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Review

Unlocking trophectoderm mysteries: *In vivo* and *in vitro* perspectives on human and mouse trophoblast fate induction

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SUMMARY

In recent years, the pursuit of inducing the trophoblast stem cell (TSC) state has gained prominence as a compelling research objective, illuminating the establishment of the trophoblast lineage and unlocking insights into early embryogenesis. In this review, we examine how advancements in diverse technologies, including *in vivo* time course transcriptomics, cellular reprogramming to TSC state, chemical induction of totipotent stem-cell-like state, and stem-cell-based embryo-like structures, have enriched our insights into the intricate molecular mechanisms and signaling pathways that define the mouse and human trophectoderm/TSC states. We delve into disparities between mouse and human trophectoderm/TSC fate establishment, with a special emphasis on the intriguing role of pluripotency in this context. Additionally, we re-evaluate recent findings concerning the potential of totipotent-stem-like cells and embryo-like structures to fully manifest the trophectoderm/trophoblast lineage's capabilities. Lastly, we briefly discuss the potential applications of induced TSCs in pregnancy-related disease modeling.

INTRODUCTION

Trophectoderm establishment during early embryonic cell fate specification

After fertilization, the zygote undergoes successive cleavages, gradually losing its totipotent capacity as it differentiates into three distinct cell lineages by the blastocyst stage. The outer layer of the blastocyst consists of trophectoderm (TE) cells, while the inner cell mass (ICM) is composed of epiblast (Epi) cells and primitive endoderm (PrE) cells (Figure 1). Throughout the initial cleavage stages and until the 2–4 cell stage, the blastomeres retain their totipotent potential capable of generating both the embryo itself and the extraembryonic tissues.[1–4]

Upon reaching the blastocyst stage, the cells become primarily committed to either the extraembryonic or embryonic lineage. Aligned with their specific roles, the Epi takes charge of the subsequent development of the complete embryo, while the PrE and TE set the stage for the formation of extraembryonic tissues. The TE gives rise to the placental trophoblast cells, and the PrE contributes to other extraembryonic tissues such as the yolk sac and a portion of the chorion.[5–8]

The role of the TE in early development

The TE plays a pivotal role in early development, notably by orchestrating the crucial task of facilitating the successful implantation of the blastocyst structure into the maternal endometrium- a pivotal step for the embryo's subsequent maturation.[8,9] Although human and mouse placentation share fundamental features, distinctive mechanisms come into play during blastocyst implantation into the maternal endometrium. In mice, the mural TE surrounding the blastocoel cavity transforms into highly invasive giant cells due to specific signals, while the polar TE remains multipotent.[9] Conversely, in humans, blastocyst implantation is steered by the polar TE, which differentiates into cytotrophoblasts (CTs) and multinucleated syncytiotrophoblasts (STBs) responsible for anchoring the blastocyst within the maternal endometrium.[9] Variations in the implantation process among various species stem from an intricate interplay of factors influencing the gestational environment. These factors include the uterus detecting the embryo, finely tuned uterine peristaltic movements, temporally regulated uterine fluid reabsorption, uterine luminal closure, and the orientation of the embryo.[10] These variations in implantation processes, combined with disparities in more advanced placental maturation,[11] underscore the need to establish enduring stem cell lines derived from the TE of each species.

In vitro derivation of trophoblast stem cells

Since the first derivation of embryonic stem cells (ESCs) from the Epi of the blastocyst in the early 1980s,[12–14] there has been an ongoing need to develop an *in vitro* counterpart of the TE compartment to facilitate the study of placenta development. The subsequent successful derivation of murine trophoblast stem cells (TSCs),[15] and, more recently, human TSCs,[16] representing an *in vitro* counterpart of the post-implantation trophoblast lineage progenitors with the capability to differentiate into the various trophoblast subtypes of the placenta, marked a significant milestone in stem cell research. This achievement opened new avenues for identifying signals and molecular mechanisms of early implantation and placentation. Additionally, the successful *in vitro* derivation led to a better molecular

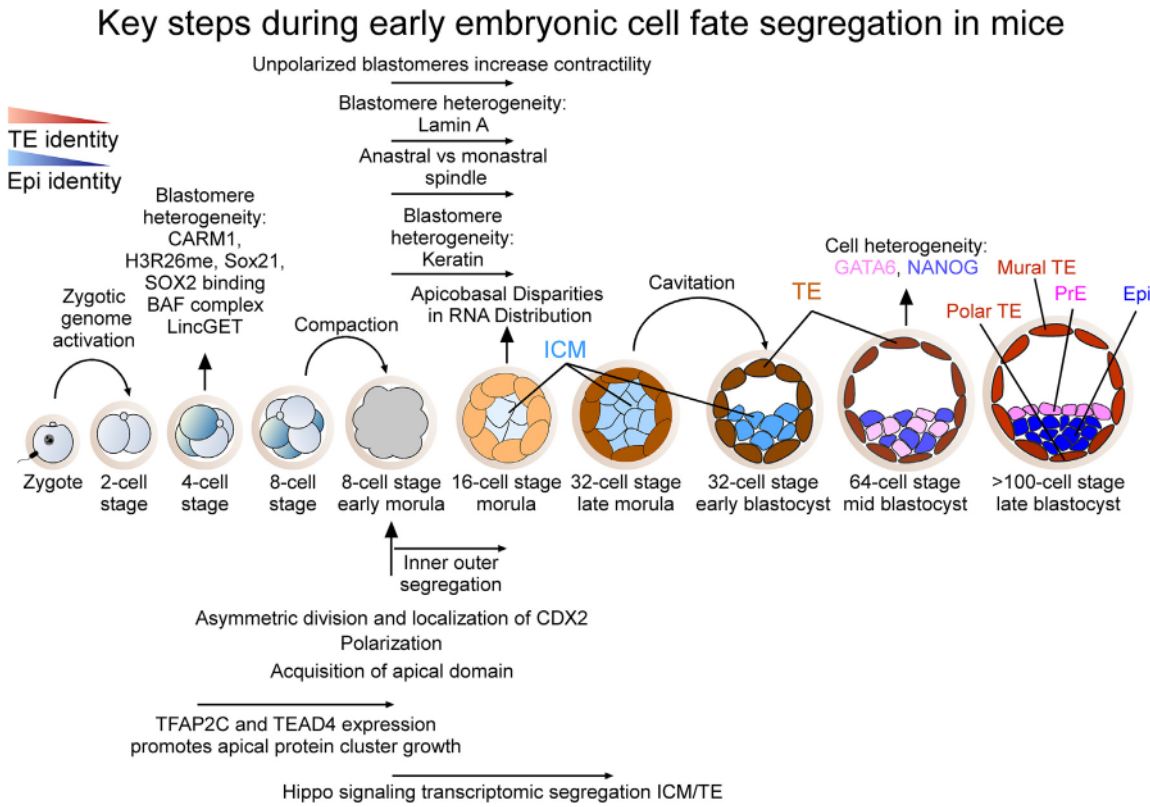

Figure 1. Key steps during early embryonic cell fate segregation in mice
A visual representation illustrating crucial factors shaping the cell fate segregation process between inner cell mass (ICM) and trophectoderm (TE) during early mouse embryonic development. PrE stands for primitive endoderm.

characterization and the identification of crucial signaling mechanisms essential for the survival of these cells. Nevertheless, the restricted availability and ethical considerations associated with stem cells representative of the pre-implantation blastocyst stage, especially in humans, presented a substantial limitation to fully unlocking their potential.

TSC state induction using *in vitro* "artificial" models

Recent breakthroughs in cellular reprogramming, induction of totipotent stem-like cell states, and the construction of stem-cell-based embryo-like models have achieved the extraordinary feat of generating various blastocyst cell types from fibroblasts and pluripotent stem cells in both mouse and human models. These advances have helped address ethical concerns and accessibility limitations associated with human embryo stem cells, providing convenient access to these crucial resources.

Although still in its infancy and warranting meticulous examination, such *in vitro* models could provide a valuable platform to explore the intricate molecular mechanisms governing TE formation, early post-implantation stages, and the dynamic communication between the pre/post-implantation TE compartment, the Epi, and the endometrium. These insights offer promising perspectives into pivotal aspects of early human pregnancy. However, despite these notable achievements, capturing the pre-implantation TE stage in culture remains a significant challenge.

In this review, we present a comprehensive summary of recent advancements in establishing human and mouse TE/TSC states. We delve into the technologies employed and the molecular mechanisms that contribute to our understanding of TE/TSC state induction and identity.

TE STATE SPECIFICATION: LESSONS LEARNED FROM SINGLE-CELL TRANSCRIPTOMIC DATA

Timing of the first cell fate segregation

Since the initial discovery of TE lineage as the precursor of trophoblast placental cells,[17] numerous studies have employed gene manipulations and gene expression analysis to delve deeper into the transcriptome segregation that takes place between the ICM and TE.

