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Antiviral Responses in Mouse Embryonic Stem Cells

DIFFERENTIAL DEVELOPMENT OF CELLULAR MECHANISMS IN TYPE I INTERFERON PRODUCTION AND RESPONSE^{*§}

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Background: mESCs are deficient in type I IFN expression.

Results: mESCs can respond to type I IFNs and express interferon-stimulated genes.

Conclusion: mESCs are unable to express type I IFNs but can respond to type I IFNs.

Significance: The findings are important for understanding the antiviral mechanisms and innate immunity in ESCs.

We have recently reported that mouse embryonic stem cells (mESCs) are deficient in expressing type I interferons (IFNs) in response to viral infection and synthetic viral RNA analogs (Wang, R., Wang, J., Paul, A. M., Acharya, D., Bai, F., Huang, F., and Guo, Y. L. (2013) *J. Biol. Chem.* 288, 15926–15936). Here, we report that mESCs are able to respond to type I IFNs, express IFN-stimulated genes, and mediate the antiviral effect of type I IFNs against La Crosse virus and chikungunya virus. The major signaling components in the IFN pathway are expressed in mESCs. Therefore, the basic molecular mechanisms that mediate the effects of type I IFNs are functional in mESCs; however, these mechanisms may not yet be fully developed as mESCs express lower levels of IFN-stimulated genes and display weaker antiviral activity in response to type I IFNs when compared with fibroblasts. Further analysis demonstrated that type I IFNs do not affect the stem cell state of mESCs. We conclude that mESCs are deficient in type I IFN expression, but they can respond to and mediate the cellular effects of type I IFNs. These findings represent unique and uncharacterized properties of mESCs and are important for understanding innate immunity development and ESC physiology.

Embryonic stem cells (ESCs)² can proliferate continuously under proper culture conditions. When induced, they can differentiate into different cell lineages. These properties, defined as self-renewal and pluripotency, respectively (1, 2), make ESCs a promising cell source for regenerative medicine. Although the benefit of ESC research in medical applications is exciting, currently there is limited understanding of the basic physiology of ESCs and their derived cells. Several recent studies (3–5),

including our own (6), have demonstrated that ESC-derived cells have limited response to inflammatory cytokines and various infectious agents. When used for cell therapy, ESC-derived cells would be placed in a wounded area of the patient that is likely to be exposed to various pathogens. Therefore, the lack of innate immunity in ESC-derived cells may conceivably affect their fate and functionality.

Cellular innate immunity is mediated by toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). TLRs, localized on the cell surface or on the membrane of endosomes, detect a wide variety of molecules that evoke immune responses (7). RLRs, including RIG-I and MDA5 (melanoma differentiation-associated gene 5), reside in the cytosol and primarily recognize viral RNA (7, 8). Upon binding with their ligands, TLRs and RLRs activate several signaling pathways, which coordinately regulate the expression of type I IFNs and pro-inflammatory cytokines that participate in various aspects of the immune responses (7, 8). Innate immunity is presumably developed in most, if not all, cell types (9). However, several recent studies have indicated that neither human ESCs (hESCs) (3) nor mESCs (4, 10) can mount effective innate immune responses to common infectious agents, including live bacteria (11) and viruses (12). Our recent study in mESCs (13, 14) and studies from other investigators in hESCs (15) demonstrated that the cellular mechanisms for expressing type I IFNs are not functional in these cells. Therefore, ESCs, normally residing in the womb, may not have active (or fully active) antiviral mechanisms as in differentiated somatic cells. These findings also suggest that the current *in vitro* differentiation methods do not effectively promote innate immunity development, which explains the defective immune responses observed in ESC-derived cells (3, 4).

In response to pathogen invasions, especially viral infections, the cells rapidly synthesize and secrete type I IFNs, a family of cytokines that include IFN α and IFN β , the two best studied members, and several other less characterized members, such as IFN ϵ and IFN ω (16). Once synthesized and secreted, type I IFNs act through autocrine and paracrine mechanisms by binding to a common cell surface receptor complex composed of the IFNAR1 and IFNAR2 subunits. The activated receptor triggers the activation of Janus tyrosine kinases (JAK1 and TYK2) in the

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§ This article contains supplemental Table S1.

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² The abbreviations used are: ESC, embryonic stem cell; mESC, mouse ESC; hESC, human ESC; ISG, IFN-stimulated gene; poly(I-C), polyinosinic-polycytidylic acid; LACV, La Crosse virus; CHIKV, Chikungunya virus; WNV, West Nile virus; TLR, toll-like receptor; RLR, RIG-I-like receptor; qPCR, quantitative PCR; m.o.i., multiplicity of infection; FB, fibroblast; RA, retinoic acid.

cytosol, which phosphorylate signal transducers and activators of transcription (STAT1 and STAT2). Phosphorylated STAT1 and STAT2 translocate to the nucleus where they induce the transcription of various genes, known as IFN-stimulated genes (ISGs), which participate in various aspects of antiviral activities and promote the cell to enter an “antiviral state” (17–19).

Although IFN production and responding systems are evolutionally conserved among different cell types in different species of mammals, recent studies suggest that the molecular mechanisms for type I IFN production and action in mESCs (13) and hESCs (15) may fundamentally differ from differentiated somatic cells. Although these studies demonstrate that both hESCs and mESCs are deficient in producing type I IFNs, the next logical question to be asked is whether or not they can respond to type I IFNs. In this report, we demonstrate that mESCs have basic functional mechanisms to detect and respond to type I IFNs, which differ from hESCs that have limited or no responses to IFN β (20).

EXPERIMENTAL PROCEDURES

Cell Culture—D3 and DBA252 mESCs were maintained in the standard mESC medium as described previously (13). C3H10T1/2 cells (10T1/2, a line of mouse embryonic fibroblasts, ATCC) were cultured in DMEM that contains 10% fetal calf serum and 100 units/ml penicillin and 100 μ g/ml streptomycin. All cells were maintained at 37 °C in a humidified incubator with 5% CO₂. Most experiments were performed with D3 cells, and key results were confirmed with DBA252 cells.

Preparation of Virus Stocks and Titer Determination—La Crosse virus (LACV, SM6 v3), West Nile virus (WNV, strain CT 2741), and chikungunya virus (CHIKV, LR 2006 OPY1 strain) were propagated in Vero cells (African green monkey kidney cell line, ATCC). Titers of virus stocks were determined by plaque assay as described previously (21).

Fibroblast (FB) Differentiation from mESCs—Retinoic acid (RA)-induced mESC differentiation was performed according to the published method with some modifications (22). Cell differentiation was initiated by adding 1 μ M RA to mESCs grown in a culture dish coated with gelatin. The medium was refreshed three times during a 10-day period of differentiation. The differentiated cells, which formed a monolayer, were trypsinized and replated in an uncoated cell culture dish where FBs quickly attach within 30–45 min. Other types of cells floating in the medium were removed. Adhered cells have morphology similar to naturally differentiated 10T1/2 FBs and were designated as mESC-FBs.

Cell Treatment—mESCs and 10T1/2 cells were plated at ~40 and ~70% confluence, respectively, and cultured for ~24 h before experiments. The conditions for cell infection with different viruses were specified in individual experiments. The cellular responses to type I IFNs were determined with mouse recombinant IFN α (IFN α -2, 1 \times 10⁸ units/mg, eBioscience) and human recombinant IFN β or IFN ω (5 \times 10⁸ units/mg, 1 \times 10⁸ units/mg, respectively, PeproTech) that are active in mouse cells (23–25). The effects of IFNs on viral replication were determined by viral titers in the media of infected cells (21). For polyinosinic-polycytidylic acid (poly(I-C), a synthetic dsRNA) treatment, the cells were transfected with poly(I-C) using

DharmaFECT reagent (Thermo Scientific). The control cells were transfected with DharmaFECT reagent alone (13).

Real Time Quantitative Polymerase Chain Reaction (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 a MX3000PTM RT-PCR system (Stratagene), as reported previously (26). The mRNA level from RT-qPCR was calculated using the comparative C_t method (27). 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.

Cell Proliferation, Viability, and Cell Cycle Analysis—Cell proliferation and viability were determined by the number of viable cells after toluidine blue staining as we described previously (28). The absorbance at 630 nm of toluidine blue-stained cells was measured with a microtiter plate reader. The absorbance 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 as described previously (28).

