Development of Pre-implantation Mammalian Blastocyst

Bhanu P. Telugu and Laramie Pence

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Abstract The preimplantation mammalian embryo is a simplistic, self-contained, and a superior model for investigating the inherent complexities of cell fate decision mechanisms. All mammals begin their humble journey from a single-cell fertilized zygote contained within a proteinaceous coat called the zona pellucida. The zygote embarks on a series of well-orchestrated events, beginning with the activation of embryonic genome, transition from meiotic to mitotic divisions, spatial organization of the cells, timely differentiation into committed trophectoderm (TE) and primitive endoderm (PrE), and ultimately escape from zona pellucida for implantation into the uterus. The entire development of preimplantation embryo can be studied in vitro using a minimalistic and defined culture system. The ease of culture along with the

B. P. Telugu (✉)
Department of Animal and Avian Sciences, University of Maryland, College Park, MD, USA

Division of Animal Sciences, University of Missouri, Columbia, MO, USA
e-mail: btelugu@umd.edu; telugub@missouri.edu

L. Pence
Department of Animal and Avian Sciences, University of Maryland, College Park, MD, USA
e-mail: lpence@umd.edu

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ability to manipulate gene expression and image the embryos makes them an ideal model system for investigation into the first two of several cell fate decisions made by the embryo that result in a pluripotent epiblast (EPI) and differentiated TE and PrE lineages. This chapter reviews our latest knowledge of preimplantation embryo development, setting the stage for understanding placental development in subsequent chapters in this Book.

Keywords Inner cell mass · Trophectoderm · Blastocyst · Epiblast · Hippo signaling

Abbreviations

Amot	Angiomotin
aPKC	Atypical protein kinase C
dnYap	Dominant-negative Yap
EPI	Epiblast
ICM	Inner cell mass
Lats-KD	Lats kinase dead
LH	Luteinizing hormone
Na^+/K^+ ATPase	Sodium potassium ATPase
PAR	PDZ-domain-containing scaffold protein
PGC	Primordial germ cell
PLC	Phospholipase C
PrE	Primitive endoderm
TE	Trophectoderm

1 Introduction

The developing mammalian embryo is a unique and valuable model system for studying cell fate decisions. One of the fundamental questions in biology is how a relatively simple single-cell zygote achieves the seemingly impossible task of generating a complex multicellular being. The embryonic cells are tasked with having to make orderly cell fate decisions in a spatiotemporal manner, differentiate into specialized cell types, self-organize into higher order tissues and organ systems, and ultimately generate a functional being. In this regard, the developing mammalian embryo, a "self-contained" system serves as a unique model system for in vitro investigation into genetic, epigenetic, and mechanochemical forces that are at the basis for regulating cell fate (White et al. 2018). Early stages of mammalian embryo development, from maturation of oocytes to blastocyst, can be recapitulated in vitro using a simplistic culture system without the need for external maternal cues (Whitten and Biggers 1968). The recent discovery of genome editors now expands the range of mammalian species (beyond mice) available for genetic manipulation

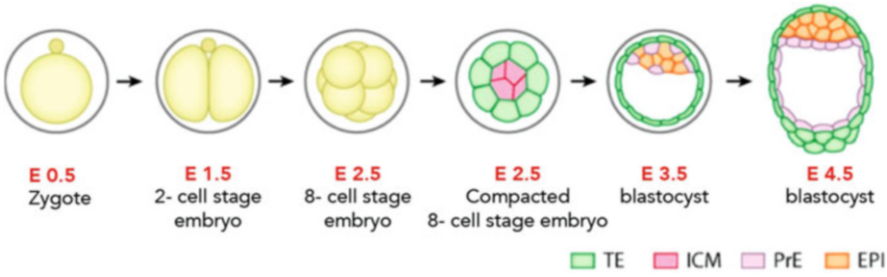

Fig. 1 Mouse embryo preimplantation development. The embryo undergoes successive and timely cleavage divisions resulting in an increased number of totipotent blastomeres with a progressively smaller cell size. In the compact morula, these blastomeres become self-organized into inside and outside cells. By embryonic day (E) 2.5 late eight-cell stage, the boundaries between the outside cells begin to disappear by "compaction." By E3.5, the embryo transforms into a fluid-filled blastocyst with outside trophectoderm (TE) and inner cell mass (ICM). By E4.5, the ICM cells differentiate further into primitive endoderm cells (PrE) lining the blastocoel cavity and pluripotent epiblast (EPI) cells at embryonic pole. TE marks the first visual and deterministic cell fate decision followed by the development of PrE making the second fate decision. In the figure, cell lineages are marked by distinctive colors to visually differentiate them

