Cytoplasmic dsRNA induces the expression of *OCT3/4* and *NANOG* mRNAs in differentiated human cells

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Cytoplasmic dsRNA is recognized by RNA helicase RIG-I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), triggering induction of the innate immune response via the mitochondrial antiviral signaling protein (MAVS). In contrast, extracellular dsRNA is internalized into endosomes and recognized by Toll-like receptor 3 (TLR3), which triggers signaling via the Toll-like receptor adaptor molecule 1 (TICAM-1). Poly(I:C) is a synthetic dsRNA analog and increases the expression of *octamer-binding protein 3/4* (*OCT3/4*), *NANOG*, and *SRY-box* (*SOX*) mRNAs during pluripotency induction. However, the mechanism underlying this increase is unclear. Here, we focused on the mechanism of poly(I:C)-induced expression of stem cell-specific genes in human somatic cells. Addition of poly(I:C) to human fibroblast culture medium did not increase *OCT3/4* mRNA expression, but poly(I:C) transfection markedly increased *OCT3/4* expression and induced nuclear localization of the OCT3/4 protein, implying that not TLR3, but RIG-I and MDA5 are required for *OCT3/4* expression. Moreover, although cytoplasmic dsRNA increased *OCT3/4* mRNA, cytoplasmic dsDNAs, such as salmon sperm DNA and poly(dA:dT), did not. Interestingly, the expression of NANOG, SOX2, Krüppel-like factor 4 (KLF4), and proto-oncogene c-Myc was also increased by cytoplasmic dsRNA. Of note, siRNAs that silenced MAVS and interferon regulatory factor 1 (IRF1) expression reduced *OCT3/4* levels after stimulation with poly(I:C); however, an NF-κB inhibitor and siRNA-mediated knockdown of proto-oncogene c-Jun did not significantly reduce the mRNA levels. We conclude that cytoplasmic dsRNA increases the expression of stem cell-specific genes in human somatic cells in a MAVS- and IRF1-dependent manner.

The innate immune system is essential for protection against viruses and bacteria at early phases of infections (1). Pattern recognition receptors (PRRs),[3] such as Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs), recognize pathogen-associated molecular patterns (PAMPs) and trigger the signals to produce type I interferon (IFN) and pro-inflammatory cytokines (2). In the case of viral infection, viral double-stranded RNA (dsRNA) in the endosomes is recognized by TLR3, whereas cytoplasmic dsRNA is recognized by RIG-I and MDA5 (3). TLR3 utilizes the TICAM-1 adaptor to trigger the downstream signaling, and RIG-I and MDA5 activate the signaling via the MAVS adaptor (4). In addition to dsRNA, exogenous dsDNA can be recognized by PRRs. TLR9 senses nonmethylated CpG DNA and induces pro-inflammatory cytokine expression via the MyD88 adaptor (5). In contrast, cytoplasmic dsDNA is recognized by cGAS, which produces a second messenger cGAMP that activates the STING adaptor and induces cytokine expression (6, 7). In addition, cytoplasmic viral nucleic acid sensors can recognize bacterial infection (8). It is notable that PRRs can recognize damage-associated molecular patterns (DAMPs), which are released from host cells via necrosis caused by cell stress and damage (9).

There are several reports showing that bacterial infection promotes the reprogramming of human somatic cells. Ohta and colleagues reported that lactic acid bacteria could induce the expression of a subset of pluripotent stem cell marker genes, such as *NANOG*, *OCT3/4*, and *SOX2* in human somatic cells (10). Moreover, *Mycobacterium leprae* infection also induces the expression of stem cell markers, CD73, CD44, Sca-1, and CD29, in Schwann cells (10). Similar to a bacterial infection, cell damage or stress also induces the expression of stem cell-specific genes. For instance, pluripotent multilineage-differentiating stress-enduring (Muse) cells are stem cells that could be isolated by treating cells with various types of stresses, such as long-term collagenase treatment, serum deprivation, low temperatures, and hypoxia (11). Severe cell stress leads to necrosis

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This article contains Fig. S1.

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[3] The abbreviations used are: PRR, pattern recognition receptor; TLR, Toll-like receptor; RLR, RIG-I–like receptor; TICAM-1, Toll-like receptor adaptor molecule 1; MAVS, mitochondrial antiviral signaling protein; IFN, interferon; DAMP, damage-associated molecular pattern; iPS, induced pluripotent stem cells; STAP, stimulus-triggered acquisition of pluripotency; qPCR, quantitative PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole; DOTAP, 1,2-dioleoyl-3-trimethylammonium propane; HCV, hepatitis C virus.

