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# Forces shaping the blastocyst

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## Abstract

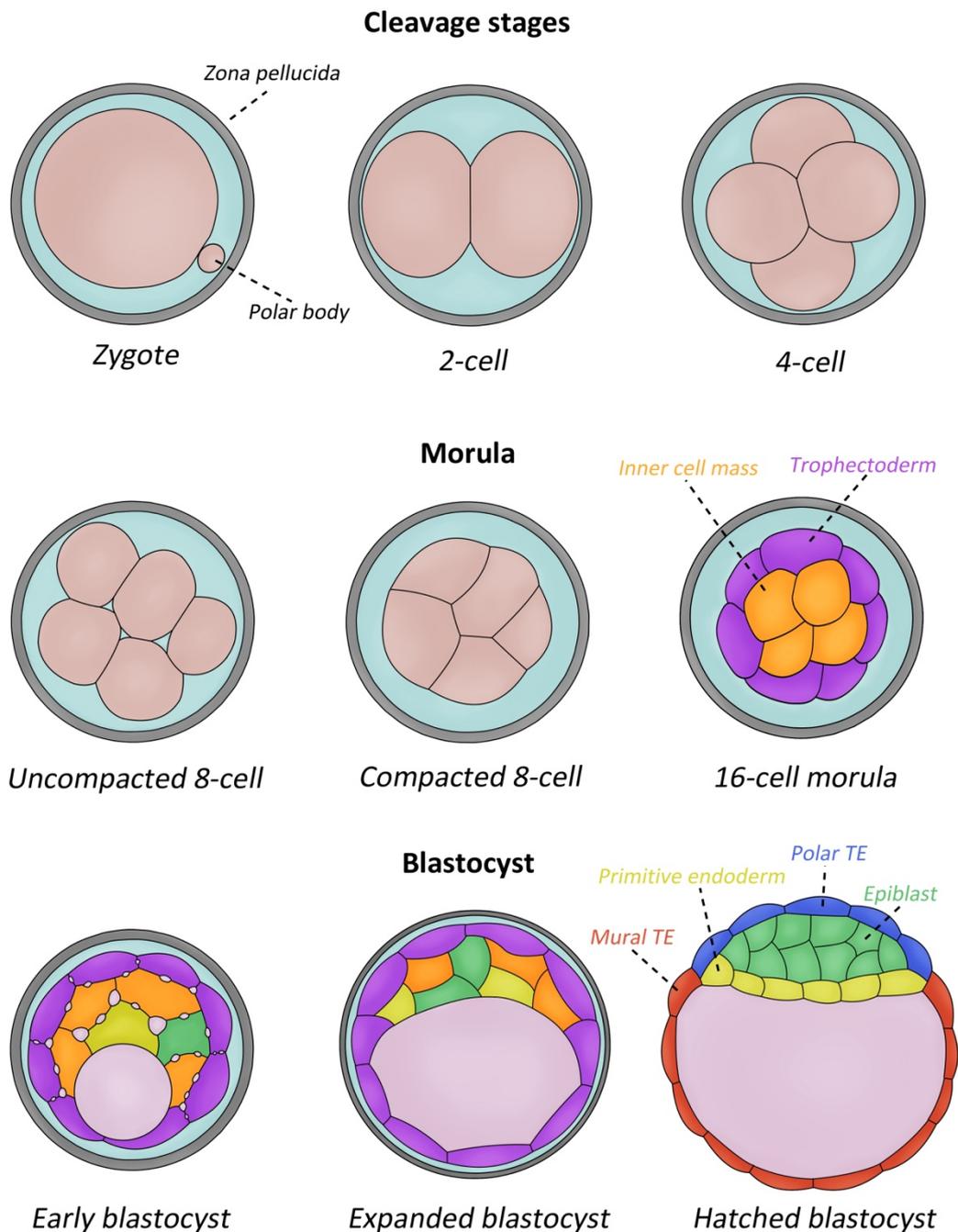
The blastocyst forms during the first days of mammalian development. The structure of the blastocyst is conserved among placental mammals and is paramount to the establishment of the first mammalian lineages. The blastocyst is composed of an extraembryonic epithelium, the trophoctoderm (TE), that envelopes a fluid-filled lumen and the inner cell mass (ICM). To shape the blastocyst, embryos transit through three stages driven by forces that have been characterized in the mouse embryo over the past decade. The morphogenetically quiescent cleavage stages mask dynamic cytoskeletal remodeling. Then, during the formation of the morula, cells pull themselves together and the strongest ones internalize. Finally, the blastocyst forms after the pressurized lumen breaks the radial symmetry of the embryo before expanding in cycles of collapses and regrowth. In this review, we delineate the force patterns sculpting the blastocyst, based on our knowledge on the mouse and, to some extent, human embryos.

## Introduction

During the first days of mammalian development, the embryo relies mostly on resources contained within the oocyte. With its limited supplies, the mammalian oocyte undergoes a transformation into a structure called the blastocyst, whose purpose is, in eutherians (also called placental mammals) and in some marsupials, to implant the embryo into the maternal uterus. The eutherian blastocyst consists of a squamous epithelium, called trophoctoderm (TE), that envelopes a fluid-filled lumen (the blastocoel) and a cluster of pluripotent stem cells named the inner cell mass (ICM, Figure 1). The TE is an extraembryonic tissue, which separates into polar and mural TEs (pTE and mTE) after the formation of the lumen, with the pTE near the ICM and the mTE lining the lumen. The TEs mediate the implantation of the blastocyst and are thought to contribute to shaping the neighboring embryonic tissues. The ICM is the origin of all embryonic tissues of the mammalian body, as well as some extraembryonic tissues. After the formation of the lumen, the ICM differentiates into epiblast (Epi) and primitive endoderm (PrE) cells, with the PrE lining the lumen and the Epi sandwiched between the TE and PrE (Figure 1). The Epi further differentiates into the definitive germ layers while the PrE contributes to extraembryonic tissues of the yolk sac. The signaling pathways and gene regulatory networks controlling the TE/ICM and PrE/Epi lineages have been dissected and discussed in several excellent reviews, while the pTE/mTE is comparably less well understood (Chazaud and Yamanaka 2016; Płusa and Piliszek 2020; Wamaitha and Niakan 2018; Posfai

et al. 2019). Studying the first lineage specifications of the mammalian embryos revealed the essential roles played by the shape of the blastocyst in their establishment and maintenance. In this review, we delineate the force patterns sculpting the preimplantation embryo, from the cleavage stages to the blastocyst stage. The forces shaping the blastocyst are generated by the cytoskeleton, as well as luminal fluid. To resist and transmit these mechanical forces, cells rely on adherens and tight junctions. Both the force generators and force transmitters are patterned by apicobasal polarity, which is instrumental to organize most preimplantation morphogenesis. This review will describe our current understanding of the shape changes in the eutherian blastocyst, a phenomenon mostly informed by research on mouse embryos. For a review on marsupial and monotreme blastocysts, see the excellent work from Frankenberg and colleagues (Frankenberg et al. 2016).

To form the eutherian blastocyst, three distinct phases were initially described during preimplantation development: cleavage stages, the formation of the morula and finally of the blastocyst (Figure 1). During cleavage stages, there is no shape change of the embryo besides the increase in cell number by cleavage divisions, i.e. without cell growth during the interphase. Unlike the cleavage stage embryo, the morula appears as a round ball of cells. To form the morula, the embryo undergoes compaction, apicobasal polarization and initiates TE/ICM differentiation by positioning cells internally while others remain at the embryo surface. The



**Figure 1: Preimplantation development**

During preimplantation development, the zygote, encapsulated in its zona pellucida (ZP, grey), undergoes cleavage divisions without any shape change before the 8-cell stage. The morula begins to form at the 8-cell stage with compaction, bringing cells in close contact and forcing out any extracellular fluid (light blue) between cells. At the 16-cell stage, the compacted morula has inner (orange) and outer (purple) cells, which begin to differentiate into inner cell mass (ICM) and trophectoderm (TE) fates, respectively. The blastocyst begins to form at the 32-cell stage with the formation of a fluid-filled lumen (lilac). The lumen physically separates some of the TE cells from some of the ICM cells: the TE cells remaining in contact with the ICM initiate their differentiation into polar TE (pTE, dark blue), whereas those in contact with the lumen become mural TE (mTE, red); meanwhile, the ICM progressively differentiates into primitive endoderm (PrE, yellow) and epiblast cells (Epi, green), which eventually segregate in space after maturing their fate (orange cells) and repositioning themselves.

blastocyst features a large fluid-filled lumen, which occupies most of its volume. To form the blastocyst, surface cells become epithelial by further maturing their apicobasal polarization with the sealing of tight junctions (TJs), along with initiation of polarized transport leading to the accumulation of luminal fluid. During the maturation of the blastocyst, the lumen continues expanding as the second lineage segregations occur within the PrE/Epi and pTE/mTE. Remarkably, the formation of the blastocyst breaks the radial symmetry of the morula with the ICM and lumen establishing the first axis of symmetry of the mammalian embryo. The side of the blastocyst containing the ICM is referred to as the embryonic pole, and the opposite side as the abembryonic pole.

As the preimplantation embryo undergoes blastocyst morphogenesis, its cells continue their initially rhythmic cleavage divisions. In the mouse, key steps take place within a specific cellular stage: with the 8-cell stage dedicated to compaction and apicobasal polarization; the 16-cell stage experiences the first wave of cell internalization and the sealing of TJs; the 32-cell stage is devoted the final wave of cell internalization resulting in TE/ICM differentiation and to lumen opening and positioning; the 64-cell stage dedicated to lumen expansion and the priming of Epi/PrE progenitors; the 128-cell stage committing Epi/PrE, and presumably pTE/mTE, positions and lineages. This view is simplified since, in fact, cell divisions become more and more asynchronous with time: at the 2-cell stage, sister cells divide within ~1 h from one another and within ~3 h at the 8-cell stage. Therefore, morulae are only briefly composed of precisely 8 or 16 cells but rather of a mixture of 8- and 16-cell stage blastomeres. This implies that while some 8-cell stage blastomeres are still maturing their apicobasal polarity, other blastomeres of the same embryo are already at the 16-cell stage and can be positioned inside the morula to initiate their ICM differentiation. Although the sequence of morphogenetic steps described above seems conserved among eutherian mammals, cell cycle durations can vary between species and this fluctuation can be uncoupled from the timing of morphogenesis. For example, compaction takes longer in human or cow embryos than in mouse and typically continues during the 16-cell stage (Gerri et al. 2020). Therefore, the synchrony between cleavage divisions and blastocyst morphogenesis can be complex.