Protein Analysis by Flow Cytometry—Cellular protein analysis by flow cytometry was performed according to our published method (29). Briefly, treated cells were incubated with the antibodies against the specific proteins to be analyzed, as specified in individual experiments. 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 as described previously (13).

Immunocytochemistry—Immunostaining was performed according to our published method (30). Briefly, cells were fixed with 4% paraformaldehyde and incubated with the following antibodies as specified in individual experiments: pSTAT1 (Cell Signaling Technology); N-cadherin (Santa Cruz Biotechnology); and NG2, metalloproteinase-14, or type IV collagen (Millipore). The cells were then incubated with rhodamine- or fluorescein isothiocyanate (FITC)-labeled secondary antibodies and examined under an LSM 510 laser-scanning confocal microscope (Zeiss).

siRNA Transfection—siRNA targeting suppressor of cytokine signaling 1 (SOCS1, from Santa Cruz Biotechnology) was transfected to cells with DharmaFECT reagent as described previously (13). The cells were then analyzed for knockdown efficiency and for mRNA levels of SOCS1.

Cell Lysate Preparation and Western Blot Analysis—Cells were 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 (28).

Statistical Analysis—Data are presented as the mean \pm S.D. derived either from three independent experiments or from a representative experiment performed in triplicate that was performed at least twice with similar results. Statistical analysis was performed using a two-tailed and paired Student's *t* test.

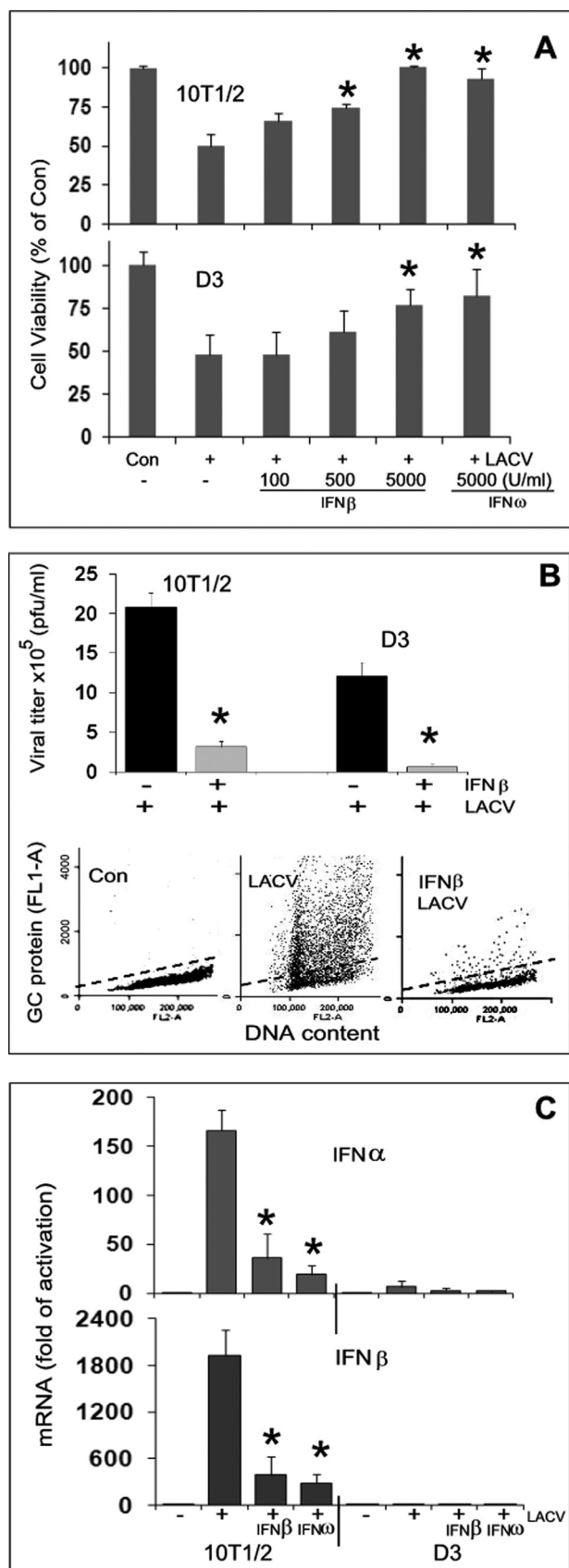


FIGURE 1. **IFN β and IFN ω protect 10T1/2 cells and mESCs from LACV-induced cell death.** A, 10T1/2 and D3 cells were pretreated with IFN β or IFN ω for 24 h or left untreated and then infected with LACV at m.o.i. of 1 and 10, respectively. Viable cells were determined at 48 h post-infection. The control

Differences are considered statistically significant when $p < 0.05$.

RESULTS

Type I IFNs Protect mESCs from LACV- and CHIKV-induced Cell Death and Repress Viral Replication—LACV is a negative sense single-stranded RNA virus that is known to cause lytic cell death of mammalian cells and is sensitive to IFN α/β (31, 32). We have previously reported that mESCs are deficient in type I IFN expression and are susceptible to LACV-induced cell death (13). Using this model, we analyzed the antiviral effects of IFN α , IFN β , and IFN ω , which represent two well studied and a less characterized type I IFNs.

We have previously shown that LACV at m.o.i. of 1 caused about 50% cell death of 10T1/2 cells within a 48-h incubation period, whereas similar cytopathic effects in mESCs were observed at much higher m.o.i. (5 and 10) (13). Although the low efficiency of LACV infection and/or replication in mESCs could be an intrinsic property of these pluripotent cells, it is noted that mESCs have a rapid proliferation rate (doubling time in ~ 6 h versus ~ 24 h in 10T1/2 cells), which may significantly alter the initial m.o.i. during the course of experiments. For this reason, mESCs were infected with high dose of LACV (m.o.i. 10). As shown in Fig. 1A, IFN β pretreatment protected both 10T1/2 and D3 cells from subsequent LACV-induced cell death in a dose-dependent manner. LACV-induced death of 10T1/2 cells was significantly attenuated by IFN β at 500 units/ml and was completely prevented at 5000 units/ml, whereas the protecting effect of IFN β on D3 cells was marginal at 500 units/ml but significantly increased at 5000 units/ml of IFN β or IFN ω (Fig. 1A).

The above results suggest that mESCs can mediate the antiviral effect of IFN β and IFN ω . To obtain further evidence, we analyzed the effects of IFN β on LACV replication. By determining the titer of virus released to the medium from LACV-infected cells, our results showed that the viral load was significantly reduced in both 10T1/2 and D3 cells that were pretreated with IFN β (Fig. 1B, graph). The repression of viral replication by IFN β was further confirmed by the reduced expression of the M-segment protein (Gc protein) encoded by the LACV genome (33). As shown in Fig. 1B (flow profiles), the expression of Gc protein was detected in a large population of D3 cells exposed to LACV, which was significantly reduced by IFN β .

We have previously shown that LACV-induced IFN α and IFN β expression precede lytic cell death in 10T1/2 cells (13). As shown in Fig. 1C, LACV-induced IFN α and IFN β expression in 10T1/2 cells was significantly reduced in the cells pretreated either with IFN β or IFN ω , which is likely due to the reduced viral replication. Conversely, D3 cells did not express IFN α or

(Con) represents cells without viral infection. B, cells were pretreated with 5000 units/ml IFN β for 24 h followed by infection with LACV for an additional 24 h under the conditions described in A. Graph shows LACV titers in the culture medium of infected cells as measured by plaque assay. Flow cytometry profile shows the cells that express LACV Gc protein (the populations above the dashed lines). Con represents cells without viral infection. C, induction of IFN α and IFN β mRNA by LACV infection (under the conditions described in B) was determined at 12 h post-infection. The mRNA level in control is designated as 1. *, $p < 0.05$, compared with virus-infected cells without IFN pretreatment.

