

Mouse Embryonic Stem Cells Are Deficient in Type I Interferon Expression in Response to Viral Infections and Double-stranded RNA^{*[5]}

Received for publication, December 28, 2012, and in revised form, April 10, 2013. Published, JBC Papers in Press, April 11, 2013, DOI 10.1074/jbc.M112.421438

Ruoxing Wang^{†1}, Jundi Wang^{†1}, Amber M. Paul[‡], Dhiraj Acharya[‡], Fengwei Bai[‡], Faqing Huang[§], and Yan-Lin Guo^{†2}

From the Departments of [†]Biological Sciences and [§]Chemistry and Biochemistry, University of Southern Mississippi, Hattiesburg, Mississippi 39406

Background: The antiviral mechanisms are not known in mESCs.

Results: mESCs are susceptible to viral infections and dsRNA-inhibited cell proliferation but do not express type I interferons.

Conclusion: mESCs have underdeveloped mechanisms for type I interferon expression.

Significance: The findings are important for understanding the development of antiviral mechanisms in ESCs and stem cell physiology.

Embryonic stem cells (ESCs) are considered to be a promising cell source for regenerative medicine because of their unlimited capacity for self-renewal and differentiation. However, little is known about the innate immunity in ESCs and ESC-derived cells. We investigated the responses of mouse (m)ESCs to three types of live viruses as follows: La Crosse virus, West Nile virus, and Sendai virus. Our results demonstrated mESCs were susceptible to viral infection, but they were unable to express type I interferons (IFN α and IFN β , IFN α/β), which differ from fibroblasts (10T1/2 cells) that robustly express IFN α/β upon viral infections. The failure of mESCs to express IFN α/β was further demonstrated by treatment with polyIC, a synthetic viral dsRNA analog that strongly induced IFN α/β in 10T1/2 cells. Although polyIC transiently inhibited the transcription of pluripotency markers, the stem cell morphology was not significantly affected. However, polyIC can induce dsRNA-activated protein kinase in mESCs, and this activation resulted in a strong inhibition of cell proliferation. We conclude that the cytosolic receptor dsRNA-activated protein kinase is functional, but the mechanisms that mediate type I IFN expression are deficient in mESCs. This conclusion is further supported by the findings that the major viral RNA receptors are either expressed at very low levels (TLR3 and MDA5) or may not be active (retinoic acid-inducible gene 1) in mESCs.

Embryonic stem cells (ESCs)³ have attracted enormous attention in recent years with the expectation that they will be

* This work was supported, in whole or in part, by National Institutes of Health Grant P20 GM103476 from NIGMS (Mississippi IDeA Network of Biomedical Research Excellence). This work was also supported in part by an Aubrey Lucas Endowment for Faculty Excellence Award (to Y.-L. G.).

[5] This article contains supplemental Table 1.

¹ Both authors contributed equally to this work.

² To whom correspondence should be addressed: Dept. of Biological Sciences, University of Southern Mississippi, 118 College Dr. 5018, Hattiesburg, MS 39406. Tel.: 601-266-6018; Fax: 601-266-5797; E-mail: yanlin.guo@usm.edu.

³ The abbreviations used are: ESC, embryonic stem cell; mESC, mouse ESC; hESC, human ESC; LACV, La Crosse virus; WNV, West Nile virus; SeV, Sendai virus; TLR, toll-like receptor; RIG-I, retinoic acid-inducible gene 1; MDA5, melanoma differentiation associated gene 5; PKR, dsRNA-activated pro-

tein kinase; ICAM1, intercellular cell adhesion molecule-1; m.o.i., multiplicity of infection; RT-qPCR, real time quantitative-PCR; ssRNA, single-stranded RNA; 3p-ssRNA, 5'-triphosphate single-stranded RNA.

used as a source for cell-based therapy. Although the benefit of this research in medical applications is exciting, currently there is limited understanding of the basic physiology of ESCs and their derived cells. When used for cell therapy, ESC-derived cells would be placed in a wounded area that is likely to be exposed to various pathogens. Therefore, their fate and functionality may depend on their innate immunity to deal with a hostile environment. However, it is not clear whether ESCs and ESC-derived cells have functional innate immunity.

Derived from an early stage embryo, ESCs can divide continuously under proper culture conditions. When induced, they can differentiate into different cell lineages, a property known as pluripotency (1, 2). Study of the molecular mechanisms that control ESC differentiation, pluripotency, and self-renewal has been the center of ESC research. Recent studies have led to the development of methods for ESC differentiation into different cell types. However, it is increasingly clear that generating clinically usable cells is a task that faces many biological and technical challenges. For instance, it is well recognized that if ESCs are not fully differentiated and transplanted to the patient, they can grow into tumor-like structures known as teratomas (3). However, there are many other concerns that have not been fully recognized. An outstanding issue is the cellular innate immunity, which is not easily recognized in the cells that are not challenged by infectious agents. This concern is illustrated with the case of ESC-derived endothelial cells. These cells have been well characterized with respect to the endothelial cell marker expression, morphology, and the ability to form vessels (4, 5). However, only a few studies (6–8), including our own (9), have investigated their innate immune and inflammatory responses. Such studies have demonstrated that ESC-endothelial cells, derived from both human and mouse ESCs (hESC and mESCs), have limited, if any, ability to respond to lipopolysaccharides and inflammatory cytokines. Apparently, the failure of *in vitro* ESC-differentiated cells to acquire active innate immunity could be a concern for clinical applications.

tein kinase; ICAM1, intercellular cell adhesion molecule-1; m.o.i., multiplicity of infection; RT-qPCR, real time quantitative-PCR; ssRNA, single-stranded RNA; 3p-ssRNA, 5'-triphosphate single-stranded RNA.

Cellular innate immunity is mediated by pattern recognition receptors that include toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors. TLRs are localized on the cell surface or on the membrane of endosomes where they detect a wide variety of molecules that evoke immune responses, known as pathogen-associated molecular patterns (10). RIG-I-like receptors, including RIG-I and MDA5 (melanoma differentiation-associated gene 5), reside in the cytosol and primarily recognize viral RNA (11). Upon binding with their ligands, these receptors activate signaling pathways, including interferon regulatory factor and nuclear transcription factor- κ B (NF- κ B), which coordinately regulate the expression of type I interferons (IFN α/β) and pro-inflammatory cytokines that participate in antiviral responses (10, 12). Another important molecule that mediates the effects of dsRNA in the cytosol is dsRNA-activated protein kinase (PKR). In addition to selectively activating the transcription of genes involved in the immune responses, PKR also causes a general inhibition of transcription, translation, and host cell proliferation that limits viral replication (13, 14). Although extensive studies have been conducted in differentiated cells, only a few studies have investigated the innate immunity in ESCs. It is speculated that ESCs, normally residing in the sterile environment of the womb, may not have active innate immunity (15). In line with this notion, recent studies indicated that hESCs do not respond to a wide range of infectious agents, including bacterial LPS and dsRNA (6, 16). Similar to hESCs, it was shown that mESCs did not respond to LPS (7) or even live bacteria (17). However, the molecular mechanisms involved have not been elucidated.

In this study, we demonstrated that mESCs are susceptible to viral infections and dsRNA-inhibited cell proliferation, but they are unable to express type I IFN. We provided molecular basis for the underdeveloped antiviral mechanisms in mESCs.

EXPERIMENTAL PROCEDURES

mESC Culture—D3 cells, a commonly used mESC line in the literature (18), were obtained from the ATCC. They were used for the majority of the experiments in this study. The key experiments were repeated in DBA252 mESCs that we previously characterized (19–21). Both cell lines were maintained in the standard mESC medium (21). Raw 264.7 (Raw) and 10T1/2 cells were cultured in DMEM that contains 10% fetal calf serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. All cells were maintained at 37 °C in a humidified incubator with 5% CO₂.

Preparation of Viral Stocks—La Crosse virus (LACV, SM6 v3) and West Nile virus (WNV, strain CT2741) were propagated in Vero cells (African green monkey kidney cell line, ATCC). Titers of virus stocks were determined by plaque assay as described previously (22). Sendai virus (SeV, Cantell strain) stock was purchased from Charles River laboratory.

Cell Treatment—mESCs and 10T1/2 were plated at ~40 and ~70% confluence, respectively, and cultured for 24 h before the experiments. For viral infection, viral stocks were added to the cell culture at the concentrations as specified in individual experiments. PolyIC (Sigma) was either directly added to the cell culture or was transfected into the cells with DharmaFECT

reagent (Thermo Scientific). For polyIC transfection experiments, control cells were transfected with DharmaFECT reagent only. The culture medium and treated cells were collected at different time periods and used for various analyses.

Real Time Quantitative-PCR (RT-qPCR)—Total RNA was extracted using TRI Reagent (Sigma). cDNA was prepared by Moloney murine leukemia virus reverse transcriptase (Sigma). RT-qPCR was performed using SYBR Green ready mix on an MX3000PTM RT-PCR system (Stratagene), as reported previously (21). The mRNA level from RT-qPCR was calculated using the comparative *Ct* method (23). β -Actin mRNA was used as a calibrator for the calculation of relative mRNA of the tested genes. The sequences of the primer sets are listed in supplemental Table 1.