The segregation between ICM and TE becomes particularly evident at the 16-cell morula stage in mice, where the YAP/TEAD4/CDX2 axis governs the process.[18] As the embryo progresses to the 32-cell stage, trophectodermal cells start to solidify their commitment in terms of their transcriptomic profile.[19–21] At the subsequent blastocyst stage, a second lineage segregation is orchestrated by the NANOG/FGF4/GATA6 axis, leading to the commitment of the ICM either to the Epi or PrE fate.[22–26]

In humans, due to limited access to early embryos, regulations prohibiting genetic modifications of human embryos, and various other challenges related to targeted genetic manipulations of embryonic material, research on early human

development remains for a long time constrained.[27,28] Nonetheless, numerous differences in gene expression timing between human and mouse have been defined. For instance, the levels of the key transcription factors OCT4 and CDX2, which dictate cell fate in mice,[22–26] show a distinct expression pattern in humans. *OCT4* is found to be expressed in both the ICM and TE during blastocyst formation, while *CDX2* expression initiates in the TE subsequent to the formation of the blastocyst stage.[29–31] Furthermore, GATA6, that drives the second lineage segregation in mice[26] is found to be segregated to the PrE only after blastocyst implantation in humans.[30] In a similar vein, notable genes like *ELF5* and *EOMES*, pivotal for mouse TE development, have been revealed to be lacking during the human blastocyst stage.[3,32]

Taking into account the factors mentioned earlier, the precise timing of early lineage segregation in humans and its parallels to mouse lineage segregation have gathered significant attention. Numerous studies have turned to single-cell RNA sequencing (scRNA-seq) to pinpoint the exact timing of this segregation process. An illustrative example comes from Petropoulos et al. who conducted single-cell transcriptome sequencing on 1,529 high-quality individual cells derived from 88 embryos, covering early development from the 8-cell stage to a time point just prior to implantation.[32]

Their findings indicate that during the late compacting morula stage (E4) in human embryos, there is an initiation of a transcriptional program associated with the TE, characterized by increased expression of key markers such as *GATA3*, *PTGES*, and *PDGFA*. This TE-related program occurs concurrently with an increased expression of Epi and PrE markers. Building on these observations, the authors propose a model for human embryos in which lineage segregation between the Epi, TE, and PrE occurs simultaneously on day 5 during blastocyst formation.[32]

However, another noteworthy study undertaken by Stirparo et al. aimed at conducting a comprehensive analysis of single-cell transcriptome datasets derived from human embryos.[33] Their work revealed a distinct developmental state within the early human ICM, which displayed a unique transcriptomic profile compared with the more mature Epi or PrE cell populations. The authors were able to differentiate early ICM, Epi, and PrE cells as separate populations based on their distinctive transcriptomic signatures. Within the early ICM network, several prominent hub genes were identified, including *MFN1*, *CYP26A1*, *NANOGNB*, *PRAMEF17*, and *PRAMEF20*. While Stirparo et al.'s findings do not completely negate the possibility of some overlap between the acquisition of ICM vs. TE identity and Epi and PrE specification, their study suggests that the divergence of Epi and PrE may commence in a subset of ICM cells on day 5 of development.

Meistermann et al. achieved a unification of prior models by recognizing the necessity for improved annotation of distinct blastocyst stages in order to accurately pinpoint the occurrence of potential cell fate segregation.[19] Through the integration of scRNA-seq and time-lapse microscopy, Meistermann et al. effectively linked pseudotime analysis of molecular events to the developmental stages of the blastocyst. This approach provided a more precise delineation of developmental stages compared with earlier models that relied on time elapsed since fertilization. This approach provided compelling evidence that human early embryonic development follows two distinct waves of lineage segregation, mirroring the observed pattern in mice. Notably, they revealed that the initial transcriptome segregation between the ICM and TE occurs after blastocyst cavitation during the transition from the early blastocyst to the blastocyst stages (B2 to B3) before reaching the expanded blastocyst stage.

Additionally, Meistermann et al. successfully identified gene signature modules associated with lineage segregation and specification.[19] For instance, the POU5F1B module, comprising 195 genes including *KLF4*, *SOX2*, and *PRDM14*, was first detected in all cells of the morula and B1 and B2 blastocysts, persisting in the Epi and subsequently downregulated in PrE and TE. On the other hand, the *GATA2* module, consisting of 595 genes such as *GATA3*, *PDGFA*, and *CDH1*, specifically marked the TE and appeared significantly at the branching point between the ICM and TE.[19] While pinpointing an early ICM signature may help in determining the onset of TE specification, in contrast to Stirparo et al., Meistermann et al. did not observe a distinct early ICM signature. This suggests that following the initial segregation between ICM and TE, ICM cells acquire a definitive Epi signature, from which PrE cells emerge during blastocyst expansion.

Recently, Radley et al. successfully demonstrated a well-defined ICM gene signature.[34] They developed a novel mathematical framework called "entropy sorting" capable of identifying genes that reflect cell identity. The authors identified a clear ICM gene signature, including genes such as *LAMA4*, *FGF1*, *PIMREG*, *BHMT*, *SPIC*, and *PRSS3*. Immunostaining confirmed the expression of LAMA4 in the mid-blastocyst stage (E5), preceding Epi-Pre segregation, as indicated by co-expression of OCT4 and SOX17 in these cells. Subsequently, as cells adopt different fates, LAMA4 becomes downregulated by the late blastocyst stage (E6–E7).[34]

Wei et al. asserted that an ICM population could be identified and marked by *EPHAM4* and *CCR8*, two novel ICM markers previously unreported.[35] However, while the authors validated these markers through immunostaining of "blastoids," it is imperative to conduct actual staining of blastocysts to confirm their validity as ICM markers.

The advent of scRNA-seq technology has undoubtedly propelled our comprehension of early cell fate determination and the consolidation of cellular identities. However, it is important to acknowledge that investigations into human early embryonic development encounter various limitations and challenges.

As highlighted in the study by Biondic et al., there persists a notable lack of precision with regard to the specific post-fertilization days under consideration and the exact cell counts within the embryos being analyzed. This ambiguity is further compounded by the presence of technical artifacts associated with mRNA expression and the time lag introduced during the thawing process, which can introduce disparities when comparing fresh and frozen embryos. Additionally, the inherent variability in the developmental trajectories of individual embryos adds an extra layer of complexity to the accurate staging of these embryos.[36] Given these challenges, it becomes evident that a comprehensive approach to embryo staging necessitates the consideration of developmental time, morphokinetics, and cell count. This is particularly crucial in research studies, where the scarcity of human embryos often compels researchers to rely on previous data as references.[36]

In conclusion, it is evident that both mouse and human embryos initiate TE commitment during the morula stage, as previously highlighted.[37] However, a notable distinction arises in terms of the timing of definitive transcriptome commitment. In mice, the TE achieves transcriptomic commitment immediately after the initiation of its fate, whereas in humans, this commitment is reached at a later stage, specifically during the blastocyst stage. Future studies are anticipated to shed light on the underlying reasons for the observed delayed fate commitment in the human developmental process.

Early TE development

Numerous studies have underscored the divergent potentials between polar and mural trophoblast cells.[19,32] These investigations have illuminated disparate mechanisms governing the implantation of trophoblasts in human and mice.[9,11] In mice, the mural trophoblast differentiates and initiates implantation, while in humans, it is the polar trophoblast that anchors into the maternal endometrium.[9] Nevertheless, the precise mechanism orchestrating the differential potential of polar vs. mural trophoblasts across human and mice remains largely unknown.

Turning attention to human TE development, a recent study identified distinct stages of TE maturation marked by specific gene modules. The authors identified an early trophoblast stage characterized by the expression of *GATA2*, *POU5F1B*, and *DNMT3L* modules. Subsequently, a second stage emerged defined by the presence of the *GATA2* module while excluding *POU5F1B*. Lastly, a third stage was identified by the co-expression of the *GATA2* and *NR2F2* modules.[19] These gene signatures could serve as valuable tools for refining the classification of human TSCs *in vitro*.

An intriguing finding from their pseudotime analysis was the emergence of *NR2F2* as a mature trophoblast marker, initially present in polar TE cells adjacent to Epi cells expressing *NANOG*. Subsequently, *NR2F2* expression extended to encompass all trophoblast cells. This observation led to further investigation, spotlighting TGFβ, IGF1, BMP2, and FGF4 as potential drivers, from Epi cells, of the molecular mechanisms orchestrating the Epi-TE dialogue. This complex interplay may play a pivotal role in cueing polar trophoblasts to embark on differentiation, a crucial step preceding the implantation stage.[19] However, whether NR2F2 and its associated gene module have a significant role in human embryo implantation remains to be assessed.

In a comprehensive comparison of polar and mural trophoblast induction between mouse and human, Liu et al. uncovered parallels in the two species.[38] Following blastocyst hatching from the zona pellucida, both polar and mural trophoblast in each model underwent distinct transcriptional changes, delineating their uniqueness. The genes signature associated with implantation-related processes—such as cell migration (*LCP1*, *EFHD2*, *FMNL2*), immune tolerance (*CSF3R*), and placentation/cell lineage specification (*RXRA*, *GATA2/3*, *TFAP2AC*, *ARID3A*)—shared commonality at the respective implantation poles in both species.[38] This intriguingly underscores the conservation of these genes' pivotal roles in embryo implantation.

Seong et al. have provided significant insights into murine development, revealing that the polar TE at the late blastocyst stage exhibits enrichment in critical transcription factors like *Cdx2*, *Esrrb*, and *Elf5*, known for their regulatory roles in trophoblast renewal.[39] In contrast, the mural TE demonstrates heightened expression of key molecules such as integrins (*Itga5/6/7/v*), laminins (*Lama1/b1/b2*), galectins (*Lgals1* and *Lgals*l), *Hb-egf*, and ephrins (*Efna1/b1/b2*), consistent with the initiation of the implantation process. The study further shows that various signaling pathways, including Hippo, fibroblast growth factor (FGF), STAT, and SMAD, are more active in the polar TE when compared with the mural TE.[39]

Seong et al.'s work unravels a delicate equilibrium within the TE maintained by a subset of molecules secreted by the Epi (i.e., Epi inducers), carefully orchestrating the balance between proliferation and differentiation. Their findings highlight the pivotal role of an optimal combination of Epi inducers (such as mitogen-activated protein kinase (MAPK), Hippo, SMAD, and STAT pathways) in sustaining the multipotent state of TE *in vitro*, closely mirroring early TE transcriptome and averting the activation of post-implantation TE genes. Through blastocyst models, the authors reveal that these Epi inducers, including lysophosphatidic acid (LPA), FGF4, NODAL, BMP4, BMP7, and IL6/11, not only maintain trophoblast multipotency but also influence the expression of *CDX2* in the polar TE, thus impacting other crucial processes such as endometrial decidualization.[39]

The findings from these studies highlight the critical significance of the communication between the Epi and TE, a crosstalk that holds paramount importance in the subsequent implantation of the blastocyst and the refinement of the trophoblast lineage. A more comprehensive understanding of the intricate implantation process in humans promises to illuminate insights that are shared between human and mouse models, while also shedding light on the fundamental divergence in Epi induction that leads to the emergence of distinct implantation poles.

