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OVERVIEW TITLE: Defined stem cell culture conditions to model mouse blastocyst development

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ABSTRACT:

The complex programme of mouse development entails specification of the embryonic epiblast (Epi) as well as of the extra-embryonic trophectoderm (TE) and primitive endoderm (PrE). These three lineages of mouse blastocyst can be modelled in vitro using stem cells derived from primary tissues. In these cultures, cells self-renew while retaining their developmental potential if put back into a developing embryo. Indeed, embryonic stem cells (ESC) when injected into a blastocyst readily contribute to all embryonic lineages. Similarly, trophoblast stem cells (TSC) will give rise to all TE-derived trophoblast lineages and extraembryonic endoderm cells (XEN) will contribute to the PrE-derived yolk sack. These model systems are a powerful tool to study early development, lineage specification and placenta formation. Only recently reproducible and chemically defined culture systems of these cells have been described. This overview discusses such novel methods for culturing ESC/TSC/XEN as well as their molecular signatures and developmental potential. Recent strides in expanding the developmental potential of stem cells as well as achieving models more reminiscent of their in vivo counterparts are discussed. Finally, such in vitro stem cells can self-assemble into structures reminiscent of embryos when used in novel 3D-culture systems. This article discusses the strengths and limitations of such “synthetic embryos” in studying developmental processes.

KEYWORDS:

Embryonic stem cells, trophoblast stem cells, extraembryonic endoderm, mouse development, blastocyst

INTRODUCTION:

Shortly after fertilization the embryo embarks on a complex sequence of dramatic changes constituting the developmental programme (Figure 1A). Initial stages of development allow for cell proliferation but also the formation of first lineages (for review see (Chazaud & Yamanaka, 2016)). Indeed, for the development to succeed three lineages need to be specified within the first four days of mouse development. The first fate decision is made between the inner cell mass (ICM) and the trophectoderm (TE). These cell lineages are fully specified by the early blastocyst stage at embryonic day (E) 3.5. At which point, the external TE cells are destined to form most placental tissues. On the other hand, the ICM will give rise to both the embryonic and extra-embryonic tissues. A day later (E4.5) the ICM specifies into two separate lineages: the epiblast (Epi) and primitive endoderm (PrE). PrE will form mainly the extraembryonic yolk sack, while the Epi is restricted to the embryonic lineages. Thus both ICM and later the Epi are truly pluripotent as they are able to contribute to all three embryonic lineages (endoderm, ectoderm and mesoderm) as well as the germline. These early events are vital to form a functional embryo, which is able to implant.

Developmental biologists have been studying pre-implantation mouse development for decades, however their work was hampered by the limited numbers of cells available. Luckily, in vitro cultured stem cell models of all three lineages (TE, Epi and PrE) were developed allowing for the expansion of lineage-restricted cells. The most widely-used of those are pluripotent embryonic stem cells (ESCs)(Bradley, Evans, Kaufman, & Robertson, 1984). The potency of such cells was tested by generating chimeras i.e. injecting them into blastocysts and transferring such embryos into pseudo-pregnant mothers. In this assay ESCs will contribute mainly to the embryonic lineages. On the other hand, trophoblast stem cells (TSCs) and extraembryonic endoderm (XEN) cells are restricted to lineages originating from the TE and PrE respectively (Kunath et al., 2005; Tanaka, Kunath, Hadjantonakis, Nagy, & Rossant, 1998). ESC/TSC/XEN cells are available for a long time, only recently very complex culture components were substituted with chemically defined reagents. Apart from using foetal calf serum (FCS) initial protocols often depended such ill-defined components as mitotically-inactivated mouse embryonic fibroblasts (feeders) or conditioned media from cultures of mouse embryonic fibroblasts. This article will provide an overview of the most up-to date methods to grow stem cells modelling pre-implantation development. It will also discuss the developmental potential of such cultures and their molecular properties. Chemically defined conditions allow for unprecedented control over signalling pathways allowing for the development of reproducible differentiation protocols. Another important use of such methods is the possibility to measure how the environment affects stem cell states. Finally, these stem cell models can be used in 3-dimentional (3D) cultures to model processes normally taking place only within the embryo e.g. blastocyst formation, gastrulation or even morphogenesis of post-implantation embryo. This article will discuss such novel 3D culture methods, their use and their limitations.

