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The mammalian embryo's first agenda: making trophectoderm

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ABSTRACT One of the bottlenecks for a successful pregnancy in mammalian species is the implantation of the early embryo into the wall of the mother's uterus. The first cell lineage the embryo sets aside following fertilization is the trophectoderm – a specialized cell type that establishes contact with the mother and mediates embryo implantation. We summarize the events that lead to the formation of the trophectoderm lineage in the preimplantation embryo and highlight key features of this cell type, which could be useful in the clinical setting for prediction of implantation outcomes.

KEY WORDS: *preimplantation embryo, trophectoderm, inner cell mass, implantation*

Introduction

During the first few days of mammalian development (~4 days in mouse and ~7 days in human) the embryo is freely floating along the oviduct until it arrives at its site of implantation in the uterus. The embryo is provided with sufficient nutrients to propel through this phase of development. Mammalian eggs do not have yolk stores, like other non-mammalian species, and in order to thrive need to establish physical contact with the mother to ensure continued nutrient flow. Therefore preimplantation development is dedicated to setting aside so-called extraembryonic cells within the embryo, which function to establish maternal contact and nurture the developing fetus in the uterus.

The very first cell fate decision in the embryo produces two cell types: the trophectoderm (TE) and the inner cell mass (ICM), creating a structure called the blastocyst. The TE will form as an epithelial layer of cells on the surface of the embryo, engulfing a fluid filled blastocoel cavity and a group of internal cells - the ICM. ICM cells will give rise to the embryo proper, as well as additional extraembryonic membranes, while TE cells are the precursors of most of the future embryonic portion of the placenta. The placenta is a peculiar organ – absolutely essential for fetal survival, but with an expiry date much shorter than the lifespan of the organism. As such, it is also more permissive towards accumulating genetic mutations compared to the embryo itself (Greco, Minasi & Fiorentino, 2015, Munne *et al*., 2017, Spinella *et al*., 2017).

Here we review the events that lead to the formation of the TE in the preimplantation embryo and the key properties of this tissue, which facilitate implantation. We compare, where possible, mouse and human.

Trophectoderm development in mouse

Compaction and intracellular symmetry breaking by polarization

The first three cleavage divisions in the early preimplantation embryo produce loosely associated, morphologically indistinguishable, totipotent blastomeres, which have the potential to give rise to any cell type (Fig. 1). Two concomitant events take place at the 8-cell stage: polarization and compaction, both of which serve as the basis for blastocyst formation (Fig. 2). Each blastomere becomes polarized along the apical-basal axis, which involves uneven distribution of both cell surface proteins and cytoskeletal components between apical and basolateral domains (Fleming & Johnson, 1988; Johnson & Maro, 1985). Compaction describes the process during which loosely associated cells increase cell-cell contacts, flatten their surfaces and condense into a sphere.

The first intracellular rearrangements involve the apical localization of myosin (Sobel, 1983; Zhu, Leung, Shahbazi, &

Abbreviations used in this paper: AD, apical domain; AJ, adherens junction; ICM, inner cell mass; TE, trophectoderm; TS, trophoblast stem cell.

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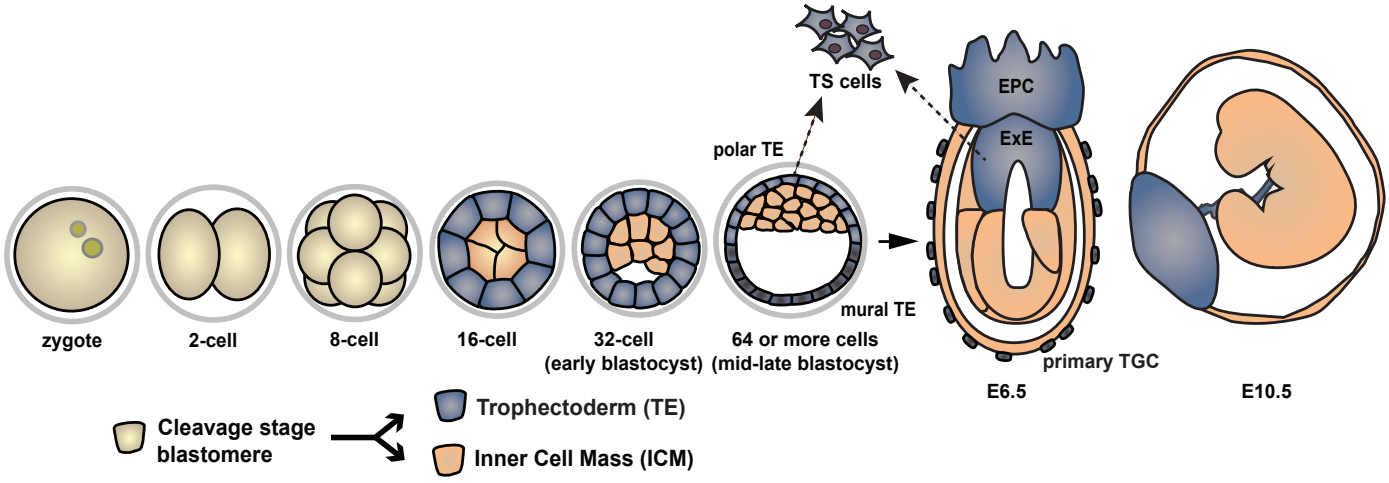

Fig. 1. Overview of early mouse development. *Early divisions of the fertilized egg produce cleavage stage blastomeres, which have unlimited developmental potential. Starting at the 16-cell stage, cells gradually start to differentiate to form two lineages: the trophectoderm (TE), which will give rise to the foetal portion of the placenta and the inner cell mass (ICM), which will give rise to the embryo proper and other extra-embryonic membranes. By the end of preimplantation development the blastocyst is formed with the TE organised into a surface epithelial layer, engulfing the ICM and a fluid filled blastocoel cavity. Two TE subpopulations can also be distinguished by the mid-late blastocyst stage: the polar TE, which is in contact with the ICM and the mural TE around the blastocoel cavity. The late blastocyst implants into the uterine wall. The mural TE gives rise to primary trophoblast giant cells (TGCs), while the polar TE rapidly proliferates to form the ectoplacental cone (EPC) and the extra-embryonic ectoderm (ExE) by embryonic day (E) 6.5. The polar TE and the ExE are the source for trophoblast stem (TS) cells.*

Zernicka-Goetz, 2017) where it assembles with the subcortical actin cytoskeleton. This is followed by apical localization of a number of other components, which build an apical domain (AD) at the center of the exposed surface of each cell. The AD consists of atypical protein kinase C (aPKC) isoforms, Par proteins, activated Ezrin-Radixin-Moesin (ERM) and microvilli (Ajduk & Zernicka-Goetz, 2016). Finally the underlying actomyosin network is excluded from the region of the mature AD and forms a ring around it (Zhu *et al.*, 2017).

The major components of basolateral cell-cell contacts that form during the 8-cell stage are adherens junctions (AJs). AJs are composed of transmembrane E-cadherin molecules, which link neighboring cells together in a Ca^{2+}-dependent manner and intracellular alpha and beta catenins, which engage cortical actin filaments. Embryo compaction requires AJs, as lack of E-cadherin or Beta-catenin leave blastomeres loosely associated (De Vries *et al.*, 2004; Stephenson, Yamanaka, & Rossant, 2010). Maître *et al.,* showed that AJs likely act as passive anchoring points between neighboring blastomeres and pulsing contractions generated by the apical actomyosin cortex are the main force generators that drive compaction (Maître *et al.*, 2012). Additionally, and not excluding the previous model, E-cadherin- and actomyosin-containing contractile filopodia, emerge from the regions where cell-cell contacts meet the embryo surface and extend to contact the apical surface of neighboring cells (Fig. 2). These filopodia are only present during compaction and have been proposed to play a role in pulling cells together (Fierro-González, White, Silva, & Plachta, 2013).

