Troika of the Mouse Blastocyst: Lineage Segregation and Stem Cells

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Abstract

The initial period of mammalian embryonic development is primarily devoted to cell commitment to the pluri-potent lineage, as well as to the formation of extraembryonic tissues essential for embryo survival *in utero*. This phase of development is also characterized by extensive morphological transitions. Cells within the preimplantation embryo exhibit extraordinary cell plasticity and adaptation in response to experimental manipulation, highlighting the use of a regulative developmental strategy rather than a predetermined one resulting from the non-uniform distribution of maternal information in the cytoplasm. Consequently, early mammalian development represents a useful model to study how the three primary cell lineages; the epiblast, primitive endoderm (also referred to as the hypoblast) and trophoblast, emerge from a totipotent single cell, the zygote. In this review, we will discuss how the isolation and genetic manipulation of murine stem cells representing each of these three lineages has contributed to our understanding of the molecular basis of early developmental events.

Keywords

Blastocyst; mouse development; preimplantation; cell fate; lineage allocation; trophectoderm; epiblast; primitive endoderm; stem cells; ES cell; TS cell; XEN cell; pluripotency

I. THE BLASTOCYST EMBRYO COMPRISES THREE CELL LINEAGES

Eutherian embryonic development takes place in a protective and nutritive environment. Unlike other phyla, mammalian oocytes are alecithal eggs that lack the large nutrient reserves that are usually accumulated during oogenesis. Early mammalian development is comparatively slow, taking place within the maternal uterine fluid and is mainly devoted to the establishment of tissues specialized for interactions with the maternal uterus. As a consequence, the first cell fate decisions occur early during embryogenesis before embryo implantation into the uterus. These cell fate decisions involve the specification and spatial segregation of extraembryonic tissues from the pluripotent epiblast (EPI) that will give rise to most of the embryo-proper. The first extraembryonic tissue specified is the trophectoderm (TE), which forms the fetal portion of the placenta, thereafter the primitive endoderm (PrE) will be specified which gives rise to the visceral (VE) and parietal (PE) endoderm layers of the yolk sac (Fig. 1). Importantly, the VE, which encapsulates the epiblast after implantation, is critical for axis formation [1, 2]. At gastrulation, the distal VE (also referred to as emVE) is incorporated into the gut endoderm tissue layer of the fetus in contrast to it previously being ascribed as having an exclusively extraembryonic fate [3].

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By the end of the preimplantation period, the embryo is composed of three distinct cell lineages that are defined by their position within the embryo, their developmental potential and their expression of molecular markers (Fig. 1 and 4). During the last decade, several factors exhibiting lineage-specific expression have been identified, many of them required for lineage establishment, maintenance or differentiation. The trophectoderm emerges as an outer epithelial cell layer and expresses *Cdx2*, *Eomes*, *Gata3* and *Krt8*. The PrE also forms an epithelial cell layer at the interface between EPI and the blastocoel cavity and expresses *Dab2*, *Fgfr2*, *Gata4*, *Gata6*, *Pdgfra*, *Sox7* and *Sox17*. The pluripotent EPI is encapsulated by these two nascent extraembryonic tissues and expresses markers such as *Fgf4*, *Nanog*, *Oct4* and *Sox2*.

Early mammalian development can therefore be viewed as a process by which totipotency is progressively lost, and where initial asymmetries reinforced by positional information, direct the formation of the first three cell lineages. Lineage-specific markers have been extensively used to follow cell lineage commitment and segregation. Our current view of the segregation of the first lineages, TE *vs.* ICM and subsequently within the ICM EPI *vs.* PrE, has suggested that early lineage segregation occurs in two successive phases. In the first phase, markers of derivative lineages are co-expressed in individual cells at variable levels. In the second phase, marker expression becomes mutually exclusive as cells are lineage committed. In the following section, we review our current understanding on how these lineages might be specified and segregated during mouse embryonic development.

2. TE *vs.* ICM SPECIFICATION

Upon fertilization, the first three cell divisions (1 -> 2 -> 4 -> 8 cell stage) give rise to 8 blastomeres that generally appear morphologically indistinguishable. At the 8-cell stage, embryos undergo compaction, a process where cell-cell contacts between blastomeres increase. E-cadherin relocalization plays a major role in this process. Zygotic E-cadherin mutant embryos compact normally [4], while maternal removal of E-cadherin delays compaction [5]. Removal of both maternal and zygotic contribution prevents compaction [6]. As compaction proceeds, blastomeres become polarized along their apical-basal axis, such that the apical surface of blastomeres which faces outside is free of cell-cell contacts, contains microvilli and is enriched in aPKC isoforms (such as PKCζ, PKCδ and PKCλ), PAR3, PAR6 and EZRIN. By contrast, the basolateral regions of compacting blastomeres are enriched in LGL1, JAM1 and PAR1. This localization of proteins is associated with the formation of gap junctions, adherens and tight junctions between neighboring blastomeres, and occurs concomitant with epithelialization.

It has been proposed that, during the next two rounds of cell division (8 -> 16 -> 32 cell stage), cleavage plane orientation underlies the emergence of two cell types that differ in their position within the morula, such that outside cells will form the TE, whereas inside cells will form the ICM (Figure 2). Symmetric cell divisions (with a cleavage plane parallel to the basolateral axis) will give rise to two identical polarized daughter cells having an outside position, whereas an asymmetric cell division (with a cleavage plane perpendicular to the basolateral axis) will generate an outer polarized cell and an inner non-polarized cell.

2.1. Inside-Outside Model

To explain this first lineage divergence, it had been proposed that fate might be determined by cell position, this being inside *vs.* outside, within the morula. Inside cells adopt an ICM fate, whereas outside cells become TE [7]. Such a divergence could result from differences in the microenvironment and promote two distinct cell fate decisions. Alternatively, differences in the nature of the cell-cell contacts could impact on lineage commitment, since

inner cells are encapsulated by neighboring cells, whereas outer cells have a free apical surface which is exposed to the outside environment.

This "inside-outside" model is supported by the observation that cell fate can be altered by experimentally repositioning cells within the embryo [8, 9]. Furthermore, ICMs isolated from early blastocysts are able to regenerate an outer TE epithelial layer, and go on to form a blastocyst structure [10–12]. By demonstrating that early blastomeres are not irreversibly committed to particular fates, and that topological position within the developing embryo may be important for lineage determination, these observations reveal an intrinsic cell fate plasticity present within the early mouse embryo.

2.2. Polarity Model

In an alternative, but not necessarily mutually exclusive model, Johnson and colleagues proposed that allocation of TE *vs.* ICM takes place between the 8 to 16-cell stage, prior to the emergence of inside and outside cells [13]. They suggested that one determinant likely results from the acquisition of cell polarity. Support for this model comes from the demonstration that experimental perturbation of cell polarity affects cell position, and presumably cell fate. Indeed, down-regulation of aPKC or PAR3 promotes cell localization to the inside of the embryo [14]. However, it is still not clear how polarity is established at the time of compaction.

