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# The Underdeveloped Innate Immunity in Embryonic Stem Cells: The Molecular Basis and Biological Perspectives from Early Embryogenesis

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## Abstract

Embryonic stem cells (ESCs) have been intensively studied as a promising cell source for regenerative medicine. The rapid advancements in the field have not only proven the feasibility of ESC-based cell therapy, but also led to a better understanding of pluripotent stem cells (PSCs) as a unique cell population at an early stage of embryogenesis. Recent studies have revealed that both human and mouse ESCs have attenuated innate immune responses to infectious agents and inflammatory cytokines. These findings raise interesting questions about the rationale for ESCs, the PSCs experimentally derived from preimplantation stage embryos, to not have an innate defense mechanism that has been adapted so well in somatic cells. All somatic cells have innate immune systems that can be activated by pathogen associated molecular patterns (PAMPs) or cellular damage-associated molecular patterns (DAMPs), leading to production of cytokines. The underdeveloped innate immunity represents a unique property of PSCs that may have important implications. This review discusses the immunological properties of PSCs, the molecular basis underlying their diminished innate immune responses, and the hypothesis that the attenuated innate immune responses could be an adaptive mechanism that allows PSCs to avoid cytotoxicity associated with inflammation and immune responses during early embryogenesis.

### Keywords

embryonic stem cells; innate immunity; embryogenesis; interferons; inflammatory cytokines

## 1. Introduction

Immune reaction and inflammation are prominent events constituting a complex immunological condition that can dynamically affect the different stages of pregnancy.[1] Throughout the process of pregnancy, there must be mechanisms to orchestrate the interaction between the embryo and the maternal immune system. Disturbance of the immunological balance in the uterus by microbial infection and sterile inflammation induced by non-infectious cellular components can lead to various pregnancy complications.[2–4] In

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CONFLICT OF INTEREST

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particular, implantation of an early embryo to the uterus represents the most critical event for the initiation of pregnancy, but it also elicits immune and inflammatory responses at the maternal–fetal interface. It is estimated that about 30% of naturally fertilized eggs do not successfully implant.[5] While genetic abnormalities of the embryo are the major cause of implantation failure, dysregulated immunological and inflammatory responses are also important contributing factors.[6] Currently, we have limited knowledge about the immunological properties of embryonic cells at the blastocyst stage and how they respond to the immunological and inflammatory stimuli under physiological and pathological conditions.

The blastocyst is the structure developed from a fertilized egg before implantation (embryonic days 3.5-4.5 [E3.5-4.5] in mice and E5-6 in humans). The early blastocyst consists of two major components: the inner cell mass (ICM) and the trophectoderm, which give rise to the embryo and placenta, respectively.[7] Cells in the blastocyst have a dedicated task of rapid cell proliferation and differentiation for embryogenesis, but they may encounter high concentrations of inflammatory cytokines in the reproductive tract and uterus that are known to negatively affect the viability and proliferation of somatic tissue cells. How embryonic cells in a blastocyst deal with inflammatory and infectious challenges during implantation is an important yet poorly understood question, especially in humans due to the lack of experimental data. The recent in vitro studies of embryonic stem cells (ESCs), the pluripotent stem cells (PSCs) experimentally derived from the ICM, and trophoblast stem cells (TSCs), the multipotent stem cells that give rise to different placental cell lineages, have provided important insights into this fundamental question in developmental and reproductive biology.

The recent intensive research on ESCs is primarily driven by our interest in using these cells for regenerative medicine.[8,9] Successful derivation of various cell types from ESCs has now demonstrated the principle and feasibility of their therapeutic application; however, recent studies of both mESCs and hESCs,[10–16] including a series of studies from our laboratory,[17–21] have revealed that they have attenuated innate immune responses to bacterial and viral pathogens and inflammatory cytokines.[22] This may represent an intrinsic property of all types of PSCs since similar observations were also made in induced pluripotent stem cells (iPSCs).[14,15] This finding challenges the concept of innate immunity, an evolutionarily conserved defense mechanism that is presumably developed in most, if not all, cell types.[23] This review discusses the immune properties of ESCs, the molecular basis for their underdeveloped innate immune system, and the physiological relevance of the findings derived from in vitro cultured ESCs to in vivo PSCs residing in early embryos. It will also discuss the hypothesis that attenuated immune responses could be an adaptive mechanism that allows PSCs to avoid negative impacts from immunological and inflammatory challenges that these cells may encounter during early embryogenesis at the blastocyst stage.

## 2. The innate immune system and its development in early embryonic cells

The vertebrate immune system consists of innate and adaptive immunity. The innate immunity responds to a broad range of pathogens and provides the first line of defense via mechanisms including inflammation and innate immune cell response.[23,24] The innate immune system includes all forms of nonspecific defense mechanisms, but antiviral, antibacterial, and inflammatory responses carried out by innate immune cells and tissue cells play the central roles. The adaptive immunity utilizes highly specialized cells, including T cells and B cells, to provide the organism with the ability to recognize and eliminate specific pathogens.

