Lineage commitment in the mammalian preimplantation embryo

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Summary

Early embryo mortality during the first two weeks of development is an important factor in the decreasing fertility of dairy cattle. During this period the embryo needs to correctly establish the first three lineages, namely epiblast, hypoblast and trophoblast so as to survive. The morphological and molecular events underlying these lineage decisions are discussed, drawing on information derived predominantly from mice and cattle.

Introduction

In cattle, gestation lasts around 280 days (average range 273-291 days) with the first six weeks constituting the embryonic period (Winters *et al*. 1942). In this period the body plan is established and most of the organ systems are formed (Fig. 1), while the subsequent foetal period is characterised by growth and modelling. The embryonic period has been divided into two time windows of three weeks, appropriately termed "early" and "late", with the beginning of implantation straddling this transition. Thus starting at Day 19, in what is termed the apposition phase of implantation, cell to cell contact is established between the trophoblast of the conceptus and the uterine epithelium (Guillomot 1995). While the term "attachment" has been suggested to supplant that of "implantation" because of the minimal invasiveness of the ruminant placenta, we shall use the latter term based on its more generic applicability.

There has been a resurgence of interest in cattle gestation owing to declining conception rates in dairy cattle (Diskin & Morris 2008) and the high financial costs associated with this. For example, Australian reproductive management programmes estimated (in 2007) that a 1% increase in the 6-week in calf would be worth $400 per 100 cows in the herd while a 1% reduction in empty rate amounted to $570 per 100 animals (Burke *et al*. 2008).

The events affecting conception rate can be separated into four categories. These are, in chronological order, (i) fertilisation, (ii) early embryonic, (iii) late embryonic and (iv), foetal development. Fertilisation failure is estimated to lie between 5 and 17% (Sartori *et al*. 2010, Walsh *et al*. 2011). Early embryonic losses (Fig. 1), encompassing the first three weeks of postfertilisation development range from 28% (beef and dairy cows 1980 genetics) to 40% (N.American genetics high producing dairy cows) (Diskin & Morris 2008, Diskin *et al*. 2011). Late embryo losses occurring between Days 22 and 42 contribute an estimated 5-10% to conception failures whereas foetal deaths during the remainder of gestation are also relatively

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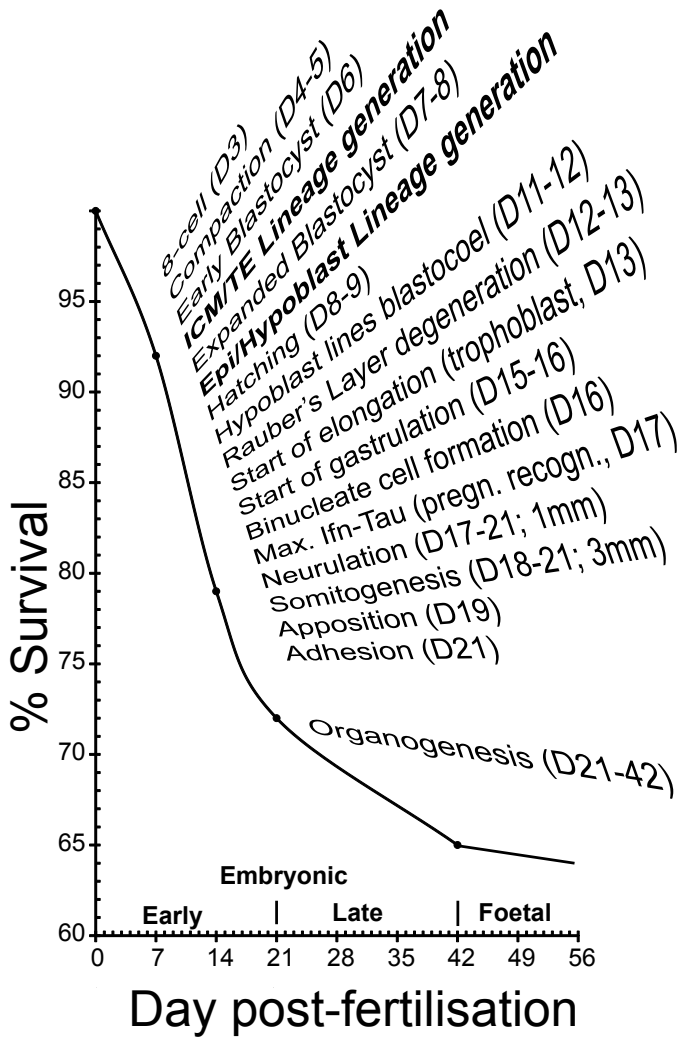

Figure 1. Developmental events in cattle in relation to embryo mortality rates. Days subsequent to fertilisation are plotted against the typical percentage of fertilised embryos surviving (references in main text). The greatest drop in survival is seen during the early embryonic period (developmental periods are shown along the x-axis). The critical nature of this period is highlighted by the number of key developmental events occurring in this phase, as listed along the survival curve in the graph. Events discussed in detail in this review are shown in bold.

low (6%) (McDougall *et al*. 2005, Diskin *et al*. 2011). The major part of early embryo loss in cattle occurs in the first two weeks after breeding (Boyd *et al*. 1969, Ayalon 1978, Diskin & Sreenan 1980, Roche *et al*. 1981, Dunne *et al*. 2000, Berg *et al*. 2010), with some evidence suggesting that high losses are already evident by Day 8 (Maurer & Chenault 1983, Wiebold 1988, Sartori *et al*. 2010).