If cell-cell contacts are a key determinant for cell polarization, the adhesion molecule E-cadherin is likely to be involved. Analysis of zygotic mutant embryos has revealed that maternal stores are clearly sufficient to polarization. However, it was recently reported that in maternal/zygotic E-cadherin mutants polarization still occurs, at least partially, leading to the establishment of both TE and ICM cells even if the allocation of blastomeres to the inside or outside compartments is impaired [6].

2.3. Key Lineage Determinants of TE *vs.* ICM Identity

Presently, the earliest identified signaling event involves components of the Hippo signaling pathway and specifically requires the TEAD transcription factor family member TEAD4 (Fig. 2). Mutants lacking *Tead4* die at preimplantation; they fail to form TE and never reach the blastocyst stage [15, 16]. Even so, polarity is maintained in *Tead4* mutant embryos indicating that TEAD4 acts either downstream or in parallel to the mechanisms that establish cell polarity. Interestingly, CDX2 was detected in early morula stage *Tead4* mutant embryos, but its expression was not maintained (in Nishioka study [15] but not Yagi study [16]). In addition, all cells of *Tead4* mutant embryos expressed the epiblast-specific OCT4 and NANOG transcription factors, but blastomeres exhibited heterogenous levels of these proteins [15]. This is intriguing, and if confirmed, would indicate that the initial period of stochastic marker expression may be initiated in *Tead4* mutants, but that subsequent marker restriction concomitant with cell fate choice is impaired.

Surprisingly at these early stages, *Tead4* is widely expressed and TEAD4 protein localizes to the nucleus of all blastomeres, indicating that its role in TE specification might be mediated by its co-factor(s). Indeed, the TEAD co-activators YAP and TAZ (also called WWTR1) specifically localize to the nuclei of outside blastomeres, not to inside blastomeres. The current model proposes that the Hippo pathway is regulating the subcellular localization of YAP/TAZ proteins. The pathway is active in inside cells, leading to the phosphorylation and subsequent degradation of YAP/TAZ. Conversely, the pathway is inactive in outside cells, leading to the nuclear accumulation of YAP/TAZ and regulation of TEAD4 activity. This leads to the question of how might the Hippo pathway, which plays a major role in the regulation of tissue growth [17], be involved in early lineage

determination? A recent study suggests that the Hippo pathway is regulated by positional information and cell polarity, and that therefore it might function to integrate major developmental signaling pathways, including TGFβ signaling. This process may rely on a cell's ability to sense its environment, for example cell density inputs through the Crumbs polarity complex [18]. In turn, active TEAD4 regulates the expression of two TE-specific transcription factors: CDX2 and GATA3 [15, 16, 19].

Cdx2 encodes a *Caudal*-type homeodomain transcription factor essential for TE maintenance. Indeed, zygotic *Cdx2*-deficient embryos progress to the blastocyst stage but the blastocoel, even though initially formed, is not maintained due to a failure to maintain epithelial integrity [20, 21]. A recent report described the presence of maternal Cdx2 mRNAs at the 8-cell stage [22]. However the role of this maternal pool remains unclear. Jedrusik and colleagues reported that *Cdx2* knockdown affects cell polarization, leading to preferential contribution to inside cells suggesting a feedback loop between *Cdx2* and cell polarity. Consequently, the phenotype of maternal/zygotic *Cdx2* mutants is more severe than zygotic mutants [22]. This finding contrasts with other studies which suggest that *Cdx2* knockdown does not affect polarization, and that the phenotypes of zygotic and maternal/ zygotic mutants are undistinguishable, thus arguing against the idea that *Cdx2* may be involved in TE specification [23, 24]. Interestingly, Jedrusik and colleagues reported the asymmetric localization of Cdx2 mRNA at the apical side of the 8-cell stage blastomeres which may be inherited by outside as opposed to inside cells [22].

Even so, it is widely accepted that CDX2 and OCT4 exert mutual repressive effects leading to the establishment of a CDX2-positive; OCT4-negative outside compartment (the future TE) and a CDX2-negative; OCT4-positive inside compartment, that will give rise to the ICM. Thus in the absence of *Cdx2*, outside cells maintain *Oct4* expression. However, *Oct4* mutant blastocysts initially exhibit a normal restriction of *Cdx2* expression in the TE outside cell layer, but this pattern is not maintained by the time of implantation, and mutant ICM cells upregulate TE markers [19, 25]. Taken together, these observations suggest that additional mechanisms must be in place to restrict the localization of key lineage-specific regulators to their appropriate compartments [19, 26]. Of note, the kinetics of establishment of a mutually exclusive expression between *Cdx2* and *Oct4* varies among mammals, and the co-repression between OCT4 and CDX2 does not seem to be conserved in cattle, suggesting that mechanisms described in the mouse may perhaps be unique to rodents, but not exploited by other mammals [27]. Recently, live imaging of the kinetics of an OCT4 protein fused to a phosphoactivatable PA-GFP predicted division and cell allocation to the outside and the inside compartments [28] arguing in favor of early asymmetries, existing in 4-cell to 8-cell stage embryos, that might influence cell fate specification (reviewed in [29, 30]).

GATA3, a member of the GATA transcription factor family, is involved in this first cell lineage decision. GATA3 is first expressed at around the 4-cell stage and becomes progressively restricted to the TE lineage concomitant with the restriction in *Cdx2*, in a process which, by contrast, does not appear to require repression by OCT4 [19]. Interestingly, while *Gata3*-deficient embryos survive until mid-gestation [31, 32], *Gata3* knockdown by RNA interference at the 2-cell stage impairs the morula-to-blastocyst transition suggesting an early role for GATA3 in TE formation [33]. Thus, one might predict that a maternal/zygotic *Gata3* mutant will exhibit an earlier lethal phenotype.

3. ACQUISITION OF EPI OR PrE CELL FATE WITHIN THE ICM

3.1. Specification of PrE *vs.* EPI

The divergence within the ICM between EPI and PrE lineages is thought to occur at the early blastocyst stage and leads to the formation of a morphologically-distinct epithelial PrE

cell layer in contact with the blastocoel. The fact that ICMs isolated from blastocyst stage embryos generate a PrE layer on their surface has led to the suggestion that cell position may also play a role in this cell fate choice [34]. However, the identification of lineage-specific markers, as well as the use of live imaging, have revealed that lineage commitment may occur earlier than previously thought, and that cell position may be involved in later refining lineage commitment.