IFN β and Cytokine Assays—The culture medium collected from treated cells was used to determine secreted IFN β and cytokines. IFN β was quantified with an ELISA kit that detects mouse IFN β (PBL Biomedical Laboratories) according to the manufacturer's instruction. IL6 and TNF α were analyzed with a Luminex cytokine assay kit (Millipore) and determined with a MAGPIX instrument. The data were analyzed according to the method described by Prabhakar *et al.* (24).

Cell Proliferation, Viability, and Cell Cycle Analysis—Cell proliferation was determined by colony size and by cell number after toluidine blue staining as we described previously (25). The absorbance at 630 nm of toluidine blue-stained cells was measured with a microtiter plate reader. The values, which correlate with the number of viable cells, were used as an indirect measurement of cell proliferation or viability. Cell cycle analysis by flow cytometry was performed after the cells were stained with 50 μ g/ml propidium iodide. The cell cycle profiles were generated with the CFlow software (25).

Protein Analysis by Flow Cytometry—Cellular protein analysis by flow cytometry was performed according to our published method (26). Briefly, treated cells were incubated with the antibodies against the specific proteins to be analyzed. The cells were then incubated with secondary antibodies conjugated with fluorescein isothiocyanate (FITC) and examined by an Accuri C6 flow cytometer. The fluorescence intensity, which correlates with the protein level, was determined with the CFlow software. In some experiments, cells were doubly stained with propidium iodide so that the expression level of the protein in different phases of the cell cycle could be simultaneously determined.

siRNA Transfection—siRNAs targeting PKR and RIG-I or negative control siRNA (Santa Cruz Biotechnology) were transfected to mESCs with DharmaFECT reagent at a final concentration of 100 nM. The cells were then analyzed for siRNA knockdown efficiency or used for the experiments as specified.

Cell Lysate Preparation and Western Blot Analysis—Cells were directly lysed with SDS sample buffer that contains 150 mM NaCl, 10 mM NaF, and 0.25 mM NaVO₄. Western blot analysis was carried out as described previously (25).

Immunostaining Assay—Virus-infected cells were fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were washed with PBS containing 0.3% hydrogen peroxide to block endogenous peroxidase activity for 30 min. Cells were blocked with 2% normal goat serum with 0.4% Triton X-100

mESCs Are Deficient in Type I IFN Expression

and incubated overnight at 4 °C with affinity-purified and anti-flavivirus envelope protein monoclonal antibody (4G2, ATCC). The cells were washed with PBS and then incubated with secondary antibodies conjugated to HRP (horseradish peroxidase) for 2 h at room temperature. Positive cells were identified using a TrueBlue detecting kit (KPL) under a phase contrast microscope.

RESULTS

mESCs Are Susceptible to the Cytopathic Effects of LACV Infection—To investigate the responses of mESCs to viral infection, we first infected cells with LACV (a negative sense ssRNA virus) that is known to cause lytic cell death of mammalian cells (27). 10T1/2 cells, a mouse cell line with fibroblast properties (28), were used as a positive control. Following the infection with LACV (m.o.i. = 1), cytopathic effects were observed at 48 h post-infection in 10T1/2 cells. These effects included cell morphological changes, such as rounding up and detachment from the culture dish. Similar effects were observed in mESCs at a longer incubation time (>60 h) (Fig. 1A, indicated by arrows). Viral infection of mESCs was confirmed by expression of the LACV gene that encodes the M-segment protein (Gc protein) (29). The infected cells were immunostained monoclonal antibodies against the Gc protein (a gift from Dr. Samantha Soldan, University of Pennsylvania School of Medicine) followed by flow cytometry analysis (29). As shown in Fig. 1B, the expression of Gc protein was detected at 30 h and was significantly increased at 40 h post-infection.

The cytopathic effect of LACV was proportional to viral load as indicated by the reduced number of viable cells at different multiplicities of infection (Fig. 1, C and D). LACV infection also caused cell cycle inhibition as indicated by an apparent reduction of cells in the G₂ phase (Fig. 1E, indicated by arrow) and a reduced expression level of cyclin E (Fig. 1F). Therefore, the reduced cell number of mESCs by LACV infection could be due to the lytic cell death as well as a general inhibition of cell proliferation associated with viral infection.

mESCs Are Deficient in Expressing Type I IFN in Response to Viral Infection—Under the normal conditions, very low mRNA levels of IFN α and IFN β were detected in 10T1/2 cells and were nearly undetectable in mESCs. LACV infection induced about a 1900-fold increase of IFN β in 10T1/2 at an m.o.i. of 1, but the induction in mESCs was negligible even at an m.o.i. of 10 (Fig. 2A). In addition, LACV infection up-regulated the expression of IFN α / β and the major viral RNA receptors, including RIG-I, PKR, MDA5, and TLR3, to different degrees in 10T1/2 cells, but such effects were not observed in mESCs (Fig. 2A). These results indicated that mESCs are susceptible to LACV infection, but they are unable to effectively express IFN α and IFN β .

To determine whether this observation is a general property of mESC or it is an event specific to LACV infection, we tested two additional viruses, WNV (a positive sense ssRNA virus) and SeV (a negative sense ssRNA virus). In both cases, viral infection induced a large increase of IFN β transcription in 10T1/2 cells, but induction in mESCs was less than 5-fold (Fig. 2, B and C). Viral infectivity in both mESCs and 10T1/2 cells was confirmed by expression of viral proteins. WNV E glycoprotein (30) was detected with monoclonal antibodies (4G2, ATCC) by

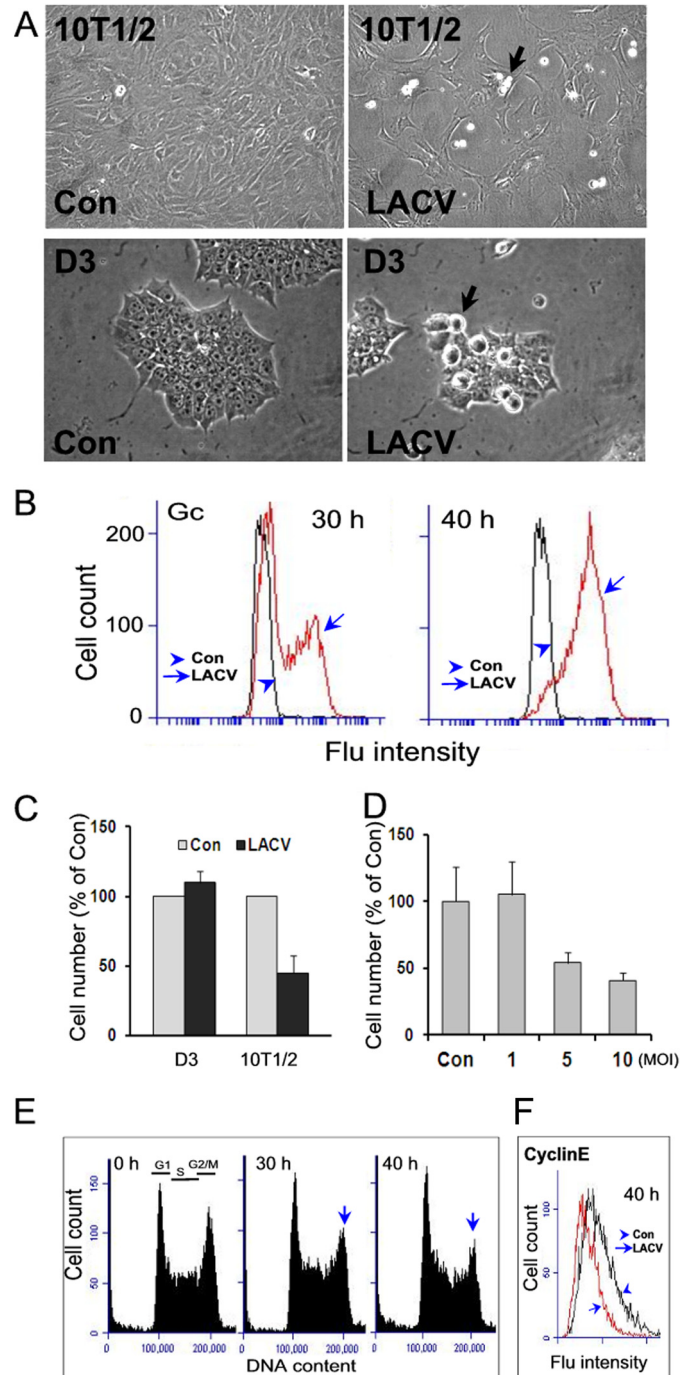


FIGURE 1. Infection of mESCs and 10T1/2 cells with LACV. A, mESCs (D3) and 10T1/2 cells were infected with LACV (m.o.i. = 1). The cells were examined under a phase contrast microscope and photographed ($\times 400$) at 48 h for 10T1/2 cells and 60 h for D3 cells. Arrows denote detaching dead cells. B, detection of LACV Gc protein in D3 cells infected with LACV (m.o.i. = 10) by flow cytometry. The expression level was proportional to fluorescence (Flu) intensity. C, D3 and 10T1/2 cells were infected with LACV at an m.o.i. of 1. Viable cells were determined at 48 h post-infection by toluidine blue cell staining. The values are means \pm S.D. of a representative experiment performed in biological triplicates. D, D3 cells were infected with LACV at a different m.o.i. as indicated. Viable cells were determined at 48 h post-infection as described in C. E, D3 cells were infected with LACV (m.o.i. = 10). The cell cycle profiles and cell count were determined with CFlow software. Reduction of the G₂ phase cells was indicated by the arrow. F, cyclin E expression level in LACV-infected D3 cells described in E (40 h) was determined by flow cytometry. The control (Con) represents cells without viral infection. All experiments were performed at least twice with similar results.

