CHAPTER FIFTEEN

Making the Mouse Blastocyst: Past, Present, and Future

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Contents

Abstract

The study of the preimplantation mouse embryo has progressed over the past 50 years from descriptive biology through experimental embryology to molecular biology and genetics. Along the way, the molecular pathways that lead to the establishment of the three cell lineages of the blastocyst have become more clearly understood but the fundamental questions of lineage commitment remain the same as those laid out in early studies. With new tools of genome manipulation, *in vivo* imaging and single-cell analysis, the mouse blastocyst is an excellent model system to understand how organized cell fate decisions are made in a self-organizing developmental context.

1. INTRODUCTION

The last 50 years have encompassed enormous progress in our understanding of the processes of embryonic development in many different model organisms. The genetic programs underlying the initial establishment of different cell lineages have become clearly defined and the complexities of

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morphogenesis and organogenesis are being explored with ever more refined technological tools. And yet, the fundamental questions of development remain the same and still drive our studies today.

Experimental studies of the preimplantation mouse embryo span this 50-year period and have advanced from cellular description to refined lineage modeling and molecular analysis. They were made possible initially by the development of defined culture systems to grow mouse eggs to the blastocyst stage (Brinster, 1963; Whitten & Biggers, 1968), embryo transfer techniques (Mc & Biggers, 1958), and chimera approaches (Mintz, 1965; Tarkowski, 1961). Several questions were posed early on in the field and still are being explored today.

- What is the later fate of the lineages of the blastocyst: trophectoderm (TE) and inner cell mass (ICM), and epiblast (EPI) and primitive endoderm (PE)?
- When are cells restricted to their fate?
- What are the cellular and molecular mechanisms that establish cell fate?

In this essay, rather than provide a broad-based review of the field, I have taken five early papers that addressed some of these issues and asked whether the conclusions stand the test of time, or whether newer technologies have enhanced, modified, or nullified the conclusions. In general, we can still find lessons to learn from the early papers, although maybe not from the rather wordy, discursive style in which they were written!

2. WHAT IS THE LATER FATE OF THE INNER CELL MASS AND TROPHECTODERM?

Gardner, R. L., Papaioannou, V. E., & Barton, S. C. (1973). Origin of the ectoplacental cone and secondary giant cells in mouse blastocysts reconstituted from isolated trophectoderm and inner cell mass. *Journal of Embryology and Experimental Morphology 30*, 561–572. (Gardner, Papaioannou, & Barton, 1973)

In 1968, Richard Gardner published the production of mouse chimeras by injection of inner cell mass (ICM) cells into the blastocyst (Gardner, 1968). Here he took this approach one step further and, by a heroic feat of micromanipulation, generated reconstituted blastocysts, in which the entire ICM was of one genotype and the trophectoderm of another. Using isozymal variants of glucose phosphate isomerase, the most sensitive genetic marker available at the time (Chapman, Ansell, & McLaren, 1972), to distinguish the two genotypes in later tissues, they attempted to define the boundaries

of ICM and trophectoderm contributions to the fetus and its extraembryonic membranes. The results were indicative but not clear-cut:

> *"The trophoblast of the implanted embryos was almost exclusively of the trophoblast donor GPI type. The embryos plus membranes were mainly of the ICM-donor type but most also showed a substantial proportion of trophoblast-donor type."*

Papaioannou followed up in 1982 (Papaioannou, 1982) with a more sensitive GPI assay and obtained more convincing evidence of separation of cell fates. However, definitive results required the development of *in situ* genetic markers so that the origin of specific cell types within a complex structure like the placenta or the yolk sac could be identified. Gardner and Johnson generated interspecific chimeras with rat ICMs in mouse blastocysts and used species-specific antibodies to distinguish the resulting chimeras (Gardner & Johnson, 1973). Rossant et al. used *in situ* hybridization against repetitive DNA differences between two species of mice (Rossant, Vijh, Siracusa, & Chapman, 1983) and also used DNA *in situ* hybridization against a large repeat transgene (Rossant & Croy, 1985), thus avoiding issues of potential rat-mouse incompatibilities. All these experiments gave the same clear results; the ICM gave rise to the entire fetus, the extraembryonic yolk sacs, and the allantois, while the trophectoderm produced the trophoblast giant cells, the extraembryonic ectoderm, and the ectoplacental cone, which make up the majority of the placenta (Fig. 1).

