CHAPTER SIX

The role of polarization and early heterogeneities in the mammalian first cell fate decision

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Abstract

The first cell fate decision is the process by which cells of an embryo take on distinct lineage identities for the first time, representing the beginning of patterning during development. In mammals, this process separates an embryonic inner cell mass lineage (future new organism) from an extra-embryonic trophectoderm lineage (future placenta), and in the mouse, this is classically attributed to the consequences of apical-basal polarity. The mouse embryo acquires this polarity at the 8-cell stage, indicated by cap-like protein domains on the apical surface of each cell; those cells which retain polarity over subsequent divisions are specified as trophectoderm, and the rest as inner cell mass. Recent research has

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advanced our knowledge of this process – this review will discuss mechanisms behind the establishment of polarity and distribution of the apical domain, different factors affecting the first cell fate decision including heterogeneities between cells of the very early embryo, and the conservation of developmental mechanisms across species, including human.

1. Introduction

1.1 Establishing first cell fates in the developing embryo

A fundamental step in embryonic development is the first cell fate decision, where cells start to differ from one another. This process lays the groundwork for future patterning of the organism, through cell signaling and further amplification of this initial difference. How is it possible for an initially symmetric organism to establish different cell fates?

In many developmental systems, the first cell fate decision is established when an initial molecular polarity in the early embryo is distributed unequally amongst daughter cells, such that each cell receives a different molecular constituent and subsequently establishes a different identity. In the *Caenorhabditis elegans* embryo, for example, molecular asymmetries as a consequence of fertilization result in a difference in identity between cells after the first cleavage (Fig. 1A): the zygote, cell P0, divides to form an anterior founder cell, AB, and a posterior founder cell, P1 (Munro & Bowerman, 2009). Sperm entry provides the egg with centrioles, which cause cessation of local actomyosin network contractions in the vicinity of the sperm through a molecular cascade. A result of this cessation of cortical contractions is that the region of sperm entry localizes posterior patterning proteins whilst the rest of the embryo localizes anterior proteins. Further maintenance of this initial symmetry breaking means that these patterning proteins are asymmetrically distributed upon division, leading to the first differential cell types in the embryo (Munro & Bowerman, 2009; St Johnston & Ahringer, 2010).

The *C. elegans* embryo is a good example of anterior-posterior polarity in the zygote, defining the future head-tail axis of the organism (Fig. 1A). Another important type of polarity found across many organisms is apical-basal polarity, which is a key component of epithelial cells that form tissues and the lining of many organs (Buckley & St Johnston, 2022; Fig. 1B). The apical-basal axis of epithelial cells is divided into three specialized sections: the apical domain, facing the lumen or outside environment; the lateral domain in-between adjacent cells; and the basal

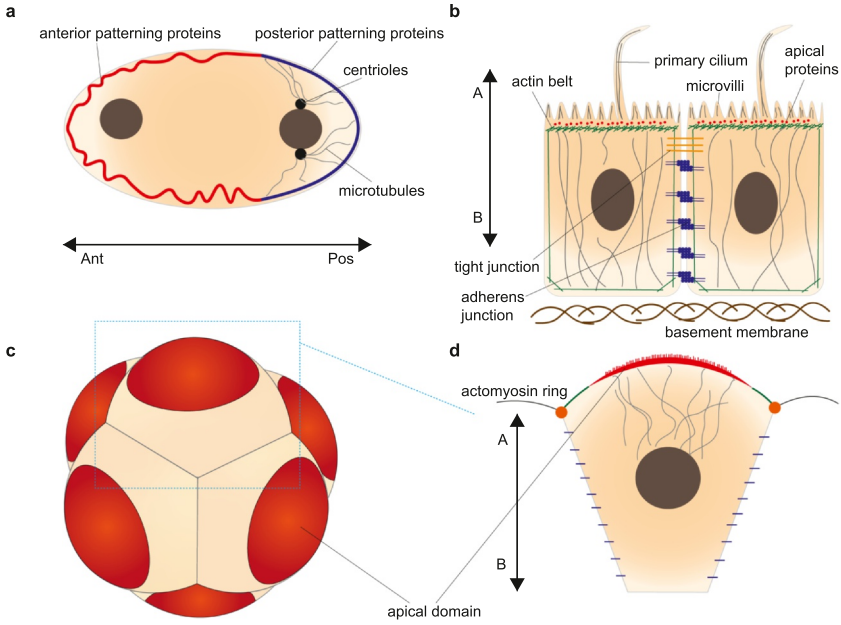

Fig. 1 Polarity across different organisms. (A) Schematic of anterior-posterior polarization of a *C. elegans* single celled embryo, directions indicated by the axis at the bottom. Red lines at the anterior represent anterior patterning proteins such as Par3 and Par6, whereas the blue lines represent posterior patterning proteins. Nuclei are shown as brown circles, whilst the symmetry breaking cue – the sperm centrosomes – are indicated in black. Microtubules emerge from these centrosomes. (B) Schematic of a vertebrate epithelial cell, with apical-basal polarity indicated by the axis alongside. Microtubules are oriented apico-basally. At the apical surface, microvilli and a primary cilium emerge, with apical proteins such as Par6 and ERM proteins (red) and an actin belt (green) underneath. The basal side contacts the basal membrane via integrins, and the lateral domain between cells contains tight junction contacts (orange, top) and adherens junctions (blue, below). (C) Schematic of apical-basal polarization in the mouse 8-cell stage embryo, 3D view, with apical domains indicated in red. (D) 2D cross section of a single polarized cell, with axis reference. Apical domains are indicated in red and are composed of many factors including Pard6 and ERM proteins, and have microvilli protrusions. Apical domains are surrounded by polarized actomyosin belts, and lateral domains consist of tight junctions (orange) and adherens junctions (blue).

domain, which interacts with the basement membrane. Interestingly, components of the *C. elegans* anterior-posterior system – such as Par proteins – are conserved in apical-basal polarity (Buckley & St Johnston, 2022; St Johnston & Ahringer, 2010).