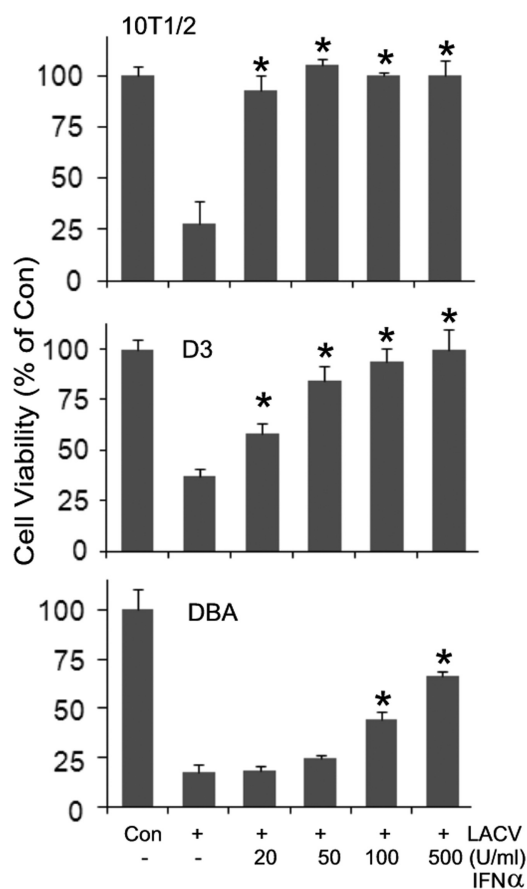


FIGURE 2. **IFN α protects 10T1/2 cells and mESCs from LACV-induced cell death.** Cells were pretreated with IFN α for 24 h or left untreated, followed by LACV infection at m.o.i. of 5. Viable cells were determined at 55 h post-infection. The control (Con) represents cells without viral infection. *, $p < 0.05$, compared with LACV-infected cells without IFN pretreatment.

IFN β in response to LACV infection as expected, and pretreatment with either IFN β or IFN ω had no additional effect. Therefore, IFN β or IFN ω can protect mESCs from the cytopathic effect of LACV, but they do not alter deficiency of these cells in expressing type I IFNs. Additional experiments with West Nile Virus (WNV) also showed that IFN β inhibited replication of WNV-infected D3 cells, although the effect of IFN β was less potent than in 10T1/2 cells (data not shown).

To confirm the results obtained from the experiments with IFN β , we further analyzed the antiviral activity of IFN α to LACV infection under conditions that differed from those used for IFN β , where both 10T1/2 cells and mESCs were infected with LACV at m.o.i. of 5 and were incubated for a longer incubation period (55 h). As shown in Fig. 2, LACV-induced cell death of 10T1/2 cells was effectively attenuated in the cells that were pretreated with 20 units/ml IFN α . LACV-induced death of D3 cells was also attenuated by IFN α in a dose-dependent manner, but the maximal effect was achieved at a much higher concentration (500 units/ml). The antiviral effect of IFN α was further confirmed in DBA mESCs (Fig. 2).

We further tested the antiviral effect of IFN α on CHIKV, a positive sense single-stranded RNA virus that is particularly effective in infecting fibroblasts and is sensitive to type I IFNs (34, 35). Infection of 10T1/2 cells with CHIKV at m.o.i. of 2 caused the death of almost all cells within a 48-h incubation

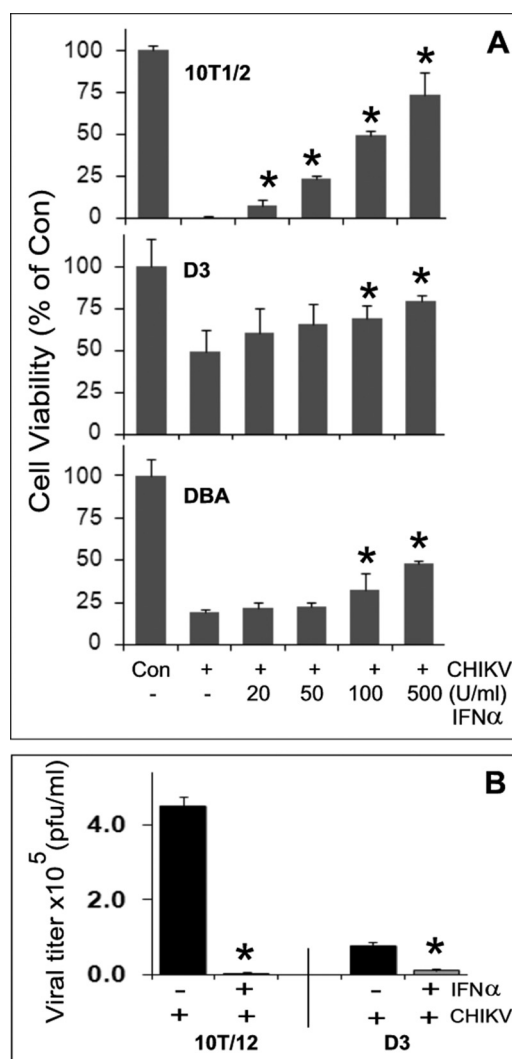


FIGURE 3. **IFN α protects 10T1/2 and mESCs from CHIKV-induced cell death.** A, 10T1/2 cells and mESCs (D3 and DBA) were pretreated with different concentrations of IFN α for 24 h or left untreated. The cells were then infected with CHIKV at an m.o.i. of 2. Viable cells were determined at 48 h post-infection. The control (Con) represents cells without viral infection. B, IFN α represses CHIKV replication in 10T1/2 and D3 cells. The cells were pretreated with 500 units/ml IFN α for 24 h followed by infection with CHIKV for an additional 24 h under the conditions described in A. The CHIKV titers in the culture medium of infected cells were measured by plaque assay. *, $p < 0.05$, compared with CHIKV-infected cells without IFN pretreatment.

period, which was attenuated by IFN α pretreatment in a dose-dependent manner (Fig. 3A, 10T1/2). CHIKV infection caused death of mESCs; however, the effect was less dramatic, and the protecting effect of IFN α in these cells is marginal at low concentrations but statistically significant above 100 units/ml (Fig. 3A, D3 and DBA). IFN α inhibited CHIKV replication in both 10T1/2 and D3 cells, which correlated with its antiviral activity (Fig. 3B).

Viral Infection-induced Antiviral Molecules in 10T1/2 Cells and mESCs and the Effects of IFNs—Induction of IFN-stimulated genes (ISGs) in response to viral infection plays key roles in host antiviral defense (17). We examined three representative ISGs as follows: 2'-5'-oligoadenylate synthetase 1 (OAS1), which activates ribonuclease L (RNase L), thereby hydrolyzing cellular and viral RNA; PKR, which inhibits protein synthesis

Type I IFN-induced Responses in mESCs

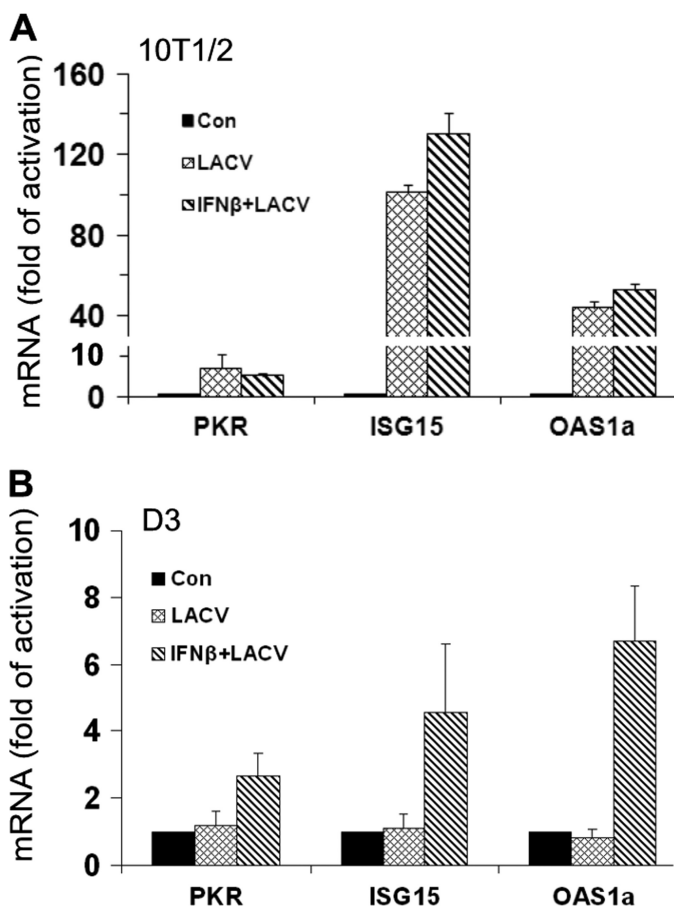


FIGURE 4. LACV infection-induced antiviral molecules and the effects of IFN β . 10T1/2 cells (A) and D3 cells (B) were infected with LACV at m.o.i. of 1 and 10, respectively, or they were pretreated with 5000 units/ml IFN β for 24 h followed by LACV infection (IFN β + LACV) for 12 h. The results are expressed as fold-activation where the mRNA level in control cells (Con, cells without viral infection) is designated as 1.

and host cell proliferation, thereby limiting viral replication; and ISG15, which is a ubiquitin-like protein that leads to the degradation of both host and viral proteins (17, 36, 37). As shown in Fig. 4A, LACV infection induced the expression of *Pkr*, *Oas1a*, and *Isg15* in 10T1/2 cells by 6, 44, and 100 times, respectively, with the expression of *Oas1a* and *Isg15* being further potentiated in the cells that were pretreated with IFN β . However, none of these genes in D3 cells were induced by LACV infection alone, but they were induced 3–8-fold in infected cells that were pretreated with IFN β (Fig. 4B). Similar observations were made when the experiments were performed with CHIKV and IFN α (data not shown).