and for comparative studies. Advances in sequencing technologies, including the ability to sequence single cells, now allow us to capture genetic and epigenetic signature at a higher resolution within the embryos. Finally, breakthroughs in live cell imaging permit tracking of respective morphological fates in vitro (Nagy et al. 2003; Brinster 1963). Cumulatively, the advantages offered by the use of the embryo as a model system and numerous technological advantages are facilitating major breakthroughs in our understanding of first two cell fate decisions: (1) the emergence of trophectoderm (TE) and inner cell mass (ICM) and (2) the differentiation of ICM into pluripotent epiblast (EPI) and committed primitive endoderm (PrE). In this chapter, key landmark events in the development of blastocyst are discussed (Fig. 1). These include awakening of the oocyte by sperm penetration and subsequent transition from meiotic to mitotic divisions (Fig. 2a–f), reductional cleavage divisions resulting in the progressive decline in the size of the embryonic cells and the emergence of heterogeneities in the cleaved blastomeres (Fig. 2g), self-organization of cleaved blastomeres into inside and outside cells (Fig. 2h), compaction of the outer cells and the erasure of distinct cell boundaries (Fig. 2h–j), development of trophectoderm and fluid-filled blastocyst cavity (Fig. 2k), generation of extraembryonic endoderm, and ultimately hatching and implantation of the embryo (Fig. 2l–n). Among the mammalian embryos, the mouse zygote has been studied extensively and will be the basis for discussion below. Several recent articles on mammalian blastocyst development have been published, and the readers are encouraged to peruse the literature for additional information (White et al. 2018; White and Plachta 2020; Zhang and Hiiragi 2018; Leung and Zernicka-Goetz 2015).

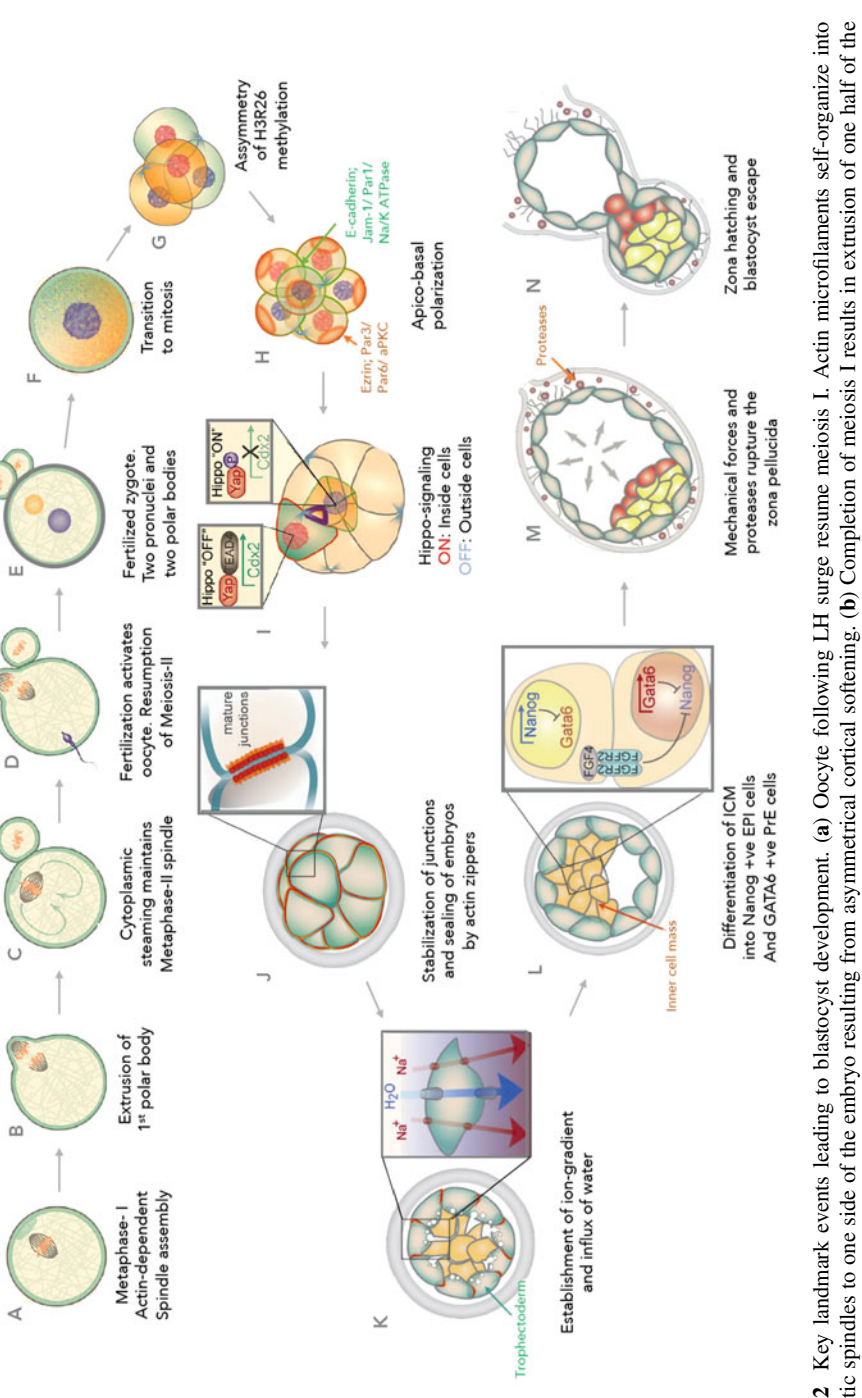

Fig. 2 Key landmark events leading to blastocyst development. (**a**) Oocyte following LH surge resume meiosis I. Actin microfilaments self-organize into meiotic spindles to one side of the embryo resulting from asymmetrical cortical softening. (**b**) Completion of meiosis I results in extrusion of one half of the chromosomes. (**c**) Extruded chromosomes with small amounts of cytoplasm result in the formation of residual cell, first polar body. Cytoplasmic streaming

