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Attenuated Innate Immunity in Embryonic Stem Cells and Its Implications in Developmental Biology and Regenerative Medicine

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Abstract

Embryonic stem cells (ESCs) represent a promising cell source for regenerative medicine. Intensive research over the past two decades has led to the feasibility of using ESC-differentiated cells (ESC-DCs) in regenerative medicine. However, increasing evidence indicates that ESC-DCs generated by current differentiation methods may not have equivalent cellular functions to their *in vivo* counterparts. Recent studies have revealed that both human and mouse ESCs as well as some types of ESC-DCs lack or have attenuated innate immune responses to a wide range of infectious agents. These findings raise important concerns for their therapeutic applications since ESC-DCs, when implanted to a wound site of a patient, where they would likely be exposed to pathogens and inflammatory cytokines. Understanding whether an attenuated immune response is beneficial or harmful to the interaction between host and grafted cells becomes an important issue for ESC-based therapy. A substantial amount of recent evidence has demonstrated that the lack of innate antiviral responses is a common feature to ESCs and other types of pluripotent cells. This has led to the hypothesis that mammals may have adapted different antiviral mechanisms at different stages of organismal development. The underdeveloped innate immunity represents a unique and uncharacterized property of ESCs that may have important implications in developmental biology, immunology and in regenerative medicine.

Keywords

embryonic stem cells; innate immunity; antiviral response; type I interferons; regenerative medicine

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Introduction

Embryonic stem cells (ESCs) are characterized by their potential to differentiate into a variety of cell lineages (pluripotency) and unlimited capacity for proliferation (self-renewal). These properties make them a promising cell source for regenerative medicine [1, 2]. Intensive research over the past two decades has led to the development of methods for ESC differentiation into various cell types. However, generating ESC-differentiated cells (ESC-DCs) suitable for clinical application is a challenging task. In addition to the difficulty in obtaining sufficient quantity and quality of specific cell types, we have limited knowledge of whether ESC-DCs are fully functional as their *in vivo* counterparts.

Recent studies indicate that certain types of structural tissue cells such as endothelial cells and smooth muscle cells derived from both human and mouse ESCs (hESCs and mESCs) have limited or no response to a wide range of infectious agents and inflammatory cytokines [3–6]. These findings raise questions for the therapeutic use of these cells since they would be implanted to a wound site of a patient, where they would likely be exposed to pathogens and inflammatory cytokines. The immune and inflammatory responses play key roles in an organism's defense against infectious agents. Conceivably, the fate and functionality of transplanted cells could be compromised if they do not have such vital mechanisms. At the cellular level, infected cells produce type I interferons (IFNs) and inflammatory cytokines, which are critical in initiating innate immune response and priming the adaptive immune system [7, 8]. Although innate immunity is presumably developed in most types of somatic cells [9], it is not well understood in ESCs. Interestingly, studies dating back 40 years have indicated that pluripotent murine teratocarcinoma (embryonic carcinoma derived from germ cells) have altered susceptibility to viral infection [10, 11] and do not produce type I IFNs, which are a family of cytokines with antiviral properties, in response to viral infection [12]. The lack of IFN response in ESCs was also noted when they were used as a model to develop RNA interference (RNAi) techniques [13], but this phenomenon was not appreciated at that time with respect to immunity development and its implications in ESC physiology. However, the lack of innate immune responses in ESC-DCs, largely driven by our interest in the medical application of these cells, inspired the revisiting of this subject. Several recent studies have revealed that both hESCs and mESCs lack an immune response to viral infection, bacterial endotoxins and other pathogens [3, 4, 14, 15]. Therefore, underdeveloped innate immunity represents a previously noted but uncharacterized property of ESCs as well as other types of pluripotent cells, including induced pluripotent stem cells (iPSCs) [16, 17].

