Chapter 11
Formation of Distinct Cell Types in the Mouse Blastocyst

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Abstract Early development of the mouse comprises a sequence of cell fate decisions in which cells are guided along a pathway of restricted potential and increasing specialisation. The first choice faced by cells of the embryo is whether to become trophectoderm (TE) or inner cell mass (ICM); TE is an extra-embryonic tissue which will form the embryonic portion of the placenta, whilst ICM gives rise to cells responsible for generating the foetus. In the second cell fate decision, the ICM is further refined into pluripotent cells forming the future body of the embryo, epiblast (EPI) and extra-embryonic primitive endoderm (PE), a tissue essential for patterning the embryo and establishing the developmental circulation. Understanding this early lineage segregation is critical for informing attempts to capture pluripotency and direct cell fate in vitro. Unlike the predictability of nonmammalian cell fate, development of the mouse embryo retains the flexibility to adapt to changing circumstances during development. Here we describe these first cell fate decisions, how they can be biased whilst maintaining flexibility and, finally, some of the molecular circuitry underlying early fate choice.

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11.1 Introduction

Embryonic development comprises a series of fate decisions whereupon cells decrease in potential and increase in specialisation. In contrast to nonmammalian species where first fate decisions are predictable, development of the mouse embryo is regulative; it has the flexibility to adapt to changing circumstances. For example, one or two cells (blastomeres) from an eight-cell stage human embryo can be removed for pre-implantation genetic diagnosis without negatively impacting on developmental outcome (Handyside et al. 1992). The same is true in more drastic circumstances: following destruction of one blastomere of the two-cell stage mouse embryo, the surviving blastomere can develop to term (Tarkowski 1959). To further demonstrate this developmental flexibility, the position of cells can be altered experimentally and they adapt to their new position, taking on the appropriate fate (Suwinska et al. 2008). This is a unique feature of mammalian development relying upon the fact that cells at this stage are not yet fully committed to a specific lineage (Rossant and Lis 1979; Rossant and Vijh 1980). This flexibility in early development is thought to facilitate formation of the essential first three lineages, which will support implantation and continued development of the embryo to term. Although there remain many unanswered questions as to how the embryo is endowed with this remarkable regulative capacity, we shall see in this chapter that we have a grasp of the molecular circuitry rooted in this early event and later mechanisms which help maintain flexibility in development, thus creating a robust system to establish the first lineages.

Three distinct cell types arise in the 4.5 days of development between the time of fertilisation and implantation: the epiblast (EPI), primitive endoderm (PE), and trophectoderm (TE). EPI will give rise to all the cells of the body; PE forms the yolk sac, a structure responsible for patterning the embryo and initiating developmental circulation prior to establishment of an internal circulatory system; the TE will form the embryonic portion of the placenta (Fig. 11.1). The priority of the embryo at this early stage centres on implantation where the TE takes on the role of providing chemical and physical integration with the uterus. The TE is the first distinct lineage, whereas the EPI and PE are initially mixed within the inner cell mass (ICM) of the embryo. ICM and TE lineages are physically separated by 3.5 days after fertilisation in a structure referred to as the blastocyst (from Greek *blastos*, meaning *bud*). Visual inspection of the blastocyst reveals a fluid filled ball of cells, inside which lies a mass of cells asymmetrically positioned to one side. These inside cells constitute the pluripotent ICM, whereas the surrounding outside cell population forms the differentiated extra-embryonic TE. The formation of these inside and outside cell populations are the foundation of the first cell fate decision, but how do cells become different? To understand the establishment and properties of the first three lineages, we must understand the developmental events leading up to and contributing to their formation.

