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Perspective

Defining pathways that enforce cell lineage specification in early development and stem cells

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Abbreviations: EC cells, embryonal carcinoma cells; ERK, extracellular signal regulated kinase; ES cells, embryonic stem cells; ICM, inner cell mass; MAPK, mitogen activated protein kinase; PE, primitive endoderm; TE, trophectoderm; TS cells, trophoblast stem cells

Key words: blastocyst, cell fate, development, early embryo, lineage commitment, pluripotency, stem cells, transdifferentiation, trophoblast

The molecular processes that govern the first cell lineage decisions after fertilization also dictate the developmental potency of stem cells derived from the early mouse embryo. Our understanding of these mechanisms is therefore instrumental for stem cell biology and regenerative medicine. A number of transcription factors are known that determine a cell's fate towards either the embryonic or extraembryonic trophoblast lineages. Recent insights have shown that the definitive fixation of cell lineage fate is achieved by an epigenetic restriction through DNA methylation of the transcription factor *Elf5*. Lineage crossover can be induced, however, by manipulation of lineage determinants and gatekeepers, or their epigenetic regulation. Here we summarize the accumulating number of experimental conditions where such 'transdifferentiation' is observed that shed light onto the genetic and epigenetic pathways involved in lineage separation and the developmental potential of stem cells.

Introducing the First Differentiation Events in the Early Mouse Embryo

By the expanded blastocyst stage of mouse development, three distinct cell populations, or lineages, have been established that will go on to form all of the embryonic and extraembryonic tissues of the conceptus. The first definitive differentiation event produces the trophectoderm (TE), which consists of a monolayer of epithelial cells surrounding the inner cell mass (ICM) and the fluid-filled blastocoel. Slightly later, the ICM differentiates into the epiblast and an overlying layer of primitive endoderm (PE) cells. The significance of the establishment of these three cell populations is that, perhaps with the exception of the PE, they remain committed in their differentiation potential towards their lineage throughout all subsequent cell divisions.[1,2] Thus, while they retain a pluri- or multipotent differentiation capacity into various cell types of a given lineage, they do not normally cross these lineage boundaries during later development.

First evidence for the separation of developmental fates in the earliest two cell populations, the ICM and TE, was provided by the observation that ICM fragments are unable to induce a decidualization reaction in recipient uteri. In contrast, trophoblast fragments implant as effectively as intact blastocysts.[3] This result is explained by the fact that ICMs have lost the ability to form trophoblast cells[4] and instead contribute solely to embryonic structures and extraembryonic membranes in aggregation chimeras.[5] The trophoblast of implanted conceptuses is, by contrast, entirely produced by the TE.[6]

A series of experiments have addressed the question as to when precisely cells become stably committed to one of the two earliest cell lineages. Establishment of 'inside' and 'outside' cells and cell polarization in the 8- to 16-cell transition introduces some bias toward fate allocation.[7] However, while the outer cells of morula-stage embryos tend to form most or all of the TE, they also give rise to a significant proportion of the inner cell mass and hence have not yet acquired fixed fates.[8] A small proportion of early blastocyst stage embryos also showed some lineage crossing in a similar experiment.[9] In keeping with this observation, isolated early blastocyst ICMs can give rise to trophoblast giant cells in culture[10] and are still able to contribute to the trophoblast in vivo when aggregated with morula stage embryos. However, by the late blastocyst, the contribution of isolated ICMs to trophoblast in vivo is extremely rare.[11] Thus, a definitive and irreversible commitment to the embryonic and trophoblast lineages seems to be established only by the late blastocyst stage.

After implantation of the blastocyst into the uterus, the TE gives rise to all trophoblast cell types of the conceptus: these include parietal trophoblast giant cells that line the implantation site, extraembryonic and chorionic ectoderm, the ectoplacental cone and later the various trophoblast cell types of the mature

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chorioallantoic placenta. The PE goes on to form the parietal and visceral endoderm layers of the yolk sac that, together with the trophoblast derivatives, contributes to providing adequate nutrition of the embryo. The embryo proper is derived from the epiblast. Upon gastrulation, the epiblast also gives rise to the extra-embryonic mesoderm that forms the allantois and the mesodermal components of the yolk sac, as well as the fetal vasculature of the placenta (Fig. 1).

Stem Cell Types of the Early Embryo

The existence of pluripotent stem cell populations was first demonstrated by the derivation of embryonal carcinoma (EC) cells from teratocarcinomas.[12] These malignant germ cell tumors harbour a small EC cell population and contain multiple differentiated cell types. The pluripotent nature of EC cells was demonstrated as single cell transplants are sufficient to generate tumors consisting of multiple and varied differentiated somatic cell types in recipient mice.[13] Additionally, EC cells can form embryoid bodies[14,15] and contribute to a range of developmentally-unrelated tissues following blastocyst injection.[16] EC cells were therefore thought to reflect the pluripotency of ICM cells. However, EC cell usage was limited to a few specific cell lines as the majority showed more limited developmental potential and contributed poorly to chimeric mice.