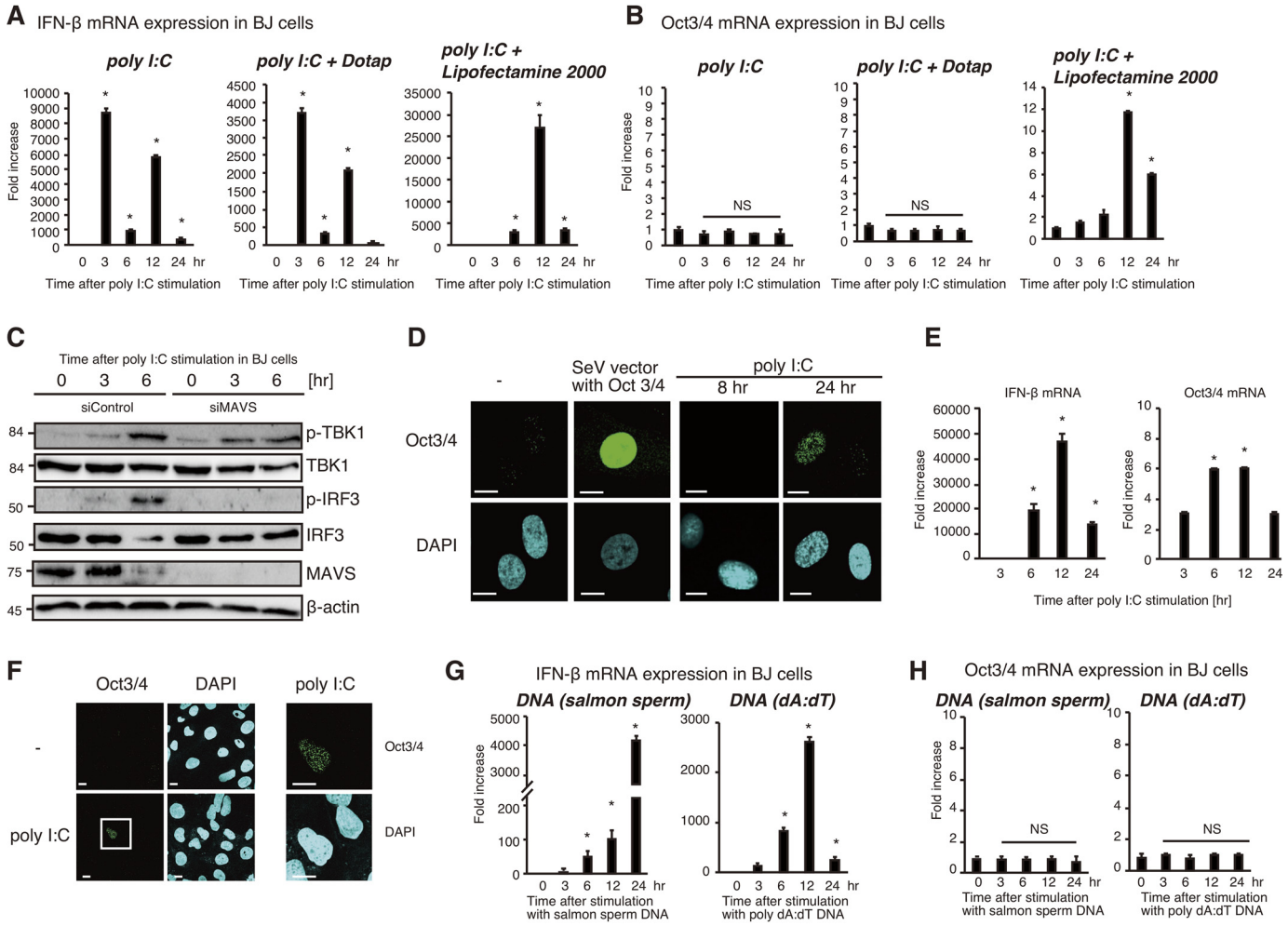

Figure 1. Cytoplasmic poly(I:C) induces the expression of Oct3/4 mRNA. *A* and *B,* BJ cells were stimulated by adding 50 μg/ml of poly(I:C) to the cell culture medium (poly(I:C)), transfecting 1 μg/ml of poly(I:C) using DOTAP (*Dotap*), and transfecting 1 μg/ml of poly(I:C) using Lipofectamine 2000 reagent. Total RNA was extracted at the indicated time points, and the expression levels of IFN-β (*A*) and Oct3/4 (*B*) mRNA were determined by RT-qPCR and normalized to those of GAPDH. *C,* siRNAs for control and MAVS were transfected into BJ cells. 2 days later, 1 μg/ml of poly(I:C) was transfected into BJ cells with Lipofectamine 2000, and whole cell extracts were prepared at the indicated time points. Proteins were detected with the indicated antibodies. *D,* 1 μg/ml of poly(I:C) was transfected into BJ cells with (−) or without Lipofectamine 2000 (poly(I:C)). For control, SeV vector expressing Oct3/4 was transfected into BJ cells. 0, 8, and 24 h after transfection, cells were fixed and stained with anti-Oct3/4 antibody. The protein was detected by an Alexa Fluor 488-conjugated secondary antibody (*green*). Nuclei were stained with DAPI (*blue*) to compare the localization of Oct3/4 and nuclei. The *white bar* represents 10 μm. *E* and *F*, LX-2 cells were transfected with 1 μg/ml of poly(I:C) using Lipofectamine 2000. Total RNA was extracted at the indicated time points, and mRNA levels were determined by RT-qPCR and normalized to those of GAPDH (*E*). Twenty-four hours after stimulation, cells were fixed and labeled with anti-Oct3/4 antibody. The proteins were detected with secondary antibody conjugated to Alexa Fluor 488 (*green*), and nuclei were stained with DAPI (*blue*) (*F*). The *white bar* represents 10 μm. *G* and *H,* 1 μg/ml of salmon sperm DNA and poly(dA:dT) were transfected into BJ cells using Lipofectamine 2000, and total RNA was extracted at indicated time points. IFN-β (*G*) and Oct3/4 (*H*) mRNA levels were determined by RT-qPCR and normalized to those of GAPDH.

or necroptosis, resulting in the release of DAMPs, which can be recognized by PRRs.

The relationship of the activation of PRRs and efficient reprogramming of cells has been previously implied. Lee *et al.* (12) reported that poly(I:C) promotes the expression of Oct4, Sox2, and NANOG, and that is required for efficient nuclear reprogramming in the induction of pluripotency. The role of RLRs on the expression of Oct3/4 was also reported (13). However, their underlying mechanisms have not yet been fully elucidated. In this study, we investigated the molecular mechanism of poly(I:C)-induced expression of the stem cell-specific genes. Our data indicate that cytoplasmic poly(I:C) as well as dsRNA activate the MAVS adaptor of RLRs and induces the expression of stem cell-specific genes via the transcription factor IRF1.

Results

Cytoplasmic poly(I:C) induces Oct3/4 expression via MAVS

To investigate the effect of poly(I:C) on the expression of stem cell-specific genes, human fibroblast BJ cells, which are frequently used to generate induced pluripotent stem cells (iPS cells) (14), were stimulated with poly(I:C) by several methods as follows. Addition of poly(I:C) to cell culture medium is known to induce type I IFN expression via the TLR3-mediated signaling pathway (15). When poly(I:C) was added to the cell culture medium, IFN-β mRNA expression was increased upon stimulation (Fig. 1*A*); however, the Oct3/4 mRNA levels were not increased (Fig. 1*B*). DOTAP is a transfection reagent and is known to enforce endosomal translocation of nucleic acids (16–18). Poly(I:C) transfected using DOTAP were delivered to

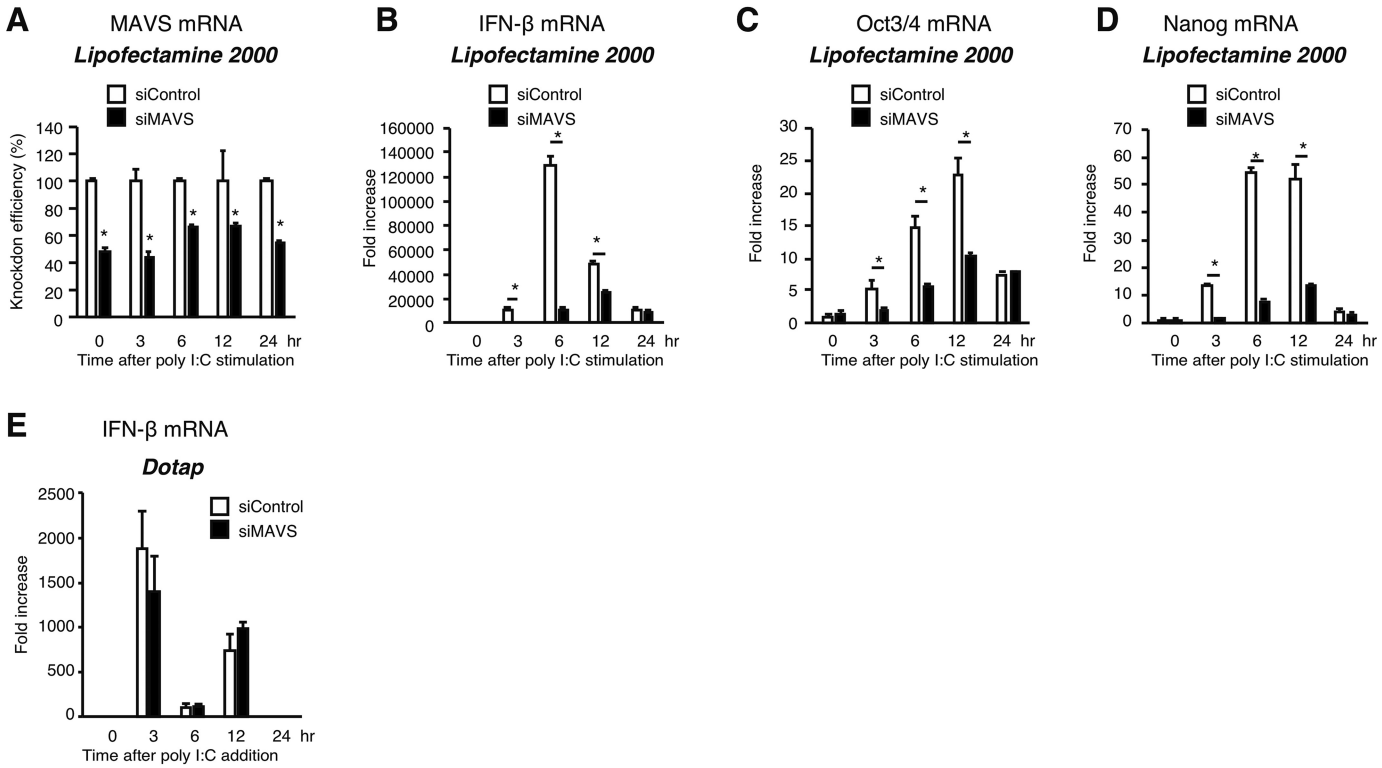

Figure 2. MAVS is required for poly(I:C)-mediated Oct3/4 mRNA expression. *A–E,* siRNAs for MAVS (*siMAVS*) and negative control (*siControl*) were transfected into BJ cells; 2 days after transfection, BJ cells were stimulated by 1 μg/ml of poly(I:C) transfection using Lipofectamine 2000 (*A–D*) or using DOTAP (*E*), and total RNA was extracted at the indicated time points. The expression levels of MAVS (*A*), IFN-β (*B* and *E*), Oct3/4 (*C*), and NANOG (*D*) mRNA were determined by RT-qPCR and normalized to those of GAPDH.

the endosomes, thus stimulating TLR3, and leading to type I IFN expression (3, 18). Similar to the addition of poly(I:C) to the cell culture medium, poly(I:C) transfection using DOTAP induced IFN-β mRNA expression (Fig. 1*A*) but did not increase the Oct3/4 mRNA levels (Fig. 1*B*).