The only real caveat to these experiments was that the reconstituted blastocyst might differ in behavior from the undisturbed embryo (Georgiades, Cox, Gertsenstein, Chawengsaksophak, & Rossant, 2007). In particular, the polar trophectoderm overlying the ICM is excluded from a reconstituted blastocyst. With today's refined lineage tracing technologies, we can easily reconfirm the chimera results by direct labeling of the intact blastocyst. For example, infection of the blastocyst with a GFP-expressing lentivirus labels all TE cells and only TE cells and later concept uses show GFP expression restricted to the extraembryonic ectoderm and ectoplacental cone (Georgiades et al., 2007).

3. WHAT IS THE LATER FATE OF THE EPIBLAST AND PRIMITIVE ENDODERM?

Gardner, R. L. & Rossant, J. (1979). Investigation of the fate of 4.5 day post-coitum mouse inner cell mass cells by blastocyst injection. *Journal of Embryology and Experimental Morphology 52*, 141–152. (Gardner & Rossant, 1979)

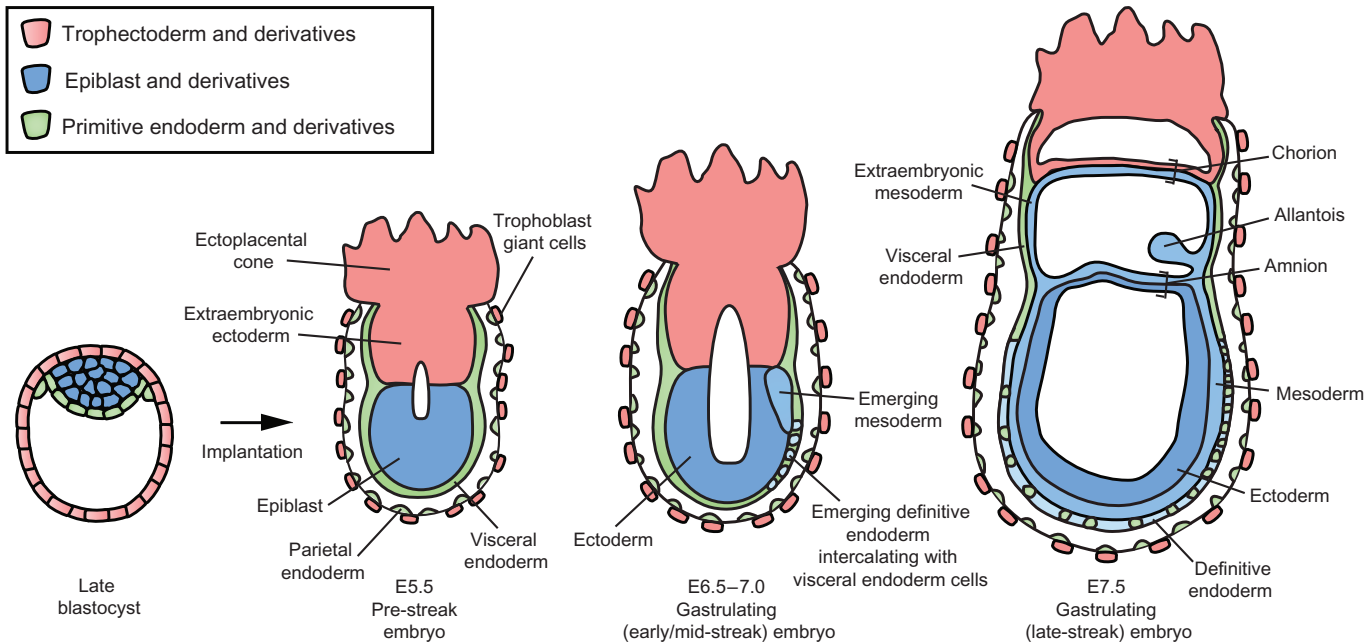

Figure 1 Postimplantation lineage derivatives of the epiblast, primitive endoderm, and trophectoderm. Trophectoderm (red) gives rise to all the later trophoblast lineages that contribute to the developing placenta, as well as to the trophoblast giant cells. Primitive endoderm (green) gives rise to the extraembryonic visceral and parietal endoderm, as well as providing a minor contribution to the developing gut along with the definitive endoderm. The epiblast (blue) gives rise the post-implantation primitive ectoderm, and to the mesoderm and definitive endoderm that arise at gastrulation. Extraembryonic mesoderm arises from the posterior of the primitive streak and forms the allantois and the mesoderm layers of the developing yolk sac, where primitive hematopoiesis occurs.