This high mortality during early embryo development underscores the functional importance of the preimplantation phase of development and in particular the first two weeks during which the major embryological events concern the establishment of the first three lineages (Fig 1).

From an evolutionary aspect, it is advantageous for the species to impose the greatest selection pressure for embryo viability at early gestational stages as it minimises the reproductive cost for the mother. From a developmental view, errors occurring during early developmental tend to have more dire consequences due to the progressive nature of development. This review will focus on the first two lineage determining events occurring during cattle early embryogenesis, presenting this in comparison to knowledge derived from other mammals.

Creating asymmetry

The earliest stages of eutherian mammalian development are characterised by the absence of asymmetry and up to the morula stage, cells appear to be homogeneous in terms of morphology and molecular characteristics. This cell to cell equivalency has been borne out in functional experiments showing that all cells of the 2- to 8-cell stage embryo are totipotent. For example, in cattle as well as mice, separated individual cells (blastomeres) of the 4-cell embryo can develop into adulthood (Tarkowski & Wroblewska 1967, Johnson *et al.* 1995). In sheep this has been shown even for individual cells of the 8-cell embryo (Willadsen 1981). How then is asymmetry or difference, necessary for establishing different lineages, introduced? And once introduced, how are these differences fixed or, in other words, how do the cells of different lineages become committed to their eventual fate?

In the mouse cell polarity, as well as inside/outside location within the cleavage stage embryo has been linked to cell lineage determination. In this species all blastomeres of the 8-cell stage embryo develop radial polarity concomitantly with compaction, the process by which blastomeres huddle together mediated by apical depletion of Cadherin-1 (E-cadherin; Ovumorulin) (Johnson & Ziomek 1981, Peyrieras *et al.* 1983). During subsequent mitosis, dependant on the division plane, daughter cells will either remain polar and located at the outside of the compacted 16-cell morula or, after tangential divisions, half will become apolar and situated on the inside. Lineage tracing has shown inside apolar cells to contribute predominantly to the inner cell mass (ICM) (Pedersen *et al.* 1986). Conversely, after a further round of division the remaining polarised outside cells of the 32-cell embryo are fated to form trophoblast (Pedersen *et al.* 1986).

Initially it was suggested that the relative position (inside versus outside) and thus environment of a cell was the symmetry-breaking event generating the two lineages (Tarkowski & Wroblewska 1967). Later on, the polarity model was proposed, emphasising cell polarisation with subsequent unequal partitioning of apical domain components as deterministic for differential lineage generation (Johnson & Ziomek 1981). Both models are experimentally supported (Nishioka *et al.* 2009, Stephenson *et al.* 2010) and are indeed non-exclusive. More recent advances in imaging technologies and sensitivities have however uncovered unexpected heterogeneity and fluctation in the distributions of lineage-associated transcription factors such as Cdx2, Nanog and Gata6 (Dietrich & Hiiragi 2007, Ralston & Rossant 2008, Plachta *et al.* 2011), as well as extensive cell rearrangements during cleavage stages (Kurotaki *et al.* 2007, Dard *et al.* 2009). This opens up the possibility that symmetry breaking is a stochastically initiated self-organising system dependent on not a single factor (such as either polarity or cell position), but on multiple factors including cell characteristics such as polarity and gene expression as well as cell-cell interactions that may be either mechanical or biochemical (Wennekamp *et al.* 2013). This model posits i) that individual factors contribute in a quantitative manner to cell fate in a manner dependent on a cell's specific temporal and spatial context and ii) that factors are interconnected, leading to positive and negative feedback loops.

Such a framework is more suited to explaining differential timings of early events among mammals. In cattle, using scanning electron microscopy, differences in microvilli distribution in individual blastomeres were seen in nearly all 16-cell cattle embryos with approximately 40% polar cells per embryo. No embryos contained more than 57% polar cells (Koyama *et al.* 1994). The polar sides were not always aligned to the outside indicating that the blastomere could rotate and so their position was not yet fixed. Indeed, compaction and the first appearance of apolar distributions of Cadherin-1 and Beta-catenin specifically in outer cells was only seen at the 32-cell stage (Betteridge & Flechon 1988, Van Soom *et al.* 1997, Barcroft *et al.* 1998), two cell divisions later than in the mouse. Yet cattle embryos can already contain inner cells from the 16-cell stage onward with compaction and inner cell allocation being largely independent from each other (Van Soom *et al.* 1997). Thus the potential causative agents of symmetry breaking, namely cell position and polarity, occur in the opposite temporal sequence to the mouse. Cattle embryos would therefore be an interesting alternate system to test predictions of the various symmetry breaking models. To date however no such lineage studies have been performed.