Next, we tested the stimulation of BJ cells by transfecting with poly(I:C) using Lipofectamine 2000, which results in the activation of cytoplasmic viral RNA sensors, RIG-I and MDA5 (19). Oct3/4 mRNA levels were markedly increased by transfection with poly(I:C) using the Lipofectamine 2000 reagent (Fig. 1*B*). TBK1 and IRF-3 phosphorylation are required for type I IFN expression after poly(I:C) stimulation, and phosphorylation of the proteins was detected in BJ cells upon transfection with poly(I:C) using Lipofectamine 2000 reagent, and knockdown of MAVS suppressed phosphorylation (Fig. 1*C*). To confirm the protein expression of the Oct3/4 upon stimulation, we performed confocal microscopy. Although the Oct3/4 protein was not detected in unstimulated BJ cells, nuclear localization of the protein was detected 24 h after poly(I:C) transfection using Lipofectamine 2000 as observed in cells infected with Sendai virus vector carrying Oct3/4 (SeV with Oct3/4) (Fig. 1*D*). LX-2 is a human hepatic stellate cell line, and transfection with poly(I:C) using Lipofectamine 2000 also increased Oct3/4 mRNA expression; moreover, the nuclear localization of Oct3/4 was detected in stimulated LX-2 cells (Fig. 1, *E* and *F*), suggesting that poly(I:C)-mediated Oct3/4 expression is not specific to BJ cells.

We next investigated the effect of other PAMPs on Oct3/4 expression. Salmon sperm DNA and synthesized oligo poly(dA:dT) were transfected into BJ cells, and the expression levels of type I IFN and Oct3/4 were determined. Unlike poly(I:C), transfection with salmon sperm DNA and oligo(dA:dT) using Lipofectamine 2000 failed to increase Oct3/4 mRNA levels; however, it successfully induced IFN-β mRNA expression (Fig. 1, *G* and *H*). These data suggest that cGAS and/or TLR9 are not required for the Oct3/4 expression.

Cytoplasmic poly(I:C) is recognized by cytoplasmic viral RNA sensors, RIG-I and MDA5. Both RIG-I and MDA5 require the MAVS adaptor molecule to induce downstream signaling. Therefore, we next investigated the effect of MAVS knockdown on Oct3/4 expression upon stimulation. siRNA for MAVS reduced MAVS mRNA levels in BJ cells (Fig. 2*A*). We confirmed that siRNA for MAVS significantly reduced IFN-β mRNA levels in cells transfected with poly(I:C) using Lipofectamine 2000 (Fig. 2*B*). siRNA for MAVS significantly reduced Oct3/4 mRNA levels (Fig. 2*C*). *NANOG* is a stem cell-specific gene, and its expression was also increased by transfection with poly(I:C) using Lipofectamine 2000 (Fig. 2*D*). Similar to Oct3/4 expression, NANOG expression was reduced by siRNA for MAVS (Fig. 2*D*). siRNA for MAVS hardly reduced IFN-β mRNA expression upon poly(I:C) stimulation using DOTAP (Fig. 2*E*). These data indicate that cytoplasmic poly(I:C) increases Oct3/4 and NANOG expression in a MAVS-dependent manner.

The expression of stem cell-specific genes induced by cytoplasmic dsRNA

Takahashi and Yamanaka (20) selected 24 genes as candidates for factors that induce pluripotency in somatic cells, and Oct3/4, Sox2, c-Myc, and Klf4 were revealed to be crucial for

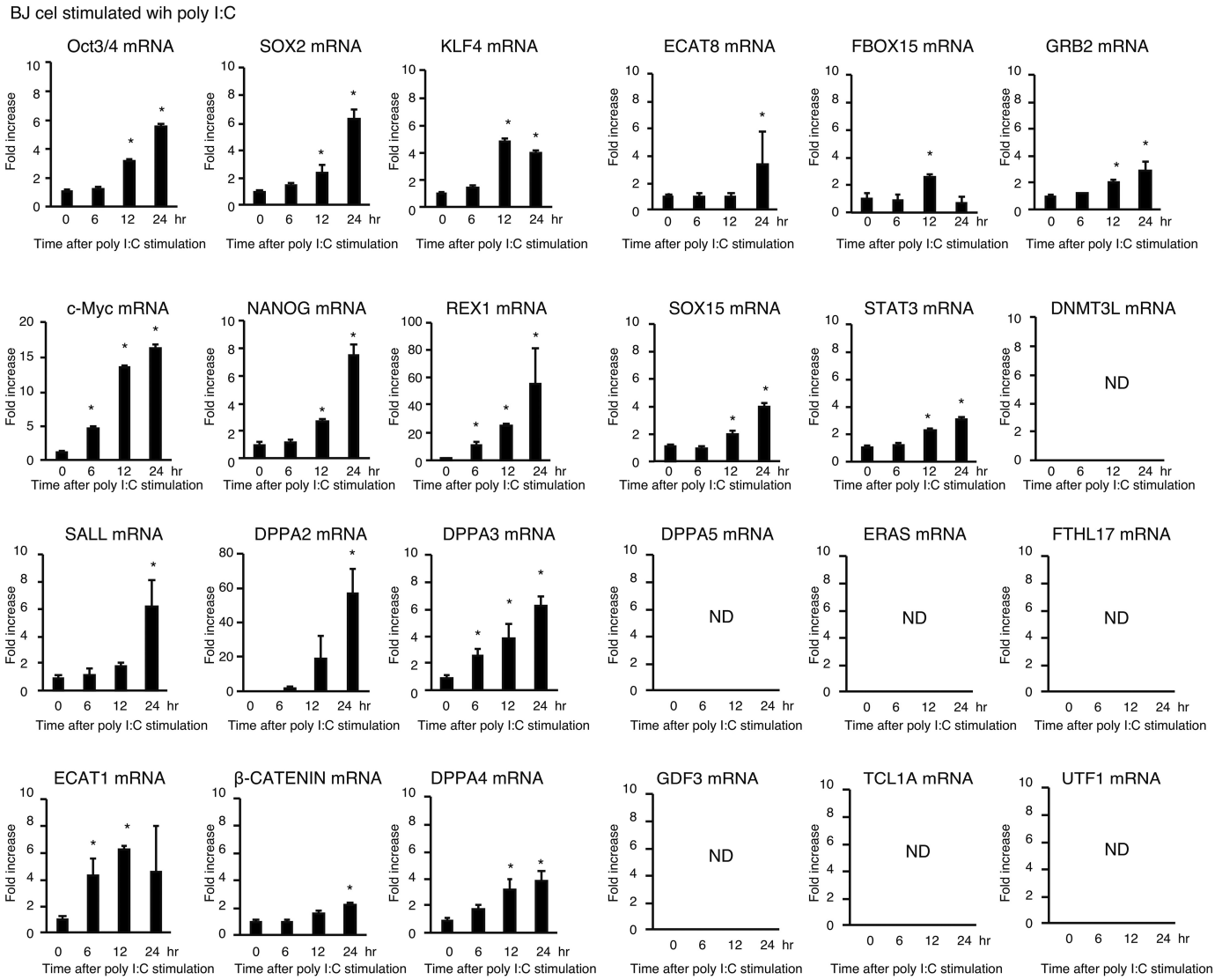

Figure 3. Cytoplasmic poly(I:C) increases the expression levels of stem cell-specific genes. 1 μg/ml of poly(I:C) was transfected into BJ cells using Lipofectamine 2000, and total RNA was extracted at the indicated time points. The expression levels of the indicated genes were determined by RT-qPCR and normalized to those of GAPDH. *ND,* not detected.

inducing pluripotent stem cells. Later studies showed that the other candidate genes also contribute to the efficient generation of pluripotent stem cells (21). Because poly(I:C) transfection using Lipofectamine 2000 induced the expression of Oct3/4 and NANOG as described above, we next measured the expression levels of the 24 genes upon stimulation.