These studies on EC cells paved the path for the derivation of embryonic stem (ES) cells from blastocysts.[17-19] When grown under appropriate conditions, ES cells are able to self-renew indefinitely. ES cells are able to contribute to all tissues in chimeric mice following blastocyst injection and even to generate entirely ES cell-derived mice in tetraploid complementation assays and therefore reflect the pluripotent nature of the ICM.[20,21]

Stem cells of the trophoblast lineage can also be derived from blastocysts, as well as from early post-implantation conceptuses, using mouse embryonic fibroblast (MEF)-conditioned media containing the growth factor FGF4.[22] These trophoblast stem (TS) cells are present as a minority in the polar TE of the blastocyst and in the extraembryonic and chorionic ectoderm of wild-type embryos until at least E8.5.[23] Recapitulating the developmental potency of their parental cell lineage, TS cells contribute exclusively to trophoblast tissues following blastocyst injection and can differentiate into all trophoblast cell types of the chorioallantoic placenta.

The possibility of deriving a stem cell line representative of the primitive/extraembyonic endoderm (ExE) was first suggested by the finding that the EC cell line F9 could be differentiated into cells with properties indistinguishable from definitive parietal endoderm.[24,25] The derivation of extraembryonic endoderm (XEN) stem cells from blastocysts and ICMs was achieved using the same conditions as for TS cell derivation. XEN cells express markers of extraembryonic endoderm but not of trophoblast or embryonic derivatives and contribute exclusively to extraembryonic endoderm cell types following blastocyst injection.[26]

Hence all three cell lineages of the mouse blastocyst give rise to a distinct type of stem cell that can be cultured indefinitely in vitro and retain the lineage restriction imposed on its parental cell population.

Cytokines Critical for Stem Cell Maintenance

Each stem cell type depends on a unique set of cytokines to retain its full developmental potency. Leukemia inhibitory factor (LIF) and its downstream signaling components, the LIF receptor GP130 and the transcriptional regulator STAT3, are of key importance for the derivation of ES cells and for the maintenance of their pluripotent state.[27-32] In the absence of LIF, ES cells lose the ability to self-renew and instead differentiate. However, the importance of LIF in early development is less clear. As expected for this signaling interaction, LIF transcripts are detected in the TE and *gp130* transcripts primarily in the ICM.[33] Despite this, *gp130* is dispensable for this stage of development, with mutant embryos instead dying between E12.5 and term.[34] It has since been found that LIF signaling is essential for epiblast maintenance following delayed implantation, suggesting that this may explain the necessity of LIF for prolonged ES cell culture.[35]

TS cell derivation and self-renewal is dependant on FGF4 and MEF-conditioned medium. Withdrawal of both components causes TS cells to differentiate into various trophoblast subtypes, predominantly trophoblast giant cells.[36] The identification of FGF4 as the critical cytokine was based on the observation that FGF signaling is indispensable in the early embryo. *Fgf4* is expressed in the ICM of late blastocysts[37] and in ES cells,[38] whilst the FGF receptor *Fgfr2* is expressed in a TE-specific manner from the early blastocyst stage.[39] Mutations in *Fgf4* and *Fgfr2*, as well as the downstream signaling transmitters *Frs2a* and *Erk2*/*Mapk1*, all lead to lethality soon after implantation, consistent with trophoblast proliferation defects.[40-43] These data have supported the hypothesis that FGF4 produced by the embryo provides a niche for TS cells in the neighboring trophoblast. More recently, the active components of the MEF-conditioned medium were identified as TGFβ and the related protein activin, and TS cells can be derived and maintained in a self-renewing state in media only supplemented with serum, FGF4 and TGFβ.[44] Consequently, TGFβ inhibition in normal media interferes with TS cell self-renewal and causes them to differentiate into trophoblast giant cells.

XEN cells were originally derived in the presence of the same media as used in TS cell derivation and culture. However, XEN cell derivation and maintenance does not require FGF4 or other purified cytokines, and is equally effective in MEF-conditioned medium alone without the need of a feeder cell layer. Hence, XEN cells appear to be the most 'robust' stem cell type of the blastocyst, and they are frequently co-derived in early stages of the ES and TS cell derivation process.[26]

Key Transcription Factors in Early Development and in Stem Cells

Recent years have seen major advances in our knowledge of transcription factors that are required for blastocyst formation as well as for the establishment and maintenance of the three stem cell types. Establishment of the ICM and epiblast depends on the mutually interacting transcription factors OCT4 (encoded by the *Pou5f1* gene), NANOG, SALL4 and SOX2,[45-48] and these four factors also fulfil a key requirement in maintaining ES cell