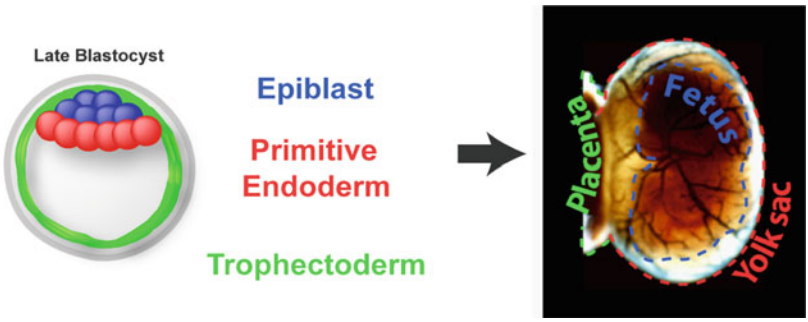

Fig. 11.1 Three distinct lineages of the pre-implantation mouse embryo: epiblast, primitive endoderm and trophectoderm, giving rise to the foetus, yolk sac and placenta, respectively

11.2 Events Leading to Formation of the Blastocyst

Prior to implantation, the first seven cell cycles are cleavage divisions in which cells halve in size, due to the absence of growth (Lehtonen 1980). After fertilisation in the ampulla of the oviduct, a region in close proximity to the ovary, the first cleavage begins around a day later as the embryo travels towards the uterus.

Mammalian cleavage is unique; in addition to the slow, asynchronous divisions (mammalian embryos frequently contain odd numbers of blastomeres) and early activation of the genome (Schultz 2002), the second cleavage in particular has been a subject of much interest due to its unique orientation. The first cleavage is a regular meridional division, following the direction of the animal–vegetal axis. However, in the second cleavage, it is common for one blastomere to divide meridionally, and the other to divide equatorially, this is termed *rotational cleavage* (Fig. 11.2) (Gulyas 1975). The order and orientation of the second cleavage divisions has been found to influence later patterning. This was a provocative finding as the traditional view of early mammalian development considered it to be random, since pre-implantation development can withstand perturbation and there is no clear morphological axis determination until after implantation.

Following a further round of cleavage to the eight-cell stage, where all blastomeres are in contact with the extracellular space between the embryo and zona pellucida (ZP: a glycoprotein membrane surrounding the embryo, produced during oogenesis, functioning to prevent polyspermy), the embryo begins a transformation marked by the formation of an inside cell population as it transitions to the 16-cell stage. After a fifth round of cleavage to produce a 32-cell stage embryo, there is an osmotic accumulation of water in-between cells which establishes the blastocyst cavity, driven by a trans-trophectoderm sodium ion gradient (Watson 1992). The ICM is asymmetrically positioned to one end within this cavity (Fig. 11.3). Concerning the formation of the blastocyst, three models exist to account for its generation, which we will discuss in detail. Each model centres on the establishment of the inside and outside cell populations—is this totally random, or do initial differences between cells exist which influence their final fate?

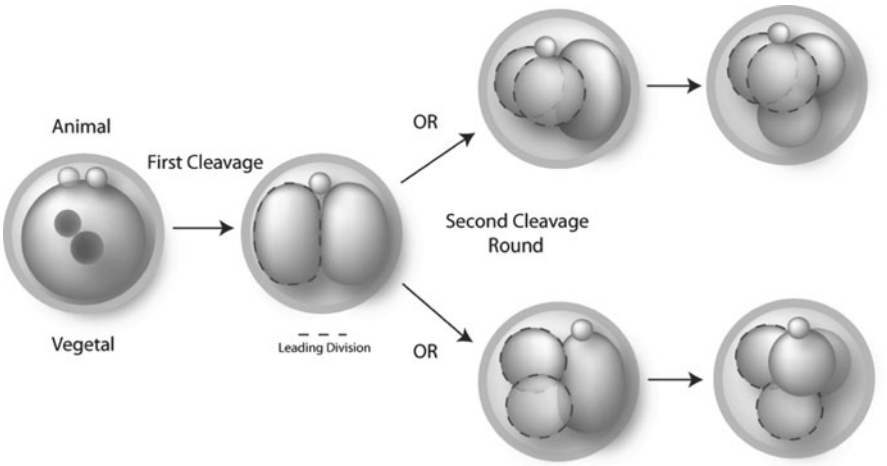

Fig. 11.2 Rotational cleavage. The first cleavage is a regular meridional division, following the direction of the animal–vegetal axis. In the second cleavage, it is common for one blastomere to divide meridionally, and the other to divide equatorially