In mammalian embryos, the onset and maintenance of apical-basal polarity (Fig. 1C and D) has been attributed as the important asymmetric

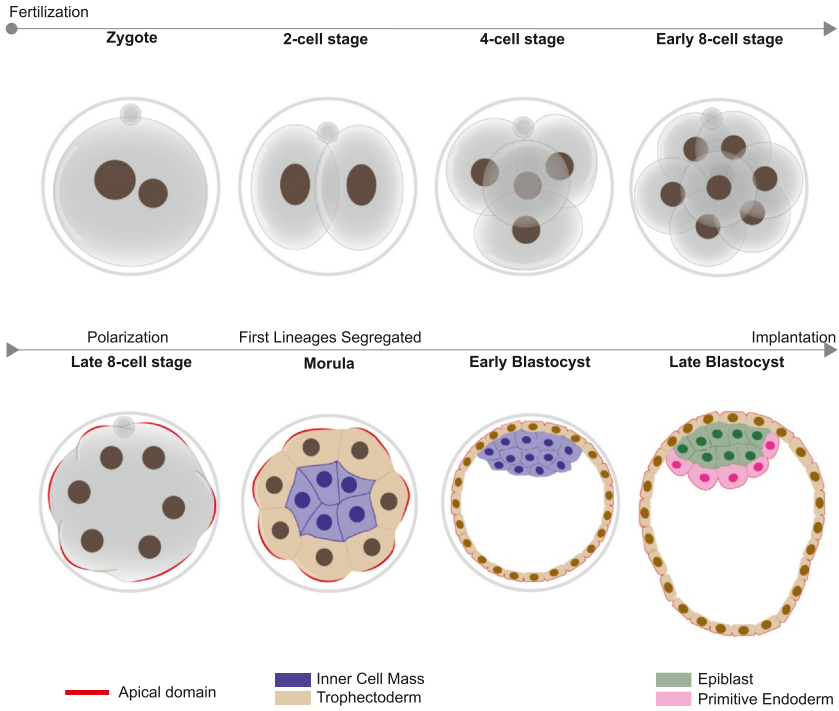

Fig. 2 Pre-implantation mouse development. Schematic of pre-implantation mouse development, indicating cell lineage segregations that make up the blastocyst. The first cell fate decision results in separation of the trophectoderm from the inner cell mass, whereas the second lineage segregation separates the epiblast from the primitive endoderm. Pre-implantation development lasts 4.5 days in the mouse embryo.

cue leading to partitioning of the first cell lineages (Leung et al., 2016). Using mouse embryos as a primary model, this review will address our current knowledge of apical-basal polarity in early mammalian embryos, and its consequence on the first cell fate decision.

1.2 Mammalian pre-implantation development

Pre-implantation development of mouse embryos comprises the period from fertilization, at embryonic day 0, to embryonic day 4.5, when the embryo is ready to implant into the mother's uterus (Fig. 2). During this time, a series of morphogenetic events and cell fate decisions transform the unicellular zygote into a blastocyst comprised of three tissues: trophectoderm (TE), the future placenta; primitive endoderm (PE), the future yolk

sac; and epiblast (EPI), the future fetus. However – unlike in the *C. elegans* system – even at the 8-cell stage, cells of the mouse embryo have the developmental potential to contribute to embryonic and extra-embryonic lineages of the organism (Chuva de Sousa Lopes & Mummery, 2009; Tarkowski, 1967). After this stage, developmental potential is restricted as the first cell lineages are established: TE and the inner cell mass (ICM), the latter of which later gives rise to the EPI and PE lineages. Human embryos, and those of other mammals, similarly separate an embryonic ICM from an extraembryonic TE lineage during pre-implantation development. Human pre-implantation development results in a blastocyst of the same three cell lineages but at embryonic day 6.0.

In the mouse, the process of embryonic polarization occurs at the 8-cell stage and is critical to the first cell fate decision (Ziomek & Johnson, 1982). At polarization, each blastomere gains a defined 'apical domain' on the surface exposed to the environment, consisting of a conserved set of apical proteins and enclosed by an actomyosin ring (Johnson & Ziomek, 1981; Plusa et al., 2005; Vinot et al., 2005; Zhu & Zernicka-Goetz, 2020a; Fig. 1C and D). At the 8–16 and 16–32 cell stage divisions, polarized cells can either divide asymmetrically, with the plane of division more perpendicular than parallel to the apical-basal axis, or symmetrically, with the plane of division more parallel than perpendicular to the axis. An asymmetric division results in only one of the two daughter cells inheriting the apical domain, and a symmetric cell division results in both daughters inheriting the apical domain (Anani, Bhat, Honma-Yamanaka, Krawchuk, & Yamanaka, 2014; Korotkevich et al., 2017; Ziomek & Johnson, 1982). Ultimately, the embryo is befitted with a proportion of polar cells inheriting the apical domain, and apolar cells that do not; those cells which retain the apical domain are ultimately specified as TE, and the apolar cells as ICM, through differential activity of Hippo signaling (Anani et al., 2014; Nishioka et al., 2008, 2009; Sasaki, 2015) and differential inheritance of Cdx2 mRNA, that localizes to the apical domain (Skamagki et al., 2013).

The given description of ICM and TE segregation outlines the first lineage segregation event in mouse embryos with regard to apical-basal polarization. However, our understanding of the first cell fate decision is much deeper and more complex than just described. In this review, we will detail the establishment and components making up apical-basal polarity in mammalian embryos and show how this process results in the segregation of ICM and TE lineages with the right proportions.

2. Establishment of apical-basal polarity

2.1 Key components of apical-basal polarity

The features of the apical, lateral and basal domains that characterize apical-basal polarity are highly conserved and stereotyped across species (Fig. 1); many of the factors involved were discovered from genetic screens in *C. elegans and Drosophila melanogaster*, and although mutations in polarity genes have more significant and lethal effects in those systems than in mammalian organisms, substantial similarity exists between invertebrate and vertebrate embryos (Buckley & St Johnston, 2022).

The apical domain faces the outside or luminal environment, and often contains specialized projections such as microvilli or cilia. Depending on the nature of the cell, these structures can have a number of specialisms relating to epithelial cell function, which include increasing surface area for absorption, acting as a node for sensory transduction, or serving as a barrier for invasive agents (Apodaca, 2018). These protrusions develop after, and as a consequence of, the establishment of the distinct apical polarity proteins that define the plasma membrane's structure and function on its apical side. A key protein involved in the establishment of polarity is atypical protein kinase C (aPKC), and it is typically considered the main effector involved in building the apical domain by exclusion of lateral and basal polarity factors (Hong, 2018). The role of aPKC, however, is dependent on its cooperation with other apical proteins such as Par6, Cdc42 and Crumbs (Assémat et al., 2008).

