos cultured in the presence of the MEK inhibitor contained a significantly higher percentage of NANOG-positive cells than those cultured in the control condition (Fig. 2D). Importantly, GATA6-positive cells were still observed in the ICMs of embryos cultured in the presence of the MEK inhibitors. We concluded from this observation that bovine embryonic cells are heterogeneous in their response to MEK inhibition and that in some cells GATA6 expression is independent of activated MEK.

To obtain more insight into the temporal role of MEK signaling in bovine development, the MEK inhibitor PD0325901 was added at various time points (days 1, 3, 5, 6 or 7 of embryo culture) and the effects were examined on the populations of NANOG-positive and GATA6-positive cells on day 8. All experimental groups that were cultured in the presence of the inhibitor contained higher median values for the number of NANOG-positive cells and lower median values for the number of GATA6-positive cells than controls (Fig. 2E). The experimental groups were similar in their responses: embryos that were cultured in the presence of the MEK inhibitor from day 1 until day 8 of embryo culture had similar ICM constitutions to embryos that were exposed to the inhibitor from day 7 to 8. This result suggests that even at day 7 of bovine development some cells of the ICM are to some degree plastic in their developmental choice between the hypoblast precursor fate and epiblast precursor fate. When the MEK inhibitor was withdrawn from the culture at day 5 and culture was continued until day 8 in the absence of the inhibitor, embryos developed comparable numbers of NANOG-positive and GATA6-positive cells to the control embryos (Fig. 2E). There were no significant differences between the different groups in the total number of ICM cells, nor in the number of cells that were double positive for NANOG and GATA6 (Fig. 2E). The above results support the idea that the observed effects of the MEK inhibitor are the result of altered cell fates and not the consequence of lineage-specific changes in apoptosis or proliferation.

FGF4 induces the formation of hypoblast precursors

From the observed effects of MEK inhibition on the constitution of the bovine ICM, we concluded that MAP kinase signaling plays a role in the segregation of the epiblast and hypoblast precursors in bovine development. Next, we investigated whether activated FGF signaling is sufficient to instruct hypoblast development in bovine embryos. Embryos were cultured in the presence of FGF4 (1 μg/ml) and heparin (1 μg/ml) from the zygote to the blastocyst stage. Heparin is required for FGFs to activate FGF receptors (Schlessinger, 2000). Embryos treated with FGF4 and heparin had greatly reduced numbers of NANOG-positive cells and enhanced numbers of GATA6-positive cells (Fig. 3A,B). The number of ICM cells in embryos cultured in the presence of FGF4 and heparin was similar to that of control embryos (Fig. 3C). Embryos that were grown in the presence of FGF4 and heparin from the morula stage onwards developed ICMs that were composed entirely of GATA6-positive cells, while no effect on the total number of ICM cells was observed (Fig. 3D-F). These findings demonstrate that FGF4 can induce the formation of hypoblast precursors and block the formation of epiblast precursors in bovine development. Embryos treated with either FGF4 or heparin did not have different numbers of GATA6-positive cells or NANOG-positive cells compared with control embryos (Fig. 3G).

In the mouse, trophoblast stem cell self-renewal depends on FGF4 (Tanaka et al., 1998). FGF4 could therefore have a positive effect on TE cell numbers in addition to its effect on the number of hypoblast precursor cells. We therefore also examined the effect of FGF4 on the expression of the TE marker CDX2. The number of CDX2-positive cells was not significantly different between ICMs that were composed entirely of GATA6-positive cells, while no effect on the total number of ICM cells was observed (Fig. 3H,I). Importantly, NANOG-positive cells and GATA6-positive cells were still observed in the ICM of embryos cultured in the presence of FGF4 (Fig. 3J). These results support the idea that FGF4 can induce the formation of hypoblast precursors and block the formation of epiblast precursors in bovine development. Embryos treated with either FGF4 or heparin did not have different numbers of GATA6-positive cells or NANOG-positive cells compared with control embryos (Fig. 3G).