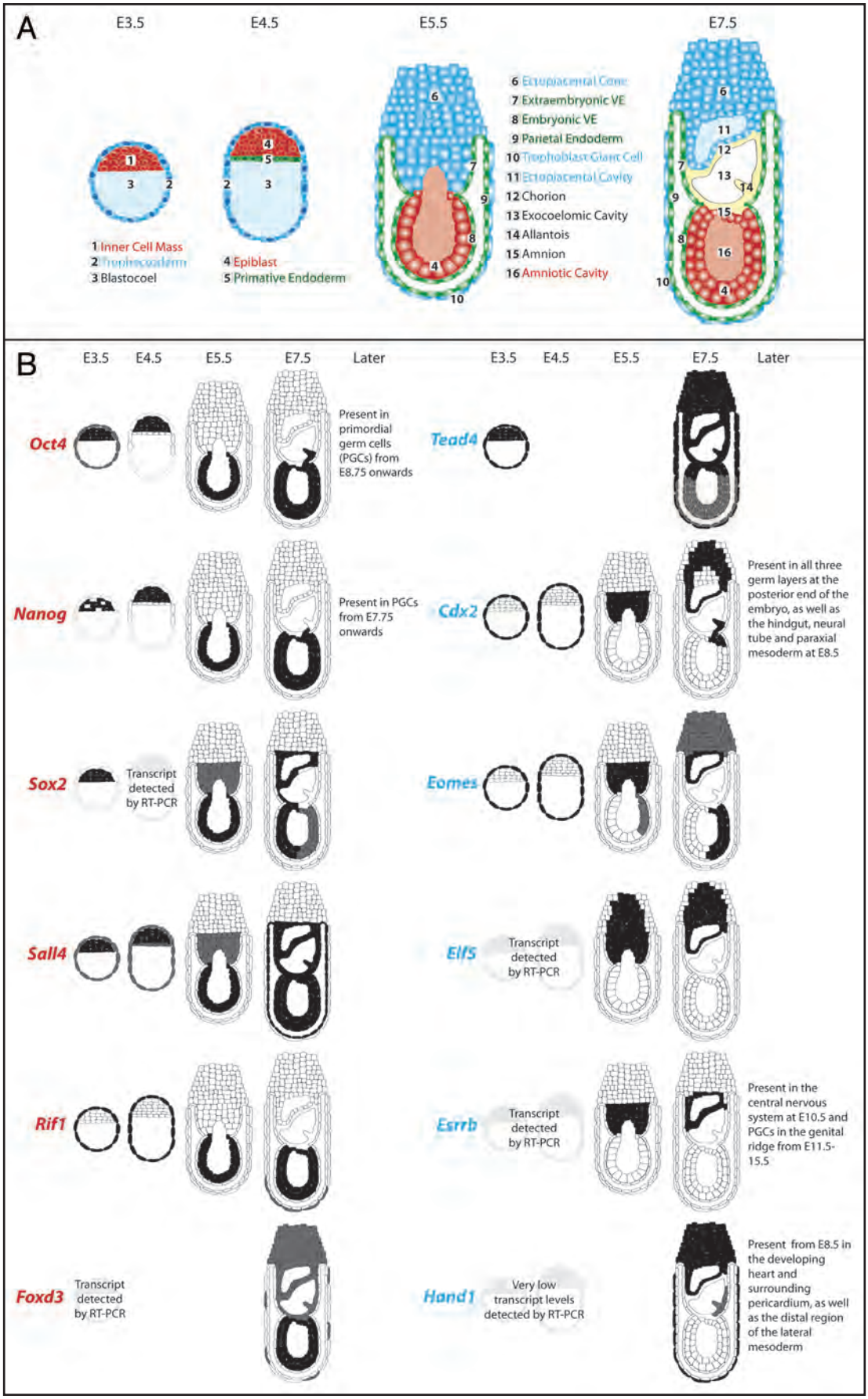

Figure 1 (See previous page). Expression dynamics of key transcription factors during early embryonic development. (A) Labelled schematic diagrams of the early blastocyst (E3.5), late blastocyst (E4.5), and post-implantation conceptuses at E5.5 (egg cylinder stage) and E7.5; with red, green and blue representing the embryonic, primitive/extraembryonic endoderm and trophoblast lineages respectively. (B) Schematic diagrams shaded to show the expression patterns of the transcription factors *Oct4/Pou5f1*,[53,103-107] *Nanog*,[62,103,108-110] *Sox2*,[48,111-113] *Sall4*,[47,111,114-116] *Rif1*,[117] *Foxd3*,[118-120] *Tead4*,[55,56,121,122] *Cdx2*,[53,81,82,106] *Eomes*,[54,82,123-125] *Elf5*,[69,126] *Esrrb*[111,127-130] and *Hand1*.[83-85,111] Black shading represents strong expression, dark grey shading significantly lower expression, and white a lack of expression. Light grey colouration of embryos indicates expression with an unknown distribution pattern at that stage. *Esrrb* is also expressed in ES cells. Onset of expression in cell types and tissues crossing the main embryonic-extraembryonic boundaries (i.e., extraembryonic expression of *Oct4/Pou5f1* and *Nanog*; embryonic expression of *Cdx2*, *Esrrb* and *Hand1*) immediately after E7.5 is also given. VE; visceral endoderm.

pluripotency. Notably, all four factors auto-regulate their own transcription and activate each other and thereby form a self-reinforcing transcriptional network of pluripotency. In addition, they can either directly heterodimerize, as in the case of OCT4 and SOX2, or are found in shared protein complexes.[49-51]

A key transcription factor for the trophoblast lineage is the caudal-type homeodomain protein CDX2. *Cdx2*$^{-/-}$ embryos die before implantation[52] because the TE fails to maintain trophoblast identity and its epithelial integrity, resulting in the collapse of the blastocyst.[53] Additionally, ES but not TS cells can be derived from *Cdx2*-mutant blastocysts, showing that CDX2 is indispensable for TS cell self-renewal. Trophoblast failure is also observed in the absence of the T-box gene *Eomesodermin (Eomes)*.[54] From genetic data, EOMES is positioned just downstream of CDX2.[53] However, while both CDX2 and EOMES are essential for TE maintenance and trophoblast function, initial formation of the TE layer can occur in their absence. A factor further upstream in the trophoblast specification sequence has been identified recently as TEAD4, which can activate CDX2 and may thus be the gene on top of the trophoblast-defining transcription factor cascade.[55,56]