The current model posits at least two distinct steps involving lineage specification and segregation. The first step corresponds to the specification of cell identity and occurs around the 64-cell stage. It involves a progressive restriction in the expression of key transcription factors such as GATA6 (PrE-specific) and NANOG (EPI-specific). By the 64-cell stage, EPI and PrE markers that were initially co-expressed by all ICM cells become mutually exclusive, such that the ICM comprises a mixture of EPI and PrE precursor cells organized in an apparent "salt-and-pepper" distribution (Fig. 3) [35–37]. The idea that lineage commitment likely occurs around this time has been suggested by testing the developmental potential of isolated ICM cells from early or late stage blastocysts [35]. While cells from early stage blastocysts could contribute to both EPI and PrE lineages, cells from late stage blastocysts were restricted to either EPI or PrE lineage.

It has been suggested that, akin to TE *vs.* ICM specification, a reciprocal inhibition between different lineage-specific transcription factors might provide the mechanistic determinant for lineage commitment and divergence. The initial analysis of *Nanog* knockout embryos suggested that NANOG is involved in maintaining pluripotency by inhibiting PrE formation. Outgrowths from *Nanog* mutant ICMs could not be maintained in culture as they differentiated into PE-like cells [38]. Recently however, an in depth analysis of *Nanog* mutant embryos revealed that PrE formation is impaired in absence of *Nanog* [39, 40]. This phenotype could be rescued when wild type ES cells were injected into mutant recipient embryos. These observations therefore suggest that *Nanog* acts non-cell autonomously in the EPI to promote PrE formation [39].

Gata4 and *Gata6* encode GATA transcription factors expressed in the PrE. GATA6 is one of the earliest markers of the cells of the developing embryo, whereas GATA4 is expressed starting at the 64-cell stage in cells likely to be committed to the PrE lineage [37]. Surprisingly however, *Gata4* and *Gata6* mutants affect PrE differentiation rather than formation [41–43]. However, given potential redundancy between these two GATA factors, the double mutant may reveal an earlier phenotype but it has yet to be reported. Even so, one study has reported a complete absence of a PrE layer in E4.5 *Gata6* mutant embryos supporting the idea that GATA6 may play an earlier role [44].

So far, the only reported mutants that exhibit severely impaired PrE formation are in genes encoding components of the FGF signaling pathway. These include the FGF ligand FGF4 ([45, 46]; A. Piliszek, J.A., M. Kang and A.-K.H. unpublished data), the FGF receptor FGFR2 [47] and the adaptor molecule GRB2 [35, 48]. PrE commitment of ICM cells is strictly dependent on the level of FGF activity (Fig. 3). Perturbation of this activity directly affects the ratio of EPI and PrE cells formed within the ICM. Pharmacological inhibition of the FGFR (PD173074, SU5402) and/or MEK effector (PD0325901, PD184352) prevents PrE formation [49, 50]. Conversely, when cultured in high doses of recombinant FGF4, a majority of ICM cells will adopt a PrE fate [50]. Furthermore, modifying the heparan sulfation of FGF receptors leads to a similar phenotype [51].

Not only does FGF signaling play a role in cell lineage commitment, but it also appears to exert a mitogenic activity on both the PrE and the TE lineages [46, 52, 53]. A major paracrine source of FGF ligands within the blastocyst comes from EPI cells which express

Fgf4 as a direct target of pluri-potency factors OCT4 and SOX2 [54], while extraembryonic lineages express FGF receptors (Fig. 3). Single-cell expression analyses have revealed an inverse correlation between *Fgfr2*/*Fgf4* expression starting at the 32-cell stage. Intriguingly, this receptor/ligand reciprocal expression precedes the reciprocal expression of lineage-specific transcription factors such as *Nanog*/*Gata4* or *Nanog*/*Gata6* [55]. Thus, FGF signaling might act upstream of the lineage restriction of lineage-specific transcription factors. However, the mechanism(s) by which a mutually exclusive expression of this ligand/receptor combination might be established remains to be determined.

A relationship between the timing of formation of inner cells and a tendency towards commitment to a particular lineage has recently been suggested [56]. In this way, the expression of *Sox2* in first-born inner cells would lead to an upregulation of *Fgf4* and might affect neighboring cells. Thus, early inner cells formed by the first wave of asymmetric cell divisions (between 8-cell -> 16-cell stage) would preferentially adopt an EPI fate (due to high levels of SOX2, and resulting upregulation of *Fgf4*). By contrast, inner cells formed during the next two waves of cell divisions (16-cell -> 32-cell and 32-cell -> 64-cell stage) would preferentially adopt a PrE identity (due to lower levels of SOX2, down-regulation of *Fgf4* and upregulation of *Fgfr2*). Even though no single study has so far revealed an absolute tendency, this "time-on-outside" model contrasts with results from Yamanaka and colleagues who failed to discern a relationship between time of origin of ICM cells and their allocation to EPI or PrE lineages [50].

In addition to FGF, other receptor tyrosine kinases (RTKs) may participate in the regulation of MEK/ERK activity required for the establishment and maintenance of extraembryonic lineages. Hence, an *Egfr* mutation leads to a periimplantation lethality on a CF-1 genetic background leaving open the possibility that EGF signaling may to some extent be involved in PrE formation [57]. Importantly, *Platelet derived growth factor alpha* (*Pdgfra*) is, along with *Gata6*, the earliest expressed PrE marker [37]. A role for PDGF signaling has been demonstrated in the expansion of the PrE lineage in late and implantation delayed blastocysts [58]. It is therefore possible that sustained MEK/ERK activity is required for the specification and the maintenance of the PrE lineage, and that its activity could be initially regulated by FGF signaling, and then reinforced by PDGF signaling.

3.2. Sorting of PrE Cells

The ICM eventually undergoes cell rearrangements that ultimately lead to the localization of PrE cells on the surface adjacent to the blastocoel where they will form a polarized epithelial layer [59]. The process that drives PrE *vs.* EPI spatial segregation is often referred to as cell sorting. The current model suggests that cell sorting involves multiple cell behaviors including actin-dependent cell movements and retention of position by PrE committed cells initially in contact with the blastocoel cavity [37, 60]. The few cells expressing PrE markers which do not sort to the surface and instead retain an inside position within the ICM usually have two alternative fates. They can either downregulate PrE marker expression, and presumably acquire an EPI fate, suggesting that there is still some plasticity of the ICM cells at the late blastocyst stage. Alternatively, cells that fail to sort can undergo selective apoptosis [37, 56, 60]. PrE cells that sort to their correct location on the ICM's surface may receive positional information indicating they have reached their final destination which will eventually lead to their epithelialization. Accordingly, *Sox7* expression is acquired in PrE cells once sorted, indicating that sorted and unsorted PrE cell populations are indeed distinct from one another [61].