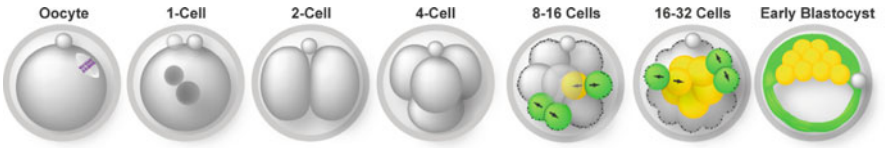

Fig. 11.3 Formation of the blastocyst. Generation of inside cells commences from the 8- to 16-cell stage transition. At the 32-cell stage, fluid accumulates in a cavity of the embryos, completing formation of the blastocyst

11.2.1 The "Early-Asymmetry" Hypothesis

The "early-asymmetry" hypothesis in its most extreme form was rooted in studies of nonmammalian embryos, where partitioning of determinants in combination with standardised cleavage patterns was held responsible for determination of cell fate. This view proposed that asymmetry of the egg would generate differences between cells that would dictate their final fate (Dalcq 1957). Support for this hypothesis came from studies where asymmetric distribution of the leptin protein hormone and STAT3 transcription factor was discovered along the animal–vegetal (AV) axis in oocytes and embryos (Antczak and Van Blerkom 1997). In addition to this, earlier studies where silicon oil droplets were injected to mark central or peripheral cytoplasm in two- and four-cell stage embryos (Graham and Deussen 1978; Wilson et al. 1972) suggested a relationship between early position and later cell fate. However, this was only correlative, and proof for the early-asymmetry hypothesis in its most drastic interpretation would rely on maintenance of cell fate following manipulation of cells into alternate positions. Rather than support the early-asymmetry hypothesis, when

such manipulative experiments were eventually performed, the hypothesis was ruled out to account for lineage establishment in the early mouse embryo.

11.2.2 The "Inside–Outside" Hypothesis

This is perhaps the most conceptually simple of the models to account for formation of inside and outside cell populations. The "inside–outside" model hypothesised that blastomeres are equivalent and totipotent until around the 32-cell stage, at which point some blastomeres are surrounded by other blastomeres, resulting in microenvironmental positional differences which would then dictate cell fate (Tarkowski and Wroblewska 1967). This model was supported by experiments where labelled blastomeres were placed into inside or outside positions within an unlabelled embryo. When positioned outside, the labelled blastomeres contributed to TE the reciprocal was true with inside-placed labelled blastomeres contributing to ICM (Hillman et al. 1972), thus demonstrating that blastomeres respond to positional cues. Still though, it remained unknown what, if anything, governed cell position, and with the discovery that eight-cell stage blastomeres become polarised along their apical–basal axes (Johnson and Ziomek 1981), prior to establishment of inside and outside populations, it seemed the model for blastocyst formation would have to be revised.

11.2.3 The Polarisation Hypothesis

Two and a half days after fertilisation, the embryo consists of eight loosely arranged blastomeres. Through the duration of the eight-cell stage, the physical properties of cell–cell contacts undergo a dramatic phenotypic change, with blastomeres transitioning from a spherical appearance to flatten against each other, giving the embryo a smooth appearance. At the cellular level, blastomere polarity is established, resulting in an apical face covered in microvilli and smooth basolateral cell–cell contacts (Handyside 1980; Reeve and Ziomek 1981; Ziomek and Johnson 1980). This polarity is maintained throughout the subsequent two cleavage divisions to the 32-cell stage. It was proposed that the polarisation of blastomeres was a critical event in creating differences between cells which would lead to lineage segregation (Johnson and Ziomek 1981); the apical pole of microvilli is structurally stable, and it is the inheritance of this pole which functions as an outer determinant, in that any cell inheriting it becomes polarised (Wiley and Obasaju 1988). Inside cells are generated when some of these eight-cell blastomeres divide to generate an inside and an outside cell in a so-called differentiative division, which can be considered as an asymmetric division since the resulting cells take different position and fate. This contrasts to conservative (or symmetric) divisions responsible for the production of two outside cells (Johnson and Ziomek 1981). The question is, which cells divide asymmetrically? What this model does not address is if it is random or pre-determined to any extent.

11.2.4 Integration of the Models

There has traditionally been an inclination to favour one of the above models over the others, but are all three models of blastocyst formation mutually exclusive? For example, even though inside and outside cells differ from the moment they are established, with polarity clearly involved, they are still able to respond to an experimental change in position. The links between polarity and position are clear, but are there any findings that early asymmetry is integrated to enhance our understanding of blastocyst development. Indeed, there is evidence that the order and pattern of the second cleavage divisions can influence cell fate.