In the extraembryonic endoderm lineage, the transcription factors GATA4 and GATA6 have been shown to have a key role. *Gata4*$^{-/-}$ and *Gata6*$^{-/-}$ embryoid bodies display a block in visceral endoderm formation and fail to express normal markers of endoderm differentiation.[57] GATA6 null embryos do not form a morphologically recognizable primitive endoderm layer, and subsequently fail to form visceral and parietal endoderm leading to embryonic lethality between E6.5 and E7.5.[58,59] Also, whilst GATA4 and GATA6 are initially co-expressed, GATA6 expression is rapidly lost in visceral endoderm after implantation, thereby discriminating parietal from visceral endoderm. Another transcription factor, SOX7, appears to lie upstream of both GATA4 and GATA6 based on experiments in the F9 EC cell line. Knockdown of *Sox7* parallels the block in parietal endoderm formation seen in *Gata4*|*Gata6* double mutants. Additionally, *Sox7* deletion leads to a decrease in both GATA4 and GATA6 expression, and upregulation of both of these downstream proteins restores normal endoderm differentiation potential.[60]

Since lineage identity is retained by stem cells derived from the early embryo, experimental situations in which this commitment is lost serve as an extremely useful model to unravel the genetic and epigenetic networks that establish lineage fate. In this context, we focus in particular on the restriction of the embryonic (i.e., ICM, epiblast, ES cell) lineage from the trophoblast (TE, TS cell) lineage. We summarize here the accumulating number of situations in which ES cells have been shown to lose their lineage commitment and transdifferentiate into trophoblast cell types (Table 1).

Aberrant Transcription Factor Expression Leads to Loss of Lineage Identity and Transdifferentiation

In line with their importance for blastocyst formation, transcription factors like OCT4, NANOG, SOX2, SALL4, CDX2 and EOMES have lineage-specifying functions in stem cells. Thus, knockout or knockdown of OCT4 and SALL4 in ES cells leads to their differentiation into trophoblast giant cells.[45,61] Similarly, knockdown of *Nanog* causes the upregulation of trophoblast genes, although *Nanog*-deficiency is predominantly associated with a differentiation into primitive endoderm.[46,62,63] The functions of SOX2 appear to be particularly sensitive to absolute protein levels as both knockdown and overexpression cause differentiation of ES cells into various cell types, including an upregulation of trophoblast genes such as *Cdx2*, *Hand1*, *Cdh3* and *Esx1*.[63-65] Together with their function in maintaining the pluripotency of ES cells, several other transcription factors also contribute to the lineage-restricted differentiation potential of ES cells, and their downregulation leads to ectopic activation of trophoblast markers (Table 1). Hence a common feature of these pluripotency factors is that they repress the transcription of differentiation-promoting genes, including those that induce trophoblast differentiation. While several factors display a more general inhibitory function, it is noteworthy that OCT4 and SALL4, and possibly also RIF1 and ZFP27, seem to specifically prevent differentiation into the trophoblast lineage.[66,67]

Opposing the role of pluripotency genes, factors with trophoblast determining capacity can induce trophoblast differentiation from cells of the embryonic lineage. Thus, constitutive overexpression of *Cdx2* and *Eomes* in ES cells leads to their conversion into fully functional TS cells that contribute exclusively to trophoblast tissues of the placenta when used in chimera experiments.[68] Intriguingly, in these experiments transdifferentiation does not depend on the downregulation of pluripotency factors such as OCT4, an observation that has also been made in other conditions that induce transdifferentiation.[69,70] These findings indicate that it is not absolute presence or absence, but instead relative abundance of lineage determining factors in proportion to each other that determine lineage specification. This suggestion is further supported by the fact that OCT4 and CDX2 can form a complex for the reciprocal repression of their target genes in ES cells. Mutual inhibition of transcription factors with opposing functions thus appears to be one of the key elements of early lineage differentiation events.[68]

Signaling Pathways with Lineage-Specifying Capacity

The unsuspected lineage-determining specificity of signaling cascades was revealed recently in experiments where activation

Table 1 **Genes/conditions associated with activation of trophoblast markers and trophoblast differentiation from ES cells and embryos**