holds the now metaphase II in place and is arrested at this stage. (**d**) Penetration of sperm following fertilization results in the completion of meiosis II and extrusion of second polar body. (**e**) The now haploid oocyte genome and sperm genomes undergo decondensation, accumulate nuclear membrane, and generate male and female pronuclei. (**f**) The now fertilized zygote begins transition to mitotic cleavage divisions. The maternally deposited messages mediate the transition until zygotic genome activation that takes place at two-cell stage in mouse embryos. (**g**) Asymmetries in epigenetic signatures among blastomeres arise at four-cell stage. Two of the four blastomeres express high levels of H3R26 methylation. (**h**) By late eight-cell stage apicobasal polarization emerges in the outside cells. On the apical surface: microvilli develop on the contact-free cell surface. Actin filaments and actin-binding protein Ezrin localize to the apical surface alongside Par3/Par6/aPkc apical polarization complex. On the basal surface, E-cadherin accumulates at the junctional complex alongside Par1/Jam1 and Na/K ATPase. (**i**) By 16-cell stage post-compaction embryo, Hippo signaling is fixed. In the outside cells, Hippo signaling is inactive, resulting in the upregulation of deterministic TE-specification gene, *Cdx2*. In the inside cells, hippo signaling is activated, resulting in the loss of Cdx2 and upregulation of pluripotency gene, *Pou5f1*. (**j**) The actin rings in the outside cells stabilize and mature junctions in a zipper-like mechanism. (**k**) Activation of sodium pumps resulting in pumping of sodium ions and diffusion of water resulting in progressive accumulation of fluids and fusion of microlumens to form blastocyst cavity. (**l**) Inside pluripotent cells differentiate into Nanog +ve EPI cells (red) and Gata6 +ve PrE cells (yellow) in a characteristic salt-and-pepper fashion. (**m**) The PrE cells via a combination of apoptosis and migration, resulting in the lining of PrE cells towards the blastocyst cavity. The blastocyst via a combination of mechanical forces resulting from expansion of blastocyst and expression of proteases result in the thinning and eventual rupture of zona pellucida. (**n**) Expanded blastocyst escapes from zona pellucida in a process known as zona hatching. (The composite figure has been generated from White et al. (2018) with permission from Elsevier Journal)

2 Setting the Stage: Fertilization Awakens the Oocyte and Jumpstarts Embryonic Development

Embryonic development begins with the fusion of a haploid sperm with the oocyte. The timeline for sperm and egg development is heterogeneous in both sexes. Both gametes start their journey as primordial germ cells (PGC)—a small cluster of cells in the primitive streak at the posterior end of the gastrulating embryo (Ohinata et al. 2009; Saitou and Yamaji 2012; Irie et al. 2014; Ginsburg et al. 1990). Upregulation of a triad of transcription factors *Prdm1* (Ohinata et al. 2005), *Prdm14* (Yamaji et al. 2008, 2013; Ma et al. 2011; Grabole et al. 2013), and *Tfap2c* (Schafer et al. 2011; Weber et al. 2010) in a subset of precursor mesodermal cells results in the suppression of somatic program, epigenetic erasure, and reactivation of pluripotency, ultimately giving rise to PGC. The PGC then make their orderly journey to their eventual resting place, the genital ridge—a mesenchyme abutting the mesonephric system. Key differences between the sexes emerge here. In males, upregulation of Nanos2 prevents progression of male germ cells to meiosis, which is resumed postnatally (Suzuki and Saga 2008). In females, the germ cells enter into prophase of meiosis I and are arrested at the diplotene stage (dictyate stage) at the time of birth and undergo sequential albeit discontinuous progression to a haploid stage. The oocyte maintains close contact with the nurse cells—the cumulus cells via transzonal cellular projections that traverse the width of zona pellucida and provide nutritional and transcriptional support, and regulate meiosis (Barrett and Albertini 2010). Postnatally, a surge in luteinizing hormone (LH) will result in the loss of cumulus-oocyte contacts, breakdown of the nuclear membrane, and resumption of meiosis (Bury et al. 2017). In what could be considered as a first departure from symmetry, the cortex of the oocyte softens at one-end promoting migration and assembly of meiotic spindle, followed by cortical thickening to hold the spindle in place (Fig. 2a) (Chaigne et al. 2013). The oocytes lack centrosomes; therefore, the first few divisions are dependent on self-organization of actin filaments (Fig. 2a) (Dumont et al. 2007; Yi et al. 2013). Asymmetrical positioning of the spindle ensures generation and expulsion of a smaller sister cell (polar body) encompassing one half of the chromosomes and a smaller volume of cytoplasm following meiosis I (Fig. 2b, c) (Yi et al. 2011; Mogessie and Schuh 2017). This ensures that bulk of cytoplasm with transcriptional and translational machinery was preserved in the oocyte for progression to mitotic divisions. Following the first meiotic division culminating in generation of the first polar body, the meiotic spindle is assembled in a similar fashion with polymerization of actin filaments, cytoplasmic streaming (Fig. 2d) (Yi et al. 2011), and is arrested at metaphase stage of meiosis II again, awaiting activation by sperm. Following entry of sperm into the oocyte, a sperm-borne oocyte-activating factor (PLC-zeta) triggers reactivation and resumption of meiosis and expulsion of second polar body (Fig. 2d, e) (Clift and Schuh 2013). This marks the first keystone event.