Inhibition of FGF signaling or proteoglycan sulfation does not alter the composition of the ICM in bovine blastocysts

FGF signaling can also bypass MEK through the phosphatidylinositol 3-kinase (PI 3-kinase) and AKT/PKB pathway. It has been demonstrated that mouse ES cells require this pathway for early differentiation (Chen et al., 2000). Because inhibition of MEK did not fully repress GATA6 expression in bovine embryos, we hypothesized that its expression could depend on FGF signaling through PI 3-kinase and AKT/PKB. Consequently, to further examine the role of FGF signaling in the segregation of the hypoblast and epiblast precursors in bovine development, we interfered with the FGF signal transduction cascade upstream of MEK. Firstly, bovine embryos were cultured in the presence of PD173074, an FGF receptor inhibitor that has been reported to block PE formation in mouse development (Nichols et al., 2009b). Surprisingly, in bovine embryo cultures, FGF receptor inhibition did not
significantly affect the composition of the ICM (Fig. 4A,B). Furthermore, simultaneous inhibition of MEK and the FGF receptor resulted in embryos with similar proportions of NANOG-positive and GATA6-positive cells to embryos that were cultured in just the MEK inhibitor (Fig. 4A,B).

To independently confirm the above result, we used an alternative method to interfere with FGF signaling. Heparan sulfate chains are crucial modulators of FGF signaling (Bernfield et al., 1999; Haltiwanger and Lowe, 2004). Interference with heparan sulfation using sodium chlorate (NaClO3), a reversible...
proteoglycan sulfation inhibitor, blocks PE formation in developing mouse blastocysts (Lanner et al., 2010). To examine the effects of proteoglycan sulfation inhibition on bovine development, embryos were cultured in the presence of NaClO3 from day 1 until day 8 or from day 5 until day 8. In either group, there were no significant differences in the proportions of NANOG-positive and GATA6-positive cells as compared with control embryos (Fig. 4A,C). Collectively, these findings indicate that, in contrast to the situation in mouse, in normal bovine development GATA6 expression is not dependent on FGF signaling.

**The effects of GSK3β and ALK5 inhibition on the constitution of the ICM of bovine blastocysts**

The above results indicate that the segregation of the hypoblast and epiblast precursors in bovine development does not depend on FGF signaling and is not entirely dependent on MAP kinase signaling. Therefore, we investigated alternative pathways that could play a role in the segregation of these lineages in bovine development. A recent study demonstrated that mouse ES cells require WNT signals to remain undifferentiated (ten Berge et al., 2011). Moreover, mouse ES cells can be maintained in an undifferentiated state in the absence of LIF, feeder cells and
serum when the cells are cultured in the presence of a MEK inhibitor in combination with CHIR99021, an inhibitor of the negative regulator of WNT signaling GSK3β (Ying et al., 2008). In combination with other factors, CHIR99021 also enables human pluripotent stem cells to adopt a so-called naïve state of pluripotency, which resembles that of mouse ES cells (Hanna et al., 2010). Therefore, we investigated whether signaling through GSK3β plays a role in the differentiation of the epiblast lineage in bovine embryos using the GSK3β inhibitor CHIR99021.

Embryos cultured from the zygote to the blastocyst stage in the presence of CHIR99021 (3 μM) had a slightly higher percentage of NANOG-positive cells than control embryos (Fig. 5A,B; two-tailed Mann-Whitney U test, \( P = 0.023 \)). Embryos were also cultured in a combination of CHIR99021 and PD0325901 (referred to as the 2i condition). In this condition, the enhanced proportion of NANOG-positive cells in the ICM was comparable to that of embryos exposed to only PD0325901 (Fig. 5B). There was no synergistic effect between CHIR99021 and PD0325901 (\( P = 0.85 \)). When the GSK3β inhibitor CHIR99021 was added to the bovine embryo cultures from the morula stage onwards, no effects were observed on ICM constitution (data not shown; two-tailed Mann-Whitney U test, \( P = 0.85 \)). We conclude from these results that GSK3β activation appears to play only a minor role in the segregation of hypoblast and epiblast precursors in bovine embryos.