Gene	Function	Phenotype	Assessment method	Reference
Transcription Factors				
Cdx2	Homeobox transcription factor	*Cdx2* overexpression in ES cells causes transdifferentiation into the trophoblast lineage. TS-like cells produced are able to contribute to normal placental structures in vivo.	Enlarged or multiple nuclei; epithelial morphology; *Cdh3* expression; upregulation of *Hand1, Fgfr2, Pl1, Tpbpa, Ets2, Psx1* and *Dlx3*; placental contribution following blastocyst injection	68
Elf5	Ets family transcription factor	Overexpression causes differentiation of ES cells into trophoblast subtypes; *Elf5* activation due to lack of DNA methylation leads to trophoblast differentiation from embryos and ES cells	Adoption of trophoblast (giant cell) morphology; upregulation of *Cdx2* and *Eomes*	69
Eomes	T box transcription factor	*Eomes* overexpression in ES cells causes cells to differentiate into the trophoblast lineage	Very small colonies of large, flat cells produced; *Cdx2, Hand1, Ets2, Esx1, Cdh3* and *Dlx3* upregulation	68
Esrrb	Nuclear receptor	*Esrrb* knockdown in ES cells causes enhanced differentiation into trophoblast, as well as into endoderm, mesoderm and ectoderm	Flattened, 'fibroblast-like' cell morphology; *Hand1* upregulation	66, 63
Foxd3	Forkhead transcription factor	*Foxd3*$^{-/-}$ ES cells display enhanced differentiation along multiple lineages including trophoblast, endoderm and mesendoderm while *Oct4, Sox2* and *Nanog* expression are maintained *Foxd3* expression is positively controlled by NANOG	Upregulation of *Cdx2, Fgfr2* and *Pl1*	99
Klf5	Kruppel-like transcription factor	*Klf5* knockdown in ES cells causes differentiation into the trophoblast, mesoderm and ectoderm lineages	Large, flattened cells produced; *Cdx2, Eomes* and *Pl1* upregulation	100, 101
Nanog	Homeobox transcription factor	Nanog Knockdown in ES cells causes differentiation into the trophoblast, endoderm, mesoderm and ectoderm lineages	Morphological changes; upregulation of *Cdx2, Hand1, Mash2, Pl1* and *Ehox*	63
Oct4	POU transcription factor	A 50% or greater *Oct4* knockdown in ES cells causes enhanced differentiation into 'trophoblastic' cells. Removal of FGF4 causes cells to adopt trophoblast giant cell-like morphology	Flattened cell morphology; often nuclei enlarged; upregulation of *Cdx2, Hand1, Mash2, Tpbp, Pl1* and *Esrrb*	45
Rif1	Telomeric protein	*Rif1* knockdown in ES cells causes enhanced trophoblast differentiation	Flattened, 'fibroblast-like' cell morphology; *Hand1* upregulation	66
Sall4	Spalt family transcription factor	*Sall4* knockdown or heterozygous knockout in ES cells causes *Oct4* downregulation in a dose-dependent manner. Such a decrease in *Sall4* mRNA is sufficient to cause ES cells to transdifferentiate in feeder-free conditions in vitro and enables them contribute to the TE of blastocysts in chimeras	Flattened cellular morphology; *Cdx2* and *Hand1* expression; ability to form Cdh3-positive giant cell-like cells; knockdown ES cells aggregated with morula stage embryos contribute to the TE of blastocysts	61
Sox2	Transcription factor	*Sox2* knockdown in ES cells promotes differentiation into trophoblast, endoderm, mesoderm and ectoderm	Flattened, epithelial cellular morphology; upregulation of *Cdx2* and *Hand1*	64, 63
		Sox2 overexpression in ES cells causes differentiation into ectoderm, mesoderm and trophoblast lineages	Flattened cellular morphology; reduced proliferation rates; increased cytoplasmic to nuclear ratio; upregulation of *Cdx2, Cdh3* and *Esx1*	65
Zfp27	Zinc finger protein	*Zfp27* knockdown in ES cells causes differentiation into the trophoblast lineage	Subtle morphological changes; upregulation of *Cdx2, Hand1, Eomes, Esx1* and *Psx1*	67
Epigenetic Factors				
Cxxc1	CpG binding protein	*Cxxc1*$^{-/-}$ ES cells differentiate into the trophoblast lineage	Upregulation of *Cdx2, Eomes, Elf5* and *Pl1*	69
Dnmt1	DNA methyltransferase	*Dnmt1*$^{-/-}$ embryos show ectopic differentiation of trophoblast cells. *Dnmt1*$^{-/-}$ ES cells differentiate into trophoblast giant cells in vitro and show increased contribution to the TE in vivo	Adoption of giant cell morphology; upregulation of *Ascl2, Tpbpa, Pl1, Pl2* (in vivo) and *Cdx2, Eomes, Ascl2, Tpbpa, Pl1, Pl2, Fgfr2c* (in vitro); contribute to the TE following aggregation with 8-cell embryos	69
Dnmt3a & Dnmt3b	DNA methyltransferases	*Dnmt3a/b*$^{-/-}$ ES cells differentiate into the trophoblast lineage	Upregulation of *Cdx2, Elf5* and *Pl1*	69

Table 1 **Genes/conditions associated with activation of trophoblast markers and trophoblast differentiation from ES cells and embryos (continued)**