MOLECULAR MECHANISMS AND SIGNALING PATHWAYS RESPONSIBLE FOR TE ACQUISITION

Chromatin modification and genes expression

While recent discoveries in this field have been somewhat limited, it is essential to briefly explore earlier research that has illuminated the influence of gene expression and chromatin modifications on the segregation of mouse cell fates. In addition to other studies that have identified critical molecular factors, such as the asymmetric localization of CDX2,[40] it is worth emphasizing the significance of histone H3R26 methylation and its associated methyltransferase, CARM1, in the context of driving cell fate determination. Torres-Padilla et al. were among the first to report in 2007 that H3R26me and its methyltransferase CARM1 exhibit significant cell-to-cell variability in mouse 4-cell stage embryos.[41] This variability suggests that the level of H3R26me may predispose cells toward distinct developmental fates. Accordingly, the overexpression of CARM1 in individual blastomeres led to a bias toward ICM fate and was accompanied by the upregulation of pluripotency factors NANOG and SOX2.[41]

A subsequent study by Goolam et al. further revealed the connection between CARM1 and the heterogeneous expression of *Sox21*, a downstream target of SOX2 and OCT4, in 4-cell stage embryos.[42] The downregulation of *Sox21* resulted in the upregulation of the TE differentiation marker CDX2. This intricate interplay suggests a model wherein CARM1-mediated

differential expression of *Sox21* influences the future fate of individual blastomeres. White et al. demonstrated that H3R26me regulates the temporal binding of SOX2 in the 4-cell stage, ultimately leading to diverse SOX2 DNA bindings in individual blastomeres. This variation in binding patterns may contribute to the divergence in blastomere fates.[43]

The enduring impact of H3R26me on cell fate has been revealed to be instigated by a range of diverse mechanisms. Wang et al. demonstrated a role for LincGET, a long noncoding RNA (lncRNA), in the early embryonic cell fate determination process.[44] LincGET was found to be transiently and asymmetrically expressed in the nucleus of 2- to 4-cell stage mouse embryos and to physically interact with CARM1, promoting its nuclear localization, thereby elevating levels of H3R26me and activating downstream targets specific to the ICM fate.[44] In parallel, Hupalowska et al. revealed the involvement of nuclear speckles, membraneless subnuclear bodies formed around scaffolds of specific lncRNA, in cell fate decisions.[45] In this scenario, the authors demonstrated that CARM1 accumulates within nuclear paraspeckles composed of p54nrb, PSPC1, PSF, and LncNEAT1 during the 4-cell stage. Moreover, the number of paraspeckles in individual blastomeres was shown to correlate with the H3R26 methylation level, suggesting a role for nuclear architecture in early cell fate decisions. Interestingly, depletion of the paraspeckles components, NEAT1 or P54nrb resulted in a more severe phenotype compared with CARM1 depletion, leading to developmental arrest at the 16–32-cell stage. These results emphasize a possible role of nuclear speckles in early cell fate decision, independently of CARM1.[45]

Despite the early influence of these molecular factors at the 4-cell stage, it is not until the 8-cell stage that the first morphological distinctions arise in mouse pre-implantation embryos. During this stage, blastomeres compact and subsequently polarize, establishing an apicobasal axis that ultimately governs the activation of the Hippo pathways and culminates in the complete transcriptomic segregation between the ICM and TE.[19–21] Recently, Zhu et al. demonstrated that the levels of transcription factors TFAP2C and TEAD4 in early stages serves as regulators of the polarization process.[46] Co-depletion of both transcription factors disrupted polarization, and premature expression of *Tfap2c* and *Tead4* was sufficient to induce early protrusion enriched in apical polarity proteins. Interestingly, the addition of activated RhoA to this combination led to the establishment of complete apical domains at the 4-cell stage, triggering the premature expression of TE transcription factors CDX2 and GATA3.[46] These findings underscore the tight relationship between zygotic activation timing and the establishment of *de novo* polarization in the mouse embryo.

Hippo signaling pathway

The Hippo signaling pathway accountable for mouse TE acquisition has undergone comprehensive review elsewhere.[22–26,28] In brief, a variety of mechanisms, encompassing cell position, cell-cell adhesion, and cell polarity during the 8-cell stage, lay the foundation for the discrete activation of the Hippo signaling pathway. In the outer cells of the morula, Hippo signaling becomes inactivated, leading to the translocation of the transcriptional co-activator YAP into the nucleus. YAP, upon interaction with the transcription factor TEAD4, instigates and maintains the expression of downstream TE targets like *Cdx2*.[2,47] Besides *Cdx2*, other TE-associated genes such as *Krt8*, *Krt18*, *Dab2*, *Lrp2*, and *Gata3* are likely to be directly activated by nuclear YAP/TEAD4, potentially contributing to the comprehensive induction of the TE compartment.[20,48]

On the contrary, the activation of Hippo signaling in inner cells hinders YAP activity, resulting in the suppression of TE transcription factors and the maintenance of pluripotency factors.[23] For instance, the activation of Hippo signaling in inner cells confines the transcription factor SOX2 specifically to inner cells, further stimulating pluripotency in the ICM.[49] Additionally, other signaling pathways have been demonstrated to influence the asymmetric activation of the Hippo pathways. Notable examples include the Rho signaling pathways in TE cells, which repress the interaction between AMOT and NF2, two critical regulators for Hippo signaling activation,[50] and the GPCR signaling, which inhibits LATS1/2 kinases, both of which are vital components for the activation of the Hippo pathways.[51]

Building on the concept of shared mechanisms between humans and mice, Gerri et al. have compellingly demonstrated that the cellular polarity and Hippo signaling pathways are not exclusive to mice. In contrast, these mechanisms are evolutionarily conserved and play a pivotal role in initiating TE segregation in embryos of diverse species, including humans, cows, rats, and mice.[37,52] Notably, Gerri and colleagues identified that, similar to mice, outer cells of human and cow embryos acquire cell polarity during the morula stage through aPKC activity. This process involves the sequestration of AMOT and subsequent inactivation of the Hippo pathway, leading to the activation of YAP1/TEAD4. Consequently, this activation prompts the initiation of TE through the segregation of key TE factors such as GATA3 to the outer cells. scRNA-seq analysis further revealed that GATA3 expression in the morula is linked to genes associated with epithelial cell formation (*KRT18*, *CLDN4*, *RAB20*, and *RAB25*) and placenta morphogenesis (*PTGES*, *TFEB*, and *PLAC8*).

It is worth noting that Regin et al. have recently reported that the TE drivers GATA3 and YAP1 co-localize within the nuclei of certain human blastomeres before polarization commences.[53] This suggests the existence of a lineage segregation mechanism independent of polarity. While Gerri et al. propose the potential existence of a broader evolutionary process governing cell fate in mammalian embryos, it is clear that further investigations spanning a diverse range of mammal species and improved access to early-stage human embryos will be essential. These efforts are necessary to uncover the functional implications of the observed discrepancies and to gain a comprehensive understanding of a possible interspecies mechanism for the initial cell fate segregation.

Cytoskeleton as a key determinant of early segregation

Despite the comprehensive characterization of the Hippo signaling pathway, the precise upstream mechanisms orchestrating autonomous specification and the stereotypical positioning of distinct cell lineages in early development through the Hippo pathway remain elusive.

In 2016, Maître et al. introduced a mechanosensory model[54] that integrates elements from both the cell polarity and cell position models,[55,56] explaining how cell contractility, regulated by the asymmetric segregation of a polarized apical domain, controls the subcellular position of the transcriptional co-activator

protein YAP.[54] Considering the role of the cytoskeleton on cell contractility, subsequent studies have underscored its pivotal role in early embryo cell lineage specification.

For instance, while asymmetric polarity's importance in early segregation had been previously extensively reported,[57] Zenker et al. used live embryo imaging to reveal that the apical domain is temporarily lost during mouse blastomere division before re-emerging in the daughter cell at the embryo's periphery,[58] raising important questions as to how cell polarity remains asymmetric during early embryo development.

Subsequently, the same research group showed that keratin, which accumulates during the interphase of cell division, was found to associate with the apical actin-rich cortex, serving as a physical memory of polarity.[59] Importantly, they observed heterogeneity in keratin within 8-cell stage blastomeres, which was correlated with heterogeneities in the BAF chromatin remodeler complex within the 4-cell stage embryo. Notably, the BAF complex has previously been shown to be regulated by CARM1,[60] highlighting an intricate interplay between BAF-CARM1 heterogeneities in 4-cell blastomeres and the asymmetrically inherited keratin in 8-cell stage blastomeres. Accordingly, the overexpression of CARM1 or downregulation of the BAF complex was demonstrated to disrupt keratin expression.[59]

Pomp et al. provided evidence that between the 8- and 16-cell stage, the embryo displays two types of mitotic spindle organization, fostering asymmetric division and cell fate determination in the early embryo.[61] Building upon a previous study demonstrating the asymmetric localization of *Cdx2* mRNA to the apical pole at the late 8-cell stage,[40] Hawdon et al. recently illustrated that the asymmetric and polarized microtubule network influences the segregation of RNA transcripts during the 16-cell stage.[62] In this model, RNA transcripts anchored to LAMP1 lysosomes by annexin 11 are guided by the polarized asymmetric microtubule network toward the basal membrane, resulting in apicobasal asymmetry of RNA transcripts and further impacting future cell fate allocation.[62]

In addition to keratins and microtubules, nuclear Lamin A, tethered to the cortex via an F-actin meshwork and responsive to cell contractility, has been shown to induce changes in actin organization, differentially regulating YAP and CDX2 to specify lineage cell fate.[63] This study also suggests the presence of a potentially similar mechanism in human embryos, but further research on human 8-cell stage embryos is imperative to validate this finding.