In addition to the FGF and WNT signaling pathways, TGFβ signaling also has major roles in early embryo development (Roelen and Mummery, 2000). Studies on human induced pluripotent stem (iPS) cells suggest that chemical inhibition of the type 1 TGFβ receptor ALK5 (TGFβR1) facilitates the reprogramming process (Lin et al., 2009). Furthermore, chemical inhibition of the ALK5 receptor has been used for the derivation of rat ES cells and iPS cells (Li et al., 2009; Kawamata and Ochiya, 2010). To investigate whether TGFβ signaling plays a role in the segregation of the epiblast and hypoblast lineages, bovine embryos were cultured in the presence of the ALK5 receptor inhibitor A-83-01. No significant differences in the number of GATA6-positive or NANOG-positive cells were observed between embryos cultured in the presence of A-83-01 and control embryos (Fig. 5A,C). Therefore, signaling through the ALK5 receptor does not appear to be essential for the segregation of the epiblast and hypoblast lineages in bovine development.
Human hypoblast development does not depend on MAP kinase signaling

The above results demonstrate that it is possible to interfere with early lineage segregation in bovine embryos using inhibitors of MEK or the growth factor FGF4. Next, we examined the effects of MEK inhibition on the specification of the hypoblast and epiblast cell lineages in human development. For this purpose, we made use of human embryos that were subjected to pre-implantation genetic diagnosis (PGD).

Human PGD embryos were cultured until day 6 of development, then fixed and whole-mount immunofluorescence was performed for NANOG and GATA6 to determine whether these markers are expressed in the epiblast and hypoblast cell lineages, respectively, as we have demonstrated for bovine embryos. Furthermore, for human embryos we included GATA4 as an additional marker for the hypoblast cell lineage.

In day 6 embryos, NANOG-positive cells were enclosed by a layer of GATA4/6-positive cells, indicating that the physical segregation of the epiblast and hypoblast cell lineages had been completed in these embryos (Fig. 6A). The majority (>95%) of GATA6-positive cells were also GATA4 positive. GATA6 and GATA4 expression was not detected in the TE lineage. In contrast to GATA6 expression, GATA4 was also expressed in the epiblast precursors, albeit at a lower level than in the hypoblast precursors (Fig. 6A).

To determine whether MAP kinase signaling is required for the segregation of the hypoblast and epiblast cell lineages in human development, human PGD embryos were grown from day 4 (morula stage) until day 6 (hatching/hatched blastocyst stage) of development in the presence of the MEK inhibitor PD0325901 (0.5 μM) or in DMSO (negative control), after which they were subjected to whole-mount immunofluorescence for NANOG and GATA6. In eight embryos (four in each condition), GATA4 was included as an additional hypoblast marker. Embryos that were cultured in the presence of the MEK inhibitor developed a clear population of hypoblast precursors (Fig. 6B). Nevertheless, it was expected that the response of human embryos grown in the presence of MEK inhibitor would be similar to that of bovine embryos, resulting in ICMs with proportionally more NANOG-positive cells and fewer GATA4/6-positive cells. Remarkably, however, the number of NANOG-positive cells was not significantly different between human blastocysts cultured in control conditions and those that were cultured in the presence of PD0325901 (Fig. 6C; two-tailed Student’s t-test, P = 0.94). Similarly, the numbers of GATA4-positive and GATA6-positive cells were not significantly different between the control and PD0325901 groups (two-tailed Student’s t-test; GATA4-positive cells, P = 0.34; GATA6-positive cells, P = 0.60) and nor were the relative numbers of NANOG-positive or GATA6-positive cells in the ICM (Fig. 6D; two-tailed Mann-Whitney U test, P = 0.95). Moreover, there were no significant differences in the total number of cells (two-tailed Student’s t-test, P = 0.16) or in the number of TE cells (two-tailed Student’s t-test, P = 0.16) between the two groups.

The lack of response in human embryos to the MEK inhibitor could be due to the inability of the inhibitor to successfully inhibit MEK at the concentration used. However, this possibility is...
unlikely because in a human ES cell line PD0325901 successfully inhibited MEK even at concentrations below 10 nM (Fig. 6E). Therefore, we conclude from these results that MAP kinase signaling has no role in the segregation of the hypoblast and epiblast cell lineages in human development and that in this respect human embryos differ from bovine and murine embryos.