3 Cleavage, Imprinting Erasure, and Embryonic Genome Activation

Following fertilization, the maternal and paternal chromosomes undergo decondensation, and assemble nuclear membranes forming male and female pronuclei (Fig. 2e). The next immediate hurdle is the transition of oocyte from meiotic to mitotic divisions when the now zygotic genome is transcriptionally silent. The maternally deposited transcripts guide this transition and predominantly code for protein transportation, localization, and cell cycle genes (Fig. 2f) (Li and Albertini 2013). Paternal and maternal DNA undergo histone exchange, global DNA methylation, gamete-specific imprinting erasure, and activation of retrotransposons, culminating in the attainment of a totipotent genome (Habibi and Stunnenberg 2017; Jachowicz et al. 2017; Burton and Torres-Padilla 2014). Even though the mammalian embryo does not go through a predetermined cell fate specification as seen in *Drosophila*, C. elegans, *Xenopus*, and other lower phyla, it is now clear that subtle differences between blastomeres emerge as early as two-cell stage and major differences are evident by four-cell stage (Fig. 2g) (Tabansky et al. 2013; Fujimori et al. 2003; Piotrowska-Nitsche et al. 2005; Littwin and Denker 2011; Antczak and Van Blerkom 1997). Following DNA replication and first mitotic cleavage division, differences in allocation of maternal transcripts between the two blastomeres, especially in the localization of ribosomal RNAs, are evident at two-cell stage (Piotrowska et al. 2001; Piotrowska and Zernicka-Goetz 2001). Likewise, differences in expression of epigenetic modifiers Prdm14 (Burton et al. 2013) and Carm1 (Torres-Padilla et al. 2007) were reported by the four-cell stage, with two of the four cells expressing high levels of Prdm14 and Carm1. Additionally, differences in H3 methylation H3R17 and H3R26 emerge in blastomeres at the four-cell stage, which tend to bias contribution to ICM (Fig. 2g) (Torres-Padilla et al. 2007; Burton and Torres-Padilla 2014). Carm1 regulates H3R26 methylation and increases expression of Sox2, ultimately biasing the cells to pluripotent ICM fate. It is not clear, what regulates increased expression of Carm1 at the four-cell stage. Prior studies that reported no differences in transcriptional profile among blastomeres until the 16-cell stage in the mouse embryo were clearly constrained by the depth and limitations of sequencing (Dietrich and Hiiragi 2008; Guo et al. 2010; Ralston and Rossant 2008; Wicklow et al. 2014; Alarcon and Marikawa 2005; Motosugi et al. 2005; Hiiragi and Solter 2004). In summary, though the blastomeres are overtly homogenous and seemingly identical morphologically, heterogeneities are built into blastomeres by the four-cell stage, setting the stage for lineage specification (Tabansky et al. 2013; Fujimori et al. 2003). Regardless, the fate of the blastomeres remains flexible and is only finalized at the blastocyst stage to accommodate for stochastic errors that are to be expected in the developing embryo.