Like other types of therapeutic cells, the immunogenicity of ESCs and their derived cells that cause immune rejection has been a subject of intensive research and has been reviewed by several excellent articles [18–20]. However, the innate immune response of grafted cells to the host environment has received less attention. The recent discovery of underdeveloped innate immunity in ESCs and ESC-DCs raises several fundamental questions in their therapeutic application as well as in basic cell biology. In this review, we discuss the immune properties of ESCs and ESC-DCs with a brief overview of their immunogenicity, followed by a focused discussion on their innate immunity, and then we discuss the

implications of attenuated innate immunity in ESCs and ESC-DCs in stem cell biology, immunology, and ESC-based regenerative medicine.

Immunogenicity of ESCs and ESC-DCs – A major challenge for ESC-based regenerative medicine

The immune system of vertebrates consists of innate and adaptive immunity. Innate immunity responds to a broad range of pathogens in a non-specific manner and provides the first line of defense through several mechanisms that include inflammation and responses of innate immune cells (such as macrophages, dendritic cells, and natural killer cells). Adaptive immunity, on the other hand, provides defense against specific pathogens and foreign cells that evade the innate immune responses through highly specialized immune cells (T cells and B cells). Immune rejection of grafted cells by the host is primarily mediated by T cells in the adaptive immune system while innate immunity is responsible for the initial inflammatory reaction towards a graft and mobilization of innate immune cells that lead to the activation of the adaptive immune system [19, 21]. Among many factors that contribute to immune responses, the major histocompatibility complex (MHC) expressed on the surfaces of the grafted cells are the key molecules that cause immunogenicity, leading to the activation of host cytotoxic T cells and immune rejection.

Initial studies indicated that both hESCs and mESCs do not express or express low levels of MHC and other co-stimulatory molecules [22, 23]. Further, both undifferentiated and differentiated hESCs did not stimulate proliferation of T cells [24]. hESC-derived mesenchyme stem cells failed to induce cytotoxic T cell degranulation [25] while mESC-derived hematopoietic cells induce transplantation tolerance [26]. Therefore, these studies seemed to suggest that ESCs and ESC-DCs are “immune-privileged” [24] and led to the notion that they may be able to evade direct killing by host cytotoxic T cells. However, other studies have found that ESC-DCs evoked a stronger immune response and were rejected faster than undifferentiated ESCs [27]. Furthermore, the expression of MHC molecules is upregulated in ESC-derived insulin-producing cells [28] and vascular progenitors [29], correlating with their accelerated immune rejection. These inconsistent results make clear conclusions about the immunogenicity in ESCs and ESC-DCs uncertain, however, a general impression is that MHC expression on ESCs is low but upregulated after differentiation, leading to accelerated immune rejection (reviewed in [18–20]).

Generation of iPSCs has made it possible for the development of “patient specific” cells to circumvent the MHC barriers encountered with ESC-derived allogenic grafts, but accumulating evidence suggests that clinical application of iPSC derivatives faces significantly more complications than initially expected. Similar to ESCs, MHC molecules are expressed at low level in iPSCs, but are upregulated after differentiation [30]. However, a particular concern for their therapeutic use stems from the rigorous/prolonged processes of reprogramming and re-differentiation, which could result in considerable genetic alterations in iPSC-derived cells due to dramatic changes in global gene expression and epigenetic landscape [31]. Accumulation of genomic mutations in iPSC-derived cells is of particular concern not only because it increases tumorogenicity, but also immunogenicity due to the possible generation of “neoantigens” that could lead to immune responses even in a MHC

matched context. Currently, we do not have sufficient knowledge about immunobiology of the cells derived from autologous iPSCs to determine whether or not they are non-immunogenic (reviewed in [19, 20, 32]).

While the immunogenicity of ESCs and their derivatives has been and will continue to be a subject of intensive study, the innate immune/inflammatory reactions of grafted cells to the host environment and their contribution to the host immune reaction have received little attention, despite the fact that infection and inflammation are almost inevitable events encountered at the site of the transplantation. This issue becomes particularly relevant to ESC/iPSC-derived cells due to the reason that they are fundamentally different from the traditional therapeutic cell sources. Depending on the methods of their differentiation, ESC-DCs could be at very different stages of maturity [33] and are heterogeneous not only in immunogenicity to the host, but also in their own innate immune response to pathogens encountered in the host. Therefore, to fully appreciate the therapeutic values of ESC-DCs, it is imperative to understand the different aspects of their immunobiology, including their immunogenicity to host and their capacity to mount immune reaction in the host environment.