The majority of embryos (over 80 %) divide in the two- to four-cell transition to form a tetrahedral structure, where one cell has divided meridionally and the other equatorially (Gardner 2002). Within this major group, several key discoveries of developmental bias have been uncovered where the first cleavage is meridional (M), and the second equatorial (E, a so called "ME" embryo). Firstly, it has been demonstrated that blastomeres in four-cell stage ME embryos are not equivalent; when chimeras of a like cells were constructed, the blastomeres inheriting material from both the animal and vegetal pole in the meridional division (AV blastomeres) were fully competent to develop into a live mouse. In contrast to this, the animal (A) and vegetal (V) blastomeres produced upon the subsequent equatorial cleavage (which divides the animal and vegetal cytoplasm) lack full developmental potential. This is demonstrated by animal chimeras producing live mice in only 25 % of cases, whereas vegetal chimeras are not viable (Piotrowska-Nitsche et al. 2005). Further support for the relation between developmental bias and early cleavages comes from live-imaging of unmanipulated embryos; AV blastomeres of ME embryos were found to contribute to the ICM whereas V blastomeres preferentially contributed to the extra-embryonic lineages of PE and TE (Bischoff et al. 2008).

As we will see in the following discussion, understanding molecular regulation helps to resolve the importance of each model in understanding blastocyst formation, and now with the advent of molecular biology and live-imaging technologies we have gained a better understanding of how all three models, early asymmetry, position, and polarity, can be integrated.

11.3 Interplay Between Cell Polarity, Position and Fate

11.3.1 Polarity: PAR Proteins

Partitioning defective (PAR) proteins, originally discovered in the worm, *Caenorhabditis elegans*, have been implicated in the regulation of cell polarisation and asymmetric division. PAR homologues, and their interactors, atypical protein kinase C (aPKCs), are expressed asymmetrically in mouse oocytes and embryos. For example, members of the Par complex, JAM1 (Thomas et al. 2004), aPKC, and

PAR3 (Plusa et al. 2005) become apically localised at the eight-cell stage, whilst Par1 is localised basolaterally (Vinot et al. 2005). Tight junctions will progressively form between the blastomeres to eventually establish the epithelium of the TE (Fleming et al. 2001). Downregulation of polarity complex members, aPKC or PAR3, in individual blastomeres drives their progeny into an inside position where they develop as ICM, either by promoting asymmetric division or engulfment by more polarised neighbours (Plusa et al. 2005). The reciprocal is also true; transplantation of inside cells to an outside position results in their polarisation and contribution to TE (Handyside 1978; Rossant and Lis 1979; Spindle 1978), thus demonstrating the interplay between polarity and position. Moreover, when expression of pluripotency-related genes is enhanced in a blastomere, its progeny are directed to the pluripotent ICM (Torres-Padilla et al. 2007), facilitated by downregulation of cell polarity (Parfitt and Zernicka-Goetz 2010). Is the same also true in that genes regulating TE formation are implicated in polarity?

11.3.2 Cdx2: Interplay of Cell Fate, Polarity and Early Asymmetry

Can polarity and position affect expression of genes driving lineage segregation in the early mouse embryo? Cdx2 is a transcription factor (TF) central to TE formation; Cdx2 protein is restricted to the TE of the blastocyst, where in the absence of Cdx2, TE identity cannot be maintained (Strumpf et al. 2005). The initiation of Cdx2 protein expression was found to be heterogeneous in the eight-cell stage embryo (Dietrich and Hiiragi 2007; Ralston and Rossant 2008), and was therefore suggested to arise at random (Dietrich and Hiiragi 2007). In a series of experiments addressing the role of Cdx2 in segregation of the TE lineage, Jedrusik et al. (2008) found that Cdx2 expression levels related to early cleavage divisions; in ME embryos, the progeny of the vegetal blastomere were found to express higher levels of *Cdx2* mRNA and Cdx2 protein. This was in agreement with earlier findings of such vegetal cells contributing more frequently to TE (Bischoff et al. 2008) and having little potential to develop to term (Piotrowska-Nitsche et al. 2005). Probing the mechanism in further detail, blastomeres in which *Cdx2* expression was experimentally enhanced, prior to inside cell generation, exhibited a higher frequency of symmetric divisions, and hence contribution to the outside TE population. Moreover, these blastomeres in which *Cdx2* was elevated appeared to be more highly polarised, on the basis of apical aPKC localisation (Jedrusik et al. 2008). In addition to this, polarity was found to influence localisation of *Cdx2* mRNA, thus creating a mutually reinforcing loop between polarity and Cdx2 expression to establish TE fate. Together, this demonstrates the interplay between early asymmetry, gene expression and polarity, which in turn directs cell allocation to either the inside or outside lineages. A question remained though: how do cells sense their position in the embryo to respond to it; by up- or downregulating polarity to elicit a position change, or by adjusting lineage-specific gene expression?

