Defective Antiviral Responses of Induced Pluripotent Stem Cells to Baculoviral Vector Transduction

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Genetic engineering of induced pluripotent stem cells (iPSCs) is important for their clinical applications, and baculovirus (BV) holds promise as a gene delivery vector. To explore the feasibility of using BV for iPSCs transduction, in this study we first examined how iPSCs responded to BV. We determined that BV transduced iPSCs efficiently, without inducing appreciable negative effects on cell proliferation, apoptosis, pluripotency, and differentiation. BV transduction slightly perturbed the transcription of 12 genes involved in the Toll-like receptor (TLR) signaling pathway, but at the protein level BV elicited no well-known cytokines (e.g., interleukin-6 [IL-6], tumor necrosis factor alpha [TNF- α], and beta interferon [IFN- β]) except for IP-10. Molecular analyses revealed that iPSCs expressed no TLR1, -6, -8, or -9 and expressed merely low levels of TLR2, -3, and -4. In spite of evident expression of such RNA/DNA sensors as RIG-I and AIM2, iPSCs barely expressed MDA5 and DAI (DNA-dependent activator of IFN regulatory factor [IRF]). Importantly, BV transduction of iPSCs stimulated none of the aforementioned sensors or their downstream signaling mediators (IRF3 and NF- κ B). These data together confirmed that iPSCs responded poorly to BV due to the impaired sensing and signaling system, thereby justifying the transduction of iPSCs with the baculoviral vector.

Induced pluripotent stem cells (iPSCs) can be derived from somatic cells by introducing a cocktail of reprogramming factors while obviating the need to destroy embryos (36). Similar to embryonic stem cells (ESCs), iPSCs can be maintained in the undifferentiated state indefinitely but can differentiate into cells belonging to all three germ layers: endoderm, mesoderm, and ectoderm. The pluripotency and avoidance of ethical issues render iPSCs a promising cell source for tissue regeneration, disease modeling, and treatment (48), and the therapeutic potentials of iPSCs can be realized by genetic manipulation (35, 38, 47). To date, iPSCs have been genetically engineered with adenovirus (43), adeno-associated virus (24), and nonviral vectors (19, 21). Since nonviral vectors usually suffer from low transfection efficiency, especially into stem cells, it is possible to genetically modify iPSCs or iPSC-derived cells using viral vectors. However, whether viral vector transduction of iPSCs induces undesirable effects and whether iPSCs respond to the viral vector and elicit antiviral effects remain unknown.

In addition to these viral vectors, baculovirus (BV) is a virus that infects insects in nature but can transduce numerous mammalian cells without appreciable cytotoxicity (4, 7). Additionally, BV effectively transduces chondrocytes (12), human (20) and rabbit (27) mesenchymal stem cells (MSCs), and adult and embryonic stem cells (16, 50) as well as neural stem cells derived from iPSCs (26). Transduction of MSCs and chondrocytes with BV expressing growth factors stimulates cellular differentiation *in vitro*. Furthermore, implantation of the BV-engineered MSCs and chondrocytes into massive bone and cartilage defects, respectively, augments tissue repair *in vivo* (11, 27, 28), thus indicating the potential of BV as a gene therapy vector for cell therapy. Importantly, BV transduction of MSCs provokes only mild and transient innate responses via the activation of the Toll-like receptor 3 (TLR3) pathway (9) although BV is known to trigger innate responses in mammalian cells via the TLR9/MyD88-dependent and -independent pathways (1, 2).

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) responsible for detecting microbial antigens. Among the TLRs, TLR2 recognizes bacterial lipoproteins while TLR3 detects virus-derived double-stranded RNA (dsRNA) and the synthetic dsRNA analog, poly(I-C) (pIC). TLR4 recognizes lipopolysaccharides (LPS) from Gram-negative bacteria, and TLR5 recognizes bacterial flagellin. TLR7 and TLR8 mediate recognition of viral single-stranded RNA (ssRNA) while TLR9 recognizes the CpG motif of bacterial and viral DNA. The engagement of TLRs with cognate ligands results in TLR activation, recruitment of adaptor molecules (e.g., MyD88), and signal transduction to downstream molecules such as NF- κ B and IRF3, leading to the secretion of cytokines such as type I interferon (alpha/beta interferon [IFN- α/β]), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF- α) (23). Aside from TLRs, other intracellular PRRs, including the RNA-sensing RIG-I (retinoic acid-inducible gene I) and MDA5 (melanoma differentiation-associated protein 5), as well as the DNA sensors AIM2 (absent in melanoma 2) and DAI (DNA-dependent activator of IFN regulatory factor [IRF]), have recently been identified (14, 34, 41). Similar to TLRs, RIG-I, MDA5, and DAI induce intracellular signaling pathways through NF- κ B and IRF3, giving rise to the expression of proinflammatory cytokines and type I IFNs, whereas AIM2 binds to cytoplasmic DNA, leading to the production of IL-1 β (22).