AD formation can proceed without cell-cell contact, however, contact was shown to be required for the proper localization of the AD (Johnson & Ziomek, 1981a; Stephenson *et al.*, 2010; Vinot *et al.*, 2005). Interestingly, it did not require E-cadherin specifically, as contact with a simple bead was sufficient to induce AD formation on the opposite pole of a blastomere, even if the blastomere itself lacked E-cadherin (Korotkevich *et al.*, 2017). These observations raise the possibility that a purely mechanical stimulus, perhaps through cell shape change may orient AD formation. Subcortical myosin localization was shown to be a prerequisite for AD formation (Zhu *et al.*, 2017) and cell-cell contacts regulate myosin localization by directing myosin towards the center of the apical cortex (Maître *et al.*, 2015; Sobel, 1983). Therefore, while AD formation and subcortical myosin accumulation can occur without cell-cell interaction, contact likely orients and concentrates these components at the center of the contact-free surface.

What are the signaling pathways that regulate these morphogenetic events? Apical myosin polarization depends on Phospholipase C (PLC)-mediated Protein kinase C (PKC) activation (Ohsugi, Ohsawa, & Yamamura, 1993; Winkel, Ferguson, Takeichi, & Nuccitelli, 1990; Zhu *et al.*, 2017). The small Rho GTPases RhoA and Cdc42 were needed for the polarization of cytoskeletal actin (Clayton, 1999) and RhoA for polarization of myosin (Zhu *et al.*, 2017). How any of these activities link to contact-induced asymmetries remains an open question.

Chemical activation of PKC or expressing active forms of RhoA or Cdc42 in 4-cell blastomeres can induce premature actomyosin polarization and compaction, but not polarization of AD components (Clayton, 1999; Zhu *et al.*, 2017). Thus, all components required for establishment of a contractile actomyosin cortex needed for compaction are present already at the 4-cell stage, however AD formation needs additional factors or signaling cues downstream of polarized actomyosin. A candidate downstream signaling factor may be the Rho-associated kinase (ROCK), as ROCK inhibition does not influence compaction but affects AD formation (Kono, Tamashiro, & Alarcon, 2014). Moreover, ROCK is known to phosphorylate and activate ERM proteins, which are required for organizing the apical cortex (Amano, Nakayama, & Kaibuchi, 2010). Further studies are needed to clarify the roles of ROCK in this context. Despite accumulating knowledge on the

sequence of signaling events leading to polarization and compaction the important question of what triggers these activities during development remains unanswered.

Intercellular symmetry breaking – divisions produce different cell types

Polarized 8-cell blastomeres divide with different orientations with respect to the AD and the axis of the embryo, producing for the first time morphologically distinct cells at the 16-cell stage. The orientation of cell divisions will influence two properties of daughter cells: whether they inherit any of the AD (resulting in polar and apolar cells) and their position in the embryo (inside cells sequestered in the core and outside cells that bear an exposed surface). What determines cleavage angle and thereby the proportions of different cell types in the embryo?

An early study found that division planes were oriented randomly with respect to the axis of cell polarity (Pickering, Maro, Johnson, & Skepper, 1988). However it noted a correlation between the size of the AD and daughter cell polarity – a larger AD was more likely to be cleaved in a randomly oriented division and thus yielded two polarized progeny. Live imaging of spindle formation reveled no tight control over spindle orientation, however the shape of cells during mitosis did to some extent have an effect (Dard, Louvet-Vallée, & Maro, 2009). Cells round up during mitosis and some bulge out of the embryo more than others. These bulging cells showed a tendency for aligning their spindles with the apical-basal axis of the cell, resulting in a polar and an apolar progeny. A more recent study observed a similar spindle-orientation phenomenon - the mitotic spindle was often aligned with the apical-basal axis, perpendicular to the AD (Korotkevich *et al.*, 2017). Interestingly, orientation was random in embryos lacking an AD. They also observed that as the AD forms, it recruits microtubule organizing centers (MTOCs) to the subapical regions of blastomeres. This suggests that the AD influences the plane of division, although it is still unclear how exactly the AD would orient the spindle. Interestingly, this would imply that the AD itself ensures its own distribution in daughter cells, favoring a pattern where one cell inherits the AD, while the other does not.

The 8-to-16-cell divisions produce three types of cells: inside cells, which are always apolar, and outside cells, which can either remain polarized or become apolar. This later population is though to arise from divisions where the exposed surface, but not the AD is cut by the cleavage furrow (Fig. 3). Regardless of whether the cleavage plane is controlled or random, there is a degree of variability reported from embryo-to-embryo both in terms of inside/outside and polar/apolar cell numbers. On average, a freshly formed 16-cell stage embryo has 1-2 apolar inside, 10 polar outside and 4-5 apolar outside cells (Anani, Bhat, Honma-Yamanaka, Krawchuk, & Yamanaka, 2014; Dietrich & Hiiragi, 2007; Fujimori, Kurotaki, Komatsu, & Nabeshima, 2009).

Of note, the recent study by Zenker *et al.,* is ruffling some feathers by reporting that the AD may in fact disassemble prior to the 8-to-16-cell divisions and reassemble *de novo* at the 16-cell stage (Zenker *et al.*, 2018). However, since the AD does not appear on all outside cells at the 16-cell stage and the frequency of asymmetric divisions are reflected in the number of apolar/polar progeny (Anani *et al.*, 2014), it is likely that some component of it is inherited in a membrane-bound form through divisions. Other members may indeed disassemble before division and then use the membrane-bound component as a seeding cue to re-establish the AD. Live imaging of endogenously tagged AD members is needed to resolve this issue in the future.

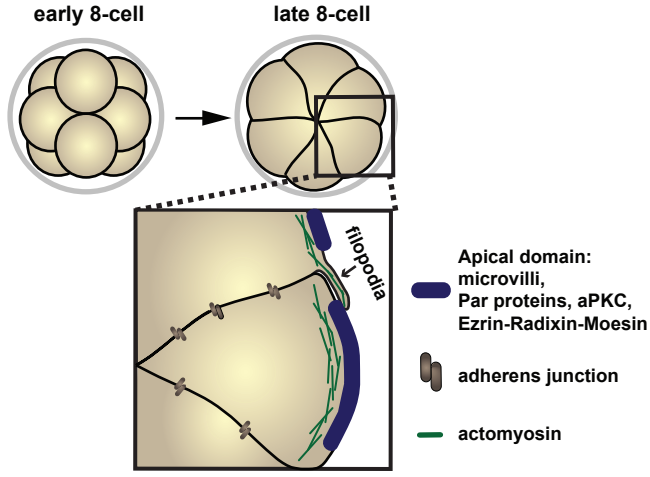

Fig. 2. Polarization and compaction. *At the 8-cell stage each blasomere becomes polarized along the apical-basal axis, forms an apical domain at the center of its exposed surface and a subcortical actomyosin network. Blastomeres also increase cell-cell contact by flattening their surfaces against one another and forming adherens junctions between them. The forces driving compaction are generated by the subcortical actomyosin network and by the pulling forces from filopodia extending from some cells and contacting neighbours.*

Dynamic sorting at the morula stage

How does an embryo acquire an outside layer of TE cells and an inside population of ICM cells with an approximate 2 to 1 ratio, as observed by the early-mid blastocyst stage (Suwińska, Czołowska, Ożdżeński & Tarkowski, 2008)? Which property - position or polarity - matter for future cell fate? Models half a century old have been proposed supporting both position (Tarkowski & Wróblewska, 1967) and polarity-driven events (Johnson & Ziomek, 1981b; 1981a). Using advanced imaging techniques, it is now clear that dynamic division-independent cell rearrangements occur during the 16-cell stage: while outside polar and inside apolar cells remain in place, most of the apolar outside cells internalize and contribute to the inner compartment (Fig. 3) (Anani *et al.*, 2014; Korotkevich *et al.*, 2017; Maître *et al.*, 2016; Samarage *et al.*, 2015).