A logical explanation for the above observations is that in 10T1/2 cells, LACV-induced type I IFNs (via autocrine signaling) were responsible for the expression of *Pkr*, *Oas1a*, and *Isg15* (Fig. 4A, LACV), whereas the expression of the three molecules in LACV-infected cells with IFN β pretreatment represents the combined effects of virus-induced IFNs and exogenously added IFN β (Fig. 4A, IFN β +LACV). However, the expression of the three genes was not induced by LACV infection due to their deficiency in expressing IFNs (Fig. 4B, LACV) (13). Therefore, the expression of *Pkr*, *Oas1a*, and *Isg15* in LACV-infected D3 cells with IFN β pretreatment

was solely induced by exogenously added IFN β (Fig. 4B, IFN β +LACV). This hypothesis was confirmed by the following experiments.

IFN-induced ISG Expression and the Priming Effect of IFN β —To determine the effects of IFNs alone on the expression of ISGs, we treated the cells with IFN β . As illustrated in Fig. 5A, IFN β induced the transcription of *Pkr*, *Oas1a*, and *Isg15* in both 10T1/2 cells (panel a) and mESCs (panel b), but the levels of their induction in mESCs (D3 and DBA cells), especially *Oas1a* and *Isg15*, were substantially lower than in 10T1/2 (panel b, note the different scales of y axis in panels a and b). Further analysis by Western blot showed that IFN β induced PKR in a dose-dependent manner in both D3 and 10T1/2 cells (Fig. 5A, panel c), paralleling its mRNA induction.

In differentiated cells, it is known that pretreatment of cells with IFNs enhances their antiviral activities against subsequent viral infection, a phenomenon known as IFN priming (38). Our results from viral infection experiments indicate that the priming mechanism is functional in both 10T1/2 cells and mESCs. Because the priming effect is partly attributed to the up-regulation of viral RNA receptors, we tested whether this is the case in mESCs. D3 cells were pretreated with IFN β (priming) followed by transfection with poly(I-C) as a viral RNA mimic. As shown in Fig. 5B, poly(I-C) or IFN β alone slightly induced the expression of *Rig-I* and *Tlr3* (two major receptors for viral RNAs). However, the effect of poly(I-C) was strongly potentiated in the cells that were pretreated with IFN β , a pattern that fits well with the IFN priming described in differentiated cells (38).

We further tested the responsiveness of D3 and 10T1/2 cells to different concentrations of IFN α , which induced the expression of *Pkr* and *Isg15* in a dose-dependent manner in both cell types. Similar to IFN β , 10T1/2 cells showed higher sensitivity and responsiveness to IFN α than D3 cells (Fig. 5C, panel a versus panel b). Although IFN α is more effective than IFN β in both cell types, the overall expression patterns of *Pkr* and *Isg15* induced by the two cytokines are similar (Fig. 5, A and C).

Relative Expression Levels of Type I IFN Signaling Molecules and IFN α -induced Activation of STAT1 in mESCs and 10T1/2 Cells—Our results thus far indicate that the mechanisms that mediate the effects of type I IFNs are operational in mESCs, but the levels of ISG induction are substantially lower than 10T1/2 cells. To determine the reasons for these discrepancies, we analyzed the expression levels of the major signaling molecules in the IFN pathway in untreated D3 and 10T1/2 cells. As shown in Fig. 6, RT-qPCR analysis indicated that the mRNAs of *Ifnar1*, *Jak1*, and *Stat1* are expressed at comparable levels in D3 and 10T1/2 cells, whereas the mRNA levels of *Tyk2*, *Stat2*, and *Irf9* are higher in D3 cells than in 10T1/2 cells. The only gene with low mRNA in D3 cells is *Ifnar2* (Fig. 6A). At the protein level, STAT1, a major transcription factor that mediates the effects of type I IFNs, was readily detected with its antibody in 10T1/2 and D3 cells, with a slightly higher expression level in D3 cells (Fig. 6B).

Activation of STAT1 transcription factor is a crucial step in cellular responses to type I IFNs. In resting cells, STAT1 is localized in the cytoplasm. Upon cell stimulation, STAT1 is phosphorylated and translocated to the nucleus where it initi-