At the cellular level, innate immune and inflammatory responses can be elicited by molecules derived from microbial pathogens (known as Pathogen Associated Molecular Patterns, PAMPs) or by non-infectious cellular molecules (known as Damage-Associated Molecular Patterns, DAMPs).[25] Inflammatory responses induced by DAMPs are termed as sterile inflammation since they take place in the absence of microbial pathogens. PAMPs and DAMPs are detected by their specific receptors known as pattern recognition receptors (PRRs). Toll-like receptors (TLRs) are membrane-bound PRRs that recognize a wide variety of pathogenic agents.[24] Cytoplasmic PRRs include retinoic acid–inducible gene I (RIG-I)–like receptors (RLRs) and nucleotide-binding oligomerization domain-like receptors (NLRs). RLRs, such as RIG-I and MDA5, play key roles in detecting viral RNA.[26] NLRs are intracellular sensors of certain PAMPs and DAMPs. Some NLRs are involved in the formation of inflammasomes that are critical for the clearance of some pathogens and damaged cellular components associated with sterile inflammation.[27] Upon binding with PAMPs or DAMPs, PRRs activate transcription factors, including IFN-regulatory factors (IRFs), AP-1, and nuclear factor κB (NFκB), leading to the expression of IFNs and other inflammatory cytokines that participate in different aspects of immune and inflammatory responses.[24,26]

The signaling mechanisms that mediate innate immune responses have been extensively studied in somatic cells, including placental cells.[3] For example, TLR3 and TLR4, which mediate antiviral and antibacterial responses, are functional in first trimester human trophoblasts.[28–30] Mouse embryonic fibroblasts isolated from E12 embryos are able to mount antiviral and antibacterial responses and express most TLRs,[31,32] indicating that the innate immune system is functional at this stage, at least in fibroblasts. While the development of the innate immune system in other embryonic cell lineages at earlier stages is not clear, one would assume that it should be functional based on the perception of innate immunity as a cognate defense mechanism. However, mTSCs isolated from the trophectoderm of mouse pre-implantation blastocysts (E3.5), but not mESCs derived from the ICM, can express IFNβ in response to viral stimuli,[33] indicating that the immunological properties of mTSCs and mESCs could be different. The effort to obtain hTSCs has not been successful until their recent derivation from first trimester cytotrophoblast cells,[34,35] and their innate immunity has not been investigated. While we have limited knowledge about the immunological properties of TSCs, in vitro studies of ESCs clearly demonstrate that their

innate immunity is not “innate,” rather, it is acquired by somatic cells and matured during differentiation as demonstrated by the evidence from in vitro ESC differentiation studies and from developmental studies.[22,36–38]

## 3. Underdeveloped innate immunity is a common feature of PSCs

The IFN system has evolved as a major innate antiviral mechanism in vertebrates.[23,39] In response to viral infection, cells rapidly synthesize and secrete IFNs. Through autocrine and paracrine mechanisms, IFNs bind to the cell surface receptor complex, which triggers the activation of the Janus tyrosine kinases-activator of transcription (JAK-STAT) pathway and induces the expression of numerous IFN-stimulated genes (ISGs) that promote the cell to enter an “antiviral state.” Therefore, the IFN system includes the capacity to both produce and respond to IFNs.[23,39] The IFN system in ESCs is underdeveloped. In particular, both mESCs and hESCs do not express IFNα and IFNβ in response to viral infection or synthetic viral RNA analogs.[14,17,18,33,40] However, the IFN response mechanism in ESCs from the two species differ to a certain degree. mESCs have an attenuated but detectable responsiveness to IFNα and IFNβ. Our recent studies[19,40] and two earlier studies[41,42] in mESCs demonstrated that exogenous IFNα and IFNβ can induce the expression of several ISGs, protect mESCs from viral infection, and repress replication of several types of viruses, but the levels of response to the two cytokines in mESCs are substantially weaker than differentiated mouse fibroblasts.[19,40] In contrast, hESCs and hiPSCs barely respond to IFNβ as judged by their lack of ISG expression.[43] However, IFNβ slightly inhibited infection of hESCs by Coxsackievirus, indicating that the IFN responding pathway in hESCs is not completely inactive.[44] In addition to inducing IFN response, viral RNA can directly activate double stranded (ds) RNA-activated protein kinase (PKR) and cause inhibition of both cellular and viral protein synthesis, thereby repressing viral replication as a separate antiviral mechanism.[45] hESCs and mESCs exhibit notable differences in the activation of the PKR pathway. While PKR is unresponsive to dsRNA in hESCs,[14] it can be activated by both poly IC (polyinosinic:polycytidylic acid, a synthetic dsRNA) and La Crosse virus infection, or by cellular dsRNA in mESCs.[18,46]

Similarly with viral infection, mESCs are susceptible to cytopathic effects of bacterial infection, but they do not mount antibacterial and inflammatory responses like those typically seen in differentiated cells.[10,11] In line with this finding, we recently demonstrated that both mESCs and hESCs do not respond to lipopolysaccharide (LPS), a bacterial endotoxin that mimics bacterial infection in eliciting the expression of inflammatory molecules. They also fail to respond to inflammatory cytokines, such as TNFα.[20] The lack of response to these agents are also reported by other investigators in mESCs[12,13] and in miPSCs.[15] Another study suggested that hESCs did not respond to LPS, like mESCs, but responded to TNFα, in contrast to mESCs.[16] TLR4, the conventional receptor for LPS, is not expressed in mESCs, but LPS at a very high concentration (10 μg/ml) may elicit response in mESCs through TLR2.[47] Despite some differences reported among different studies and the discrepancies noted between mESCs and hESCs, current data strongly suggest that the underdeveloped innate immune system is a common feature of PSCs, including iPSCs since they display similar immunological properties to ESCs,[14,15] as summarized in Table 1. It is particularly interesting to note that mouse fibroblasts lost their

antiviral response after they were reprogrammed to miPSCs, i.e. defective in IFN expression and in the major TLR signaling pathways.[15]

hESCs and mESCs share fundamental similarities in pluripotency and self-renewal, but they display a number of species-specific differences.[48] For instance, leukemia inhibitory factor is essential for the maintenance of pluripotency in mESCs but not for hESCs.[49,50] mESCs, derived from pre-implantation blastocysts, represent PSCs in a "naïve" state, whereas hESCs, derived from blastocysts produced by in vitro fertilization, are more differentiated and are considered to be "primed" PSCs (will be further discussed in section 8).[51] Currently, there is no evidence indicating that these differences are responsible for the discrepancies in the immunological properties noted in mESCs and hESCs. The attenuated response to IFNβ in hESCs and hiPSCs appears to be due to the constitutively expressed high levels of suppressor of cytokine signaling 1 (SOCS1) that negatively regulate the IFN signaling pathway.[43] However, the difference in PKR activation between hESCs and mESCs might be due to the different abundancies of cellular transcripts with dsRNA structures related to repetitive DNA elements in human and mouse cells.[52,53]