Innate immunity in ESC-DCs and its implications for ESC-based regenerative medicine

Various types of cells have been generated from ESCs by different *in vitro* differentiation strategies [33]. While these advances have proven the principles of ESC-based cell therapy, whether ESC-DCs can acquire full functions of their *in vivo* counterparts during differentiation remains poorly understood. ESC-DCs are mainly characterized by cell marker expression and cell-specific functions. However, the cellular innate immunity of ESC-DCs is not easily recognized when the cells are not exposed to infectious agents or inflammatory cytokines. A few recent studies have reported that the innate immune/inflammatory response in several types of ESC-DCs is substantially attenuated when compared with naturally differentiated cells. For example, hESC-differentiated endothelial cells express typical cell-specific markers and display basic properties similar to human aortic endothelial cells, but they lack response to a wide range of pathogens [3] and inflammatory cytokines [4–6]. This markedly contrasts to naturally-differentiated endothelial cells, which are exquisitely sensitive to infectious agents [34, 35]. The attenuated response to inflammatory cytokines was also observed in mESC-differentiated smooth muscle cells [4, 36], cardiomyocytes [3] and osteoblasts [37].

While an attenuated innate immune response seems to be a common feature among the above-mentioned ESC-derived structural tissue cells, the studies of ESC-derived innate immune cells appear to be different. Innate immune cells, including NK cells, dendritic cells, and macrophages have been generated from ESCs or iPSCs and are primarily characterized by their cell specific functions, i.e, cytolytic activity for NK cells and phagocytic/antigen presentation activity for dendritic cells and macrophages [38–45]. These cells express immune cytokines/chemokines when activated, but the assays were usually performed under different conditions, which make it difficult for a direct comparison with other types of ESC-DCs. For example, ESC-derived NK cells are able to express IFN-γ [43–

45], which indicates their capacity for cytokine production, but this is a unique property limited to certain immune cells, such as NK cells, T cells, and macrophages [7]. Nonetheless, several recent studies with macrophages and dendritic cells provided some informative data for comparison. As previously mentioned, ESC-derived endothelial cells and smooth muscle cells are insensitive to LPS [3, 4, 36]; however, macrophages derived from both hESCs and mESCs can express immune cytokines in response to this bacterial endotoxin [40–42]. In addition, mESC-derived macrophages also express inflammatory cytokines when exposed to several infectious agents, including *Salmonella* Typhimurium; whereas, undifferentiated mESCs do not respond to any of the tested stimuli [38]. Similar observations were made in mESC-derived dendritic cells, but these cells are 'primitive' since they produce low levels of certain cytokines when compared with bone morrow-derived dendritic cells [39].

The aforementioned studies suggest that the innate immunity is underdeveloped in ESC-derived structural tissue cells but appears to be more advanced in ESC-derived innate immune cells. While more studies are needed to draw general conclusions beyond the cells that have been investigated, the discrepancies found in the two classes of cells are not entirely surprising considering the processes of their differentiation. The stem cell state of ESCs is maintained by leukemia inhibitory factor (LIF) in mESCs and by feeder cells in hESCs. Removal of LIF or feeder cells triggers ESC spontaneous differentiation while the cell fate and differentiation rate can be influenced by different agents and growth conditions [33]. Based on this principle, most differentiation methods mainly use growth factors/cytokines that promote ESC differentiation toward a particular cell lineage of interest. For example, VEGF and bFGF are commonly used to promote endothelial cell differentiation [46–49], whereas PDGF and TGF-β are used to stimulate smooth muscle cell differentiation [46, 50]. We are not aware of any differentiation methods for these structural tissue cells containing factors that promote innate immunity development. This could contribute, at least partly, to their undeveloped innate immunity where they are differentiated in a cell culture environment without any immunostimuli. On the other hand, the differentiation medium for innate immune cells usually includes a cocktail of immune cytokines, such IL-3, IL-15, IL-17, and M-CSF (macrophage colony-stimulating factor) [38, 40–42], which could promote innate immunity development as a part of the differentiation program toward innate immune cells. It is conceivable that the *in vitro* differentiation conditions likely attribute to the differences in ESC-derived structural tissue cells and innate immune cells.