In conclusions, a multitude of distinct elements, ranging from gene expression to mechanical forces, wield influence over the initial segregation of cell fates (Figure 1). This endeavor is pivotal in attaining a profound understanding of the sequential events that orchestrate the process of cell fate segregation and TE establishment during embryogenesis.

TSC STATE INDUCTION: LESSONS LEARNED FROM CELLULAR REPROGRAMMING STUDIES

The successful derivation of TSCs from both mouse and, more recently, human has opened an exciting avenue for investigating the intricate molecular characteristics and signaling pathways that sustain these stem cells.[15,16]

Numerous studies have already described and reviewed the signaling pathways important for the TSC state.[28,64] In the context of mice, research has highlighted the importance of signaling pathways involving FGF4 and transforming growth factor β1 (TGF-β1) in maintaining the TSC state.[15] However, recent advancements in the derivation of human TSCs have identified a distinct signaling landscape. Activation of WNT and epidermal growth factor (EGF), coupled with the inhibition of TGF-β, histone deacetylase (HDAC), and ROCK signaling, has been identified as essential for maintaining a TSC state in humans.[16] This revealingly contrasts with the role of WNT and TGF-β1 signaling in mouse TSCs. Furthermore, in-depth characterization of human TSCs has identified a unique transcription factor network compared with their murine counterparts. Notably, the transcription factor EOMES was found to be poorly expressed, and CDX2 exhibited significantly lower expression levels in human TSCs, showcasing intriguing disparities in the regulatory elements governing these cells.[16]

Significant leaps in cellular reprogramming and direct lineage conversion have ushered in a novel pathway for delving deeply into the intricate molecular processes pivotal for acquiring distinct cellular identities.[65] Recent research delving into the somatic nuclear reprogramming of fibroblasts into TSCs, both in human and mouse systems, has illuminated a plethora of pivotal factors that underlie the attainment of the TSC state as discussed below.

In 2015, the Schorle and Buganim research groups achieved a notable milestone by demonstrating that the direct introduction of specific transcription factors—namely, GATA3, EOMES, TFAP2C, along with either MYC or ETS2—into fibroblasts could lead to the transformation of these cells into functional TSCs.[66,67] These studies thoroughly explored pluripotency acquisition and revealed its dispensability in the establishment of the murine TSC state.[66,67]

In a more recent study, the Buganim group embarked on a thorough expedition, conducting a comparative parallel multi-omics analysis. Their goal was to dissect the intricate molecular mechanisms governing the transition to the murine TSC state, contrasting it with pluripotency. The authors found that although both systems undergo analogous processes like somatic cell identity loss, proliferation, mesenchymal-epithelial transition (MET), and metabolic shifts, each system predominantly operates through distinct molecular networks encompassing transcription, chromatin accessibility and activity, and DNA methylation to meticulously orchestrate their distinct cellular fates.[68] A compelling illustration pertains to the intricate nature of the embryonic program, which is defined differently within the reprogramming processes toward the TSC and pluripotent stages. Within this context, the genes and networks associated with TSC identity orchestrate the comprehensive cessation of the embryonic program via DNA methylation. Conversely, the genes and networks responsible for sustaining pluripotency take center stage in the initial establishment of the embryonic program, executed through the deposition of the histone mark H3K4me2 to effectively delineate forthcoming active regions while simultaneously suppressing the presence of the active histone mark H3K27ac.[68]

Unlike in the mouse context, recent research has found differences in the induction process of the TSC state from human fibroblasts. While OSKM reprogramming in mice predominantly results in induced pluripotent stem cells (iPSCs) and a small side population of differentiated trophoblasts, as confirmed by gene expression signatures,[68,69] a study by the Polo group has

highlighted a distinct scenario in humans. This study demonstrated that during OSKM reprogramming, besides iPSCs, a specific subset of cells acquires a TSC state.[70] This subset could be selectively isolated and cultivated, ultimately giving rise to a stable population of TSCs.[70] A parallel study conducted by Castel et al. further substantiated the successful induction of human TSCs through OSKM induction.[71]

In these human models, unlike in mice, the same induction network initially propels both the reprogramming toward iPSCs and iTSCs. However, as the process unfolds, a distinct subset with TE potential undergoes further segregation, setting it apart from the mouse paradigm. This intricate interplay offers fresh insights into the nuances of TSC induction, particularly emphasizing the variability between species.

Drawing on the understanding that the intricate reshaping of a cell's epigenetic landscape involves the cooperative interplay among various transcription factors,[72–74] a recent study by Naama et al. has shed light on an alternative approach to induce human TSC state. By substituting the pluripotency-associated transcription factor SOX2 with the TE-specific transcription factor GATA3 (forming GOKM instead of OSKM), the authors demonstrated the feasibility of reprogramming human fibroblasts toward the TSC stage independently of pluripotency.[75]

The study, which is based on a comprehensive analysis of cells undergoing reprogramming by both OSKM and GOKM combinations, elucidates that cells transduced with GOKM adopt a distinctive trajectory toward the human TSC state in comparison with those subjected to OSKM. GOKM selectively target loci specifically associated with the TSC state, while OSKM induce the TSC state by acting upon regions shared between PSCs and TSCs.[75] Through time course transcriptomic analysis, the authors identified unique gene expression patterns emerge for OSKM- and GOKM-induced cells during the reprogramming process, shedding light on how GATA3 and SOX2 target distinct genetic domains to induce pluripotent or TSC states. Importantly, while surprising insights emerged as for the unanticipated role of the pluripotency factor OCT4 in inducing the TE/TSC state,[31,75] the study demonstrates that pivotal pluripotency factors such as SOX2, NANOG, and PRDM14 are completely dispensable for human TSC fate acquisition.

Furthermore, a comparative analysis of stable induced TSCs (iTSCs) generated via OSKM and GOKM reprogramming, alongside blastocyst-derived TSCs (bdTSCs), revealed intriguing distinctions. GOKM-iTSCs exhibited striking similarity in gene expression to bdTSCs, while OSKM-iTSCs displayed significantly reduced expression in 94 genes associated with estrogen response. Estrogen plays a pivotal role in the preparatory phase of endometrial receptivity for implantation. It achieves this by orchestrating the growth and development of the endometrium throughout the menstrual cycle.[76,77] This concerted effort results in the establishment of an ideal microenvironment conducive to trophoblast attachment and successful implantation. Furthermore, estrogen, acting in tandem with other biological processes, enhances the trophoblast cells' vitality and their capability to invade the endometrial lining. This in part is attributed to the activation of SGK1 in trophoblast cells.[78] These findings suggest potential challenges inherent in OSKM-induced TSCs, particularly in attaining fully functional TSCs.

Collectively, the growing body of research illuminates critical distinctions in the pathways guiding the induction of TSCs in mice and humans. In the murine setting, TSC establishment and its induction mechanisms function autonomously, separate from the influence of pluripotency factors. This autonomy is starkly contrasted by the human scenario, where a closer interconnection between pluripotency and TE induction is observed. Although human TSCs can attain a higher quality state independently of the pluripotent state, implying distinct induction patterns, their developmental trajectories remain intricately intertwined with pluripotent factors such as OCT4, revealing a distinct relationship compared with mice.

THE BARRIERS UNDERLYING TRANSDIFFERENTIATION BETWEEN PLURIPOTENT AND TSC STATES

TSC state induction from naive pluripotent stem cells

Within the realm of pluripotency, discernible differences in properties have emerged among PSCs representing the pre-implantation blastocyst stage, denoted as ESCs in mice, and naive-like ESCs in humans, as opposed to PSCs in the post-implantation stage, termed Epi stem cells (EpiSCs) in mice and primed ESCs in humans.[79]

The barrier between the different pluripotent states and the TSC state have captivated researchers for the past two decades. In the context of mice, ESCs necessitate the downregulation of OCT4[80] or the upregulation of pivotal TSC-associated transcription factors like CDX2, TEAD4, ELF5, GATA3, EOMES, TFAP2C or Ras-MAPK signaling[48,81–84] to comprehensively transition from the pluripotent state to the trophoblast stem-like fate. In these models, the newly emerging trophoblasts demonstrate inadequate expression of critical TSC gatekeeper genes, such as *Elf5*, *Tead4*, *Ezr*, and *Hand1*.[85] This deficiency stems from the incomplete removal of methylation in these loci, thus underscoring methylation as a significant impediment in the transition from mouse pluripotency to the TSC state.

A recent report provided evidence indicating that the segregation of the ICM and TE lineages in humans occur at later stages compared with mice.[19] This intriguing observation prompts the query of whether a similar barrier between pluripotency and TSC state exists in humans. Notably, unlike their mouse counterparts, human naive-like ESCs exhibit a remarkable capability for rapid differentiation into trophoblast derivatives *in vitro*. Several studies have illustrated that transitioning naive-like ESCs into a TSC-defined medium leads to the formation of TSCs.[71,86,87] Additionally, the resulting TSCs demonstrated abilities for proliferation and differentiation toward extravillous trophoblast (EVT) and STB fates, along with exhibiting transcriptomic profiles akin to human TSCs.