Fixation of the first lineages

Shortly after compaction, a cavity, named the blastocoel develops. As the blastocoel expands, the inner cells are pushed to one side of the embryo and are now termed the inner cell mass, or ICM. These cells are morphologically quite distinct from the outer layer of polarised epithelial cells which is termed the trophectoderm (TE). In mice the TE has been shown to give rise to all the trophoblast, namely the conceptus–derived part of the placenta, whereas the ICM cells make up the rest of the conceptus, that is the embryo proper (foetus) and the various extraembryonic membranes such as the yolk sac, amnion and allantois (Copp 1979, Dyce *et al.* 1987). Thus by the blastocyst stage the first major split in lineages has taken place. However, it is only recently was the fate of cattle TE cells has been followed. TE cells from Day 7 expanded blastocysts were labelled with the lipophilic lineage tracer DiI, then replaced into recipient cows to be flushed out a week later. At this pre-gastrulation stage embryos had started elongating and the ICM had differentiated into the epiblast and extraembryonic hypoblast. No contribution of label to the latter two lineages was detected, confirming the mouse results that the TE, once specified, maintains its trophoblast character in a minimally manipulated embryo (Berg *et al.* 2011).

Equally important though is the question as to when the fate of a cell is irreversible, or in embryological terms, when is a cell committed to its expected fate. This can be measured by various means, but the principle is to place a cell in a novel cellular context and determine whether it retains its original fate (and thus is committed) or assumes the fate of its new neighbours (not committed). In mice various types of aggregation experiments revealed that up to the 32-cell stage outer prospective TE cells were not committed to the trophoblast fate (Rossant & Vijh 1980, Tarkowski *et al.* 2010). However once embryos had started to cavitate (post 32-cell stage; early blastocysts), commitment of outer cells to their respective fates had occurred (Suwinska *et al.* 2008). Inner blastomeres of mice appear to be somewhat more plastic and, depending on the experimental set-up, seem to commit to an ICM fate somewhere between mid and late blastocyst stages (Rossant & Lis 1979, Suwinska *et al.* 2008, Szczepanska *et al.* 2011, Grabarek *et al.* 2012).

In cattle, ICM cells isolated at Day 7 or 9 post-ovulation by immunosurgery were aggregated to Day 4.5, 16-cell embryos. Chimerism was detected at Day 13 and in born calves with the aid of chromosome markers (Picard *et al.* 1990). The pre-hatching stage ICM cells (Day 7) yielded chimeras, whereas when using post hatching donor cells, only one "inconclusive" Day 13

chimera was obtained. As contribution to the trophoblast was not examined, no conclusion can be drawn as to commitment. However these experiments indicated that aggregation experiments with cells of at least 3 days difference in development were feasible, setting the stage for testing the commitment of cattle TE cells. For this experiment, DiI labelled enzymatically separated TE cells from expanded Day 7 blastocysts were sandwiched between totipotent blastomeres of 8-cell (Day 3) embryos (Fig. 2) and allowed to develop for 4 days in culture to the expanded blastocyst stage (Berg *et al*. 2011). It was found that one third of the transposed TE cells contributed to the ICM. That the Day 7 TE-derived cells that were located in the ICM were truly fated to ICM derivatives was proven by repeating the experiment with *LacZ*-transgenic host embryos and transferring the chimeras to recipient cows. When embryos were retrieved at early gastrulation stages, it was found that the blastocyst TE-derived cells had contributed extensively to inner cell mass derived hypoblast (Berg *et al*. 2011). In contrast, when TE cells from pregastrulation (Day 14) embryos were used for the sandwich chimeras, contribution was nearly exclusively to the TE, indicating that commitment of TE cells to trophoblast occurred between the expanded blastocyst stage and gastrulation, distinctly later than in mice.

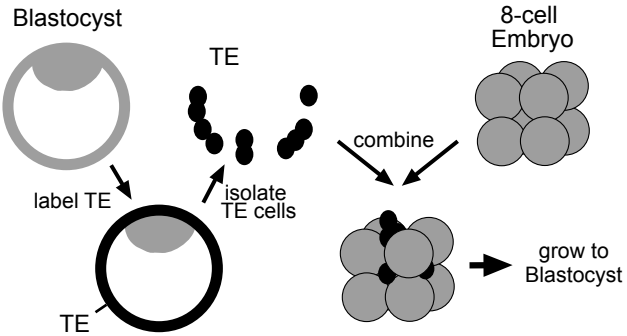

Figure 2. Experimental approach for determining the commitment of cattle trophectoderm cells. Expanded donor blastocysts are labelled with the lipophilic dye DiI (indicated in black) and TE cells are then isolated by microsurgically cutting the blastocyst followed by enzymatic dissociation. Concurrently zona-free host 8-cell embryos are grown in vitro (on right, grey) to provide an ectopic, uncommitted environment for the TE donor cells. After combining the donor and host cells, the chimeric embryos are allowed to develop to the expanded blastocyst stage and the contribution of the donor TE cells to ICM and TE can be traced using the fluorescent DiI label. If TE donor cells maintain their original fate and contribute only to TE, they would be considered to have been committed to this fate at the stage that they had been isolated. If they were to contribute to both ICM and TE, they would be considered to have been uncommitted.

Recently, TE lineage commitment has also been addressed in humans. It was found that TE cells from full (late) Day 5 blastocysts were, upon reaggregation, able to form both ICM and TE, whereas TE cells from expanded Day 5 blastocysts were not (De Paepe *et al*. 2013). Furthermore, when full blastocyst-derived TE cells were placed on the inside of Day 5 blastomere aggregates, they could express NANOG, an ICM marker, a day later. Thus human TE cells are committed at a later blastocyst stage than mice, but earlier than cattle.