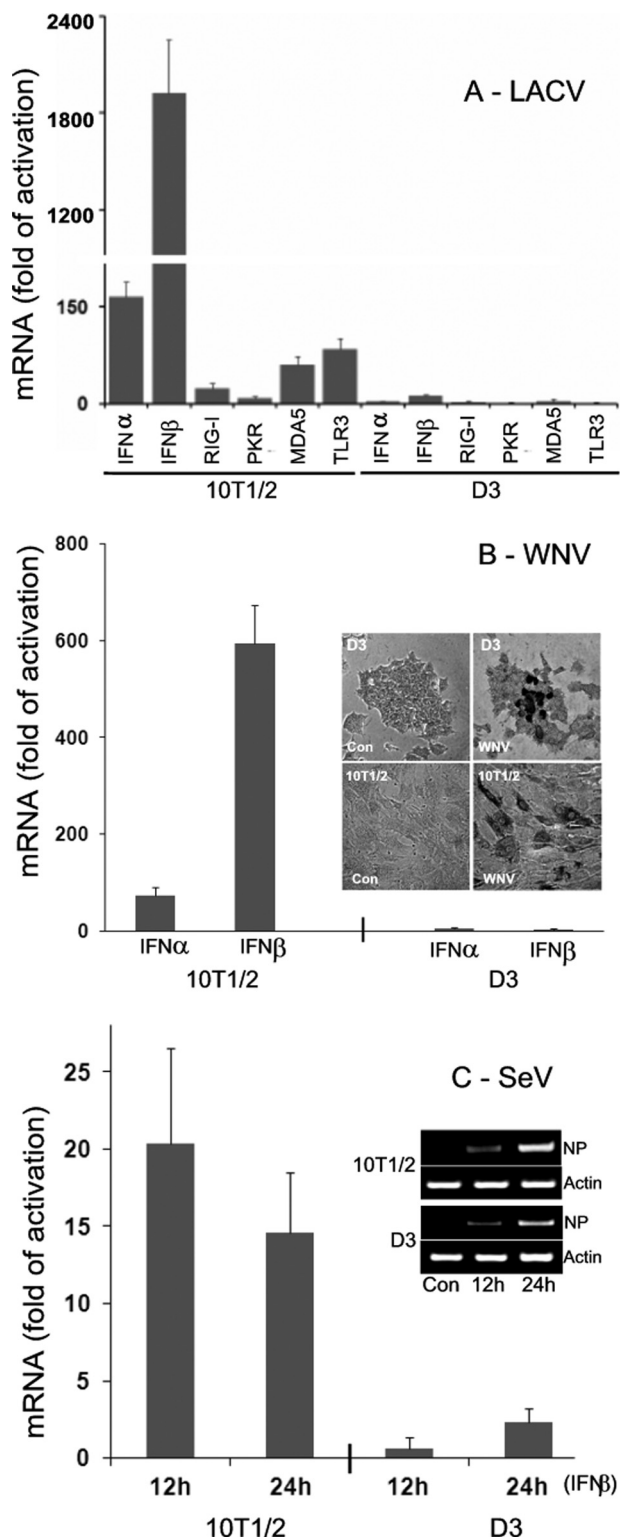


FIGURE 2. Viral infection-induced expression of IFN α and IFN β in D3 and 10T1/2 cells. A, 10T1/2 and D3 cells were infected with LACV at an m.o.i. of 1 and 10, respectively. The mRNA levels of the tested genes were determined by RT-qPCR 24 h post-infection. B, 10T1/2 and D3 cells were infected with WNV at an m.o.i. of 5 and 10, respectively. The mRNA levels of IFN α and IFN β were determined by RT-qPCR 24 h post-infection (graph). Infected cells were immunostained with antibodies against WNV E glycoprotein, and immunopositive cells were stained dark blue (inset). C, 10T1/2 and D3 cells were infected with SeV at 300 hemagglutination units/ml. The mRNA levels of IFN β were determined by RT-qPCR 12 and 24 h post-infection (graph). The transcription of SeV nucleocapsid protein in infected cells was determined by

immunostaining (Fig. 2B, inset), whereas SeV nucleocapsid protein (31) was detected by RT-PCR analysis (Fig. 2C, inset). Although WNV or SeV infection caused a slight but consistent inhibition of cell proliferation, they did not cause apparent lytic cell death as LACV (data not shown).

mESCs Can Sense and Respond to dsRNA via Cytosolic Receptors but Fail to Produce Functional IFN β and IL6—We further analyzed the responses of mESCs to polyIC, a synthetic analog of viral dsRNA that is widely used to mimic viral infection (32). When directly added to the cell culture, polyIC is expected to bind to TLR3 at the cell surface or to be internalized into the endosomes where it activates TLR3 (33, 34). This was demonstrated in Raw cells where polyIC induced the expression of IFN α and IFN β , but not in mESCs (data not shown), indicating that mESCs may not have functional TLR3 and/or a functional mechanism for polyIC internalization. However, when transfected into cells, polyIC can induce a several-fold increase of the transcription of three types of molecules typically seen in antiviral responses as follows: type I IFN (IFN α / β), inflammatory cytokines (TNF α and IL1 α), and the cell adhesion molecule (VCAM1) in D3 mESCs (Fig. 3A). Similar results were obtained in another line of mESCs, DBA252 (Fig. 3B). These results imply that mESCs can sense and respond to dsRNA via cytoplasmic receptors. However, the induction of IFN β in mESCs was negligible when compared with 10T1/2 cells, whereby polyIC induced nearly a 4000-fold increase of IFN β mRNA. PolyIC-induced transcription of IL6 and ICAM1 was also substantially higher in 10T1/2 cells than in mESCs (Fig. 3C). When examined at the protein level by ELISA, neither IFN β nor IL6 was detected in the media collected from polyIC-transfected mESCs, although they were readily detected in the samples from Raw cells and 10T1/2 cell cultures (Fig. 3D). Similar results were obtained for TNF α (data not shown). Similarly, ICAM1 protein was induced by polyIC transfection in 10T1/2 cells, but not in mESCs, as analyzed by flow cytometry of the cells immunostained with anti-ICAM1 antibodies (Santa Cruz Biotechnology) (Fig. 3E). Therefore, transfected polyIC could elicit a limited increase of transcripts of the above mentioned immune mediators in mESCs, but their corresponding proteins were not detected.

PolyIC Transfected to mESCs Inhibits Cell Proliferation—The most notable effect of transfected polyIC on mESCs at the cellular level was the inhibition of cell proliferation. As shown in Fig. 4, the colonies of polyIC-transfected cells were much smaller than the control colonies (Fig. 4A), correlating with markedly reduced cell numbers (Fig. 4B). In control cells, about 60% of cells were in the S phase, but polyIC caused an apparent reduction of cells in the S and G₂/M phases (Fig. 4C, indicated by arrow and arrowhead, respectively). A time course analysis revealed that the effects of polyIC can be detected as early as 6 h (Fig. 4D). It is noted that polyIC transfection also caused a small number of apoptotic cells (floating cells, data not shown),

RT-PCR and analyzed by agarose gel electrophoresis. β -Actin was used as a loading control (inset). All results shown in the graphs are expressed as fold activation, where the mRNA level in control cells is designated as 1 (not shown). The values are means \pm S.D. of three independent experiments. The experiments shown in the insets were performed twice with similar results.