The attenuated innate response in ESC-derived structural tissue cells may have different implications for their therapeutic application. A strong immune/inflammatory reaction of a grafted tissue to the host environment could negatively impact the transplant by augmenting the inflammatory responses in the wounded area. From this point of view, an attenuated innate immune/inflammatory response in ESC-DCs could be beneficial since it may not potentiate the inflammatory response in the injured area, thereby potentially reducing further damage of grafted cells by the host's adaptive immune response. Conversely, the fate and the functionality of transplanted ESC-DCs could be compromised if they do not have competent innate immunity to sense and combat infections and to mobilize the adaptive immunity of the host when needed. For example, the endothelium is exposed to infectious

agents in the bloodstream. Endothelial cells are not only critical for vascular function, but also act as innate immune surveillance cells [34, 35]. The use of ESC-derived endothelial cells for vascular tissue repair could be compromised by their lack of innate immune response and pathogen sensing function [3]. Therefore, whether an underdeveloped innate immunity in ESC-derived structural tissue cells is beneficial or harmful to tissue regeneration is a complex issue and may depend on the nature of the transplantation. Many important questions remain to be answered, such as whether the host innate and adaptive immune function may confer immune protection to the grafted cells that lack innate immunity, and to what extent do the signals from the host environment promote their maturation. However, the *in vivo* studies that could help to answer these questions are currently lacking. To fully understand the importance of the innate immune response in ESC-based cell therapy, it will be essential to have a complete characterization of ESC-DCs by *in vitro* and in-vivo studies.

Based on the lessons learned from ESC-derived structural tissue cells and innate immune cells, it should be possible to design strategies that generate “customized” ESC-DCs with different levels of innate immunity depending on whether or not the innate immune response is desired in tissue regeneration. For example, the innate immunity development could be accelerated by the inclusion of “immunostimulants” (such as the cytokines used for the differentiation of innate immune cells) [38, 40–42] or other epigenetic modifiers [4, 36] during differentiation of structural tissue cells. To this end, it is imperative to understand the molecular mechanisms that control immunity development and they must be sought at the level of ESCs.

Underdeveloped innate immunity – a common feature of pluripotent cells

At the cellular level, innate immune response is mediated by pattern recognition receptors, including toll-like receptors (TLRs) and retinoic acid–inducible gene I (RIG-I)-like receptors (RLRs). TLRs are membrane proteins localized on the cell surface or endosomes where they recognize a wide variety of infectious agents [51], while RLRs (including RIG-I and MDA5) detect viral RNAs in the cytosol [52]. Upon binding with their ligands, TLRs and RLRs activate the transcription factors IRF, NFκB and AP-1, which coordinately regulate the expression of type I IFNs and inflammatory cytokines that play central roles in immune responses [51]. In addition to TLR- and RLR-mediated antiviral responses, double stranded (ds) RNA-activated protein kinase (PKR) is directly activated by dsRNA and causes inhibition of both cellular and viral protein synthesis, thereby repressing viral replication as a separate antiviral mechanism [7, 53].