The lateral domain is important in cell-cell contacts, being the site of tight junctions and adherens junctions in cells. Adherens junctions are important in meditating cell-cell adhesion via the actions of nectins and cadherins, whereas tight junctions help maintain polarity and control ion passage, but a high level of interdependency and interplay is a result of the interactions between these junctions (Campbell et al., 2017). The basal domain contacting the basement membrane contains proteins such as Scribble (Scrib), Discs large (DLG) and Lethal Giant Larvae (LGL), and apical-basal polarity is maintained by mutual antagonism between the apical and basal domains (Fletcher et al., 2012).

The first sign that epithelial cell-like apical-basal polarity could be found in mouse embryonic development came from studies in the 1970s, which focused on the intracellular processes associated with the embryo as it undergoes shape changes at the 8-cell stage (Ducibella & Anderson, 1975; Johnson & Ziomek, 1981; Reeve & Ziomek, 1981). At this stage,

blastomeres of the embryo flatten and adhere more closely to one another, a process known as compaction. Concomitantly, intercellular junctions start to form at cell interfaces, and electron micrographs reveal the presence of microvilli concentrated to specific areas of the outside surface of the compacting embryo (Ducibella et al., 1975). These tiny protrusions are initially widely spread out across the surface of blastomeres during the 4-cell stage but become highly concentrated to an apical tip opposite the cell contact surface during the time of compaction (Reeve & Ziomek, 1981; Reima & Lehtonen, 1986), but their function in the early mouse embryo is not yet known. These initial observations provided first evidence of polarization of the mouse embryo, and the formation of an apical domain on the cell contact-free surface of the blastomere (Fig. 1C and D).

We now know much more about the features that characterize polarity in the 8-cell stage mouse embryo. The apical domain on the cell contact-free surface takes the form of a protein cap surrounded by an actomyosin ring, containing many of the evolutionary conserved players involved in apical polarity (Fig. 1D): the Par6-aPKC complex (in mouse embryos the Par6 homologs are known as Pard6); the Crumbs complex, some tight junction proteins; and the ERM proteins Ezrin and Radixin, which are involved in the interactions between polarized epithelia and cytoskeletal regulation amongst other functions (Hirate et al., 2015; Louvet et al., 1996; Vinot et al., 2005; Zhu & Zernicka-Goetz, 2020a). Cdc42 anchors Par6-aPKC to the apical domain, and mutually antagonistic interactions between apical factors, adherens junction proteins and basal region proteins such as LGL and Scrib maintain the integrity of apical-basal polarity in mouse embryos (Hirate et al., 2013; Zhu & Zernicka-Goetz, 2020a).

Recent research has provided insights into the nature of polarization in other mammals, including humans. In most cases, polarity is much more heterogeneously established between cells and does not form at a stereotyped stage. In rabbit and cow embryos, for example, not all blastomeres of the embryo polarize, and polarization occurs at various stages of pre-implantation development (Koyama et al., 1994). Similarly, in humans, polarization occurs between day 3 and day 4 of development and so between the 8–16 cell stages (Zhu et al., 2021). In this case, polarization occurs whilst the embryo is establishing its first lineages, and some cells remain unpolarized. Despite these differences, the composition of the apical domain is similar between human and mouse, containing many of the same proteins such as Par6 homologs and F-actin (Zhu et al., 2021).

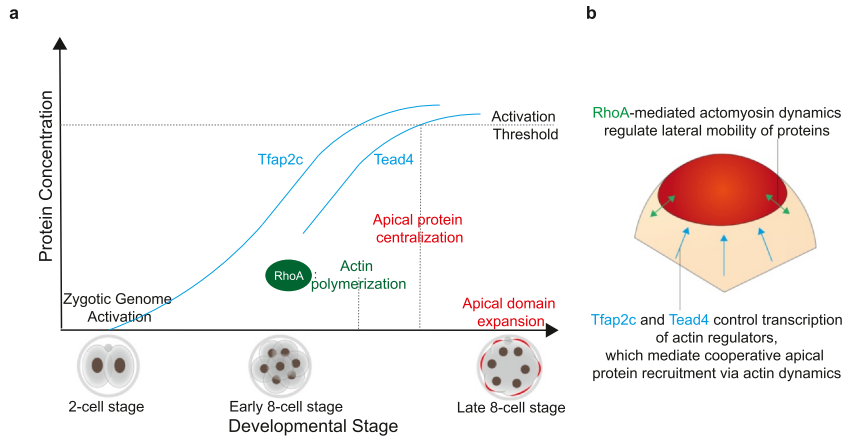

Fig. 3 Establishment of the apical domain in the mouse embryo. (A) Graph showing how accumulation of the transcription factors Tfap2c and Tead4 along with Rho-mediated actin polymerization ultimately establish different aspects of apical-basal polarity. (B) The combination of cooperative protein recruitment and regulation of lateral mobility creates a stable cap at the apical surface of the 8-cell stage embryo.

Evidence from the last ten years of study into mouse embryology has shed light on the timing of polarity establishment, and the steps that take place at the 8-cell stage that result in a polarized apical cap (Fig. 3). First, actomyosin polarity is established gradually at the 'early-mid' 8-cell stage (1–4 h post division), resulting in a circumferential distribution at the apical domain (Zhu et al., 2017). After this, apical proteins such as Pard6 form a cluster at the center of the apical surface, 5–8 h post division at the 'mid-late' 8-cell stage (Fig. 3A). Before division to the 16-cell stage (at 12 h after 8-cell stage formation), the apical proteins at the center of the cell expand to occupy the whole of the surface (Zenker et al., 2018; Zhu et al., 2017). More recent evidence has detailed the molecular pathways leading to polarization, describing in detail the upstream cellular processes that result in an 8-cell stage with a polarity axis that will define asymmetry of cell types in the embryo.

2.2 Establishment of polarity in mammalian embryos

What triggers apical-basal polarization, and why does it occupy blastomeres at the 8-cell stage? Initial ideas of polarity establishment referenced the necessity for cell-cell contact in the positioning of the apical domain, and it was inferred that cell interactions were critical for formation of polarity. Experiments in which cells were disaggregated and placed in contact with

other cells showed that two 8-cell stage blastomeres in contact result in apical domain initiation at opposite poles to that of cell contact (Johnson & Ziomek, 1981). However, further evidence has indicated that cell contact is merely instructive in guiding the location of the apical domain as single 8-cell stage cells can form apical domains too in a random location (Anani et al., 2014; Korotkevich et al., 2017).