4 Compaction: First Visual Departure

Following a series of mitotic cleavage events that lead to a progressively smaller and morphologically indistinguishable blastomeres within the zona pellucida, the first overt morphological change that disrupts the uniformity is the compaction of blastomeres soon after the eight-cell stage (Ziomek and Johnson 1980). Blastomeres on the outside establish apicobasal polarity with the accumulation of microvilli, actin-binding protein Ezrin, and actomyosin complex on the contact-free apical cell surface (Fig. 2h) (Ducibella et al. 1977; Louvet et al. 1996; Vinot et al. 2005). On the basolateral surface, E-cadherin accumulates in the junctional complexes (Vestweber et al. 1987; Ziomek and Johnson 1980). It is yet unclear as to what triggers apical polarity at the cell surface, but preliminary reports suggest that actomyosin complex is accumulated at the apical cortex by phospholipase C-mediated hydrolysis of phosphoinol phosphate 2 and activation of protein kinase C (Zhu et al. 2017). Activated protein kinase activates RhoA, which in turn triggers polarization of the actin network, accumulation of the Par3-Par6-aPKC complex (Vinot et al. 2005), and ultimately the formation of an apical domain. In the cell-cell contact basolateral surface, accumulation of Par1 (Vinot et al. 2005), Jam-1 (Fig. 2h) (Thomas et al. 2004), and Na^+/K^+ ATPase (Watson and Kidder 1988) was seen alongside E-cadherin (Fig. 2k). Accumulation of E-cadherin at the basolateral surface increases the surface area of cell contacts, resulting in a gradual flattening of the outer blastomeres and erasure of distinct cell boundaries, and establishment of a primitive epithelium-like structure in the embryo (Fig. 2j) (Johnson 2009; Ducibella and Anderson 1975). It was long believed that accumulation of E-cadherin at junctional complexes and an increase in intercellular adhesion were responsible for morphological changes during compaction. However, recent studies cast doubt on this basic assumption as to whether E-cadherin transligation will yield enough forces to achieve cellular deformation (Samarage et al. 2015; Maitre et al. 2012). Rather, the discovery of filopodia—long membrane protrusions emanating from the apical cell surface and adhering via E-cadherin to the apical surface of adjacent cells—to be likely responsible for characteristically distinct compaction event (Fierro-Gonzalez et al. 2013). The filopodia are connected to the cytoskeleton and myosin motor protein, myosin-10 (Fierro-Gonzalez et al. 2013). Laser ablation of the filopodia results in rapid rounding of the cells and loss of compaction, bringing adhesion-based compaction model into question (Maitre et al. 2015). These observations corroborate a mechanochemical model for compaction, whereby the pulsatile actomyosin contractility linking the neighboring cells is redistributed by junctional E-cadherin away from cell-cell contact surface. Future investigations are aimed at understanding how the E-cadherin-dependent filopodia emerge, and the pulsatile actomyosin contractility is initiated and regulated.

5 Specification of Inner Cell Mass (ICM) and Trophectoderm: The First Cell Fate Decision

Following compaction and between 8- and 16-cell late-morula stage embryo, the cells are organized into outer polar and inner apolar cells, which give rise to TE and ICM lineages, respectively. However, the phenotypes and lineage identities at the 16-cell stage are not stable. Isolated apolar blastomeres can become polarized if placed on the outside position (Ziomek et al. 1982); outside cells of the late morula can produce ICM derivatives when aggregated with earlier embryos (Rossant and Vijh 1980), and cells of the ICM can produce trophoblast tissue (Handyside 1978). It is not until blastocyst formation that the TE and ICM lineages are irreversibly determined. Formation of the blastocyst marks the first visual cell fate decision, with TE cells on the outside committed to placental development, and cells on the inside (ICM) committed to the development of fetus and components of extraembryonic membranes. These events as discussed below are dependent on stochastic and deterministic events involving Hippo signaling system.

5.1 Differentially Expressed Transcription Factors Lead to Lineage Specification

At the late 16-cell stage, when the TE and ICM lineages are set-aside, differential expression of specific transcription factors becomes apparent in the mouse embryo. Inner apolar cells show strong expression of Sox family member *Sox2* (Avilion et al. 2003), the homeobox gene *Nanog* (Chambers et al. 2003; Mitsui et al. 2003), and octamer-binding transcription factor gene *Pou5f1* (Niwa et al. 2000). Sox2 is the first transcription factor to be specifically upregulated in the inner cells starting at the 16-cell stage (Guo et al. 2010). *Nanog* and *Pou5f1* are found uniformly expressed in all cells, with strong expression evident at the eight-cell stage, and later restricted to the ICM of the blastocyst (Fig. 2l) (Palmieri et al. 1994; Dietrich and Hiiragi 2008).

The caudal-related homeobox gene, *Cdx2*, and the zinc-finger transcription factor *Gata3*, have an expression pattern opposite to that of *Sox2*, *Nanog,* and *Pou5f1*. *Cdx2* and *Gata3* are strongly expressed in the outside polarized cell population (Strumpf et al. 2005; Beck et al. 1995; Ralston et al. 2010). It is speculated that an imbalance between Pou5f1 and Cdx2 initiates a reciprocal inhibition system that results in the restricted pattern (Ralston et al. 2010). To support this, TE-like cells found in *Cdx2* null embryos reexpress Pou5f1 (Strumpf et al. 2005), while *Pou5f1* null embryos express TE markers in the ICM (Nichols et al. 1998). However, a more recent study shows that *Pou5f1* levels remain high during the accumulation of Cdx2 and speculates that *Pou5f1* levels therefore do not affect accumulation and maintenance of Cdx2 (Dietrich and Hiiragi 2008). It is still possible however that the reciprocal inhibition pathway is dependent on Cdx2 reaching a certain threshold before it is able to downregulate *Pou5f1*. Either way, single blastomere RNA

sequencing has shown that activation of TE-specific genes, including *Cdx2*, shows up as the first overt transcriptional difference between blastomeres (Posfai et al. 2017). To summarize, the currently accepted model for lineage specification in mouse embryo combines gene expression patterning and blastomere position. In this model, molecular differences between blastomeres are established during the eight-cell stage, and then a combination of stochastic processes restrict blastomeres with specific molecular profiles to the inside or outside populations (Dietrich and Hiiragi 2008; Lanner 2014). Following this stochastic allocation, a deterministic event led by Hippo signaling establishes the lineage commitment (Fig. 3) (Nishioka et al. 2008).