Recently, Guo et al. reported that the inhibition of ERK/MAPK and NODAL signaling in a proper medium composition can effectively convert human naive-like ESCs into TSCs.[88] Following a similar approach, Io et al. documented the successful derivation of TSCs, combining pre-treatment with BMP4 and JAK inhibitors along with the inhibition of ERK/MAPK and NODAL signaling.[89] Interestingly, in contrast to previous studies, both groups demonstrated the presence of a transient pre-implantation TE-like state, as discussed below.[88,89]

In line with the concept of unrestricted lineage potential in human naive-like PSCs, the mouse gatekeeper genes

demonstrated suitable methylation levels in transdifferentiated human TSCs.[86] However, it is important to acknowledge that beyond the mere ability of cell conversion, the culture conditions can wield a crucial influence over the eventual epigenetic state. This concept was recently substantiated by Kaiser et al., who showed that the previously identified methylation barrier between mouse ESCs and TSCs could be effectively overcome through the overexpression of *Cdx2* within a more precisely defined culture setting.[90]

Simultaneously, the extensive body of evidence affirming the unbounded potential of naive-like human PSCs has elicited inquiries regarding the potential presence of inherent molecular mechanisms within these cells that curtail their differentiation into various lineages, setting them apart from "totipotent cells." Recently, two papers demonstrated that repressive chromatin pathways were stabilizing the undifferentiated naive-like state while opposing the induction of other fates.[91,92] Their studies demonstrated the presence of the H3K27me3 repressive mark, which is laid down by PCR2,[93] on crucial genes associated with trophoblast induction. This discovery underlines the significant involvement of PCR2 in upholding the naive-like state within human PSCs and counteracting the initiation of trophoblast fate.

TSC induction from primed pluripotent stem cells

While, to the best of our knowledge, no previous studies on mice have been able to fully convertEpiSCs to TSCs,[81] the capacity of primed human post-implantation ESCs to undergo differentiation into TSCs has ignited considerable debate within the scientific community.

Over the past decade, several studies have documented the successful derivation of human TSCs from primed PSCs, with many of these studies employing BMP4-based conversion protocols.[94,95] However, the initial success of these studies faced skepticism due to the prompt expression of the mesoderm marker Brachyury shortly after the initiation of BMP4 treatment. This early expression cast doubts on whether the resulting cells were genuinely destined for a TSC fate.[96–98] Moreover, further investigations comparing TSCs derived from naive-like or primed PSCs unveiled distinctions. TSCs originating from primed PSCs exhibited features consistent with amnion-like characteristics in comparison with those originating from naive-like PSCs.[87–89]

However, building upon the earlier criticisms, a number of studies have recently presented compelling evidence in favor of the successful differentiation of primed PSCs into TSCs. These newly derived TSCs demonstrate attributes such as self-renewal, anticipated transcriptomic profile, and differentiation potential.[99–102] Additionally, in an intriguing twist, Wei and colleagues showed that, although BMP4 enhances the derivation process, it is not a prerequisite for the successful generation of hTSC from primed PSCs.[103]

Interestingly, Kobayashi et al. brought attention to the fact that primed PSCs do possess some capacity for differentiation into TSCs, although to a lesser extent when compared with naive-like PSCs. In this context, the authors have proposed that the limited differentiation of these cells into genuine TSCs might stem from the *in vitro* culture conditions and may not manifest in an *in vivo* scenario. As per their findings, while the transcriptomic and methylomic profiles strongly suggest the validity of these cells as TSCs, their restricted differentiation potential, relatively abbreviated lifespan, and the absence of expression of the trophoblast miRNA marker C19MC could potentially indicate that the *in vitro* primed state is suboptimal for TSC production.[104]

It is important to mention, however, that despite the lack of full activation of the miRNA marker C19MC, in contrast to the aforementioned study, other research groups have reported findings demonstrating that human TSCs originating from primed sources do indeed possess the capability to differentiate into various trophoblast subtypes, including STBs and, to a lesser extent, EVTs.[99–101] The only discernible distinction among these various studies lies in the specific medium in which the primed PSCs were cultured prior to the commencement of the differentiation process. Furthermore, it is worth noting that although they may not exhibit identical abnormalities, human TSCs derived from naive-like PSCs have also been found to display certain methylation irregularities, particularly in imprinted genes.[86]

These differences give rise to the possibility that a significant portion of these characteristics might be attributed to the pivotal role of varying culture conditions in facilitating the conversion of distinct cell types, rather than being solely dependent on the intrinsic potential of the cells to undergo complete transformation. Ultimately, while the evidence suggests that human PSCs possess a notably broader potential for extraembryonic fate when compared with mice, the conclusive resolution of the debate regarding the potential of primed PSCs to differentiate into TSCs hinges on forthcoming studies that can effectively demonstrate whether post-implantation Epiblasts indeed possess the capability to contribute to the TE lineage in primate species.

ACQUISITION OF A PRE-IMPLANTATION NAIVE-LIKE TSC STATE THROUGH TRANSDIFFERENTIATION OF PSCs

While TSCs offer a valuable avenue for delving into the molecular intricacies of placenta formation, it is noteworthy that mouse TSCs can be derived from the extraembryonic ectoderm up to embryonic day (E)8.5,[105] and human TSCs can be derived from the first-trimester placenta.[16] Following this observation, several studies have provided evidence that current TSCs embody a primed post-implantation state in both mouse and human models.[39,71,87]

In mice, Seong et al. have recently reported the successful cultivation of stable TE-like stem cells, termed TESCs, which more closely resemble the TE of the pre-implantation blastocyst stage.[39] Expanding upon earlier findings regarding the heterogeneity within the *in vitro* TSC population,[68,106–108] Seong et al. demonstrated that by culturing TSC on laminin-coated plates using a previously defined TSC medium (TX, [109]), supplemented with Activin, IL11, BMP7, 8-Br-cAMP, and LPA, they achieved successful cultivation of a homogeneous population of TESCs. This distinctive state was characterized by elevated expression of self-renewal transcription factors such as CDX2, EOMES, and ESRRB and the absence of differentiation markers such as *Ascl2*, in contrast to conventional TSC culture conditions.[39]

Interestingly, TESCs exhibit a heightened ability to generate blastocyst-like structures when combined with PSCs and demonstrate enhanced implantation proficiency within the endometrium. Nonetheless, these TESCs did not surpass the previous ESC-TSC embryo-like model in enabling substantial *in vivo* advancement

beyond the implantation stage.[39] This could potentially stem from the influence of various supplementary factors influencing subsequent development. This observation alludes to the notion that despite more faithfully mimicking the TE, this state may not yet fully encapsulate the authentic trophectodermal condition essential for facilitating complete blastocyst development within the maternal endometrial environment.

Within the realm of human TSCs, Castel et al. showed their resemblance to post-implantation day 8–10 CTs,[71] while Dong et al., established their similarity to human CTs at day 12 post-fertilization.[87]

Recent investigations have harnessed the distinctive expression pattern of the transcription factor CDX2 during blastocyst formation[29,110] and reported the induction of a transient pre-implantation/naive-like TSC state during the transdifferention of human PSCs to TSCs.[88,89,99,102] In support of this concept, both Guo et al. and Io et al. employed single-cell transcriptomic analysis to uncover transcriptional parallels reminiscent of pre-implantation trophoblast cells during the initial phases of differentiation.[88,89] By tracing the trajectory of these cells during differentiation and juxtaposing them with cynomolgus monkey trophoblasts, Io et al. illuminated a sequence where *HAND1*, *GATA2*, *GATA3*, *TBX3*, and *TEAD1* exhibited upregulation during the Epi-to-pre-implantation TE-like state transition.[89] Subsequently, *HAND1* experienced rapid downregulation, while *PEG3* and *PEG10* underwent upregulation as the pre-implantation TE-like cells transitioned toward CTs. This transition culminated in the complete acquisition of CT identity, marked by the upregulation of *DPP4*, *EGFR2*, and *ITGA2*.[89]

While transiently capturing pre-implantation TE-like cells during the ESC-TSC transdifferentiation, the existing culture conditions of human TSCs proved inadequate in maintaining this state, leading to the swift differentiation of cells toward post-implantation CTs.[88,89] Additionally, it remains to be determined whether such a population mirroring TE cells can be isolated from current TSC culture.

The pursuit of discovering culture conditions that enable the expansion and sustainability of pre-implantation/naive-like TSCs is currently underway. The identification of such optimal conditions holds profound implications for enhancing our comprehension of human implantation dynamics and the early stages of placental development.

THE POTENTIAL OF TOTIPOTENT STEM-LIKE CELLS TO CONTRIBUTE TO THE TROPHOBLAST LINEAGE

The concept of a single cell giving rise to an entire conceptus has captivated the imagination of many. The *in vitro* derivation and propagation of authentic totipotent cells hold the promise of unraveling new insights into the regulation of cell fate, encompassing the intricate interplay between extraembryonic and embryonic lineages, while also facilitating comprehensive manipulation of the complete conceptus. In mouse embryonic development, totipotency has been definitively established in the zygote and 2-cell stage.[111,112] Conversely, it is widely presumed that human embryos maintain totipotency until the 4–8 cell stage, although verifying this experimentally remains a challenge due to ethical constraints.[1]

Despite the confinement of totipotency to early blastomeres, which differ significantly in size and molecular characteristics from ESCs,[113] an important assertion was made by Macfarlan et al., in 2012. They identified a small subset within mouse ESC cultures that shared similarities with 2-cell-stage embryos.[114] This subset, termed 2-cell-like cells (2-CLCs), exhibited the expression of totipotency markers, including the activation of retrotransposon elements unique to early mammalian development and displayed potential to contribute to extraembryonic tissues.[114]

Following the initial discovery of 2-CLCs in mouse ESCs, subsequent studies have reported the successful induction or derivation of ESCs with "extended potential" (EPSCs) capable of contributing to extraembryonic tissues.[115,116] However, upon more rigorous scrutiny and stringent criteria described by Posafi and colleagues, it became evident that these studies lacked sufficient compelling evidence to substantiate their claims of contributing to extraembryonic lineages.[117]