Importantly, cleavage divisions and the increase in cell number are not strictly essential for blastocyst morphogenesis (Tarkowski 1959). Physically removing cells from the embryo does

not stop blastocyst formation. For example, single blastomeres isolated as late as at the 8-cell stage can continue cleavage divisions as normal and form a mini-blastocyst, composed of a small lumen and inner and outer cells (Tarkowski and Wróblewska 1967) expressing markers of ICM and TE differentiation, respectively (Dietrich and Hiiragi 2007). This is not exclusive to physical removal of cells, as multiple rounds of cytokinesis can fail and blastocyst formation still occur in a timely fashion (Schliffka et al. 2021). In one of the most extreme examples, in which all successive cytokinesis fail, the embryo will reach the blastocyst stage with a single blastomere. This single-celled embryo will have succeeded in acquiring some form of apicobasal polarity, differentiation into TE and even some aspect of lumen formation (Schliffka et al. 2021). Therefore, blastocyst morphogenesis is running on a developmental clock that is to some extent synchronized but not firmly coupled with the rhythms of cleavage divisions. This spectacular adaptive capability of the preimplantation embryo is possible thanks to the morphogenetic program that relies on cell autonomous processes including, cell polarization and luminal fluid accumulation, as we will see in greater details in the following sections.

### **Cleavage stages: the mechanical maternal-zygotic transition**

From the zygote to the 8-cell stage, the shape of the embryo is only affected by cleavage divisions, with the exception of a transient deformation during the 2-cell stage by the zona pellucida (ZP), an elastic glycoprotein shell surrounding embryo that gently squeezes cells together. Although mammalian morphogenesis has not started yet, internal remodeling of the actin and microtubule cytoskeletons lead to mechanical changes of the cells without affecting the shape of the cells and embryo.

Immediately after fertilization, the paternal genome enters the cytoplasm of the oocyte, which completes its second meiotic division expelling half of the maternal genome into the polar body (Mori et al. 2021). Polar body extrusion is mediated by a contractile cytokinetic ring assembled at the periphery of an actin cap to encapsulate the expelled maternal genome (Clift and Schuh 2013). To coordinate the formation and contraction of the actin cap, molecular signals emanate from both chromatin and the central-spindle complex, located at the periphery of the oocyte (Dehapiot et al. 2021). Chromatin triggers actin polymerization in a distance dependent manner (~20  $\mu\text{m}$ ) via Ran and Cdc42 small GTPases (Deng et al. 2007). Paternal

chromosomes can also trigger the formation of actin caps when in close proximity to the cell cortex but interestingly, these events do not lead to extrusion into polar bodies (Mori et al. 2021). This is because actin caps induced by paternal chromosomes do not form a contractile ring, which is organized by signals from the central-spindle complex involving the small GTPase Rho (Dehapiot et al. 2021). Interestingly, interfering with the anchoring of the spindle or with Rho effectors can lead to the formation of polar body-like structures during cleavage divisions of mouse embryos (Pelzer et al. 2023). These structures resemble cellular fragments formed by human and macaque embryos, which sometimes contain chromosomes and negatively affect the outcome of assisted reproductive technologies (ART) procedures (Daughtry et al. 2019; Alikani et al. 2005; Alikani 1999). In cleavage stage mouse embryos, chromatin induces the activation of Cdc42 and the formation of an actin cap when it comes within 20  $\mu\text{m}$ , indicating that the same signals used during meiosis remain active long after meiosis is complete (Pelzer et al. 2023). In somatic cells, signals from chromatin typically relax the cortex instead of stimulating it (Rodrigues et al. 2015; Sedzinski et al. 2011), yet actin cap formation near the chromatin can still be observed during the 8- to 16-cell stage division (Pomp et al. 2022; Pelzer et al. 2023). However, it is not yet clear at what point in preimplantation development this meiosis-like control of the actomyosin cortex stops.

During cleavage stage mitoses, the microtubule cytoskeleton also shows peculiar behaviors. In mouse embryos, there is no centrosome and the mitotic spindle assembles from multiple microtubule organizing centers (MTOCs), as observed during meiosis (Courtois et al. 2012), before forming a functional barrel-shaped mitotic spindle. During the first division, the local organization of MTs around parental pronuclei can lead to the formation of overlapping “maternal and paternal spindles” that maintain the parental chromosomes in distinct regions of the mitotic plate (Reichmann et al. 2018). The progressive de novo synthesis of centrosomes allows the spindle to gradually transition from a meiosis-like construction to a classical mitotic assembly by the 8- to 16-cell stage (Courtois et al. 2012). The asynchrony in centrosome assembly among blastomeres could therefore affect how the 8- to 16- and 16- to 32-cell stage divisions take place, with potentially important consequences on TE/ICM cell allocations. How relevant this behavior is to other mammals remains unclear. Indeed, in human and cow embryos, the sperm brings the centrosome and, during the first

mitosis, centrosomes position themselves around the parental pronuclei before nuclear envelope breakdown (NEBD) (Cavazza et al. 2021). This helps clustering the centromeres of parental chromosomes near one another and speeds up chromosome clustering upon NEBD. Chromosome mis-segregation is more frequent when pre-NEBD clustering does not occur and is particularly prominent in human embryos, leading to the formation of micronuclei and aneuploidy (Cavazza et al. 2021; Currie et al. 2022).

The egg-like behavior of the cytoskeleton of cleavage stage embryos is not restricted to mitosis but also affects the interphase. Oocytes are cells with high surface tensions compared to typically softer, embryonic cells (Krieg et al. 2008; Godard et al. 2020; Bischof et al. 2017; Yamamoto et al. 2023). Mouse germinal vesicle-intact and germinal vesicle breakdown stage oocytes have tensions of several  $\text{nN}/\mu\text{m}$  for oocytes in metaphase I and II, whereas 8-cell stage blastomeres at the onset of morphogenesis are much softer at  $0.2 \text{ nN}/\mu\text{m}$  (Larson et al. 2010; Chaigne et al. 2013; Maître et al. 2015). This embryonic softening is gradual over the zygote to 8-cell stages and is associated with noticeable changes in the contractile behaviors of cells (Özgüç et al. 2022). On the timescale of tens of seconds, preimplantation blastomeres show rhythmic deformations, similar to those initially described in fly embryos and as observed in a variety of developing animals (Solon et al. 2009; Martin 2010; Kim and Davidson 2011; Roh-Johnson et al. 2012; Bement et al. 2015; Maître et al. 2015). The softening of cells during cleavage stages is associated with a gradual decrease in the period of contractions from  $\sim 150$  to  $80 \text{ s}$  (Özgüç et al. 2022). Periodic contractions also increase in amplitude, can be detected in most mouse embryos by the 4-cell stage, and are likely the result of cortical remodeling by actin regulators rather than simply the gradual reduction in cell size. The microtubule cytoskeleton also adopts an unusual organization during the interphase due to the absence of centrosomes. Most of the microtubule network is poorly organized due to the absence of a strong microtubule organizing center (MTOC). Weak MTOCs can be found in undefined locations within the cytoplasm but stronger MTOCs consistently originate from cytokinetic bridges that persist after incomplete abscissions (Zenker et al. 2017; Courtois et al. 2012).

Taken together, these recent findings reveal that during cleavage stages, cytoskeletal remodeling leads to a mechanical maternal-zygotic transition (MZT) at the onset of blastocyst morphogenesis. In the mouse, zygotic gene expression begins as

early as the late zygote stage with the minor zygotic genome activation (ZGA) and grows stronger at the 2-cell stage with the major ZGA (Abe et al. 2018). Blocking gene expression during the ZGA strongly affects the initiation of compaction, suggesting that zygotic gene expression may be needed to prepare the embryonic cytoskeleton and mechanics for blastocyst morphogenesis (Levy et al. 1986; Kidder and McLachlin 1985). In other mammals such as humans or cows, ZGA occurs at the 8-cell stage (Zou et al. 2022) and, based on biochemical and biophysical studies of human embryos, cytoskeletal defects are suspected to be responsible for arrested or abnormal development (Firmin et al. 2022; Wang et al. 2024). Since relatively little is known of the cytoskeletal and mechanical changes during the cleavage stages of mammalian species other than the mouse, it remains unclear how much ZGA timing would influence a potential mechanical MZT.