5.2 *Hippo Signaling Pathway and Lineage Specification*

The Hippo signaling pathway was first discovered in *Drosophila* as a tumor suppressor-signaling pathway, but is also conserved in mice and humans (Harvey et al. 2013; Yu and Guan 2013). The Hippo pathway is regulated by a variety of stimuli including cell-cell adhesion mediated by E-cadherin and by cell polarity mediated by angiomotin (Amot) which is thought to be the stimuli that initiates Hippo signaling activity in the mouse embryo (Fig. 3) (Kim et al. 2011; Hirate et al. 2013). The main components of this pathway are the protein kinase Mst1/2 and its co-activator Sav1 that function to activate kinase Lats1/2. In turn, Lats1/2 and its coactivator Mob1a/b phosphorylate Yap1 and Taz, which are transcriptional coactivators of Tead proteins (Yu and Guan 2013). Amot interacts with the E-cadherin-a α/β-catenin complex and serves as a scaffold protein that associates with Yap and Lats proteins (Fig. 3) (Paramasivam et al. 2011). Phosphorylation of Yap1 results in cytoplasmic sequestration and in turn suppresses target gene expression. Therefore, activation of Hippo signaling suppresses expression of *Tead* target genes, and inactivation of the pathway induces the expression of those target genes.

In the mouse embryo, Hippo signaling is an upstream regulator of *Cdx2* expression and is directly involved in ICM vs. TE lineage specification (Yagi et al. 2007). In the inside cells of the mouse embryo destined to become ICM, Hippo signaling is activated as a result of increased cell-cell contacts (Nishioka et al. 2009). This activation is mediated by Lats1 and Lats2 kinase, which phosphorylates and sequesters Yap1 in the cytoplasm, preventing it from coactivating Tead4, a transcription factor that directly regulates *Cdx2* expression (Nishioka et al. 2009; Hirate et al. 2013). Transient upregulation of *Lats2* in the mouse embryo results in reduced nuclear accumulation of Yap1, downregulation of *Cdx2*, and failure to form a blastocoel because of the absence of TE cells (Fig. 3) (Nishioka et al. 2009). This phenotype is also a characteristic of *Tead4* null embryos, indicating that aberrant overexpression of Hippo component Lats2 suppresses differentiation to the TE lineage via its control of Tead4 activation (Yagi et al. 2007). In the outside cells destined to become TE, Hippo signaling is inactive, which allows Yap1 to translocate into the nucleus, activate Tead4, and upregulate *Cdx2* (Fig. 3) (Nishioka et al.