By E4.5, the inner cell mass has clearly segregated into two cell types; a monolayer of cells on the blastocoelic surface called primitive endoderm or hypoblast and an enclosed group of cells called the primitive ectoderm or epiblast (Fig. 1). In this chapter, Gardner and Rossant used cell morphology, without the aid of any molecular markers, to show that rough and smooth cells dissociated from the E4.5 ICM correspond to primitive endoderm and epiblast, respectively. They then injected isolated rough and smooth cells into host blastocysts and showed that the primitive endoderm gave rise to the endoderm but not the mesoderm of the visceral yolk sac, but that the definitive endoderm arose from the epiblast lineage.

They concluded:

> *"The present results demonstrate that morphological differentiation of the ICM in the late blastocyst into two populations of cells is accompanied by the acquisition of distinct cell states, as judged by the criterion of behaviour of the cells following transplantation. We believe* (sic)*, furthermore, that they provide an accurate description of the normal lineage of primitive endoderm and ectoderm cells. If this is the case, the former cells give rise to the extra-embryonic endoderm of the conceptus, and the latter to the entire foetus plus extra-embryonic mesoderm."*

While apparently based on belief rather than completely definitive experimental results, these conclusions stood the test of time for many years and were confirmed and extended in chimeras using more sensitive genetic markers.

However, with the advent of GFP-expressing transgenic lines and live-cell imaging, a more nuanced view of the fate of the primitive endoderm has come into play. Incidental observations from tetraploid complementation experiments with embryonic stem cells (Dufort, Schwartz, Harpal, & Rossant, 1998) had shown the persistence of some tetraploid primitive endoderm-derived cells in the definitive endoderm as it forms during gastrulation, but it was not clear whether this occurred in the intact embryo. The Hadjantonakis lab used GFP-lineage specific transgenic lines to show that not all primitive endoderm cells were displaced from the developing definitive endoderm (Kwon, Viotti, & Hadjantonakis, 2008). PE-derived progeny could persist and be observed in the later gut tube. Whether these cells take on definitive endoderm properties or whether they have distinct functions in the gut is still unclear, but the earlier proposed clear distinction between the cell fates of primitive and definitive endoderm is no longer tenable (Fig. 1). Given the similarities in molecular properties of the two lineages, including the dual roles of transcription factors like Sox17 (Artus, Piliszek, & Hadjantonakis, 2011; Kanai-Azuma et al., 2002) and

Foxa2 (Dufort et al., 1998), the boundaries are definitely getting blurred. This has relevance to the early differentiation of endoderm during embryoid body formation from ES cells. Since ES cells rarely contribute to visceral endoderm in chimeras, the identity of this early endoderm layer *in vitro* is not altogether clear. Is it definitive or primitive or maybe a mixed population similar to the early streak stage embryo?

Once the blastocyst is fully expanded and the epiblast and primitive endoderm layers separated, the three blastocyst cell lineages are clearly distinct. Consistent with this stabilization of cell fate, it is possible to isolate and maintain three different lineage-restricted self-renewing progenitor cell lines from the blastocyst: ES cells are epiblast-derived (Boroviak, Loos, Bertone, Smith, & Nichols, 2014), while trophoblast stem cells derive from the TE (Tanaka, Kunath, Hadjantonakis, Nagy, & Rossant, 1998) and XEN cells from the PE (Kunath et al., 2005).

4. WHAT IS THE MECHANISM THAT DRIVES THE FORMATION OF THE ICM AND THE TE?

4.1 The Inside–Outside Hypothesis

Tarkowski, A. K. & Wroblewska, J. (1967). Development of blastomeres of mouse eggs isolated at the 4- and 8-cell stage. *Journal of Embryology and Experimental Morphology 18*, 155–180. (Tarkowski & Wroblewska, 1967)

Early morphological and enzymatic observations had suggested that the mouse egg might contain intrinsic determinants that were segregated during cleavage into specific precursors of later lineages (Dalcq, 1957; Mulnard, 1961). This concept was attractive since it was in accord with early events in many other vertebrate and invertebrate embryos. Tarkowski himself had proposed such a mechanism based on properties of isolated 2-cell blastomeres (Tarkowski, 1961). In this chapter, Tarkowski and Wroblewska studied in detail the *in vitro* development of blastomeres isolated at the 4 and 8-cell stage. They showed that the majority of isolated 8-cell blastomeres formed trophectoderm vesicles, a result not predicted if there were early segregation of ICM and TE lineages based on cytoplasmic determinants. They realized that the predominance of TE structures from isolated blastomeres was predicted from topological considerations if blastocyst formation always occurs after a set number of cell divisions. Not enough cells would be present at the time of cavitation from an isolated 8-cell blastomere to provide any enclosed cells, thus promoting TE vesicle formation. From these results

and other observations they then proposed what has come to be known as the "inside–outside hypothesis":