The molecular basis for early lineage specification and segregation remains poorly understood but likely relies to some extent on differences in the adhesive properties of PrE *vs.* EPI cells. For example, in embryos lacking the cargo protein adaptor DAB2 [62] or the

extracellular matrix protein LAMC1 [63], PrE cells are specified, but either fail to sort, or cannot be maintained at the ICM's surface. In addition, integrins α5 and β1 have been shown to play a role in extraembryonic endoderm (ExEn) formation in embryoid bodies (EBs) [64], and *Itgb1* mutant embryos die shortly after implantation with defects in the specification or differentiation of the PrE [65, 66]. Reanalyzing the phenotype of some of these mutants with the current cohort of lineage-specific markers combined with live imaging will likely help to gain further insight into the mechanisms of cell sorting.

4. ISOLATION OF THREE STEM CELL POPULATIONS REPRESENTATIVE OF THE EARLY EMBRYONIC LINEAGES

Stem cell lines can be isolated from the three first lineages of the mouse blastocyst, these being embryonic stem (ES) cells which are likely derived from and represent the EPI [67, 68], the trophoblast stem (TS) cells from the TE [69] and extraembryonic endoderm (XEN) cells from the PrE [70] (Fig. 4). These cells are often considered as stem cells based on their ability to: (i) proliferate for extensive periods in culture, (ii) remain undifferentiated when cultured under appropriate conditions, and (iii) retain their developmental potential, and so differentiate *in vivo* and *ex vivo* into cell types that normally arise from their cognate lineage.

Stem cells provide not only a basis for regenerative medicine, but are also powerful tools for investigating the molecular mechanisms involved in specification, maintenance and differentiation of these first cell lineages. Our knowledge of these early developmental events has largely been hampered by the small size of the embryo, the limited number of embryos that can be recovered, and their inaccessibility. In this way, access to cognate stem cells overcomes some of these limitations.

In this context, evaluating the role of a given gene in these early lineages can be determined by the ability to establish stem cell lines (ES, TS, XEN) from mutant embryos. Furthermore, candidate factors involved in lineage specification, for example TE or PrE, can be validated by determining if their misexpression in ES cells can drive differentiation towards TE or PrE lineages. Elucidating the factors involved in stem cell maintenance can be resolved by two complementary approaches: loss-of-function should lead to differentiation, while forced expression should impair differentiation. Lastly, if the expression of a gene is sufficient to induce differentiation, it is likely a good indication that this gene is involved in differentiation. In the following section, we highlight the specificity and common features of three stem cell lines that can be derived from mouse blastocysts, based on the extrinsic and intrinsic molecules involved in their self-renewal, as well as discussing some emergent ideas on their stem cell properties.

4.1. Signaling Molecules Required for Maintenance and Self-Renewal of Early Stem Cells

ES cells were originally isolated on mitotically inactivated fibroblast feeder cells [67, 71]. Feeders can be replaced by supplementing the serum-containing medium with LIF, a molecule that belongs to IL-6 family [72, 73]. LIF signals through the LIFR and GP130 leading to phosphorylation of the transcription factor STAT3. STAT3 eventually translocates into the nucleus where it regulates gene expression. ES cells constitutively expressing a STAT3-ER fusion protein can be grown in the absence of LIF only when the fusion is nuclear-localized by the presence of tamoxifen [74]. By contrast, expression of a STAT3 dominant-negative protein impairs ES cell self-renewal [75]. STAT3 is a main regulator of *Myc* expression and ES cells stably expressing a stabilized form of the MYC protein can be maintained in absence of LIF [76]. Serum can be replaced by BMP4, such that ES cells can be derived and propagated in serum-free culture conditions supplemented

with LIF and BMP4 [77]. In this situation, the LIF and BMP signaling pathways cooperate to maintain pluripotency [78]. Furthermore, it has been elegantly demonstrated that self-renewal does not require activation of signaling pathways, but rather the inhibition of proteins involved in differentiation, namely FGF/ERK signaling and GSK3 [79]. This double inhibitor (also referred to as 2i) strategy permits the efficient derivation of ES cells from genetic backgrounds that are normally not permissive for ES cell establishment [49, 79, 80], as well as the establishment of ES cell from other mammalian species including rat [81, 82]. Thus, in the absence of FGF signaling, ES cells are blocked in a "naïve" state that is more refractory to differentiation. In this way, FGF signaling appears to be essential for epiblast lineage commitment [51]. The effect of GSK3 inhibition is not entirely clear, but it may facilitate cell survival during the derivation process [79].

TS cells can be derived and expanded on fibroblast feeder cells in serum-containing medium supplemented with FGF4 and heparin [69]. Moreover, feeders can be replaced with feeder-conditioned medium. Members of the Tgfβ superfamily are key regulators in TS cell maintenance such that Activin and TGFβ, but not Nodal, can replace feeder cell conditioned medium [83, 84].

In contrast to ES and TS cells, which require specific conditions for their derivation, mouse XEN cell lines have successfully been isolated using at least three different protocols [70]. XEN cell lines can be established by placing blastocysts or isolated ICMs in TS cell conditions (feeders + FGF4), and then routinely cultured on feeders in the absence of FGF4. XEN cell lines have also been derived using ES cell conditions (feeders + LIF). Moreover, XEN cells have reproducibly been derived not only from mouse embryos [58, 70, 85, 86] but also from voles [87] and rats [87–90]. It is not entirely clear which signaling pathways are required for XEN cell maintenance, but PDGF signaling is required for XEN isolation and/or cell expansion. Indeed, XEN cell lines lacking the receptor PDGFRα cannot be established and conditional inactivation of *Pdgfra* in established XEN cells impairs their propagation [58]. It is likely that several RTKs act in concert to sustain a threshold level of MEK/ERK activity necessary for XEN cell maintenance. In addition to PDGFRα, possible candidates include the FGF and KIT receptors.

4.2. Regulatory Networks of Stem Cell Self-Renewal

Unraveling the molecular mechanisms underlying "stemness" has been a longstanding challenge. The elucidated gene regulatory networks with transcription factors representing core components have revealed both divergent and common regulators between these early stem cell types.

The core regulatory network involved in ES cell maintenance has been intensively studied and is relatively well characterized. It involves a triumvirate of transcription factors: OCT4, NANOG and SOX2 each of which function to both regulate positively their own expression, as well as repressing the expression of genes promoting cell differentiation [91, 92]. OCT4 levels are critical for the maintenance of pluripotency. It was elegantly shown that OCT4 downregulation in ES cells induces TE differentiation, while OCT4 upregulation induces mesoderm and PrE differentiation [93]. NANOG was originally isolated in a screen designed to identify factors that confer, when overexpressed, an ability to promote pluripotency in the absence of LIF [38, 94].

In addition to this core circuit, other components may work in parallel. This is likely to be the case for *Ronin,* a gene that encodes a zinc finger protein. RONIN is essential during early embryonic development and in ES cell maintenance [95]. Forced RONIN expression in ES cells impairs differentiation [95]. RONIN and its essential co-factor HCF-1 regulate

the expression of a variety of genes involved in metabolism, thus sustaining the growth of undifferentiated cells [96].