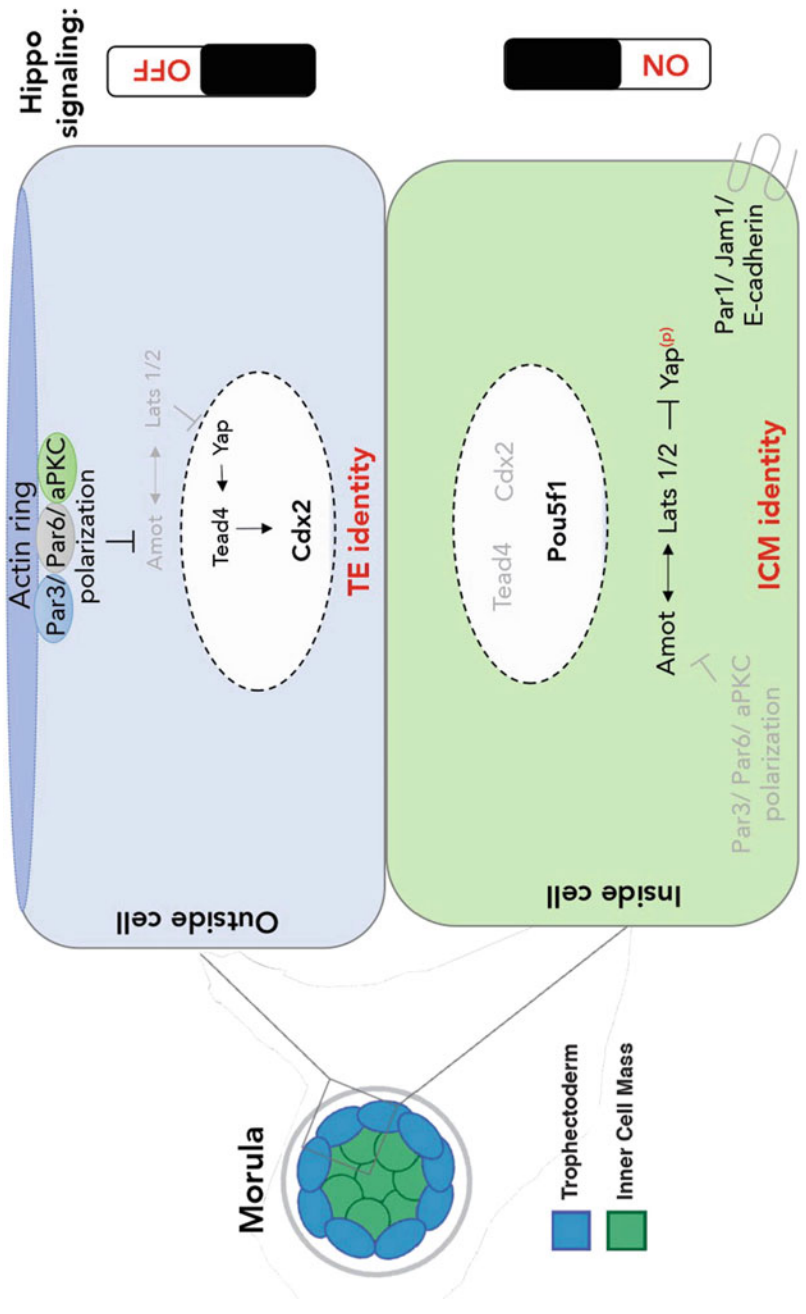

Fig. 3 Hippo signaling in the mouse embryo. Hippo signaling regulates lineage specification in the early mouse embryo. In the outside cells, Hippo signaling is active. Development of polarization complex (Par3/Par6/aPKC) in the outside cells results in tethering of junction-associate protein angiomoitin (Amot) to the apical side and subsequent lack of Lats1/2 activation. Yap protein is available for translocation into the nucleus, which in turn activates Tead4. Tead4 in turn

2008, 2009). Null mutation of either Lats1 or Lats2 does not appear to disrupt normal development during the preimplantation stages (McPherson et al. 2004; St John et al. 1999; Yabuta et al. 2007). However, overexpression of a catalytically inactive form of Lats2 (Lats kinase dead, or Lats-KD) is able to dominantly inhibit both Lats1 and Lat2, resulting in an embryo that expresses Cdx2 in inside cells (Nishioka et al. 2009). Furthermore, a double knockout of both *Lats1* and *Lats2* exhibits the same phenotype, strongly suggesting that Lats1/2 kinase is necessary to activate expression of the TE lineage marker, *Cdx2* (Nishioka et al. 2009).

The importance of Hippo signaling in TE vs. ICM lineage specification has been confirmed in several mouse models: double knockdown and knockout of *Lats1* and *Lats2* genes (Lorthongpanich et al. 2013; Nishioka et al. 2009), overexpression of a catalytically inactive form of Lats2 (Lats2 kinase dead) (Nishioka et al. 2009), and depletion of *Amot* and *Amot12* (Hirate et al. 2013) all show nuclear accumulation of Yap1, strong expression of *Cdx2* in both outside and inside cells, failure to develop ICM-derived tissues, and TE-like blastomeres that populate both the inner and outer cell positions. Overexpression of Lats2, overexpression of a dominant negative form of Yap (Nishioka et al. 2009) (dnYap), and *Tead4* null embryos (Nishioka et al. 2008, 2009; Yagi et al. 2007; Ralston et al. 2010) show cytoplasmic accumulation of Yap1, reduced expression of *Cdx2*, and failure of embryonic cells to differentiate into trophectoderm.

5.3 *Hippo Signaling as It Relates to Cell Polarity and Cell Position*

Apicobasal polarity in mouse embryos is dictated by the PAR-aPKC system (Vinot et al. 2005). This system involves a set of evolutionarily conserved proteins that include PDZ-domain-containing scaffold proteins (PARs) and atypical protein kinase C (aPKC) which dictate polarity in a variety of both invertebrate and vertebrate species (Fig. 3) (Suzuki and Ohno 2006). As discussed above, polarization begins at the eight-cell stage in the mouse embryo and is marked by apically restricted microvilli (experimentally visualized with phosphorylated-Ezrin) (Louvet et al. 1996) and accumulation of PARD6b and later aPKCζ at the apical surface (Vinot et al. 2005). Establishment of polarity in turn has been shown to directly suppress Hippo signaling activity in embryos (Hirate et al. 2013). Disruption of the PAR-aPKC system via injection of RNAi constructs in mouse zygotes causes Hippo

Fig. 3 (continued) activates trophectoderm gene expression, including *Cdx2*. In the inside cells, Hippo signaling is inactive. Lack of polarization complex allows for Amot to activate Lats kinase. Lats kinases phosphorylate Yap and subsequently sequester Yap protein in the cytoplasm. Lack of Yap translocation in the nucleus results in loss of expression of TE specification genes and upregulation of pluripotent genes including *Pou5f1* and establishment of pluripotent inner cell mass cells