MAJOR TOPIC: Embryonic stem cells – modelling the ICM and the epiblast

ESCs are extensively used not only to study early mouse development but also adult tissues. This is because, ESCs can be easily genetically engineered using CRISPR/CAS9. Such modified ESCs are often used as the starting point of many experiments and can be used to generate either in vivo (through chimeras) or in vitro models (through targeted differentiation protocols). This topic will treat on the culture systems available for mouse ESCs (Figure 1B), their molecular characteristics as well as developmental potential.

Subtopic: Establishment of defined ESC culture conditions

ESCs are derived from the ICM of a blastocyst (Figure 1A), self-renew in vitro and maintain the developmental potential of the tissue of origin. These lines were first established 1981 by Martin Evans, Matthew Kaufman and Gail R. Martin (Evans & Kaufman, 1981; Martin, 1981). Initially, culture conditions were relatively complex with the use of specific feeder cells and conditioned media. These conditions could be simplified after the discovery of leukaemia inhibitory factor (LIF), which promotes ESC self-renewal through the activation of JAK/STAT3 signalling (Niwa, Burdon, Chambers, & Smith, 1998; A. G. Smith et al., 1988; Williams et al., 1988). This breakthrough allowed for the establishment of feeder-free culture conditions, with ESCs relying on LIF and FCS for their propagation (FCS+LIF culture)(Nichols, Evans, & Smith, 1990). Next step towards the establishment of highly reproducible conditions for ESC growth was the substitution of FCS with a cocktail of chemically-defined factors. Indeed, FCS can be substituted by a complex culture media called N2B27 and BMP (BMP+LIF culture)(Q. L. Ying, Nichols, Chambers, & Smith, 2003). LIF and BMP together allow for self-renewal of ESCs and prevent their differentiation. On the other hand, N2B27 provides necessary sugars, amino acids, fatty acids, insulin, progesterone, sources of iron, regulators of redox potential and other compounds. One problem with this initial chemically-defined condition was the high levels of heterogeneity within the culture. In order to prevent this variability, cells can be treated with an inhibitor of the FGF signalling pathway (PD0325901) and an inhibitor of GSK3 (CHIR99021), which leads to activation of WNT signalling (Q.-L. Ying et al., 2008). This double inhibitor (2i) treatment in N2B27 medium allows for highly efficient derivation and expansion of relatively-homogenous ESCs (for ESC culture protocol see (Mulas et al., 2019); for ESC derivation protocol see (Nichols & Jones, 2017)). One important aspect to consider when working with ESCs is their genetic background. Initial culture conditions could not be easily applied to all mouse strains. However 2i seem to work for all mouse backgrounds tested so far (Nichols et al., 2009; Q.-L. Ying et al., 2008). All in all, the development of a highly reproducible 2i (+/-LIF) culture system allowed for stable expansion ESCs, while maintaining their full developmental potential.

Subtopic: Molecular properties of different ESC culture conditions

In general ESCs express high levels of pluripotency markers such as SOX2, OCT4, KLF2, NANOG and low levels of genes typically associated with the post-implantation epiblast (e.g. OTX2, FGF5). However, the precise molecular signature of ESCs is largely defined by the signalling regime of the culture condition. Indeed, any two signalling components of 2i+LIF can sustain stable ESC culture (Wray, Kalkan, & Smith, 2010) but resulting cells differ significantly when it comes to their transcriptional state (Hackett, Kobayashi, Dietmann, & Surani, 2017). The full cocktail of 2i+LIF results in a transcriptional state that is most closely related to the tissue of origin (E3.5 ICM) (Hackett et al., 2017). The omission of any of the three signalling components results in the