## 4. The molecular basis underlying the underdeveloped innate immunity in ESCs

The molecular basis for the underdeveloped innate immunity in ESCs is not completely understood, but the current data suggest that the deficiencies are at multiple levels. The receptors for viral RNA (TLR3, RIG-I, and MDA5), LPS (TLR4), and TNFα (TNFR1) are expressed at low levels or are not functional in hESCs or mESCs.[12–14,17,18,20] In mESCs, the mRNA of TLR4 and TNFR1 were not detected due to DNA hyper-methylation of their promoters and histone hypo-acetylation.[12,13] The inactive status of NFκB further explains the failure of ESCs to express IFNs and inflammatory cytokines and their lack of response to LPS and TNFα.

NFκB is a transcription factor essential in mediating immune and inflammatory responses.[54] Viral RNA, LPS, and TNFα are among the most potent stimuli that activate NFκB in differentiated cells, but they all fail to do so in hESCs, mESCs, and miPSCs.[15,19,20,40,55,56] Although there is an indication that NFκB might not be completely inactive in hESCs or hiPSCs,[16,57,58] the findings from most studies demonstrated that NFκB is kept in an inactive state that cannot be turned on by immunological and inflammatory stimuli. In both mESCs and hESCs, RelA (p65) and p50, the subunits of the NFκB transcription factor, are expressed at relatively low levels but are upregulated upon differentiation.[55,56] Nanog, one of the key pluripotency markers, can directly bind to and inhibit NFκB transcriptional activity in mESCs.[59] Furthermore, the mRNA of RelA is silenced in mESCs by the miR-290 cluster, a class of ESC-specific miRNA.[60] Therefore, the activity of NFκB is repressed at both the protein and mRNA level. Blocking the activity of Nanog and miR-290 resulted in NFκB activation, concurrent with the onset of mESC differentiation.[60] Together, these findings point to a unique model of NFκB activation in ESCs where NFκB acts as a molecular switch that controls innate immunity response and differentiation. It is important to point out that the NFκB pathway is also critical for other developmental processes during

embryogenesis, as demonstrated by the fact that RelA knockout mice die after 15–16 days of gestation.[61] Thus, NFκB activity is essential for differentiation and survival of differentiated cells but not for the viability of cells in a pre-implantation blastocyst. This conclusion is in line with the transition of NFκB from the inactive state to the active state observed during in vitro ESC differentiation.

## 5. Innate immunity is not innate to ESCs but is acquired by somatic cells during differentiation

If innate immunity is not (or at least not completely) "innate" to ESCs or to their in vivo counterpart PSCs, then it must be developmentally acquired by somatic cells. Fibroblasts are highly responsive to various immune stimuli and are a major cell type responsible for maintaining tissue immunity.[62,63] Using mESC-differentiated fibroblast-like cells (mESC-FBs) as a model system, we investigated innate immunity development during in vitro differentiation and demonstrated that mESC-FBs can acquire the ability to express IFNα and IFNβ and to respond to TNFα during differentiation.[19,40] Studies from other investigators have reported similar findings in several types of tissue cells differentiated from both hESCs and mESCs, including endothelial cells, smooth muscle cells, cardiomyocytes, and osteoblasts.[12,13,16,64,65] These studies proved the concept that innate immunity in somatic cells is acquired during organismal development.

It is noted that although mESC-EBs have gained the ability to respond to viral stimuli and inflammatory cytokines, their levels of responsiveness are substantially lower than their naturally differentiated counterparts but can be further developed along with their continued in vitro propagation.[19,40] At the molecular level, cytokine receptors and PRRs, such as TNFR1, TLR3, and MDA5, are upregulated in ESC-differentiated cells.[13,20,40] A defining feature after differentiation is the transition of NFκB from the inactive state in mESCs to the active state in mESC-FBs in response to viral infection and TNFα. This is elegantly demonstrated with a co-culture model in which mESCs and mESC-FBs are cultured in the same dish and subjected to identical experimental conditions.[19,20,40] However, mESC-FBs, mESC-differentiated smooth muscle cells, and hESC-differentiated endothelial cells still do not respond to LPS.[12,16,20,21] This is, at least partly, due to the lack of expression of a functional TLR4 at the protein level.[12,16,20] Consequently, LPS was unable to activate NFκB and failed to induce inflammatory genes. Apparently, in vitro differentiation can initiate but not effectively promote the development of innate immunity.