What are the mechanisms that regulate these dynamic cell rearrangements? An isolated polar/apolar cell couplet from a 16-cell stage embryo mimics this internalization event – the polar cell envelopes the apolar neighbor. Measuring the curvatures of the cell membranes at cell-cell contacts on such isolated couplets revealed that apolar cells had higher cortical tension than polar cells (Anani *et al.*, 2014). Direct measurement of surface tensions using micropipette aspiration confirmed the asymmetry between apolar and polar blastomeres and a mathematical model was used to predict that cell internalization would take place if the tension asymmetry exceeded 1.5-fold (Maître *et al.*, 2016). The increased cortical tension of apolar cells was generated by subcortical contractile actomyosin. Phosphorylated myosin light chain II, a marker of activated myosin was enriched at the cortex of apolar cells and inhibiting contractility genetically or using an inhibitor resulted in failure to internalize (Anani *et al.*, 2014; Maître *et al.*, 2016; Sa-

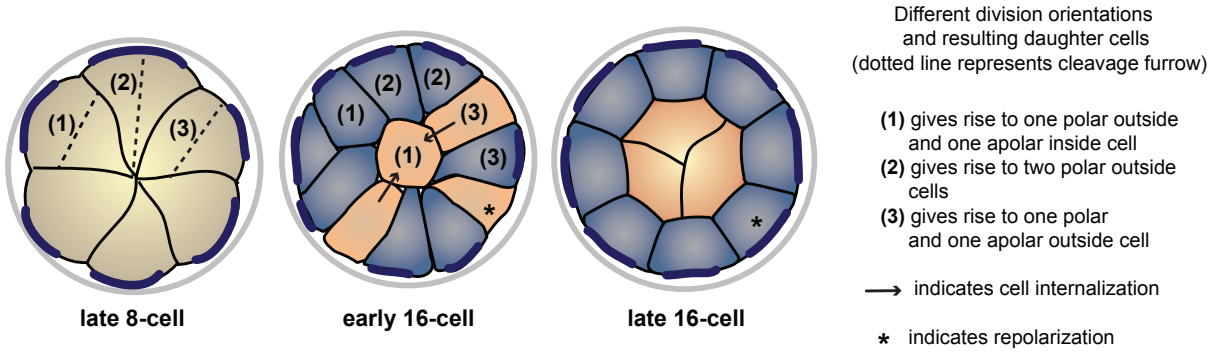

Fig. 3. Dynamic cell rearrangements at the morula stage. *The cells of the 8-cell embryo divide with various angles – examples of different division orientations are shown as (1), (2) and (3). Dotted lines represent the cleavage planes of different divisions. Depending on the cleavage plane different daughter cell types are formed at the 16-cell stage – daughter cells can remain in contact with the outside surface or become engulfed by other cells. Additionally, they may or may not inherit part of the apical domain (in dark blue), resulting in polar (blue) and apolar (orange) cells, respectively. (1), (2) and (3) also indicate the progeny arising from different division orientations. During the 16-cell stage most apolar outside cells internalize, while the occasional apolar outside cell acquires* de novo *polarity (*).*

marage *et al.*, 2015). The presence of a contractile actomyosin cortex in turn was reversely correlated with the presence of an AD (Anani *et al.*, 2014; Maître *et al.*, 2016) and transplanting an AD together with the underlying subcortical material onto an apolar cell kept it from getting internalized by a polar neighbor (Korotkevich *et al.*, 2017). How exactly the AD regulates actomyosin contractility, however, remains to be solved.

These data argue that polarity dictates cellular rearrangements at the 16-cell stage, which for majority of cells is indeed the case. There are however, rare, apolar outside cells, which instead of internalizing stay on the surface and acquire a new AD (Anani *et al.*, 2014; Korotkevich *et al.*, 2017), thus in some cases a positional cue can override polarity. It is currently not known how the choice is made in an apolar outside cell to internalize or to repolarize. Prolonged exposure of apolar cells of an early blastocyst by removing the outside TE layer is known to result in repolarization (Handyside, 1978; Hogan & Tilly 1978; Rossant & Lis, 1979; Spindle, 1978; Stephenson *et al.*, 2010). It may therefore be simply a matter of timing – is internalization or reestablishment of polarity faster?

Just prior to the next round of divisions, a roughly 4-5 apolar inside and 10-11 polar outside cell configuration is achieved, which more closely resembles ICM/TE ratios at the early-mid blastocyst stage. The 16-to-32-cell divisions can still perturb cell position and therefore future cell fate. Specifically, some outside cells can divide with an orientation that will push one of the daughters inside (Morris *et al.*, 2010; Strnad *et al.*, 2015; Watanabe, Biggins, Tannan, & Srinivas, 2014; Yamanaka, Lanner, & Rossant, 2010). However, there are conflicting data about how frequent such divisions are and whether the daughter cell pushed in by the division actually stays within or sorts back out to the surface (Strnad *et al.*, 2015; Watanabe *et al.*, 2014).

Initiation of lineage-specific expression programs

There are a number of transcription factors (TFs) characteristic of both ICM and TE lineages (Bissiere, Gasnier, Álvarez, & Plachta, 2018). Notable examples include pluripotency factors Oct4, Sox2 and Nanog expressed in the ICM and Tead4, Cdx2, Gata3, Eomes, Tcfap2c and Elf5 in the TE. Examining protein expression by antibody localization showed that many of these lineage-specific TFs were expressed initially at low levels in all cells of the embryo, followed by a gradual restriction and increase in expression level in respective lineages (Mitsui *et al.*, 2003; Palmieri, Peter, Hess, & Scholer, 1994; Ralston *et al.*, 2010; Strumpf *et al.*, 2005). Such gradual restriction in expression were also noted in single-cell gene expression profiling studies (Deng, Ramskold, Reinius, & Sandberg, 2014; Guo *et al.*, 2010; Posfai *et al.*, 2017). Lineage specific transcriptional profiles of both ICM and TE emerged concomitant with the onset of morphogenetic changes among cells: at the 16-cell stage cells still expressing a mixed signature are detected, however a number of cells already initiate ICM or TE expression profiles (Posfai *et al.*, 2017). What initiates the divergence of these transcriptional programs?

To date the earliest ICM/TE segregation phenotype is the failure of TE lineage formation due to *Tead4* disruption (Nishioka *et al.*, 2008; Yagi *et al.*, 2007). In *Tead4* mutants ICM markers such as Oct4 (Nishioka *et al.*, 2008; Yagi *et al.*, 2007) and Sox2 (Wicklow *et al.*, 2014) are ectopically expressed in outside cells and generation of an epithelial TE layer is compromised. In various other systems Tead proteins function together with co-activators Yes-associate protein (Yap) and Taz, that are regulated by the Hippo signaling pathway. When the Hippo pathway is active, the serine/threonine kinases Lats1/2 phosphorylate Yap/Taz, causing their cytoplasmic retention (Fig. 4). On the other hand when the pathway is inactive, unphosphorylated Yap/Taz shuttle into the nucleus and interact with Tead4.

In the TE lineage the Hippo pathway is inactive, resulting in nuclear Yap localization and activation of TE-associated TFs, *Cdx2* (Nishioka *et al.*, 2008; Yagi *et al.*, 2007) and *Gata3* (Ralston *et al.*, 2010), as well as other TE-associated genes, such as *Dab2*, *Lrp2*, *Krt8*, *Krt18* (Posfai *et al.*, 2017). Cdx2 plays a particularly important role in TE formation – embryos deficient in Cdx2 develop into blastocysts, however the blastocoel cavity collapses due to the lack of TE epithelial integrity and embryos fail to implant (Blij, Frum, Akyol, Fearon, & Ralston, 2012; Strumpf *et al.*, 2005). Cdx2 is required to repress Oct4 and Nanog expression, as *Cdx2* mutant embryos continue to express these pluripotency factors in outside cells (Strumpf *et al.*, 2005). In wild type 16-cell stage embryos initially low levels of Cdx2 are detected in all cells in a Tead4/

Yap-independent manner (Posfai *et al*., 2017; Yagi *et al*., 2007). However, as soon as Yap localization differences are detected amongst blastomeres, Cdx2 levels start to show a positive correlation with nuclear Yap levels, suggesting that Hippo signaling differences ignite lineage segregation by inducing target gene expression in TE progenitors. On the other hand Hippo signaling is active in the ICM lineage, Yap is retained in the cytoplasm and cannot activate Tead4-dependent transcription.

A number of studies have reported various molecular differences present between 2 or 4-cell stage blastomeres, and have initiated a debate whether these differences are functionally relevant for biasing ICM/TE cell fate decisions later on (Burton *et al*., 2013; Goolam *et al*., 2016; Plachta *et al*., 2011; Torres-Padilla, Parfitt, Kouzarides, & Zernicka-Goetz 2007; White *et al*., 2016; and recently reviewed in Chazaud, & Yamanaka, 2016). While the possibility of a pre-existing bias is intriguing, it is clear that up to the 8-cell stage blastomeres are plastic and can respond to cues promoting either cell fate.