upregulation of a specific set of lineage markers. Namely the germline, endoderm and ectoderm programmes become upregulated after the removal of LIF, PD0325901 or CHIR99021 respectively (Hackett et al., 2017). Consistently with this, the addition of BMP-rich FCS to the medium results in the partial derepression of mesodermal genes (Hackett et al., 2017). Thus, while multiple conditions for ESC culture exist they all result in a distinct state on the pluripotency spectrum.
Apart from expressing a specific transcriptional programme, the ICM also shows global reduction in the repressive chromatin mark: DNA methylation. This can be recapitulated in vitro in 2i cultures (Ficz et al., 2013; Hackett et al., 2013; Leitch et al., 2013). In this case it seems that simultaneous 2i treatment is required for global DNA hypomethylation, while LIF addition does not play a major role (Hackett et al., 2017). Another factor regulating DNA methylation levels is Vitamin C (VitC) (Blaschke et al., 2013). Addition of VitC allows for robust activation of TET enzymes, which in turn promote DNA demethylation. By combining 2i treatment with VitC it is possible to nearly completely demethylate the genome, a state that never exists during pre-implantation development (Walter, Teissandier, Perez-Palacios, & Bourc'his, 2016). Changes to the DNA methylation levels have direct consequences on other chromatin marks e.g. on the Polycomb Repressive Complex 2-dependent histone H3 lysine 27 trimethylation (H3K27me3). Indeed, 2i treatment of ESCs results in spreading of H3K27me3 along the genome (Marks et al., 2012). In summary, culture conditions of ESCs result in dramatic differences to not only transcriptional status of cells but also their epigenome. So far 2i+LIF conditions seem to most closely mimic the *in vivo* ICM.

Subtopic: Developmental potential of ESC

The defining feature of ESCs is their ability to contribute to chimeras when injected back into embryos. ESCs grown under 2i (+/-LIF) conditions retain their potential to contribute to the embryonic lineages (endoderm, ectoderm, mesoderm, germline) (Q.-L. Ying et al., 2008) but unlike their in vivo ICM counterpart do not readily contribute to the yolk sack (derived from PrE). This indicates that at least when put back into the embryo, ESCs typically have a limited developmental potential more reminiscent of the E4.5 Epi. Interestingly however, under 2i+LIF culture conditions a subpopulation of cells seems to stochastically upregulate *Hex*, a marker of PrE (Morgani et al., 2013). These cells when injected back into the embryo can contribute to both the embryonic as well as all extraembryonic lineages. This indicates that at a low level, 2i+LIF conditions support transient establishment of cells with expanded potency. Another interesting subpopulation can arise in FCS+LIF condition, which also seems to show expanded potency (Macfarlan et al., 2012). These cells transiently express markers of an early embryonic transcriptional state reminiscent of the 2-cell stage embryo (2C). 2C-like cells can also be isolated from chemically defined conditions but when cells are grown without LIF (2i-LIF)(Hackett et al., 2017). It has been reported that, when injected back into the embryo, these cells can contribute to all embryonic and extraembryonic lineages (Macfarlan et al., 2012). However, it still remains to be shown if a single 2C-like cell can actually contribute to both embryonic and extraembryonic lineages. All in all, chemically defined culture conditions can support varying numbers of cells with some expanded potency, nevertheless it seems that both *Hex+ve* or 2C-like cells contribute best to the embryonic rather than extraembryonic lineages. In recent years there has been significant effort to identify culture conditions which would support such expanded potency in the whole population of cells. One such culture condition is based on N2B27+ CHIR99021 + LIF supplemented with (S)-(+)-dimethindene maleate (DiM) and minocycline hydrochloride (MiH), which most probably target PARP1 (Yang et al., 2017). Under these so called LCDM conditions cells show expanded potency since when injected into embryos they can contribute to all embryonic and extraembryonic lineages (Yang et al., 2017). Importantly, the authors were able to validate that even single cells grown under LCDM conditions can give rise to both ICM and TE. Thus a chemically defined culture system exists to support self-renewal of cells with expanded potency. Interestingly however, the transcriptome of such cells differs strikingly from early blastomeres. This indicates that such in vitro cultured cells likely need to be significantly reprogrammed after injection into the embryo to embark on normal development.

MAJOR TOPIC: Trophoblast stem cells – modelling trophectoderm

TSCs can be used as a model for trophectoderm development as they can differentiate towards all trophoblast subtypes of the placenta. As such, they constitute an important model system to study how an embryo prepares itself for implantation and later how it interacts with the tissue of the mother (for review on TSCs see (Latos & Hemberger, 2016)). This topic will treat on the culture systems available for mouse TSCs (Figure 1B), their molecular characteristics as well as developmental potential.