Although developed in various differentiated somatic cells, innate immunity is not, or at least not fully, developed in ESCs. hESCs are unresponsive to a wide range of infectious agents [3, 17], including lipopolysaccharide (LPS, a well-studied endotoxin that induces antibacterial responses) [54] and polyinosinic:polycytidylic acid (polyIC, a synthetic dsRNA widely used as a viral dsRNA analog) [55]. Although different results have been reported regarding their responsiveness to LPS and polyIC [4, 15, 56], mESCs do not show immune responses typically seen in differentiated cells when they are infected with live bacteria or viruses [14, 57]. These studies suggest that the innate immunity deficiency seems to be a

common feature of hESCs and mESCs. Our recent studies in mESCs [58] and in hESCs [17] have provided the molecular basis that underlies defective IFN expressing mechanisms in ESCs. The key signaling components in antiviral pathways, including the major viral RNA receptors (MDA5, TLR3 and RIG-I) are expressed at very low levels in both hESCs [17] and in mESCs [58], which may partly explain the failure of these cells to express IFNs in antiviral response. In addition to polyIC, short ssRNA, long ssRNA, and live viruses, all of which induced robust expression of IFNα/β in fibroblasts, failed to do so in mESCs [58, 59].

It was recently reported that mouse fibroblasts lose their antiviral responses when they are converted to iPSCs [16], which show defective IFN expression in response to viral infection similar to mESCs [57, 58]. Comparative analysis of hESCs and hiPSCs revealed similar findings [17]. Together with the early observations made in pluripotent embryonic carcinoma cells [12], the aforementioned studies suggest that the lack of a functional innate antiviral immunity, at least in the case of deficiency in IFN induction, is a common feature of pluripotent cells. While we do not yet know the physiological implications of this inverse relationship between antiviral response and pluripotency, one can speculate that the underdeveloped in ESCs (or repressed in iPSCs) antiviral activities might be needed to maintain the stem cell state, or alternatively, the stem cell state may restrict the development of antiviral mechanisms.

Differences between hESCs and mESCs in antiviral responses

Type I IFNs are a family of cytokines that include IFNα and IFNβ and several less characterized members, such as IFNε and IFNω [60]. 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 Janus tyrosine kinases (JAK) that phosphorylate signal transducers and activators of transcription (STAT1 and STAT2). Phosphorylated STAT1 and STAT2 translocate to the nucleus where they induce the transcription of IFN-stimulated genes (ISGs), which participate in various aspects of antiviral activities and promote the cell to enter an “antiviral state” [7, 8].

The IFN system, which includes both IFN expression and response mechanisms, has evolved as a central part of antiviral mechanisms in vertebrates [7–9]. Since ESCs are deficient in type I IFN expression, a logical question is whether or not they can respond to these cytokines. In hESCs and hiPSCs [17], we found that these cells have substantially attenuated responses to IFNβ, as judged by their failure to express ISGs [61]. The major signaling molecules in the IFN pathway are expressed at relatively lower levels than in differentiated cells, but the failure of hESCs and hiPSCs to respond to IFNβ seems to be mainly attributed to the high expression level of suppressor of cytokine signaling 1 (SOCS1) [61]. In differentiated cells, SOCS1 is expressed at a low basal level in resting state, but it is rapidly upregulated and acts as a negative regulator of JAK/STAT signaling pathway, thereby limiting excessive actions of IFNs [62]. However, SOCS1 is constitutively expressed at a high level in hESCs and hiPSCs, and may repress IFNβ action in those cells [61]. For hESCs, the lack of IFN response might be beneficial for the maintenance of pluripotency since IFN has been shown to stimulate differentiation [63]. In addition, pluripotent cells undergo rapid cell division, and may mute the IFN response to avoid its

antiproliferation effects [63]. Moreover, hESCs are primed to undergo rapid apoptosis [64]. Since there is a well-established association between STAT1 activation and apoptosis, it would make sense for human pluripotent cells to limit STAT1 signaling by a high level of SOCS1.