The network that governs TS self-renewal remains to be dissected more precisely. CDX2 and GATA3 are thought to be at the top of the hierarchy as each of which, when misexpressed in ES cells, is sufficient to drive differentiation towards a TE identify [19, 97]. Interestingly, comparative transcriptome analyses of *Cdx2*-misexpressing and *Gata3*-misexpressing ES cells has revealed that while they share common targets, GATA3 and CDX2 can act in parallel pathways [19, 97]. In addition, several studies have determined a role of various transcription factors in TS self-renewal including EOMES, ELF5, ETS2, TCFAP2C and SMARCA4 [98–100]. Recent analyses of promoter binding sites has established that SMARCA4, TCFAP2C and EO-MES are likely to be part of the core transcriptional machinery in TS cells [98].

For XEN cells, even less is known, but several studies have suggested a role for the transcription factors GATA4, GATA6, SOX7 and SOX17 in cell maintenance [101, 102].

An emerging concept is that these early stem cell types may require some common regulators. This for example is the case for SOX2, which is expressed by both ES and TS cells. In ES cells, a critical role of SOX2 is to regulate the level of *Oct4* [103]. *In vivo*, removal of both maternal and zygotic *Sox2* expression leads to developmental arrest at the morula stage and a failure to form TE [104]. The exact role of SOX2 in TS cell remains to be determined, but Avilion and colleagues reported a failure to establish *Sox2*-deficient TS cells, suggesting a critical requirement for TS cell derivation [105]. Similarly, SALL4 is expressed in ES and XEN cells where it regulates the expression of distinct lineage regulators [101]. *In vivo* SALL4 is essential for the development of ICM lineages [106].

Taken together, recent studies suggest some regulators are co-opted to participate in different gene regulatory networks, and that specificity is likely to be context dependent, for example, though the availability of co-factors as well as through accessibility of binding sites.

4.3. Pluripotent Stem Cells from Periimplantation and Early Postimplantation Stage Mouse Embryos

Various pluripotent cells have been isolated from early postimplantation stage mouse embryos, for example epiblast stem cells (EpiSC) have been isolated from the epiblast [107, 108]. Mouse EpiSCs differ from mouse ES cells not only by their flattened colony morphology, molecular signature and ability to contribute cellular progeny to chimeras but also in the extrinsic conditions (Activin and bFGF) necessary for their isolation and propagation. Interestingly, EpiSCs have also been isolated from mouse blastocyst embryos [80, 109] leading the question of whether EpiSC lines are established from a particular subset of epiblast cells resident within the ICM or result from the progressive maturation of ICM cells into an epiblast-like state in culture (Fig. 4).

Interestingly, Bao and colleagues reported the isolation of pluripotent reprogrammed epiblast ES cell-like cells (rESCs) from the epiblast of perigastrulation stage mouse embryos resembling to ES cells [110] indicating that the mature state of the epiblast around the time of gastrulation may still be reversible to a more immature state. Additional types of pluripotent stem cells have been isolated from mouse blastocyst embryos cultured in the presence of bFGF, Activin, BIO (a GSK3 inhibitor) and a LIF blocking antibody [111]. These cell lines, referred to as FAB-SCs according to the culture conditions in which they were isolated, express pluripotent markers, but fail to differentiate in teratoma or embryoid body assays if not stimulated with LIF and BMP4 beforehand.

Lastly, embryonic germ (EG) cells which have been isolated from primordial germ cells at later stages of postimplantation development and share common properties to other pluripotent cell types [112–117].

Notably, human ES cells which are derived from blastocyst embryos [118] share greater similarities to mouse EpiSCs than ES cells. hES cells, like mouse (m)EpiSCs, grow as flattened colonies and require FGF and Activin signaling to self-renew. Interestingly, while SMAD2/3 directly controls *Nanog* expression in both mEpiSC and hES cells, FGF2 only supports self-renewal in hES cells [119]. However, hES cells also share some features with mES cells but not mEpiSCs, suggesting they may represent an intermediate pluripotent state. For example, they also express some ICM markers (such as *REX1* or *ZFP42*), but not the epiblast marker *FGF5,* in contrast to mEpiSCs. For more details, we invite the readers to dedicated reviews on this topic [120, 121].

4.4. Heterogenous and Interconvertible Stem Cell Sub-populations Coexist in Culture

It is becoming increasingly apparent that stem cells are heterogenous in culture. This is relatively well documented in mouse ES cells based on detection of various markers including *Nanog* [122–124], *Pecam* [125, 126], *Rex1* [127] and *Stella* [128]. Interestingly, these sub-populations reflect cell states that are interconvertible, but share distinct patterns of gene expression and exhibit distinct differentiation properties. For example, expression profile comparisons of NANOG-high expressing cells *vs.* NANOG-low expressing cells revealed that NANOG-low cells expressed genes enriched in the PrE [123, 124]. Similarly, PECAM-positive ES cells preferentially contributed to the epiblast when injected into recipient embryos whereas PECAM-negative cells expressed markers of differentiation and primarily contributed to extraembryonic lineages [125, 126]. More recently, Canham and colleagues reported low levels of expression of *Hex* (a marker of distal/anterior visceral endoderm) in ES cells in culture [129]. Using a sensitive reporter, they showed that *Hex*-positive and *Hex*-negative cell populations are interconvertible. When allowed to differentiate, these cells have different properties: *Hex*-negative cells contribute to embryonic lineages, while *Hex*-positive cells differentiate into extraembryonic lineages, suggesting they likely represent an early PrE state. Similar observations have been made with the endoderm marker *Sox17* [102] and the mesoderm marker *Brachyury* [130].

Of note, several subpopulations have also been described in mouse EpiSCs [131, 132] and in hES cells [133–136]. An emerging idea is that pluripotency is associated with metastable states, which are usually interconvertible, and which are defined by differences in gene expression and developmental potential. These fluctuations of (or metastable) cell states in a cultured population may extend to other early stem cell types, including TS and XEN cells. Indeed, XEN cells undergo morphological transitions [70] and heterogenously express some markers such as *Sox7* [137]. These observations may reflect fluctuations in cell states and an inherent metastability. Moreover, we recently demonstrated that activation of BMP signaling in XEN cells drives their differentiation toward a stable state resembling the extraembryonic VE [138]. In this state, we observed fluctuations of a VE reporter transgene, indicating that at least two subpopulations coexist in BMP-treated XEN cell cultures, and that they are interconvertible. Additional studies will be required to address the origin and significance of the fluctuations that are observed and stably maintained in XEN cells, as well as establishing whether a similar heterogeneity exists in TS cell cultures.