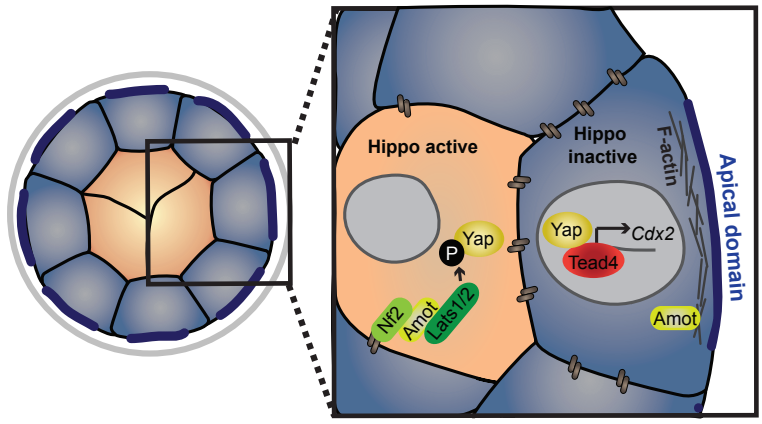

Fig. 4. Differential Hippo signalling activity in trophectoderm (TE) and inner cell mass (ICM) progenitors. *In apolar ICM cells several Hippo pathway members (Nf2, Amot and Lats1/2 kinases) assemble at adherens junctions (AJs) and activate signaling. Lats1/2 phosphorylate the transcriptional coactivator Yap, which retains Yap in the cytoplasm. In TE cells Amot is sequestered away from AJs by binding the F-actin network underlying the apical domain and therefore an active Hippo complex can not assemble. Unphosphorylated Yap shuttles into the nucleus and together with Tead4 activate transcription of key TE-specific genes, such as* Cdx2.

Linking position and polarity with Hippo signaling

How are different Hippo activities achieved in ICM and TE progenitor cells? A scaffolding protein called Angiomotin (Amot) is necessary to recruit Hippo pathway members to AJs, leading to Hippo activation (Cockburn, Biechele, Garner, & Rossant, 2013; Hirate & Sasaki, 2013; Leung, & Zernicka-Goetz, 2013). All cells in the embryo express Amot and have AJs along cell-cell contacts, therefore this alone does not explain Hippo signaling differences. Interestingly, Amot is also able to bind subcortical F-actin underlying the AD (Hirate & Sasaki, 2013). In fact, in polarized outside cells Amot is sequestered from AJs, and becomes exclusively localized to the apical membrane. At the apical membrane Amot does not assemble Hippo members resulting in no Hippo activity. Therefore polarity differences dictate Amot localization and Hippo activity. Indeed, a closer look at the 16-cell stage revealed that apolar outside cells had cytoplasmic Yap, thus Yap localization correlated with polarity status rather than cell position (Anani *et al*., 2014; Maître *et al*., 2016). The molecular explanation for this polarization-dependent F-actin-binding of Amot is a key missing piece.

Cavitation

During the 32-cell stage cavity formation is initiated in the embryo (Fig. 5). First, small intracellular fluid-filled vacuoles appear in the TE layer, which are then deposited by exocytosis into intercellular spaces (Watson, Natale, & Barcroft, 2004). Fluid initially accumulates in multiple small cavities between inner cells, which then fuse to form one large cavity that compresses the ICM to one side. A tightly sealed epithelial TE, Na^+/K^+ ATPase pumps and aquaporins are needed for fluid accumulation (Watson, Natale, & Barcroft, 2004). Na^+/K^+ pumps in the basolateral membranes of TE cells establish an ion gradient, which then drives water uptake through aquaporins in the TE. The sealing of the TE layer has long been known to involve AJs and tight junctions (TJs). Recently, live imaging was used to show that F-actin rings form under the apical membranes of cells at the morula stage (Zenker *et al*., 2018). A polarized microtubule network, originating from the disassembled spindle from the previous division, underlays the AD and inhibits F-actin accumulation, forcing it to build a ring around the AD. Down-regulating *Pard6b* disrupted ring formation (Zenker *et al*., 2018) and inhibiting aPKC delayed cavitation (Eckert *et al*., 2004). At the morula stage, the F-actin rings expand towards cell-cell junctions, meet, and couple between neighboring cells and then zipper along the entire length of the junction. Both ring binding and zippering was found to be dependent on local myosin II accumulation, suggesting these processes are tension-dependent. Moreover, zippering was found to be necessary for junction maturation and sealing of the epithelium. It will be important to distinguish the molecular composition of non-contractile actin rings of polar cells required for embryo sealing from the contractile subcortical actin network driving compaction and internalization of apolar cells at the 8- and 16-cell stages, respectively.

Mechanical forces

An area that has so far received little attention in the embryo is the effect the mechanical environment has on lineage development and patterning. This is a particularly relevant question, as Hippo signaling and Yap have been shown to regulate differentiation by transducing mechanical stimuli through the actomyosin cytoskeleton (Halder, Dupont, & Piccolo, 2012). It is clear that position and polarity-dependent inputs regulate Hippo signaling in the embryo, but is there more to this regulation?

At the 16-cell stage dynamic cell rearrangements are due to differential intracellular contractility of cells. Inhibiting myosin contractility increases cytoplasmic Yap and mimics ICM-like Yap distribution; therefore contractility in principle has an effect on Yap localization (Maître *et al*., 2016). However, in intact embryos Yap was found to be mainly cytoplasmic in internalizing contractile cells and nuclear in outside cells with low contractility. It is therefore currently unclear whether Yap is indeed regulated by intracellular contractility at this stage.

As cells sort to the inner compartment, the remaining outside cells need to deform and stretch to form a continuous epithelial layer

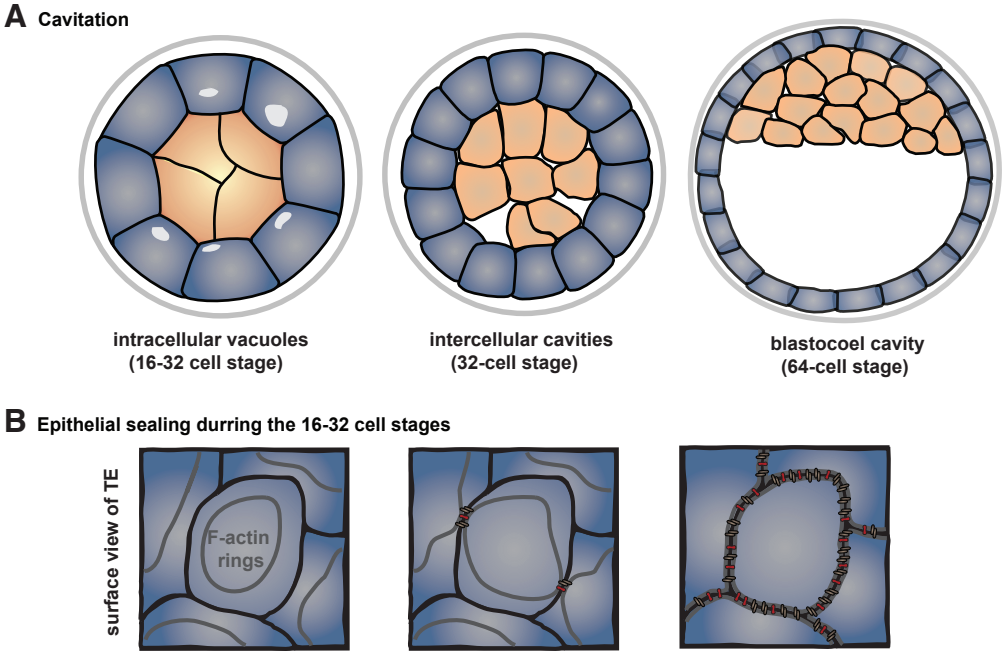

Fig. 5. Embryo cavitation. (A) *The first signs of fluid accumulation in the embryo appear as intracellular vacuoles in TE cells. These vacuoles are emptied into spaces between ICM cells and eventually fuse to form one large blastocoel cavity.* **(B)** *For fluid to accumulate a tightly sealed epithelial TE layer is needed. Sealing of the TE involves non-contractile actin rings forming under the apical cortex. These rings expand until they reach the cell boundary and rings of neighbouring cells couple by binding to adherens (brown) and tight (red) junctions. Rings zipper along the entire cell boundary and recruit additional adherens and tight junctions.*

on the surface. Cell stretching is further boosted during cavitation, when hydrostatic pressure builds up in the fluid-filled cavity and presses on the TE layer. It will be interesting to examine whether stretching influences Yap localization and therefore cell fate. Cells which develop without any contact, develop a gene expression profile that more closely resembles TE rather than ICM cells (Lorthongpanich, Doris, Limviphuvadh, Knowles, & Solter, 2012). A TE-like state is in line with cells being exposed to the outside environment. Curiously however, the gene expression pattern of these cells is still distinct from TE cells, raising the possibility that mechanical stretch may indeed be needed for the acquisition of a full TE profile.