Since genetic engineering of iPSCs is important for their appli-

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cations and since BV holds promise as a gene therapy vector, we sought to evaluate the feasibility and safety of using BV to transduce iPSCs. In this study, we first assessed whether BV was able to transduce iPSCs and whether BV transduction mitigated iPSC pluripotency and differentiation and elicited antiviral responses. The molecular pathway governing the antiviral responses of iPSCs was also examined.

MATERIALS AND METHODS

Mouse iPSC culture and transduction. Mouse iPSCs (20D17) expressing green fluorescent protein (GFP) under the control of *Nanog* promoter were kindly provided by Shinya Yamanaka (Center for iPS Cell Research and Application, Kyoto University) and routinely cultured on mouse embryonic fibroblasts ([MEF] Food Industry Research and Development Institute, Taiwan) as described previously (40). For iPSC expansion, the cells were seeded onto MEF cells in T75 flasks and were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (without sodium pyruvate; Gibco) containing 15% fetal bovine serum (FBS; Gibco), 0.1 mM nonessential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), and 1,000 U/ml leukemia inhibitory factor (LIF; Millipore). iPSCs on MEF feeder layers were routinely passaged every day after trypsinization. Before transduction, iPSCs and MEFs were trypsinized, seeded to T75 flasks coated with 0.1% gelatin, and incubated at 37°C for 40 min. The floating iPSCs were transferred to gelatin-coated six-well plates (1×10^4 cells/cm²) or 96-well plates (1×10^3 cells/well). The iPSCs were transduced as described previously (10), with a recombinant BV harboring no mammalian transgene cassette (9) or with a recombinant BV expressing DsRed under the control of elongation factor 1 α (EF1 α) promoter (7). The BV was amplified and harvested, and titers were determined by end-point dilution assay as described previously (39).

Cell proliferation, apoptosis, and EB formation. For proliferation assays, iPSCs cultured in 96-well plates were transduced and analyzed at 3 days posttransduction (dpt) using a bromodeoxyuridine (BrdU) cell proliferation assay kit (Millipore) as described previously (8). The apoptosis of iPSCs was quantified by two-dimensional flow cytometry, using an Annexin V-PE (phycoerythrin) Apoptosis Detection Kit I (BD Biosciences). For embryonic body (EB) formation, the mock- and BV-transduced cells were cultured in suspension at a density of 800 cells per 20 μ l, in a nonadhesive petri dish by the hanging-drop method. The EBs were observed under a phase-contrast microscope or fluorescence microscope at day 2.

RT-PCR, qRT-PCR, and PCR array. Total RNA was extracted from the cells using a NucleoSpin RNA II purification kit (Clontech) and reverse transcribed to cDNA using an MMLV (Moloney murine leukemia virus) Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre Biotechnologies). The reverse transcription-PCRs (RT-PCRs) were performed using *Taq* DNA polymerase (Promega) in a Px2 Thermal Cycler (Thermo Electron) under the conditions of 30 s at 95°C, 45 s at 60°C, and 30 s at 72°C, and the amplicons were subjected to 2% agarose gel electrophoresis. The quantitative RT-PCRs (qRT-PCRs) were performed using 10 μ l of diluted cDNA ($\approx$ 400 ng) with a Power SYBR Green PCR Master Mix and ABI Prism 7300 (Applied Biosystems) according to the manufacturer's instructions. The sequences of primers used for qRT-PCR and RT-PCR are listed in Tables S1 and S2, respectively, in the supplemental material. For RT-PCR analysis of TLR transcription, the cDNA was analyzed using murine TLR RT-Primers (Invivogen). To simultaneously detect 84 TLR-associated genes, the cDNA was analyzed using a mouse TLR pathway-focused RT² Profiler PCR Array (SABiosciences).