Even though the molecular mechanisms and significance of such heterogeneity is not fully understood, it reveals that some cells in a cultured population may be more undifferentiated than others. This concept was first developed by Austin Smith and colleagues who defined pluripotency as a "ground state" in which, in the absence of GSK3 and FGF activity, ES cells remain undifferentiated. ES cells can be isolated and maintained in this naïve state of

pluripotency in 2i conditions. FGF signaling is required to convert naïve cells to a state where they are primed to differentiate, a state that may resemble the more mature periimplantation epiblast and its *ex vivo* counterpart, the EpiSC. Indeed, ES cells cultured in the presence of an FGF inhibitor (or lacking *Fgf4*) are generally refractory to differentiation [139, 140]. Thus, both naïve ES cell subpopulation, as well as one poised for differentiation, may coexist in culture. Interestingly, ES cells can be converted into EpiSC simply by changing their culture conditions to those for EpiSCs [141]. Reciprocally, EpiSC can be reprogrammed to a more naïve ES-like state by overexpression of various factors such as Klf2, Klf4, Nanog, Nr5a2, Nr5a1 or Stat3 [40–144] or by culturing in ES cell conditions [80, 110, 119].

5. EMBRYOID BODY FORMATION: AN *EX VIVO* MODEL OF PERIIMPLANTATION DEVELOPMENT

Pluripotent ES cells have the tremendous potential to generate virtually all the cell types of the body, and this feature directly impacts their use in regenerative medicine. Two key differentiation protocols have been defined and reflect the culture of cells in two or 3 dimensions (3D). In 3D dimensions, cells are cultured in non-adherent conditions promoting the formation of small aggregates called embryoid bodies (EB). EBs can also be generated from embryonal carcinoma (EC) cells derived from teratocarcinoma tumors [71, 145]. EB differentiation follows a stereotypical sequence of events that mimics the differentiation of the ICM, and apposition of tissue layers during normal periimplantation and early postimplantation stages of embryonic development (Fig. 5). As a consequence, EBs are commonly used as a first step in ES cell differentiation protocols.

In this section, we review the first events occurring in the process of EB formation in relation to early mammalian development *in vivo*. The first morphologically distinct differentiation within EBs, taking place between 3 to 4 days, involves the formation of an outer layer with extraembryonic endoderm (ExEn) characteristics. By 8 days of differentiation, a cavity has formed and internal cells are arranged into a pseudostratified columnar epithelium. These cystic embryoid bodies resemble early postimplantation embryos in which the proamniotic cavity is surrounded by embryonic ectoderm (epiblast) encapsulated by a layer of VE. Cells eventually differentiate into the three germ layers of the embryo-proper (ectoderm, mesoderm and gut endoderm).

5.1. Formation of the Outer Layer of ExEn

As previously discussed, a recent model of *in vivo* PrE formation and segregation involves: (i) positional inductive signals that may reinforce fate, (ii) sorting of cells already committed to PrE *vs.* EPI lineages, and (iii) selective apoptosis. If this model could be adapted to ExEn formation during EB differentiation, then one might predict that EBs are composed of a heterogeneous population of cells committed to either EPI- or ExEn-like fates that would eventually sort and resolve. The fact that ES cells in culture fluctuate between different states might lend support to such a model. Therefore, monitoring the dynamics of these fluctuations during the early steps of EB differentiation will likely be interest with respect modeling PrE formation.

It is difficult to find evidence suggesting that EBs can be used to model cell sorting. In EBs generated from ES cells lacking *Dab2* [146] or *Sox17* [102], ExEn cells differentiate but are found inside the EB. In contrast, in EBs comprised of wild-type cells, ExEn cells comprise the outer layer. These observations can be interpreted as a failure of ExEn cells to sort and/ or be maintained in an outer location. However, a mixed population of undifferentiated and RA-treated ES cells segregates into two distinct compartments, suggesting differential

adhesive properties [147]. Transmission electron microscopy analyses has not revealed any endodermal cells at the early stages of EB differentiation arguing in favor of the positional model, where outside cells would acquire ExEn identity [145]. By contrast, Rula and colleagues reported some DAB2-positive cells inside EBs preceding outer ExEn layer differentiation [147].

Thus, the question of a positional effect in EBs and its relevance to the embryo remains to be addressed. Interestingly, it was reported that an outer layer of ExEn cells is formed upon aggregation of GATA6-overexpressing ES cells [148]. Similarly, RA-treated EBs (from ES or EC cells) are composed by an outer layer of ExEn (see for review [149] indicating that the position of cells within the EB may be important for fate decision. Strikingly, some studies reported the transient expression of ExEn markers including *Afp* [150] and *Gata6* [58] in almost all cells within the EB prior their specific localization of endodermal cells. Therefore, the formation of the ExEn layer during EB formation is a multistep processes which might reflect aspects of the *in vivo* segregation of PrE and EPI lineages within the ICM of the blastocyst.

5.2. Differentiation of the ExEn Layer

The identity of endodermal cell types that constitute the outer layer of an EB was originally described through ultra-structural studies by transmission electron microscopy [145]. Cells that form the outer layer of the EB are usually composed of a mixed population of both PE and VE cells. PE cells contain rough endoplasmic reticulum (ER) usually filled with a substance resembling the Reichart's membrane. VE cells have a thinner ER, microvillosities and vacuoles. In addition, the outer layer exhibits non-homogenous expression of some VE markers such as *Afp* (and a reporter trans-gene *Afp::GFP* [151, 152]), suggesting that several ExEn subtypes may co-exist within an EB. Our recent analysis of EBs over a period of 3 to 7 days of differentiation revealed a lack of expression of *Pdgfra* or *Sox7*, both markers of nascent PrE, PE and proximal VE [58]. Taken together, these results suggest that the outer layer if EBs might most closely resemble the distal VE overlying the epiblast, which is also referred to as emVE.

Several signaling pathways have been proposed to be important for ExEn differentiation; they include BMP, FGF, LIF and Retinoic Acid (RA). When EBs are cultured in presence of LIF, the outer layer acquires a PrE-like identity, but cells do not differentiate into either PE or VE-like derivatives indicating that LIF pathway prevents PrE differentiation [153]. Exogenous addition of BMP4 triggers differentiation of the outer layer of EB towards a VE-like fate [154]. In 2D, RA promotes differentiation of F9 EC cells into PrE while when combined with dbAMPc cells adopt a PE-like identity. In contrast, RA-treated F9 EBs differentiate into VE (reviewed in [149]). ES cells stably expressing a dominant negative FGFR2 or mutant for *Grb2* neither differentiate into VE-like, or form a columnar epithelium and cavitate [48, 155]. A similar phenotype was also observed when PI3K activity is inhibited [155] and the effect observed in ES cells expressing a dominant negative FGFR2 can be rescued when cultured in matrigel or in presence of Laminin-1 [156].

5.3. Cavitation

Cavitation in EB occurs by apopototic cell death and is thought to mimic the formation of the proamniotic cavity in the embryo soon after implantation. Cavitation occurs at the periphery of an EB and then progress inward. Cavitation is concomitant with the formation of a columnar epithelium resembling the embryonic ectoderm (Fig. 5). A comparison between two EC lines that differ in their ability to form cystic EBs has revealed some insight into the mechanisms of cavitation, and highlighted the intimate dialog between neighboring

tissues [154, 157]. Notably, these studies have identified the BMP signaling pathway as important for cavitation and ExEn differentiation [154].