mESCs Are Deficient in Type I IFN Expression

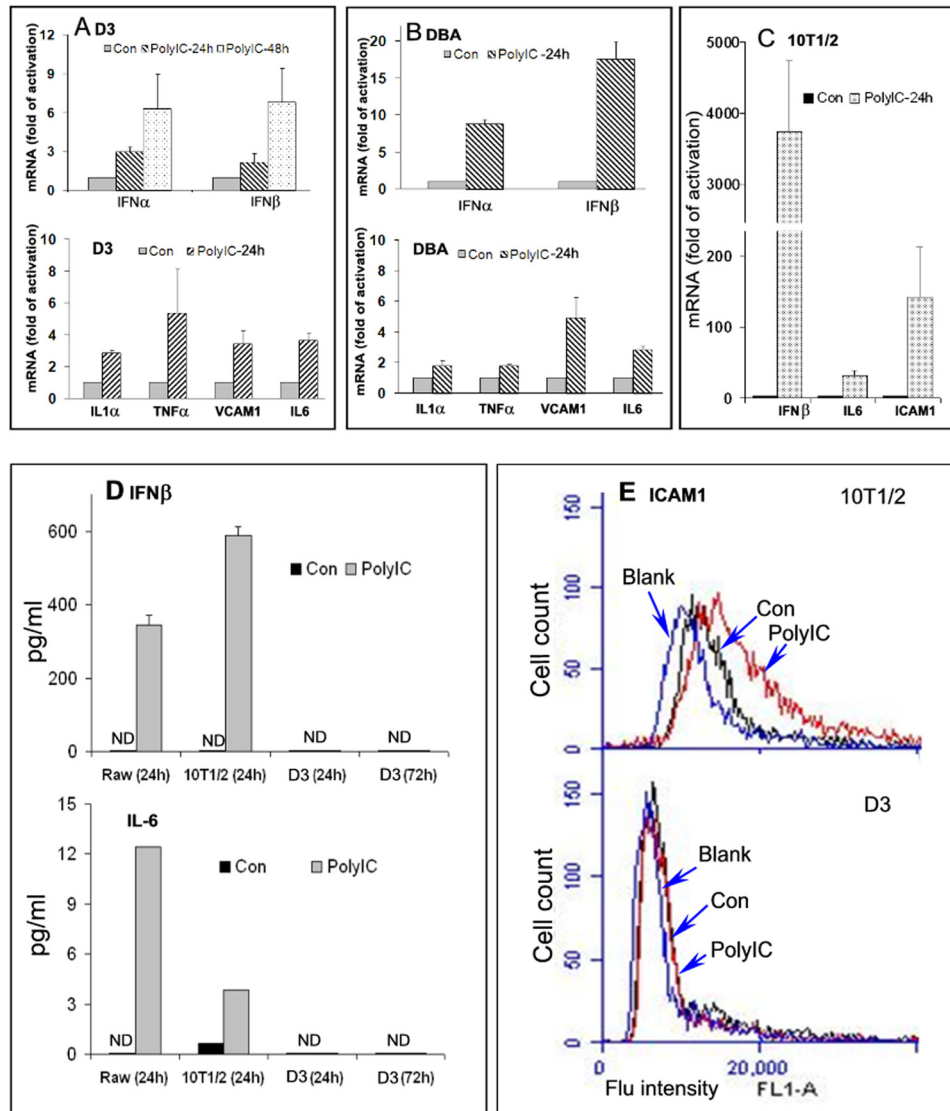


FIGURE 3. PolyIC transfected to mESCs can induce low level transcription of immune mediators but fail to produce detectable proteins. A–C, D3, DBA252, and 10T1/2 were transfected with 300 ng/ml polyIC. mRNA was analyzed by RT-qPCR. The results are expressed as fold-activation where the mRNA level in control cells is designated as 1. The values in A and C are means \pm S.D. of three independent experiments. The values in B are means \pm S.D. of a representative experiment performed in biological triplicates. D, D3 and 10T1/2 were transfected with polyIC as described in A and C. For Raw cells, polyIC (25 μ g/ml) was directly added to the culture medium. The culture medium collected at different time points was analyzed for IFN β by ELISA (detectable range 15.6–1000 pg/ml) and IL6 by Luminex assay (detectable range 0.64–10,000 pg/ml). ND, not detected. The values for IFN β are means \pm S.D. of a representative experiment performed in triplicate. The values for IL6 are average of a representative experiment performed in duplicate. E, expression profiles of ICAM1 in 10T1/2 and in D3 cells determined by flow cytometry. The representative experiments in all panels were repeated at least twice with similar results. Con, control.

which may also contribute to the reduced cell number and colony size.

Because the constitutive activation of CDK2 by cyclin A and E is the key factor that drives the rapid cell proliferation in mESCs (35), we examined the effects of polyIC on the expression of these cell cycle regulators. The reduced expression of cyclin E was apparent at 6 h after treatment, and this trend lasted for 24 h, as determined with its antibodies (Santa Cruz Biotechnology) by flow cytometry (Fig. 4E). Although cyclin A was not affected at 6 and 12 h, it was reduced at 24 h. A similar pattern was also demonstrated in the expression levels of CDK1 and CDK2 (data not shown). The CDKs, including p16, p19, and p21, are expressed at very low levels in control cells, and polyIC did not affect their expression (data not shown). These results suggest that down-regulation of cyclin E, and to a lesser

extent cyclin A, CDK1, and CDK2, contributed to polyIC-inhibited cell proliferation.

Effect of PolyIC on the Stem Cell State—To test if the stem cell state of mESCs was affected by polyIC, we examined the mRNA levels of pluripotency markers in polyIC-transfected cells. They were transiently down-regulated at 6 and 12 h but recovered nearly to the level of the controls by 24 h (Fig. 5A). In agreement with mRNA change, the protein level of OCT4, a key gene in the pluripotency control network (1), displayed a similar pattern with an initial slight decrease but returned to the normal levels at 24 h (Fig. 5B). Interestingly, the transcription of several early differentiation markers showed a similar dynamic pattern (Fig. 5C) to the pluripotency markers (Fig. 5A). Because of polyIC-induced cell cycle inhibition, the colonies formed from polyIC-transfected cells were smaller than those formed from control

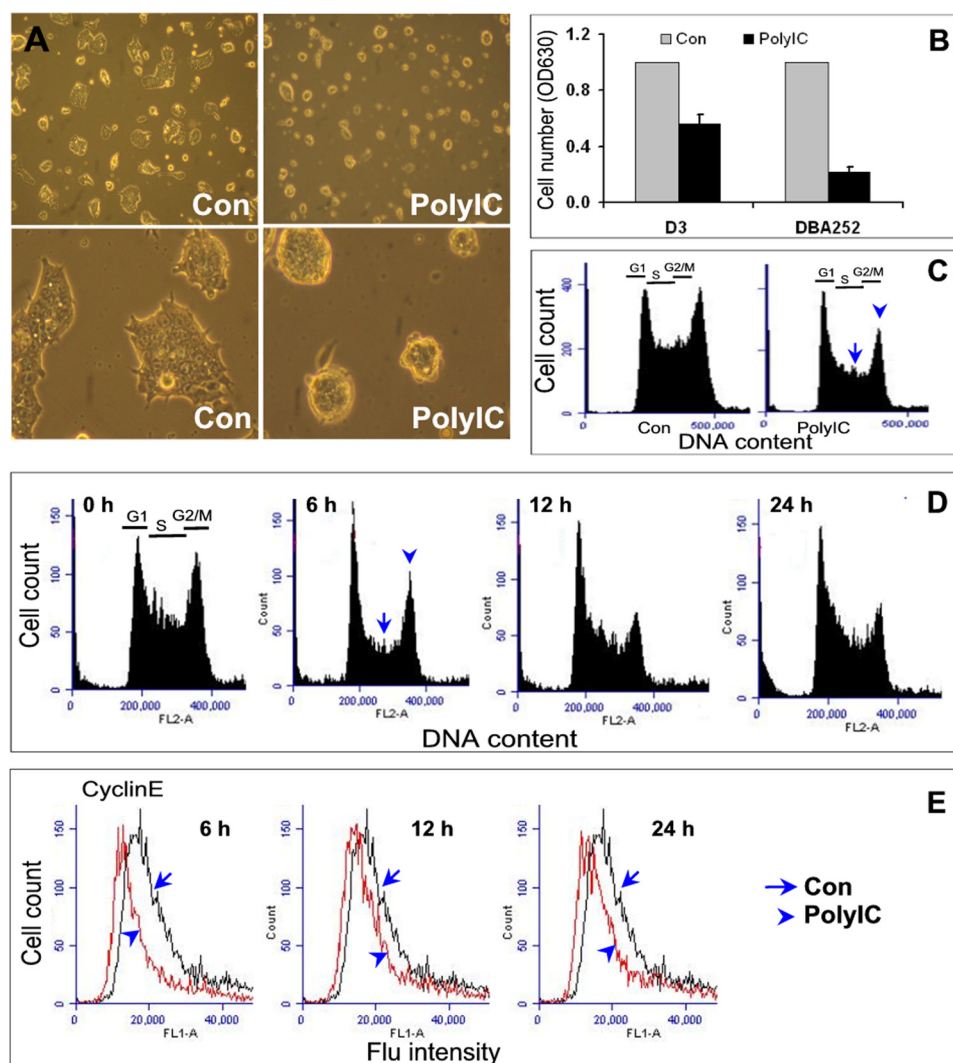


FIGURE 4. Effects of polyIC on mESC colony formation and cell proliferation. A–C, D3 cells were transfected with 300 ng/ml polyIC. Control (Con) represents cells transfected with DharmaFECT without polyIC. After incubation for 40 h, the colonies were examined under a phase contrast microscope and photographed with a digital camera ($\times 100$, upper panels; $\times 400$, lower panels) (A). Cell proliferation was measured by cell number indirectly determined from toluidine blue staining (absorbance at 630 nm). The values are means \pm S.D. of an experiment performed in biological triplicate (B). Cell cycle was analyzed by flow cytometry. The cell cycle profiles and cell count were determined with a computer software. Reduction of the S and G₂/M phase cells were indicated by the arrow and arrowhead, respectively (C). D, D3 cells were transfected with polyIC and incubated for the times as indicated. The cell cycle progression was analyzed by flow cytometry as described in C. E, effect of polyIC on cyclin E expression was determined by flow cytometry after immunostaining of the cells with antibodies against cyclin E. All representative experiments were performed at least twice with similar results.

cells, but their overall morphology (round with densely packed undifferentiated cells) was not significantly changed, as examined at 40 h (as shown in Fig. 4A) or at 72 h post-transfection (Fig. 5D).