Subtopic: Establishment of defined TSC culture conditions

TSCs are self-renewing stem cells derived from the pre-implantation TE or post-implantation extraembryonic tissues (Tanaka et al., 1998). The original protocol for TSC derivation and culture depended on the use of feeder cells (or conditioned media), FCS as well as treatment with FGF4 and heparin. FGF4 treatment in this case mimics the signalling cues originating in the ICM, which promotes specification and self-renewal of the TE. On the other hand, the addition of heparin presumably aids in the uptake of

FGF4. The first step in simplifying these culture conditions was substituting the feeder cells for either TGFβ or Activin A polypeptides (Erlebacher, Price, & Glimcher, 2004). These factors bind distinct receptors on the cell surface but both lead to the activation of SMAD2/3/4 transcription factors. Based on these advances, two defined culture conditions have been identified, which allow for the maintenance of TSC self-renewal and differentiation capacity (Kubaczka et al., 2014; Ohinata & Tsukiyama, 2014). Firstly, the "FAXY" culture conditions substituted the FCS with chemically defined N2B27 medium (Ohinata & Tsukiyama, 2014). In order to activate the FGF and TGFβ signalling cascades this protocol uses FGF2 and Activin A respectively. However, to prevent spontaneous TSC differentiation the addition of a WNT inhibitor (XAV939) was necessary. Finally, such cells could be either grown on an ill-defined extracellular matrix: matrigel or on defined fibronectin. In the latter case however it was necessary to prevent cell death by the addition of an inhibitor of Rho-associated protein kinase p160ROCK (Y27632). This indicates that such culture conditions are still somewhat suboptimal as they depend on the use of pro-survival drugs. An alternative "TX" culture media published by Kubaczka et al., utilised a simpler basal medium, previously used for human stem cell cultures (Chen et al., 2011). In order to activate FGF and TGFβ signalling pathways FGF4 and TGFβ were added to the cultures as well as heparin (Kubaczka et al., 2014). However, such cultures also depended on matrigel coating which is relatively ill-defined and can lead to batch-to-batch variability. A synthetic substrate Synthemax was tolerated but lead to lower colony forming efficiency. Thus further optimization of both chemically-defined TSC culture conditions is still required.

Subtopic: Molecular properties of different TSC culture conditions

In general TSCs express high levels of TE markers such as CDX2, TFAP2C, EOMES and low levels of markers typically associated with trophoblast differentiation (e.g. PL1, PL2, TPBPA). However, the precise molecular signature of TSCs cultured under different conditions is not extensively studied. Nevertheless, when compared to standard culture conditions (conditioned media+FGF4+heparin) the TX media results in a strikingly similar transcriptional state with very few genes being differentially expressed (Kubaczka et al., 2014). On the other hand, the FAXY condition results in significant transcriptional changes. While TSC marker genes remain equally expressed the trophoblast differentiation genes were significantly repressed. This indicates that FAXY conditions prevent spontaneous TSC differentiation. Significant transcriptional differences between culture conditions could also be a consequence of drastically increased proliferation rate in FAXY conditions (doubling time: 9 vs 24hrs). Such transcriptional analysis indicates that the FAXY medium likely leads to less differentiated and more robustly self-renewing population.

In pre-implantation development both the ICM and TE are globally hypomethylated (Z. D. Smith et al., 2017), shortly afterwards embryonic lineage undergoes dramatic de novo DNA methylation. This process of de novo DNA methylation is less pronounced in the TE-derived cells. Interestingly, global levels of DNA methylation in TSCs grown in standard conditions are relatively high, probably higher than what is observed in vivo (Oda, Oxley, Dean, & Reik, 2013). This seems to also be the case for TX culture system (Kubaczka et al., 2014). Unfortunately data is still lacking when it comes to the FAXY protocol. All in all, it seems that current TSC culture conditions do not recapitulate exactly the epigenetic status of the in vivo TE or cells derived thereof.