Formation of an ExEn epithelial cell layer on the surface of an EB leads to the deposition of ECM proteins basally. ECM appears to be required for the formation of an inner columnar ectoderm and EB cavitation, as absence of *Lamc1* or *Itgb1*, results in a failure of cavitation and epithelialization, but does not affect ExEn layer formation [158, 159].

6. CONCLUDING REMARKS

In this review, we have discussed some of the parallels which can be drawn between pluripotent stem cell biology and early mammalian embryology. Understanding the mechanisms governing stem cell self-renewal *vs.* differentiation is not only key for facilitating progress in regenerative medicine, but also provides clues about the critical early cell fate decisions taking place *in vivo* within the embryo. Conversely, knowledge gained from dissecting the processes involved in lineage specification and diversification during embryonic development is likely to directly impact our understanding of stem cell biology. We have chosen to highlight studies in the mouse, the premier experimentally tractable mammalian model. However, one long-standing question in the field concerns the inherent similarities and notable differences taking place across mammalian species. We do not currently know how far, and at what level, events correlate, and how relevant knowledge gained from mouse model will be in understanding events taking place in higher mammals, including humans. Clearly, future studies involving cross-species comparisons will be required both in embryos and in stem cells for validating molecules and signals involved in cell lineage commitment, self-renewal and differentiation.

Acknowledgments

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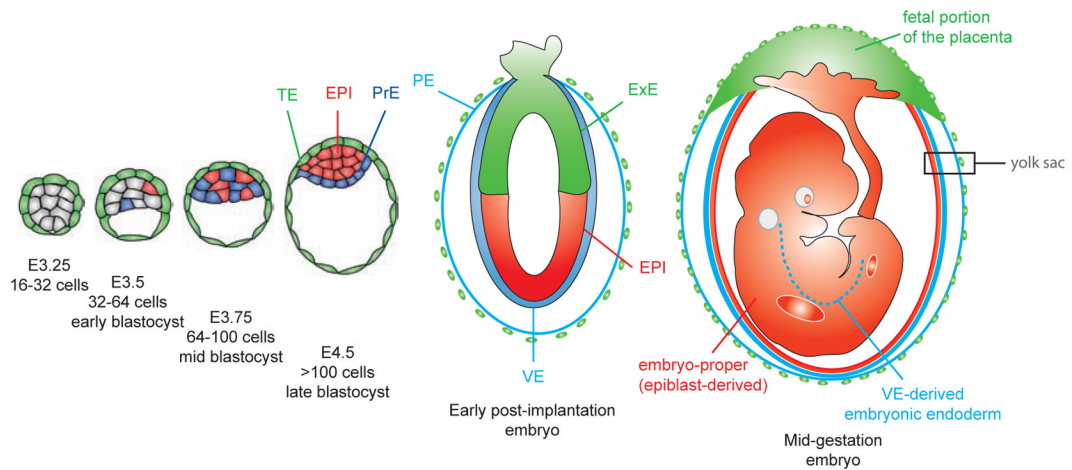
Fig. 1. Schematic representation of the formation and fate of the first cell lineages of the mouse embryo

The pluripotent epiblast (red) will form the embryo proper. The trophectoderm (green) will give rise to the fetal portion of the placenta and contribute to the yolk sac. The primitive endoderm (blue) will differentiate into the parietal and visceral endoderm that will constitute the yolk sac. The visceral endoderm will also be incorporated into the embryonic endoderm (blue dashed line). EPI, epiblast; ExE, extraembryonic ectoderm; PE, parietal endoderm; PrE, primitive endoderm; TE, trophectoderm; VE, visceral endoderm.
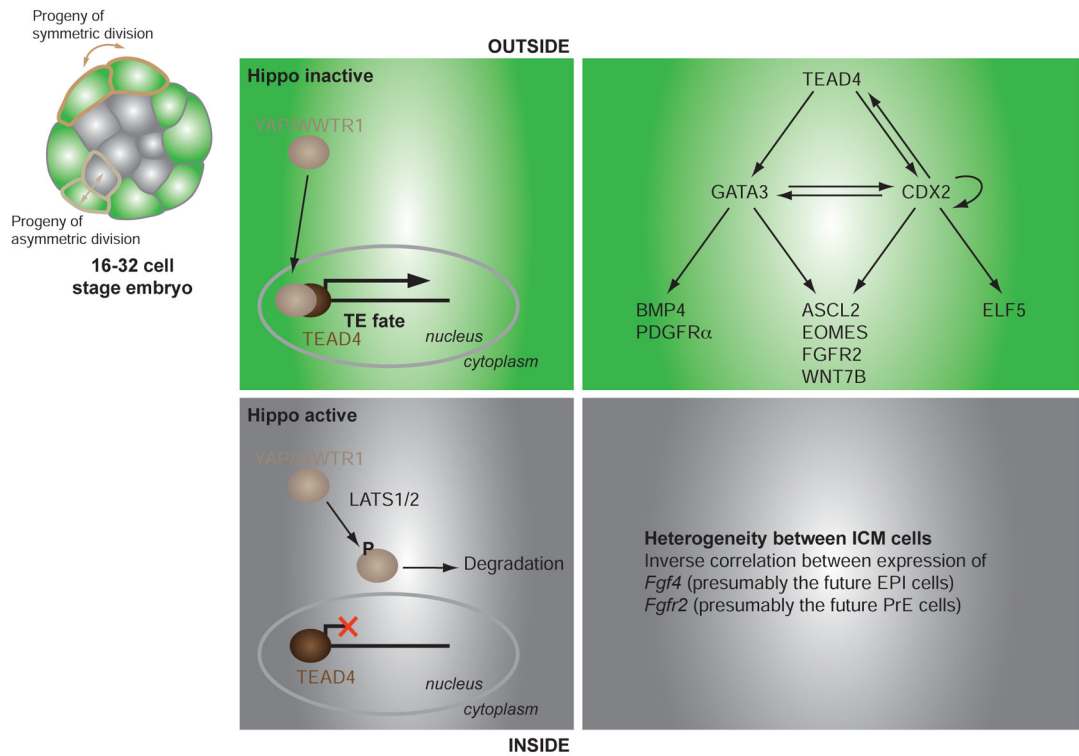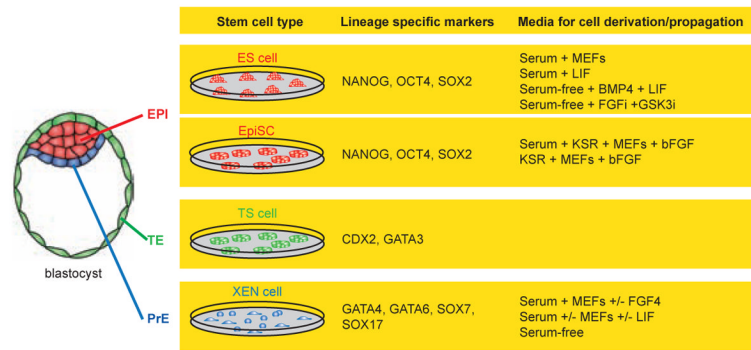
Fig. 2. The first cell lineage decision in the mouse embryo