Differing from the observations in hESCs, two early studies reported that mESCs could respond to IFNα and IFNβ [65, 66]. It is intriguing that hESCs and mESCs are common in defective expression of type I IFNs, but yet they differ in response to these cytokines. These paradoxical observations led to our recent study [67] in which we demonstrated that mESCs indeed have basic functional mechanisms to detect and respond to type I IFNs [65, 66]. We further showed that IFNα, IFNβ and IFNω can protect mESCs from viral infection-induced lytic cell death and repress viral replication. mESCs express the major signaling components in the IFN pathway and are able to express ISGs, which is the hallmark of IFN action. Furthermore, the mRNA of *Socs1* in mESCs is expressed at a similar level to that of mouse fibroblasts, suggesting that SOCS1 may not be a major repressor that limits IFN response in mESCs [67]. While these results differ from hESCs[61], it is noted that the response of mESCs to IFNs is much weaker than that of fibroblasts, as judged by ISG induction and antiviral activity [67]. Therefore, the response of mESCs to IFNs is attenuated in comparison with differentiated cells. Currently, we do not know the reasons underlying this observation. Since ISG expression is regulated at multiple levels, it is likely that mESCs may not yet have developed fully functional mechanisms required for maximal ISG expression, or they may utilize different regulatory mechanisms from differentiated cells.

Another notable difference between hESCs and mESCs is the activation of the PKR pathway. Although PKR can selectively activate the transcription of some genes involved in immune responses, the major cellular effect of its activation is to inhibit translation and proliferation in host cells, thereby limiting viral replication [7, 53]. Unlike other viral RNA receptors in hESCs and mESCs that are expressed at low levels, PKR is expressed at a comparable level to that of differentiated cells. However, PKR is unresponsive to dsRNA in hESCs [17], but can be activated by both polyIC and La Crosse virus infection in mESCs, resulting in inhibition of cell proliferation as in differentiated fibroblasts [58]. Although we are not sure the reasons behind this difference between hESCs and mESCs, we speculate that the abundance of cellular transcripts with dsRNA structures within human and mouse cells may affect the activation status of PKR. Repetitive *Alu* elements are unique to primates and account for almost all of the human SINEs (short interspersed elements) comprising more than 10% of the human genome. Since most of these are closely related to one another in their sequences and lie predominantly within euchromatic and gene-rich regions, their abundance leads to the frequent occurrence of inverted repeat structures (IR*Alu*s) in gene regions [68]. We have previously reported that mRNAs containing IR*Alu*s in their 3’-UTRs are retained in the nucleus [68, 69]. This retention is mediated by a long noncoding RNA, NEAT1, which is not expressed in hESCs and iPSCs [69]. This means that many transcripts with dsRNA structures in their 3’-UTRs may be exported to the cytoplasm, where innate immune responses might be triggered if this system were not strongly attenuated. Since inverted repeats are less common in murine transcripts, this could be one explanation for the difference in PKR activation in hESCs and mESCs.

While hESCs and mESCs share fundamental similarities in pluripotency and self-renewal, they nevertheless display a number of notable species differences [70]. First, activation of the JAK/STAT3 pathway by leukemia inhibitory factor (LIF, which shares a similar signaling paradigm with type I IFN) is essential for the maintenance of stem cell state in mESCs [71], but LIF is not required for hESCs [72]. Second, mESCs are characterized by a shortened cell cycle, whereas hESCs have a cell cycle time frame similar to that of differentiated cells [73, 74]. The differences in PKR activation [17, 58] and in responding to type I IFNs [61, 67] represent further distinctive features in ESCs from the two species, although both have attenuated innate antiviral mechanisms in comparison with differentiated cells.

The biological implications of an underdeveloped IFN system in ESCs and in developmental biology

The underdeveloped IFN system in ESCs raises an intriguing question: What is the rationale for ESCs not to have such an effective innate antiviral mechanism that is well developed in most differentiated cells? While we do not yet have answers to this question, we can speculate from different perspectives. ESCs normally reside in the womb where they have limited exposure to pathogens [75]. From this point of view, an underdeveloped innate immunity in ESCs is not entirely surprising since the mother's immune system can offer necessary protection to ESCs. However, a different conjecture could be made based on the pleotropic effects of IFN-based antiviral responses. It is well known that multiple forms of antiviral activities triggered by the IFN system can cause various adverse effects to the infected cells, such as cell cycle inhibition or cell death [7, 53]. While these negative effects on infected cells in a tissue may not cause much damage to a developed organism, the consequence could be detrimental to ESCs if infected since they are the progenitors for all ensuing tissues of a developing organism. On the other hand, viral infections would be equally disastrous if ESCs lack an effective antiviral mechanism as their descendant cells would be infected as well. The recent discovery of a functional RNAi mechanism in mESCs may offer a plausible solution to this dilemma [76].