11.3.3 Hippo Signalling: Unlocking Position, Polarity and Fate?

How are differences translated into differential gene expression in inside and outside cells? An ideal candidate could function by sensing cell position through cell–cell contacts. In mammals, Hippo signalling controls growth through cell contact-mediated control of proliferation (Pan 2007). Cell–cell contact regulates the nuclear accumulation of Yes-associated protein 1 (Yap1) through Hippo signalling and controls cell proliferation by regulating transcriptional activity of Tead proteins (Ota and Sasaki 2008). Interestingly, Tead4 null mice die shortly after implantation due to reduced cell proliferation and increased apoptosis (Sawada et al. 2008) and Cdx2 expression is controlled by the transcriptional regulator, TEAD4 (Nishioka et al. 2008; Yagi et al. 2007), thus establishing a link between cell contact/position and lineage-specific gene expression. Prior to the blastocyst stage, Yap1 is localised to the nucleus of outside cells (cytoplasm of inside cells), regulated by phosphorylation via the Hippo signalling pathway member kinases Lats1/2. Here, in the nucleus, Yap can directly interact with its transcriptional co-activator Tead4 to stimulate transcription of *Cdx2* (Nishioka et al. 2009). As yet, the identity of the Yap-regulating signals that can sense cell position remain unknown, but likely involve the Hippo signalling pathway and possibly proteins involved in cell contact such as cadherins. Nonetheless, this recent advance represents a promising direct link between cell position and gene expression.

Taken together, the above examples demonstrate the interplay between early asymmetry, position, polarisation and cell fate. First of all, blastomeres are not identical at their inception; gene expression relates to developmental history, with Cdx2 expressed in a distinct blastomere population. This gene expression is in turn able to effect a change in polarisation, which itself is able to translate into specific positioning within the embryo, either executed through orientation of division or physical movement of entire cells. Furthermore, polarity feeds into gene expression, reinforcing cell identity. We must remember though that developmental flexibility is a hallmark of the mouse embryo, thus this sequence of events as described is not absolute. This is demonstrated by the capacity for polarity and gene expression to adapt to a change in blastomere position within the embryo, and vice versa. We have recently been able to garner a deeper understanding of this elegant interplay through improved technologies to uncover the molecular circuitry underlying developmental flexibility. To understand the segregation of embryonic (ICM) and extra-embryonic fates (TE) in greater detail, we must address the second cell fate decision in pre-implantation development where the ICM is further refined into the EPI and PE lineages.

11.4 The Second Cell Fate Decision: Lineage Segregation of the Inner Cell Mass

A second cell fate decision distinguishes two ICM cell types: pluripotent EPI, stem cells for the future foetus; and PE, the second extra-embryonic tissue that becomes visceral endoderm and parietal yolk sac after implantation (Gardner 1982). Of the

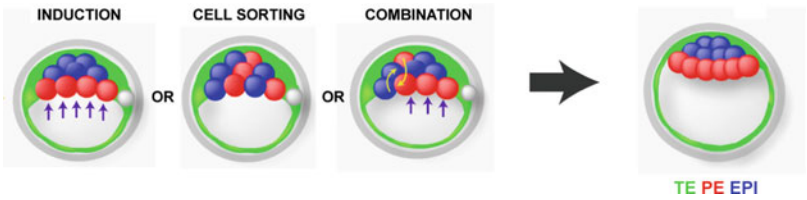

Fig. 11.4 Models of PE and EPI segregation in the second cell fate decision. Induction: PE is induced in the cells contacting the blastocyst cavity, where they will finally reside. Cell sorting: EPI and PE are specified randomly, or in relation to developmental history and subsequently sort into their correct positions. Alternatively, the induction and cell sorting models may be combined