The fact that cell contact is not needed to build an apical domain provides evidence for the remarkable fact that cell intrinsic processes can generate asymmetry alone. An obvious candidate for intracellular generation of asymmetry are microtubules, which have an inherent difference between ends of each polymer. Microtubules have long been known to be involved in symmetry breaking mechanisms from yeast to *D. melanogaster* (Cha et al., 2001; Roth & Lynch, 2009; Tsai & Ahringer, 2007). In mouse embryos, however, delivery of microtubule inhibitors such as Nocadozole and Colcemid have minimal effects on the apical domain (Maro & Pickering, 1984). Instead, microtubules emanate from the nucleus to the apical domain and are organized by the apical domain itself once established (Fleming et al., 1986). The extracellular matrix (ECM) is also critical for directing polarity in epithelial cells but components of the ECM, such as integrin, fibronectin and collagen are only established later on in mouse development (Johnson, 2009; Zhu & Zernicka-Goetz, 2020a).

Recent studies have revealed the mechanisms behind the dynamic changes of the actin cytoskeleton which appear to initiate polarity establishment. At the beginning of the 8-cell stage mouse embryo, the motor protein myosin II becomes phosphorylated and activated, then associating with actin filaments, initiating actomyosin contractility (Zhu et al., 2017). Actomyosin contractility is involved in processes such as apical constriction of cells during tissue invagination, and muscle constrictions. In mouse blastomeres it is essential to the establishment of a proper apical domain, as inhibition of active myosin II by ML-7 results in defunct apical-basal polarity (Svitkina, 2018; Zhu et al., 2017). Contractile actin initially locates to the apical surface and is excluded from the cell-contact domains (for unknown reasons), before further centralizing to the middle of the apical surface. Whilst at first forming a cap, this contractile actomyosin is negatively regulated by the clustering of apical proteins at the center of the apical domain. As a consequence, actomyosin forms a ring at the periphery of the apical domain that regulates the lateral mobility of proteins in and out of the apical domain (Zhu & Zernicka-Goetz, 2020b). Importantly, it has been shown that this actomyosin activity is dependent on

phospholipase signaling and Rho-GTPAses: phospholipase C-mediated PIP_2 hydrolysis causes activation of RhoA, which in turn causes activation of myosin II (Zhu & Zernicka-Goetz, 2020a; Zhu et al., 2017; Fig. 3).

Actomyosin polarization is followed by polarized accumulation of proteins associated with the apical domain, such as Ezrin or Pard6. This process is distinct from actin polymerization as, although the integrity of the apical cap is dependent on the actomyosin network forming its ring-like topology, inhibition of actomyosin contractility does not disrupt the apical accumulation of these proteins (Zhu et al., 2017). How does polarization of apical proteins occur? Early experiments placing blastomeres of different stages together showed that interaction of a 2-cell stage blastomere and an 8-cell stage blastomere could result in induction of polarity in the 8-cell blastomere – this led to the suggestion that some property of 2-cell embryos was important to polarization (Johnson & Ziomek, 1981), but this property was not held by the zygote. It was then hypothesized that transcriptional activation of the zygotic genome (ZGA), the major wave of which occurs at the 2-cell stage in mouse, was a critical factor for apical-basal polarization, as opposed to maternally inherited proteins present in the zygote (Johnson & Ziomek, 1981; Schulz & Harrison, 2019; Zhu et al., 2020).

Despite this initial hypothesis, the role of ZGA upstream of polarity establishment was only recently revealed. The association between transcription and polarization can be shown through treating the early 8-cell stage embryo with transcriptional inhibitors, demonstrating that transcription itself is required for embryo polarization (Zhu et al., 2020). Further experiments in which some cytoplasm was removed from blastomeres through resection (thus resulting in an increase in nuclear-to-cytoplasmic ratio, and concentration of transcripts in the cell) were able to advance polarization, demonstrating that a critical threshold of certain transcripts is required to trigger formation of an apical domain (Zhu et al., 2020). To identify the identity of these transcripts, an RNA interference screen was carried out for 124 transcriptional or cytoskeletal regulators that were found to be upregulated between the 2-cell and 8-cell stages. These functional screens revealed that Tfap2c (transcription factor AP-2 gamma) and Tead4 (TEA domain transcription factor 4) are critical factors upstream of polarity establishment (Zhu et al., 2020; Fig. 3A and B). It was shown that these two factors control transcription of actin regulators, and thus promote apical protein recruitment through the coordination of actin dynamics (Zhu et al., 2020).

Injection of Tfap2c and Tead4 mRNA into a cell is enough to trigger the clustering of apical proteins such as Ezrin at the apical surface, but only in

combination with an injection of active RhoA – leading to activation of myosin II, actomyosin contractility and establishment of actomyosin polarity – can polarization truly be advanced. Furthermore, functional studies and high-resolution measurements using photo-activatable constructs, together with computer modeling, have begun to allow us to define the role of each of the key factors involved in polarity establishment. Tead4 and Tfap2c accumulation above a threshold, downstream of ZGA, leads to expression of actin regulatory proteins such as Arpc1b. This results in actin remodeling, leading to recruitment of apical proteins such as Ezrin to the apical surface. The actomyosin cytoskeleton, regulated by RhoA, coordinates mobility of the apical proteins, whilst it is excluded from the cap center itself due to antagonistic interactions with other apical factors (Zhu et al., 2020). All in all, the activity of these factors can establish a cap of apical proteins surrounded by an actomyosin ring, at the 8-cell stage. Although many further details of the process are yet to be uncovered, this process represents a remarkable example of spontaneous symmetry breaking in a cell (Zhu & Zernicka-Goetz, 2020a, 2020c; Zhu et al., 2020; Fig. 3A and B).

Together, these recent results have shed light on how the apical domain is established in the mouse embryo, and subsequently there has been investigation into the conservation of polarity factors in humans. Interestingly, ZGA is known to occur at different time in different mammals, which may play a role in the differences between organisms in establishment of embryonic apical-basal polarity (Schulz & Harrison, 2019). In humans, polarity establishment also occurs in a two-step process – F-actin polarization before PARD6 accumulation (Zhu et al., 2021). Furthermore, downregulation of phospholipase C isoforms shows that, like in mouse, phospholipase C signaling is important for actomyosin polarity (Zhu et al., 2021). The genetic components and signaling pathways upstream of human polarization, however, are yet to be elucidated – and downstream of polarization, leading to the first cell fate decision, differences are present between human and mouse embryos.