pathway activation as evidenced by disruption of apical localization of Amot, exclusion of Yap1 from the nucleus, and TE development failure (Hirate et al. 2013). However, when the same embryos are dissociated, Hippo signaling is not activated despite the disruption of polarity (Hirate et al. 2013). This suggests that activation of Hippo signaling is not only dependent on the apolar status of individual blastomeres but is also dependent on cell-cell adhesion. Furthermore, in apolar cells where the PAR-aPKC system is inactive, Amot is found associated with basolateral adherens junctions via binding to the E-cadherin complex (Hirate et al. 2013). Activation of Amot at adherens junctions is thought to potentiate the activation/function of Lats kinases and results in Hippo pathway activation (Hirate et al. 2013). Together, these observations indicate that Hippo signaling responds to a combination of inputs: cell-cell adhesion and cell polarization. In the inner cells, Hippo signaling is activated by increased cell-cell contacts whereas in the outer cells, cell-cell contact-dependent Hippo signaling is suppressed by the polarization status of the outside cells mediated by the PAR-aPKC system (Sasaki 2015). To relate this finding to TE-specific expression of *Cdx2*, a study analyzing localization of aPKC in *Cdx2* mutant embryos showed that aPKC localization to the apical surface was not affected by the lack of *Cdx2*. This suggests that cell polarization is independent of Cdx2, and *Cdx2* upregulation is genetically downstream of cell polarization (Ralston and Rossant 2008).

From these studies, progression of events can be summarized as follows: (1) cell polarization begins at the eight-cell stage, (2) from the 16-cell stage onwards final polarity status in individual blastomeres is established, (3) Hippo signaling is suppressed in the polarized outer cells and is activated by cell-cell adhesion in the apolar inner cells (Fig. 3), and (4) this leads to lineage specific gene expression to stabilize the TE and ICM fates in the blastocyst (Fig. 3). Hippo signaling is therefore a key driver for segregation of ICM and TE lineages; however, it is still unknown at what time point Hippo signaling is sufficient to establish cell fate (Posfai et al. 2017).

6 Blastocyst Maturation and Hatching

Following the stabilization of Hippo signaling and the establishment of outer TE cells and ICM cells, the embryo transitions to a fluid-filled blastocyst stage. Blastocyst cavity is formed by active transport of sodium ions across the TE cells which create an osmotic gradient and an influx of liquid to create a fluid-filled blastocyst (Fig. 2k) (Aziz and Alexandre 1991; Benos et al. 1985; Watson and Barcroft 2001). The expanding blastocyst cavity is stabilized by tight junctions between the outer cells resulting in the generation of first embryonic epithelium the TE (Wang et al. 2008). Recent studies have highlighted the emergence of atypical apical actin rings that extend to the junctions between the outer cells and subsequent stabilization of adherens and tight junctions in a zipper-like mechanism in the TE (Fig. 2j) (Zenker et al. 2018). The expanding blastocyst cavity results in marginalization of ICM cells towards one end of the blastocyst (embryonic pole). The pluripotent ICM cells

express Pou5f1, Sox2, Nanog, and Gata6 (Fig. 2l) (Ohnishi et al. 2014). Soon after, a reciprocal expression is established between Nanog expressing EPI cells producing FGF4 in an Sox2/Pou5f1-dependent fashion and Gata6 and Ffgr1/2 expressing FGF4-responsive PrE cells—in a characteristic "salt-and-pepper" fashion (Fig. 2l) (Chazaud et al. 2006). Following the establishment of two distinct populations, the PrE cells line up the blastocyst cavity through a combination of migration, apoptosis, and adhesion (Fig. 2m) (Saiz and Plusa 2013). The blastocyst at this stage will need to escape from zona pellucida to initiate implantation with uterine epithelium. The blastocyst escapes from zona in still a poorly understood process but likely includes a combination of mechanical forces from an expanding blastocyst, and proteolytic activity (Fig. 2m) (Seshagiri et al. 2009; Perona and Wassarman 1986). The escape of blastocyst (Fig. 2n) from zona pellucida will herald an implantation phase, and subsequent development is contingent on maternal inputs.

7 Concluding Remarks

More than one-third of pregnancies are lost in the preimplantation phase of embryo development, and close to 50% of human pregnancies fail during the first few weeks of pregnancy (Hyde and Schust 2015). The prevalence is especially worse for recipients in ovum donation programs, and in patients undergoing in vitro fertilization (McDonald et al. 2009; Margalioth et al. 2006; Abdalla et al. 1998). These huge rates in embryonic losses are also true in livestock, where embryonic and preimplantation loses remain major contributors of infertility (Berg et al. 2010). Majority of our current understanding on blastocyst development comes from studies in rodent models. Traditionally, this may have to do with ease of husbandry and the ability to perform sophisticated genetic modifications. However, key differences in the expression of lineage specification genes in non-rodent models remain and necessitate investigation into alternative model species. For example, there is a lack of mutually exclusive and antagonistic expression of *POU5F1* and *CDX2* in TE and ICM cells in embryos from livestock, a hallmark of rodent embryos (Rossant 2011). As discussed in subsequent chapters in this book, key differences also emerge post-hatching in the implantation phase between mouse, human, and other livestock species. In this regard, comparative studies involving livestock and other non-rodent model systems will likely contribute to a greater understanding of lineage specification, and in bridging gaps left by the rodent models. Livestock offer several key advantages, including a well-established culture system, unlimited supply of oocytes (slaughterhouse), and in the post-CRISPR and genome editing era, an ability to perform genetic modification. Ultimately, cross species investigations will unlock conserved mechanisms, highlight key differences, and will have a greater impact on animal and human health.

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