Subtopic: Developmental potential of TSCs

The defining feature of TSCs is their ability to contribute to chimeras when injected back into embryos. However unlike ESCs, TSCs contribute only to the development of various TE-derived trophoblast lineages and not the PrE-derived yolk sack or Epi-derived embryonic lineages (Tanaka et al., 1998). TSCs grown in all aforementioned conditions retain this developmental potential thus are functionally reminiscent of the in vivo TE cells. However on the molecular level it remains unclear how similar they are to their in vivo counterparts. Single cell RNA-seq analysis of TSCs cultured under TX protocol revealed striking heterogeneity of such cells (Frias-Aldeguer et al., 2019). Indirect comparison to the in vivo TE cells and differentiated trophoblasts suggested that in vitro TSCs oscillate between different transcriptional programmes: that of pre-implantation TE and more differentiated post-implantation cell types. Importantly, the authors have identified new culture conditions which promoted self-renewal of more primitive TSCs reminiscent of the pre-implantation TE. These conditions apart from using FGF4, TGFβ, Activin A also contained Il11, BMP7, cAMP and LPA. What is more, matrigel was successfully substituted by laminin L521-coating. Resulting cells express higher levels of self-renewal markers (e.g. CDX2 and EOMES) and lower levels off differentiating trophoblast markers (e.g. ASCL2, GCM1). Together this data indicates that it is possible to trap TE-like cells in vitro while keeping their full developmental potential. Future experiments however should try to perform direct transcriptome and epigenome comparison between TE and TSCs.

MAJOR TOPIC: Modelling primitive endoderm development – XEN and nEnd cells

The third and last lineage of a pre-implantation blastocyst is PrE (Figure 1B). PrE, like the Epi, arises from the ICM at E4.5. First stem cell models of the PrE were reported only in 2005 and were named XEN (eXtraembryonic Endoderm) cells (Kunath et al., 2005). Such cells, when injected back into the embryo, will contribute only to the PrE-derived tissues. This topic will treat on the culture systems available for mouse PrE-derived stem cells, their molecular characteristics as well as developmental potential.

Subtopic: Establishment of defined PrE culture conditions

XEN cells constituted first PrE-derived, self-renewing stem cells (Kunath et al., 2005). In the original report, XEN cells were derived from the ICM using either TSC or ESC growth conditions on feeder cells. Subsequent optimization steps allowed for more efficient propagation of XEN cells in such mixed cultures (for XEN cell derivation protocol see (Niakan, Schrode, Cho, & Hadjantonakis, 2013)). Once derived, XEN cells can grow in very simple conditions with FCS but without feeders or any additional grow factors. First chemically defined conditions for XEN cells expansion were reported in 2012 and comprised of just N2B27 (Anderson et al., 2017; Paca et al., 2012). The authors also noted that the addition of BMP4 resulted in drastically altered cell morphology reminiscent of what happens during post-implantation development. Recently the most pre-implantation-like state has been achieved in another chemically defined culture (Anderson et al., 2017). By treating ESCs with Activin A and WNT3a the authors managed to induce a PrE-like cell type named nEnd (naïve endoderm). In principle, the derivation of nEnd should also be possible from the blastocyst but this has not yet been shown. All in all, chemically-defined culture conditions for PrE-derived stem cell lines are relatively simple and rely on very few (or none) additional growth factors.

Subtopic: Molecular properties of different PrE culture conditions

In general XEN and nEnd cells express high levels of PrE markers such as GATA4, GATA6 and SOX17. However, the precise molecular signature of these cells depends on their signalling milieu. First of all, the transcriptome of XEN cells cultured in N2B27 as well as of nEnd cells correlates with PrE of E4.5 embryos (Anderson et al., 2017). However, the XEN cells showed increased levels of some genes typically expressed only after the embryo has implanted. An even more differentiated transcriptional phenotype is observed after XEN culture in N2B27+BMP4 (Anderson et al., 2017; Artus et al., 2012). Together this indicates that distinct extraembryonic endoderm states can be captured in vitro. Unfortunately, the epigenetic landscape of the PrE in vivo is largely unknow and it is unclear how it relates to different in vitro stem cell cultures.

Subtopic: Developmental potential of nPrE cells

The defining feature of XEN and nEnd is their ability to contribute to chimeras when injected back into embryos. These cells contribute only to the development of PrE-derived yolk sack (Anderson et al., 2017; Kunath et al., 2005). Interestingly, in vivo PrE has been shown also to contribute at low levels to some embryonic endodermal tissues (Kwon, Viotti, & Hadjantonakis, 2008). Whether XEN/nEnd are able to contribute to embryonic lineages remains unclear. Transcriptomic analysis indicates that nEnd is reminiscent of the PrE of E4.5 blastocyst, while XEN cells relate more closely to post-implantation PrE-derived tissues (Anderson et al., 2017). An interesting point relating to the developmental potential of these cells is the fact that they can be derived from ESCs (Anderson et al., 2017; Niakan et al., 2013). Even in normal ESC cultures (FCS+LIF) there is a subpopulation of cells, which express a PrE marker PDGFRα (Lo Nigro et al., 2017). When injected into blastocysts, such cells preferentially contribute to PrE-derived lineages rather than to the Epi. This data suggests that ESCs can show remarkable plasticity in certain culture conditions and that their lineage potential is not necessarily limited to the Epi. They can either be induced into the PrE-like cells or even the spontaneously oscillate through such a cell-state.