3. Linking polarity and cell fate

3.1 Evidence for the polarity model of cell fate specification

Theories of the first cell fate decision predate knowledge of the existence of an apical domain in the embryo. Consequently, the first hypothesis for the

initial segregation of lineages in the embryo came from the observation that cell fates correlated with position: at the late morula stage, the TE cells occupy the outside of the embryo whilst ICM cells are positioned on the interior. More importantly, when inside cell is positioned outside of the early embryo, it will follow fate of the outside cell towards the trophectoderm; and vice versa. Thus, an 'inside-outside' model was proposed, suggesting that the cells in the embryo had some mechanism of sensing their position in relation to their neighbors (Balakier & Pedersen, 1982; Suwińska et al., 2008; Tarkowski, 1967).

The 'polarity' model instead suggested that cell fate was determined by the inheritance of the apical domain (Fig. 4A; Johnson, Ziomek', 1983; Mihajlović & Bruce, 2017; Ziomek & Johnson, 1981; Ziomek & Johnson, 1982). First, it was found that downregulation of important polarity factors, such as Par3 and aPKC, in individual cells in the embryo directs cells towards ICM fate (Plusa et al., 2005). Also, downregulation of Pard6b arrests development (Alarcon, 2010). Moreover, transplantation of apical domains to the inside cells has shown that this protein cap is necessary and sufficient for TE differentiation (Korotkevich et al., 2017). However, live imaging studies have also shown that the initial inheritance pattern of the apical domain during the 8- to 16 cell stage division does not correspond exactly with final position or cell fate in the embryo (Anani et al., 2014). How is it possible to reconcile these different theories?

During the 8- to 16- cell stage division, many divisions are asymmetric with respect to the apical domain, meaning that the 16-cell stage embryo is befitted with cells which inherit the apical domain and retain polarity, usually located to the outside of the embryo, as well as apolar cells that do not and locate to the inside (Bischoff et al., 2008; Morris et al., 2010; Niwayama et al., 2019; Watanabe et al., 2014). However, some apolar cells remain on the outside of the embryo, after which these cells internalize as a result of differences in cortical tension between blastomeres (Samarage et al., 2015; Watanabe et al., 2014). During the transition from the 8- to the 16- cell stage, some cells remain outside and adopt a wedge shape, whereas other cells undergo a cuboidal shape change and are pushed to the interior of the embryo (Samarage et al., 2015). Examination of the tensile forces on the cell surface using laser ablation shows high tensile forces along the apical surface of the constricting cell and low tension along the apical surface of the wedge-like cells. This indicates that some form of apical constriction causes cell internalization. The process is driven by myosin II, inhibition of which results in a reduction of inner cells and downstream

effects on cell fate (Samarage et al., 2015). The differences in cortical tension are driven by differences in cell polarity, with outer apolar cells having higher cortical tension and thus being preferentially internalized (Anani et al., 2014). In this way, cell internalization can ensure that cells which are not properly polarized remain on the interior whilst polar cells occupy the outside space of the embryo. Together, these processes help ensure that the embryo has the right number of cells of each lineage, in the right positions (Anani et al., 2014; Zhu & Zernicka-Goetz, 2020b). The 16-cell embryo has between 1 and 5 inner apolar cells, which also depends on the genetic background of the mouse (Morris et al., 2010).

Ultimately, in order to ensure that the correct number of cells are specified to each lineage, the proportion of symmetric and asymmetric divisions from 8- to 16-cell stage and 16- to 32-cell stage need to be regulated ensuring the right number of polar and apolar cells in the embryo, along with the aforementioned process of internalization, as well as further polarization (Morris & Zernicka-Goetz, 2012; Morris et al., 2010).

How are division orientations determined? Division of a mouse blastomere at the 8- to 32-cell stage is dependent on the combination of two cues: the orientation of the mitotic spindle pointing towards the apical domain, and the geometry of the cell, specifically the relative dimensions of the cell axes (Kim et al., 2018; Korotkevich et al., 2017; Niwayama et al., 2019). These cues influence the division of each individual cell and ultimately drive the proportion of cells allocated to each lineage in the embryo.

It was found that spindle orientation in *mzCdc42*$^{-/-}$ embryos (embryos in which both maternal and zygotic Cdc42 protein were eliminated) with disrupted apical domains are randomized, in comparison with control embryos in which cell fate specification – but not the apical domain – is inhibited (Korotkevich et al., 2017). However, 3D analysis of cell geometry and division planes show that this orientation towards the apical domain is only present when longest and shortest axes are similar in length (Niwayama et al., 2019). By contrast, when a mouse blastomere is stretched in one axis, the division plane is instead influenced by geometry and the cell divides along its longest axis, a rule commonly seen in biological systems – Hertwig's rule (Dumollard et al., 2017; Hertwig & Hertwig, 1884; Gray et al., 2014; Plusa et al., 2005). In an unperturbed embryo, lack of geometric asymmetries at the 8-cell stage favors an asymmetric division of cells, whereas the elongation of cells at the 16-cell stage leads to symmetric divisions. Indeed, applying compressive forces to cells and embryos can adjust the conventional division proportions but

still result in a blastocyst with the correct number of each cell type (Niwayama et al., 2019).

It should be not surprising that a number of processes are involved in the allocation of two lineages in the mammalian embryo, as its development is 'regulative', indicating a high level of flexibility and redundancy in each process (Zhu & Zernicka-Goetz, 2020c). Nevertheless, through these processes, the apical domain is distributed in such a way that the embryo ultimately results in an inside proportion of ICM cells and an outer proportion of TE cells. Further evidence for the role of polarity in driving these differences, and not some other position-related mechanism, comes from an understanding of the molecular processes that lead to differences in cell fate from segregation of the apical domain.

3.2 How polarity allocates cell lineages

The Hippo signaling pathway has been shown to be responsible for apolar cells being specified as ICM, whilst polar cells are specified as TE (Nishioka et al., 2009). The presence of the apical domain in polar cells results in nuclear localization of the protein Yap, whereas Yap remains phosphorylated and cytoplasmic in apolar cells (Anani et al., 2014; Cockburn et al., 2013; Hirate et al., 2013; Nishioka et al., 2009; Figs. 4 and 5). This difference in Hippo signaling is due to the apical domain of outer cells sequestering proteins such as Angiomotin (Amot) and Large kinase tumor suppressor (Lats), which are inactivated and thus cannot bind and phosphorylate Yap, while at the adherens junction of apolar cells, Amot and Lats form a complex that phosphorylates Yap, targeting it for degradation (Hirate & Sasaki, 2014). Other potential mechanisms of segregating Yap localization are present at this stage, as Amot can also phosphorylate Yap independently of the Hippo pathway, bypassing Lats kinase (Leung & Zernicka-Goetz, 2013). In the nucleus of polar cells, Yap binds to Tead4 and activates expression of *Cdx2*, which mediates the exit from pluripotency and specifies TE fate (Nishioka et al., 2009; Ralston & Rossant, 2008; Strumpf et al., 2005). Conversely, lack of Cdx2 in apolar cells results in upregulation of pluripotency genes such as *Nanog* and *Oct4*, specifying ICM fate (Mitsui et al., 2003; Niwa et al., 2000).