> *"It seems reasonable to assume that the conditions in which external and internal cells find themselves are diametrically different. The internal cells, being completely cut off from the exterior, develop in a micro-environment created by the external cells. In our opinion, the position of a cell in the morula and, a consequence of the position, the different environmental conditions play a decisive role in the differentiation of cells in one of the two directions (trophoblast versus inner mass). For the formation of the inner mass it is necessary that certain blastomeres should become isolated from the exterior before the moment when blastocoelic fluid starts to accumulate between the cells."*

This hypothesis, dating back to 1967, has been extremely influential in all the intervening years and underlies much current thinking on the molecular mechanisms promoting ICM versus TE fate. Although there may be some early predisposition of certain blastomeres to give rise to ICM or TE (Piotrowska-Nitsche, Perea-Gomez, Haraguchi, & Zernicka-Goetz, 2005), the overwhelming evidence suggests that formation of the blastocyst can be achieved as a fully self-organizing system, based on the gradual development of differences between inside and outside cells. In the past few years, focus has been placed on components of the Hippo signaling pathway as candidates driving this segregation (Fig. 2). Sasaki and colleagues first showed that a key coactivator of TEAD transcription factors, YAP, is phosphorylated by the Hippo complex serine–threonine kinase, Lats2, in inside cells of the morula (Nishioka et al., 2009). This leads to Yap's exclusion from the nucleus and prevents TEAD4 activity and expression of the TE-specific transcription factor, Cdx2, in the inside cells. In outside cells, the Hippo pathway is inactive, Yap enters the nucleus and Cdx2 expression is upregulated. Other studies have shown that Cdx2, in turn, represses the expression of the ICM-specific pluripotency genes, *Oct4* and *Nanog* (Strumpf et al., 2005), thus leading to restricted expression of key lineage regulators in the appropriate cell layers by the blastocyst stage. Other players in the Hippo pathway, have also been shown to play necessary roles in ICM/TE segregation, including Neurofibromatosis2 (Nf2) (Cockburn, Biechele, Garner, & Rossant, 2013) and Angiomotin (Hirate et al., 2013). Interestingly, Nf2 protein is inherited from the oocyte: maternal/zygotic loss of function leads to morulae/early blastocysts in which Yap is nuclear in all cells, whether inside or outside (Cockburn et al., 2013). Cdx2 is then expressed in the ICM and transforms those cells into a TE fate. Thus, position-dependent activation of Hippo signaling in inside cells is a prerequisite for formation of a functional ICM.