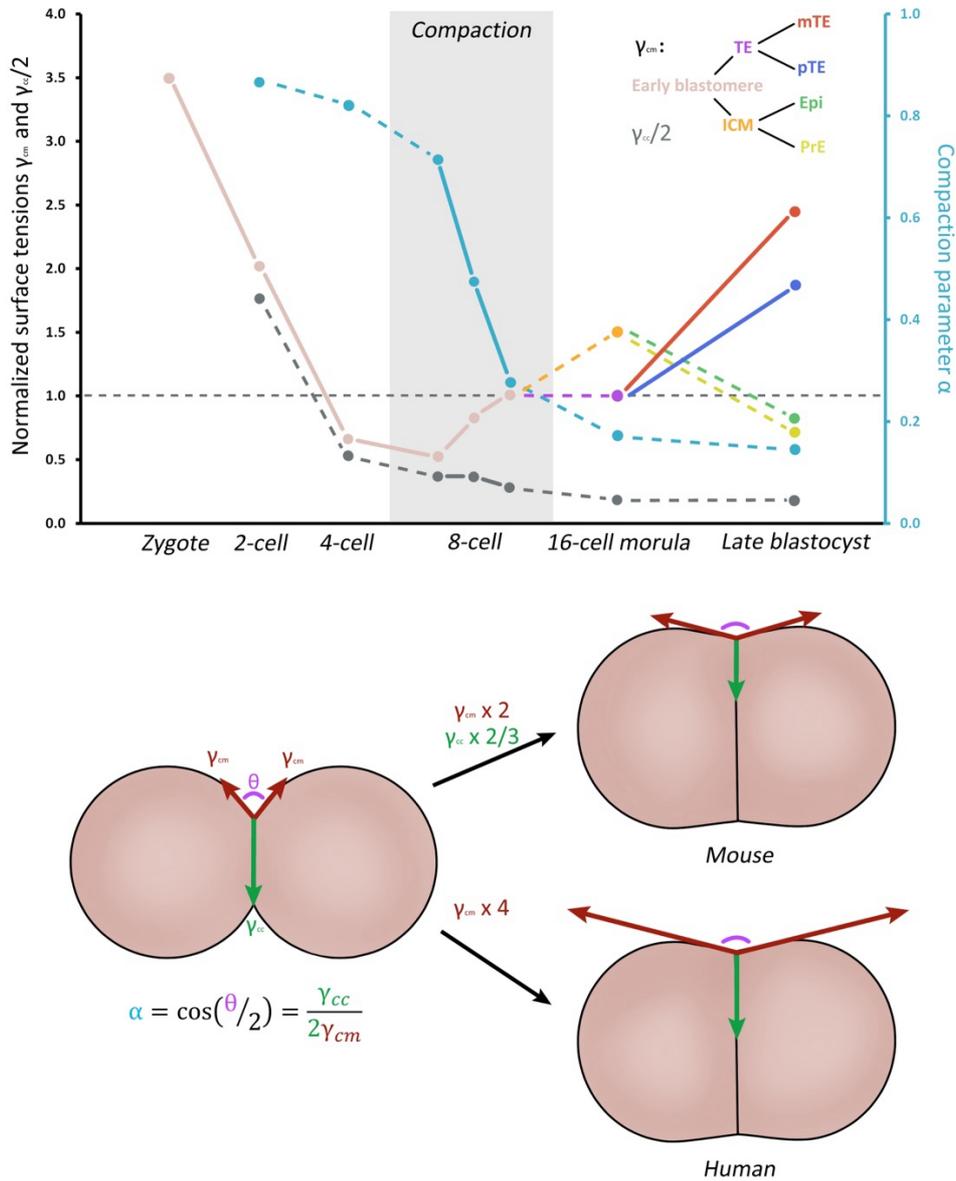
### **Morula: from a cluster of individual cells to an embryo**

At the beginning of the 8-cell stage, cells do not have any particular spatial arrangement, are almost completely spherical, and can contribute to all tissues of the mammalian embryo. After the formation of the morula, cells are polarized, squashed together and the developmental potential of individual cells is reduced, with some of them being committed to extraembryonic tissues. The formation of the morula is the critical time when cells cease to be individual “totipotent” cells and start to become part of an embryo.

### **Compaction, the first morphogenetic movement, what is it for?**

Cleavage stage embryos have small cell-cell contacts. During compaction, blastomeres come closer to one another and spread their contacts until the embryo forms a compact mass (Figure 1). Since their discovery in the compacting mouse embryo (Hyafil et al. 1980), it is well established that, to hold onto one another, preimplantation blastomeres rely on cadherin-mediated adhesion (Larue et al. 1994; Stephenson et al. 2010). This led to the proposal that compaction would be mediated by adhesion molecules zipping up cell-cell contacts, a theory still prominent in the biomedical field (Coticchio et al. 2019). Although cadherin adhesion molecules are essential to mechanically couple cells and to signal the cytoskeleton (Maître and Heisenberg 2013), adhesion molecules may not be able to provide

much energy to reshape contacts compared to other morphogenetic engines (Maitre et al. 2012; Stirbat et al. 2013; Chan et al. 2017). Instead, cell-cell contact rearrangements typically rely on actomyosin contractility, which pulls at the surface of cells and onto the edges of cell-cell contacts (Lecuit and Yap 2015). Blocking actomyosin, using inhibitors such as blebbistatin or ML7, or by knocking out non-muscle myosin IIA, has been shown to prevent compaction (Maître et al. 2015, 2016; Zhu et al. 2017; Schliffka et al. 2021). Thus, both contractility and adhesion need to be functional for compaction. To increase cell-cell contact size, cells can pull stronger onto the contact (by contracting their cell-medium interface) and/or relax their cell-cell contacts (by contracting them less or by providing more adhesion energy) (Maître and Heisenberg 2011; Winklbaauer 2015). This physical phenomenon is formally captured by the Young-Dupré equation (Figure 2) and thus one can determine the relative contributions of contractility and adhesion to compaction by measuring the surface tensions at the cell-medium and cell-cell interfaces (Turlier and Maître 2015). The tension at the surface of cells increases by a factor 2 in mouse embryos and by a factor 4 in human embryos throughout compaction in a contractility-dependent and adhesion-independent manner (Maître et al. 2015; Firmin et al. 2022). In mouse embryos, increased contractility is associated with prominent periodic contractions that propagate around the cell surface, termed periodic contractile waves of contraction (PeCoWaCo) (Maître et al. 2015; Özgüç et al. 2022). As an alternative to contractility, it was proposed that surface protrusions could increase tension at the cell-medium interface in non-cell autonomous and Myo10-dependent manner (Fierro-González et al. 2013). This is ruled out by the facts that the surface tension increase is cell-autonomous and that Myo10 mutants do not seem to have compaction problems (Maître et al. 2015; Crozet et al. 2023). Concerning tensions at cell-cell contacts, they do not change in human embryos and decrease by one-third in mouse embryos, playing at most a minor contribution to compaction (Maître et al. 2015; Firmin et al. 2022). Therefore, compaction for both mouse and human embryos results from an increase in contractility at the cell-medium interface. Importantly, high tensions at the cell-medium interface are ineffective if tensions are also high at cell-cell contacts (Figure 2), as inferred from tension measurements during cleavage stages (Özgüç et al. 2022).



**Figure 2: Cell surface tensions throughout mouse preimplantation development and comparative analyzes of mouse and human embryo compaction**

Taking measurements from multiple studies (Özgüç et al. 2022; Maître et al. 2015, 2016; Chan et al. 2019; Yanagida et al. 2022), the dynamic changes in cell surface tensions can be plotted over the entirety of preimplantation development. All values are normalized to the surface tension at the cell-medium interface  $\gamma_{cm}$  of compacted 8-cell stage embryos. Solid lines show measurements directly taken from the studies while dashed lines indicate that values were inferred from the provided measurements. Values of surface tensions at the cell-medium interface are plotted in pink from the zygote to the 8-cell stage (Özgüç et al. 2022; Maître et al. 2015), in orange for the ICM and in purple for the TE (Maître et al. 2016), in red for the mTE and in dark blue for the pTE (Chan et al. 2019), in green for the Epi and in yellow for the PrE (Yanagida et al. 2022). Values of surface tensions at the cell-cell interface are plotted in grey. The right-hand side axis indicates the scale for the compaction parameter (light blue), which describes the shape of the contact and corresponds to the ratio of surface tensions at the cell-cell contact and at the cell-medium interface.

Schematics of the compaction of a cell doublet based on the surface tensions measured in mouse and human embryos (Maître et al. 2015; Firmin et al. 2022). The shape of a cell doublet is characterized by its external contact angle  $\theta$ , which results from the balance of the tensions at the cell-medium  $\gamma_{cm}$  (red) and cell-cell interfaces  $\gamma_{cc}$  (green) (Turlier and Maître 2015). This is formally characterized by a simplified form of the Young-Dupré equation where the compaction parameter  $\alpha = \cos(\theta/2) = \gamma_{cc}/2\gamma_{cm}$ . Both mouse and human embryos compact by reducing their compaction parameter from  $\sim 0.7$  to 0.25. While this results from increasing  $\gamma_{cm}$  by a factor 4 without changing  $\gamma_{cc}$  in human embryos, mouse embryos compact by doubling  $\gamma_{cm}$  and by reducing  $\gamma_{cc}$  by 1/3.

During cleavage stages, the actomyosin cortex is rather homogeneous around the cells and becomes preferentially localized at the cell-medium interface in mouse and human embryos as compaction progresses (Maître et al. 2015; Firmin et al. 2022; Zhu et al. 2017). Actomyosin reduction at cell-cell contacts depends on cadherin adhesion molecules, which remodel the cytoskeleton locally via small GTPases (Yamada and Nelson 2007; Wildenberg et al. 2006). However, the specific molecules that control the increase in contractility and the signals from cadherins that occur specifically at the 8-cell stage, remain unknown. It has previously been hypothesized that this could involve phospholipid signaling, since interfering with protein kinase C and phospholipases can induce transient premature compaction at the 4-cell stage (Winkel et al. 1990; Zhu et al. 2017).

Finally, although compaction defects are associated with poor outcome in human embryos (Mizobe et al. 2017; Lagalla et al. 2017), the necessity of compaction is unclear. Indeed, mutants lacking maternal adhesion molecules or non-muscle myosin II fail to compact but form viable blastocysts (Stephenson et al. 2010; Maître et al. 2016; Dumortier et al. 2019; Schliffka et al. 2021, 2023). Therefore, while compaction constitutes the first sign that blastomeres are part of a single embryo, its function is nebulous.