Recent studies using high resolution live-imaging indicate that the traditional apical domain factors become disassociated prior to the 8–16 cell stage division and, instead, other factors such as keratins may be responsible for separating cell fates (Lim et al., 2020). It has been found that keratins become anchored at the apical domain and retain their localization

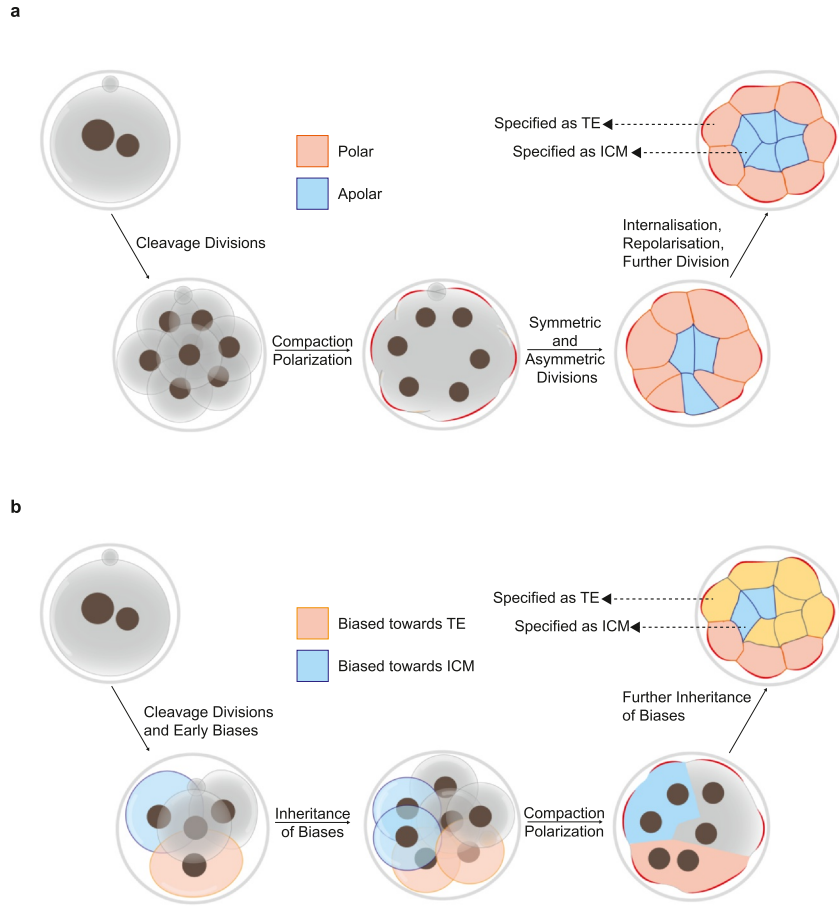

Fig. 4 Different models of the first cell fate decision. (A) The polarity model of cell fate specification. The embryo remains unspecified until the 8-cell stage, after which each cell gains an apical domain. This domain is distributed in such a way that only some daughter cells are polar and others are apolar. Further patterning mechanisms establish the polar cells on the outside of the embryo, and apolar cells on the inside, in right proportions. Polar cells become TE and apolar cells become ICM. (B) The early heterogeneity model of patterning suggests that biases between cells – whilst not determining their fate one way or the other – increase the probability that a cell will reach one lineage. These biases are dependent on mechanisms that take place in the very early embryo, perhaps even as early as the zygote or egg.

following disaggregation of the domain, and, consequently become asymmetrically inherited at the 8–16-cell stage division. Those cells which inherit keratins upon asymmetric division re-establish the apical domain and form TE, and those cells which do not inherit keratins become ICM

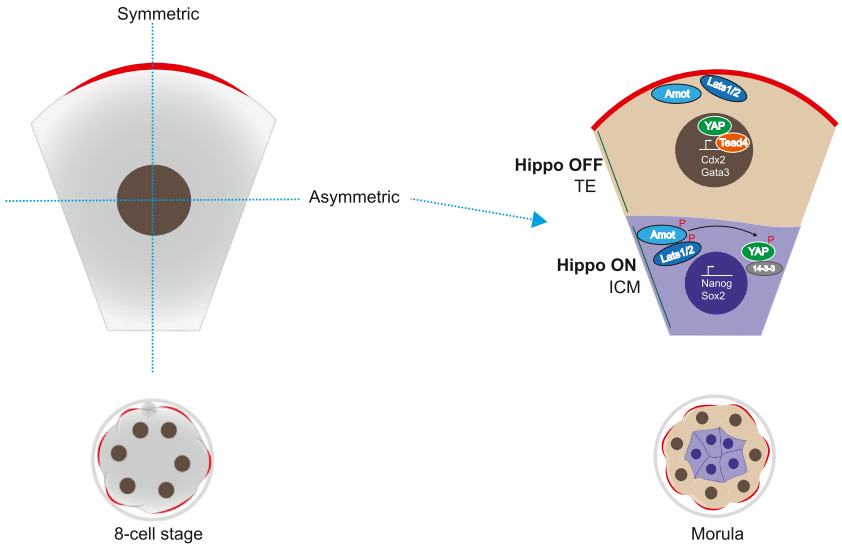

Fig. 5 Segregating the apical domain, and its consequence on cell fate. At the 8-cell stage, cells can divide symmetrically (both daughters inherit the apical domain) or asymmetrically (one daughter inherits the apical domain, one does not). Differential inheritance of the apical domain results in differential activation of Hippo signaling, such that apolar cells have phosphorylated Yap which fails to enter the nucleus, and polar cells have nuclear Yap accumulation. Nuclear Yap activates *Cdx2* and *Gata3* transcription in polar cells, but in apolar cells *Nanog* and *Sox2* are upregulated instead.

(Lim et al., 2020). In this way, whilst much of the polarity model remains the same, keratin filaments appear to provide the critical asymmetrically distributed factor, although they are not present in every polarized cell at the 8-cell stage so other factors may also help re-establish the apical domain. The mechanism of this re-establishment is not yet fully known, however, it has been shown that keratins promote F-actin stability and thus promote apical-basal polarization (Lim et al., 2020).