It is tempting to speculate that the timing of trophoblast commitment (normalised to the morphological stage of development) is determined by the timing of implantation. Mouse blastocysts implant a day, humans two to three days and cattle 12 to 13 days after blastocyst formation. Implantation requires trophoblast proliferation as well as differentiation so as to establish the necessary contact and interaction with the maternal epithelium. Hence the shorter

the interval between TE specification and implantation, the sooner TE commitment has to take place.

Molecular determinants of lineage specification in mice

Much effort has gone into the elucidation of the critical lineage determining factors and their reciprocal interactions with symmetry breaking events, such as differential cell-cell signalling (inside versus outside cells) and distributions of cellular components (via polarity generating events). As the state of a cell as well as the reversibility or lability of that state is dependent on the gene regulatory network prevailing in that cell and its susceptibility to spatially and temporally changing signals, it is clear that transcription factors and signalling pathways play a paramount role in lineage fixation.

It is likely that the early gene expression program runs according to a developmental clock as mouse embryos halved at the 2-cell stage maintain the same timing of polarization, compaction, generation of inside cells, cavitation and cell fate allocation as whole embryo controls (Morris *et al.* 2012). In mice, single cell RNA-seq revealed enormous changes between matured oocytes and blastomeres of the 2-cell stage with 8000 down-regulated and 2000 up-regulated genes. This probably reflects maternal RNA degradation and embryonic genome activation. During the following 2 cell divisions, a further 5400 and 3400 genes were upregulated (Tang *et al.* 2011). Yet blastomere transcriptomes from individual 2-cell embryos were highly similar to each other (Tang *et al.* 2011). From the 8-cell (compaction) stage, mRNA (Guo *et al.* 2010) and nuclear protein (Dietrich & Hiiragi 2007, Plusa *et al.* 2008) of lineage specific markers such as Oct4/Pou5f1 (ICM), Nanog (ICM/epiblast), Gata6 (ICM/hypoblast) and Cdx2 (TE) could be detected. Importantly, these factors remain coexpressed, though sometimes at varying levels until at least the 16-cell stage but begin to resolve by the early (32-cell, ca E3.25) blastocyst stage. Measuring RNA expression of 48 genes in individual cells of mouse preimplantation embryos also revealed a progressive refinement of differential gene expression. From the 32-cell (nascent blastocyst) stage, inner (prospective ICM) and outer (TE) cells exhibited distinct expression profiles, while from the 64 cell stage cells epiblast and hypoblast signatures were apparent (Guo *et al.* 2010). Interestingly, the positional allocation of a cell (inside/outside) preceded the generation of differential (ICM/TE) gene regulatory networks. That cell-cell contact, and thus positional information, is important to establishing a state-specific gene network, was demonstrated directly by keeping blastomeres segregated from the 2-cell stage to the 32-cell stage. Such isolated individual 32-cell blastomeres resembled the normal 32-cell gene expression pattern, but assumed intermediate levels of lineage-specific genes, with overall a more TE-like gene expression profile (Lorthongpanich *et al.* 2012). Thus the gene expression programs are set into motion independently of symmetry-breaking events but have to be refined via cell-extrinsic signals.

A good candidate for driving the first resolution of TE and ICM gene expression programs is the Hippo signalling pathway which is believed to use cell polarity and position to modulate the TE-specific gene regulatory network as follows. In nonpolar cells such as the inner cells of the postcompaction morula, the Angiomotin proteins (Amot/Amot-like2) interact through Nf2A with Cadherin-1 at adherens junctions. Cell-cell contact, presumably via Cadherin-1 crosslinking causes the phosphorylation and stabilisation of Amot by the hippo pathway kinase Lats2. The resultant adherens junction-linked complex leads to the Lats2 mediated phosphorylation of Yes-associated protein (Yap) which prevents it from shuttling into the nucleus to activate its partner protein Tead4 (Nishioka *et al.* 2009, Cockburn *et al.* 2013, Hirate *et al.* 2013). Importantly Tead4

is crucial for trophoblast development and the establishment of the TE-specific transcriptional program including expression of *Cdx2*, *Eomes* and *Fgfr2* (Nishioka *et al.* 2008). Thus in inner cells cell contact leads to the repression of the TE-gene regulatory network, allowing the co-expressed ICM/pluripotency network to gain the upper hand.

However as all these factors are also expressed in outer cells which also display Cadherin-1 mediated cell-cell interactions along their mediobasal surfaces, why is Yap not retained in the cytoplasm as well? From the 8-cell stage onwards, outer cells are polarised containing an apical domain and a basolateral domain, separated by tight junctions. This is mediated by the polarity regulator complex aPKC-Par6-Par3. Disruption of the polarity complex destroys polarity increases cytoplasmic Yap in outer cells and shuts down the Tead4 directed TE gene regulatory network (Plusa *et al.* 2005, Alarcon 2010, Hirate *et al.* 2013). Interestingly from the 8-cell stage onward Amot is found only in the apical domain in polarised outer cells, whereas in inner apolar cells it is dispersed across the plasma membrane. This sequestering of Amot away from basolateral adherens junctions abrogates Hippo signalling resulting in nuclear Yap and Tead4 dependent TE transcription in outer cells (Hirate *et al.* 2013). Thus both cell polarity (via Tead4 activation) and subsequent inside-outside location (cell-cell contact in the respective absence-presence of polarity) are required to direct the differential gene expression programs.