In addition to Oct3/4 and NANOG, the expression levels of SOX2, KLF4, c-Myc, REX1, SALL, DPPA2, DPPA3, ECAT1, DPPA4, and SOX15 were markedly increased by poly(I:C) transfection using Lipofectamine 2000 (Fig. 3). We could not detect the expression of DNMT3L, DPPA5, ERAS, FTHL17, GDF3, TCL1A, and UTF1 in BJ cells even in the presence or absence of poly(I:C) transfection (Fig. 3). Stimulation with poly(I:C) exhibited a marginal effect on the expression of β-catenine, ECAT8, FBOX15, GRB2, and STAT3 (Fig. 3). These data suggest that cytoplasmic poly(I:C) enhanced the expression of genes that contribute to the efficient generation of pluripotent stem cells.

Poly(I:C) is a synthetic analog of viral dsRNA, and the 3′ UTR of HCV RNA is well-known to be recognized by RIG-I (22). Therefore, we prepared two types of HCV RNAs. First, 3′ UTR dsRNA was synthesized *in vitro* by T7 RNA polymerase and used for stimulation. Second, we prepared total RNA samples of O cells that contained HCV replicons, which are HCV genomic RNA replicating in host cells (23). Total RNA of Oc cells, in which HCV replicons were removed by type I IFN treatment, was used for a negative control (24). We confirmed that *in vitro* synthesized 3′ UTR of HCV RNA increased IFN-β mRNA levels (Fig. 4*A*). Interestingly, the expression levels of Oct3/4 as well as SOX2, KLF4, and c-Myc were increased by transfection with 3′ UTR HCV RNA using Lipofectamine 2000, as cytoplasmic poly(I:C) did (Fig. 4, *B–E*). In contrast, transfection with 3′ UTR HCV RNA using DOTAP failed to increase Oct3/4, SOX2, KLF4, and c-Myc, but successfully increased IFN-β mRNA expression levels (Fig. 4, *F–J*). Nuclear localization of the Oct3/4 protein was detected by transfection with 3′ UTR HCV

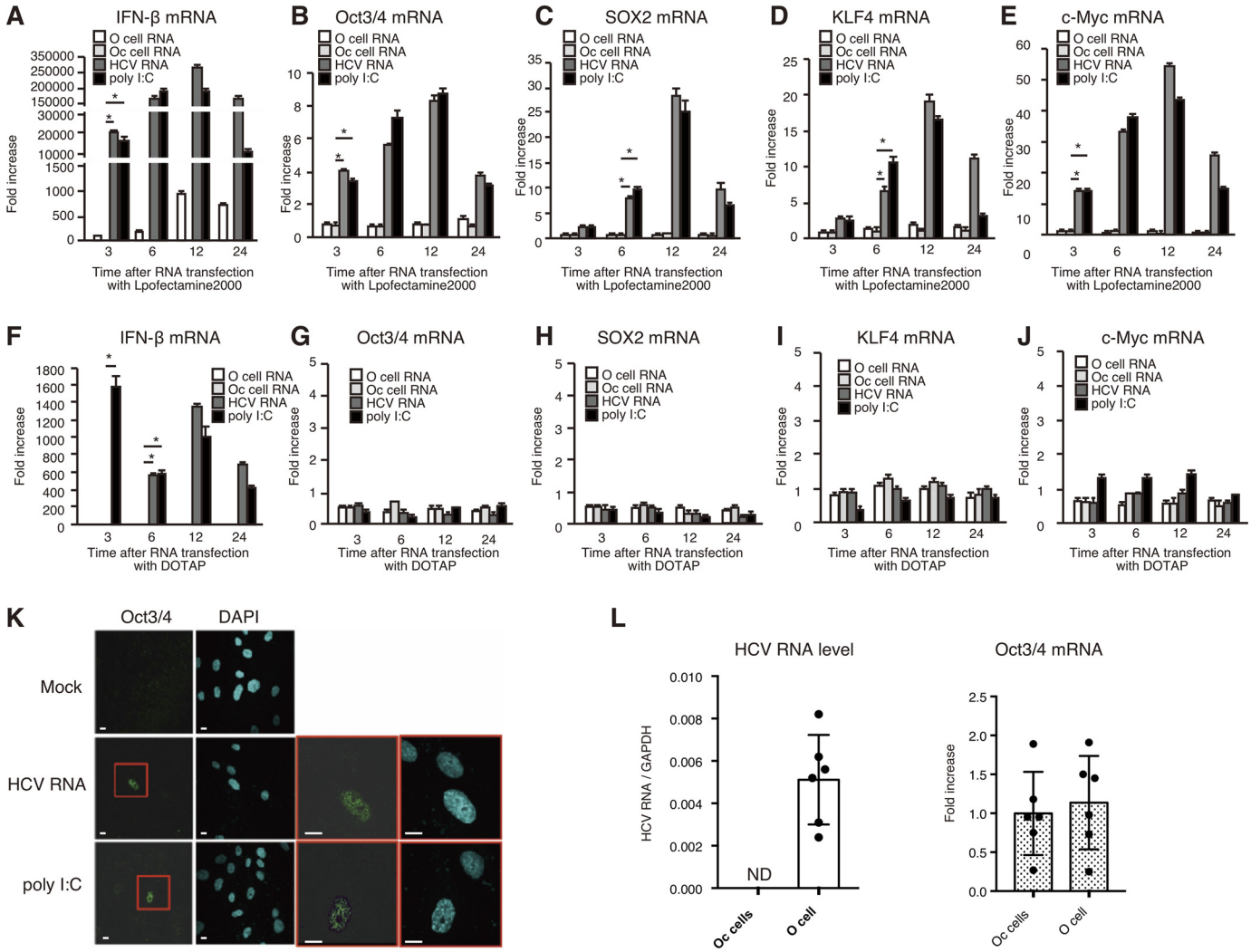

Figure 4. *In vitro* synthesized 3′ UTR of HCV RNA induces Oct3/4 mRNA expression. *A–J,* 3′ UTR of HCV RNA (HCV RNA) was *in vitro* synthesized by T7 RNA polymerase. Total RNA samples were extracted from O and Oc cells; 1 μg/ml each RNA sample was transfected into BJ cells using Lipofectamine 2000 (*A–E*) and DOTAP (*F–J*), and then total RNA was extracted at the indicated time points. The expression levels of IFN-β, Oct3/4, Sox2, KLF4, and c-Myc were determined by RT-qPCR and normalized to those of GAPDH. *K,* 1 μg/ml of 3′ UTR of HCV RNA and poly(I:C) was transfected into BJ cells using Lipofectamine 2000; 24 h after transfection, cells were fixed and labeled with anti-Oct3/4 antibody. The protein was stained with a secondary antibody conjugated to Alexa Fluor 488 (*green*). Nuclei were stained with DAPI (*blue*). The *white bar* represents 10 μm. *L,* HCV RNA and Oct3/4 mRNA levels in O and Oc cells were determined by RT-qPCR.

RNA using Lipofectamine 2000 (Fig. 4*K*). These data indicate that not only poly(I:C), but also *in vitro* synthesized HCV RNA can induce Oct3/4 expression in human BJ cells.

In contrast to *in vitro* synthesized HCV RNA, transfection with total RNA extracted from O cells using Lipofectamine 2000 did not increase the expression of Oct3/4, although O cell total RNA increased the expression of IFN-β mRNA (Fig. 4*A*). Because O cells contain HCV replicons, these data imply that the amount of HCV RNA in O cells is not sufficient to induce the Oct3/4 expression. When we compared the expression of Oct3/4 mRNA levels in O cells with those of Oc cells, we could not detect any significant difference (Fig. 4*L*). These data suggest that some specific conditions, such as large amounts of cytoplasmic dsRNA, are required for RIG-I and MDA5 to induce Oct3/4 mRNA expression (see "Discussion").