In humans, the consequences of cell polarity on the first cell fate decision are less well known. However, recent evidence suggests that there is conservation in the molecular pathways involved in the first cell fate decision across mammalian species. The Hippo signaling pathway might also have a role in the human embryo ICM/TE differentiation as the outer cells of the human morula contain YAP, TEAD4, and express the TE lineage marker GATA3, and some of these markers are also present in the cow embryo (Gerri et al., 2020). In addition, pharmacological inhibition and protein depletion of aPKC impairs human TE differentiation, suggesting that polarity plays a role in the first cell fate decision in human

embryos too. Not all mechanisms appear to be conserved between mouse and human. Recent studies show that, unlike in the mouse, GATA3 is present in both polarized and unpolarized human morula cells suggesting that the TE-differentiation program can be initiated independently of apical-basal polarization (Zhu et al., 2021). On the other hand, polarized cells express GATA3 more strongly, suggesting that whilst polarity might not drive first steps of lineage segregation, it certainly plays a major role in reinforcing and determining the lineages of the human embryo (Gerri et al., 2020; Zhu et al., 2021).

4. The first cell fate decision beyond apical-basal polarity

4.1 Introducing early heterogeneities in the embryo

Since the inception of the polarity model, it has been proposed that aspects of the first cell fate decision can precede the segregation of an apical domain, and perhaps start to be initiated as early as the zygote or oocyte stage itself.

The first evidence that symmetry breaking mechanisms are initiated early in the embryo came from tracking lineages of embryos from the 2-cell stage with live embryo markers. These cell tracking studies revealed that the first cleavage plane predicts the embryonic-abembryonic axis of the blastocyst, which separates the side containing the ICM (embryonic side) from its opposite side containing only TE (abembryonic side; Gardner, 1997, 2001; Piotrowska & Zernicka-Goetz, 2001; Zernicka-Goetz, 2002). These results opened the possibility that the embryo might be pre-patterned to a certain extent, as early as the zygote.

Subsequent studies related the orientation and order of the second cleavage division with the embryonic-abembryonic axis of the blastocyst. It was found that embryos in which the first blastomere at the 2-cell stage divides meridionally – in line with the location of the second polar body that remains attached to the embryo – whilst the second blastomere divides equatorially, results in a bias of the earlier dividing blastomere towards the embryonic part of the embryo, and consequently the ICM lineage (Piotrowska & Zernicka-Goetz, 2001; Piotrowska-Nitsche et al., 2005). It was also found that at the 4-cell stage the blastomere furthest away from the polar body, the so-called the 'vegetal' blastomere, is biased towards contributing to the TE lineage (Piotrowska-Nitsche et al., 2005). Together,

these results strongly imply that a pre-patterning system of segregating factors may play a part in the first cell fate decision of the mouse embryo (Fig. 4B).

Of course, this challenge to the dogma was not met without resistance. A pre-patterning model somewhat akin to that of other invertebrate and vertebrate model embryos might at the first quick look to be at odds with the regulative nature of the mouse embryo. This would seemingly contradict chimera experiments which have shown that cells of the embryo up until the 8-cell stage remain able to contribute to all lineages of the implantation blastocyst (Hiiragi et al., 2006; Motosugi et al., 2005; Tarkowski et al., 2005, 2010; Tarkowski, 1967). A reconciliation of these issues comes from an appreciation for the differences between pre-patterning in other, simpler model systems, as compared to the mouse. In many embryos studied to date, pre-patterning relates to the factors that establish an initial polarity in the egg or zygote and become segregated between cells during divisions. Such factors are classified as 'determinants' because their inheritance fixes the fate of the daughter cells. In contrast, factors that segregate between cells of the early mouse embryo do not fix the fate of the daughter cells, ensuring that the cells retain the flexibility to specify into different cell types. Instead, these factors bias lineage commitment one way or the other (Piotrowska-Nitsche et al., 2005; Plusa et al., 2006).

These early biases might provide an alternate mechanism of ensuring fidelity of cell fate segregation, contributing to the redundancy of the mouse embryo. Alternatively, they could be a vestige of a pre-patterning that have existed in mammalian ancestors, given the high level of conservation that exists in molecular specification between different animals. Recent lineage tracking studies in human embryos indicate that biases may exist between early blastomeres also in humans (Casser et al., 2017; Coorens et al., 2021; Fasching et al., 2021; Krawczyk et al., 2021), but the molecular underpinnings of this in human are yet to be determined.

4.2 Characterizing early heterogeneities in the embryo

The first molecular factor identified as segregating its activity between early blastomeres of the mouse embryo, and thus contributing to the first cell fate decision, is the activity of H3-specific arginine methyltransferase CARM1 (Torres-Padilla et al., 2007). Discovery of this role for CARM1 came from the realization that differences in epigenetic markers may be underlying the differences in potential of each blastomere at the 4-cell stage, and that the vegetal blastomere has low levels of H3 methylation at arginine 26

(H3R26me2), which is methylated by CARM1 (Torres-Padilla et al., 2007). This observation suggested that low levels of CARM1 activity may result in bias to the TE lineage, which was supported by CARM1 overexpression in one blastomere of the 2-cell embryo which resulted in bias towards the ICM lineage and upregulation of pluripotency markers such as *Nanog* and *Sox2* (Burton et al., 2013; Torres-Padilla et al., 2007; Wu et al., 2009).

More recently, single cell transcriptomics and following the dynamics of specific transcription factors in live embryos have verified the role of CARM1 in early mouse development, by showing that heterogeneity in H3R26me2 underlies the ability of the pluripotency regulators Oct4 and Sox2 to bind to DNA and activate lineage specific genes. In the vegetal blastomere, low CARM1 activity means low Oct4 and Sox2 binding, which favors activation of TE genes such as *Cdx2* over pluripotency genes such as *Sox21* associated with ICM differentiation (Goolam et al., 2016; White et al., 2016). The relationship between CARM1 activity and nuclear organization has also been revealed and has shed further light on the nature of early heterogeneities in the mouse embryo. It was found that paraspeckles, small nuclear foci of non-coding RNAs enriched in RNA-binding proteins are key to the action of CARM1 (Hupalowska et al., 2018). CARM1 accumulates in nuclear paraspeckles in order to enact its methylation activity, and negative and positive feedback loops between paraspeckle components such as the long non-coding RNA Neat1 and CARM1 itself act to regulate CARM1 function (Hupalowska et al., 2018).