Tead4 is ubiquitously expressed from the 2 cell stage (Yagi *et al.* 2007). Once activated via nuclear Yap, it accentuates expression of *Cdx2* and, independently of *Cdx2* expression, *Gata3* and *Eomes* (Nishioka *et al.* 2009, Ralston *et al.* 2010). Cdx2 is one of the most central players in mouse TE lineage commitment and maintenance, as shown by a variety of in vivo and in vitro assays (reviewed in (Pfeffer & Pearton 2012)) and its importance for TE maintenance has also been demonstrated in cattle, using a knock down approach (Berg *et al.* 2011). Aside from the establishment of the TE-specific gene regulatory network, Cdx2 is necessary to turn off the ICM-specific pluripotency network in outer cells by inhibiting Oct4 and Nanog (Niwa *et al.* 2005, Chen *et al.* 2009). These genes, as well as Sox2, stabilise each other's expression in ICM-derived embryonic stem cells, while repressing other lineages including that of the trophectoderm (Boyer *et al.* 2005). In the absence of Cdx2 expression, Oct4 is expressed at high levels in outer cells with concomitant loss of TE characteristics (Niwa *et al.* 2005, Strumpf *et al.* 2005). Cdx2 downregulates *Oct4* expression via the conserved region 4 (*CR4*) of the distal *Oct4* enhancer (Niwa *et al.* 2005, Wang *et al.* 2010). Importantly, Cdx2 can also inactivate Oct4 at the post-transcriptional level (Niwa *et al.* 2005, Nishiyama *et al.* 2009). Thus TE commitment may occur before *Oct4* transcription is fully shut down as long as Cdx2 protein levels are in functional excess of Oct4 protein levels.

The protein-protein interaction between Cdx2 and Oct4 is mutually inhibitive (Niwa *et al.* 2005). In inner cells therefore, where Cdx2 is not maintained via Yap/Tead4, remaining Cdx2 activity is repressed by Oct4 and the Cdx2-dependent TE-gene regulatory network is rapidly shut down with Cdx2 protein restricted to outer cells by the 32-cell early blastocyst (Dietrich & Hiiragi 2007, Ralston & Rossant 2008). The outer cell restriction of Cdx2 may also be accentuated by a recently discovered motif within the 3′ coding part of the Cdx2 transcript. This motif directs Cdx2 RNA to the apical region of polarised cells such that upon asymmetrical divisions outer cells inherit larger amounts of transcript (Skamagki *et al.* 2013).

Molecular determinants of lineage specification in cattle

In non-rodent mammals for which data are available, namely humans, monkeys, rabbits, pigs and cattle (van Eijk *et al.* 1999, Cauffman *et al.* 2005, Kuijk *et al.* 2008, Kobolak *et al.* 2009,

Sritanaudomchai *et al*. 2009, Khan *et al*. 2012), Oct4 protein is seen in outer TE cells at later developmental stages than in mice. Expressing a GFP reporter under the control of either mouse or cattle *Oct4* regulatory sequences in embryos of both mammals, inherent differences of *Oct4* regulation in TE were shown (Fig. 3) (Berg *et al*. 2011). Cattle expanded blastocysts were unable to shut down in the TE a GFP-expressing mouse *Oct4* reporter. At that developmental stage (expanded blastocyst) cattle *Oct4* mRNA levels were shown to exceed those of *Cdx2* ten-fold, suggesting that Cdx2 was unable to counteract Oct4 autoregulation. This was indeed shown by the lack of *Oct4* upregulation upon *Cdx2* knock-down. Thus high endogenous cattle Oct4 activity was mediating via a known autoregulatory element the transcription of the introduced mouse *Oct4* reporter in the TE (Berg *et al*. 2011). By inference it would indicate that the TE-gene regulatory network had not achieved prominence, which correlated well with the observed lack of commitment of cattle TE derived from such expanded blastocysts.

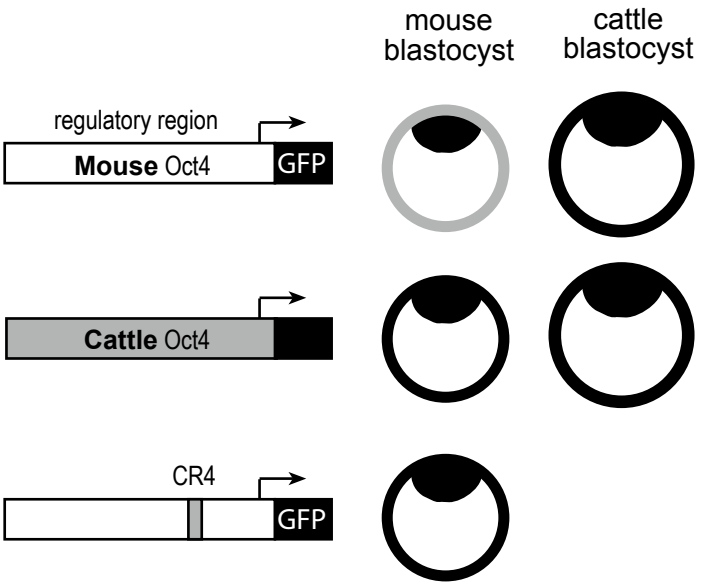

Figure 3. Differential trophectoderm regulation of *Oct4* reporters in transgenic cattle and mouse blastocysts. **A**. A *GFP* reporter construct linked to 18 kbp of mouse *Oct4* regulatory sequences results in fluorescence (shown in black) restricted to the ICM of transgenic mouse but not cattle blastocysts. **B**. The equivalent cattle *Oct4* regulatory construct continues to be expressed in the TE and ICM in both cattle and mouse embryos. **C**. The difference in mouse and cattle *Oct4* regulation is shown to lie within the CR4 region.