Plasticity

An interesting problem that has engaged developmental biologists for decades is when and how the emerging lineages lose their ability to morph into the other cell type. The timing of commitment has been explored by numerous studies (Fig. 6), and has recently been revisited using new tools to identify progenitors of the two developing lineages (Posfai *et al.*, 2017). Interestingly, specification and commitment arises simultaneously in the developing TE, with some TE cells emerging already at the 16-cell and most set aside by the early 32-cell stage. In the developing ICM lineage however, a developmental window separated specification and commitment: most ICM cells specified by the early 32-cell stage, however they were still able to convert to TE up to the 64-cell stage. Commitment of the TE lineage first may reflect the developmental urgency of producing a cell type that will ensure implantation. On the other hand, retaining plasticity in the ICM up to the mid blastocyst stage could serve as a backup mechanism to replenish the TE, if necessary. Loss of plasticity in the ICM coincided with the initial appearance of EPI and PE transcriptional profiles, suggesting a functional relationship between loss of TE potential and initiation of EPI/PE differentiation. Indeed, Wigger *et al.,* showed that by inhibiting Fgf and downstream Extracellular signal-regulated kinase (ERK) signaling, the pathway that is required for the segregation of EPI and PE cells, plasticity of ICM cells could be extended towards the TE lineage (Wigger *et al.*, 2017). Further studies are clearly needed to identify the molecular mechanisms underlying cell fate commitment.

Lineage crosstalk and trophoblast stem cells

Trophoblast vesicles can form from cells that are committed to the TE lineage. These empty vesicles can occasionally initiate implantation, albeit at low frequencies (Surani & Barton, 1977). This highlights the importance of sustained crosstalk between the ICM and TE lineages. A number of differentially expressed receptor and ligand pairs have been observed between ICM and TE lineages, providing clues to the molecular mechanisms underlying inter-lineage communication. Most notably, Fibroblast growth factor (Fgf) ligands produced by the ICM, in particular Fgf4, activate ERK signaling in the TE through Fgf receptors and stimulate TE proliferation (Nichols, Silva, Roode, & Smith, 2009). Mutations in Fgf4 or its associated receptor Fgfr2, lead to peri-implantation embryonic lethality and poor trophoblast development (Arman, Haffner-Krausz, Chen, Heath, & Lonai, 1998; Feldman, Poueymirou, Papaioannou, DeChiara, & Goldfarb, 1995). In addition, treatment of blastocysts that lack an ICM with Fgf4 increases TE proliferation (Chai *et al.*, 1998; Nichols *et al.*, 1998). Thus, proliferation of the TE is maintained, in part, by a source of Fgf4 originating in the ICM. Given its propensity to divide, the TE overlaying the ICM and its later derivative, the extraembryonic ectoderm (ExE), contain a pool of trophoblast stem (TS) cells (Fig. 1). Indeed, it is possible to derive TS cells *in vitro* from blastocyst outgrowths as well as from the ExE in the presence of Fgf4 (Tanaka *et al.*, 2002).

Maintenance of TS cells also requires additional stimulants such as members of the TGF-β superfamily, Activin and Nodal (Erlebacher, Price, & Glimcher, 2004) and embryos that lack *Nodal* show developmental defects in the placenta (Ma *et al.*, 2001). Bone morphogenetic protein (Bmp) ligands and TGF-β family members

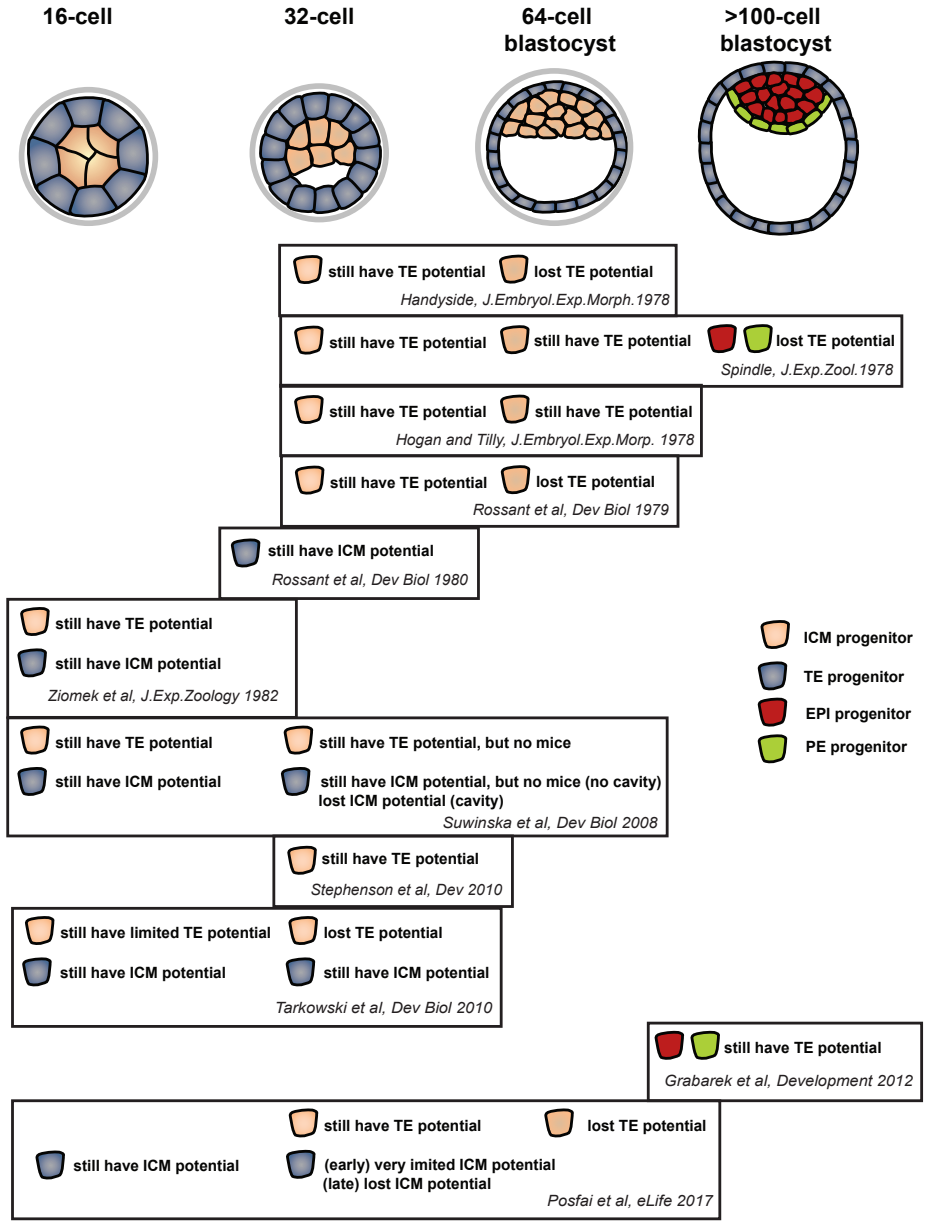

Fig. 6. Summary of studies examining the potential of inner cell mass (ICM) and trophectoderm (TE) cells. *Overview showing the developmental potential of ICM (later epiblast (EPI) and primitive endoderm (PE)) and TE progenitors at different embryonic stages. Developmental potential was examined by either adding a single cell to a host morula or to host cells and assaying which lineage the single cell contributed to (morula aggregation assay) (Grabarek* et al.*, 2012; Posfai* et al.*, 2017; Rossant & Vijh, 1980; Tarkowski, Suwinska, Czołowska, & Ożdżeński, 2010) or by creating an entire embryo from the same type of progenitors and assaying whether the other cell type could be re-formed (Handyside, 1978; Posfai* et al.*, 2017; Rossant & Lis, 1979; Spindle, 1978; Stephenson* et al.*, 2010; Suwińska, Czołowska, Ożdżeński, & Tarkowski, 2008; Ziomek, Johnson, & Handyside, 1982). Creating an embryo from only ICM cells can be achieved by removing the TE layer using immunosurgery. Alternatively single ICM or TE cells can be re-aggregated to make entire embryos.*

produced by the ICM are needed for generating correct TE cell numbers in the embryo (Graham *et al.*, 2014).