MAJOR TOPIC: 3D stem cell cultures for modelling mouse embryogenesis

Three cell lineages build a functional blastocyst allowing for its implantation and further development. This overview has discussed available systems to maintain lineage-specific stem cells in vitro allowing for their self-renewal and maintenance of distinct developmental potentials. Recently these stem cell lines have been used to self-assemble into structures reminiscent of mouse embryos. Such “synthetic embryo” systems always depend on 3D cultures. This topic will briefly discuss recent advances in constructing structures resembling pre-implantation as well as post-implantation embryos.

Subtopic: Building a blastocyst from ESCs and TSCs

The availability of in vitro cultured stem cells makes them useful models for studying early mouse development. However such 2D cultures do not recapitulate the morphogenetic changes occurring during development. It is thus tempting to implement 3D cultures to exploit the developmental potential of such cells as ESC and TSC. Indeed, a recent report revealed that upon WNT and cAMP stimulation ESCs and TSCs can aggregate and self-organise to form blastocyst-like structures (Rivron et al., 2018). The starting point of such cultures were ESCs grown in 2i+LIF conditions and TSCs grown in chemically defined medium with

TGFβ+FGF4+heparin. In rare cases these blastocyst-like structures showed PrE specification as judged by PDGFRα expression. While the morphology of these structures was strikingly similar to that of E3.5 blastocysts they were not able to develop past implantation. More recently it was reported that the PrE fate could be first induced in ESC aggregates in N2B27+cAMP+Retinoic Acid+FGF4+ CHIR99021+LIF and then these clusters could be further aggregated with TSCs (Vrij et al., 2019). This final aggregation in defined conditions supplemented with Y72632+CHIR99021+cAMP+FGF4+TGFβ+IL11 allowed for the formation of blastocyst-like structures containing cells reminiscent of the TE, Epi and PrE. Whether these structures could be successfully implanted remains to be shown. The Zernicka-Goetz group has recently used cells cultured in LCDM medium supporting extended pluripotency of ESCs {Sozen, 2019 #2731}. When such cells were aggregated with TSCs under reduced oxygen conditions in previously published medium {Rivron, 2018 #2717}, blastocyst-like structures formed. These contained three lineages (PrE, Epi and TE). Transcriptomic analysis from such structures showed that the PrE-like cells were reminiscent of their in vivo counterpart in E4.5 blastocysts. These structures were able to initiate implantation but could not develop past E7.5 {Sozen, 2019 #2731}. All in all, ESC together with TSCs show remarkable propensity to self-organise in 3D structures resembling blastocysts. When cultured under specific conditions ESCs can give induce proper PrE formation. Further analysis is required to identify optimal conditions to allow these "synthetic embryos" to progress further.

Subtopic: Building post-implantation embryos

ESC have been used to study the differentiation processes occurring after implantation for a very long time. ESCs can be readily aggregated to form embryoid bodies (EB), which contain a disorganised array of differentiated and pluripotent cells (Doetschman, Eistetter, Katz, Schmidt, & Kemler, 1985). More recently however, aggregating a precise number of ESCs in N2B27 and exposing them to a pulse of CHIR99021 resulted in the formation of so called gastruloids (Beccari et al., 2018). Cells within these large aggregates undergo differentiation but in a spatio-temporal organized manner similar to gastrulation in vivo. Indeed, gastruloids break symmetry and reproducibly specify some neuronal, endodermal and mesodermal cell types. However they never fully repress pluripotency markers (e.g. OCT4, NANOG) indicating that they are only able to recapitulate early developmental stages. The strength of this approach is its simplicity and reproducibility. However, the lack of extraembryonic lineages can be seen as downside.