Secondly, it was found that the *Oct4* regulatory regions of these species had diverged (Fig. 3). This was demonstrated in a reciprocal approach, that is, placing a cattle *Oct4* reporter construct into mouse embryos. Expression of the cattle *Oct4*-reporter could not be extinguished in the mouse TE, even though these cells contained the necessary factors to switch off the endogenous mouse *Oct4* gene. Noting sequence divergence in the distal *Oct4* enhancer *CR4*, the authors replaced the mouse *CR4* region with that of cattle. Changing these 400 bp in the context of the 18 kilobase mouse *Oct4* reporter resulted in continued reporter expression in the mouse TE, in a cattle-like fashion. The regulatory difference was pinpointed to the exclusive presence in mice (as opposed to cattle, primates, rabbits and pigs) of two binding sites for the Tcfap2 family of transcription factors. The functional significance of these sites was supported by the ability, in vitro, of Tcfap2 proteins to inhibit transcription from an *Oct4-CR4* luciferase construct (Berg

et al. 2011). A recent report has questioned this based on the absence of Oct4 downregulation upon Tcfap2 knockdown (Choi *et al.* 2013). However this conclusion is questionable as only a modest knockdown of 60% was achieved and yet protein levels of Oct4, while restricted to the ICM in control embryos, failed to be extinguished in the TE of the Tcfap2c knockdown embryos shown (Choi *et al.* 2013), thus actually supporting a requirement for Tcfap2c in the TE-specific shut-down of Oct4. In summary, cattle and mouse embryos follow different trajectories in terms of lineage commitment based on differential Cdx2 levels and a change in the regulation of the ICM lineage specifier Oct4, as illustrated conceptually in Figure 4.

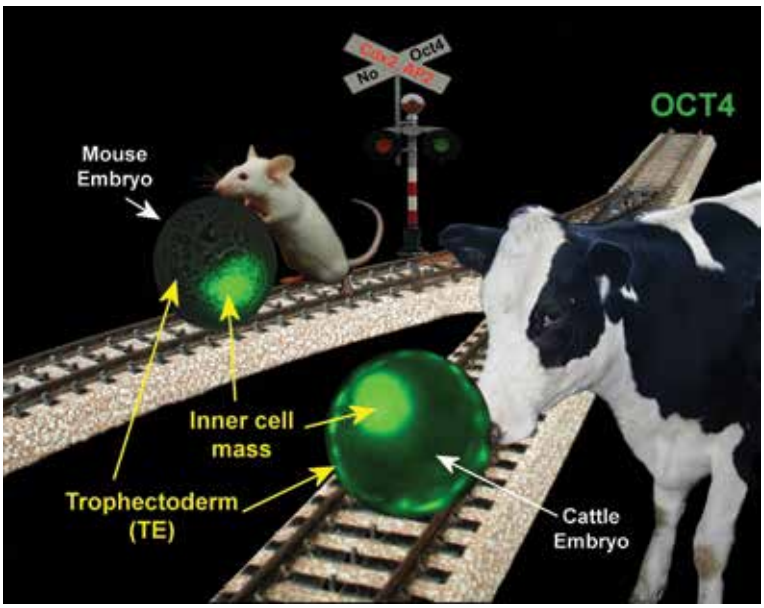

Figure 4. Conceptual visualisation of trophoblast commitment in cattle and mice. Cattle embryos stay on their developmental track with Oct4 expression ubiquitously activated and maintained in both the ICM and TE. In contrast, mouse embryos switch track via signals mediated through CDX2 and AP2 proteins, leading to Oct4 restriction to the ICM and an early commitment of TE tissue.

At later developmental stages, cattle *Oct4* expression in the TE diminishes while *Cdx2* levels remain constant. By E9 when the ICM has differentiated into epiblast and hypoblast layers, *Cdx2* and *Oct4* RNA levels are similar in the TE and by E11 Cdx2 is in 10 fold excess (Berg *et al.* 2011). The relatively delayed downregulation of cattle Oct4 in TE prompted the question as to whether this was causal for the corresponding delay of cattle TE commitment. To address this the 18kb of cattle Oct4 regulatory region was used to drive expression of a bicistronic Oct4-IRES-GFP construct in a cattle-like fashion in mouse embryos (Fig. 5). Transgenic pronuclear injected embryos could be recovered at a frequency of 27% (12/45) at E4.5 to E6.5 with expression levels varying between weak and strong. Notably though, embryos with stronger expression exhibited a mosaic expression pattern at postimplantation stages and we were only able to generate 2 founders (5%) from 42 pups born after pronuclear injection, with both lines showing exceedingly weak fluorescence at blastocyst stages. This suggests, but does not prove, that extended uniformly high *Oct4* expression in the mouse TE is deleterious to mouse development.