## 6. The underdeveloped innate immunity in PSCs – biological perspectives from early embryogenesis

Our studies of innate immunity in ESC-differentiated cells were originally intended to determine how their attenuated innate immune responses may affect their application in regenerative medicine. However, subsequent findings in ESCs have inspired us to ask basic biological questions about the physiological implications of their underdeveloped innate immunity. In particular, it reignited a highly controversial subject about RNA interference (RNAi), a well-known antiviral mechanism in plants and invertebrates that doesn't seem to

exist in vertebrates.[66] Recent studies suggest that RNAi may operate in mESCs and tissue cells at an early developmental stage,[67,68] which has led to the hypothesis that mammals may have adapted distinct antiviral mechanisms at different stages of organismal development: the IFN system is utilized by differentiated somatic cells, whereas RNAi may be used in PSCs as an alternative antiviral mechanism to the IFN system.[69] Although plausible, this hypothesis remains hotly debated.[70,71] Furthermore, it was recently reported that both mESCs and hESCs constitutively express a subset of ISGs (IFN-induced transmembrane proteins, IFITM). Unlike other ISGs that depend on induction by IFNs in differentiated cells, preexisting IFITM proteins in ESCs effectively protect ESCs against viral infection.[72,73] These findings strongly suggest the existence of alternative antiviral mechanisms in ESCs. While the question of why ESCs choose not to have the conventional IFN-based antiviral system and antibacterial mechanisms that are so well adapted by differentiated somatic cells could be speculated from different views, it could be better understood from the perspectives of reproductive immunology and embryogenesis.

Theoretically, the underdeveloped innate immune defense mechanism might put PSCs at risk of being infected, but a pre-implantation embryo is enclosed in a zona pellucida (ZP), which provides a physical barrier to microbial pathogens. Together with the protection by maternal immunity and the potential alternative antiviral/bacterial mechanisms yet to be further investigated in PSCs (such as RNAi and IFITM), pathogenic infection may not pose a major threat to PSCs in a pre-implantation blastocyst. However, immunological and inflammatory responses, the dynamic events that take place during and shortly after implantation, could have significant impacts on the embryo. Immune and inflammatory responses have been viewed as a double-edged sword. On one hand, they defend the organism against pathogens; on the other hand, they can cause collateral damage to tissue cells through cell cycle inhibition and even cell death.[45,74] Generally, IFNs and inflammatory cytokines are primarily produced for the purpose of defense and are known to negatively impact cell proliferation and viability.[75] These negative effects could be tolerated in a tissue of a developed organism, but the consequences could be detrimental to PSCs in an early embryo where rapid cell proliferation is their dedicated task essential for embryogenesis. From this perspective, it would be beneficial for PSCs not to produce and respond to inflammatory cytokines when the blastocyst is exposed to high concentrations of inflammatory cytokines and increased populations of immune cells resulting from the implantation process or from maternal systemic or intrauterine infection.[76,77] The attenuated inflammatory cytokine production and response could serve as an adaptive mechanism that not only allows PSCs to avoid the cytotoxicity of inflammatory cytokines, but also to prevent further aggravation of immune and inflammation reactions associated with the implantation process. Therefore, the diminished innate immune responses could help PSCs maintain the rapid rate of cell proliferation essential for early embryo development.

## 7. ESCs and ESC-FBs with attenuated innate immunity are less vulnerable to the cytotoxicity of inflammatory cytokines

Our recent studies demonstrated that mESCs and mESC-FBs are indeed less vulnerable than their fully differentiated counterparts to the cytotoxicity associated with inflammatory

responses. TNFα and IFNγ are two of the best-characterized inflammatory cytokines. Their excessive production can lead to deleterious immunologic consequences, including pregnancy complications.[77] The cytotoxic effect of TNFα is mainly associated with its apoptosis-inducing activity.[78] TNFα alone did not cause detectable toxicity in mESCs and mESC-FBs.[40] However, in the presence of transcription inhibitor actinomycin D that is known to potentiate TNFα toxicity,[79,80] TNFα caused cell death of mESC-FBs (which have gained the ability to respond to TNFα) but not mESCs that do not respond to TNFα. Furthermore, infection with Chikungunya virus made mESC-FBs more sensitive to TNFα cytotoxicity than mESCs.[40] This study suggests that the lack of response to TNFα enables mESCs to avoid TNFα cytotoxicity. IFNγ, a type II IFN mainly secreted by T cells and NK cells, is known to inhibit cell proliferation and induce apoptosis of tissue cells.[81] It has been reported that TNFα and IFNγ synergistically potentiate each other's cytotoxicity.[82] Like TNFα, IFNγ alone does not cause apparent toxicity in either mESCs or mESC-FBs, but the combination of the two cytokines causes cell cycle inhibition in mESC-FB and not in mESCs (unpublished data). Therefore, mESCs are insensitive to the cytotoxic effects of TNFα and IFNγ that otherwise significantly reduce the viability and proliferation of differentiated tissue cells.

LPS-induced inflammatory responses, resembling certain aspects of bacterial infection, can cause serious cell and tissue damage. Its cytotoxicity can be further potentiated by IFNγ.[83,84] As previously mentioned, mESC-FBs have gained limited responsiveness to poly IC and TNFα, but they are still insensitive to LPS like mESCs.[19,40] In a recent study, we compared the effect of LPS on mESC-FBs to mouse bone marrow mesenchymal stem cells (BM-MSCs), which have a functional TLR4 signaling pathway that mediates LPS effects. LPS alone did not affect the viability of either cell type within a period of 4 day treatment; however, in the presence of IFNγ, LPS caused apparent cell death of BM-MSCs but not mESC-FBs.[21] The relative sensitivity of BM-MSCs and mESC-FBs to an inflammatory environment was further determined by an in vitro macrophage-induced inflammation model in which macrophages were stimulated with LPS. The conditioned medium collected from LPS-stimulated macrophages (LPS-CM), which contains various inflammatory cytokines,[85] caused massive cell death of BM-MSCs, but only moderately reduced viability of mESC-FBs.[21] All inflammatory stimuli that caused the cell death of BM-MSCs or mESC-FBs, including LPS/IFNγ, LPS-CM, and heat-killed bacteria, had no apparent effect on the viability of mESCs (unpublished data). These results suggest that the lack of response to LPS allows mESCs, and to a lesser extent mESC-FBs, to minimize the cytotoxicity of this endotoxin. Together with our previous studies with poly IC and TNFα,[19,40] the observations from this study reveals a pattern indicating that the cells' sensitivity to the cytotoxicity of inflammatory agents is correlated to the level of cellular response to these agents.