**DISCUSSION**

Here we performed functional studies on bovine and human embryos to gain a better understanding of the developmental processes that lead to the segregation of the pluripotent epiblast precursors and the hypoblast cells in these species. We demonstrate the ability to influence lineage development in bovine embryos through modulation of the FGF/MAP kinase signal transduction pathway. The addition of a MEK inhibitor at as late as day 7 of bovine development significantly affects the balance between GATA6-positive and NANOG-positive cells in the ICM without significant effects on ICM size or the number of ICM cells. It is therefore unlikely that the effects we observed are due to changes in the speed of development or to lineage-specific changes in apoptosis. The described effects of MEK inhibition or FGF4/heparin stimulation on the constitution of the ICM of bovine embryos are not identical to those described for mouse embryos (Nichols et al., 2009b; Yamanaka et al., 2010). MEK inhibition during early mouse embryo development results in ICMs that are completely NANOG positive and GATA4/6 negative (Nichols et al., 2009b; Yamanaka et al., 2010). However, in the current study, GATA6 expression was not fully ablated upon MEK inhibition in bovine embryos (Fig. 2). It appears that the ICM cells of bovine embryos are heterogeneous in their response to MEK inhibition, but the underlying cause for this heterogeneity is as yet unknown. Possible mechanisms include differential epigenetic regulation, cell-specific differences in the target genes of phosphorylated ERK, or a lack of activated MEK in a subset of ICM cells.

**Fig. 6. Expression of NANOG, GATA6 and GATA4 in human blastocyst stage embryos and the effect of MEK inhibition on this expression.**
(A) Immunofluorescent detection of NANOG, GATA6 and GATA4 in hatching human blastocysts. NANOG-positive cells are enclosed by a layer of GATA4/6-positive cells, indicating that physical segregation of the epiblast and hypoblast cell lineages has initiated. The boxed areas are enlarged in the bottom row. (B) Immunofluorescence for NANOG, GATA6 and GATA4 on human PGD embryos that were cultured from the 8-cell stage to the blastocyst stage in the presence of the MEK inhibitor PD0325901 or DMSO (negative control). (C) Box-whisker plots showing quantification of the number of NANOG-positive, GATA6-positive and GATA4-positive cells in PGD embryos were cultured from the 8-cell stage to the blastocyst stage in the presence of PD0325901 or DMSO. (D) Box-whisker plots showing the quantified proportion of NANOG-positive (green) and GATA6-positive (red) cells in the ICM of human embryos grown in PD0325901 or DMSO. (E) Immunoblot for ERK and phosphorylated ERK on lysates of human ES cells that were treated with decreasing concentrations of the MEK inhibitor PD0325901. In all experiments, equal concentrations of solvent (DMSO) served as negative controls. n.s., not significant. Error bars comprise the whiskers that extend to the maximum and minimum value data sets. Scale bars: 50 μm.
Bovine embryos that were cultured in the presence of FGF4 and heparin developed ICMs composed entirely of GATA6-positive and NANOG-negative cells, suggesting that FGF signaling directs GATA6 expression in bovine embryos. However, contrary to our expectations, chemical inhibition of the FGF receptor or interference with FGF signaling through inhibition of proteoglycan sulfation did not result in fewer GATA6-positive cells (Fig. 4). We hypothesize that the FGF4-induced expression of GATA6 in bovine embryos (Fig. 3) is an indirect effect caused by the repressive effect of activated MEK on NANOG (Fig. 7), as has been described for mouse ES cells (Hamazaki et al., 2006). Such regulation (Fig. 7) could explain why GATA6 expression in developing bovine embryos is independent of FGF signaling, although its expression is induced upon NANOG repression by exogenous FGF4.

The incomplete block of GATA6 expression upon MEK inhibition that we observe indicates that GATA6 expression is not dependent on MAP kinase signaling. Moreover, the lack of detectable effects following the inhibition of FGF signaling suggests that GATA6 expression in developing embryos is not regulated by the FGF signal transduction cascade. Using small chemical compounds, we also investigated the roles of signaling through GSK3β and ALK5 in early bovine development. Chemical inhibition of GSK3β or of the ALK5 receptor failed to block hypoblast formation in developing bovine embryos. It therefore remains unknown which pathway lies upstream of GATA6 in early bovine development. In developing bovine embryos, all cells progressively become GATA6 positive until day 7, after which GATA6 expression is rapidly downregulated in the TE and epiblast precursors (Fig. 1). This indicates that GATA6 expression is the default state in all cells and that from day 8 of development GATA6 expression is excluded from the TE cells and epiblast cells, for example by the expression of lineage-specific transcription factors. Which factors repress the initial expression of GATA6 and what induces the expression of these factors can as yet only be speculated upon. The transcription factors CDX2 and NANOG are good candidates for the exclusion of GATA6 expression in the TE and epiblast lineages, respectively. This hypothesis would also fit with the observed expression dynamics of NANOG and with the initially high level of NANOG/GATA6 double-positive cells at day 7 of bovine development.