Gene	Function	Phenotype	Assessment method	Reference
Eed	Polycomb-group family protein	*Eed*$^{-/-}$ ES cells express markers of multiple lineages including trophoblast, mesoderm and endoderm	Upregulation of *Cdx2* and *Hand1*	78
Ezh2	Polycomb-group family protein	*Ezh2* knockdown in ES cells causes differentiation into the trophoblast, endoderm, mesoderm and ectoderm lineages	Subtle morphological changes; upregulation of *Cdx2, Hand1, Eomes, Esx1* and *Psx1*	67
Jmjd2c	H3K9me3 demethylase	*Jmjd2c* knockdown in ES cells causes differentiation along the trophoblast, endoderm, mesendoderm and ectoderm lineages	Flattened, fibroblast-like cellular morphology; upregulation of *Cdx2* and *Hand1*	80
Mbd3	CpG binding protein	*Mbd3*$^{-/-}$ ES cells are unable to proceed past an early stage of differentiation into embryonic lineages in embryoid bodies but instead differentiate along the trophoblast lineage	Upregulation of *Pl1* and *Tpbpa*	102
Np95	Ring-finger type E3 ubiquitin ligase	*Np95*$^{-/-}$ ES cells differentiate into the trophoblast lineage	Cells adopt trophoblast-like morphology; upregulation of *Cdx2, Eomes* and *Elf5*	69
Parp1	Poly(ADP-ribose) polymerase	*Parp1*$^{-/-}$ ES cells produce trophoblast giant cells in teratocarcinoma-like tumours in vivo, and in cultured cells in vitro	Production of cells with enlarged nuclei and giant cell-like morphology; upregulation of *Pl1, Pl2, Plf, Plfr, Tpbp*	79
Signaling Factors				
Hras1	GTP-binding protein	Conditional activation of *Hras1*^{Q61L} (and *Kras*^{G12V}) in ES cells causes differentiation into the trophoblast and endoderm lineages. TS-like cells derived can differentiate into trophoblast giant cells and also colonize placental tissues in vivo. Conversely, the inhibition of downstream MAPK signaling, but not PI3K signaling, abrogates trophoblast differentiation	*Hras1* activation produces flat colonies of epithelial-like cells and cells of giant cell morphology; upregulation of *Cdx2* and *Hand1*; cells contribute to the polar TE and later the placenta following aggregation with 4–8 cell embryos. MAPK inhibition reduces TE outgrowth from embryo explants	70
Lef1	Signal regulatory protein; transcription factor	ES cells exposed to WNT3A show increased transdifferentiation when *Lef1* is overexpressed. Conversely, *Lef1* knockdown reduces transdifferentiation.	*Lef1* overexpression increased *Cdx2* induction, whereas *Lef1* knockdown reduced *Cdx2* expression	76
Wnt3a	Signalling protein	Exposure of ES cells to WNT3A increases differentiation into the trophoblast and mesoderm lineages	TS cell-like morphology and subsequent trophoblast giant cell-like cells observed; *Cdx2, Eomes, Hand1, Mash2, Gcm1, Pl1* and *Tpbp* upregulation	76
Other Factors				
CollV	Structural protein	ES cells cultured on Collagen type IV differentiate along the trophoblast, cardiovascular and hematopoietic lineages. *Cdx2*-positive clones can be cultured as TS cells and give rise to trophoblast derivatives in vitro	Large, flat, cuboidal-shaped cells with enlarged nuclei; upregulation of *Cdx2, Hand1, Eomes, Esrrb, Cdh3, Err2, Mash2, Tpbp, Pl2, Psx1, Psx2, Plac1, Plac8, Esx1, Dlx3, Tpbg, Idb2* and *Gcm1*	77
Slc25a36	Solute carrier	*Slc25a36* knockdown in ES cells causes differentiation into the trophoblast but not ectoderm, mesoderm or endoderm lineages	Giant trophoblast cell-like morphology; upregulation of *Cdx2, Hand1, Eomes, Esx1* and *Psx1*	67

of the *Ras* proto-oncogene in ES cells induced the expression of trophoblast markers and allowed the derivation of TS cell lines.[70] It was found that differentiation into the trophoblast lineage specifically depends on the RAS-MAPK-ERK2 pathway. Inhibition of MAPK signaling in cultured mouse embryos compromises *Cdx2* expression, delays blastocyst development and reduces TE outgrowth from embryo explants. These findings link the RAS-MAPK signaling pathway to the establishment and/or maintenance of the trophoblast lineage. In line with this observation, interference with FGF or ERK activity favours the pluripotent state of ES cells and restricts their ability to differentiate.[71] Since trophoblast proliferation critically depends upon the FGF4 signal transmitted through the FGFR2 receptor leading to ERK (predominantly ERK2/MAPK1) activation,[72] it is likely that FGF4 provides the extracellular signal to activate the RAS/MAPK/ERK2 signal transduction cascade in the trophoblast lineage (Fig. 2). Consistent with these data is the finding that knockouts of many of the pathway components exhibit trophoblast- or placenta-specific defects.[73,74] Interestingly, mutation of the nonreceptor protein-tyrosine phosphatase SHP2, which is required for ERK activation downstream of receptor tyrosine kinases, has revealed that the RAS-MAPK pathway is also important for trophoblast survival through the inhibition of the pro-apoptotic protein BIM.[75] Therefore the same pathway couples lineage specification with pro-survival signals to specifically reinforce trophoblast cell fate (Fig. 2).

Other signaling pathways have also been implicated in stable lineage fate maintenance, although their effects are comparatively