Relative Expression Levels of dsRNA Receptors in mESCs and 10T1/2 Cells—To identify the receptor(s) that mediate the effects of transfected polyIC, we analyzed the relative expression levels of the four dsRNA receptors. The mRNA level of each molecule was determined by RT-qPCR and compared with that of β -actin. In D3 cells, TLR3 is expressed at a very low level, and MDA5 is barely detected, although RIG-I and PKR are expressed at higher levels (Fig. 6A). A similar pattern was seen in DBA252 cells (Fig. 6B). When compared with 10T1/2 cells, the mRNA of TLR3 and MDA5 in D3 cells are negligible, although RIG-I is about one-third of that in 10T1/2 cells. However, PKR is expressed at similar levels in the two types of cells (Fig. 6C).

PKR Plays a Key Role in Mediating the Effects of PolyIC in mESCs—The higher basal expression levels of PKR and RIG-I in mESCs make them the likely candidates that mediate the effects of polyIC. We first investigated PKR by its ability to phosphorylate eukaryotic initiation factor 2 α (eIF2 α). It is known that phosphorylation of eIF2 α by PKR attenuates its function in translation, thereby resulting in protein synthesis inhibition (13). PolyIC indeed stimulated eIF2 α phosphorylation in mESCs as determined by flow cytometry and confirmed by Western blot analysis using antibodies that specifically recognize phosphorylated eIF2 α (p-eIF2 α , Cell Signaling), although the expression of PKR did not change significantly. p-eIF2 α was detected as early as 30 min after polyIC transfection (Fig. 7A, boxed areas), which preceded cell cycle inhibition. Pretreatment of the cells with a PKR inhibitor C16 (Sigma) blocked polyIC-induced eIF2 α phosphorylation (data not shown), indicating that PKR is the responsible kinase. These

mESCs Are Deficient in Type I IFN Expression

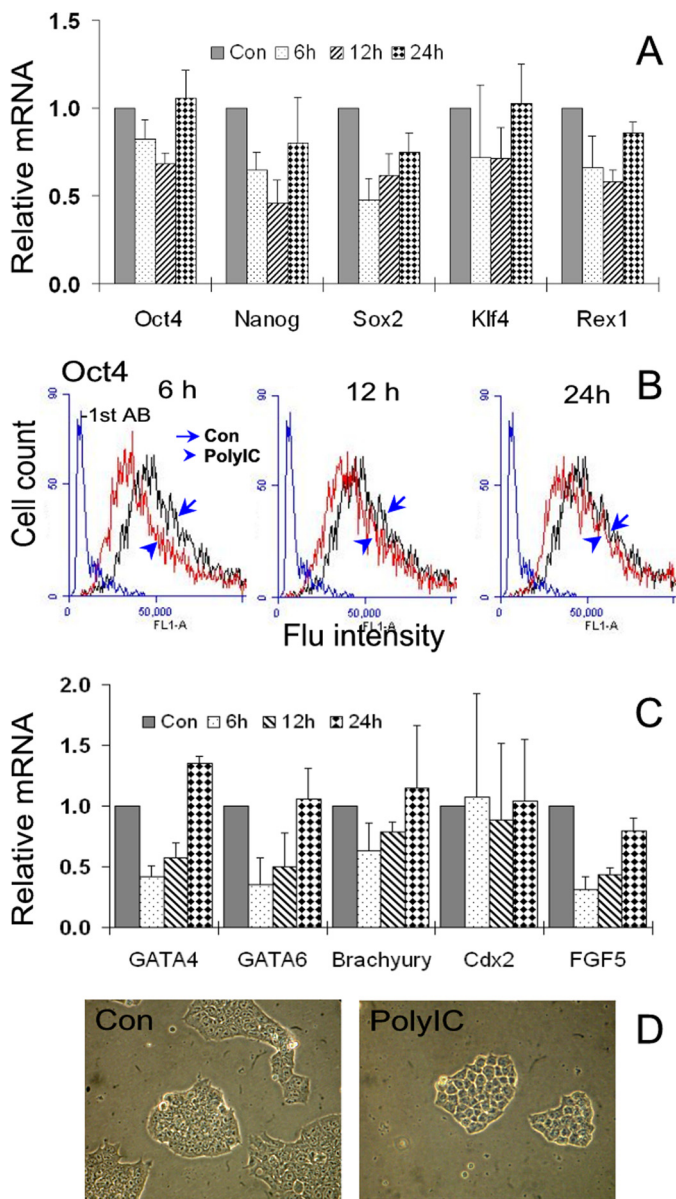


FIGURE 5. Effects of polyIC on the expression of pluripotency/differentiation markers and cell/colony morphology of mESCs. D3 cells were transfected with 300 ng/ml polyIC and incubated for a time course of 24 h. The mRNA levels of pluripotency markers (A) and early differentiation markers (C) were determined by RT-qPCR. The results are means \pm S.D. of three independent experiments. The effect of polyIC on the protein level of Oct 4 was determined by flow cytometry. – 1st AB represents the experiments where the cells were not incubated with anti-Oct4 antibodies (1st antibodies) as the control (B). The effect of polyIC on cell/colony morphology was examined under a phase contrast microscope and photographed with a digital camera at 72 h after treatment ($\times 400$). The images are representative experiments performed at least three times (D). Con, control.

results were confirmed with siRNA targeting PKR, which attenuated eIF2 α phosphorylation, although the negative control siRNA had no effect (Fig. 7B, upper panels, boxed areas). Importantly, silencing PKR reversed polyIC-induced reduction of S and G₂/M phase cells (Fig. 7B, middle panels) as well as reduced colony size (Fig. 7B, lower panels). However, knocking down RIG-I did not show such effects. These data demonstrate that PKR, not RIG-I, plays a key role in mediating polyIC-induced cell cycle inhibition.

We then analyzed the effects of silencing PKR and RIG-I on polyIC-induced transcription of IFN β and IL6. siRNA targeting PKR and RIG-I specifically knocked down their targets as expected (Fig. 7C). Silencing PKR significantly reduced mRNA levels of both IL6 and IFN β , whereas silencing RIG-I slightly attenuated transcription of IL6 but not that of IFN β (Fig. 7D).

PKR Is Activated by LACV but Not by WNV or SeV Infection in mESCs—We analyzed whether or not PKR was activated in virus-infected cells. As shown in Fig. 8A, a time course study of LACV-infected cells indicated PKR was activated at 11 h post-infection and lasted up to 30 h as indicated by eIF2 α phosphorylation, although the protein level of PKR was not altered as determined by Western blot with PKR antibodies (Santa Cruz Biotechnology). Knocking down PKR expression did not have an apparent effect on LACV-induced cell death (Fig. 8B). Similar results were obtained in the experiments with the C16 PKR inhibitor (data not shown). PKR activation by LACV was further confirmed by flow cytometry with antibodies against p-eIF2 α ; however, p-eIF2 α was not detected in the cells infected with WNV or SeV in parallel experiments (Fig. 8C). These results suggested that PKR activation is virus-dependent in mESCs.

DISCUSSION

In this study, we investigated the responses of mESC to three types of live viruses and two synthetic viral dsRNA analogs. Although all of these stimuli induced a robust IFN α/β expression in 10T1/2 cells, they only induced very limited or no detectable transcription of IFN α/β . These results suggest that mESCs are deficient in type I IFN expression, a central component of the antiviral mechanisms in most types of somatic cells.

It is known that many viruses have developed certain mechanisms to avoid the host antiviral responses. Repressing IFN α/β induction in host cells is one such mechanism (36). However, the capacity of these viruses to repress IFN α/β induction seems to depend on the types of host cells, as we demonstrated in 10T1/2 cells in which LACV can induce strong transcription of *Ifn α/β* as well as the key components involved in IFN α/β expression, such as *Rig-I*, *Mda5*, and *Tlr3*. The induction of IFN β by WNV and SeV in 10T1/2 cells was also significant. Therefore, the failure to express IFN α/β in mESCs is not virus-specific or due to the IFN evasion strategies of viruses. This conclusion is in line with the observations recently reported by Wash *et al.* (37) that herpes simplex virus type I (HSV-1, a DNA virus) and influenza virus (a negative sense ssRNA virus) caused cytopathic effects without evoking type I IFN induction. Together with results from the experiments with polyIC and 3p-ssRNA,⁴ which mimic viral RNA in inducing a robust IFN α/β expression in 10T1/2 cells but not in mESCs, we conclude that deficiency in type I IFN expression is an intrinsic property of mESCs.

We have provided the molecular basis that explains the defective IFN α/β expression in mESCs. In some cell types, polyIC can induce IFN α/β via activation of TLR3 at the cell surface or in the endosomes (11, 38, 39). When transfected into the cells, polyIC can induce robust IFN α/β expression and

⁴ R. Wang, F. Huang, and Y.-L. Guo, unpublished data.