Alternative methods to mimic the development of early post-implantation embryos have also been developed. In one approach TSCs and ESCs were allowed to aggregate within a 3D matrigel matrix (Harrison, Sozen, Christodoulou, Kyprianou, & Zernicka-Goetz, 2017). The starting point were ESC grown in FCS+2i+LIF, while TSCs were grown in conventional culture conditions (feeders+FGF4+heparin). Cell aggregates were formed in a complex culture media containing both FCS and N2B27 as well as FGF4 and heparin. Under such conditions, cells spontaneously formed structures resembling E5.5 post-implantation embryos but lacked the PrE-derived visceral endoderm. In comparison to the gastruloid cultures, these aggregates were more organized and contained TE-derived cells. Such structures were also reported to break symmetry in some cases. An updated version of this protocol allows for the aggregation of not only ESC, TSCs but also of XEN cells (Sozen et al., 2018). In this case the aggregation does not take place in matrigel but in suspension in "AggreWell". The starting point for these cultures is also somewhat different. Both ESCs and TSC were cultured on feeders in a FCS containing medium supplemented with either 2i+LIF (for ESCs) or FGF2+FGF4+Heparin (for TSCs). In the case of XEN cells they were cultured in conditioned media. Such ESC/TSC/XEN cells were aggregated in conditioned media containing Y27632 to prevent cell death. After two days of aggregation, media was changed to chemically-defined embryo culture conditions (Bedzhov, Leung, Bialecka, & Zernicka-Goetz, 2014). In this protocol cells self-assemble into embryo-like structures. The ESC derived cells form a cup-shaped epithelium which is in contact with the TSC-derived cells. These lineages become encapsulated in XEN-derived cells reminiscent of visceral endoderm. Such "synthetic embryos" not only can break symmetry but also initiate gastrulation. Crucially, transcriptomic analysis revealed that they are patterned similarly to E7.0 embryos. Another team has recently reported a simplified protocol for the culture of such structures (Zhang et al., 2019). In this case ESC/TSC/XEN cells were aggregated in simple FCS containing medium in normal dishes but with constant agitation. This allowed the self-assembly of embryo-like structures which were able to break symmetry. Such structures when transferred into pseudo-pregnant mothers could implant but not develop further.

CONCLUSION:

Stem cells of the three blastocyst lineages can be stably maintained in vitro. While ESCs have been extensively characterised, the stem cells of extraembryonic lineages still remain somewhat enigmatic. This is, in part, because we do not fully understand the molecular signatures of in vivo TE and PrE. Extensive chromatin characterisations of these lineages will help in identifying optimal culture conditions in vitro. The use of chemically defined ESC/TSC/XEN and nEnd cultures allowed the field to unravel the signalling pathways mediating self-renewal and differentiation. In the future significant focus will be placed on the

extraordinary capabilities of stem cells to self-assemble into embryo-like structures. It seems likely, that these studies will be greatly facilitated by the use of tightly controlled chemically-defined culture conditions. In principle, it should be possible to identify in vitro stem cells extremely closely relating to the in vivo TE and PrE. Once this is achieved a fully functional "synthetic embryo" might one day develop past gastrulation and become born. In the meantime these methods allow us to study not only the process of self-organisation but also how distinct stem cell populations interact, programme each other and create a niche for the development of specific lineages.

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FIGURE LEGENDS:

Figure 1. Stem cells of three lineages from the mouse blastocyst can be maintained in vitro. A. Early mouse development entails the specification of three linages. Around E2.5 between 8 to 16-cell stage the outer cells become specified into the trophectoderm (TE-green) while the inner cell mass (ICM-red) retain pluripotency. By early blastocyst stage (E3.25) TE and ICM have fully specified. By late blastocyst stage (E4.5) ICM becomes specified into the pluripotent epiblast (Epi-dark red) and primitive endoderm (PrE-orange). By E6.0 the embryo has implanted and embryonic lineage of Epi formed a cup shaped epithelium. At this point TE has formed the extraembryonic ectoderm (ExE) and, at the far end of the embryo, the ectoplacental cone (round cells). The PrE gives rise to visceral endoderm surrounding both the Epi and ExE. ESCs can be derived from both the ICM and the Epi. TSCs are established from the TE at the blastocyst stage, while XEN cells arise from PrE. nEnd can be induced in vitro from ESCs by treating them with WNT3a and Activin A. All in vitro stem cell lines can be maintained indefinitely in vitro thanks to their ability to self-renew. **B.** Table summarising various culture conditions for ESCs, TSCs and XEN/nEnd cells. Shown are the main signalling pathways activated or repressed in these conditions.