### **Formation of the apical domain, master organizer of preimplantation development**

As compaction proceeds a parallel event occurs: the establishment of bona fide apicobasal polarity. From the 2-cell stage, cells have cell-cell contacts where the cell-cell adhesion molecule Cdh1 shows some enrichment. While cell-cell contacts may constitute a basolateral interface, it is only at the 8-cell stage that cells form an actual apical interface. Initially, the apical membrane does not occupy the entire cell-cell contact-free interface and instead concentrates into a small cap or domain (Figure 3). The formation of the apical domain is critical for continued development as it provides instructions for cells inner and outer positioning (Korotkevich et al. 2017; Maître et al. 2016), ensuing ICM and TE differentiation (Hirate et al. 2013), and for polarized transport during lumen formation (Dumortier et al. 2019). Therefore, the apical domain constitutes a master organizer of preimplantation development.

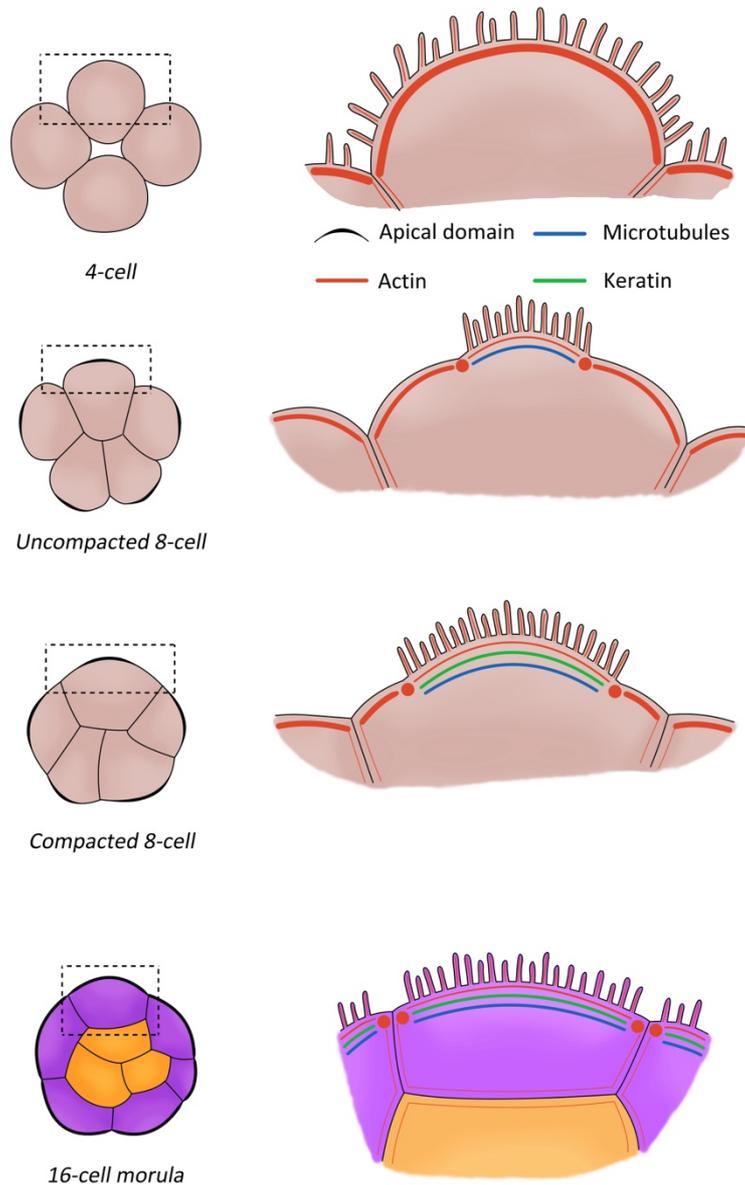
Like for other epithelia, apicobasal polarity of the mouse preimplantation embryo relies on the Par complex to determine the molecular specificity of the contact and non-contact interfaces (Campanale et al. 2017). The mouse Par

homologs, Pard3 and Pard6b, are scaffolding proteins that localize to the apical domain at the onset of polarization and are required for the apical recruitment of atypical protein kinase C (aPKC) (Plusa et al. 2005; Vinot et al. 2005; Alarcon 2010). Concomitant to the recruitment of apical determinants, previously existing structures start to be reorganized (Figure 3). For example, the surface of the embryo is covered in microvilli throughout cleavage stages (Ducibella et al. 1977). During apical domain formation, microvilli concentrate at the apical pole of the blastomere, which is how the apical domain was initially identified (Ducibella et al. 1977; Ziomek and Johnson 1980; Johnson and Ziomek 1981b). The role of microvilli in the mouse embryo has not been explored, but they could act as membrane reservoirs to facilitate growth as observed in other systems (Figard et al. 2013, 2016; LaFoya and Prehoda 2023). Formation and maintenance of microvilli depends on the action of Ezrin-Radexin-Moesin (ERM) proteins, an actin-plasma membrane linker (Sauvanet et al. 2015). ERM proteins must be phosphorylated and bound to phosphatidylinositol biphosphate (PIP<sub>2</sub>) to relieve their autoinhibitory folding (Sauvanet et al. 2015). During the 8-cell stage, PIP<sub>3</sub> becomes enriched at basolateral interfaces (Halet et al. 2008), suggesting that PIP<sub>2</sub> could localize at the apical domain and promote ezrin recruitment. Ezrin is phosphorylated by aPKC and its activation has been linked to microvilli clustering (Louvet et al. 1996; Dard et al. 2004; Liu et al. 2013). Although ezrin is not part of the apical machinery, its role in structuring the membrane of the apical domain makes it the focus of several studies on apical domain formation and maturation.

The precise mechanisms responsible for organization of the apical domain have yet to be elucidated. Apicobasal polarization has been shown to depend on feedback between proteins specifically enriched at cell-cell junctions and the nascent apical domain in other model systems (Morais-de-SA et al. 2010; Klompstra et al. 2015; Campanale et al. 2017). In mouse preimplantation embryos, cell-cell contacts are dispensable for de novo polarization. Isolated 8-cell stage blastomeres are capable of polarization, albeit with a delay of ~3 hours compared to doublets (Ziomek and Johnson 1980). This delay may suggest that cell-cell contacts expedite polarization, however, placing the cell in contact with a bead is enough to trigger polarization at a similar speed to doublets (Ziomek and Johnson 1980; Korotkevich et al. 2017). Importantly, the molecular specificity of the adhesive contact is not important since beads coated with or without Cdh1 have the same effect,

which is also supported by the fact that *Cdh1* mutants polarize normally (Korotkevich et al. 2017; Stephenson et al. 2010). Further, as observed in doublets and more complicated cell assemblies (Ziomek and Johnson 1980; Johnson and Ziomek 1981a), physical contact with a bead causes the apical domain to form opposite to the contact, indicating that physical contact is enough to bias the axis of polarization (Korotkevich et al.

2017). The ability of isolated 8-cell stage blastomeres to polarize implies that formation of the apical domain is under tight temporal control. This has recently been suggested to depend on a wave of transcription at the early 8-cell stage, as inhibition of transcription at this stage delayed polarization (Zhu et al. 2020). This model would suggest that the machinery required to form the apical domain is newly synthesized upon



**Figure 3: Surface rearrangement during apical domain formation and expansion**

During the formation of the morula, surface cells reorganize their cell-medium interface into an apical surface, as found in epithelia (Campanale et al. 2017). Left shows, from top to bottom, a 4-cell stage embryo, an uncompact 8-cell stage embryo, a compact 8-cell stage embryo and a 16-cell stage morula with inner (orange) and outer (purple) cells. On the right, the cell surface shows surface protrusions, called microvilli, that become clustered into the apical domain (Ducibella et al. 1977), harboring a distinct curvature and progressively expands (Korotkevich et al. 2017; Zenker et al. 2018). Cytoskeletal elements associated with the cell surface are shown with the actin filaments in red (Maître et al. 2016), microtubules in blue (Zenker et al. 2018) and keratin filaments in green (Lim et al. 2020). Line thickness indicating how and where these elements are enriched. The cytoskeletal elements present in the cytoplasm are not depicted.

reaching the 8-cell stage. This comes in contradiction with previous observations showing that inhibition of translation at the early 4-cell stage leads to premature compaction and polarization as early as the late 4-cell stage, thereby suggesting that compaction and polarization need the removal from an inhibitor rather than de novo synthesis (Levy et al. 1986; Kidder and McLachlin 1985).

The formation of the apical domain is concomitant with compaction and the two processes are often presented as being coupled. Since actomyosin drives compaction of mouse and human embryos (Maître et al. 2015; Firmin et al. 2022) and apicobasal polarization relies heavily on actomyosin in other settings such as the *c. elegans* zygote (Munro et al. 2004), it was proposed that actomyosin may also be important for apical domain formation in the mouse. This was concluded after contradicting results with treatments of embryos with blebbistatin and ML7, which, respectively, did and did not form apical domains (Zhu et al. 2017). Clarification came from mutant embryos lacking both homologues of non-muscle myosin II, that were able to polarize despite chronically defective contractility (Schliffka et al. 2021). Thus, demonstrating that actomyosin contractility is not required for apicobasal polarization of preimplantation embryos. On the other hand, it has been previously shown that apicobasal polarity does in fact control actomyosin activity (Blankenship et al. 2006; Padmanabhan and Zaidel-Bar 2017; Campanale et al. 2017). In the mouse embryo, this is clearly visible by the decreased periodic contractions at the apical domain (Maître et al. 2016). This could result from the reduced levels of actomyosin at the apical domain as a consequence of aPKC signaling. Additionally, Cdh1-mediated signaling reduces actomyosin levels at cell-cell contacts in mice embryos (Maître et al. 2015) and in other systems (Wildenberg et al. 2006; Yamada and Nelson 2007; David et al. 2014; Padmanabhan and Zaidel-Bar 2017; Chan et al. 2017). Importantly, the reduced actomyosin levels at cell-cell contacts are independent of the formation of the apical domain, since actomyosin is also reduced at cell-cell contacts of double knockout of zeta and iota aPKC paralogs (Maître et al. 2016), or after knockdown of Pard6b (Alarcon 2010). Inversely, blocking cell adhesion through incubation in Ca<sup>2+</sup>-free media or knockout of Cdh1 prevents compaction and homogenizes actin across each blastomere without preventing the formation of the apical domain (Stephenson et al. 2010; Anani et al. 2014). Therefore, while concomitant and partially relying on common