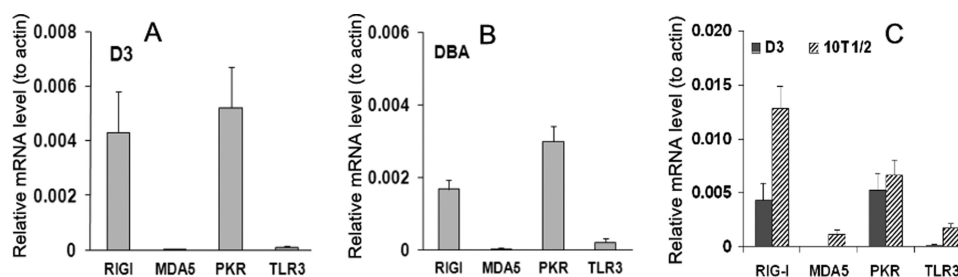


FIGURE 6. **Relative mRNA levels of dsRNA receptors in mESCs and 10T1/2 cells.** The mRNA levels of dsRNA receptors in D3 (A), DBA252 (B), and 10T1/2 (C) were determined by RT-qPCR. Their relative abundance in each cell was determined by comparing with the mRNA level of β -actin (designated as 1). For a direct comparison, the relative mRNA level of each molecule in D3 cells and 10T1/2 cells is presented in C. The results are means \pm S.D. of three independent experiments in all panels.

other responses that are similar to those evoked by viral infection via cytoplasmic dsRNA receptors (32, 40). mESCs were unresponsive to polyIC that was directly added to the medium, likely due to the very low level expression of TLR3. However, polyIC transfected into the mESCs showed a profound inhibitory effect on proliferation, a known effect of polyIC on many types of differentiated cells, indicating that the cytoplasmic receptors for polyIC are active in mESCs.

One of the distinctive features of mESCs is their rapid cell proliferation that is driven by constitutively active Cdk2-cyclin A/E complexes (41, 42). It is conceivable that actively dividing mESCs are more sensitive to polyIC-inhibited translation because cell proliferation critically depends on *de novo* protein synthesis. We had expected that polyIC-inhibited cell proliferation could significantly affect their stem cell state. However, morphology of the individual cells and colonies appeared to be maintained at the undifferentiated state despite their transient decrease in transcription of pluripotency markers. Because polyIC caused similar changes to the transcription of differentiation markers, the effect is not gene-specific, but rather a general transcription disturbance similar to that caused by viral infection (13). We speculate that the nonspecific transcription interference might help mESCs maintain their stem cell state by preventing the expression of genes that promote differentiation, at least for a short period of time under our experimental conditions. PolyIC-induced cell cycle inhibition is transient because polyIC-treated cells can resume a normal rate of proliferation in subculture when they were passed to new culture dishes. Thus, the transient change of pluripotency and differentiation markers is not surprising because the experiments were performed in the presence of leukemia inhibitory factor, which maintains the pluripotency and prevents differentiation of mESCs. This observation is in line with our recent study demonstrating that transient inhibition of cell proliferation does not compromise the stem cell state of mESCs (26).

It is generally believed that MDA5 and RIG-I play primary roles in mediating viral RNA-induced IFN α/β expression in the cytoplasm (11, 39), although PKR also contributes to and modulates this process (13, 14). Because MDA5 is expressed at a negligible level in mESCs, it is conceivable that PKR and/or RIG-I may mediate the effects of transfected polyIC. However, the RIG-I signaling pathway seems to be inactive because silencing RIG-I did not affect the effects of polyIC and because 3p-ssRNA (5'-triphosphate single-stranded RNA), the best studied ligands of RIG-I (43, 44), failed to induce IFN β in

mESCs. Nevertheless, substantial evidence indicates that PKR plays a key role in mediating the effects of polyIC, as demonstrated by the phosphorylation of eIF2 α and the results observed in the PKR silencing experiments. It is known that IFN-induced activation of ribonuclease L (RNase L) can hydrolyze both cellular and viral RNA (14). Although polyIC could lead to RNase L activation in some cells (45), this does not seem to be the case in mESCs because polyIC did not cause rRNA degradation (data not shown), which is a commonly used indicator of RNase L activation (45). The result further supports our conclusion that polyIC could not induce a sufficient level of IFN α/β in mESCs for RNase L activation.

LACV infection also activated PKR in mESCs, similar to the finding in murine embryonic fibroblasts (46). However, silencing or inhibition of PKR did not significantly affect LACV-induced cell death of mESCs, which is in line with the observation that PKR activation has little impact on viral replication as demonstrated in PKR^{-/-} murine embryonic fibroblasts (46). PKR activation has been implicated in apoptosis, but its roles are stimulus- and virus-dependent (47). The effects of PKR activation by LACV on mESCs could be more complex than polyIC due to the fact that many other cellular and viral factors are involved during infection. Unlike LACV infection, we did not detect PKR activation in the mESCs infected with WNV or SeV. Failure to activate PKR by these viruses has been noted in other cells. As recently reported in rodent cells, WNV infection does not induce PKR activation, possibly by a mechanism that sequesters viral dsRNA from host PKR (48). In the case of SeV, it is proposed that PKR activation is repressed by the C protein encoded by the virus (49). Whether or not these mechanisms are utilized in mESCs remains to be investigated.

It is interesting to note that hESCs do not express IFN α/β even at the mRNA level when transfected with polyIC (6, 16). mESCs can express limited transcripts of *Ifn α/β* , *Il6*, and *Icam1*, but they could not produce detectable levels of their respective proteins. Thus, the transcription of these immune mediators in mESCs may have limited function, if any, as an antiviral mechanism. In speculating the physiological significance of dsRNA-induced cellular events in ESCs, it is worth mentioning that in differentiated cells, dsRNA can be formed under some circumstances, but they are retained in the nucleus for editing and rarely exported to the cytoplasm (50). However, the nuclear retention mechanism in ESCs is less effective. As a result, dsRNAs may be exported to the cytoplasm in ESCs where they activate dsRNA receptors (51). In addition, other