ADDITIONAL INSTRUCTIONS:

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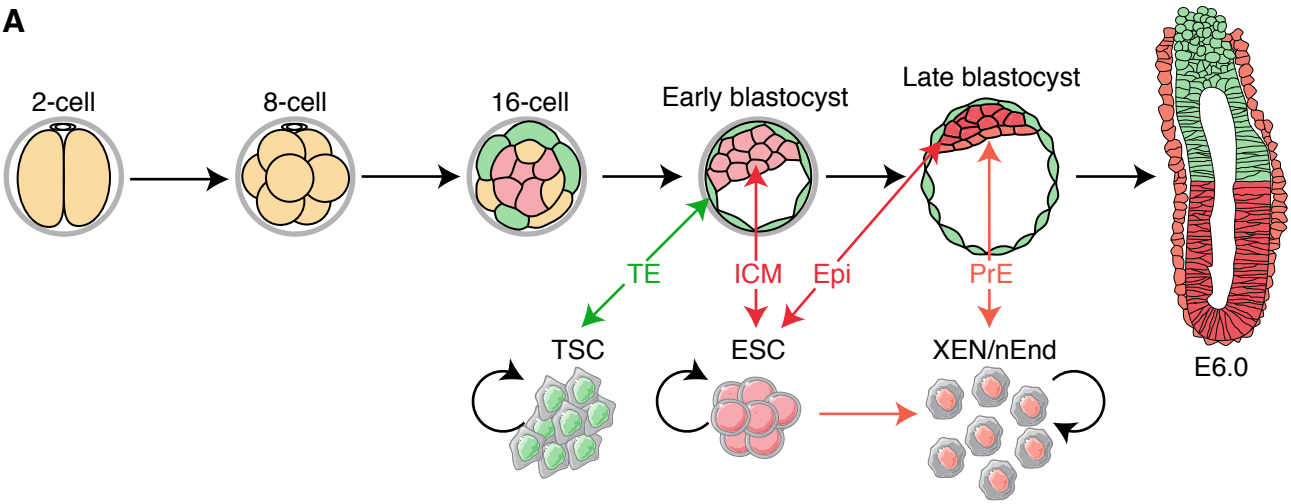
A



B

	Name	Reference	Chemically defined	JAK/STAT3	BMP	WNT	FGF	TGFβ	Coating	Other
ESC	FCS+LIF	Nichols, Evans, & Smith, 1990	NO	LIF	FCS				gelatin	
	BMP+LIF	Q. L. Ying, Nichols, Chambers, & Smith, 2003	YES	LIF	BMP4				gelatin	
	2i	Q. L. Ying et al., 2008	YES			CHIR99021	PD0325901		gelatin	
	2i+LIF	Q. L. Ying et al., 2008	YES	LIF		CHIR99022	PD0325902		gelatin	
	FCS+2i+LIF	Hackett, Kobayashi, Dietmann, & Surani, 2017	NO	LIF	FCS	CHIR99023	PD0325903		gelatin	
	LCDM	Yang et al., 2017	NO	LIF		CHIR99024			feeders	PARP1i (MiH) +DiM
TSC	TSC-feeders	Tanaka, Kunath, Hadjantonakis, Nagy, & Rossant, 1998	NO				FGF4+ Heparin	Feeders	feeders	
	TSC-FCS	Erlebacher, Price, & Glimcher, 2004	NO				FGF4+ Heparin	TGFβ	-	
	FAXY	Ohinata & Tsukiyama, 2014	YES			XAV939	FGF2	Activin A	fibronectin	p160ROCK (Y27632)
	TX	Kubaczka et al., 2014	YES				FGF4+ Heparin	TGFβ	matrigel	
	pTSC	Frias-Aldeguer et al., 2019	YES		BMP7		FGF4+ Heparin	TGFβ ActivinA	L521	Il11, cAMP, LPA
PrE	XEN-FCS	Niakan, Schrode, Cho, & Hadjantonakis, 2013	NO						gelatin	
	XEN-N2B27	Artus et al., 2012; Paca et al., 2012	YES						gelatin	
	XEN-BMP	Artus et al., 2012; Paca et al., 2012	YES		BMP4				gelatin	
	nEnd	Anderson et al., 2017	YES			WNT3a		Activin A	fibronectin	