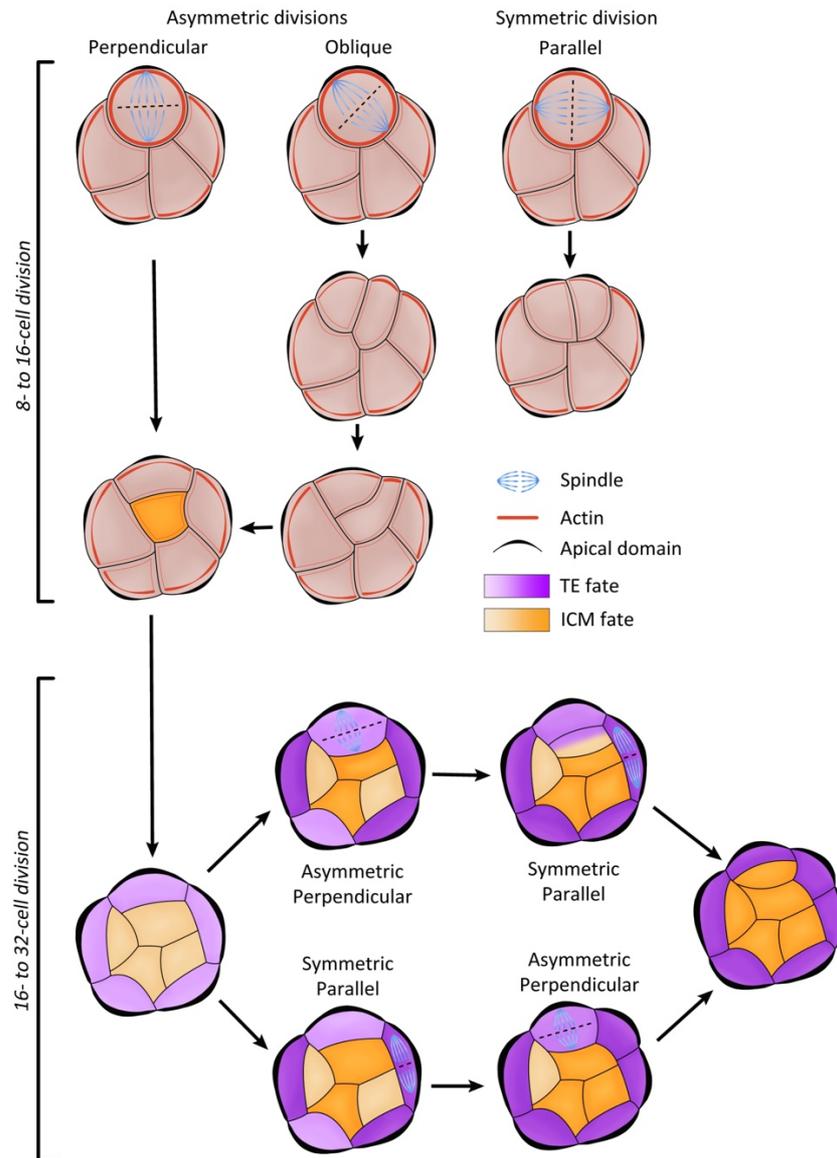
molecular components, compaction and apicobasal polarization can occur independently from one another.

While what initiates apical domain formation remains unclear, its maturation is better understood. Upon formation of the apical domain, the cytoskeleton is extensively remodeled. Both microtubules (Zenker et al. 2018; Houliston et al. 1987) and keratin intermediate filaments (Lim et al. 2020; Chisholm and Houliston 1987; Schwarz et al. 2015) are enriched at the apical domain (Figure 3). On the other hand, actomyosin is excluded from the apical pole due to the action of aPKC and microtubules (Maître et al. 2016; Zenker et al. 2018). Instead, actin forms a ring around the recruited apical components (Anani et al. 2014; Korotkevich et al. 2017; Zenker et al. 2018). The ring expands throughout the 8- and 16-cell stages until it eventually reaches cell-cell junctions where it is stabilized by adherens and tight junction proteins, at which point the apical domain occupies the entirety of the contact free surface (Korotkevich et al. 2017; Zenker et al. 2018). Apical cytoskeletal remodeling is sometimes associated with the apical domain giving the appearance of 'bulging out' from the cell surface (Ducibella et al. 1977), where the adoption of this distinct curvature could result from the line tension of the actin ring. However, laser ablation experiments suggest that the ring may not be contractile (Zenker et al. 2018). Therefore, apical domain curvature is most likely the result of a lower surface tension from the non-apical part of the contact-free surface, which contains more actomyosin (Maître et al. 2016) and could drive apical domain expansion.

### **Inner-outer cell positioning, a movement of a lifetime**

The presence of the apical domain at the 8-cell stage introduces the possibility that the following cell divisions may be asymmetric, with one daughter cell inheriting more apical material than the other. In some cases, one daughter cell inherits all of the apical material and its sister cell receives none. These cells are often referred to as polar and apolar, respectively, while noting that the apolar cell conserves some polarity provided by its cell-cell contacts and contact-free interfaces.

Early observations found that 80% of isolated 8-cell blastomeres would divide asymmetrically (Johnson and Ziomek 1981b), with similar rates later being observed in embryos (Anani et al. 2014; Watanabe et al. 2014; Korotkevich et al. 2017). Further, following asymmetric divisions, which tend to occur perpendicular to the embryo surface (Figure 4), the polar cell could remain at



**Figure 4: Cell inner and outer positioning from the 8- to the 32-cell stage**

Schematics of cellular events leading to the formation of the ICM (orange) and TE (purple). 8-cell stage blastomeres divide with 3 distinct outcomes: left, the mitotic spindle (light blue) is perpendicular to the embryo surface, which asymmetrically divides the apical domain (black bump) among sister cells and pushes the apolar cell inside the embryo; middle, the mitotic spindle is obliquely oriented relative to the embryo surface, which asymmetrically divides the apical domain among sister cells and leaves both the polar and apolar cell at the surface; right, the mitotic spindle is parallel to the embryo surface, which symmetrically divides the apical domain among sister cells and leaves two polar cells at the surface. In the case of oblique asymmetric divisions, the apolar cell displays a stronger actomyosin cortex (red) at its cell-medium interface than neighboring polar cells and are subsequently internalized. Thus, at the 16-cell stage, asymmetric divisions lead to cell internalization as a result of division orientation relative to the surface of the embryo (Dard et al. 2009; Watanabe et al. 2014; Korotkevich et al. 2017) or as result of contractility-mediated cell sorting (Maître et al. 2016). 16-cell stage surface blastomeres divide with 2 distinct outcomes: top, the mitotic spindle, oriented by the apical domain, is perpendicular to the embryo surface, which asymmetrically divides the apical domain among sister cells and pushes the apolar cell inside the embryo; bottom, the mitotic spindle, oriented by the longest axis of the cell, is parallel to the embryo surface, which symmetrically divides the apical domain among sister cells and leaves two polar cells at the surface (Niwayama et al. 2019). Competition between both modes of orientation ensures a consistent number of inner cells: consecutive symmetric divisions will crowd the outer layer leading to a less elongated morphology, allowing the next cell division to be oriented by the apical domain and thus produce an outer an inner cell.

the surface while the apolar cell becomes internalized as a direct result of the division orientation. Alternatively, symmetric divisions tend to produce two outer cells due to their orientation parallel to the embryo surface. Hence, symmetric and asymmetric divisions tend to have distinct orientations relative to the surface of the embryo and this mostly explains how cells internalize during the 8- to 16- and 16- to 32-cell stage divisions.

Though this could suffice to explain how all cells adopt inner and outer positions, it was noted that during the 8- to 16-cell stage transition some asymmetric divisions leave some apolar cells at the surface of the embryo, which subsequently internalize (Anani et al. 2014; Watanabe et al. 2014; Samarage et al. 2015). Interestingly, this also occurs in isolated 8-cell stage blastomeres after their division to 16-cell stage doublets as apolar cells are engulfed by polar cells (Johnson and Ziomek 1983; Korotkevich et al. 2017; Maître et al. 2016). Differences in contractility can drive engulfment of a tenser tissue by a less tensed one (Krieg et al. 2008) and similar engulfment can result at the level of a cell doublet by a process called entosis (Overholtzer et al. 2007). It was experimentally validated that symmetric divisions produce cells with similar surface tension whereas asymmetric divisions generate polar cells with reduced tension compared to apolar ones (Maître et al. 2016). This results from the asymmetric inheritance of aPKC that negatively regulates actomyosin activity and is clearly visible with apolar cells showing prominent periodic contractions while polar cells do not. Importantly, the process of cell internalization is distinct from apical constriction, as proposed initially (Samarage et al. 2015), since the cells which internalize do not have an apical domain and the surrounding cells expand rather than constrict their apical domain (Zenker et al. 2018). Instead, differences in contractility between polar and apolar cells results in their sorting (Krieg et al. 2008; Maître et al. 2012), as shown by mosaic embryos made of WT and non-muscle myosin II KO cells in which WT cells sort in to form the ICM (Maître et al. 2016; Dumortier et al. 2019). Most inner cells internalize during the 8- to 16- and 16- to 32-cell stage divisions as a result of oriented cell divisions, and while cell sorting may only have a minor contribution to the allocation of inner cells, this backup mechanism ensures the correct positioning of apolar cells. Therefore, cell positioning and cell fates are coupled by the apical domain, which signals to regulate both cell mechanics and gene expression (Maître et al. 2016).