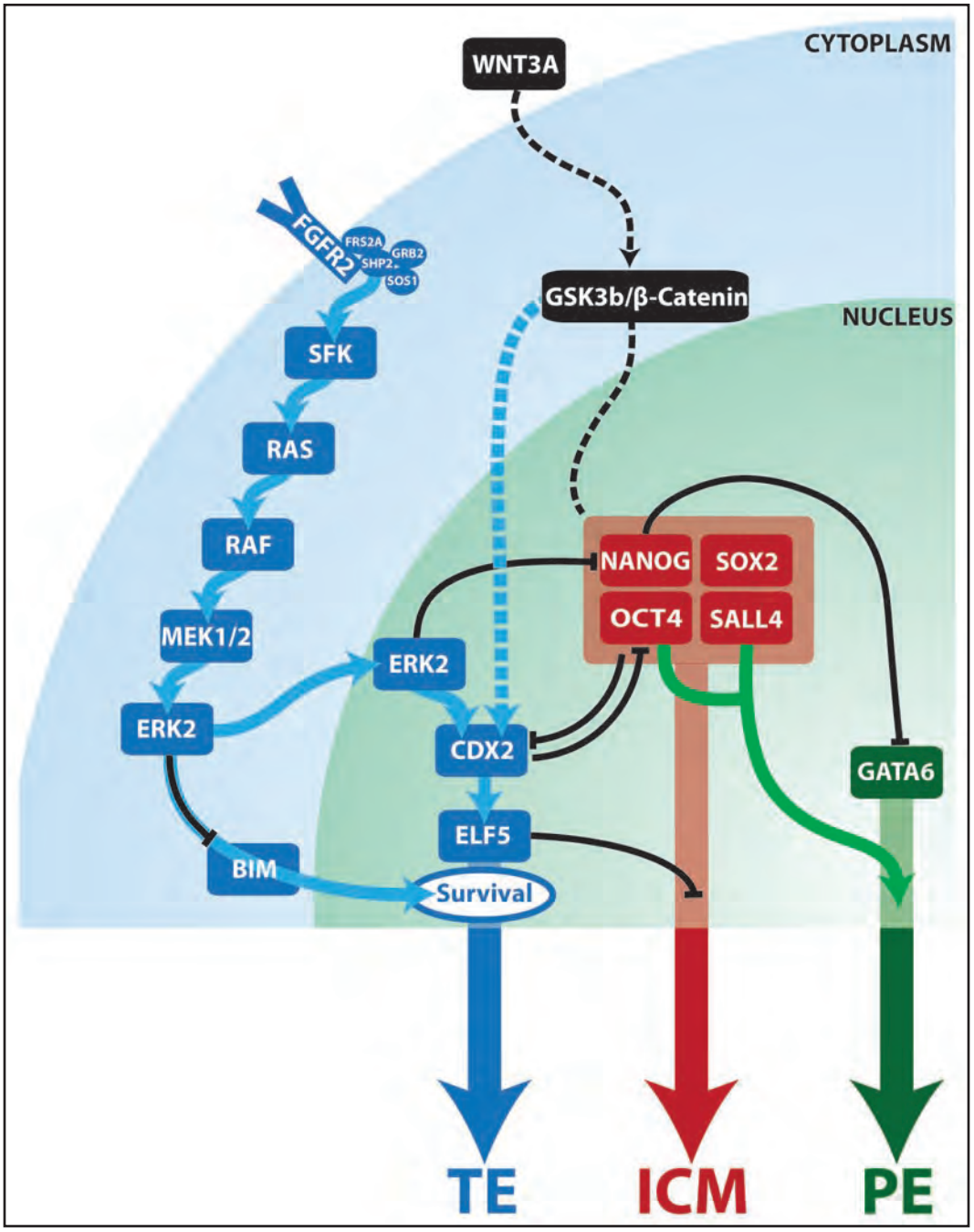

Figure 2. Model of signalling pathways and transcription factors that contribute to cell lineage specification and/or maintenance as revealed mainly by transdifferentiation phenotypes. RAS-MAPK-ERK2 activation is of major importance for trophoblast proliferation and survival, and RAS activation in ES cells induces a strong and dominant transdifferentiation phenotype into TS cells. Because of the importance of FGF signalling for the trophoblast lineage, FGF4/FGFR2 is the likely extracellular signal that activates this pathway. Specific trophoblast defects are also observed in intermediate transmitters of the extracellular signal such as FRS2a, SHP2, GRB2, SOS1 and GAB1.[42,73-75] The RAS-MAPK pathway leads to rapid induction of *Cdx2*, while low levels of RAS activation induce primitive endoderm differentiation (and the PE marker *Gata6*) from embryonic cells by inhibiting *Nanog*.[70] SHP2-RAS-ERK2 activation also provides pro-survival signals for the trophoblast lineage as ERK2/MAPK1 phosphorylation leads to degradation of the pro-apoptotic protein BIM.[75] Wnt signalling is important to maintain pluripotency of ES cells but also to direct their differentiation. In the context of lineage fate stability, WNT3A has been shown to activate *Cdx2* and induce some trophoblast differentiation from ES cells grown in the absence of leukemia inhibitory factor LIF.[76] ELF5 is required downstream of initial lineage specification in trophoblast cells to maintain *Cdx2* and *Eomes* expression. *Elf5* is also the key target gene for the epigenetic restriction of embryonic versus trophoblast lineage fate that is imposed by DNA methylation.[69] OCT4, SALL4, NANOG and SOX2 are required for embryonic lineage identity and pluripotency of ES cells. Deficiency of either of these factors induces trophoblast markers,[45,61,63,64] whereas overexpression of OCT4 and SALL4 leads to primitive endoderm formation.[45,61] In contrast, NANOG inhibits endoderm differentiation by repressing GATA6.[131,132] ICM, inner cell mass; ES, ES cells; PE, primitive endoderm; TE, trophectoderm; TS cells, trophoblast stem cells.

minor and seem subordinate to the robust transdifferentiation induced by RAS-MAPK activation. Wnt signaling, for example, is important for pluripotency and differentiation in ES cells, but specific components of the pathway can also promote trophoblast differentiation from ES cells. In this context, WNT3A functions synergistically with LEF1 to induce expression of trophoblast (and mesodermal) lineage genes.[76] It has also been observed that trophoblast differentiation is initiated when ES cells are plated on Collagen IV in the presence of FGF4 and feeder cells, implying that collagen IV-specific integrin receptor signaling (mainly through the $\alpha_1\beta_1$ integrin receptor) can also alter lineage fate.[77]