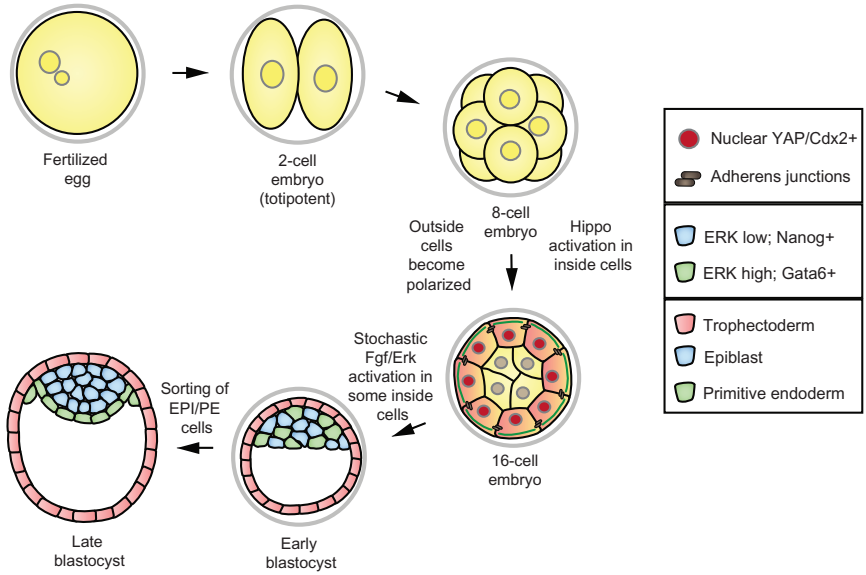

Figure 2 Mechanisms of cell fate specification during preimplantation development. Between the 8- and 16-cell stage, outer blastomeres become polarized and enclose a group of apolar cells. Cell contact and cell polarity lead to differential activation of the Hippo-related Lats kinase in the inside versus the outside cells. Lats phosphorylates Yap in inside cells which causes its exclusion from the nucleus. Hippo signaling is inactive in the outside, polarized cells, Yap is nuclear and activates expression of trophectoderm-specific genes such as *Cdx2*. Absence of *Cdx2* in inside cells allows expression of pluripotency genes and establishment of the inner cell mass. Once the inner cell mass is established at the early blastocyst stage, stochastic differences in the activation of FGF/ERK signaling occur in a mosaic manner within it. Active ERK signaling promotes primitive endoderm formation while absence of ERK signaling promotes epiblast. As the two lineages gradually become specified, they sort out within the ICM so that the primitive endoderm ends up on the blastocoelic surface.

4.2 The Polarization Hypothesis

> Johnson, M. H. & Ziomek, C. A. (1981). The foundation of two distinct cell lineages within the mouse morula. *Cell 24*, 71–80. (Johnson & Ziomek, 1981)

While activation of Hippo signaling in inside cells clearly drives ICM/TE segregation, it begs the question of how Hippo signaling is constrained to inside cells. A major distinction between inside and outside cells of the morula is that the outside cells show apico-basolateral polarity and eventually form a tight junction coupled epithelium, while enclosed cells lack apical polarity (Fig. 2). Martin Johnson's group in the late 1970s and 1980s carried

out extensive cellular studies of the process of polarization, which begins at the 8-cell stage when all cells develop an apical pole. In this chapter and several others (reviewed Johnson & McConnell, 2004), they studied the formation and inheritance of the apical pole during cell divisions from the 8–16 cell stage, mostly using isolated blastomeres and morphology or lectin staining to distinguish the polar region. From these studies, they proposed the "polarization hypothesis":

> *"Two propositions of the polarization hypothesis are … First, each constituent blastomere of the 8-cell mouse embryo …becomes structurally and functionally polarized along a radial axis through the embryo.*
> *Second, elements of the cellular polarity established within each blastomere may be conserved during its division to 16 cells. Such a conservation could, with appropriate cleavage planes, generate two cell populations distinct from the moment of their formation as a result of the inheritance of distinctive regions of cytoplasm and membrane."*

This hypothesis has often been stated as an alternative to the inside–outside hypothesis. However, again more recent studies would suggest a more nuanced interpretation. Johnson and colleagues observed that isolated polarized 8-cell blastomeres often divided such that one cell would inherit most of the apical pole and that these kinds of polarized cell divisions were more common than generation of two polarized progeny. The concept that polarized cell divisions generated inside cells in the intact embryo made sense. However, a set of recent papers using detailed live imaging and 3D reconstruction analysis has suggested that some inner cells in the intact embryo at the 16-cell stage arise not by polarized cell division, but by a process of internalization, involving apical constriction, from an original outside position (Anani, Bhat, Honma-Yamanaka, Krawchuk, & Yamanaka, 2014; Samarage et al., 2015; Watanabe, Biggins, Tannan, & Srinivas, 2014). There is no absolute relationship between the angle of cell division and the later internal or external position of the progeny.

However, the idea that the formation of polarized outside and apolar inside cells is key to the segregation of the ICM and TE still holds. Once an outer polarized epithelium is formed it is apparently rare for progeny of inside cells to emerge again into the outer position, although outside cells can still generate more inside progeny at the 16–32 cell stage. Thus, the physical formation of the outer epithelium will provide a mechanism to allow stable generation of an enclosed environment in which Hippo signaling can lead to long-term transcriptional changes in inside cells. Blocking cell adhesion prevents activation of Hippo signaling (Nishioka et al.,

2009; Stephenson, Yamanaka, & Rossant, 2010), while conversely, blocking cell polarization while leaving cells in close contact (Laeno, Tamashiro, & Alarcon, 2013), promotes active Hippo signaling in both inside and outside cells. While the full mechanisms linking cell position, cell–cell contact and cell polarity with ICM/TE fate remain to be determined, elements of both the inside–outside and the polarization hypotheses are still relevant.