The continued expression of Oct4 in cattle TE as well as the lack of commitment of this tissues at expanded blastocyst stages predicts that the TE-specific gene regulatory network has

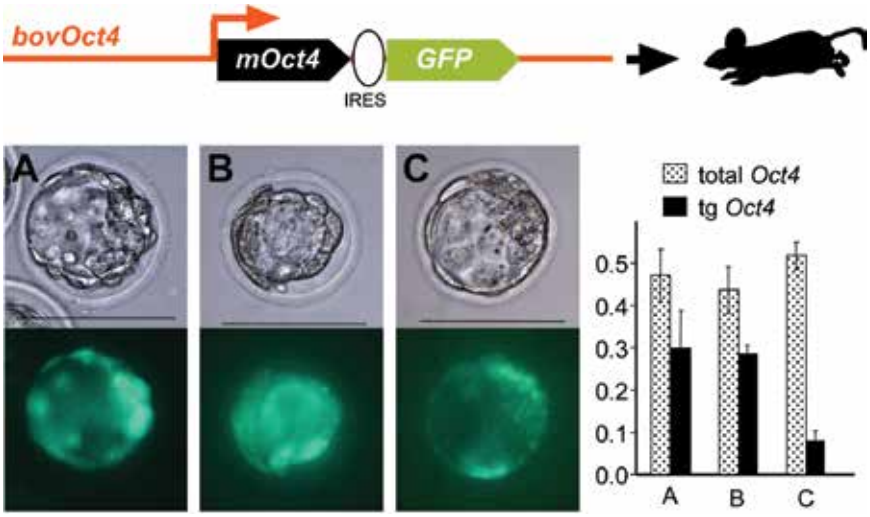

Figure 5. Expressing *Oct4* in a cattle-like fashion in mice. An 18 kbp cattle *Oct4* regulatory region equivalent to that used by Berg *et al.*, 2011, was modified, using recombineering technology, so as to splice in the mouse *Oct4* codons followed by an internal ribosome entry site (IRES) followed by *GFP* codons, thus allowing bicistronic expression of both mouse *Oct4* and *GFP* in a cattle *Oct4*-like fashion. This construct was injected into the pronuclei of mouse zygotes to create transgenic embryos which were retrieved from recipients at E4 (late blastocyst stage). Panels **A-C** represent bright field and fluorescent images of three embryos which were subsequently analysed by quantitative real-time PCR for mRNA expression of the introduced and introduced + endogenous mouse *Oct4* and normalised to three housekeeper genes. Non-restricted expression as expected from using the cattle *Oct4* regulatory region was seen at these expanded blastocyst stages. Overall *Oct4* levels were elevated relative to endogenous *Oct4* expression, particularly in embryo C. The resultant phenotype of such transgenic embryos is discussed in the main text.

not been fully established. A global transcriptome comparison between Day 8 ICM and TE cells revealed 870 differentially expressed genes. However when comparing this data against a set of 120 curated mouse and human ICM/ES specific genes, only eight were found to be upregulated in cattle ICM while 6 were actually upregulated in the TE. The curated TE-specific dataset fared better with 12 of 49 genes upregulated in cattle TE and only 1 exhibiting a reciprocal expression pattern (Ozawa *et al.* 2012). While some mouse lineage commitment genes such as *Nanog*, *Sox2* and *Gata3* were higher in the equivalent bovine cell compartment, others such as *Oct4* and *Sall4* were not (Ozawa *et al.* 2012). Along similar lines, a second such study noted novel genes with ICM and TE restriction (Nagatomo *et al.* 2013). Two non-exclusive interpretations are possible. First, that similar to the *Oct4* gene, the regulatory wiring has diverged among mammals. In support of this, a transcriptomic comparison of whole zygote to blastocyst stages among cattle, mice and humans led to an estimation that 40% of orthologous genes are differently expressed (Xie *et al.* 2010). Secondly, that the timing of the TE/ICM gene regulatory network establishment differs: Analysis of individual blastomere transcriptomes from 1-cell to morula stages in humans identified 9 developmental-stage specific modules of co-expressed genes of which 7 are preserved in mice, with the timing of these modules differing, mirroring the difference in EGA timing (Xue *et al.* 2013).

The second lineage decision

The second lineage decision during mammalian embryogenesis involves the ICM lineage and follows close on the heels of the TE/ICM lineage split. This is the decision as to whether cells form the epiblast which will form the foetus and some extraembryonic mesoderm, and the hypoblast, which is the progenitor of the yolk sac but also contributes some endodermal cells. As the process has been well examined in mice, I will detail this model system first, using "hypoblast" as opposed to the equivalent term "primitive endoderm" throughout this discussion.