Interestingly, in a recent publication researchers devised a protocol to assemble TS and embryonic stem (ES) cells, a stem cell type derived from the ICM, into structures resembling a blastocyst (Rivron *et al.*, 2018). Comparing expression profiles of "TE" and "ICM" cells from these so-called blastoids with cultured TS and ES cells identified Bmp and Nodal signaling as inductive cues sent by the ICM to the TE for the generation of an implantation-competent TE.

Mural/polar differentiation of trophectoderm

By the mid blastocyst stage of murine embryo development, the already specified TE is clearly segregated into two distinct subtypes based on its proximity to the ICM – the polar TE that is in direct contact with the ICM, and the mural TE, which surrounds the blastocoel. The mural TE of the late blastocyst attaches to the maternal endometrium and initiates embryo implantation. Following implantation, mural TE cells progressively stop dividing and instead begin to endoreduplicate DNA, forming large, polyploid primary trophoblast giant cells (TGCs) (Varmuza, Prideaux, Kothary, & Rossant, 1988). Primary TGCs are highly invasive cells that facilitate implantation by secreting proteases, including cathepsins and metalloproteinases, which help to invade the surrounding maternal tissue (Screen, Dean, Cross, & Hemberger, 2008, Alexander *et al.*, 1996). In contrast, the polar TE cells overlying the ICM continue to proliferate and give rise to all of the trophoblast lineages that make up the mature placenta (Copp, 1979). Withdrawal of Activin/Nodal as well as Fgf4 *in vitro* results in down-regulation of TS cell-specific TFs and terminal differentiation into TGCs. This supports the no-

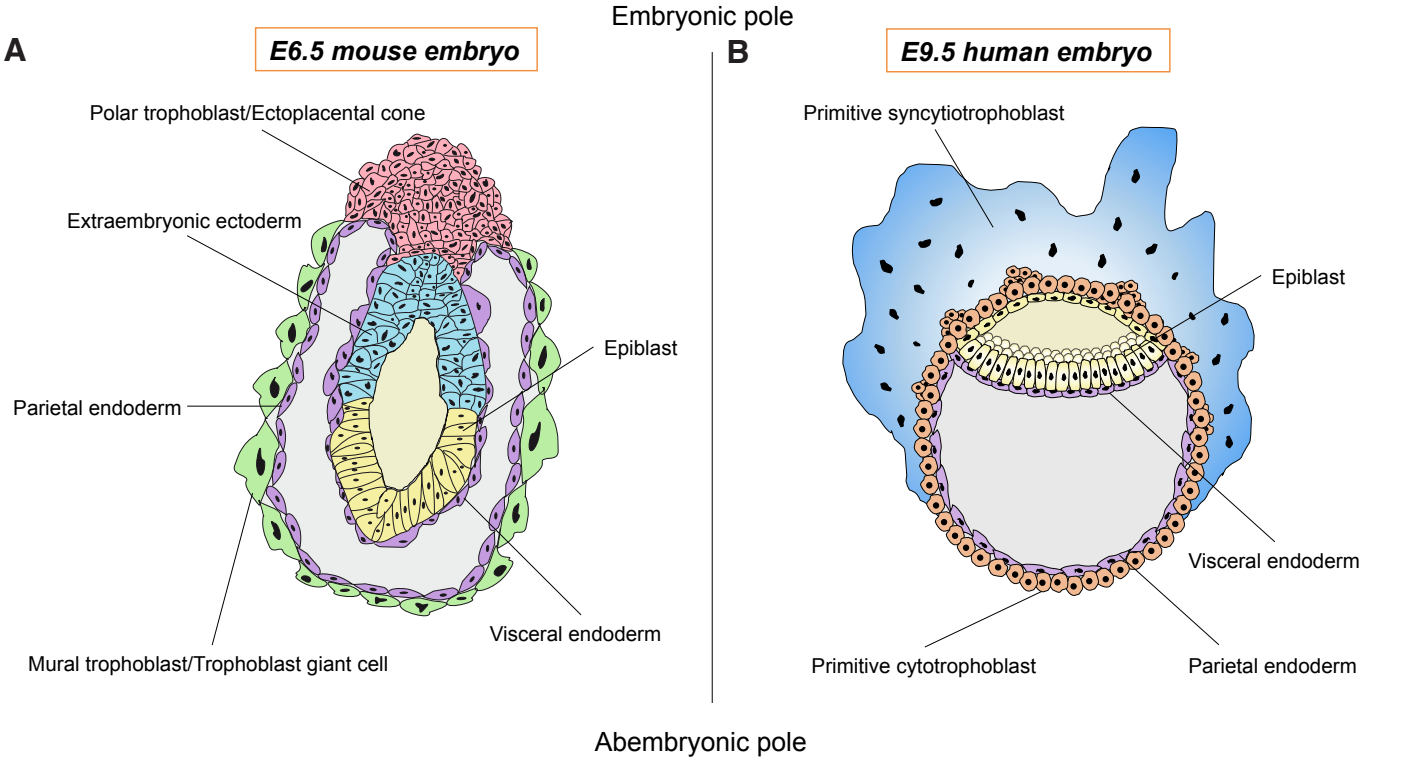

Fig. 7. Comparison of trophoblast lineages of the mouse and human early post-implantation embryo. (A) *Egg cylinder stage mouse embryo at embryonic day (E)6.5. Continuous proliferation of the polar TE gives rise to the extraembryonic ectoderm (ExE) and invading ectoplacental cone. The mural TE stops dividing and differentiates into trophoblast giant cells (TGCs). Note the absence of syncytiotrophoblast cells in the post-implantation mouse embryo.* **(B)** *Implanted human embryo at (E)9.5. In humans, the TE gives rise to the primitive cytotrophoblast (CT) that surrounds the entire embryo and the multinucleated primitive syncytiotrophoblast (ST) that start forming from the TE at the embryonic pole.*

tion that *in vivo,* the mural TE cells that are further away from the ICM stop proliferating and differentiate into TGCs partially due to insufficient activation of Fgf, Activin and Nodal signaling.

While there have been extensive studies analyzing postimplantation development of polar and mural TEs, not much is known about the differences between these two lineages prior to implantation. Analysis of the transcriptional profile obtained from polar and mural cells of the late blastocyst found hundreds of genes differentially expressed between these two lineages (Nakamura *et al.*, 2015). While polar TE cells were enriched in GO terms such as 'mitotic cell cycle' and 'cell division', mural TE cells were characterized by 'lipid storage', 'membrane organization', and 'cell death' pathways. Such gene signatures suggest that the mural TE already exhibits transcriptional characteristics of differentiated TGCs, including cell cycle exit. A recent study identified several TS cell-enriched microRNAs (miRNAs), including miR-15b, that are capable of inducing trans-differentiation of ES cells into a trophoblast-like lineage with a mural-TE phenotype (Nosi, Lanner, Huang, & Cox, 2017). These induced trophoblast cells showed exclusive localization to the mural TE when injected into preimplantation embryos. Furthermore, gene signature of these cells confirmed expression of primary TGC markers. This again emphasizes the idea that TE differentiation is compartmentalized and influenced by signals from the ICM.