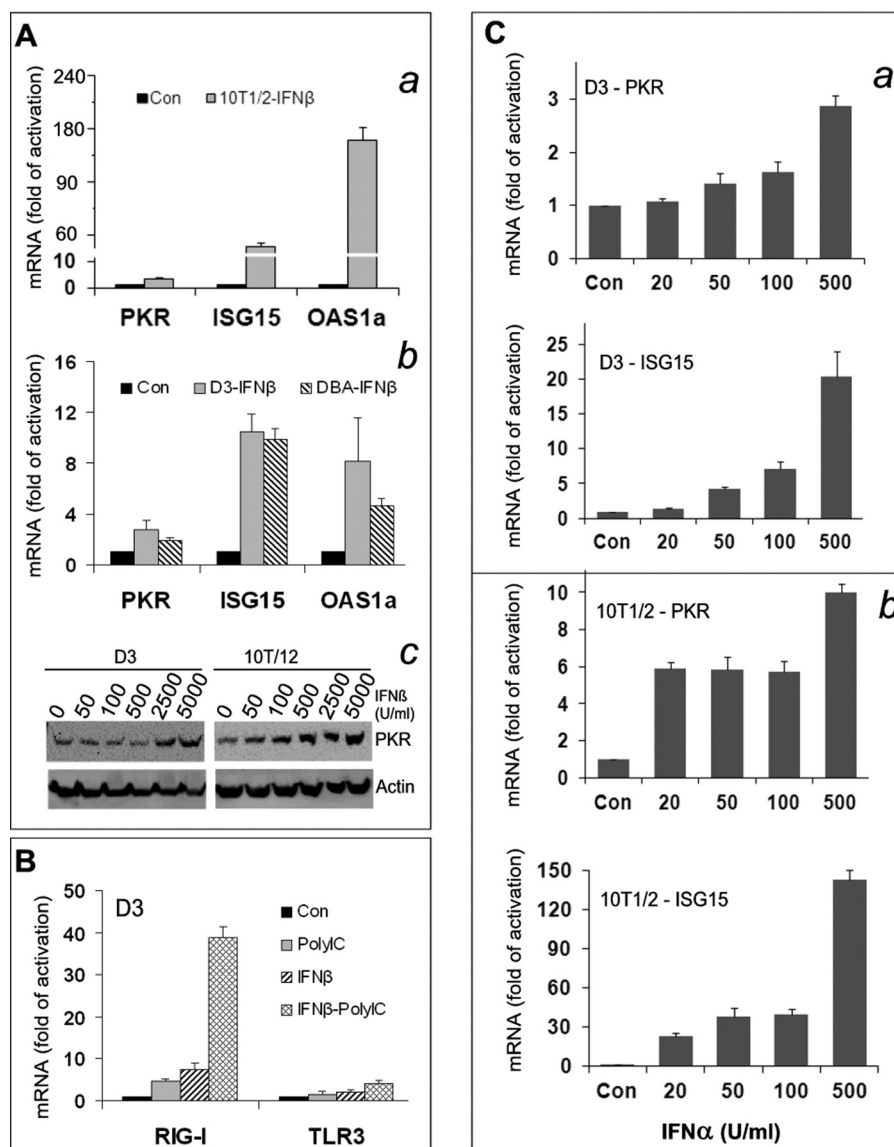


FIGURE 5. IFN β - and IFN α -induced ISG expression in 10T1/2 cells and mESCs. *A*, 10T1/2 cells (*panel a*) mESCs (D3 and DBA) (*panel b*) were treated with 5000 units/ml IFN β for 12 h. The mRNA levels of PKR, ISG15, and OAS1a are expressed as fold-activation where the mRNA level in control cells (Con, cells without IFN β treatment) is designated as 1. IFN β -induced PKR was analyzed by Western blot. β -Actin was used as a control for protein loading (*panel c*). *B*, D3 cells were transfected with poly(I-C) (300 ng/ml), or treated with IFN β (5000 units/ml) separately, or pretreated with IFN β for 24 h followed by poly(I-C) transfection. The mRNA levels of RIG-I and TLR3 were determined at 12-h incubation, and are expressed as fold-activation where the mRNA level in control is designated as 1. *C*, D3 and 10T1/2 cells were treated with different concentrations of IFN α for 12 h. The mRNA levels of PKR and ISG15 are expressed as fold-activation where the mRNA level in control cells (Con) is designated as 1.

ates transcription activity. Thus, nuclear translocation of STAT1 is commonly used as an indicator of its activation (39). As shown in Fig. 6C, 10T1/2 cells are large flattened cells with clearly defined nuclei, although mESCs are characterized by their small size and grow in compact clusters. No pSTAT1 was detected in the nuclei of control cells (Fig. 6C, Con, indicated by arrowhead), but an intensive signal was detected in the nuclei of IFN α -treated 10T1/2 cells. Translocation of pSTAT1 to the nuclei of IFN α -treated mESCs was apparent, although the signal is moderate in comparison with 10T1/2 cells (Fig. 6C, 15 min and 60 min).

Different Induction Patterns of ISGs in mESCs and 10T1/2 Cells—To further characterize ISG expression, we compared their induction patterns in IFN β -treated D3 and 10T1/2 cells in a 24-h time course. As shown in Fig. 7, the mRNAs of *Pkr* and

Isg15 were quickly induced in both cells by IFN β at 4 h. However, they quickly declined to ~50% of the maximal activation in 10T1/2 cells at 8 h. In D3 cells, the expression levels of the two genes, especially *Isg15*, were substantially lower than in 10T1/2 cells, but the duration of their induction was slightly sustained (Fig. 7, A and B, note the different scales in the y axis). We further tested the expression patterns of *Socs1*, a negative regulator of ISG induction. As shown in Fig. 7C, *SOCS1* was induced by IFN β at 4 h in 10T1/2 cells, which coincided with the decline of PKR and ISG15. However, *SOCS1* induction was not obvious until 24 h in D3 cells. To determine whether low level expression of *Pkr* and *Isg15* in D3 cells is due to the induction of *Socs1*, we transfected the cells with siRNAs that target *Socs1* before the stimulation with IFN β . As shown in Fig. 7D, IFN β -induced ISG15 was not affected at either 4 or 24 h of

Type I IFN-induced Responses in mESCs

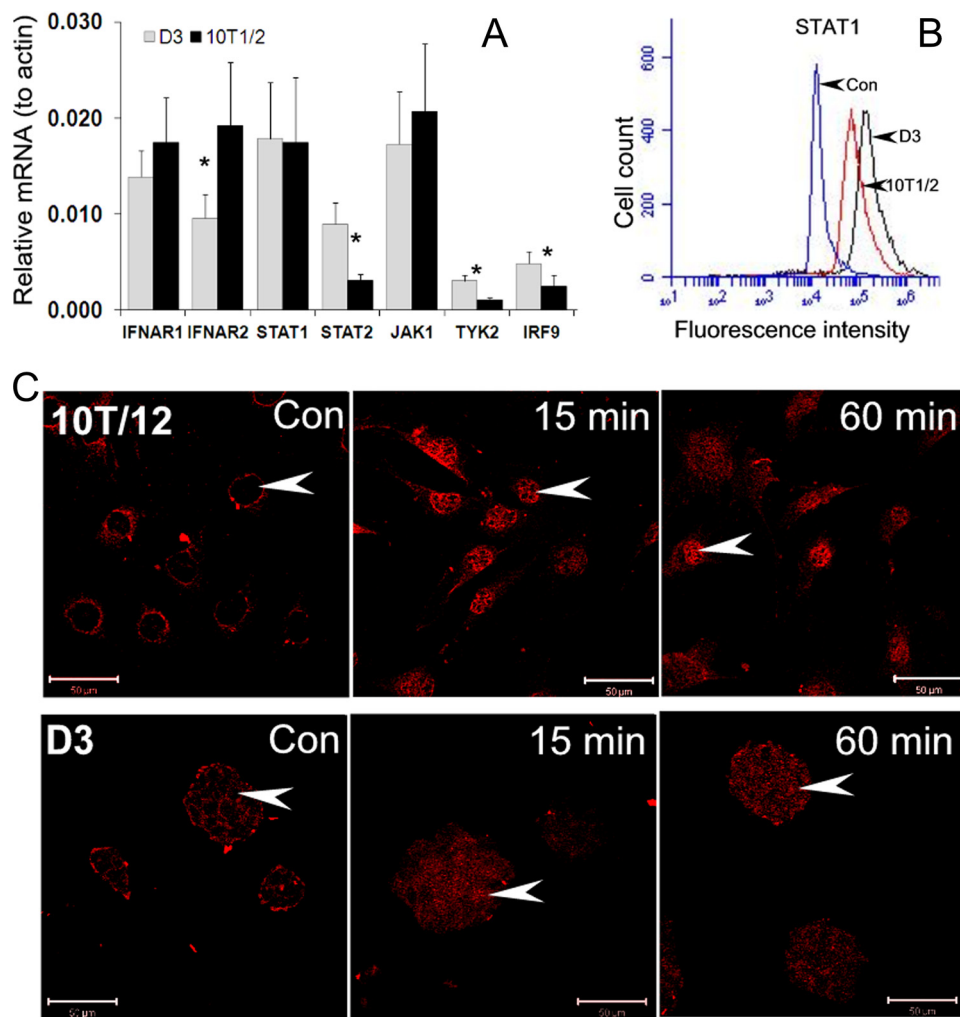


FIGURE 6. Expression of type I IFN signaling molecules and activation of STAT1 in 10T1/2 cells and mESCs. *A*, relative mRNA level of each gene in untreated D3 and 10T1/2 cells was compared after normalization to β -actin mRNA in each cell type. *, $p < 0.05$, the same gene compared in the two cell types. *B*, expression of STAT1 in untreated D3 and 10T1/2 cells analyzed by flow cytometry. The control (*Con*) represents cells without STAT1 antibodies. *C*, immunodetection of STAT1 nuclear translocation. 10T12 cells and D3 cells were treated with IFN α (500 units/ml) for 15 and 60 min. The cellular location of pSTAT1 was detected by its antibody under an LSM 510 laser-scanning confocal microscope. The control (*Con*) represents cells without IFN α treatment. In 10T1/2 cells, an arrowhead denotes the nucleus of a single cell (*upper panels*). In D3 cells, an arrowhead denotes the nucleus of a cell within a colony (*lower panels*).

treatment by SOCS1 knockdown. Therefore, SOCS1 may not be a critical repressor responsible for the low level ISG induction in mESCs.

IFN β Potentiates dsRNA-inhibited Proliferation of mESCs—We have previously shown that poly(I-C) cannot induce IFN α/β in mESCs, but it can activate PKR, thereby inhibiting mESC proliferation (13). Because IFN β induces PKR expression, we assumed that treatment of mESCs with IFN β would augment the effect of poly(I-C). To test this hypothesis, we determined PKR activity by the phosphorylation of eukaryotic initiation factor 2 α (eIF2 α , a known substrate of PKR) (13). As shown in Fig. 8, poly(I-C) transfection caused eIF2 α phosphorylation in D3 cells at 6 and 24 h, which was potentiated in the cells that were pretreated with IFN β (Fig. 8*A*, *PolyIC* and *IFN β /PolyIC*, respectively, boxed areas). Accordingly, poly(I-C) by itself caused a significant reduction of cells in the G₂/M phases, which was further augmented by IFN β (Fig. 8*B*, *PolyIC* and *IFN β /PolyIC*, indicated by arrows). The inhibitory effect on the cell cycle was reflected by reduced cell proliferation, where IFN β pretreatment caused a slight

additional effect to poly(I-C) alone (Fig. 8*C*). These results further confirm functional molecular mechanisms that mediate the effects of IFN β in mESCs.