Immunofluorescence labeling and confocal microscopy. The cells were fixed and permeabilized as described previously (9) and then subjected to extensive washing and primary antibody staining (1:150 dilution) for 1 h at 4°C in the dark. The primary antibody was specific for mouse Nanog (ab80892; Abcam), Sox2 (ab97959; Abcam), phosphorylated NF- κ B (3033; Cell Signaling Technology), or IRF3 (sc-9082; Santa Cruz Biotechnology). After being washed, the cells were incubated with goat anti-rabbit antibody conjugated with Alexa 543 (Invitrogen) or donkey anti-goat IgG-R (for IRF3) (sc-2094; Santa Cruz Biotechnology) for 1

h at 4°C in the dark. After being washed, the cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI; Vector Labs) and visualized with a confocal microscope (Nikon TE2000 equipped with a confocal upgrade laser kit).

Teratoma formation. Mouse iPSCs were transduced with BV at a multiplicity of infection (MOI) of 100 for 4 h, cultured for 3 days to expand the cell number, trypsinized, resuspended in phosphate-buffered saline ([PBS] 4×10^6 cells/ml), and injected (4×10^5 cells in 100 μ l) subcutaneously into the back of nude mice (8 to 10 weeks old). Four weeks later, teratomas were surgically dissected from mice. The cryostat sections (10 μ m thick) were stained with hematoxylin and eosin (H&E) and observed by a light microscope (Nikon).

Flow cytometry. To characterize TLR expression, cells were fixed and permeabilized with 4% formaldehyde and 0.5% Tween 20. After being washed, the cells were incubated with different primary antibodies (1:150 dilution) for 1 h at 4°C in the dark. For TLR2 and TLR4 detection, the primary antibody was Alexa 647-conjugated monoclonal antibody (MAb) specific for mouse TLR2 (12-9924; eBioscience) or phycoerythrin (PE)-conjugated MAb specific for mouse TLR4 (51-9021; eBioscience). After being washed, the cells were collected for flow cytometry (FACSCalibur; BD Biosciences) analysis. For TLR3 detection, cells were incubated with the primary antibody specific for mouse TLR3 (NB100-565715; NOVUS) and then incubated with Alexa 543-conjugated goat anti-rabbit IgG (Invitrogen) for 1 h at 4°C in the dark, followed by flow cytometry analysis. As controls, cells were incubated for 30 min with 500 μ l of NaHCO₃-deficient minimal essential medium, alpha modification (α -MEM), containing 10 μ g/ml pIC (Sigma) or 10 ng/ml LPS (Sigma) and then analyzed for TLR expression as described above.

Cytokine measurement. Conditioned medium from the BV-transduced iPSC cultures was collected at 24 posttransduction (hpt) and analyzed with a multiplex bead immunoassay kit (Cytokine Mouse 20-Plex Panel; Invitrogen). The optical densities were read and analyzed using a dual laser detection system (Luminex 100; Luminex Corporation). IP-10 (IFN- γ -inducible protein 10) was measured using a murine enzyme-linked immunosorbent assay (ELISA) development kit (PeproTech).

Western blotting. The transduced and mock-transduced cells were lysed for Western blotting. The primary antibodies (1:1,000 dilution) were specific for MDA5 (4110; Cell Signaling Technology), RIG-I (4520; Cell Signaling Technology), AIM2 (sc-137967; Santa Cruz Biotechnology), or β -actin (A-2066; Sigma). The secondary antibody was species-specific horseradish peroxidase (HRP)-conjugated IgG (1:5,000 dilution; Amersham Biosciences). The images were developed using a GeneGnome HR scanner (Syngene).

Statistical analysis. All data represent the mean $\pm$ standard deviation or the mean values of at least three independent culture experiments. The data were statistically analyzed by one-way analysis of variance (ANOVA). A *P* value of <0.05 was considered significant.

RESULTS

Effects of BV transduction of iPSCs on EB formation, cell proliferation, and apoptosis. To ascertain that BV was able to transduce iPSCs, we transduced mouse iPSCs with BV expressing the red fluorescent protein (DsRed) and determined that BV transduced approximately 45% of iPSCs at a multiplicity of infection (MOI) of 100 (see Fig. S1 in the supplemental material), which is commonly used for BV transduction of mammalian cells without appreciable cytotoxicity (29). The transduced iPSCs remained capable of forming embryonic bodies (EBs) as culture time increased and concomitantly expressed DsRed (Fig. 1A).