In the context of human cells, recent studies have identified a transient population (8-CLCs) among naive-like human PSCs, expressing well-known markers of zygotic gene activation (ZGA), including *ZSCAN4*, *LEUTX*, and 8-cell stage-specific genes such as *HERVL* and *MLT2A1*.[118–120] These populations, transcriptionally resembling the 8-cell stage embryo, can be induced further by overexpression of the early embryonic transcription factor DUX4[120] or by epigenetic modulations and splicing inhibitors.[118–120] However, assessing the totipotent potential of 8-CLCs through *in vitro* experimentation is challenging due to the already existing unrestricted lineage potential of human naive-like PSCs[71,86,88,89] Furthermore, evaluating their *in vivo* potential is hindered by ethical considerations. Consequently, no meaningful experiment was conducted to explore their bi-directional potential in contributing to both embryonic and extraembryonic tissues.[121]

Subsequent to the establishment of totipotency criteria, a series of studies have asserted the induction of totipotency in murine ESCs through diverse combinations of small molecules that modulate pluripotency and totipotency. While one study emphasized the inhibition of spliceosomes as sufficient for driving ESCs to a totipotent stem-cell-like state,[122] others have asserted the activation of totipotency via distinct chemical cocktails. This includes manipulating the cell's epigenetic regulation by inhibiting histone demethylase (KDM5B), HDAC, or methyltransferase (G9A, DOT1L), as well as affecting crucial signaling pathways such as WNT.[123–125] These investigations effectively demonstrated cellular resemblance to early pre-implantation cells at transcriptome, epigenetic, or metabolome levels. However, it is vital to recognize that the true benchmark for totipotency transcends the transcriptome, methylome, or metabolome, lying within the inherent capacity of a single cell to contribute equally to both embryonic and extraembryonic tissues upon division.

TSC derivation from totipotent stem-like cells

Using the illustration of the extensive potential seen in human naive-like PSCs and their efficient conversion into TSCs,[88,89] one might anticipate that mouse totipotent cells could readily differentiate into TSCs upon transfer to a TSC-defined medium *in vitro*. However, despite effectively sustaining these totipotent stem-like cells, studies have not given sufficient emphasis to demonstrating the derivation of functional TSCs from these cells.

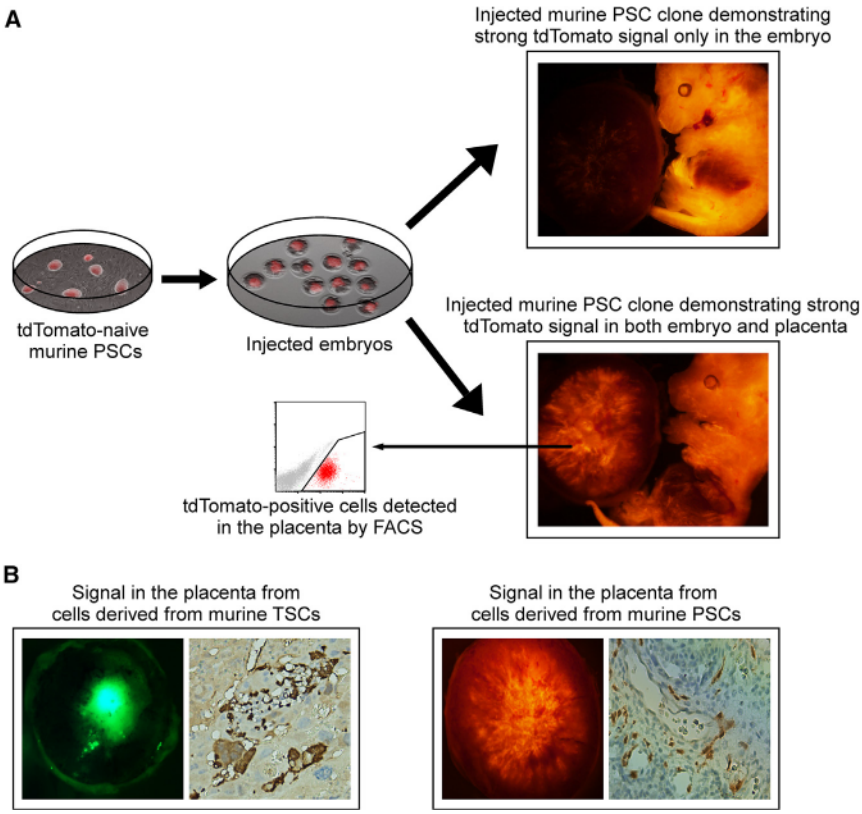

Figure 2. Naive murine pluripotent stem cell clones demonstrate strong signal in the placenta

Schematic illustration of the procedure undertaken and the phenotype observed in the placenta after injecting murine naive PSCs or TSCs into developing embryos

(A) Stably tdTomato-expressing murine naive pluripotent stem cells (PSCs) are microinjected into embryos at either the 8-cell or blastocyst stage and subsequently transferred to surrogate mothers. At 13.5 days post-coitum (dpc), embryos and placentas are dissected and examined under a fluorescent stereoscope. While some PSC clones display a strong tdTomato signal only in the embryo, others demonstrate a strong signal in the placenta as well.

(B) Representative images of placentas, displaying either a GFP signal, originating from embryos injected with GFP-labeled murine TSCs (left), or a tdTomato signal, originating from tdTomato-labeled murine naive PSCs (right). Immunohistochemistry is performed on sectioned placentas to detect GFP (left) and tdTomato (right) expression patterns, providing further insight into cell morphology and cellular localization. Figure was generated from the data described in Benchetrit et al.[73]

In Shen et al.'s work, there is no evidence of directly deriving TSCs from their totipotent stem-like cells,[122] while Xu et al. and Yang et al. report TSC derivation based on morphological observations and limited gene expressions.[124,125] These studies lack comprehensive evidence confirming the validity or functionality of the resultant cells as well as the efficacy of the differentiation process. Furthermore, Hu et al. only show the expression of key trophoblast markers after a few days in TSC culture, with no evidence supporting the capability of their totipotent stem-like cells to effectively differentiate into functional TSCs.[123]

Contribution to the trophoblast lineage following injection

Although the spatial distribution of cells within the early embryo (E3.5 and E7.5) can possibly provide insights into their forthcoming development, establishing the potential of totipotent stem-like cells to generate subsequent generations of placental cells has been a fundamental criterion for claims of totipotency. However, the placenta is a multifaceted organ composed of both trophoblast and embryo-derived cell types.[126] Furthermore, the placenta's rich metabolite content results in elevated autofluorescence levels.[4,127,128] Additionally, the scarcity of techniques available for extracting and culturing placenta cells during later embryo development further complicates the challenge of demonstrating the capability of these totipotent stem-like cells to yield functional placental trophoblast cells in subsequent stages of development.

Constrained by those limitations, the authors injected fluorescent-labeled totipotent stem-like cells into early stages embryos, tracked the cells' development, identified them in the placenta, and successfully confirmed the presence of numerous placental markers from different placental cell types using scRNA-seq analysis.[122–125] However, it has been demonstrated, through the use of fluorescence reporters, that both embryos and placentas derived from naive murine PSCs exhibit discernible signals under the microscope (Figures 2A and 2B).[73] Although these placental contributions likely originate from embryonic cells, such as endothelial cells of the vascular portion of the placenta originating from the extraembryonic mesoderm of the embryo,[126] a thorough examination needs to be conducted to exclude small and spontaneous contributions to the trophoblast lineage by naive PSCs.

Interestingly, among all those studies above, only Hu et al. demonstrated some contribution of murine ESCs to the placenta. Yet, they dismissed it as being of embryonic origin and did not conduct single-cell analyses to definitively assert the absence of trophoblast markers in a small number of cells. This essential evidence, while not aligning with previous findings,[129,130] remains essential to confirm that the observed contribution of totipotent stem-like cells or bi-directional cells truly arises from their totipotency potential and not merely from a broader potential of some PSC clones or culture conditions that mildly affect the epigenetic state of the cells, leading to sporadic contribution of a limited number of placental cells.

Furthermore, while it is universally accepted that PSCs do not have the potential to contribute to trophoblast tissues, it is worth noting that, in former days, several reports have hinted at some weak contribution of ESCs to trophoblast giant cells and parietal endoderm.[129,130] While subsequent research has not definitively validated those prior findings, it is important to note the limited contribution of totipotent stem-like cells to placental trophoblast cells, as demonstrated by the studies' scRNA-seq data from

more advanced embryonic stages. This stands in contrast to their substantial contribution to the embryo. Given this discrepancy, it becomes imperative to conduct comprehensive explorations into the potential of PSCs to sporadically generate placental trophoblast cells across different defined conditions.

Differentiation of trophoblast cells in teratomas

While exploring the placenta for evidence of totipotent potential has its inherent limitations as discussed above, the injection of ESCs under the skin of immunodeficient mice has emerged as a valuable strategy to replicate cellular differentiation potential.[13,131,132] In this context, the injection of totipotent stem-like cells in immunodeficient mice could serve as an additional rigorous test to demonstrate their potential in giving rise to both embryonic and trophoblast lineages. Surprisingly, this approach has been underutilized as a means to further characterize the potential of these totipotent-like cells. Among the recent articles discussing the induction of totipotent stem-like cells, the studies of Hu et al. and Xu et al. stand out as the most recent works that demonstrated such potential following injections into NOD-SCID mice.[123,125]

In their study, Xu et al., injected their totipotent stem-like cells into immunodeficiency mice and analyzed the teratomas using scRNA-seq. In addition to multiples embryonic lineages, Xu et al., revealed that the teratomas had signatures of extraembryonic lineages. It is worth mentioning that several of the genes examined as representative markers of extraembryonic ectoderm (such as *Ttr*, *Trh*, *Sox17*, and *Gsc*) are not exclusive to the placenta but are also shared with PrE derivatives.[133] Furthermore, the same cluster of extraembryonic ectoderm does not provide evidence for the expression of major trophoblast markers such as *Tfap2c*.