The RNA interference (RNAi) pathway is an antiviral mechanism by which a cell uses small double-stranded RNA molecules (siRNAs) derived from viral RNA to silence the virus's own RNAs [77]. RNAi is a major antiviral mechanism in plants and invertebrates, which lack IFN-based innate antiviral immunity. It has been uncertain whether RNAi functions in mammals, in which a well-developed IFN system can mount multiple forms of powerful antiviral activities [78, 79]. The expression of type I IFNs in differentiated mammalian cells elicited by viral infection makes it difficult to assess the RNAi effect (as opposed to in plants and invertebrates). However, using mESCs as a model system, Voinnet and colleagues recently demonstrated that RNAi may indeed function in mammalian cells [76]. This finding was in part attributed to the lack of IFN expression in mESCs, which avoids the complications from IFN responses encountered in differentiated cells. Further data suggest that RNAi is also functional in differentiated cells, but is significantly attenuated in comparison with mESCs. A separate study by Li et al. [80] also demonstrated that siRNAs were detected in tissues of newborn mice infected with viruses.

The results from the above-mentioned studies strongly support the role of RNAi in mammals that has been uncertain for a long time and led to the notion that mammals may retain a functional RNAi pathway. However, a recent study by Backes et al. [81] questions the physiological contribution of RNAi to the antiviral responses in mice that lack an IFN system. Therefore, the relevance of RNAi mechanism to the overall antiviral activities in mammals at the organismal level is still a debatable issue. Nonetheless, the existence of RNAi in mESCs provides a rational explanation for their lack of functional IFN system; by utilizing viral specific and short-lived siRNA derived from invading viruses, ESCs may prevent viral infection, thereby avoiding potential negative effects associated with the IFN-based antiviral mechanism as previously discussed. An emerging paradigm is that mammals may have adapted different antiviral strategies at different stages of development whereby the IFN-based system is mainly utilized by differentiated somatic cells, whereas the RNAi mechanism could play a role in antiviral response in ESCs. This paradigm appears to fit, especially for humans, where both IFN production and response systems in hESCs are inactive [17, 61]. However, the utilization of two antiviral mechanisms in mice may be slightly different, since mESCs are partially responsive to IFNs [67] and the IFN-based antiviral mechanism is not completely inactive in these cells. It appears that mESCs are unable to produce type I IFNs, but they are able to acquire protective antiviral effects to a certain degree from type I IFNs secreted from other cells via a paracrine mechanism. While this seems to be an advantage for mESCs during early development, the question of how mESCs manage to avoid the potential adverse effects associated with IFN response remains to be determined. We speculate that IFN-induced low level cellular responses (yet still providing antiviral activity) may limit the excessive IFN action [67]. Much work is needed to elucidate this seemingly paradoxical phenomenon in mESCs.

In addition to its activation by viral dsRNA, PKR can also be activated by cellular dsRNA and abnormal RNAs, such as those derived from cell death or from RNA processing [82, 83]. Therefore, PKR is considered a “sentinel kinase” [84]. Recently, it has been proposed that PKR may function as a mitotic regulator of the cell cycle in several cell lines, including hESCs [85]. This finding provides a rationale for ESCs to express PKR. The difference in abundance of cellular transcripts with dsRNA structures (such as IR*Alu*s) and in their mRNA processing/nuclear retention mechanisms as previously discussed may suggest that PKR in hESCs and mESCs could be regulated differently under certain conditions.