The negative effects of type I IFNs on cell proliferation and survival has been noted in somatic cells, especially in malignant cells and infected cells.[86] The reason for the lack of a functional IFN-based antiviral mechanism in hESCs can be postulated as a protective scenario similar to the case discussed for inflammatory cytokines since hESCs barely respond to IFNβ.[43] However, mESCs are partially responsive to IFNα or IFNβ and express ISGs.[19,41,42] This means that mESCs could benefit from the antiviral effects of IFNs secreted from trophoblasts via a paracrine mechanism, but how they manage to avoid the

potential adverse effects of IFNs, if there are any, is unclear. However, we did not see IFNα and IFNβ cause detectable negative effects on proliferation and self-renewal at a wide concentration range in mESCs.[18] One can speculate that IFN-induced low-level cellular responses in ESCs may limit excessive IFN action or that ESCs may have additional mechanisms to counterbalance the cytotoxicity of IFNs. Currently, there is no experimental evidence to prove or disprove these possibilities.

## 8. Existence of PSCs during early embryogenesis

The idea that the underdeveloped innate immunity in PSCs could be an adaptive mechanism to avoid the negative effects of immune and inflammatory responses is largely based on studies of in vitro cultured ESCs. Developmentally, however, PSCs only exist transiently during early embryogenesis. As such, the physiological relevance of the results obtained from ESCs will rest on the premise that PSCs with similar properties to ESCs indeed exist in the early embryo and that they could be subjected to immunological and inflammatory insults during the time of their existence. Prior to implantation, the fertilized egg undergoes a series of cell divisions and develops into a blastocyst. This process is similar in mice and humans, but the time needed for blastocyst formation in humans is longer than in mice (Fig. 1). The ICM in the blastocyst segregates into the epiblast (EPI) and the primitive endoderm layer. The blastocyst hatches out of the ZP (at E4.5 in mice and E5-6 in humans) and starts implantation by invading uterine endometrium. As the implanted blastocyst further develops, the late EPI cells give rise to all the tissues of the developing fetus in both mice and humans while the extraembryonic endoderm formation and placentation take place through different mechanisms in the two species (Fig. 1).[87,88]

mESCs were first isolated from preimplantation blastocysts (E3.5-4.5), which represent a naïve state of pluripotency of epiblast cells.[89] Cells with pluripotency similar to mESCs were isolated from the late EPI of post-implantation mouse embryos (E5.5-6.5) and named as epiblast stem cells (EpiSCs, Fig.1).[90,91] These EpiSCs represent more developed or “primed” PSCs. Therefore, PSCs exist in early mouse embryos at least up to 3-4 days, but data about whether or not they exist in the embryo beyond E6.5 is not available. hESCs were derived from pre-implantation blastocysts produced via in vitro fertilization.[92] The studies that have examined the properties of hESCs in culture suggest that their pluripotency is more similar to the primed state of mEpiSCs.[87] Since experimental data about post-implantation human embryos is lacking, our understanding of early human post-implantation development is primarily based on the studies of other organisms, especially non-human primates.[93] A recent study with cynomolgus monkeys demonstrates that the late EPI in post-implantation embryos (E13, E14, and E16) retains a stable expression profile of pluripotency-related genes, including Oct4 and Nanog. Comparative transcriptome analysis indicates that hESCs and hiPSCs show the highest similarity to post-implantation late EPI cells of the monkey embryo.[94] Based on the developmental correlation of non-human primate and mouse PSCs, current data suggest that hESCs in conventional culture are in a “primed state” (vs the native state of mESCs) and are likely to be developmentally equivalent to mEpiSCs.[51,93] Therefore, PSCs are present in pre-implantation blastocyst and are present up to about 12 days in the late post-implantation embryos (Fig.1) of cynomolgus monkeys, which are closely related to humans.

## 9. Potential infection of early embryos by microbial pathogens

The placenta acts as an effective physical and immunological barrier to microbial pathogens, but nevertheless, certain bacteria and viruses can still breach this barrier and may lead to pregnancy complications.[1,95,96] Experimental and clinical data about the susceptibility of human embryos at the blastocyst stage to microbial infection is lacking, but the findings from limited studies with animal models have provided valuable insights. One early study reported that infection of mice with murine cytomegalovirus (MCMV) 7 days before and 1 day after mating (around ovulation and implantation) led to retarded embryo development and a decreased implantation rate[97] However, the retarded embryos collected from the infected mice could develop normally when cultured in vitro. Therefore, the inflammation in the uterus caused by viral infection, rather than direct embryo infection, was likely the reason for the observed results.[97] In another study, preimplantation mouse embryos at the 4-8 cell stage were infected with Moloney murine leukemia virus (M-MuLV), cultured to the blastocyst stage, and then transferred to foster mothers. Of 29 transferred embryos, 15 developed into young mice (a 50% survival rate similar to uninfected embryos), but only one mouse developed lymphatic leukemia, suggesting that the infection frequency of preimplantation blastocyst with M-MuLV was rather low but nevertheless possible.[98]