The expression patterns of GATA4/6 and NANOG in the ICM of human blastocysts are mutually exclusive, as has been described for mouse (Chazaud et al., 2006; Nichols et al., 2009b) and bovine blastocysts (this study). We also observed low expression levels of NANOG and GATA4 in the hypoblast and epiblast cell lineages, respectively, of human embryos, which could indicate subtle reciprocal regulation of NANOG and GATA4 expression levels. Furthermore, the low expression of GATA4 in epiblast cells suggests that its expression is independent of GATA6.

Surprisingly, in contrast to the observed effects of MEK inhibition on bovine embryos described in this study and the previously reported effects on mouse embryos (Nichols et al., 2009b; Yamanaka et al., 2010), PD0325901-mediated inhibition of MEK did not significantly alter the numbers of NANOG-positive and GATA4/6-positive cells in human embryos. This lack of effect cannot be explained by a decreased potency of PD0325901 to inhibit human MEK because even at concentrations below 10 nM we observed decreased ERK phosphorylation in human ES cells. We therefore conclude that MEK signaling is not the driving force behind hypoblast formation in human development and that other signaling pathways probably regulate the mosaic expression of GATA4/6 and NANOG in human embryos (Fig. 7).

While this manuscript was in preparation, another study was published in which the role of FGF/MAP kinase signaling in human lineage segregation was investigated (Roode et al., 2011). Embryos that were cultured in the presence of a MEK inhibitor with or without an FGF receptor inhibitor developed GATA4-positive hypoblast precursors (Roode et al., 2011). Although this observation is very similar to ours, there is an important difference between the two studies that is worth considering. Our experiments with bovine embryos clearly demonstrate that interference with MAP kinase signaling does not necessarily lead to an all or nothing response, highlighting the need for quantification and statistical analysis in these kinds of studies. Compared with the Roode et al. study, we have examined more embryos, which allowed us to quantify and subsequently perform statistical analysis on the proportion of epiblast and hypoblast precursors in the ICMs of human embryos cultured with or without the MEK inhibitor. In support of the study by Roode et al., we conclude that inhibition of MEK in human development cannot prevent the formation of hypoblast precursors. Moreover, we can extend these findings and conclude that inhibition of MEK in human development does not significantly alter the relative proportions of epiblast and hypoblast precursors in the ICM. Another difference between the two studies is that Roode et al. used frozen-thawed embryos, whereas we used fresh embryos that were determined unsuitable for transfer after PGD analysis. Freeze-thawing usually results in a delay in development of ~1 day. Indeed the embryos that we used were expanded at day 6, whereas the frozen-thawed embryos were expanded at day 7 (Roode et al., 2011). Independently, the overall conclusions of both studies are the same: endoderm differentiation in human development is not driven by MAP kinase signaling.
Mouse and human ES cells differ strikingly in colony morphology, growth factor requirements, cell cycle, gene expression and epigenetic status (Buecker et al., 2010; Kuijk et al., 2011). Constitutive activation of STAT3 signaling is sufficient to maintain mouse ES cells in an undifferentiated state (Matsuda et al., 1999). By contrast, STAT3 is not involved in maintaining human ES cells in the undifferentiated state, suggesting that cell signaling is regulated differently in mouse and human embryonic cells (Daheron et al., 2004). Indeed, as the results of the current study suggest, MEK signaling has divergent roles in the early development of mouse and human embryos.

Recent studies have provided accumulating evidence for striking differences between mammalian species in early embryo development in processes such as X-chromosome inactivation and TE lineage determination (Berg et al., 2011; Okamoto et al., 2011). In the current study, we demonstrate that developing mammalian embryos also differ in their response to disrupted FGF/MAP kinase signaling. In contrast to mouse embryos, inhibition of MEK only partially interferes with the formation of the hypoblast in bovine embryos, resulting in a less severe phenotype, and in human embryos MEK inhibition does not interfere with the formation of the hypoblast.

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Competing interests statement

The authors declare no competing financial interests.

Author contributions

E.W.K. designed and conducted the experiments and wrote the manuscript; L.T.A.T., H.V. and M.W. designed and conducted the experiments; R.W. contributed to confocal imaging and imaging analysis; N.G. provided reagents and materials, analyzed results and supervised writing of the manuscript.

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