IRF1 is required for Oct3/4 expression in BJ cells

To reveal the underlying mechanism, we investigated the role of the factors of the innate immunity in cytoplasmic dsRNA-mediated Oct3/4 expression. Because cytoplasmic poly(I:C) leads to the production of type I IFN, it is possible that type I IFN induces Oct3/4 expression after poly(I:C) stimulation. To test this hypothesis, siRNA for STAT1 was used to inhibit type I IFN receptor signaling. siRNA for STAT1 substantially reduced STAT1 mRNA expression (Fig. 5*A*). Contrary to what we expected, siRNA for STAT1 hardly reduced poly(I:C)-mediated Oct3/4 mRNA expression (Fig. 5*A*), suggesting that type I IFN signaling does not play a major role in the Oct3/4 expression. siRNA for STAT1 moderately reduced IFN-β mRNA expression upon poly(I:C) stimulation (Fig. 5*A*). Considering that RIG-I is an interferon-inducible gene, it is possible that basal expression of type I IFN affected RIG-I expression, thereby STAT1 knockdown reduced RIG-I–mediated type I IFN expression.

To further test whether type I IFN signaling is responsible for Oct3/4 mRNA expression, we next used siRNA for STAT2, anti-IFNAR2 neutralization antibodies, and a JAK

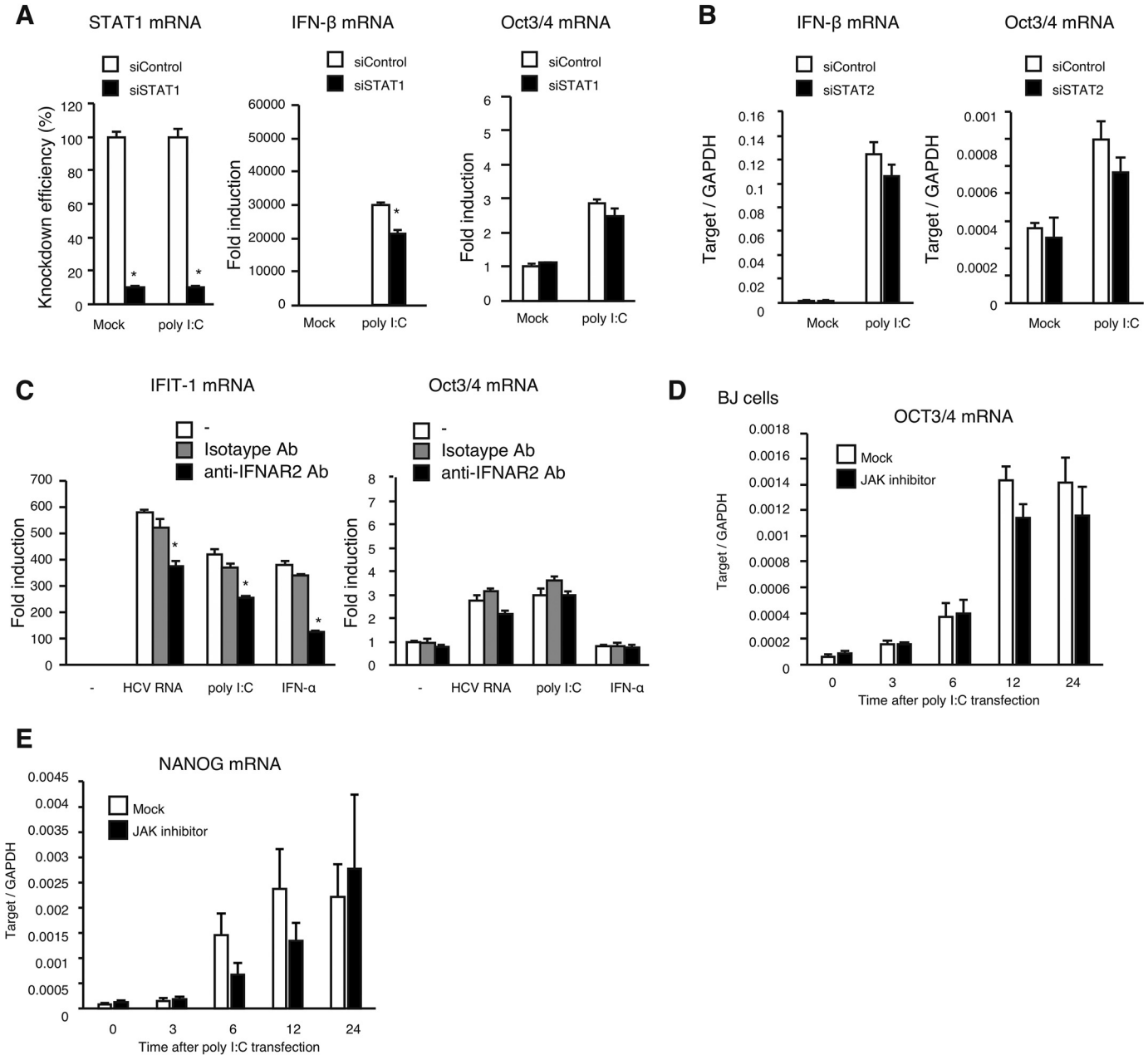

Figure 5. Type I IFN was dispensable for poly(I:C)-mediated Oct3/4 expression. *A* and *B*, siRNAs for STAT1 (siSTAT1) (*A*), STAT2 (siSTAT2) (*B*), and negative control (siControl) (*A* and *B*) were transfected into BJ cells; 2 days after transfection, transfected cells were stimulated by transfection with 1 μg/ml of poly(I:C) using Lipofectamine 2000 for 12 h, and then total RNA was extracted. The expression levels of STAT1, IFN-β, and Oct3/4 were determined by RT-qPCR and normalized to those of GAPDH. *C*, BJ cells were treated with mock, isotype control IgG, and anti-IFNAR2 antibodies, and then transfected with HCV RNA and poly(I:C) using Lipofectamine 2000 or treated with type I IFN for 12 h. The expression levels of IFIT-1 and Oct3/4 mRNA were determined by RT-qPCR and normalized to those of GAPDH. *D* and *E*, BJ cells were treated with 3 nM JAK inhibitor, Ruxolitinib for 12 h, and then cells were stimulated with poly(I:C) using Lipofectamine 2000 in the presence of the inhibitor, and the mRNA expression of Oct3/4 (*D*) and NANOG (*E*) was determined by RT-qPCR.

inhibitor. siRNA for STAT2 exhibited only a marginal effect on the Oct3/4 expression (Fig. 5*B*). Anti-IFNAR antibodies reduced the expression of an IFN-inducible gene IFIT-1; however, it failed to reduce Oct3/4 mRNA expression (Fig. 5*C*). In addition, stimulation with IFN-α itself could not increase Oct3/4 mRNA expression (Fig. 5*C*). Moreover, a JAK inhibitor failed to reduce the expression of Oct3/4 and NANOG mRNAs (Fig. 5, *D* and *E*). These data also suggest that type I IFN does not play a major role in poly(I:C)-mediated Oct3/4 mRNA expression.