Zika virus (ZIKV) has recently caused serious public health concerns due to its implication in causing microcephaly in newborns.[99] Using a mouse model with vaginal infection with ZIKV, a recent study demonstrated that when pregnant mice were infected at E4.5, the developing embryo examined at E18.5 showed mild but notable growth defects, correlating with the infection of neural progenitors in the brain.[100] When the same experiments were performed with IFN receptor 1 deficient mice, ZIKV infection was much more serious and led to the demise of the embryo. This study demonstrates the susceptibility of early embryos to ZIKV infection and highlights the importance of the IFN system in protecting against ZIKV infection. In a recent study, we demonstrated that congenital ZIKV infection of pregnant mice at E8.5 by intraperitoneal injection could lead to postnatal neurobehavioral deficits of ZIKV-infected newborn mice. The head tissue of ZIKV-infected fetuses examined on E10.5 have reduced expression of the neural development- and microcephaly-related genes.[101] However, these in vivo studies could not reveal the time point when the virus had gained access to the embryo or the differentiation stage at which the cells were initially infected. Interestingly, a recent in vitro study reported that hESCs are susceptible to ZIKV infection.[102] Furthermore, ZIKV can infect hESC-derived trophoblasts (presumably similar to the cellular component of trophectoderm) with much higher efficiency than cytotrophoblasts and syncytiotrophoblasts derived from term placenta.[102] These findings suggest that both PSCs and the trophectoderm at the blastocyst stage can be targets of ZIKV. Whether or not these observations reflects what happen in vivo remains to be investigated.

It is interesting to note that in vitro culture of embryos has been used as a model to study embryonic development dating back to three decades ago.[103] Early studies have indicated that the susceptibility of preimplantation embryos to viral infection is dependent on types of viruses, age of the embryo, and the presence of ZP. For example, mouse morulae or blastocysts with ZP are not susceptible to Herpes Simplex Virus-1, Rubella virus, or Sendai virus, but they became sensitive to Rubella virus and Sendai virus when ZP was removed.

[104–106] In the case of Rubella virus, infected cells were mainly in the trophectoderm, not in the ICM cells.[105] Similarly, mouse embryos at the 4-8 cell stage or the blastocyst stage could be infected with Semliki Forest virus after ZP removal, leading to rapid virus production and cytolysis of the embryos.[105] However, Simian virus 40 can infect and rapidly kill mouse embryos at the two-cell, morula, or blastocyst stage even with an intact ZP. While Polyoma virus infection was not deleterious to blastocysts, the outgrowths of blastocysts disintegrated after infection, and viral proteins were only detected in trophoblast cells but not in ICM cells.[107] Therefore, ZP can be an effective barrier that prevents infection from some but not all viruses. Conceivably, hatched blastocysts could be particularly vulnerable to microbial infection. However, it is important to point out that protection provided by maternal immunity cannot be assessed by in vitro studies.

In mimicking bacterial infection at the time of conception, a mouse model was used in which LPS was administered to the mice. LPS caused upregulated expression of TNF, IFNγ, and TNF-related apoptosis-inducing ligand (TRAIL) in oviduct and uterine tissues.[108] Depending on concentrations and time of LPS administration, it caused failed pregnancy, impaired blastocyst development, or defective embryo development at late gestation.[108–110] However, LPS had no direct effect on in vitro cultured blastocysts at the dosages that caused embryo defects in mice.[108] Therefore, the effects of LPS observed in mice were most likely caused by the elevated levels of embryotoxic cytokines resulting from maternal systemic or intrauterine inflammation, rather than direct fetal sensitivity to LPS.[108,111] How the results from in vitro studies with ESCs reflect PSCs in the blastocyst and the likelihood that they may get infected in vivo remains to be further investigated. However, the inflammatory responses, either resulting from implantation and/or from microbial infection, likely have profound effects on embryonic cells.

## 10. The effects of embryotoxic cytokines on pre-implantation embryos

The pre-implantation blastocyst in the reproductive tract may not face as dramatic immunological and inflammatory challenges compared to an implanting blastocyst in the uterus. However, it experiences fluctuations in the composition of growth factors.[77] The pre-implantation blastocyst depends on “embryotrophic factors” for normal development and successful implantation; it is also subject to stringent scrutiny by “embryotoxic cytokines.” It is proposed that, by integrating information on environmental and embryonic parameters, embryotoxic cytokines act as a “quality control” system that help determine whether or not an embryo should progress to implantation and pregnancy.[77] Excessive embryotoxic cytokine production under certain situations, such as systemic or reproductive tract inflammation, infection, or metabolic disorders, could lead to implantation failure. This hypothesis is in part based on clinical studies demonstrating that embryotoxic cytokines, including TNFα, IFNγ, and TRAIL, are increased in the endometrium of women with implantation failure and miscarriage.[77] IFNγ is considered a contributing factor to recurrent spontaneous abortion in women and to in vitro fertilization failure.[112–114] In the case of TNFα, it is detected in oviductal and uterine tissues throughout the preimplantation period in women and is elevated in peritoneal fluid of patients with endometriosis.[115,116]

In vitro culture of early embryos ranging from the two-cell stage to pre-implantation blastocyst stage have been used to study embryo response to various immunological and inflammatory conditions. It was reported that secreted products from concanavalin A-activated lymphocytes and human sera from infertile patients could inhibit the growth of cultured blastocysts.[114,117] The embryotoxic effects could be replicated with IFNγ, which seemed to mainly target trophoblasts since trophectoderm growth, but not the ICM was affected.[114,118] However, studies with TNFα reported different observations, ranging from no effect to detrimental effect on embryo development.[117–125] The different results could be related to the wide range of TNFα concentrations (5-10000 ng/ml) and developmental stages of embryos used in these studies. In the studies that showed embryotoxic effect of TNFα, the most notable observation was reduced ICM size while the trophectoderm was less affected.[122–125]

Both in vivo and in vitro studies have demonstrated that embryotoxic cytokines at certain concentrations can impair embryo development, but the mechanisms involved at the molecular and cellular levels were poorly understood. Even in relatively simple in vitro embryo culture models, none of the above-mentioned studies investigated the specific effects of tested cytokines on ICM or trophectoderm. Nevertheless, in vitro embryo culture models can replicate certain aspects of embryo responses to environmental cues and capture some important developmental features that can help interpret the data obtained from in vitro studies of ESCs.