Type I IFNs Do Not Affect Unique Properties of mESCs—Type I IFNs are best characterized as immunomodulators, but they also regulate other cellular processes, such as cell differentiation (40). However, we did not detect notable morphological changes in D3 and DBA mESCs that were treated with IFN α , IFN β , or IFN ω . To test whether they have any effects on the unique properties of mESCs, we examined the expression of pluripotency markers and cell proliferation of D3 cells that were treated with IFN β or IFN ω . mESCs are characterized by their rapid cell proliferation rate with about 60% of cells in S phase and growth in compact colonies (41). As shown in Fig. 9, neither IFN β nor IFN ω affected the cell cycle profile (*A*), cell proliferation (*B*), or colony morphology (*C*) of D3 cells. Similarly, the expression levels of the pluripotency markers unique to mESCs were not affected by a 48-h period single treatment with IFN β (Fig. 9*D*) or by two consecutive 48-h treatments (Fig. 9*E*), although *Pkr* (a ISG positive control)

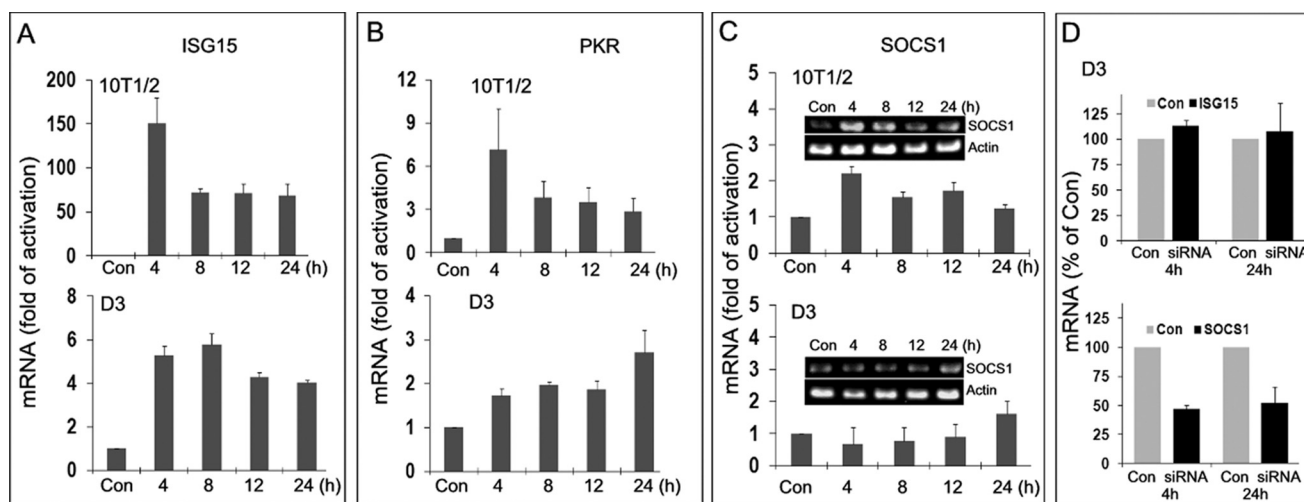


FIGURE 7. **Expression patterns of ISGs in 10T1/2 and mESCs.** D3 and 10T1/2 cells were treated with 5000 units/ml IFN β for the indicated time points. A–C, relative mRNA levels of the three indicated genes were analyzed by RT-qPCR. The results are expressed as fold-activation where the mRNA level in control cells is designated as 1. The *gel insets* in C are PCR products of SOCS1 analyzed by agarose gel electrophoresis. The control (Con) represents cells without IFN β treatment. D, D3 cells were transfected with siRNA targeting SOCS1 or with control siRNA (Con). After 24 h, the cells were treated with IFN β for 4 or 24 h. The mRNA levels of ISG15 and SOCS1 were determined by RT-qPCR. The mRNA in the control cells was expressed as 100%.

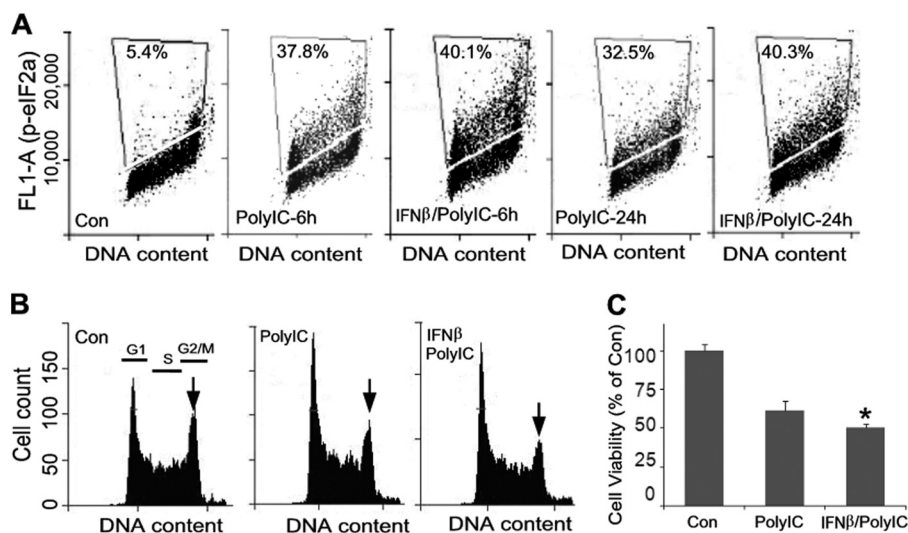


FIGURE 8. **IFN β potentiates poly(I-C)-inhibited proliferation of mESC.** A, D3 cells were transfected with 300 ng/ml poly(I-C) alone (PolyIC) or pretreated with IFN β (5000 units/ml) for 24 h followed by poly(I-C) transfection (IFN β /PolyIC). The cells with phosphorylated eIF2 α (*p-eIF2 α*) were quantified by flow cytometry at 6 and 24 h after poly(I-C) transfection (boxed areas, white slash lines were used to help identify the bottom sides of the boxes). B, cells treated under the condition described in A at 24 h were analyzed for cell cycle by flow cytometry. The change in G₂/M phase cells is indicated by the arrow. C, numbers of viable cells in the samples described in B were determined by toluidine blue cell staining. The control (Con) represents cells without any treatment. *, *p* < 0.05, compared with the cells treated with poly(I-C) alone.

was induced. Therefore, type I IFNs do not affect the stem cell state of mESCs.

mESC-FBs Are More Responsive to Type I IFN than mESCs—The lower IFN response in mESCs suggests that the mechanisms mediating the effects of IFN are not fully developed in these cells. We hypothesized that the differentiation process may promote the IFN system development. Therefore, we generated mESC-FBs through RA-induced differentiation. RA is a vitamin A derivative that regulates several developmental processes during embryogenesis and strongly induces ESC differentiation *in vitro* (42). As shown in Fig. 10A, undifferentiated mESCs (DBA) grew in colonies and differentiated cells formed a continuous monolayer (10dRA). FBs are highly adhesive to a growth surface and quickly attach to an uncoated culture dish. Based on this property, we obtained rather homogeneous prep-

arations of mESC-FBs (DBA-FBs) that are similar to 10T1/2 cells (naturally differentiated FBs in an early embryo) (43) in morphology (Fig. 10A) and cell marker expression (Fig. 10B). In addition to smooth muscle actin that is commonly expressed in FBs (44), several other markers expressed in 10T1/2 cells, including N-cadherin (45), NG2, a chondroitin sulfate proteoglycan (46), metalloproteinase-14 (MMP14) (47), and type IV collagen (CO14) (48), are all expressed in DBA-FBs with similar patterns (Fig. 10B). FBs derived from D3 mESCs (designated as D3-FBs) showed similar properties to DBA-FBs (data not shown).