In addition to the transcriptional differences between polar and mural TEs, functional differences between these two TE subtypes also exist. This is elegantly reflected in cellular responses of the TE during diapause. Embryonic diapause, a state when implantation is suspended for a period of time (dormancy) until optimal conditions for implantation are achieved (reactivation), has been used for mapping metabolic responses in the blastocyst. Proteins found to be up-regulated in reactivated, implantation competent blastocysts, most related to mitochondrial function (Atp5b) and endo-lysosomal activity (CtsD), were significantly enhanced in the mural TE (Fu *et al.*, 2014). In addition, the mural TE contains more multi vesicular bodies, indicative of increased phagocytosis (Rassoulzadegan, Rosen, Gillot, & Cuzin, 2000), needed to clear up endometrial cell debris during implantation. Exposure of the blastocyst to Fgf4 in culture resulted in the inhibition of phagocytosis in the mural TE, suggesting that Fgf signaling may also control functional behaviour of the TE beyond regulation of stemness. These studies highlight the heterogeneous nature of the TE, and reveal differences between polar and mural TE function.

Trophectoderm development in human embryos

Trophectoderm and inner cell mass segregation

Fertilization and preimplantation development is an accessible phase of embryonic development, making it a time frame when interventions can be made to help couples with fertility issues. Improvements have been made in human assisted reproduction techniques (ART) over the past decades, however the implantation rate of *in vitro* generated embryos still remains relatively low and is considered the main hurdle for the success of ART (Munne *et*

al., 2017, Spinella *et al*., 2017).

Overall blastocyst morphology and the events of compaction, formation of an epithelial TE layer and cavitation all resemble the morphogenetic events observed in the mouse, with the general rule that everything happens at a slightly higher cell number in human - for example compaction takes place at the 16-cell stage, cavitation around the 64-cell stage (Cockburn, & Rossant, 2010). However, examining the expression of key lineage regulators has already revealed fundamental species-specific differences. CDX2 is expressed markedly later in the human TE, only after blastocoel formation (Niakan & Eggan, 2013). *EOMES* and *ELF5* were undetectable and *TCFAP2C* was expressed also in the ICM (Blakeley *et al*., 2015). Instead, human TE identity was characterized by robust expression of *GATA 2/3, DAB2, EFNA1, PPARG, FHL2, KRT18/8 and TEAD3* (Stirparo *et al*., 2018*).* The pluripotency marker OCT4 persisted longer in the TE, and was only restricted to ICM shortly before implantation (Niakan & Eggan, 2013). A recent study used CRISPR/Cas9 editing to generate *POU5F1* (gene encoding OCT4) deficient human embryos and found that unlike in mouse where only the ICM is compromised without Oct4, in human both TE and ICM lineages were affected (Fogarty *et al*., 2017). This study exemplifies that different expression patterns reported in human lineages will likely have different functional roles as well.

Consistent with the later restriction of CDX2/OCT4 to appropriate lineages, single cell RNA-sequencing on human embryos revealed that segregation of lineage-specific profiles similarly only took place after blastocyst formation (Petropoulos *et al*., 2016). Thus there is a clear difference in timing of lineage segregation. Moreover, all three lineage profiles segregated nearly synchronously, unlike the two consecutive lineage decisions in mouse. These observations question whether the central signaling pathways identified in mouse, Hippo/Yap in TE/ICM and Fgf/ERK signaling in EPI/PE segregation, function in human. While Yap is expressed throughout human preimplantation development (Yan *et al*., 2013; Yu *et al*., 2016), it appears nuclear in both TE and ICM cells (Qin *et al*., 2012). Inhibiting Fgf signaling had no apparent effect on EPI and PE segregation (Kuijk *et al*., 2012; Roode *et al*., 2012). Further studies are clearly needed to address human-specific regulatory mechanisms of preimplantation lineage formation.

Trophectoderm maturation

Unlike in the mouse embryo, in which implantation is initiated by the mural TE, attachment and implantation of the human blastocyst occurs via the polar TE, near the ICM (Gamage, Chamley, & James, 2016). Following implantation, human TE cells begin to differentiate into primitive mononuclear cytotrophoblast (CT) and primitive multinucleated syncytiotrophoblast (ST). Based on existing images of early postimplantation human embryos (Gasser *et al*., 1975), the primitive syncytium is believed to originate from fusion of cytotrophoblast cells all over the TE in the implanted human blastocyst. Eventually syncytial cells continue to be replenished from underlying proliferative cytotrophoblast cells in a perpetual cycle of fusion and cell debris extrusion. Thus, it is clear that primitive syncytial cells in human are morphologically distinct from ExE cells of mouse, which are formed by rapid proliferation of polar TE (Fig. 7). Why distinct cellular behaviour patterns exist between these two species is a mystery. However, such divergent biology begs the question as to whether what we currently know about TE differentiation and implantation in mice also pertains to human embryos.

At the late blastocyst stage the human TE also contains two transcriptionally distinct populations of cells; presumably polar and mural TE cells (Petropoulos *et al*., 2016). Of the genes that were most differentially expressed, several have previously been associated with ST differentiation*,* and likely reflect the polar TE population. However, this study did not perform RNA sequencing on individually isolated polar and mural TE cells, thus we cannot exclude the possibility that within both of these distinct groups there are clusters of cells with high stem cell potential.

The TE of human blastocysts has also been shown to have functional properties that differ between polar and mural cells. Phagocytic activity was increased at the polar TE (Y. Li, Xu, Zhou, Zhang, & Zhuang, 2016) and corresponded with the site of attachment, with several cell adhesion and extracellular matrix proteins showing localized increase in TE expression (Aberkane *et al.*, 2018).

It is clear that the polar and mural TE cells—both human and mouse—exhibit transcriptional and functional characteristics that presage the functional aspects of their post-implantation trophoblast derivatives. In both cases, the implanting regions of the TE exhibit heightened phagocytic properties that promote embryo attachment and implantation into the uterus. From transcriptome studies it is obvious that while the murine TE exhibits increased expression of TGC genes, the human TE has increased expression of ST genes. This likely reflects difference in the biology of initial stages of adhesion (mural vs polar TE) and subsequent immediate differences in implantation (syncytialization vs. robust proliferation).

Human trophoblast stem cells

The derivation of mouse TS cells 20 years ago has allowed for further and more detailed investigation of the molecular events that underlie normal murine placental development. Establishment of a human counterpart of these cells has proven to be difficult. Early attempts to derive human TS cells using mouse protocols have been unsuccessful (Kunath *et al*., 2014). This is likely due to differences in the signaling pathways that control early TE differentiation and maintenance of the TS population in human versus mouse embryos. Indeed, *in vitro* culture of human blastocysts with FGF4/heparin and human embryonic fibroblasts failed to support derivation of TS cells (Kunath *et al*., 2014). In addition, FGFR2 has been shown to be virtually undetectable in human blastocysts (Kunath *et al*., 2014). Thus, unlike in mouse, maintenance of human TS cells is not dependent on Fgf4 signaling. Additional attempts to derive human TS cells have focused on culturing isolated primary CT cells from first-trimester placentas. CT cells are a proliferative, undifferentiated population of cells that give rise to two terminally differentiated cell types– the extravillous cytotrophoblast (EVT) and the ST (Soncin, Natale, & Parast, 2014) and have some stem-like properties. EVTs are invasive cells, often containing polyploid nuclei that can facilitate embryo implantation or remodel maternal arteries later in gestation. The ST are multinucelated cells that are in direct direct contact with the maternal blood and mediate gas, nutrient and waste exchange between the mother and fetus. These two cell types are analogous to the murine TGCs and syncytial trophoblast cells of the labyrinth, respectively. However, while CT can be isolated from early placentas, they cannot be maintained *in vitro* (L. Li & Schust, 2015*).* In light of this problem, several groups have attempted to create trophoblast cell lines using other methods, including immortalization of isolated CT cells

or differentiation of human embryonic stem cells into trophoblast (Gamage *et al.*, 2016). Presumptive trophoblast progenitor lines were established from the first-trimester chorion (Genbacev *et al.*, 2011), and from stem cells derived from pre-compaction human blastomeres (Zdravkovic *et al.*, 2015). However, all of these lines failed to resemble human trophoblast cells transcriptionally, molecularly and/or morphologically.