Developing mRNA-directed differentiation strategies based on the attenuated antiviral response in ESCs

The landmark achievement in generating iPSCs has not only alleviated some ethical and social concerns associated with hESCs, but also led to the new concept of cell reprogramming [86]. However, the fact that viral vectors are commonly used for effective expression of reprogramming factors prevents the therapeutic use of the reprogrammed iPSCs in humans [87, 88]. Extensive effort to avoid this problem has led to the development of several alternatives to viral vectors, among which mRNA-mediated gene expression has shown great promise due to the non-integrating and transient nature of mRNA [89]. This method directly introduces synthetic mRNA into the host cells to express reprogramming

factors, thereby eliminating the need of viral or DNA vectors. The successful generation of RiPSCs (RNA-induced iPSCs) from fibroblasts [90] demonstrated the great promise of this strategy. A major biological challenge, however, is that a synthetic mRNA is detected as a viral RNA by host cells and induces strong antiviral responses, resulting in IFN induction and reduced viability of host cells [90–93]. As a result, synthetic mRNAs must be modified via a complex process to minimize their immunogenicity [90, 93]. The lack of antiviral responses in mESCs prompted us to investigate the feasibility of developing an mRNA-based gene expression strategy. As expected, mESCs can tolerate repeated transfection with synthetic mRNA prepared by *in vitro* transcription [94] and effectively express the expected proteins [59]. Therefore mRNA-based gene expression may open a new field for its application in mRNA-directed ESC differentiation.

Although methods to promote ESC differentiation to specific cell lineages have been developed, these strategies primarily depend on the spontaneous differentiation potential of ESCs under the influence of certain growth factors and/or cytokines as previously discussed. The efficiency of these methods is usually so low that isolating a pure cell population is difficult. The lack of efficient differentiation methods to obtain specific types of cells in sufficient quality and quantity is another major challenge that limits clinical applications of ESC-derived cells. Since cell lineage specification is mainly driven by the activation of cell-specific transcription programs, the inefficiency of the existing *in vitro* differentiation methods is, at least in part, due to insufficient transcription activation. A recent study reported that hESCs can be effectively differentiated into endothelial cells by viral vector-mediated expression of transcription factors that control vascular differentiation [95], demonstrating that intervention at the transcription level can provide a strong driving force for ESC differentiation into a specific cell fate. However, the use of viral vectors in that study means the derived cells inherited all safety concerns associated with viral vectors, as in the generation of iPSCs. mRNA-mediated gene expression can bypass the viral vector, as we recently demonstrated that synthetic mRNA encoding ETV2, a transcription factor that promotes vascular differentiation, can be effectively expressed in mESCs with expected transcription activity [59], therefore, demonstrating the feasibility of using synthetic mRNA as an alternative to viral vectors in directed ESC differentiation.

Conclusions and perspectives

While the molecular mechanisms that control ESC pluripotency and self-renewal have been at the center of ESC research for the past two decades [74, 86, 96], we are starting to witness its expansion not only to translational medicine but also to many areas of basic cell biology. The lack of innate antiviral responses in ESCs challenges the traditional view in cell biology that all cells have innate immunity, and this discovery has led to new insights of innate immunity in developmental biology and regenerative medicine. Although in theory ESC-DCs may be used for clinical applications, in reality these cells would not be beneficial if they do not have essential functions of their *in vivo* counterparts. While the immunogenicity of ESC-DCs that causes immune rejection of grafted cells has been a major issue in ESC-based cell therapy, the innate immune response of ESC-DCs in the host environment apparently adds another dimension of complications, as it raises several fundamental questions, such as whether or not transplanted ESC-DCs could be at risk if they lack a

competent innate immunity against infections, whether and to what degree the host immunity can protect the grafted cells, and whether the attenuated immune response in ESC-DCs is beneficial or harmful to the interaction between the host tissue and grafted cells. The clinical significance of these questions will need to be assessed eventually by translational studies, but finding the answers to these compelling questions overlapping innate immunity, developmental biology, and regenerative medicine will also depend on understanding the immunobiology of ESCs and ESC-DCs at the molecular and cellular levels.

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