Using ISG15 expression as a measurement of IFN response, we compared mESC-FBs with mESCs and 10T1/2 cells. As shown in Fig. 10C, IFN α - and IFN β -induced ISG15 expression in mESC-FBs (D3-FBs and DBA-FBs) is significantly higher

Type I IFN-induced Responses in mESCs

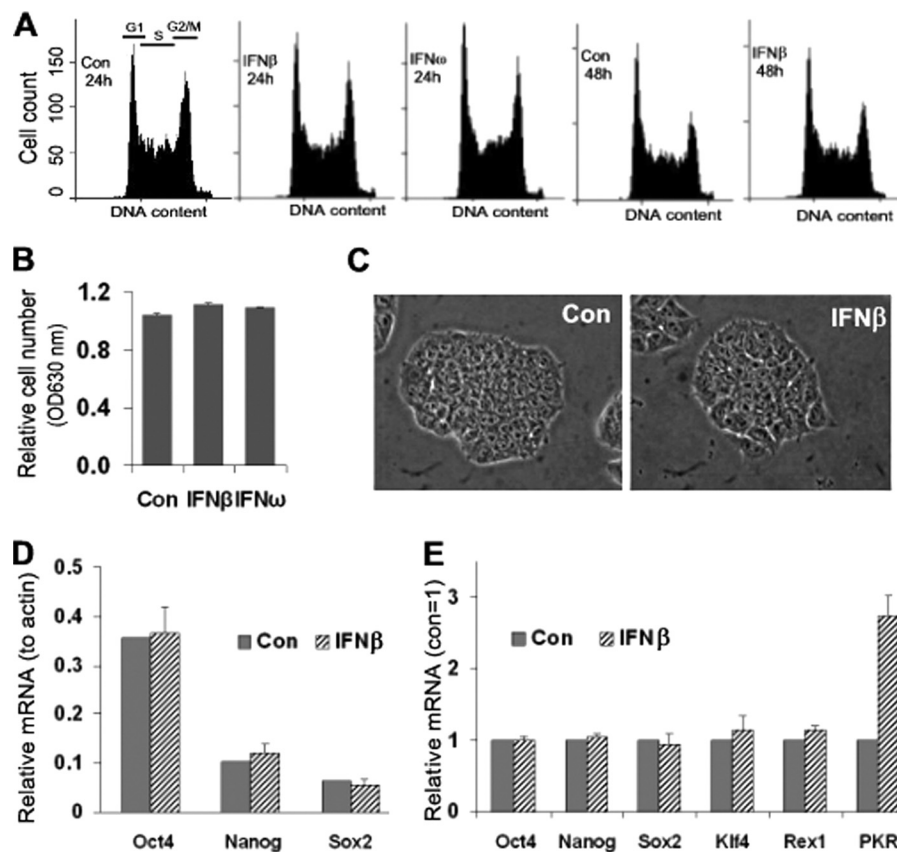


FIGURE 9. **Type I IFNs do not affect the stem cell state of mESCs.** D3 cells were treated with IFN β or IFN ω (5000 units/ml) for 24 or 48 h. The cells were then analyzed for the following. *A*, cell cycle progression by flow cytometry; *B*, cell proliferation by toluidine blue cell staining (24-h treatment); *C*, cell/colony morphology analysis by microscopy (48 h treatment); *D* and *E*, mRNA levels of pluripotency markers by RT-qPCR in the cells that were treated with IFN β for 48 h once (*D*) or twice (two times for 48 h) (*E*). PKR was used as a positive control. The control (*Con*) represents cells without IFN β treatment.

than in mESCs but that represents only about 50–60% of ISG15 induction in 10T1/2 cells. These results indicated that the differentiation process promotes the responsiveness of mESC-FBs to type I IFNs but not to the level observed in 10T1/2 FBs. In line with increased ISG expression, D3-FBs and DBA-FBs showed increased sensitivity to the antiviral effect of IFN α . As shown in Fig. 10*D*, IFN α at 20 units/ml significantly reduced cell death of D3-FBs and DBA-FBs caused by CHIKV infection, which differs from undifferentiated mESCs where the protecting effect of IFN α was only observed at concentrations above 100 units/ml (Fig. 3*A*, D3 and DBA).

DISCUSSION

The type I IFN system is considered to be a critical part of innate immunity in mammalian cells (17). However, the deficiency in expressing type I IFNs in both mESCs (13, 14) and hESCs (15) suggest that “innate immunity” is not, or at least not fully, developed in ESCs. To further characterize this unique property of ESCs, we determine how mESCs respond to type I IFNs.

A recent study (20) reported that hESCs have substantially attenuated responses to IFN β as judged by their failure to express ISGs. However, this finding differs from two early studies that showed mESCs could respond to IFN α and IFN β (49, 50). These brief studies in mESCs attracted little attention in the early days of ESC research, and the physiological implications of these findings were not investigated. Based on multiple

criteria, here we demonstrate that mESCs have the basic mechanisms to mediate the effects of type I IFNs. At the cellular level, IFN α , IFN β , and IFN ω can protect mESCs from LACV- and CHIKV-induced cell death and repress replication of these viruses. At the molecular level, mESCs express the major signaling components in the IFN pathway and are able to express ISGs, which is the hallmark of IFN action and the molecular basis for the antiviral activity of type I IFNs (17–19).

Although we provide strong evidence for the responsiveness of mESCs to type I IFNs, it is important to note that mESCs are significantly less sensitive to IFNs than 10T1/2 cells. We used fibroblasts as a reference for comparison because they robustly express and respond to IFNs (51). 10T1/2 fibroblasts are particularly relevant to our study because they were derived from early embryonic tissues (43). However, it should be pointed out that a direct quantitative comparison of the antiviral activity in mESCs and 10T1/2 cells is difficult due to their different growth behaviors, as discussed previously. Furthermore, viral infectivity and replication in ESCs may also differ from differentiated cells due to their altered cellular processes, such as an underdeveloped glycosylation mechanism in ESCs (12, 52). Therefore, the low level of ISG induction by type I IFNs could be an intrinsic nature of mESCs, but the overall antiviral activity of IFNs could be affected by multiple factors in these cells.