numerous possibilities to account for this fate decision, one of the earliest was the Induction (or Positional) hypothesis. In this, the fate of ICM cells was thought to reflect their position: surface ICM cells next to the blastocyst cavity would become PE, and deeper cells, epiblast. Consistent with this, the outside cells of isolated ICM or embryoid bodies formed from embryonic stem (ES) cells differentiate into endoderm when cultured in vitro (Martin and Evans 1975; Rossant 1975; Solter and Knowles 1975; Dziadek 1979; Murray and Edgar 2001). Later studies of the differential distribution of cytokeratin filaments in blastomeres suggested that inner cells generated in two successive rounds of asymmetric divisions might physically differ (Chisholm and Houliston 1987) leading to speculation, without definitive evidence, that this might be the route towards PE and epiblast formation (Rossant et al. 2003). The subsequent finding that the PE marker Gata6 and the epiblast marker Nanog "were expressed in a random 'salt-and-pepper' pattern" in early blastocysts in advance of PE formation (Chazaud et al. 2006; Kurimoto et al. 2006) gave rise to the idea of a mixed population of epiblast and PE progenitors that would then segregate into their composite layers: the Sorting model. The origins of this pattern had never been addressed, but it was widely taken to have stochastic origins. The apparent lack of any relationship of this heterogeneity to cell position seemed to signal the demise of the Positional/Induction model. However, neither the positional nor the sorting model alone appears sufficient to account for PE origins because when single surface ICM cells were injected with lineage markers in the early blastocyst, they gave rise predominantly to PE but a minor proportion gave epiblast or even both epiblast and PE lineages (Fig. 11.4) (Perea-Gomez et al. 2007; Weber et al. 1999).

Despite this multitude of theoretical proposals, a definitive explanation of the heterogeneity of the early ICM had been lacking because all interpretations were of fixed rather than living, dynamic preparations. The first, recently published time-lapse study, starting at the early blastocyst stage, demonstrated that at least some deep ICM cells expressing Pdgfrα, a marker of the late PE, move to the ICM surface (Plusa et al. 2008). Unfortunately, this work could not follow the behaviour of all ICM cells and thus the question of genesis and behaviour of the epiblast was left open. The implication was, however, that the intermingling of epiblast and PE progenitors was the consequence of a stochastic process. There are reasons to be

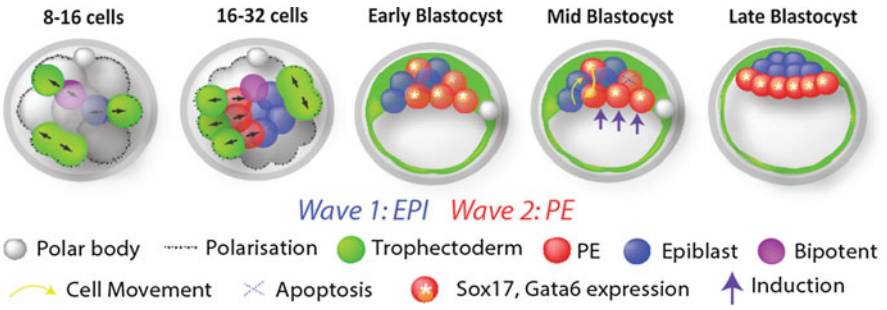

Fig. 11.5 The influence of developmental history on lineage segregation in the ICM. EPI is derived from wave 1 internalised cells, whereas PE is derived from the later, wave 2 internalisations

cautious about this interpretation. First, these particular time-lapse studies commenced when it was only possible to observe the resolution of the "salt-and-pepper" pattern and not how the pattern originated. Second, the "PE-reporter" gene used is initially expressed in a broad domain before becoming restricted to PE, making it impossible to unambiguously identify PE progenitors by this criterion. Thus, it had remained unknown how intermingling of epiblast and PE progenitors first arises and exactly how it becomes resolved into both epiblast and PE lineages.