5. WHAT ARE THE MECHANISMS DRIVING SEGREGATION OF EPI AND PE WITHIN THE ICM?

> Rossant, J. (1975). Investigation of the determinative state of the mouse inner cell mass. II. The fate of isolated inner cell masses transferred to the oviduct. *Journal of Embryology and Experimental Morphology 33*, 991–1001. (Rossant, 1975)

In the absence of lineage-specific markers, the mechanism by which the apparently homogenous ICM of the E3.5 blastocyst is transformed within 24 h into two cell types, distinguished by morphology, position, and fate, was somewhat mysterious. Informed by the fact that the PE formed on the surface of the ICM and by comparison with the inside–outside hypothesis of ICM/TE formation, it was usually assumed that cell position somehow drove this process also. With the primitive tools of direct microdissection of ICMs from blastocysts (prior to the development of immunosurgery (Solter & Knowles, 1975)) and promoting development by transferring them back to the oviduct inside empty zonae pellucidae, Rossant reported on the development of isolated ICMs and concluded:

> *"Although no trophoblast was formed, $3^{1/2}$-day ICMs formed an outer endoderm layer after 1 day in the oviduct, as judged by light and electron microscopical evidence. It is suggested that cell position may be important in endoderm differentiation."*

The conclusion about lack of TE formation is consistent with our knowledge of the restricted potential of the ICM. However, the positional interpretation of the fact that all ICMs produced a full layer of PE on the outside has turned out to be not correct. Thirty years later, the Rossant lab produced evidence that the formation of EPI/PE is a much more interesting and challenging process. With the development of lineage-specific gene markers and technologies to observe expression *in situ* in the early embryo, they were able to show that genes such as *Nanog* and *Gata6*, which were thought to be

specific to the EPI and PE, respectively, could already be seen to show variable and complementary "salt and pepper" expression at the E3.5 ICM (Chazaud, Yamanaka, Pawson, & Rossant, 2006). Expression of the markers gradually segregated into the two defined PE and EPI cell layers by E4.5. This suggested that initial activation of lineage specification was position-independent and that cell sorting then generated the two lineages. More detailed and nuanced interpretation of these events has been provided by live-cell imaging of a PE-specific GFP reporter line, showing both cell sorting and death of cells in the wrong position, leading to the final segregation of EPI and PE (Plusa, Piliszek, Frankenberg, Artus, & Hadjantonakis, 2008).

In the 2006 paper from the Rossant lab, they also provided the first clues as the mechanism of the initiation of lineage specification of PE and EPI. In the ICM of embryos mutant for *Grb2*, a key player in signal transduction linking receptor tyrosine kinase signaling to the Ras–MAP kinase pathway, cell numbers were normal but all cells expressed EPI markers and none expressed PE markers (Chazaud et al., 2006). It is now clear from many different studies that FGF4 produced autonomously by cells of the ICM is the driver of the differentiation of the PE (Fig. 2). Blocking FGF/ERK signaling at the blastocyst stage promotes EPI versus PE fate (Nichols, Silva, Roode, & Smith, 2009; Yamanaka, Lanner, & Rossant, 2010) and activating FGF signaling promotes PE versus EPI fate (Yamanaka et al., 2010). Cells within the ICM somehow read out local variation in FGF levels and respond accordingly to generate approximately the right number of EPI versus PE cells. Changes in cellular properties induced by expression of EPI versus PE transcription factors then lead to physical segregation and stabilization of the EPI and PE cell layers. Whether this is entirely a stochastic process or whether the origin of ICM cells from different rounds of cell divisions during cleavage predisposes to EPI or PE fate is somewhat unclear (Morris et al., 2010; Yamanaka, 2011) and the downstream events leading to lineage segregation are not well understood at all.

6. CONCLUSIONS

Building on 50 years of research, the preimplantation mouse embryo is now poised to enter into a new phase as the most powerful system to explore the fundamental processes of lineage development at a single-cell systems level. The entire process can be observed live and imaged *in vitro* in a fully defined culture medium with all the tools of modern imaging and

fluorescent tracers. The process of going from one cell to the 100-cell blastocyst with three distinct lineages takes place slowly over 4 days, so that events of lineage specification can be observed evolving in real time. The advent of single-cell transcriptomics allows detailed correlation of gene expression changes with cell behavior. All the tools of mouse genetic manipulation, including newly developed genome editing technologies, are available to explore gene function. New tools of optogenetics promise to allow manipulation of cell signals and responses at the single-cell level in the living embryo. The future is bright!

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