The mechanism of asymmetric inheritance of apical material has recently been questioned, with claims that the apical domain would disassemble prior to division (Zenker et al. 2018; Lim et al. 2020; Pomp et al. 2022). Multiple lines of evidence refute this claim. First of all, while proposing that the apical domain would disassemble before division (Pomp et al. 2022), the same study proposes that the apical domain would somehow persist to control mitotic spindle orientation. Second, several studies have observed that, despite weakening of the actin ring structure, both Pard6b (Vinot et al. 2005; Anani et al. 2014; Niwayama et al. 2019) and ezrin (Louvret et al. 1996; Anani et al. 2014; Korotkevich et al. 2017) remain apically enriched throughout cell division in both fixed and live embryos. Another recent study found that the apical domain regulate the rate of cleavage furrow progression during symmetric divisions and measured that the apically localized furrow progressed significantly slower than the basal one (Paim and FitzHarris 2022). This further demonstrates that the apical domain is able to influence actomyosin driven processes throughout mitosis and can thus be symmetrically or asymmetrically divided, as initially proposed (Johnson and Ziomek 1981b). Further attesting of its persistence throughout division is discussed below with the direct influence of the apical domain on the mitotic spindle.

During the 8- to 16-cell stage divisions, the mitotic spindle can be oriented by the apical domain, biasing it along the apical-basal axis and accounting for the high proportion of asymmetric divisions (Korotkevich et al. 2017). However, it has consistently been observed that there are 0-6 inner cells at the 16-cell stage (Dietrich and Hiiragi 2007; Morris et al. 2010; Yamanaka et al. 2010; McDole et al. 2011; Anani et al. 2014; Watanabe et al. 2014; Samarage et al. 2015), which is fewer than expected if 80% of divisions were asymmetric. Two models have been proposed to explain this discrepancy: subclasses of asymmetric divisions and repolarization of apolar cells. The first is based on the observation that a subset of blastomeres at the 8-cell stage from a specialized 'monoastal' spindle that would lead to a stronger apicobasal bias in division than those with anastral spindles (Pomp et al. 2022). This could result in the inheritance of different amounts of apical domain, as measured previously (Korotkevich et al. 2017), which could influence cell mechanics and cell sorting (Maître et al. 2016). Alternatively, apolar cells who would be slow to internalize could instead be forced to remain at the surface for long enough to form an apical domain and become TE (Korotkevich et al.

2017). Indeed, after removal of the TE, ICM cells can polarize and redirect into a TE fate (Hogan and Tilly 1978; Korotkevich et al. 2017). To visualize the apical domain, many studies relied on the overexpression of ezrin (Korotkevich et al. 2017; Zhu et al. 2020; Zenker et al. 2018; Pomp et al. 2022), which is known to influence the size of the apical domain (Zenker et al. 2018). Therefore, physiological approaches may be required to distinguish the relative contributions of these scenarios.

Divisions from the 16-32 cell stage are mostly symmetric but can still occur asymmetrically and with an orientation that ensures that the cell without apical domain is pushed inside (Watanabe et al. 2014; Niwayama et al. 2019). Manipulation of embryo geometry revealed that the apical domain and cell shape compete to bias the orientation of cell division (Niwayama et al. 2019), as observed in other species (Minc et al. 2011; Pierre et al. 2016; Tanimoto et al. 2018; Sallé et al. 2019; Godard et al. 2021). While cell shape is homogenous at the 8-cell stage, at the 16-cell stage the TE are stretched as they cover the ICM leading to oriented symmetric division along the long axis of the cell to give rise to two TE cells. Interestingly, this competition reinforces the robustness of cell positioning. When several sequential TE divisions are asymmetric, the TE will be less stretched thus allowing the apical domain to orient division and produce a TE and ICM cell (Figure 4). This could contribute to the consistent allocation of about ten cells to the ICM of 32-cell stage embryos.

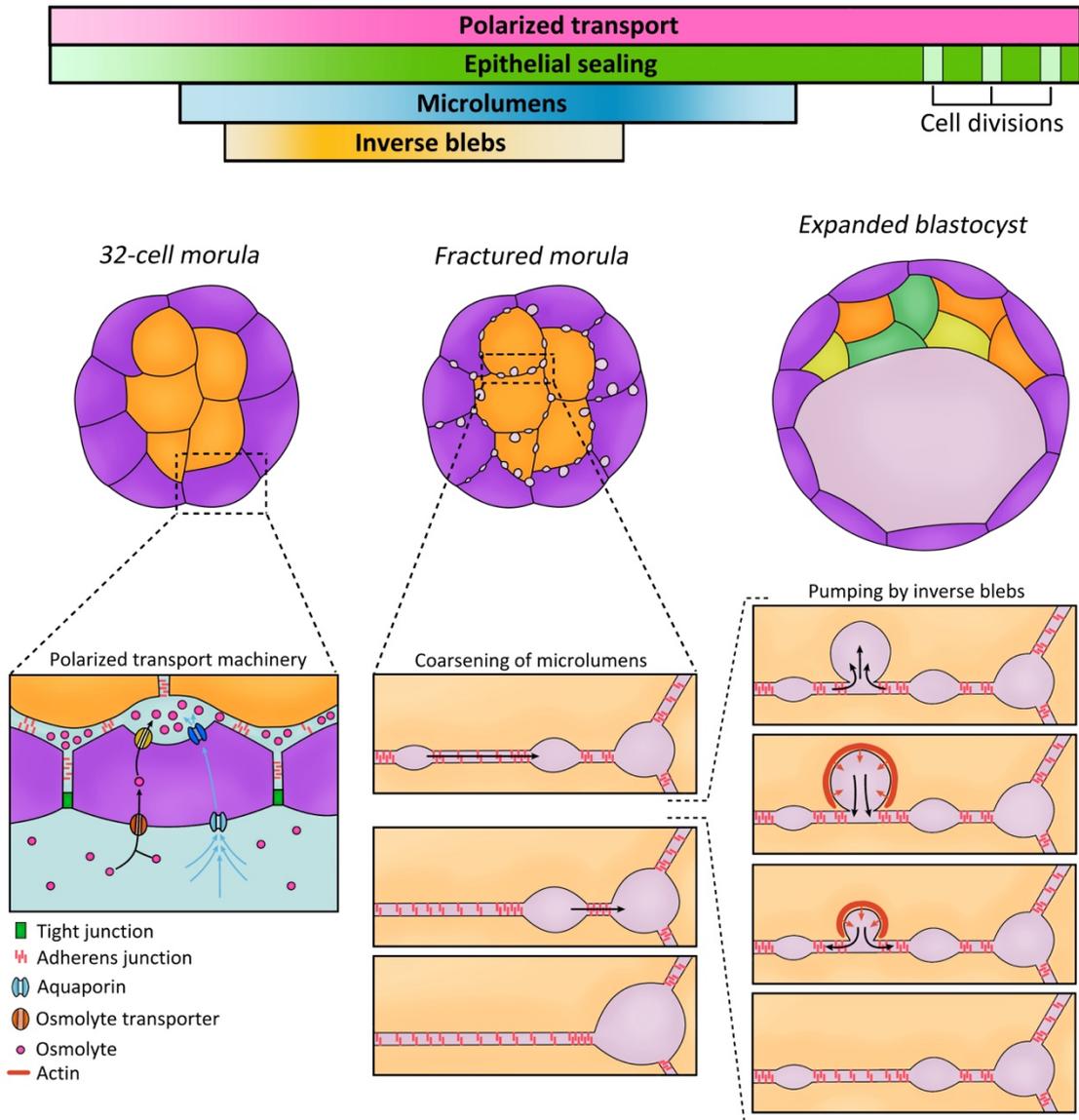
As cells adopt an outer or inner position, they begin to differentiate into either TE or ICM fate (reviewed in (Chazaud and Yamanaka 2016; Posfai et al. 2019; Firmin and Maître 2021)). Briefly, in polar outer cells, the Hippo signaling component AMOT is sequestered at the apical domain preventing phosphorylation by the junction localized LATS1/2 (Hirate et al. 2013). This prevents phosphorylation of the co-transcriptional activator YAP, leaving it to enter the nucleus and initiate the TE transcription program (Hirate et al. 2012). In apolar inner cells AMOT is free to localize to cell-cell junctions where it is phosphorylated by LATS1/2 (Cockburn et al. 2013). This in turn leads to the phosphorylation of YAP which blocks its nuclear translocation, leaving the inner cell fate to be influenced by Sox2 (Wicklow et al. 2014). Therefore, this bifurcation is tightly coupled to each blastomeres apicobasal polarity, shape and position in the embryo (Royer et al. 2020; Otsuka et al. 2023).

### **Blastocyst: prepare for landing**

Implantation into the maternal uterus is critical for the continued development of eutherian mammals. This is ensured by the TE, which attaches to the luminal epithelial cells of the uterus. Whether it is on the mural or polar side of the TE, the specific site of attachment is dictated by the position of the lumen, which defines the first axis of symmetry of the mammalian embryo. Like preparing the landing of a rocket, the implantation of the blastocyst requires exquisite control over its assembly.