What is the origin of molecular heterogeneities that bias cell fate in the embryo? The earliest known molecular factor so far that influences cell fate is shown to be heterogeneous already at the 2-cell stage. The long non-coding RNA LincGET is asymmetrically expressed in the 2 and 4-cell embryo nucleus, and fluorescent in-situ hybridization combined with immunofluorescence has shown that it forms a complex with CARM1, with increased LincGET levels leading to enhanced H3R26me2 (Wang et al., 2018). Many potential origins of this early heterogeneity have been suggested: a role for the sub-cortical maternal complex, sperm-donated factors or mechanical cues (Chen et al., 2018), but this exciting question still remains to be addressed.

4.3 Different models of the first cell fate decision

The two models of the first cell fate decision that have been proposed so far are seemingly distinct. The polarity model attributes the decision of a cell to become either TE or ICM based on the segregation of the apical domain

after the 8-cell stage and subsequent differential activity of Hippo signaling (Fig. 4A). The early heterogeneity model, on the other hand, attributes initiation of the first cell fate decision to molecular biases that exist between blastomeres in the very early embryo, as early as the 2–4 cell stage (Fig. 4B). However, these two models can be integrated. It can be imagined that segregation of the apical domain drives cell fate, but the decision of a blastomere to divide in a symmetric or asymmetric manner and the mechanics of segregation are influenced by molecular heterogeneities. Evidence for this comes from experiments that show blastomeres in which CARM1 is overexpressed contribute preferentially to ICM by being biased towards asymmetric cell divisions (Parfitt & Zernicka-Goetz, 2010). Similarly, cell tracking has shown that the progeny of the vegetal blastomere of the 4-cell stage embryo are biased towards symmetric divisions (Bischoff et al., 2008). Despite this evidence, the molecular link between early heterogeneities and apical-basal polarity are yet to be truly revealed.

Keratin filaments, known to interact with the apical domain, have also been shown to be linked to molecular heterogeneities in the early embryo. Keratin was shown via selective photoactivation and live imaging to arise from the vegetal blastomere at the 4-cell stage and be downstream of BAF155 expression, which is highly present in the vegetal blastomere and a target of CARM1 (Lim et al., 2020; Panamarova et al., 2016). How keratin links the heterogeneity model and polarity model is yet to be determined.

Apical-basal polarity and early heterogeneities are not the only factors that influence the first cell fate decision. Recent evidence has also implicated metabolic pathways in lineage segregation. It has been known that embryonic glucose metabolism is initiated at the morula to blastocyst transition, but its role in cell fate decisions was unclear until it was uncovered that *Cdx2* expression and so TE lineage differentiation is affected by glucose depletion (Chi et al., 2020; Zhu & Zernicka-Goetz, 2020b). This is because two glucose-dependent metabolic pathways are implicated in Hippo pathway signaling: the pentose phosphate pathway regulates Tfap2c synthesis and the hexosamine biosynthetic pathway regulates Yap nuclear translocation. Together, Tfap2c and Tead4/Yap form a complex to activate *Cdx2* expression, paralleling the synergistic function of Tfap2c and Tead4 in polarity establishment. The interaction between metabolism, polarity and fate regulation is an intriguing area of study requiring further investigation (Chi et al., 2020; Zhu & Zernicka-Goetz, 2020b).

5. Summary and discussion

5.1 The first cell fate decision in mammalian embryos

Apical-basal polarization is established at the 8-cell stage in the mouse, and the molecular factors associated with this polarity have largely been discerned and are conserved across species. More recently, the mechanism behind the establishment of polarization has also been fleshed out. Downstream of ZGA, the accumulation of Tead4 and Tfap2c mRNA transcripts results in transcription of actin regulators that result in clustering of apical proteins, which – together with RhoA-mediated formation of a surrounding actomyosin ring – establish the apical domain.

Segregation of the apical domain in the mouse embryo defines the TE and ICM lineages, as retention of the apical domain prevents Hippo signaling. This segregation is based on cell divisions, and the right number of each lineage is formed as a result of division orientations being guided by embryo geometry and the apical domain itself. Furthermore, the lineages are adjusted by internalization and further polarization mechanisms so that the outer layer is populated by polar TE, and the inner layer by ICM.

In many organisms, the first cell fate decision is determined by a pre-patterned axis of molecular determinants laid down in the early embryo, often in the egg, so the first cleavage division already separates two cell types. It has been shown that in the mouse, cell fate biases exist between blastomeres of the embryo earlier than the 8-cell stage, and although these do not definitively distinguish each cell type, they bias the future fates of each blastomere and their developmental potential. In this way, the mouse embryo has multiple symmetry breaking mechanisms that combine in order to specify final cell fate.

In the case of the human embryo, the role of polarity is less clear. Recent studies have suggested a high level of conservation between the pathways leading to the first cell fate decision in human, and polarity has been implicated in reinforcing the TE program. However, a role for molecular heterogeneities and other symmetry breaking mechanisms is unknown, and it is likely that a high level of redundancy is present given the regulative nature of the mammalian embryo (Arias et al., 2013; Lawrence & Levine, 2006).

5.2 Where next on polarity and cell fate?

Our understanding of the mechanisms that influence the first cell fate in mammalian embryos is continuously improved but whilst we have a good

understanding of how the ICM and TE lineages are segregated, important questions remain.

One key question that has been brought up multiple times in this review is how different aspects of the first cell fate decision come together. How does molecular heterogeneity at the 2- and 4-cell stage, such as that of CARM1, combine with the role of cell polarization at the 8-cell stage? What role does metabolism play in the first cell fate decision? A key question relates to the origin of symmetry breaking, and as more mechanisms involved in the first cell fate decision become uncovered, it is increasingly important to unify them in a working model for lineage segregation of the mammalian embryo. Live-imaging techniques, new-omics technologies, as well as computational modeling and artificial intelligence-based technologies, can all aid in our understanding of these theories.

The last few years has seen a significant advancement in our understanding of apical-basal polarity in the mammalian embryo. With the improvement in our ability to manipulate embryos and record their developmental progress, we have reached an understanding of the transcription factors and molecular effectors that initiate formation of the apical domain, and we have begun to extrapolate our understanding of polarity to other organisms and investigate human embryos. Understanding the first cell fate decision in human embryos is an exciting avenue of research, given that it represents the origin of patterning that leads to our own development.

In addition to the open questions of polarity and cell fate in human embryos, fundamental questions about the mouse apical domain remain unanswered: what is the purpose of an apical cap and not an apical surface akin to other epithelia? Is there a function for surface microvilli? Early mammalian development remains a fascinating field of study with incredible potential for future research on symmetry breaking mechanisms and the first cell fate decision.

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