Considering that Oct3/4 expression in BJ cells requires MAVS, we reasoned that transcription factors that are downstream of MAVS would be responsible for Oct3/4 expression. The transcription factors IRF1, IRF3, AP-1, and NF-κB are reported to be activated by MAVS-dependent signaling. We identified the binding motifs of IRF1, AP-1, and NF-κB on the promoter region of Oct3/4, SOX2, KLF4, c-Myc, and NANOG (Fig. 6*A*). To test the role of NF-κB in Oct3/4 expression, we used BAY11-7082, an inhibitor for NF-κB. Although the inhibitor markedly reduced IFN-β and

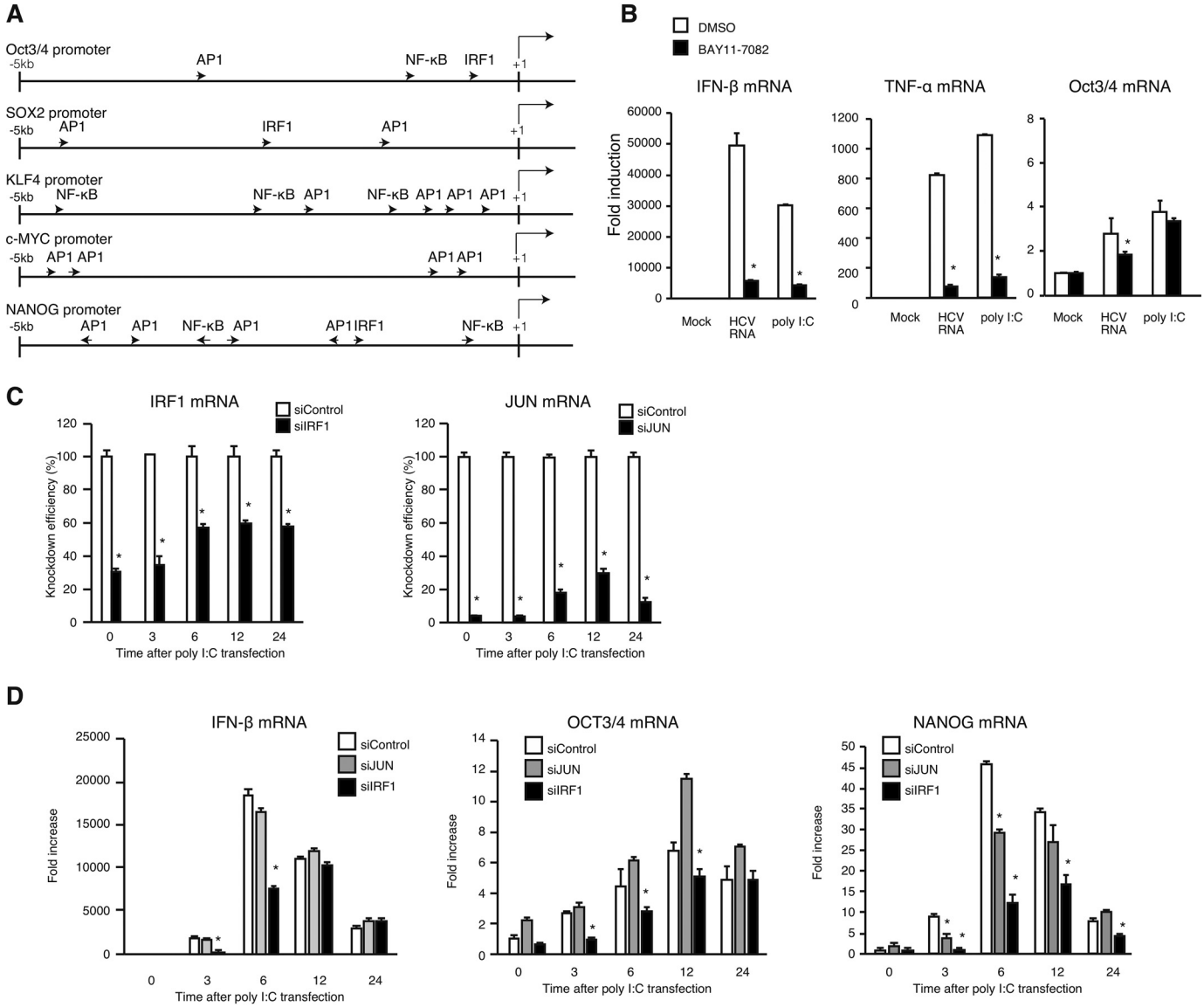

Figure 6. IRF1 is involved in poly(I:C)-mediated Oct3/4 expression. *A,* schematic representation of the binding sites of transcription factors on the promoter regions of Oct3/4, SOX2, KLF4, c-Myc, and NANOG. *B,* BJ cells treated with BAY11-7082, an inhibitor for NF-κB, were stimulated by transfecting with 1 μg/ml of poly(I:C) and 3′ UTR of HCV RNA using Lipofectamine 2000 for 6 h. Total RNA was extracted, and the expression levels of IFN-β, TNFα, and Oct3/4 mRNA were determined by RT-qPCR and normalized to those of GAPDH. *C* and *D,* siRNAs for negative control, IRF-1, and Jun were transfected into BJ cells. 48 h after transfection, cells were stimulated by transfecting with 1 μg/ml of poly(I:C) using Lipofectamine 2000. Total RNA was extracted at the indicated time points. The expression levels of IRF1, JUN, IFN-β, Oct3/4, and Nanog mRNA were determined by RT-qPCR, and normalized to those of GAPDH.

TNFα expression upon stimulation with the 3′ UTR of HCV RNA and poly(I:C), it exhibited a marginal effect on Oct3/4 expression (Fig. 6*B*).

Next, we tested the roles of IRF1 and c-Jun by using siRNAs, which substantially reduced their mRNA levels (Fig. 6*C*). Knockdown of c-Jun hardly reduced the expression of IFN-β, Oct3/4, and NANOG upon poly(I:C) stimulation (Fig. 6*D*). In contrast, knockdown of IRF1 reduced the mRNA levels of Oct3/4 and NANOG after poly(I:C) stimulation (Fig. 6*D*), suggesting that IRF1 is involved in Oct3/4 expression upon RLR stimulation. In contrast to Oct3/4 and NANOG, siRNA for IRF1 reduced KLF4 mRNA expression only at later time points (24 h post-stimulation) and failed to reduce the mRNA expression of SOX2 and c-Myc (Fig. 7*A*). These data imply that not only IRF1 but also other transcription factors might be required for the expression.

To further investigate the role of IRF1, subcellular localization of IRF1 was observed before and after stimulation, because IRF1 is known to translocate to the nuclei after its activation. IRF1 nuclear localization was observed after stimulation with poly(I:C) using Lipofectamine 2000 (Fig. 7*B*). These data suggests that IRF1 was up-regulated and translocated into the nuclei after RLR stimulation. TLR3 activation also induced IRF1 nuclear localization (Fig. S1), implying that other transcription factors as well as IRF1 are involved in Oct3/4 expression. To further investigate the involvement of IRF1 in Oct3/4 expression, we performed a chromatin immunoprecipitation (ChIP) assay with anti-IRF1 antibody. Poly(I:C) stimulation using Lipofectamine 2000 increased the amount of precipitated Oct3/4 promoter region with anti-IRF1 antibody (Fig. 7*C*). Taken together, our data indicate that RIG-I and MDA5-in-