### **Lumen formation**

The most striking shape change of preimplantation development is the formation of the blastocoel, often referred to as cavitation. Within a day, the formation of the first mammalian lumen inflates the embryo by several times its original volume and disrupts its radial symmetry. Lumen formation leaves the ICM positioned at one pole of the embryo thus establishing the first anatomical axis in the embryo: the embryonic and abembryonic, marked by the ICM and blastocoel, respectively (Figure 1). This axis determines the site of implantation, on the side of the lumen for the mouse embryo and of the ICM in humans (Siriwardena and Boroviak 2022). In the mouse, this axis will later on coincide with the dorsoventral axis (Rossant and Tam 2009). In order for lumen formation to proceed, two key steps must first occur: sealing of intercellular space by TJs and polarized transport of osmolytes to draw water into the intercellular space (Figure 5). First, cell-cell junctions at the surface of the embryo must be sealed in order to prevent uncontrolled diffusion between cells. This requires the formation of TJs, the principal components of which are the transmembrane adhesive proteins occludin and claudin, and adaptor protein Tjp1 (previously called ZO1) that links TJs to the actin cytoskeleton (Zihni and Matter 2016). Interference with TJ components impairs blastocyst formation (Sheth et al. 2000; Thomas et al. 2004; Kim et al. 2004; Moriwaki et al. 2007; Xu et al. 2008; Sheth et al. 2008). TJ formation starts as Tjp1 is recruited to the junction after the actin ring of the apical domain is fully expanded to the cell-cell junctions at the 16-cell stage (Zenker et al. 2018), and therefore requires intact apicobasal polarity (Alarcon 2010; Kono et al. 2014; Hirate et al. 2013). Second, the embryo must generate an osmotic gradient between the intercellular space and its external environment. The osmotic gradient is built by the accumulation of osmolytes such as sodium ions and glucose. This is thought to be driven by the polarized



**Figure 5: Formation and positioning of the blastocoel**

During the 32-cell stage, cell-cell contacts are transiently fractured by luminal fluid accumulating into pockets, which exchange their fluid until they coarsen into a single large lumen (lilac), called blastocoel. At the start of the 32-cell stage (left), the TE (purple) is a functional epithelium with polarized transport (purple) and sealed tight junctions (green) (Schliffka et al. 2021; Zenker et al. 2018), until TE cells start to divide to the 64-cell stage (interruptions in the sealing) (Leonavicius et al. 2018; Chan et al. 2019). As seen in the bottom inset, cell-cell contacts among and between TE and ICM (orange) cells are kept shut by cadherin adhesion molecules (pink rods) until fluid accumulates throughout the contacts of the embryo. Osmolytes (pink dots) are transported via pumps and exchangers (red and yellow channels), which creates an osmotic gradient across the epithelium and draws water into the embryo via aquaporins (light blue and orange pores) (Barcroft et al. 2003; Manejwala et al. 1989). Osmolytes and water cannot flow back out of the embryo due to the presence of functional tight junctions (TJ, green square) found at the apical portion of the cell (Chan et al. 2019). In the fractured embryo (middle), fluid fills up microlumens and later inverse blebs, which persist only for a few tens of seconds. In the bottom inset, fluid flows from small microlumens to larger ones, which act as pressure sinks (Dumortier et al. 2019). In the bottom right inset, fluid movement is facilitated when fluid inflates large asymmetric intrusions into the cell, which are retracted by actomyosin contraction (red line), effectively pumping fluid back into the intercellular network (Schliffka et al. 2023). In the expanded blastocyst, microlumens have coarsened into a single lumen, the blastocoel, which sets the conditions for the TE to differentiate into pTE (dark blue) and mTE (red) and for the PrE (yellow) and Epi (green) cells to position themselves. The boxes on top represent a relative timeline of the cellular processes required for blastocyst formation, and the gradient of colour represents each process' peak activity.

localization of Na<sup>+</sup>/K<sup>+</sup>-ATPase and Na<sup>+</sup>/H<sup>+</sup> exchanger at basolateral and apical membranes, respectively (Vorbrodt et al. 1977; Watson and Kidder 1988; Barr et al. 1998; MacPhee et al. 2000; Kawagishi et al. 2004). Chloride anions are also imported but since they have a lower osmotic power than sodium cations, this import seems to serve mostly to neutralize electric charges (Manejwala et al. 1989). As a consequence of the accumulation of osmolytes in the intercellular space, water flows into the intercellular space via aquaporins, three of which are expressed in the blastocyst with specific apicobasal localizations (Barcroft et al. 2003; Bell et al. 2009). Like for the establishment of TJs, apicobasal polarity is required for polarized transport (Tao et al. 2012). Chemical inhibition of osmolyte transporters or of aquaporins interferes with blastocoel formation (Wiley 1984; Kawagishi et al. 2004; Barcroft et al. 2003). Interestingly, the route taken by osmolytes and water is mostly through the cells rather than cell-cell contacts. Indeed, fluid accumulation rate does not depend on the number of contacts and even single-celled embryos inflate at a similar rate to whole embryos (Schliffka et al. 2021). Fluid can accumulate in a cell-autonomous manner into cytoplasmic vacuoles as observed in non-muscle myosin II or Cdh1 mutants (Schliffka et al. 2021; Stephenson et al. 2010). Notably, while both polarized transport and a sealed intercellular space are required for fluid to accumulate, it is not clear which one of the two is first to become functional. If polarized transport becomes functional in the absence of a sealed space, transport is not effective, while if TJs seal the embryo but it does not pump fluid, no lumen forms.

Barring any perturbations, these conditions are typically met by the 32-cell stage and fluid starts to accumulate in the intercellular space (Figure 5). This inundation of fluid is opposed by adhesion molecules of the adherens junction. This builds up hydrostatic pressure, which hydraulically fractures cell-cell contacts, leading to the formation of hundreds of micron-sized lumens distributed across all contacts of the embryo (Dumortier et al. 2019). Initially described *in vitro* (Casares et al. 2015), hydraulic fracturing of tissues was also observed in zebrafish (Munjal et al. 2021; Kennard et al. 2023). Adhesion strength influences lumen positioning: less adhesive contacts are easier to fracture biasing the lumen position to less adhesive cells. This was recently investigated in more detail using a reconstituted *in vitro* system, in which the density and the length of adhesive bonds was found to play a critical role (Dinet et al. 2023). After fracturing, microlumens begin to coarsen into

larger lumens until the primary lumen invariably forms at the interface between the TE and ICM. Theoretically, coarsening is driven by differences in pressure between microlumens, with higher pressure microlumens emptying into their lower pressure neighbors (Le Verge-Serandour and Turlier 2021). Both osmotic and hydrostatic pressures could in theory affect microlumen coarsening, but only hydrostatic effects were investigated experimentally. Microlumen hydrostatic pressure is influenced by microlumen size (larger being lower pressure) and the tension of the cells surrounding them (i.e., microlumens between higher tension ICM cells have higher pressure). This phenomenon contributes to the robustness of lumen positioning, as the final lumen will always form between lower tension cells (Dumortier et al. 2019). The movement of fluid between microlumens can rely on specialized protrusions known as inverse blebs (Schliffka et al. 2023). Like outward blebs, inverse blebs inflate as a result of differences in hydrostatic pressure between the cell and its surrounding fluid, which in the case of inverse blebs drives inflation into the cell (Tinevez et al. 2009; Gebala et al. 2016). Inverse blebs inflate into the cells with interstitial fluid and within tens of seconds contract with actomyosin to empty their contents into neighboring microlumens that persists for tens of minutes. Therefore, inverse blebs act as fast hydraulic pumps to flush interstitial fluid and coarsen the blastocoel (Schliffka et al. 2023).

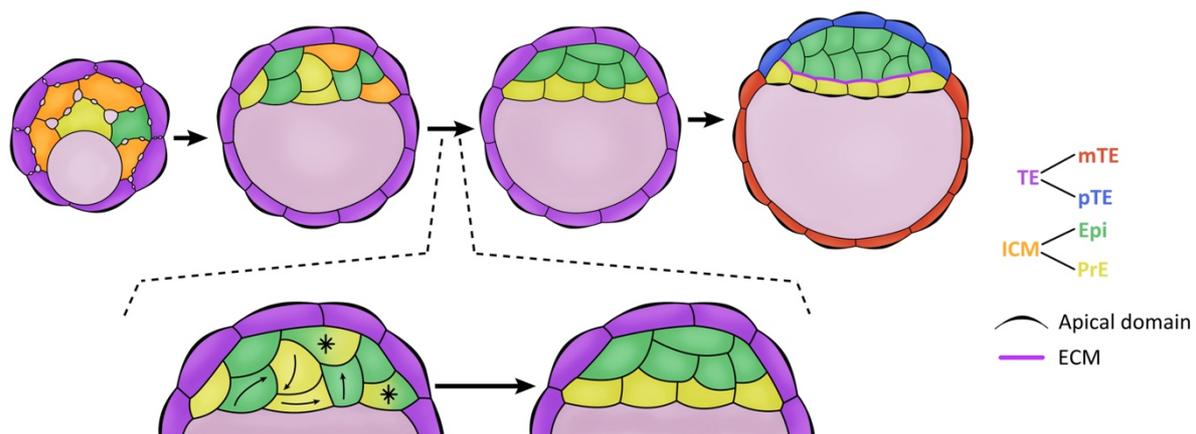
After the position of the main lumen is set, it continues to inflate as the blastocyst matures increasing the total volume of the mouse embryo by a factor of 5. This growth is punctuated by cycles of fast collapses (few minutes) followed by slower regrowth (few hours) (Cole 1967). Collapses coincide with cell divisions in the TE lining the blastocoel (Leonavicius et al. 2018; Chan et al. 2019). In agreement with this, a recent study following divisions in a single human embryo found that collapses systematically occur in the presence of mitosis (Domingo-Muelas et al. 2023). Continued inflation increases the hydrostatic pressure in the lumen (Leonavicius et al. 2018), which is thought to be important for maintaining TJ integrity (Chan et al. 2019; Kinoshita et al. 2022) and setting the average size of the embryo (Ruiz-Herrero et al. 2017). This building pressure ultimately contributes to hatching of the embryo from its ZP (Leonavicius et al. 2018). The ZP is an elastic glycoprotein shell, which, with elastic moduli ranging from 0.2 to 40 kPa depending on the species and technique used, is much more rigid than cells (Andolfi et al. 2016; Khalilian et al. 2010, 2013; Yu

Sun et al. 2003) but eventually yields with sufficient pressure from the lumen (~1.5 kPa (Leonavicius et al. 2018)). The ZP can be degraded by enzymes produced by the TE (Perona and Wassarman 1986; Sawada et al. 1992; O'Sullivan et al. 2001) and uterus (O'Sullivan et al. 2002), which makes it unclear whether pressure driven hatching would actually occur *in vivo*. Nevertheless, the presence of an elastic shell is essential to buildup pressure (Leonavicius et al. 2018) and this may be key in other species such as rabbits, which inflate their lumen up to 5000 times their initial volume (Gamow and Daniel 1970). Such increased elasticity in the ZP of rabbit embryos could be conferred by the presence of the protein, ZP4, which is also present in humans but absent in mice (Lamas-Toranzo et al. 2019). However, much remains to be understood when it comes to the regulation of the size of the lumen in blastocysts from different species.