## 11. The reduced sensitivity of ESCs to embryotoxic cytokines – biological relevance to PSCs in vivo

The insensitivity of cultured blastocysts to LPS toxicity and the tolerance of ICM in a blastocyst to IFNγ cytotoxicity [108,111,114,118] are in line with the refractory nature of ESCs to inflammatory insults (discussed in section 7). However, the effect of TNFα on cultured blastocysts is intriguing since the reduced size of ICM suggests that ICM cells are susceptible to TNFα cytotoxicity.[122–125] This is somewhat unexpected since pluripotent ICM cells are presumably less sensitive to TNFα based on in vitro studies of ESCs. A major difficulty in interpreting the results from in vitro embryo culture is the lack of knowledge about immunological properties of trophoblasts at the blastocyst stage. None of the cited studies have specifically investigated the signaling events that mediate the effect of TNFα and IFNγ in the ICM cells or trophoblasts. In the case of TNFα, reduced ICM size could be a secondary effect exerted by activated trophoblasts (if they have a functional signaling pathway for TNFα) via paracrine signaling. However, a more plausible scenario could be that a cultured embryo is a highly dynamic structure with rapidly proliferating/differentiating ICM and trophoblast cells and that these cells (or a certain population of these cells) may have gained the ability to respond to TNFα and become sensitive to its cytotoxic effects during the course of in vitro culture. This hypothesis is supported by the finding that TNFR1 (the receptor that mediates the effect of TNFα) is not expressed in mESCs or in E4 blastocysts but is induced in E6.5-9.5 blastocysts after in vitro culture.[13,122,122] Whether TNFR1 is expressed in the ICM or trophectoderm or both in cultured blastocysts was not investigated, but in situ hybridization analysis of the placenta at E7.5 embryo in the uterus

revealed that TNFα mRNA was detected only in trophoblast cells and not in the embryo proper or ectoplacental cone.[122] This finding suggests that TNFα signaling mechanisms are more developed in the trophectoderm.

The transient existence of PSCs during early embryogenesis makes it difficult to capture their immunological behavior in vivo. This is possible in ESCs, which represent PSCs that are artificially “arrested” in the pluripotent state at a time point when their innate immunity has not developed. From this perspective, it is rational to question the physiological relevance of the findings from in vitro cultured ESCs with respect to the transient existence of PSCs in vivo, but this narrow window probably represents one of the most critical times for embryo development and a successful pregnancy. As illustrated by in vivo mouse models, although low dosage of LPS given to mice before implantation only caused little overt adverse impact on blastocyst development, its embryotoxic effect is long-lasting since LPS-treated mice showed developmental defects in late gestation.[108,111] Therefore, the lack of response of mESC-FBs, mESC-derived smooth muscle cells, and hESC-derived endothelial cells to LPS is of particular significance.[12,16,21,40] Furthermore, the mechanisms that mediate innate immune response are gradually developed. ESC-FBs are still relatively insensitive to the cytotoxicity of poly IC and TNFα compared to fully differentiated fibroblasts.[19,40] This is likely the case for other ESC-differentiated tissue cells since they also have attenuated innate immune response.[12,13,16,64,65,126] This means that the attenuated innate immunity makes PSCs as well as their newly differentiated cells less vulnerable to embryotoxic cytokines. While more studies will be needed to demonstrate the physiological relevance of immunological properties of ESCs to PSCs and differentiating cells in an embryo, the current data present a compelling case for the hypothesis that attenuated innate immune responses could be a protective mechanism for PSCs to avoid cytotoxicity resulting from inflammation and immune responses.

## 12. Future perspectives

Disturbances of the immunological environment in the uterus are well-known factors that cause failed implantation and pregnancy complications.[2,3] Currently, we have limited knowledge about how an early embryo at the blastocyst stage deals with immunological and inflammatory challenges. Technically, in vivo studies are difficult due to the limited number of embryonic cells that are rapidly dividing and differentiating in a blastocyst. The ethical issues make it even more difficult to study in humans. Therefore, in vitro culture of embryos, ESCs, and TSCs has become an indispensable tool in the field. In particular, ESCs and TSCs can be propagated in unlimited amounts and kept in an undifferentiated state, essentially “freezing” them at a state that only exists for several days in vivo. TSCs can be induced to differentiate into different placental cell lineages while ESCs can be differentiated into specialized tissue cells on demand by controlling differentiation conditions.[8,127] While we have gained ample knowledge in recent years about the immunological property of ESCs, much less is known about TSCs. With the availability of mTSCs and newly established hTSC lines, understanding the immunological properties of these cells and their differentiated cells will further uncover the potential of in vitro embryo culture models that have already been utilized to study other aspects of embryonic development. It is exciting that recent advances in three-dimensional organoid culture techniques has led to the

generation of primitive “organs” from ESC-differentiated cells.[128] It is now feasible to reconstitute a blastocyst-like structure with ESCs and TSC-derived trophoblasts that can recapitulate certain developmental events of early embryogenesis.[129,130,131] This model could be extended to investigate innate immunity development at the blastocyst stage. With the knowledge and resources that have been obtained from stem cell research, the emergence of new tissue culture models and molecular tools, such as gene editing, will provide more exciting interdisciplinary opportunities for a deeper understanding of early embryogenesis, immunology, and reproductive biology, which is otherwise difficult or impossible with in vivo studies.