mESCs Are Deficient in Type I IFN Expression

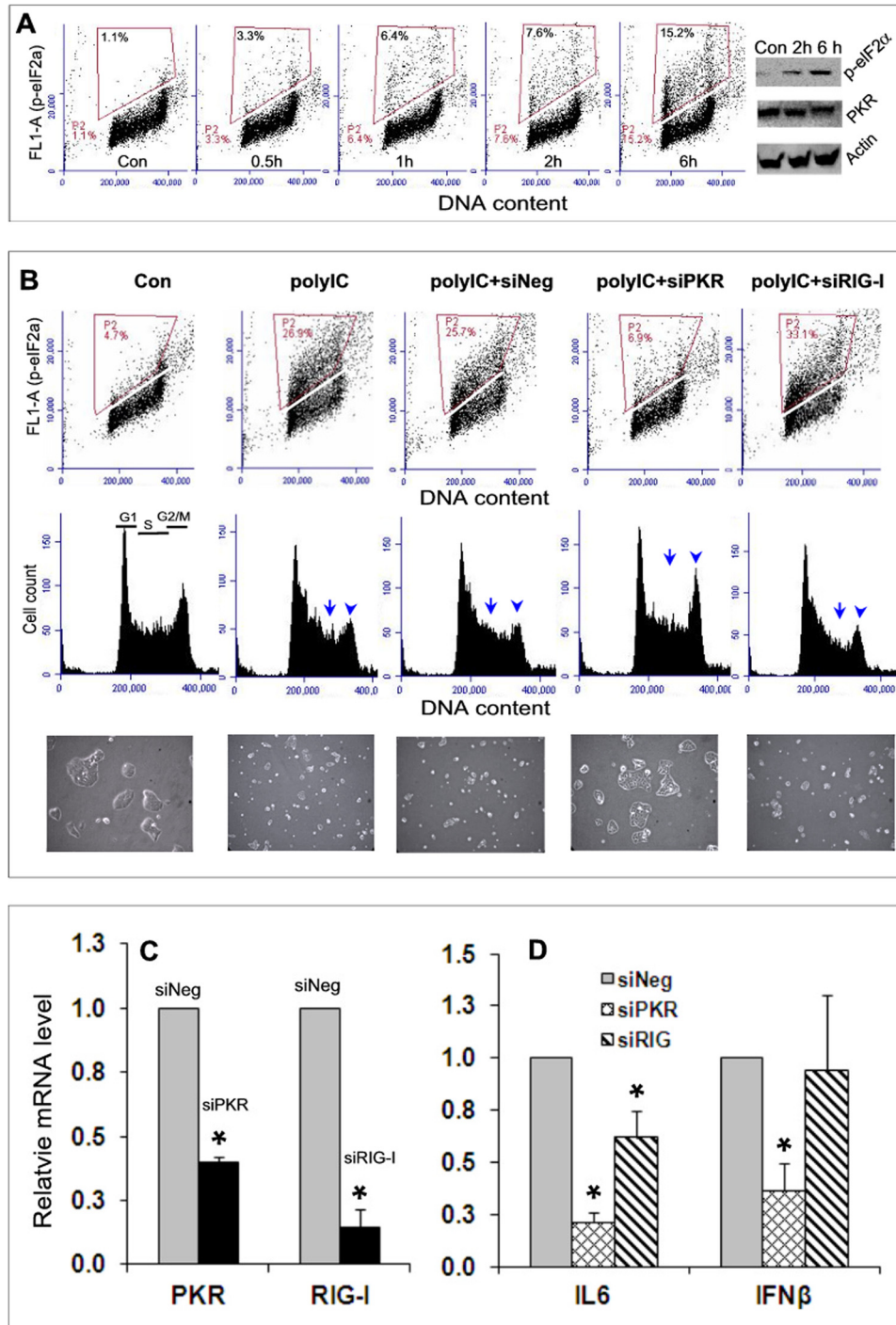


FIGURE 7. PKR plays key roles in mediating the effects of polyIC. *A*, polyIC-induced phosphorylation of eIF2 α . D3 cells were transfected with 300 ng/ml polyIC and incubated for the times indicated. The cells with phosphorylated eIF2 α (*p-eIF2 α*) were quantified by flow cytometry (boxed areas). White slash lines were used to help identify the bottom sides of the boxes. The blot insets illustrate the levels of p-eIF2 α and PKR determined by Western blot. Actin was used as a loading control. *B*, effects of PKR and RIG-I silencing on polyIC-induced cellular events. D3 cells were transfected with siRNA against PKR (siPKR), RIG-I (siRIG-I), or negative control siRNA (siNeg) for 24 h. The cells were split into new dishes. After 24 h, the cells were transfected with polyIC for 24 h and analyzed for p-eIF2 α by flow cytometry (upper panels), cell cycle by flow cytometry (middle panels, the changes in S and G₂/M phase cells are indicated by the arrow and arrowhead, respectively), and cell proliferation/colony formation by microscopy (lower panels). *C* and *D*, relative contributions of PKR and RIG-I to polyIC-induced mRNA of IL6 and IFN β . D3 cells were transfected with siPKR, siRIG-I, or negative control siRNA followed by polyIC treatment as described in *B*. The mRNA levels of PKR and RIG-I (*C*) or IL6 and IFN β (*D*) were determined by RT-qPCR and compared with the mRNA in the cells that were transfected with negative control siRNA (defined as 1) in each group. The results are means \pm S.D. of three independent experiments. The difference is considered to be statistically significant when $p < 0.05$ (*). All representative experiments in *A* and *B* were performed at least twice with similar results.

types of abnormal RNAs, such as those derived from cell death or from RNA processing, could activate PKR (52, 53). In particular, PKR is considered to be a “sentinel kinase for stress” for its

involvement in stress response in addition to antiviral response (54). Therefore, it appears that dsRNA-induced inhibition of translation and cell proliferation in mESCs could be an impor-

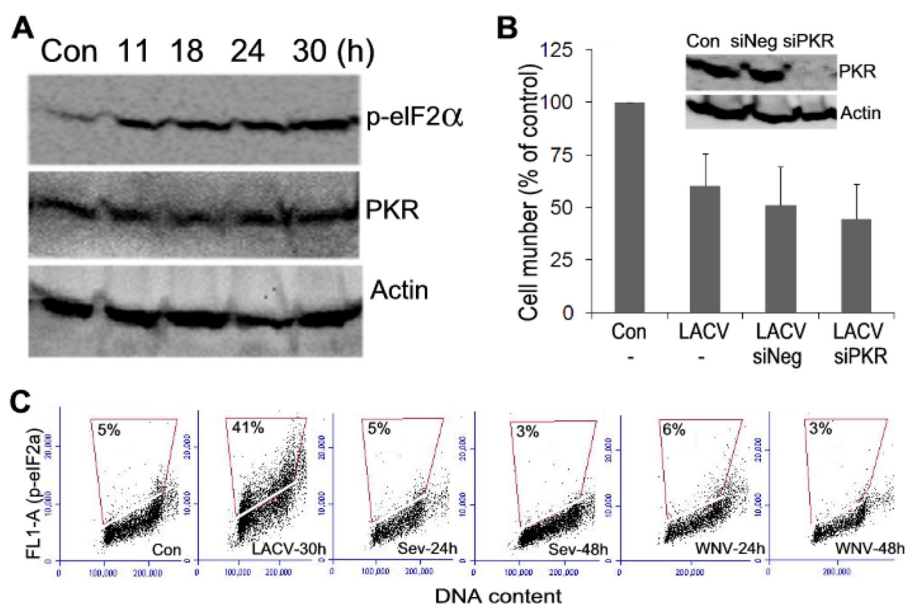


FIGURE 8. Effects of viral infections on PKR activation. *A*, activation of PKR by LACV infection in mESCs. D3 cells were infected with LACV (m.o.i. = 10) for the different time periods as indicated. Activation of PKR was analyzed by Western blot with antibodies against p-eIF2 α . Actin was used as a control for protein loading. *B*, silencing PKR did not have an apparent effect on LACV-induced cell death. D3 cells were transfected with siRNA against PKR (*siPKR*) or control siRNA (*siNeg*) as described in Fig. 7C. The efficiency and specificity of PKR knockdown were analyzed by Western blot (*blot inset*) after 36 h of transfection. *Con* represents the cells without transfection. The cells, either transfected with siRNA (negative control siRNA or *siPKR*) or without (*Con*), were infected with LACV at an m.o.i. of 10. The cell viability was determined at 48 h post-infection as described in Fig. 1. *C*, WNV and SeV infection did not activate PKR in mESCs. D3 cells were infected with WNV (m.o.i. = 10) or SeV (300 hemagglutination units/ml) for the specified times. The cells with p-eIF2 α were quantified by flow cytometry (*boxed areas, white slash lines* were used to help identify the bottom sides of the boxes). Cells infected with LACV (m.o.i. = 10) for 30 h were used as a positive control for comparison. The values shown in the graph (*B*) are means \pm S.D. of three independent experiments. The results in all other panels are representative of experiments performed at least twice with similar results.

tant mechanism that helps cells deal with immunogenic stresses.

In summary, our study demonstrates that expression of type I IFN as a crucial part of antiviral responses is underdeveloped in mESCs. Our study may open up an important area in ESC research for understanding the development of antiviral mechanisms during embryogenesis and how the stress caused by immunogenic signals affects ESC physiology.

Acknowledgments—We thank Dr. John F. Anderson (Connecticut Agricultural Experiment Station) for providing LACV and WNV; Dr. Samantha Soldan (University of Pennsylvania School of Medicine) for providing LACV Gc antibodies; Joseph Spangler for providing 3p-ss-RNA; and Baobin Kang for assistance in microscopy. We also thank Mississippi-IDEA Network of Biomedical Research Excellence for the use of the imaging facility. We are grateful to Rachel Payne for critical reading of the manuscript.

REFERENCES

- Niwa, H. (2007) How is pluripotency determined and maintained? *Development* **134**, 635–646
- Wobus, A. M., and Boheler, K. R. (2005) Embryonic stem cells: prospects for developmental biology and cell therapy. *Physiol. Rev.* **85**, 635–678
- Hentze, H., Soong, P. L., Wang, S. T., Phillips, B. W., Putti, T. C., and Dunn, N. R. (2009) Teratoma formation by human embryonic stem cells: Evaluation of essential parameters for future safety studies. *Stem Cell Res.* **2**, 198–210
- Balconi, G., Spagnuolo, R., and Dejana, E. (2000) Development of endothelial cell lines from embryonic stem cells: a tool for studying genetically manipulated endothelial cells *in vitro*. *Arterioscler. Thromb. Vasc. Biol.* **20**, 1443–1451
- Vittet, D., Prandini, M. H., Berthier, R., Schweitzer, A., Martin-Sisteron, H., Uzan, G., and Dejana, E. (1996) Embryonic stem cells differentiate *in vitro* to endothelial cells through successive maturation steps. *Blood* **88**, 3424–3431
- Földes, G., Liu, A., Badiger, R., Paul-Clark, M., Moreno, L., Lendvai, Z., Wright, J. S., Ali, N. N., Harding, S. E., and Mitchell, J. A. (2010) Innate immunity in human embryonic stem cells: comparison with adult human endothelial cells. *PLoS ONE* **5**, e10501
- Zampetaki, A., Xiao, Q., Zeng, L., Hu, Y., and Xu, Q. (2006) TLR4 expression in mouse embryonic stem cells and in stem cell-derived vascular cells is regulated by epigenetic modifications. *Biochem. Biophys. Res. Commun.* **347**, 89–99
- Glaser, D. E., Gower, R. M., Lauer, N. E., Tam, K., Blancas, A. A., Shih, A. J., Simon, S. I., and McCloskey, K. E. (2011) Functional characterization of embryonic stem cell-derived endothelial cells. *J. Vasc. Res.* **48**, 415–428
- Rajan, S., Ye, J., Bai, S., Huang, F., and Guo, Y. L. (2008) NF- κ B, but not p38 MAP kinase, is required for TNF- α -induced expression of cell adhesion molecules in endothelial cells. *J. Cell. Biochem.* **105**, 477–486
- Kawai, T., and Akira, S. (2011) Toll-like receptors and their cross-talk with other innate receptors in infection and immunity. *Immunity* **34**, 637–650
- Stetson, D. B., and Medzhitov, R. (2006) Type I interferons in host defense. *Immunity* **25**, 373–381
- Kato, H., Takahashi, K., and Fujita, T. (2011) RIG-I-like receptors: cytoplasmic sensors for non-self RNA. *Immunol. Rev.* **243**, 91–98
- García, M. A., Meurs, E. F., and Esteban, M. (2007) The dsRNA protein kinase PKR: virus and cell control. *Biochimie* **89**, 799–811
- Samuel, C. E. (2001) Antiviral actions of interferons. *Clin. Microbiol. Rev.* **14**, 778–809
- Levy, O. (2007) Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nat. Rev. Immunol.* **7**, 379–390
- Chen, L. L., Yang, L., and Carmichael, G. G. (2010) Molecular basis for an attenuated cytoplasmic dsRNA response in human embryonic stem cells. *Cell Cycle* **9**, 3552–3564
- Yu, J., Rossi, R., Hale, C., Goulding, D., and Dougan, G. (2009) Interaction of enteric bacterial pathogens with murine embryonic stem cells. *Infect. Immun.* **77**, 585–597