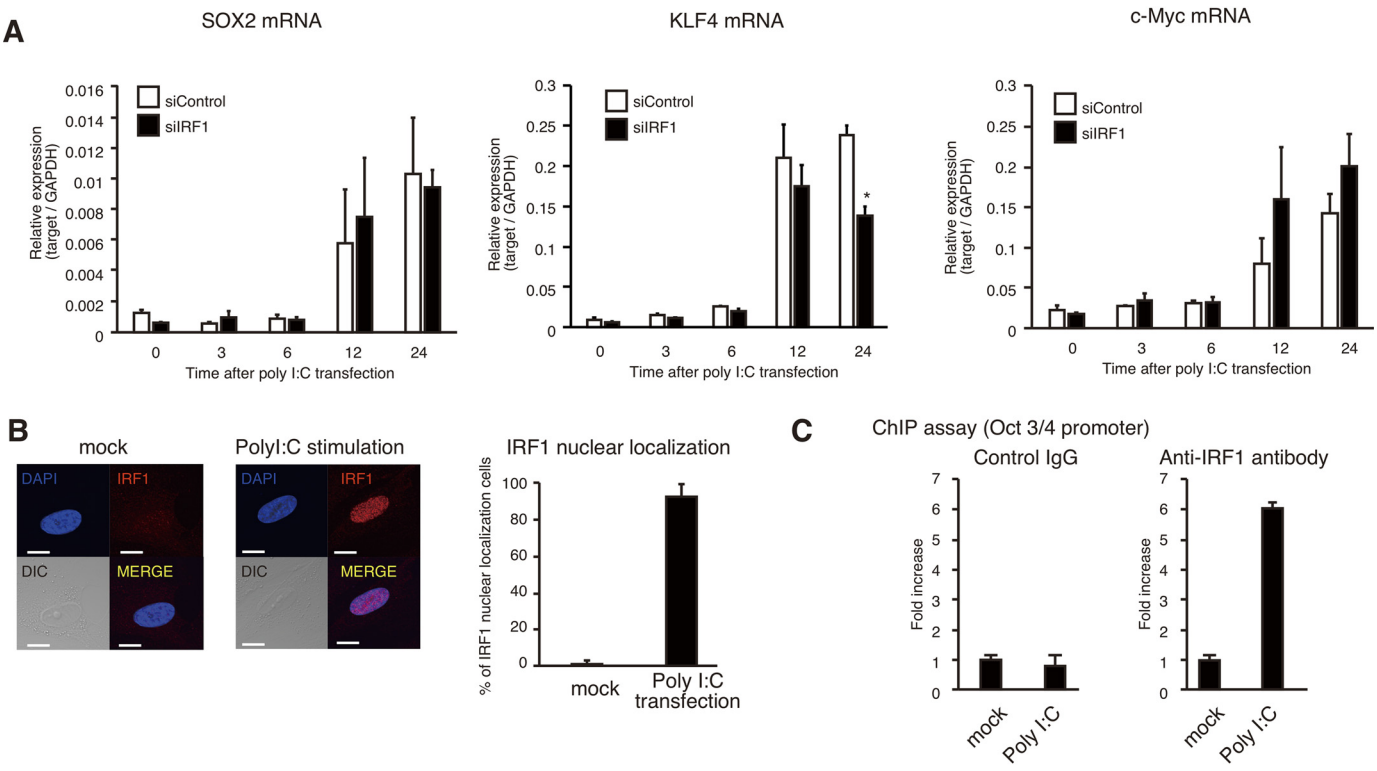

Figure 7. IRF1 activation after poly(I:C) stimulation. *A,* siRNAs for IRF1 and negative control were transfected into BJ cells. 48 h after transfection, cells were stimulated by transfection of 1 μg/ml of poly(I:C) using Lipofectamine 2000. RT-qPCR was performed to determine the expression levels of SOX2, KLF4, and c-Myc mRNAs. *B,* BJ cells were stimulated with 1 μg/ml of poly(I:C) using Lipofectamine 2000. Cells were fixed and stained with DAPI and anti-IRF1 antibody at 0 and 5 h after stimulation. IRF1 (*red*) and nuclei (*blue*) were observed by fluorescence confocal microscopy (*left panel*). % of the cells with IRF1 nuclear localization was measured (*right panel*). The *white bar* represents 10 μm. *C,* BJ cells were stimulated with 1 μg/ml of poly(I:C) using Lipofectamine 2000. Cells were then fixed and a ChIP assay was performed with control IgG and anti-IRF1 antibody. Oct3/4 promoter DNA fragments were detected by PCR, and their amounts were normalized to those of mock samples.

duced IRF1 activation is involved in the expression of Oct3/4 mRNA.

Discussion

The role of PRRs in the reprogramming of somatic cells has been reported; however, the underlying mechanism has not yet been fully elucidated. In this study, we investigated the effect of extracellular and cytoplasmic poly(I:C) and dsDNA, and found that cytoplasmic poly(I:C) increased Oct3/4 expression and induced its nuclear localization in human somatic cells more efficiently than extracellular/cytoplasmic dsDNA or extracellular poly(I:C). Interestingly, cytoplasmic poly(I:C)-induced Oct3/4 expression requires MAVS and IRF1 transcription factors. These observations indicate that the cytoplasmic poly(I:C), which is recognized by RIG-I and MDA5, activate the MAVS protein, leading to Oct3/4 expression via the activation of IRF-1 transcription factor. IRF1 was critical for the Oct3/4 expression. However, since IRF1 activation was also detected after TLR3 expression, we do not exclude the possibility that not only IRF1 but also other transcription factors are involved in Oct3/4 expression.

MAVS is a solo adaptor of RIG-I and MDA, and it localizes on the mitochondria and peroxisome (25, 26). In the innate immune response, mitochondrial MAVS activates IRF3 and NF-κB, which results in the expression of type I IFNs and other pro-inflammatory cytokines. In contrast, peroxisomal MAVS activates IRF1 and induces the interferon-stimulated genes (26). Considering that IRF1 is required for poly(I:C)-mediated Oct3/4 expression, we hypothesize that the activation of peroxisomal MAVS is required for the Oct3/4 expression.

Previous studies reported that poly(I:C)-mediated expression of Oct3/4 is required for TLR3 and its adaptor TICAM-1 (12). Based from our results, poly(I:C)-mediated Oct3/4 expression was largely dependent on the MAVS adaptor but not TICAM-1. However, these observations do not exclude the possibility that the TLR3 and TICAM-1 pathways induce the expression of the stem cell-specific genes in other types of somatic cells or in other experimental conditions. Several innate immune responses might be involved in the efficient reprogramming in the induction of pluripotency.

There are several reports on how severe cell stress triggers the expression of stem cell-specific genes. Isolation of Muse cells requires several severe cell stresses, and cells referred to as stimulus-triggered acquisition of pluripotency (STAP) cells require acidic conditions (11, 27). Severe cell stress would induce necrosis and necroptosis, leading to the release of DAMPs, such as RNA and DNA. Recent studies reported that host RNA functions as a ligand for RIG-I and MDA5 (28, 29), and myeloid cells have the ability to incorporate extracellular RNA into their cytoplasm, thereby activating the innate immune responses via RIG-I and MDA5 (30, 31). Therefore, it is possible that severe cell stresses lead to the

release of host RNA, which results in its internalization into the cytoplasm, thereby activating the MAVS-IRF1 signaling pathway and leading to Oct3/4 mRNA expression. However, further studies are still required to reveal the underlying mechanism.

In our experimental conditions, cytoplasmic poly(I:C) and *in vitro* synthesized viral RNA induced Oct3/4 mRNA expression, but Oct3/4 mRNA did not increase by stimulation with a physiological amount of viral RNA. These observations imply that mere viral infection hardly increases Oct3/4 expression. Considering that extracellular RNAs are easily internalized into the cytoplasm of myeloid cells, leading to activation of RIG-I and MDA5 (31), it is expected that RIG-I and MDA5 require some specific conditions, such as release of a large amount of dsRNA from viral-infected dead cells, to increase Oct3/4 mRNA expression.

Niwa (27) showed that the acidic conditions markedly increased Oct3/4 expression in a small population of cells; however, he could not reproduce the STAP phenomenon. We also could not detect any STAP phenomenon by using poly(I:C), but we detected a substantial increase in the expression of stem cell-specific genes. Thus, unlike the induction of four Yamanaka factors, the activation of the innate immune system would be insufficient for inducing pluripotency. Although the physiological significance of cytoplasmic dsRNA-induced expression of stem cell-specific genes is still unclear, we expect that chemicals targeting of the molecules involved in the MAVS-IRF1 pathway, such as RIG-I, MDA5, MAVS, IRF1, Riplet, and ZNF598 (32, 33), may be useful in increasing the efficiency of the production of iPS cells.