Due to the lack of proper guidelines for identifying trophoblast cells *in vitro*, *Lee et al.,* compiled a list of criteria used to aid the classification of primary trophoblast/CT cells (Lee *et al.*, 2016). These include the expression of trophoblast-specific proteins (GATA3, TFAP2C and KRT7), an appropriate HLA class I profile, observed hypomethylation of the ELF5 promoter, and the expression of microRNAs (miRNAs) from the chromosome 19 miRNA cluster (C19MC). Previously reported human TS-like cell lines did exhibit some feature of CT cells, however they failed to display all four required hallmarks. Recently, however, human TS cells that fall within all of these guidelines have been derived. Successful derivation of human TS cells from both blastocysts and isolated placental CT cells has been accomplished (Okae *et al.*, 2018). These cells were capable of proliferating in culture for at least 5 months as mononuclear epithelial cells, and could differentiate into EVTs and STs under specific growth conditions. The culture conditions for these TS cells were selected based on transcriptomic analysis of CT cells isolated from first-trimester placentas. The analysis revealed an overrepresentation of Wingless/Integrated and epidermal growth factor pathways, indicating that these factors may be essential for maintaining CT cells in their undifferentiated proliferative state. In addition, ROCK, TGF-β and histone deacetylase inhibitors were used to enhance attachment and boost proliferation. As expected, the signalling pathways required for maintaining human TS cells differ substantially from those that regulate mouse TS cell renewal. Considering differences in the behaviour of mouse and human TE during implantation, it is not surprising that distinct signaling pathways are used to achieve the same biological outcome.

A clinical outlook

Post-implantation development is a considerable investment from the mother's side, thus quality control of embryos before implantation may be a good way to avoid sacrificing resources for nonviable embryos. Several factors contribute to the success of implantation of human embryos, and can be used as predictors of implantation success in clinical settings A combination of hallmarks are taken into account when making a decision which embryo to transfer including the rate of development (timing of cavitation and expansion), appearance and size of the ICM, size of blastocoel and zona thickness. However, morphological appearance of TE cells alone has been used as a successful indicator of live birth after the transfer of a single human blastocyst (Ahlstrom, Westin, Reismer, Wikland, & Hardarson, 2011) and a few molecules produced by TE were explored as possible biomarkers, albeit with variable success.

TE cells are a source of crucial pregnancy-associated hormones, otherwise associated with the mature trophoblast of the placenta. β-hCG and pregnancy specific β1-glycoprotein (Jurisicova, Antenos, Kapasi, Meriano, & Casper, 1999) are used to signal the arrival of the conceptus to the mother, and are detected in culture medium of human embryos, albeit at variable levels. In addition, the mural TE also maintains patchy expression of HLA-G (De Paepe *et al.*, 2013; Jurisicova, Casper, MacLusky, Mills, & Librach, 1996), a molecule involved in the immunoprotection of invasive trophoblast against maternal natural killer cells. It is tempting to speculate that HLA-G could be marking regions with early invasive extravillous trophoblast buds, which initiate proliferation at syncytialized implantation sites. Secreted HLA-G has been shown to correlate with implantation potential, however a large study failed to find such association at all participating fertility centres (Tabiasco *et al.*, 2009). Expression of chemokine receptor type 4 (CXCR4) by the TE has been shown to contribute to implantation success of human embryos (Bao *et al.*, 2016). Although the mechanism has not been fully elucidated, bioinformatic analysis suggested that CXCR4 may activate Rho pathway to promote TE apoptosis, migration, or may affect polarity to maintain TE fate.

Furthermore, implantation-competent TE modulates endometrial receptivity by communicating with uterine cells. TE cells are a source of at least 38 miRNAs, two of which (miRNA20a and miRNA30) can be identified in spent culture medium of human blastocysts with high implantation potential, but not earlier cleavage stage embryos (Capalbo *et al.*, 2016). These miRNAs are predicted to modulate expression of genes involved in endometrial cell growth and are implicated in the process of implantation.

In addition, euploidy rates are used to eliminate those embryos that are chromosomally imbalanced. For this procedure, a few cells from the TE are biopsied by herniation at the site opposite to the ICM. The most predictive marker of euploidy even at the blastocyst stage is maternal age - higher the age, higher the proportion of aneuploid embryos (Piccolomini *et al.*, 2016). However, the biggest headache for most of the clinicians when interacting with patients is the decision whether to use mosaic embryos, containing TE cells with variable genetic rearrangements, which are not always concordant with ploidy of the ICM (J. Huang, Yan, Lu, Zhao, & Qiao, 2017; Taylor *et al.*, 2016). The degree of mosaicism in the TE is a relatively poor predictor of ongoing pregnancy outcomes (Kushnir, Darmon, Barad, & Gleicher, 2018). Whether these inconsistencies reflect technical limitations of detection or whether they speak of the biology of TE function, is currently unclear. TE cells have a forgiving nature towards their DNA content. Moreover, the TE, which later gives rise to the CT of the placenta, is not a homogenous compartment of cells. These CT progenitors acquire aneuplodies as they differentiate into a more invasive phenotype (Weier *et al.*, 2005). From studies of murine tetraploid embryos used to rescue a placental phenotype as well from human chorionic villi sampling (Grati *et al.*, 2014) it is clear that aneuploid cells can function in extraembryonic tissues without interfering with euploid embryo development. Giant cells in mouse placentas have a high nuclear DNA content (~162N) (Sher *et al.*, 2013) and specific chromosomal regions containing genes relevant to placental function are amplified in trophoblast cells (Hannibal & Baker, 2016). This unique cellular approach utilized by a small number of invasive cells serves as a mechanism used during early placentation to produce large quantities of transcripts driving hormonal production needed for maintenance of pregnancy. Recently, amplified/lost regions on several chromosomes were found in human placentas with normal babies but with placental pathologies (Leavey *et al.*, 2016). However, it is not clear when these changes start occurring in development and whether they could be detected already in the mural TE of the blastocyst. It is also possible that a population of invasive trophoblast progenitors in the TE may already exhibit signs of aneuploidy as they begin to differentiate. The question therefore

still remains as to whether or not a relatively small sample of TE can accurately represent all the different cell types of the embryo.

In addition to nuclear DNA, cell biopsies contain smaller sized (16Kb), but much more abundant mitochondrial (mt)DNA. mtDNA copy number in TE biopsies (so called Mitoscore) became the latest trendy diagnostic tool used by some clinics to determine which blastocyst is the most likely to implant. Several independent studies found an association between maternal age, ploidy, implantation potential and mtDNA content in TE biopsies. mtDNA copies become elevated with increasing maternal age in both chromosomally normal and abnormal blastocysts (Fragouli *et al*., 2015). The cause of elevated mtDNA has not been established, however it may reflect the higher energy demands of compromised embryos (Fragouli & Wells, 2015). At the present time it is unclear if similar differences occur in the ICM compartment or if the mtDNA copies are increased only in TE lineages of developmentally less competent embryos. In addition, it is also unclear if increased mtDNA reflects increased mitochondrial organellar number, premature onset of mtDNA replication, defective distribution during earlier cleavage stages or elevated endowment in oocytes from which they were conceived. However, it is clear that with TE commitment, mitochondria, which are immature and relatively quiescent during early stages of development, transition to elongating, less electron dense organelles with better defined cristae. This morphological transition is associated with higher mitochondrial activity, initiation of mtDNA replication and increased energy demands of the TE compared to the ICM (Sun & St John, 2016). Interestingly, at least in mouse not all TE cells appear equal in terms of mitochondria. TE cells further away from the ICM contain more active mitochondria, compared to the polar TE with elevated expression of nutrient transporters and ATP generating pathways (Fu *et al*., 2014; Houghton, 2006). If these changes are similarly reflected in human TE or if species differences exist due to initial contact during implantation is currently unknown.

Many questions related to TE lineage commitment and biology are known in mouse but remain unexplored in human due to ethical concerns around the use of human embryos in research. A comprehensive study of cleavage patterns and localization of molecules regulating TE commitment to transfer the knowledge from mouse to human will likely not be feasible, but key factors should be assessed. Despite differences in signaling pathways used by early TE between these two species, studies assessing impact of chromosomal mosaicism and TE mitochondrial function in mouse would help to validate biological interpretations of these outcomes in human. Understanding the processes that regulate lineage formation, with the emphasis on understanding TE development and functionality could improve implantation rates by translating this knowledge into optimized culture conditions during ART or providing guidelines for selecting the fittest embryo for implantation.

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