### Sorting of the primitive endoderm from the epiblast

As the lumen forms, the cells of the ICM bifurcate towards different fates: the Epi which will form the embryo proper, and the PrE that contributes to the extra-embryonic yolk sac. The relative levels of the transcription factors Gata6 and Nanog are commonly used to mark the differentiation of PrE and Epi (Plusa et al. 2008; Guo et al. 2010). Though both TFs can simultaneously be expressed by ICM cells, as the two populations diverge, PrE progenitors lose Nanog and the Epi

progenitors lose Gata6, thought to be driven by the secretion of Fgf4 by Epi progenitors (Chazaud et al. 2006; Guo et al. 2010; Yamanaka et al. 2010; Frankenberg et al. 2011; Ohnishi et al. 2014). Fgf4 promotes ERK activity, which is consistently higher in PrE progenitors while only sporadically activated in Epi progenitors (Pokrass et al. 2020; Simon). This occurs asynchronously among blastomeres transiently leaving the once homogenous ICM as a mix of cell types in a 'salt and pepper' pattern (Gerbe et al. 2008; Plusa et al. 2008; Saiz et al. 2013; Bessonard et al. 2014). Given that Fgf4 is secreted in the luminal fluid (Ryan et al. 2019) and that multiple microlumens persist for hours before the dominant lumen is established (Dumortier et al. 2019), fate heterogeneity within the ICM may result from the variations in exposure of cells to microlumens. This mixed pattern is resolved when all PrE cells line the blastocoel while the Epi is apposed between the pTE and PrE (Bassalart et al. 2018). This is achieved by the commitment of cells to one lineage and to some extent by the relocation of cells within the ICM (Figure 6). Tracking of cells within live embryos revealed that the majority of PrE cells already lined the lumen before their commitment, a quarter of PrE cells come into contact with the lumen due to its expansion and the remaining quarter of PrE cells changes neighbor or undergoes apoptosis (Plusa et al. 2008). Therefore, only a couple of PrE cells may sort within developing embryos. However, the exact mechanism driving cell sorting has not yet been elucidated.



**Figure 6: Maturation and positioning of the Epi and PrE**

As the lumen expands (lilac), some ICM (orange) cells secrete Fgf4 in the microlumen network (Ryan et al. 2019). This induces PrE (yellow) fate while Epi (green) cells will continue secreting Fgf4. Cells leaning towards PrE and/or Epi fates are initially mixed within the ICM. Eventually, all cells facing the lumen are PrE and those away from the lumen are EPI. In the bottom inset, uncommitted cells (asterisk) adopt the fate corresponding to their position or change their position (arrows) in a cell sorting process (Plusa et al. 2008; Yanagida et al. 2022). The position of PrE and Epi cells is then secured by the apicobasal polarization of PrE cells and the deposition of extracellular matrix (purple) (Kim et al. 2022).

Several potential sorting mechanisms have been proposed. Based on Steinberg's differential adhesion hypothesis (Steinberg 1962), it was suggested that the relative adhesiveness of the Epi and PrE cells may drive their segregation. Although, it has been measured that levels of E-, N-, and P-cadherin are similar in both cell types (Yanagida et al. 2022; Filimonow et al. 2019). Unlike for the sorting of TE and ICM progenitors by contractility differences (Maître et al. 2016), AFM measurements of isolated PrE and Epi progenitor cells revealed identical surface tensions, potentially ruling out this mechanism (Yanagida et al. 2022). As an alternative, it was proposed that different levels of surface fluctuations can drive Epi and PrE sorting. PrE cells display dynamic surface fluctuations, which manifest as blebs in isolated cells or cell shape fluctuations within explants (Yanagida et al. 2022). Altering the levels of surface fluctuations in ES cells could affect their sorting when injected into host embryos (Yanagida et al. 2022). Additional fluctuations may arise from the cycles of lumen inflation and collapse that were proposed to promote Epi and PrE sorting (Guo et al. 2021). Alternatively, *in silico* modelling indicated a potential role for the Eph-ephrin receptor-ligand pairs based on a multiscale three-dimensional model informed by transcriptomics data (Cang et al. 2021). Eph-ephrin pairs can drive rapid and robust cell sorting even in the absence of differences in the mechanical properties between cell types (Canty et al. 2017), and are involved in cell sorting and boundary formation in several contexts (Xu et al. 1999; Rohani et al. 2011). It remains to be experimentally tested whether they may play a role in PrE/Epi sorting.

As they mature, PrE cells acquire apicobasal polarity and become a proper epithelium with extracellular matrix at its basal interface (Bassalart et al. 2018). Initially displaying enrichment of DAB2 and LRP2 at their future apical surface (Gerbe et al. 2008), this is later reinforced as aPKC is recruited to the apical surface and prevents remixing with Epi (Saiz et al. 2013). Interestingly, when the ICM is isolated and cultured in suspension, the PrE is able to polarize and coat Epi cells (Kim et al. 2022). This is akin to internalized apolar 16-cell stage blastomeres, which can polarize if forced to be exposed to culture medium (Korotkevich et al. 2017), indicating a general ability of preimplantation blastomeres to epithelialize once exposed to liquid medium. However, if these isolated ICMs are cultured in matrigel containing laminin this pattern is disrupted. Though the PrE and Epi cells still segregate, the PrE does not polarize and is

not able to coat the Epi cells (Kim et al. 2022). Furthermore, Integrin b1 mutants fail to form a single-layered PrE, indicating that contacts to the extracellular matrix are required to orient the epithelium correctly (Kim et al. 2022).

### **Formation of the polar and mural trophoctoderm**

As the lumen forms, the TE effectively becomes separated into two distinct populations: TE cells contacting the ICM and TE cells lining the lumen. Progressively, these two types of TE cells will differentiate into the pTE that coats the ICM and the mTE which lines the lumen. In the mouse, the mTE will attach to and invade the uterus, notably by entosis of luminal cells (Li et al. 2015). Meanwhile, the pTE will contribute to sculpt the Epi during implantation (Ichikawa et al. 2022). Compared to the PrE/Epi differentiation process, characterization and direct comparisons of the pTE and mTE are sparse, though in recent years these tissues have started to receive more attention (Posfai et al. 2019). Single cell RNAseq revealed that the gene expression profiles of the two tissues diverge at the time of implantation (Nakamura et al. 2015). With over 128 cells, this is also the time when the transcription factor *Cdx2*, a general mark for TE, becomes visibly restricted to pTE while it is downregulated in mTE (Saiz et al. 2016).

The physical segregation of pTE and mTE by the lumen imposes at least two principal differences in their environments that could contribute to the divergence in cell fate: first, Epi cells secrete *Fgf4* to which the pTE is likely more exposed due to its proximity; second, lumen expansion is associated with growing hydrostatic pressure (Leonavicius et al. 2018) to which the mTE is fully exposed while the pTE may be cushioned by ICM. Addition of *Fgf4* to blastocysts reduced the phagocytic activity of their TE cells (Rassoulzadegan 2000), a feature of the mTE that is conserved in other extraembryonic epithelia (Hojjman et al. 2021), supporting the idea that *Fgf4* is involved in pTE/mTE specification. Although this was not investigated in details, addition of *Fgf4* or inhibition of its signaling cascade through ERK seemed to homogenize the expression of *Cdx2* throughout the TE (Saiz et al. 2016). Consistent with the action of *Fgf4* on the TE, ERK activity is higher in pTE than in mTE cells (Simon et al. 2020). Together, these observations support the idea that distancing TE cells from the source of *Fgf4* will lead to their differentiation into mTE. However, studies in different systems also linked ERK signaling to mechanical stretch (Aoki et al. 2017; Hino et al. 2020; Boocock et al. 2020), including by regulating endocytosis and thus *Fgf4*

responsiveness (De Belly et al. 2021). Since mTE cells seem to be under a higher tension than pTE cells (Chan et al. 2019), an effect of TE stretching by the lumen on pTE/mTE differentiation remains a possibility.

### Concluding remarks

In the past ten years, the mouse preimplantation embryo has become one of the best mechanically characterized model systems to study morphogenesis. This has answered outstanding questions about how the ICM and TE cells are positioned, how the first axis of symmetry is set, or how the size of the blastocyst is regulated. We now understand some of these mechanisms so well that we can engineer embryos to direct cells to the TE/ICM or set the axis of symmetry by tuning mechanical properties (Maître et al. 2016; Dumortier et al. 2019). With a nearly complete spatiotemporal map of surface tensions throughout mouse preimplantation development (Özgüç et al. 2022; Maître et al. 2015, 2016; Chan et al. 2019; Yanagida et al. 2022), we can start to compare mechanical strategies between species and gain unique insights into the evolution of morphogenesis (Firmin et al. 2022). However, other physical properties will need to be considered in the future to understand what shapes the blastocyst. In the morula, the formation of the apical domain remains unclear and looking into the mechanical changes of the plasma membrane may reveal some clues (Yanagida et al. 2022; De Belly et al. 2021), or how much adhesive coupling changes throughout compaction will require testing cells' mechanical resistance to separation (De Plater et al. 2023). During the formation of the lumen, we will need better tools to measure and/or control osmolarity (Vian et al. 2023), estimate the electric effects of ion charges on lumen opening (Shim et al. 2022), or the effects of the extensive stretching of the TE (Latorre et al. 2018).

Beyond preimplantation development, technological advances now allow to study mammalian development *ex vivo* over longer periods. Peri-implantation, post-implantation and gastrulation are becoming accessible to high resolution live imaging (McDole et al. 2018; Ichikawa et al. 2022; Francou et al. 2023; Mathiah et al. 2020; Bondarenko et al. 2023). This will reveal the dynamic shape changes transforming the blastocyst into the gastrula and will raise new questions about the forces shaping the mammalian embryo.

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