To resolve these different views and to uncover the genesis of ICM lineages, the origin, behaviour and fate of each and every individual cell was followed as it developed into either PE or epiblast. This revealed that the developmental timing with which cells become set aside in the inside part of the embryo by successive waves of asymmetric division dictates their fate. Thus, inside cells generated by the first wave during the 8- to 16-cell transition give rise to the majority of epiblast cells. Conversely, the majority of PE cells are progeny of inside cells generated by the second wave (Morris et al. 2010). This relationship between cell fate and developmental history is most apparent when the ICM is generated from equal proportions of early and late asymmetric divisions, as is most often the case. When the ICM is derived mostly from the early first wave, these cells have the flexibility to generate both the EPI and PE (Morris et al. 2010) (Yamanaka et al. 2010). Thus, the asymmetric divisions, which play such an important part in the first cell fate decision, also have a major impact upon the second cell fate decision (Fig. 11.5).

11.4.1 Molecular Circuitry of the Second Cell Fate Decision

The transcription factors Gata4 and Gata6 contribute to development of extra-embryonic endoderm after implantation (Morrisey et al. 1998; Koutsourakis et al. 1999). Both genes are expressed at the early blastocyst stage (Wang et al. 2004) and drive PE formation when overexpressed in ES cells (Fujikura et al. 2002; Shimosato et al. 2007). However, these might not be the sole transcription factors required to specify the PE and indeed the Sox17 protein appears important for endoderm differentiation in different model systems (Kanai-Azuma et al. 2002) (Niakan et al. 2010). Sox17 levels rise dramatically when PE becomes specified at the

blastocyst stage (Wang et al. 2004) beginning in PE progenitor cells of the second wave of asymmetric divisions (Morris et al. 2010). Consistently, downregulating levels of Sox17 in individual blastomeres prevents their forming PE and promotes their development to EPI. Conversely, overexpression of Sox17, enhanced by Gata6, directs cells to the PE lineage.

11.4.2 The Role of FGF Signalling in the Second Cell Fate Decision

Intact FGF signalling is essential for PE formation, where loss of the FGF receptor, or ligand, FGF-4 results in the absence of PE differentiation and peri-implantation lethality (Arman et al. 1998; Feldman et al. 1995). The exact mechanism by which FGF signals participate in ICM patterning is currently unknown, although attempts to integrate it into stochastic and origin-dependent models have been made.

In the stochastic model of ICM pattering—it is proposed that individual ICM cells randomly respond to different levels of FGF signalling, leading to the "salt-and-pepper" distribution of EPI and PE progenitors as described above. This model was developed from the finding that chemical inhibition of FGF signalling with small molecules blocks differentiation into PE and promotes the formation of an ICM consisting purely of EPI cells (Nichols et al. 2009; Yamanaka et al. 2010). Conversely, when embryos are treated with high-level exogenous FGF-4 ligand, the ICM differentiates entirely into PE (Yamanaka et al. 2010). This lead to the conclusion that cells of the early ICM have the potential to become either EPI or PE—and stochastic fluctuations in FGF signal intensity drive the switch between pluripotency and differentiation.

The finding that ICM cells possess early flexibility does not preclude the influence of developmental history on the capacity of a cell to transduce FGF signals. For example, the FGF4 receptor, FGFR2, has been found to be expressed at higher levels in outside cells of the 16-cell stage embryo (Guo et al. 2010). As these are the cells which generate the second wave divisions giving rise to PE progenitors, raising the possibility that such cells inherit FGFR2 conferring competence to form PE. In contrast to this, those early wave 1 internalised cells would have little or no FGFR2 receptor, rendering them "blind" to FGF4 ligand and fated to EPI. In support of this, in the early blastocyst, PE markers Sox17 and Gata6 expression cluster with FGFR2 expression whereas Nanog expression clusters with that of FGF4 (Kurimoto et al. 2006).

In summary, the first and second cell fate decisions both demonstrate the remarkable capacity for the early mouse embryo to maintain the flexibility to adjust to changing circumstances during its development. Even though these adaptive mechanisms exist, it does not rule out the existence of biases, in which developmental history influences cell fate. Through examining these facets and flexibilities of patterning, we may build towards a more complete understanding of patterning in the early embryo, and learn how to direct it in vitro in stem cells for clinical use in regenerative medicine.

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