mESCs Are Deficient in Type I IFN Expression

18. Toumadje, A., Kusumoto, K. I., Parton, A., Mericko, P., Dowell, L., Ma, G., Chen, L., Barnes, D., and Denry Sato, J. (2003) Pluripotent differentiation *in vitro* of murine Es-D3 embryonic stem cells. *In Vitro Cell Dev. Biol. Anim.* **39**, 449–453
19. Chakraborty, S., Kang, B., Huang, F., and Guo, Y. L. (2009) Mouse embryonic stem cells lacking p38 α and p38 δ can differentiate to endothelial cells, smooth muscle cells, and epithelial cells. *Differentiation* **78**, 143–150
20. Guo, Y. L., and Yang, B. (2006) Altered cell adhesion and cell viability in a p38 α mitogen-activated protein kinase-deficient mouse embryonic stem cell line. *Stem Cells Dev.* **15**, 655–664
21. Guo, Y. L., Ye, J., and Huang, F. (2007) p38 α MAP kinase-deficient mouse embryonic stem cells can differentiate to endothelial cells, smooth muscle cells, and neurons. *Dev. Dyn.* **236**, 3383–3392
22. Bai, F., Wang, T., Pal, U., Bao, F., Gould, L. H., and Fikrig, E. (2005) Use of RNA interference to prevent lethal murine West Nile virus infection. *J. Infect. Dis.* **191**, 1148–1154
23. Pfaffl, M. W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45
24. Prabhakar, U., Eirikis, E., and Davis, H. M. (2002) Simultaneous quantification of proinflammatory cytokines in human plasma using the LabMA assay. *J. Immunol. Methods* **260**, 207–218
25. Guo, Y. L., Chakraborty, S., Rajan, S. S., Wang, R., and Huang, F. (2010) Effects of oxidative stress on mouse embryonic stem cell proliferation, apoptosis, senescence, and self-renewal. *Stem Cells Dev.* **19**, 1321–1331
26. Wang, R., and Guo, Y. L. (2012) Transient inhibition of cell proliferation does not compromise self-renewal of mouse embryonic stem cells. *Exp. Cell Res.* **318**, 2094–2104
27. Verbruggen, P., Ruf, M., Blakqori, G., Överby, A. K., Heidemann, M., Eick, D., and Weber, F. (2011) Interferon antagonist NSs of La Crosse virus triggers a DNA damage response-like degradation of transcribing RNA polymerase II. *J. Biol. Chem.* **286**, 3681–3692
28. Haas, A. R., and Tuan, R. S. (2000) Murine C3H10T1/2 multipotential cells as an *in vitro* model of mesenchymal chondrogenesis. *Methods Mol. Biol.* **137**, 383–389
29. Soldan, S. S., Hollidge, B. S., Wagner, V., Weber, F., and González-Scarano, F. (2010) La Crosse virus (LACV) Gc fusion peptide mutants have impaired growth and fusion phenotypes but remain neurotoxic. *Virology* **404**, 139–147
30. Hanna, S. L., Pierson, T. C., Sanchez, M. D., Ahmed, A. A., Murtadha, M. M., and Doms, R. W. (2005) N-Linked glycosylation of West Nile virus envelope proteins influences particle assembly and infectivity. *J. Virol.* **79**, 13262–13274
31. Ye, J., and Maniatis, T. (2011) Negative regulation of interferon- β gene expression during acute and persistent virus infections. *PLoS ONE* **6**, e20681
32. Fortier, M. E., Kent, S., Ashdown, H., Poole, S., Boksa, P., and Luheshi, G. N. (2004) The viral mimic, polyinosinic:polycytidylic acid, induces fever in rats via an interleukin-1-dependent mechanism. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **287**, R759–R766
33. Matsumoto, M., Funami, K., Tanabe, M., Oshiumi, H., Shingai, M., Seto, Y., Yamamoto, A., and Seya, T. (2003) Subcellular localization of toll-like receptor 3 in human dendritic cells. *J. Immunol.* **171**, 3154–3162
34. Vercammen, E., Staal, J., and Beyaert, R. (2008) Sensing of viral infection and activation of innate immunity by Toll-like receptor 3. *Clin. Microbiol. Rev.* **21**, 13–25
35. Stead, E., White, J., Faast, R., Conn, S., Goldstone, S., Rathjen, J., Dhingra, U., Rathjen, P., Walker, D., and Dalton, S. (2002) Pluripotent cell division cycles are driven by ectopic Cdk2, cyclin A/E and E2F activities. *Oncogene* **21**, 8320–8333
36. Haller, O., Kochs, G., and Weber, F. (2006) The interferon response circuit: induction and suppression by pathogenic viruses. *Virology* **344**, 119–130
37. Wash, R., Calabressi, S., Franz, S., Griffiths, S. J., Goulding, D., Tan, E.-P., Wise, H., Digard, P., Haas, J., Efstathiou, S., and Kellam, P. (2012) Permissive and restricted virus infection of murine embryonic stem cells. *J. Gen. Virol.* **93**, 2118–2130
38. Meylan, E., and Tschopp, J. (2006) Toll-like receptors and RNA helicases: two parallel ways to trigger antiviral responses. *Mol. Cell* **22**, 561–569
39. Nasirudeen, A. M., Wong, H. H., Thien, P., Xu, S., Lam, K. P., and Liu, D. X. (2011) RIG-I, MDA5 and TLR3 synergistically play an important role in restriction of dengue virus infection. *PLoS Negl. Trop. Dis.* **5**, e926
40. Matsumoto, M., and Seya, T. (2008) TLR3: Interferon induction by double-stranded RNA including poly(I:C). *Adv. Drug. Deliv. Rev.* **60**, 805–812
41. Singh, A. M., and Dalton, S. (2009) The Cell cycle and Myc intersect with mechanisms that regulate pluripotency and reprogramming. *Cell Stem Cell* **5**, 141–149
42. Burdon, T., Chambers, I., Stracey, C., Niwa, H., and Smith, A. (1999) Signaling mechanisms regulating self-renewal and differentiation of pluripotent embryonic stem cells. *Cells Tissues Organs* **165**, 131–143
43. Pichlmair, A., Schulz, O., Tan, C. P., Nöslund, T. I., Liljestrom, P., Weber, F., and Reis e Sousa, C. (2006) RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* **314**, 997–1001
44. Yoneyama, M., and Fujita, T. (2007) Function of RIG-I-like receptors in antiviral innate immunity. *J. Biol. Chem.* **282**, 15315–15318
45. Castelli, J. C., Hassel, B. A., Wood, K. A., Li, X. L., Amemiya, K., Dalakas, M. C., Torrence, P. F., and Youle, R. J. (1997) A Study of the interferon antiviral mechanism: apoptosis activation by the 2–5A System. *J. Exp. Med.* **186**, 967–972
46. Streitenfeld, H., Boyd, A., Fazakerley, J. K., Bridgen, A., Elliott, R. M., and Weber, F. (2003) Activation of PKR by Bunyamwera virus is independent of the viral interferon antagonist NSs. *J. Virol.* **77**, 5507–5511
47. Zhang, P., and Samuel, C. E. (2007) Protein kinase PKR plays a stimulus- and virus-dependent role in apoptotic death and virus multiplication in human cells. *J. Virol.* **81**, 8192–8200
48. Elbahesh, H., Scherbik, S. V., and Brinton, M. A. (2011) West Nile virus infection does not induce PKR activation in rodent cells. *Virology* **421**, 51–60
49. Takeuchi, K., Komatsu, T., Kitagawa, Y., Sada, K., and Gotoh, B. (2008) Sendai Virus C protein plays a role in restricting PKR activation by limiting the generation of intracellular double-stranded RNA. *J. Virol.* **82**, 10102–10110
50. Zhang, Z., and Carmichael, G. G. (2001) The fate of dsRNA in the nucleus: A p54nrb-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs. *Cell* **106**, 465–475
51. Chen, L. L., and Carmichael, G. G. (2009) Altered nuclear retention of mRNAs containing inverted repeats in human embryonic stem cells: functional role of a nuclear noncoding RNA. *Mol. Cell* **35**, 467–478
52. Amarante, M. K., and Watanabe, M. A. (2010) Toll-like receptor 3: involvement with exogenous and endogenous RNA. *Int. Rev. Immunol.* **29**, 557–573
53. Yu, L., Wang, L., and Chen, S. (2010) Endogenous toll-like receptor ligands and their biological significance. *J. Cell. Mol. Med.* **14**, 2592–2603
54. Williams, B. R. (1999) PKR; a sentinel kinase for cellular stress. *Oncogene* **18**, 6112–6120