Hu et al. focus was confined to validating the presence of trophoblast giant cells and the three germ layers, overlooking other trophoblast subtypes such as STB and spongiotrophoblast.[123] While this oversight might be unintentional or attributed to an accelerated differentiation toward trophoblast giant cells, a pivotal aspect of comprehending the totipotency potential of these cells lies in discerning whether they can equally give rise to all trophoblast subtypes or if they show a specific inclination toward trophoblast giant cells.

While we did not delve into the molecular intricacies of the totipotent stem-like cells, the evidence presented in these studies undeniably validates the successful induction of specific totipotent characteristics in ESCs (see Table 1), greatly enhancing our comprehension of the molecular mechanisms underpinnings of totipotency. Nevertheless, the relatively modest contribution of these cells to the trophoblast cells of the placenta in comparison with their *in vivo* totipotent counterparts, coupled with the challenges in definitively confirming their complete potential, necessitates careful consideration when attributing them the title of totipotent cells with the ability to generate the entire spectrum of the TE lineage.

STEM-CELL-BASED EMBRYO-LIKE STRUCTURES: DOES THE TE LAYER REALLY REPRESENT AUTHENTIC TE?

The successful derivation and utilization of each blastocyst lineage as individual models have facilitated the exploration of cell fate decisions and differentiation mechanisms.[28] However, creating an *in vitro* model that encompasses the coordinated interactions of all three lineages and accurately replicates the blastocyst's structure and the early post-implantation process remains a persistent and long-standing challenge.

Mouse stem-cell-based embryo-like models

In 2017, the Magdalena Zernicka-Goetz group reported that the aggregation of mouse ESCs and TSCs within a 3D scaffold led to the development of a cylindrical structure reminiscent of a post-implantation embryo.[134] These structures successfully underwent critical morphogenetic events, including the formation of an amniotic cavity, the development of embryonic and placental tissue, and precise cellular regional specialization into mesoderm.[134] Later on, the same group further revealed that addition of PrE cells facilitated the formation of embryonic-trophoblast-extraembryonic endoderm (ETX) embryoids without reliance on exogenous extracellular matrix.[135] This alteration permitted enhanced development, providing a more accurate representation of the essential morphological events that transpire in post-implantation embryos.

In 2018, Rivron et al. reported that the aggregation of mouse ESCs and TSCs in microwells led to the formation of a structure termed blastoids resembling the E3.5 mouse blastocyst.[136] Although resembling a blastocyst at the structural level and undergoing implantation into the decidua, those blastoids failed to develop after implantation.[136] Since then, improved blastoids resembling the blastocyst more accurately were claimed to be successfully generated from the aggregation of TSCs with EPSCs[137] or by the aggregation of EPSCs alone.[138] However, despite the touted enhancements in blastoid development, they remained incapable of progressing beyond implantation. Notably, concerns about the blastoids generated exclusively from EPSCs were also raised due to their questionable trophoblast identity. An alternative study revealed that a minor fraction of the blastoid's TE population truly resembled trophoblast cells, while the dominant cell type within the blastoids exhibited a stronger association with a mesodermal identity, as indicated by the mesendodermal marker, T.[117]

One intriguing aspect lies in the contrast between the incapacity of blastoids to achieve adequate *in vivo* development and the recent reports demonstrating advanced *ex utero* development of stem-cell-based embryos.[139–141] Given the utilization of high-quality ESCs, which have successfully passed stringent tetraploid complementation assays in some of these studies, coupled with the *ex utero* development of equivalent stem-cell-based embryos facilitated by the diffusion of essential minerals and growth factors directly provided in the culture medium, it strongly suggests that the generation of proper and fully functional TE compartment remains elusive through these techniques.

The importance of a well-functioning and properly developed TE compartment in ensuring comprehensive fetal development is clearly evident in a recent study by Liao et al.[142] The authors identified irregularities in methylation levels and the loss of maternal imprinting, both within the TE compartment, and later in the term placenta, of somatic-cell-cloned rhesus monkeys. Notably, normal development and the birth of a live fetus achieved only through the strategic replacement of the cloned TE layer with a normal TE compartment.[142] This underscores

Table 1. Current approaches to validating extra-embryonic contribution capacity of totipotent stem-like cells

Article	Method for triggering totipotency	Potential to give rise to TSCs in vitro	Placenta contribution by control ESCs	Trophoblast markers used to detect contribution to the placenta	Trophoblasts subtypes identified in the placenta based on scRNA-seq	Teratoma formation and examinations	Techniques used to validate extraembryonic contribution
Shen et al., 2021[122]	Splicing inhibitor	Not reported	No contribution	TPBPA, PROLIFERIN	All of placenta subtypes	Not reported	Chimeric blastocysts, confocal immunofluorescence of E7.5 embryos, Immunohistochemistry analysis of E13.5 embryos and placentas, scRNA-seq of E.13.5 placentas
Yang et al., 2022[124]	KDM5B inhibitor, DOTL1 inhibitor and G9a inhibitor.	Yes, from blastoids structures. Primarily assessed morphology and a few TSC markers. No functional examination was conducted.	No contribution	TFAP2C, MCT4	All of placenta subtypes	Not reported	Chimeric blastocysts, confocal immunofluorescence of E7.5 embryos, Immunohistochemistry analysis of E13.5 placentas, scRNA-seq of E.13.5 placentas
Xu et al. 2022[125]	RARγ agonist, HDAC inhibitor, DOTL1 inhibitor and GSK-3 inhibitor	Yes, primarily assessed morphology and a few TSC markers. No functional examination was conducted.	No contribution	CK8, TFAP2C, MCT4	All of placenta subtypes	scRNA-seq of teratomas shows the presence of extraembryonic ectoderm. No reference to any specific placenta subtypes.	Confocal immunofluorescence of E7.5 embryos, Immunohistochemistry analysis of E10.5 embryos, teratoma formation, scRNA-seq of E.17.5 placentas
Hu et al., 2023[123]	Retinoic receptor agonist, GSK-3β inhibitor and IκB/IKK modulator	Reported the induction of a few trophoblast genes following transition into TSC medium.	Small contribution, mostly in the labyrinth area. No single-cell analysis was performed to assess the absence of trophoblast markers in the contributed cells.	CK8, PROLIFERIN	All of placenta subtypes	Reported the presence of trophoblast giant cells with no mention of other subtypes.	Teratoma formation, chimeric blastocysts, confocal immunofluorescence of E7.5 embryos, Immunohistochemistry analysis of E13.5 placentas, scRNA-seq of E.13.5 placentas.

the indispensable nature of a fully functioning TE compartment for the proper embryonic development. One could speculate that surpassing the existing constraints of blastoid development *in vivo* might necessitate the attainment of authentic pre-implantation TE cells capable of effective and appropriate uterine implantation, thereby facilitating regular placental growth.

The endeavor to generate a live embryo *in utero* and *ex utero* from stem-cell-based mouse embryos is actively underway. Successfully achieving either of these milestones would undoubtedly mark a significant breakthrough in both stem cell research and embryology. However, it is important to note that the true validation of TE lineage development lies in the successful in utero development of blastoids.

Human stem-cell-based embryo-like models

Unlike in mice, where the formation of appropriate blastoids requires the aggregation of at least ESCs and TSCs or the manipulation of ESCs to overexpress lineage inducers such as CDX2 for TE and GATA4 for PrE,[139,141] the aggregation of naive-like human PSCs alone, in combination with a cocktail of small molecules, has led to the creation of blastoids that closely mimic the morphology and gene expression profile of blastocysts.[143–145]

Surprisingly, the aggregation of both human naive-like PSCs and TSCs has been found to result in poorly formed blastoids, lacking the proper TE-like layer and featuring multiple cavities instead of one.[146] The capacity of naive-like human PSCs to independently generate blastoids, in contrast to naive mouse ESCs, could originate from the inherent unrestricted potential of human naive-like PSCs, as previously discussed. Alternatively, this discrepancy could be attributed to a lack of comprehensive understanding concerning the specific culture conditions necessary to fully unleash the unrestricted potential of mouse ESCs. In contrast to the aforementioned claim, it is intriguing to note that while both human and mouse TSCs represent a primed post-implantation state,[39,71,87] the successful amalgamation of ESCs and TSCs to create blastoids has been accomplished solely in the mouse model. Nevertheless, it is worth noting that blastocyst-like structures have been reported to emerge during OSKM reprogramming,[147] representing a subsequent observation of OSKM's capacity to generate human TSCs during reprogramming.[70] However, even in this scenario, criticism has been raised regarding the validity of the TE-like layer expressing an amniotic ectoderm signature similar to that observed in gastrulating human embryos.