While symmetric divisions starting from the 8-cell stage give rise to two outside (green) polarized cells, asymmetric divisions give rise to an outside cell and an inside (grey) apolar cells. In outside cells, TEAD4 transcription factor and its cofactors YAP/WWTR1 regulate the expression of a TE-specific transcription program that ultimately commits outside cells to a trophectoderm fate. This program is not achieved in inside cells because of the activity of the Hippo pathway that leads to the degradation of YAP/WWTR1. ICM cells are not homogenous and an inverse correlation of the expression of *Fgf4* and *Fgfr2* may presage the divergence between PrE and EPI lineages.
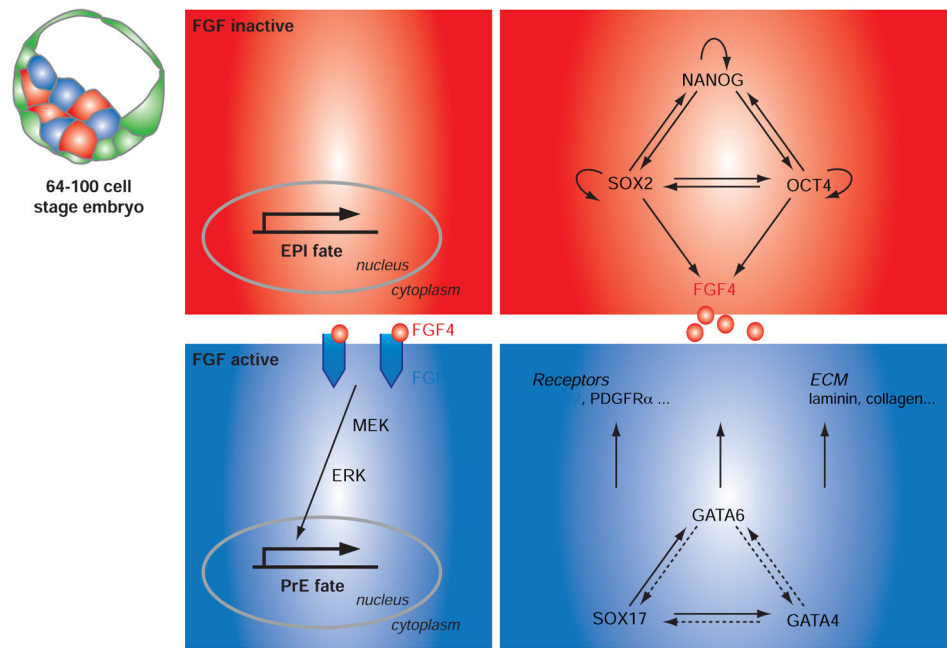
Fig. 3. Lineage specification within the inner cell mass of the preimplantation mouse embryo
Lineage commitment of PrE and EPI cells is thought to occur around the 64-cell stage and is driven by a core-regulatory network of lineage-restricted transcription factors. FGF4 signaling pathway is a major determinant of PrE specification. FGF4 ligand is synthesized by EPI cells whereas FGFR2 is expressed by PrE cells. Activation of ERK signaling is predicted to be necessary for PrE commitment.

Stem cell type	Lineage specific markers	Media for cell derivation/propagation
ES cell	NANOG, OCT4, SOX2	Serum + MEFs Serum + LIF Serum-free + BMP4 + LIF Serum-free + FGFi +GSK3i
EpiSC	NANOG, OCT4, SOX2	Serum + KSR + MEFs + bFGF KSR + MEFs + bFGF
TS cell	CDX2, GATA3	
XEN cell	GATA4, GATA6, SOX7, SOX17	Serum + MEFs +/- FGF4 Serum +/- MEFs +/- LIF Serum-free

Fig. 4. Stem cells that can be derived from blastocyst stage mouse embryos
Three stem cells can be isolated from a mouse blastocyst embryo. Embryonic stem (ES) cells and Epi stem cells (EpiSCs) from the epiblast (red), trophoblast stem (TS) cells from the trophectoderm (green) and extraembryonic endoderm (XEN) cells from the primitive endoderm (blue). These stem cells are characterized by their distinct morphologies, their developmental potential in chimeras, as well as the expression of different sets of lineage-specific transcription factors (the transcription factors listed can be recognized by commercially available antibodies) and by the conditions used to isolate and propagate them in culture.
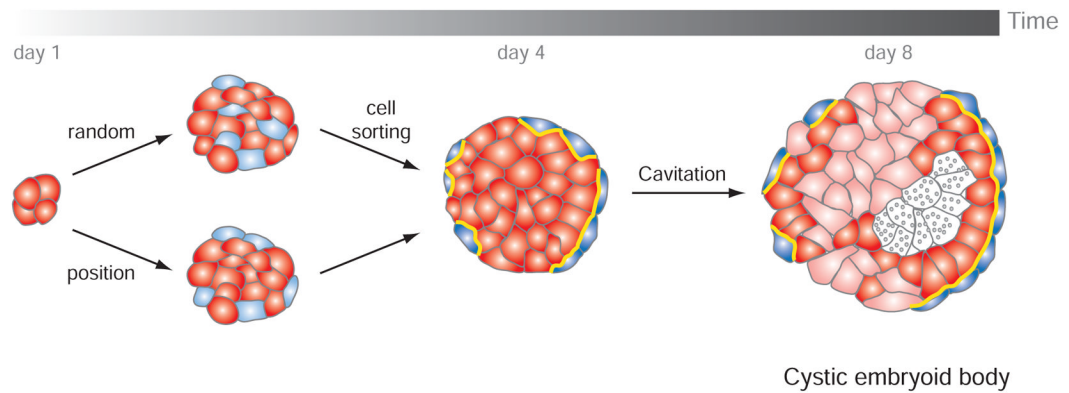
Fig. 5. Schematic representation of the first differentiation events occurring in an embryoid body
An initial morphological event is the formation of an outer layer of extraembryonic endoderm cells (blue). This could be the result from the random differentiation of ES cells (red) within the embryoid body and subsequent cell sorting, and/or from the differentiation of cells residing at the periphery and exposed to the outside environment. Extraembryonic endoderm cells secrete ECM components that will participate in the maturation of an epithelial epiblast and in the cavitation (grey dots indicate cell death). This process is usually not homogenous within an embryoid body and is accompanied by cell differentiation (pink cells).