In mice, following compaction at the 8-cell stage, apolar cells located on the inside of the morula/blastocyst, are generated via asymmetric (radial) cleavage during two successive waves (8-16 and 16-32) of cell divisions. These inner cells coexpress both epiblast (Nanog) and hypoblast (Gata6) lineage markers, while the TE expression network is progressively shut down. By the mid-blastocyst 64-cell stage, a "salt and pepper" intermingling of Nanog and Gata6 positive cells in the ICM was observed. These cells were fated, but not fully committed, to epiblast or hypoblast as predicted by respectively Nanog or Gata6 expression (Chazaud *et al*. 2006). As reports sometimes differ as to exact timings, it should be pointed out that a mouse-strain dependency of when mutual exclusions occur, has been demonstrated (Frankenberg *et al*. 2011). Over the next few hours the mutually exclusive Nanog and Gata6 positive cells segregate such that the Gata6 hypoblast progenitors line the ICM/blastocyst cavity, whereas the "inner" ICM cells are Nanog positive (Chazaud et al., 2006; Plusa et al., 2008; Meilhac et al., 2009; Frankenberg et al., 2011). The end of sorting correlates with epiblast/hypoblast commitment (Grabarek *et al*. 2012). Implantation occurs by E4.5 (> 100 cells). Over this period a progressive series of hypoblast markers is switched on, starting with Gata6 at the 8-cell stage, followed by Pdgf (onset 16-32 cell), Sox17 (32-64 cell), Gata4 (> 58 cell) and Sox7 (> 64 cell) (Plusa *et al*. 2008, Artus *et al*. 2011).

How is the heterogenic distribution of epi-and hypoblast progenitors established? After some initially opposing conclusions (Morris *et al*. 2010, Yamanaka *et al*. 2010), the following model has emerged (Morris *et al*. 2013, Krupa *et al*. 2014). The first wave of cells to be internalised have a tendency to contribute to epiblast, particularly when the total number of such cells are low. Conversely the second wave of cells tend to contribute more hypoblast cells. These tendencies appear to be related to Fgf signalling where first wave internalised cells show higher Fgf4 expression than second wave inner cells, while the reverse is true for the Fgf receptor, Fgfr2. This reciprocity had been previously seen between inner and outer cells at the 32-cell stage (Guo *et al*. 2010). It is thus possible that the longer exposure of second wave inner cells to an outside environment had predisposed them to increased Fgfr2 and decreased Fgf4 expression. The bias in Fgf signalling and/or response would be expected to modulate the initially co-expressed Nanog and Gata6 genes. In line with this, inhibition of Fgf signalling switches all ICM cells to Nanog+, Gata6- epiblast, while Fgf4 exposure results in Gata6+, Nanog- hypoblast progenitors (Yamanaka *et al*. 2010). Fgf signals via the Map kinase (MAPK) cascade. The onset of Gata6 expression was shown to be only initially directly dependent on MAPK signalling (Frankenberg *et al*. 2011), though such signalling may not be exclusively dependent on Fgf4 (Kang *et al*. 2013). In cells less responsive to MAPK signalling, Nanog cell-autonomously counteracts Gata6 expression via direct binding and repression in the proximal promoter (Singh *et al*. 2007) and/or by negatively regulating the Gata6-stabilising Bmi protein (Lavial *et al*. 2012). Conversely, in cells expressing higher amounts of Fgfr2, Fgf leads to downregulation of Nanog, thus lifting Gata6 repression (Frankenberg *et al*. 2011). From the 64-cell stage, when reciprocal expression of Nanog and Gata6 is established, Fgf4 expression becomes dependent on and restricted to Nanog expressing cells. This signal is required to maintain Sox17 and Gata4 expression in the surrounding Gata6 positive cells so as to allow these to acquire full hypoblast character (Messerschmidt & Kemler 2010, Frankenberg *et al*. 2011).

Interestingly, cattle epiblast/hypoblast segregation shows some substantial differences. Gata4, which is expressed subsequent to Gata6 and restricted to prospective hypoblast cells in mice, instead exhibits widespread expression in cattle expanded blastocysts (Kuijk *et al*. 2012). Secondly, although Nanog and Gata6 are expressed in a mutually exclusive salt and pepper pattern within the ICM of cattle expanded blastocyst, similar to what is seen in 64-cell mid-blastocyst mouse embryos, Fgf receptor inactivation via small molecule inhibitors had no effect on lineage segregation. Yet inhibiting MAPK signalling did increase the number of Nanog+ cells and reduced Gata6+ cells albeit not quite as efficiently as in the mouse system (Kuijk *et al*. 2012). An increase in ICM-specific Nanog mRNA upon MAPK inhibition was independently verified (Harris *et al*. 2013). Stimulation of MAPK signalling via FGF/heparin treatment elicited the opposite result, again in concurrence with mouse work (Kuijk *et al*. 2012). This strongly suggests that in cattle another signalling molecule (such as Igf, Egf or Pdgf) working through the MAPK signalling cascade may work in parallel or instead of FGF to specify hypoblast formation.

Notably, in human embryos the difference in hypoblast formation is even more pronounced in that MAPK inhibition had no effect on the establishment of reciprocal Nanog and Gata4/6 expression (Kuijk *et al*. 2012, Roode *et al*. 2012). However in both studies the effect of exogenous Fgf treatment was not tested in human embryos, leaving open the possibility that MEK signalling is involved but works in parallel to another signalling mechanism. Some care has to be taken in the interpretation of small molecule inhibitor treatments, as in mouse Fgfr inhibitors produces a phenotype that resembles but is not identical to that caused by lack of endogenous Fgf4 (Kang *et al*. 2013).

Conclusion

While the study of lineage segregation in mice is leading to an increasingly clear understanding of the basic cell mechanical and molecular events driving these processes, it is similarly becoming increasingly apparent from recent work in cattle that these events differ substantially among different mammals. Therefore if the problem of high embryo mortality in cattle is to be understood, let alone ameliorated, it is necessary to specifically learn more about this system by direct experimentation in cattle as opposed to inferences from the mouse model system which may or may not be correct.

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