Experimental procedures

Cells and reagents

Human foreskin fibroblast BJ cells (early passage, p6) were purchased from Stemgent. Human hepatic stellate cell line LX2 cells were provided by T. Shimotohno (NCGM) (34). Human hepatocyte Oc and O cells that contained HCV 1b replicons were provided by N. Kato (Okayama University) (35). All cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum and penicillin-streptomycin (Gibco). Additionally, L-glutamine (Gibco) and NEAA (Gibco) were supplemented for both O and Oc cells, and G418 (Roche Applied Science) was added for O cells. NEAA and pyruvate sodium (Gibco) were supplemented for LX2 cells. Poly(I:C) was purchased from GE Healthcare and dissolved in saline. Human IFN-α and TGF-β were purchased from Miltenyi Biotec and R&D Systems, respectively. An anti-OCT3/4 antibody was purchased from Cell Signaling Technology, Inc. An anti-IFNAR2 antibody was purchased from PBL. Anti-MAVS and anti-IRF1 antibodies were purchased from Cell Signaling Technology. MAVS, AP1, IRF1, STAT1, and STAT2 siRNAs were purchased from Thermo Fisher Scientific. Ruxolitinib was purchased from Chemscene LLC (Funakoshi). Detection of phosphorylation of TBK1 and IRF were performed as described previously (32).

Table 1
Primer sequences for PCR

		Sequence
GAPDH	F[a]	5′-GAGTCAACGGATTTGCTCGT-3′
	R	5′-TTGATTTTGCAGGGATCTCG-3′
IFN-β	F	5′-CAACTTGCTTGGATTCCTACAAAG-3′
	R	5′-TATTCAAGCCTCCCATTCAATTG-3′
OCT3/4	F	5′-CCGTGAAGCTGGAGAAGGAG-3′
	R	5′-GCAGATGGTCGTTTGGCTGA-3′
SOX2	F	5′-CCCCTTTATTTTCCGTAGTTGTATTT-3′
	R	5′-TGGATTCTCGGCAGACTGATT-3′
KLF4	F	5′-TACCAAGAGCTCATGCCACC-3′
	R	5′-GCGAATTTCCATCCACAGCC-3′
c-MYC	F	5′-GCCACGTCTCCACACATCAG-3′
	R	5′-TCTTGGCAGCAGGATAGTCCTT-3′
NANOG	F	5′-AGAACTCTCCAACATCCTGAACCT-3′
	R	5′-TGCCACCTCTTAGATTTCATTCTCT-3′
β-CATENIN	F	5′-TGCAGTTCGCCTTCACTATG-3′
	R	5′-GATGTGCACGAACAAGCAAC-3′
REX1	F	5′-ACAGAACAGAAGAGGCCTTCAC-3′
	R	5′-GTCTTTGCCCGTTTCTTCAG-3′
ECAT1	F	5′-TGATCATGAACCTGGCTGAC-3′
	R	5′-GCCTCCCGGACTTCTATTG-3′
DPPA2	F	5′-GCAAGAATTGCTGCAAGAGC-3′
	R	5′-TTGTGTCTGCCGAGAGAAGTC-3′
DPPA3	F	5′-GTTACTCGGCGGAGTTCGTA-3′
	R	5′-TGAAGTGGCTTGGTGTCTTG-3′
DPPA4	F	5-GCCAGGAACATCAGCAAAGAG-3′
	R	5′-CAGAATGTCCCGGTGAATCAG-3′
DPPA5	F	5′-TCACCGAGGTCGTGGTTTAC-3′
	R	5′-TTGGCTGGAAACTGGTTCAC-3′
ECAT8	F	5′-CCTGGCAGAATGTTTCCTTG-3′
	R	5′-TCTCGGCAGAAGTCAATGTG-3′
GDF3	F	5′-CGGGAATGTACTTCGCTTTC-3′
	R	5′-TGGGCCAATGTCAACTGTTC-3′
FTHL17	F	5′-TATCCGCCATTCTTGTCACC-3′
	R	5′-TCGCAGTTGGTGTCGTACTTC-3′
SOX15	F	5′-AGCAGAGGCTTTGGGTACAG-3′
	R	5′-TATAGGTGGGCAGCAGTTCC-3′
STAT3	F	5′-TTTCACTTGGGTGGAGAAGG-3′
	R	5′-GGCTACCTGGGTCAGCTTCAG-3′
UTF1	F	5′-ACCAGCTGCTGACCTTGAAC-3′
	R	5′-TTGAACATACCCAAGAACGG-3′
ERAS	F	5′-TCATGGAGCTGCCAACAAAG-3′
	R	5′-TGGTTCAGCTGGATGGTCAG-3′
DNMT3L	F	5′-ATGCTACTGCTTCGAGTGTGTG-3′
	R	5′-CGGTTTCGAACATCTCAAGG-3′
GRB2	F	5′-ACGAGCTGAGCTTCAAAAGG-3′
	R	5′-TTTCTTCTGCCTTGGCTCTG-3′
FBXO15	F	5′-CCATGCCTAGCATCATTGTC-3′
	R	5′-AGGCAGAAGATCCGAATGAG-3′
TCL1A	F	5′-GGCTCTGGCTCTTGCTTCTTAG-3′
	R	5′-GCACCCGTAACTGTAACCTATCC-3′
SALL4	F	5′-CCAATAGTCAAGCCGAAAGC-3′
	R	5′-ATGTGCCAGGAACTTCAACC-3′
STAT1	F	5′-TTCAGGAAGACCCAATCCAG-3′
	R	5′-TGAATATTCCCCGACTGAGC-3′
JUN	F	5′-ACCTTGAAAGCTCAGAACTCG-3′
	R	5′-GTTTGCAACTGCTGCGTTAG-3′
IRF1	F	5′-ACATCCCAGTGGAAGTTGTG-3′
	R	5′-TTCCCTTCCTCATCCTCATC-3′
Oct3/4 promoter	F	5′-CACTCCAGTCTGGGCAACAAAG-3′
	R	5′-AAACCGGGAGACACAACTGG-3′

[a] F, forward; R, reverse.

RNA preparation

Total RNA from human hepatocyte O and Oc cells were extracted using TRIzol reagent (Thermo Fisher Scientific). HCV genotype 1b 3′ UTR RNA including the poly(U/UC) region was synthesized using T7 and SP6 RNA polymerase and purified with TRIzol, as described previously. These RNAs were transfected into cells using either Lipofectamine 2000 or DOTAP (Roche Applied Science).

Quantitative PCR

For quantitative PCR (qPCR), total RNA was extracted using TRIzol reagent (Invitrogen), after which 0.5 μg of RNA was

reverse transcribed using a high capacity cDNA transcription kit with an RNase inhibitor (Applied Biosystems) according to the manufacturer's instructions. qPCR was performed using a Step One Real-Time PCR system (Applied Biosystems). The expression of mRNA was normalized to that of GAPDH mRNA, and the fold-change was determined by dividing the expression levels in each sample by that in the control samples in each experiment. The PCR primers sequences are listed in Table 1.

Confocal microscopy

Anti-OCT4 rabbit mAb was purchased from Cell Signaling Technology, Inc. Cells were fixed with 3% formaldehyde in 1× PBS for 30 min, and permeabilized with 0.2% Triton X-100 in 1× PBS for 15 min. For blocking, 1% BSA in PBS was used for 30 min. The cells were labeled with the anti-OCT4 antibody overnight at 4 °C. After washing four times with 1% BSA in PBS, cells were incubated with an Alexa 488-conjugated secondary antibody and 1% BSA in PBS for 60 min at room temperature, and then washed four times with 1% BSA in PBS. Samples were mounted on glass slides using Prolong Gold (Invitrogen). Cells were visualized at a magnification of ×63 using an LSM510 META microscope (Zeiss).

ChIP assay

4×10^6 of BJ cells were transfected with short poly(I:C). Zero and 6 h after transfection cells were subjected to ChIP assay, according to the manufacture's instructions (Cell Signaling Technology number 9002). Briefly, cells were fixed with formaldehyde and lysed, and genomic DNA was partially digested with Micrococcal Nuclease treatment. Immunoprecipitation was performed with anti-IRF1 antibody and control IgG. To elute genomic DNA from the protein-DNA complex, immunoprecipitates were treated with 1× ChIP Elution Buffer over 2 h at 65 °C. Eluted DNA was purified with spin columns. PCR was performed with primers, Oct3/4 promoter F and R.

Statistical analysis

Student's *t* test were performed using MS-Excel software. Error bars represent S.D. ($n > 3$, *, $p < 0.05$). All experiments were repeated at least two times. ND represents "not detected," and NS represents "not significant."

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