A critical concern when evaluating the resemblance of human blastoids to blastocysts lies in the challenge of distinguishing between trophoblast and amnion tissue. Amnion tissues share several common markers with villous CTs, including *GATA3*, *GATA2*, *TP63*, and *KRT18*,[148,149] which are often utilized to identify TE. Furthermore, the distinction between human trophoblasts and amnion remains relatively underexplored,[150] underscoring the significance of establishing valid markers to effectively differentiate between these two cell types. Recently, Zheng et al. highlighted the constraints of current transcriptome comparisons relying on scRNA-seq data. They proposed that, in upcoming research, it is essential to provide explicit validation of the expression of key cell fate markers to establish TE identity conclusively. Importantly, Zheng et al. identified that markers such as *ISL1*, *GCM1*, and *HAVCR1* effectively distinguished between trophoblast-like cells and amnion-like cells, while markers like *GATA3*, *TFAP2A*, and *TFAP2C* did not exhibit the same discriminatory ability.[151]

Notwithstanding the importance of gene expression, relying solely on morphology and gene expression is insufficient to ascertain the functional capability of the TE compartment in blastoids. While ethical limitations hinder *in vivo* experimentation, Kagawa et al. have demonstrated that blastoids derived from human naive-like PSCs possess the ability to attach to a 2D layer of endometrial cells *in vitro* and interact with receptive endometrial cells.[144] However, while this functional assay of the trophoblast compartment is essential, it is crucial to include a direct comparison with a genuine blastocyst for a comprehensive assessment. Furthermore, the capacity to attach to the endometrium encompasses only one aspect of the TE compartment, primarily mediated by a specific subtype of trophoblast cells.[110]

Recently, Yu et al. and Karvas et al. achieved notable advancements in enhancing the generation of human blastoids from human naive-like PSCs.[152,153] Intriguingly, although both studies produced blastoids capable of modeling the late blastocyst stage (E6–E7), Yu et al. demonstrated that their blastoids could adhere to immortalized primary endometrial stromal cells, resulting in trophoblast outgrowth and trophoblast syncytialization similar to blastocysts.[153] Conversely, Karvas et al. presented culture conditions on a 3D extracellular matrix that allowed the development of blastoids until gastrulation stages, enabling the study of extraembryonic lineage development. In line with this, the authors showed that 3D culture of blastoids initiated embryonic germ layers' induction, accompanied by continuous expansion and differentiation of the trophoblast compartment toward trophoblast progenitors such as CTs, EVTs, and STBs.[152] Through trajectory analysis, Karvas et al. proposed a model wherein early STBs originate from polar TE cells during the implantation window, while EVTs and late STBs originate from CTs.[152]

It is important to note that recently, bypassing the blastocyst-like stage, studies on human stem-cell-based embryos demonstrated that aggregating PSCs and directing a portion of them toward TSC and PrE fates through distinctive culture conditions or overexpression of specific transcription factors resulted in the formation of embryo-like structures. These structures have the capability to partially replicate post-implantation processes and give rise to developmental pathways leading to the formation of amnion and primordial germ-like cells.[154,155]

While such models present exciting opportunities for studying early human post-implantation development, ISSCR guidelines limit the culture of human embryos to the onset of gastrulation (day 14 post-fertilization), raising serious ethical dilemmas regarding the utilization of embryo models for advanced developmental stages.[156] However, it is important to emphasize that existing embryo models do not accurately represent the full complexity of human embryos.[157,158] Therefore, as proposed by Rivron et al.,[158] a more refined definition of an embryo is essential to precisely determine when an embryo model should be subject to the same regulations as human embryos.

With these advancements in the creation of stem-cell-based embryo-like models, further research utilizing advanced 3D *in vitro* techniques and conducting thorough comparisons among blastoids, blastocysts, and early post-implantation stages will be imperative to gain a comprehensive understanding of the capability

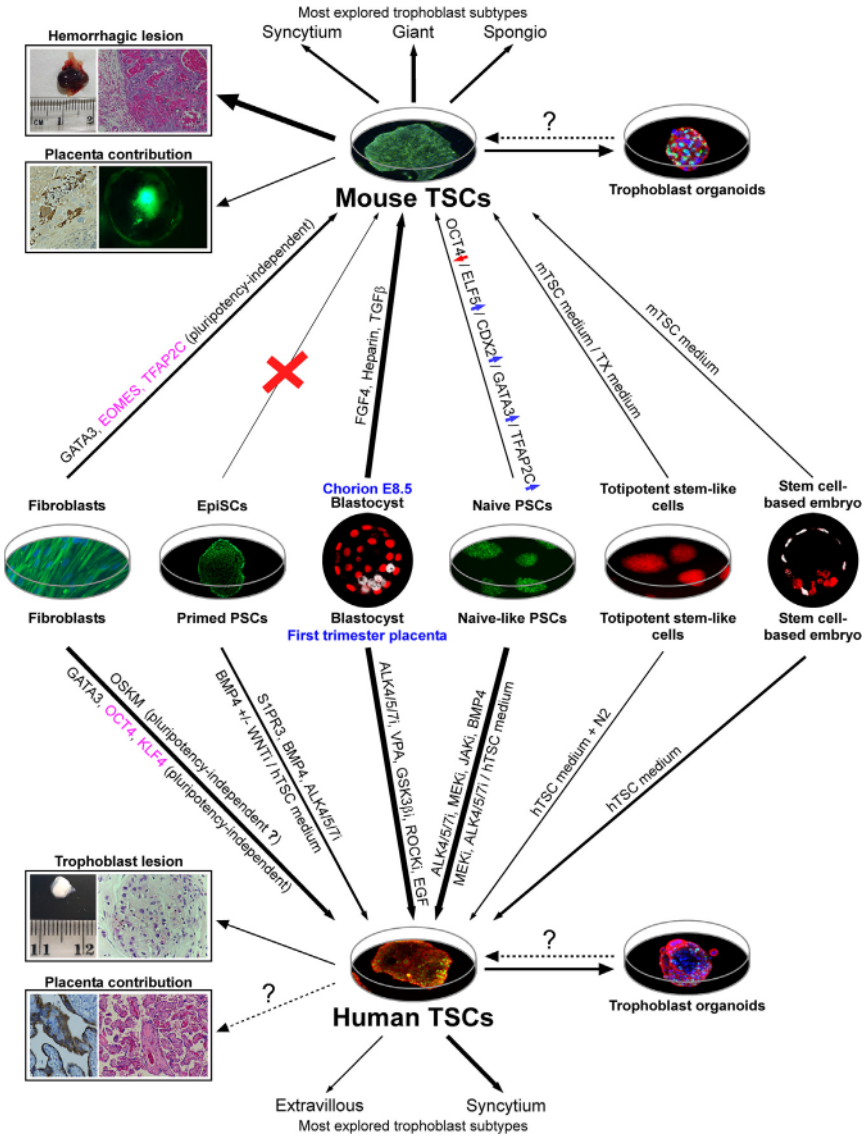

Figure 3. Distinct strategies resulting in the establishment of mouse and human TSC state *in vitro*

A schematic illustration depicting the diverse technologies and methodologies utilized to establish the TSC state in mice (top) and humans (bottom). Line width represents process efficiency, while dashed lines denote unexamined, unreported, or unknown aspects. Figure was partially generated from the data described in Benchetrit et al.,[67] Benchetrit et al.,[73] and Naama et al.[75]

of stem-cell-derived embryo-like models in accurately generating an authentic and functional pre-implantation TE compartment.

TSCs IN DISEASE MODELING

Human early placentation studies remain in their nascent stages, primarily due to limited access to human post-implantation embryonic stages and the lack of robust *in vitro* models, thus heavily relying on mouse research. Nonetheless, disparities between human and mouse placentation underscore the need for improved models. The recent advancements in producing human TSCs from various pluripotent sources, nuclear reprogramming, and stem-cell-based embryo-like models are revolutionizing our ability to model implantation and early pregnancy disorders. The successful generation of blastoids from PSCs, capable of differentiating into various trophoblast progenitors,[152,153] is enabling a more precise characterization of developmental events in the TE compartment and enhancing our comprehension of the crucial uterine/blastocyst crosstalk essential for implantation.

Furthermore, complications such as preeclampsia, often associated with early implantation even though it manifests itself in the later stages of pregnancy,[159] become evident at advanced stages when the early trophoblast progenitors are no longer present for isolation. This poses a significant challenge in terms of modeling such diseases.

The re-creation of human TSCs from iPSCs derived from individuals affected by placental insufficiency disorders or by directly reprogramming affected cells into iTSCs could play a pivotal role in modeling aberrant trophoblast behavior associated with conditions like preeclampsia.[75]

These recent advancements, albeit still in their infancy, mark significant strides toward unraveling fertility issues, early pregnancy loss, and late pregnancy disorders associated with early TE.

CONCLUDING REMARKS

Investigating TE state specification through single-cell transcriptomic data has shed light on the molecular intricacies of early cell fate segregation in both mice and humans. This exploration deepens our grasp of cell fate decisions, gene regulation, lineage commitment, and lineage-specific markers.

The activation and inactivation of the Hippo pathway in outer and inner cells, responding to mechanical forces and intracellular polarity, stand as pivotal for ICM-TE fate separation. These mechanisms largely span mammalian species, underscoring the universality of early embryonic development processes.

Advancements in TSC derivation, including transdifferentiation of PSCs, direct conversion from fibroblasts, and totipotency-like state induction, along with stem-cell-based embryo-like models (Figure 3), provide insights into the interplay between pluripotency and TSC states, elucidating species-specific nuances. Despite these strides, challenges persist in differentiating trophoblast from amnion tissues and in capturing the full range of TE lineage functionality.

The successful derivation of TSCs from pluripotent sources and fibroblasts via reprogramming has revolutionized our comprehension of these cells' molecular underpinnings.

Species-specific signaling pathways, key TSC factors, and maintenance mechanisms gain prominence. Addressing obstacles in accessing post-implantation embryonic stages and establishing robust *in vitro* models is pivotal. Notable breakthroughs like TSC-derived trophoblast organoids[160–162] and stem-cell-based embryo-like models offer promise in modeling early placental development and pathogen susceptibility. Further exploration of uterine/placental crosstalk using these models will decode vital maternal-fetal interactions for successful pregnancies.

In conclusion, the joint exploration of TE specification and TSC induction mechanisms defines the intricate cellular fate landscape. These insights not only enrich our understanding of early developmental processes but also hold the key to unlocking the mysteries of achieving accurate and fully functional TSC identity. Advancing these technologies holds potential across diverse applications, from deciphering early embryogenesis mechanisms to modeling pregnancy-related disorders.

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DECLARATION OF INTERESTS

Prof. Buganim holds a patent regarding the production of human TSCs with GOKM factors.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

During the preparation of this work, the authors used ChatGPT in order to improve language and readability. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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