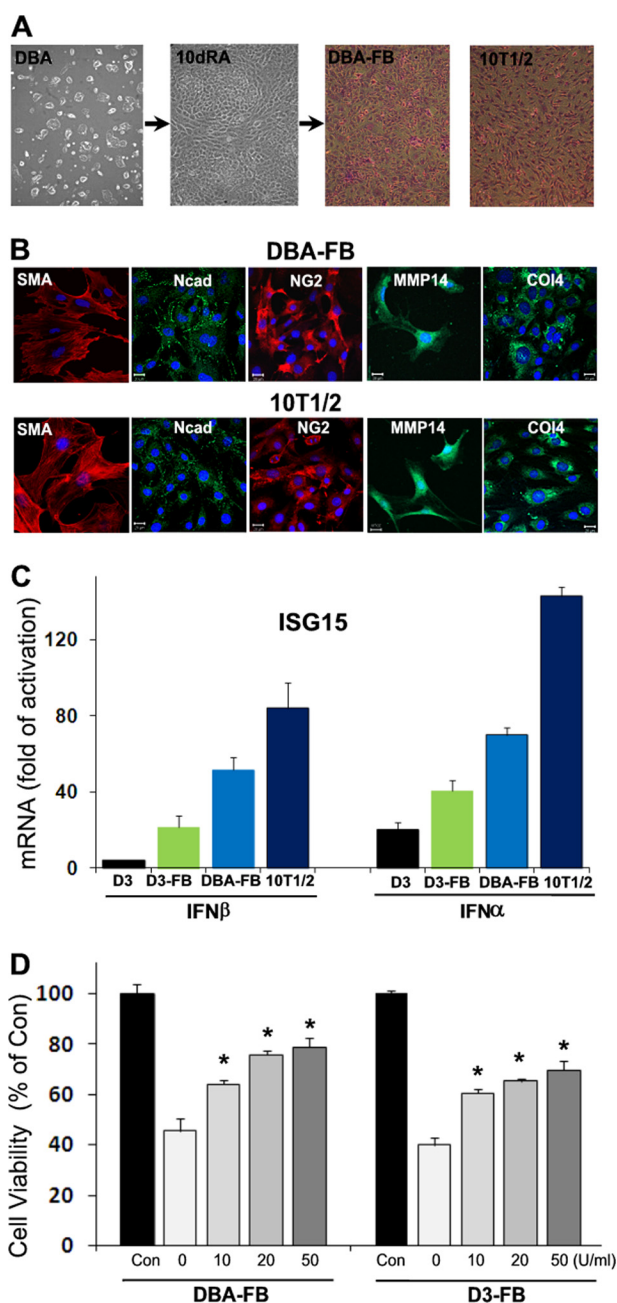


FIGURE 10. mESC-FB differentiation and response to IFNs. *A*, morphology of undifferentiated DBA mESCs (DBA), RA differentiated cells (10dRA), purified fibroblasts (DBA-FBs), and 10T1/2 cells. The images were acquired from live cells (DBA and 10dRA) and toluidine blue-stained cells (DBA-FBs and 10T1/2 cells) under a phase contrast microscope. *B*, cell marker expression in DBA-FBs and 10T1/2 cells. The cells were immunostained with antibodies against indicated cell markers and detected with rhodamine- (red) or FITC (green)-labeled secondary antibodies. The images were acquired with an LSM 510 laser-scanning confocal microscope (Zeiss). Scale bar, 20 μ m. *C*, IFN-induced ISG15 expression in 10T1/2 cells and mESC-FBs. Cells were treated with IFN α (500 units/ml) or IFN β (5000 units/ml) for 12 h. The mRNA levels of ISG15 are expressed as fold-activation where the mRNA level in their respective control cells (without IFN treatment) is designated as 1 (data not shown). *D*, IFN α protects mESC-FBs from CHIKV-induced cell death. DBA-FBs and D3-FBs were pretreated with the indicated concentrations of IFN α for 24 h or left untreated (0 units/ml). The cells were then infected with CHIKV at m.o.i. of 1. Viable cells were determined at 24 h post-infection. Control (Con) represents cells without viral infection. *, $p < 0.05$, compared with CHIKV-infected cells without IFN pretreatment.

In hESCs, the major signaling molecules in the IFN pathway are expressed at relatively lower levels than in differentiated cells. However, the failure of hESCs to respond to IFN β seems to be mainly attributed to the high expression level of SOCS1 (20). In differentiated cells, SOCS1 is expressed at a low basal level in the resting cells but is rapidly induced by IFNs and acts as a negative regulator of ISG induction (53). However, hESCs constitutively express a high level of SOCS1, thereby limiting IFN β action (20). However, our analysis in mESCs suggests that, with the exception of *Ifnar2*, the mRNAs of the major signaling molecules in the IFN pathway are expressed at comparable levels to that of 10T1/2 cells. Unlike hESCs, the mRNA of *Socs1* is expressed at a similar level to 10T1/2 cells. Furthermore, silencing SOCS1 by RNAi did not increase IFN β -induced ISG expression, indicating that SOCS1 is not a major repressor that limits IFN response in mESCs.

Although our results provided a molecular basis that explains the responsiveness of mESCs to type I IFNs, we do not know the reasons for the low levels of ISG induction in these cells. Because cellular responses to IFNs are regulated at multiple levels, one can speculate that the mechanisms required for maximal ISG expression may not have fully developed yet in mESCs. It is also possible that the low level ISG induction in mESCs may be closely related to their defective IFN-expressing mechanism. In differentiated cells, exogenous IFN-induced ISGs can be strongly potentiated by cell-expressed IFNs through autocrine signaling as a positive feedback mechanism (17, 19). Therefore, in IFN-primed (or virally infected) 10T1/2 cells, the ISG induction is the collective effects of exogenously added IFNs and cell expressed IFNs, whereas the ISG induction in mESCs is solely induced by exogenously added IFN α or IFN β because these cells are deficient in expressing type I IFNs (13). It appears that the positive feedback loop in IFN production and action established in differentiated cells is incomplete in mESCs due to the lack of the IFN expression system.

In addition to innate immune responses, type I IFNs also regulate several other important biological processes (40). Whether and how they affect the stem cell state of ESCs is of particular interest. Our results suggest that none of the three types of IFNs tested affects the distinctive features of mESCs, *i.e.* rapid cell proliferation rate, colony formation, and pluripotency. However, it should be pointed out that these experiments were performed in the presence of leukemia inhibitory factor, which represses cell differentiation. The outcome could be different if the experiments were conducted with differentiating mESCs in the absence of leukemia inhibitory factor.

hESCs and mESCs share fundamental similarities in pluripotency and self-renewal, but they also show species differences in two important aspects. First, activation of the JAK/STAT3 pathway by leukemia inhibitory factor (which in fact shares the similar signaling paradigm with IFN) is essential for the maintenance of self-renewal and pluripotency in mESCs (54), but it is not required for hESCs (55, 56). Second, mESCs are characterized by a shortened cell cycle, and hESCs have a time frame similar to differentiated cells (57–59). The difference in response to type I IFNs in mESCs (this study) and in hESCs (20) represents a new distinctive feature between the two species. However, it is noted that the cellular mechanisms that respond

Type I IFN-induced Responses in mESCs

to type I IFNs appear more developed in mESCs than in hESCs, which could be an advantage for mESCs dealing with viral infection during embryogenesis. The lack of such mechanism in hESCs is somehow surprising. This finding suggests that some differences may exist in the development of innate immunity between two species during embryogenesis.

Because the IFN system is developed in most somatic cells, the deficiency of this mechanism in ESCs suggests that it is developmentally acquired during differentiation, as indicated by the increased responsiveness of *in vitro* differentiated mESC-FBs to IFN α and IFN β . However, it is noted that, in comparison with naturally differentiated 10T1/2 cells, the *in vitro* differentiated mESC-FBs have lower IFN response, which is in line with other studies demonstrating that ESC-derived endothelial cells and smooth muscle cells cannot effectively respond to various pathogens as their naturally differentiated counterparts (3, 4). Therefore, the differentiation process could in principle promote the development of innate immunity, but the maturity of *in vitro* differentiated cells from ESCs could be affected by many factors, such as differentiation methods and cell types involved.

The underdeveloped IFN system in ESCs raises an intriguing question as follows. What is the rationale for ESCs not having such an effective antiviral mechanism that is well developed in most differentiated cells? Although we do not yet have a complete understanding of this question, we can speculate from different perspectives. ESCs normally reside in the womb where they have limited exposure to pathogens (60). From this point of view, the lack of innate immunity in ESCs is not entirely surprising because the innate and adaptive immunity of the mother may offer necessary protection to ESCs. However, a different speculation could be made based on the pleiotropic effects of IFN-based antiviral responses. It is known that multiple forms of antiviral activities mobilized by the IFN system can cause adverse effects to the infected cells, such as cell cycle inhibition or cell death (17, 36). Although these negative effects on infected cells in a tissue may not cause much damage to a developed organism, the consequence could be detrimental to a developing organism if the infected cells are ESCs because they are the progenitors for all tissues. However, viral infections of ESCs would be equally disastrous if they lack an effective antiviral mechanism as their descendant cells would be infected as well. The recent discovery of a functional RNA interference (RNAi) mechanism in mESCs (61) offers a plausible solution to this dilemma. RNAi has been known as a major antiviral mechanism in plants and invertebrates that lack the IFN-based antiviral immunity (62), but whether it plays any role in mammals has been uncertain (63–65). Using mESCs as a model system, Maillard *et al.* (61) recently provided definitive evidence for the antiviral function of RNAi in mammals. This finding has led to the conclusion that mammalian cells, especially in ESCs, retained a functional RNAi pathway. It also provides a rational explanation for the underdeveloped IFN system in ESCs. By utilizing virus-specific/short lived siRNA derived from an invading virus, ESCs can effectively prevent viral infection, thereby avoiding potentially detrimental consequences associated with the IFN system. An emerging paradigm is that mam-

mals may have adapted different antiviral strategies at different stages of development (63).

The studies using ESC models have led to the new insights of innate immunity in developmental biology and regenerative medicine. However, further details and many important questions remain to be investigated. For instance, the attenuated IFN response in ESCs may help alleviate the potential adverse effects; nonetheless, the IFN-responding system in mESCs is not completely inactive. It would be interesting to know whether mESCs have additional mechanisms to counteract the potential damage caused by IFN responses. The studies with ESC models have been and will continue to be instrumental to address the compelling questions overlapping innate immunity, stem cell biology, and regenerative medicine.

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Type I IFN-induced Responses in mESCs

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