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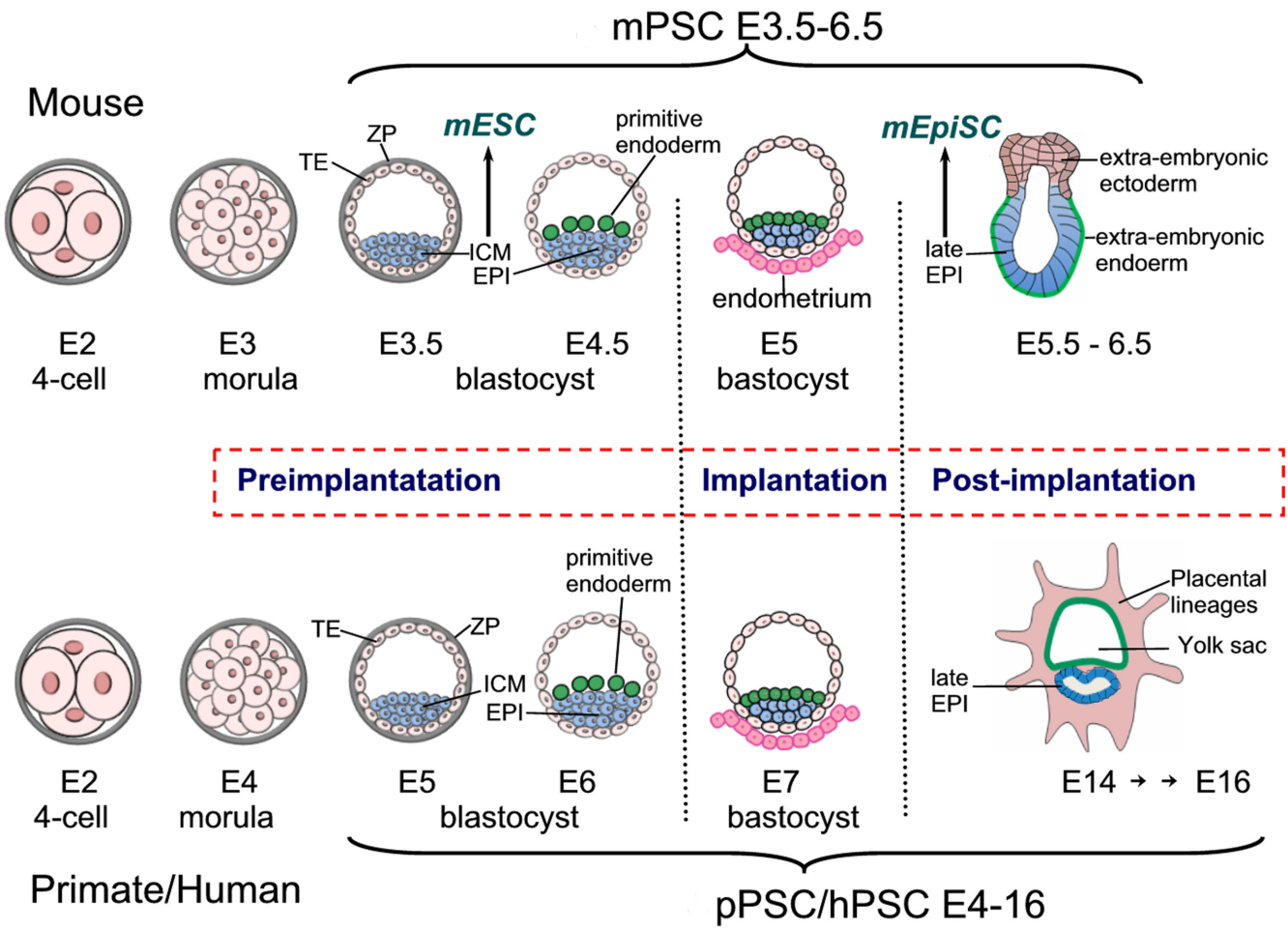


**Fig. 1. Pluripotent state of embryonic cells in mouse and human/primate early embryo.**

ZP, zona pellucida; TE, trophectoderm; ICM, inner cell mass; EPI, epiblast. PSCs at different developmental stages were indicted with blue color. PSCs in post-implantation EPI (E14-E16) were identified in cynomolgus monkey.[94] See discussion in the text for detail.

**Table 1.**

Comparison of major innate immune properties of mPSCs and hPSCs

| Innate immune property | mESCs / miPSCs | hESCs / hiPSCs |
|---|---|---|
| Expression of IFNα/β | Deficient, determined by responses to synthetic viral RNA analogs and viral infection [11,17,18,33,40] / Deficient, determined by responses to viral infection[15] | Deficient,[14] determined by responses to synthetic viral RNA analogs / Deficient,[14] determined by responses to synthetic viral RNA analogs. |
| Response to IFNα/β | Responsive, determined by ISG expression[41,42]; Weakly responsive (compared with mESC-FBs), determined by induction of ISG and antiviral activity[19,40] / Not reported | Barely responsive, determined by induction of ISG[43]; Weak antiviral replication [44] / Barely responsive, determined by induction of ISG[43] |
| Expression of TNFα | Deficient, determined by responses to bacterial infection or LPS[10,20,21] / Deficient, determined by responses to viral infection or LPS[15] | Deficient, determined by response to bacterial infection or LPS[20] / Not reported |
| Response to TNFα | Deficient, determined by TNF-induced genes or toxicity assay[13,20,55] / Not reported | Deficient[20,56]; Responsive, determined by CXCL8 induction[16] / Not reported |
| Response to LPS | Deficient, determined by LPS-induced genes[12,20] / Deficient, determined by LPS-induced genes [15] | Deficient, determined by LPS-induced genes[16,20] / Not reported |
| PKR activation | Activated by synthetic dsRNA, viral infection[18] or cellular dsRNA during cell cycle[46] / Not reported | Inactive in response to synthetic dsRNA[14]/ Inactive in response to synthetic dsRNA[14] |