Epigenetic Restriction of Cell Lineage Fate

After the embryonic and trophoblast cell lineages have been specified, a stable maintenance of lineage identity is ensured by an epigenetically imposed cellular memory. We have recently found that DNA methylation establishes a major restriction of lineage fate and is a critical epigenetic modification to enforce the clear-cut and heritable embryonic-trophoblast lineage boundary. Hence, ES cells deficient in DNA methylation due to a lack of *Dnmt1*, *Dnmt3a/b*, *Np95* or *Cxxc1*, readily transdifferentiate into trophoblast derivatives when cultured in TS cell conditions.[69] This lineage restriction is mainly mediated through epigenetic regulation of the transcription factor *Elf5*. *Elf5* is robustly methylated and repressed in ES cells, but is hypomethylated and expressed in TS cells. In the trophoblast compartment, ELF5 is necessary to maintain expression of the trophoblast stem cell genes *Cdx2* and *Eomes*, and thereby reinforces trophoblast cell fate. This pathway is aborted in the embryonic lineage due to epigenetic silencing of *Elf5*. Thus, ELF5 functions downstream of initial lineage determination as a gatekeeper to ensure the stable and irreversible canalization of embryonic and trophoblast lineage pathways. The developmental restriction imposed by epigenetic regulation of *Elf5* is likely complemented by other, albeit less stringent, epigenetic mechanisms. In this context it is interesting to note that an upregulation of trophoblast markers is also observed in ES cells that lack the NuRD repressive complex component MBD3, the Polycomb repressive complex components EED and EZH2, the histone demethylase JMJD2C, or the poly(ADP-ribose)polymerase PARP1 (Table 1).[67,78-80] Whether or not these modifications act on *Elf5* or on other factors that contribute to lineage restriction remains to be elucidated.

The emerging picture from these data is that specific transcription factors, some of which may be activated by extracellular signal-regulated kinase cascades, are able to direct differentiation into particular lineages. This lineage allocation is then fixed by epigenetic modifications, most notably DNA methylation, to ensure stable and heritable lineage commitment.

Expression Patterns of Lineage 'Markers'

With the increasing number of conditions that have been reported to cause 'trophoblast differentiation' from ES cells, it is important to reassess the lineage specificity of genes that are commonly used as marker genes (Fig. 1). In this regard, it is particularly noteworthy that all known trophoblast determining factors also have a function in embryonic development. This situation has most likely arisen from the redeployment of available genes for trophoblast differentiation during eutherian evolution. As such, the TS cell 'markers' *Cdx2* and *Eomes* are expressed in embryonic structures soon after embryonic-trophoblast lineage separation. *Cdx2* is detected at E7.5 in the mesoderm of the developing allantoic bud and posterior primitive streak. At E8.5, expression is seen in all three germ layers at the posterior end of the embryo extending into the allantois, in the endodermal epithelium of the hindgut rudiment, and in the neural tube.[52,81] Embryonic *Eomes* expression is observed even earlier, at E5.5-E5.75, in the posterior primitive streak region and overlying visceral endoderm.[54,82] Another example is the frequently used trophoblast giant cell-expressed gene *Hand1* that is also required soon after implantation for cardiac morphogenesis and is expressed from E8.5 onwards in the developing heart, pericardium and lateral mesoderm.[83-85] Thus, the conclusion of trophoblast differentiation from ES cells on the basis of 'marker' gene analyses has to be treated with some caution, as expression of many of these genes may in fact represent ES cells recapitulating their activation in embryonic lineage derivatives slightly later in development.

As the placenta represents a relatively late acquisition in evolution, many genes with important functions in trophoblast development are also expressed elsewhere, and a recurrent pattern is the placenta-testis-brain axis.[86,87] There are relatively few murine genes that are truly trophoblast-specific, for example the placental lactogen/prolactin family, some pregnancy-specific glycoproteins (Psg's), the placenta-expressed cathepsin family and the Syncytins.[88-95] In general, these genes are expressed in differentiated trophoblast cell types, and well-characterized marker genes include *Tpbpa*, characteristic of ectoplacental cone trophoblast and spongiotrophoblast;[96] Syncytins A and B (*Syna* and *Synb*), expressed in syncytiotrophoblast;[97] and the placental lactogens I and II (*Pl1*/*Prl3d1* and *Pl2*/*Prl3b1*), demarcating primary and secondary trophoblast giant cells, respectively.[95,98] In conjunction with the activation of these specific marker genes, appearance of the morphologically distinct syncytiotrophoblast and trophoblast giant cells is a unique feature that can serve as a reliable indicator of trophoblast differentiation.

The most vigorous test for the functionality of trophoblast cells transdifferentiating from ES cells is to analyze their developmental potential in chimera experiments. Of all the situations for which trophoblast differentiation and/or activation of trophoblast genes has been described, such definitive proof has only been provided for very few genes (Table 1). Namely, *Sall4* knockdown and *Dnmt1*-deficient ES cells contribute to the TE at the blastocyst stage, and *Cdx2*-overexpressing and *Ras*-activated ES cells contribute to trophoblast tissues of the mature placenta.[61,68-70]

Despite these limitations, the accumulating evidence of extracellular signals, signaling cascades, transcription factors and epigenetic modifiers that are involved in the specification and stable maintenance of lineage fate have provided significant insights into the mechanisms that underlie stem cell potency and the canalization of developmental pathways (Fig. 2).

Perspectives

Understanding how the early cell lineages are specified and then maintained is of key importance for developmental biology and regenerative medicine. Thus, the ability to remove developmental restrictions may enable us to widen a stem cell's potency towards that of a different cell lineage. At the same time, knowledge of the molecular basis for the progressive loss of developmental plasticity is fundamental to achieve directed and terminal differentiation into specific cell types. Insights into the roles of transcription factors, signaling pathways and epigenetic modifiers have highlighted their importance for the stability of cell fate determination and for the differentiative potency of stem cells. These recent findings open up new opportunities for the manipulation of lineage determinants and gatekeeper genes, or their epigenetic regulation, in experimental approaches aimed at generating appropriate cell types by transdifferentiation or by reprogramming of somatic cells.

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