To examine whether BV transduction itself affected the cell characteristics, iPSCs were untreated, mock transduced, or transduced with a BV carrying no mammalian gene cassette at an MOI of 100. A BrdU proliferation assay performed at 96 h posttransduction (hpt) revealed statistically similar (*P* > 0.05) optical densities between the untreated, transduced, and mock-transduced

FIG 1 BV transduction of iPSCs did not undermine the cell characteristics. (A) Formation of EBs from iPSCs transduced with a DsRed-expressing BV (MOI of 100). (B) Cell proliferation. (C) Cell apoptosis. (D) Colony formation. For panels B, C, and D, iPSCs in 96-well plates (1×10^3 cells/well) were mock transduced or transduced with a BV that carried no transgene cassette (no recombinant protein expression). Cell proliferation was assayed at 96 hpt by BrdU labeling, and the data are expressed as optical densities (OD). Apoptosis was analyzed at 24 dpt using an annexin V-PE apoptosis kit followed by flow cytometry. Colony formation was observed by confocal microscopy at 48 hpt. Mock-transduced cells served as the control.

cells (Fig. 1B), indicating that iPSC proliferation was not hindered by BV transduction. The apoptosis analyses using annexin V-PE/7-aminoactinomycin D (7-AAD) staining coupled with flow cytometry at 24 hpt (Fig. 1C) showed that, in comparison with mock transduction, BV transduction resulted in no significant fluctuations in the live, apoptotic, or dead cell populations. Furthermore, Fig. 1D illustrates that the BV-transduced colony remained capable of expressing green fluorescent protein (GFP) under the control of the *Nanog* promoter (which served as the pluripotency marker [36]).

Effects of BV transduction on iPSC pluripotency and differentiation. To examine whether BV transduction undermined the pluripotency and differentiation states of iPSCs, the mRNA levels of five transcription factors, *Nanog*, *Sox2*, *Oct4*, *klf4*, and *Tbx3*, in the cells were measured at 24 hpt by quantitative real-time reverse transcription-PCR (qRT-PCR) (Fig. 2A), which revealed no significant differences in the mRNA levels of all five pluripotency marker genes between the transduced and mock-transduced cells ($P > 0.05$). The immunofluorescence labeling specific for Nanog and Sox2 (Fig. 2B) illustrated evident Nanog and Sox2 expression in the BV-transduced and mock-transduced colonies, demonstrating that BV transduction did not mitigate iPSC pluripotency.

We next measured by qRT-PCR the mRNA levels of the lineage-specific genes, which were normalized against those of mock transduction. Figure 2C depicts statistically similar ($P > 0.05$) expression levels of endoderm markers (*Gata6*, *Gata4*, Lamin B1 gene, and *Ihh*), ectoderm markers (*Fgf5* and *Nestin* gene), and mesoderm markers (*Bmp4*, *mHox*, and *T*) in BV-transduced and mock-transduced cells. Furthermore, four weeks after subcutaneous injection of iPSCs into the backs of nude mice, similar teratomas containing respiratory, neural epithelium, and muscle tissues arose from the mock-transduced and transduced iPSCs (Fig. 2D).

These data together confirmed that BV transduction did not impair the pluripotency and differentiation capability of iPSCs.

Cytokine production in response to BV transduction. Recently, iPSCs were discovered to be immunogenic after implantation and differentiation (51). Because cells can sense foreign pathogens by TLRs, we next explored whether BV transduction of iPSCs elicited TLR-associated responses by mock transducing or transducing iPSCs with BV harboring no transgene (MOI of 100). Gene transcription was analyzed at 24 hpt using an RT² Profiler PCR array, which detects 84 genes involved in the TLR pathway (including *TLR1* to *TLR9*). The genes that were associated with the TLR signaling pathway and significantly perturbed by BV are summarized in Table 1, which reveals the upregulation of such genes as *TLR3* and *TLR4* and downstream genes *IL-10*, *CXCL10*, *IL-6*, *IFN- β 1*, *Ikbkb*, *PTGS2*, and *MyD88* with statistical significance. However, the magnitude of induction was considerably lower in the iPSCs than in the MSCs transduced under the same conditions (Table 1). Since TLR activation elicits downstream secretion of cytokines, we next screened the BV-induced cytokines at the protein level by analyzing the culture supernatant at 24 hpt using a 20-plex cytokine immunoassay. The data shown in Fig. 3A demonstrate that iPSCs barely expressed such cytokines as fibroblast growth factor (FGF) basic, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN- γ , IL-1 α , IL-1 β , IL-4, IL-5, IL-6, IL-12, IL-13, IL-17, and monokine induced by gamma interferon (MIG), but iPSCs indeed expressed various levels of IL-2, IL-10, keratinocyte-derived chemokine (KC), monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 α (MIP-1 α), TNF- α , vascular endothelial growth factor (VEGF), and IP-10. Nonetheless, BV transduction did not significantly provoke the secretion of these cytokines except for IP-10 ($P <$

FIG 2 BV transduction of iPSCs did not impair the pluripotency and differentiation. (A) mRNA levels of pluripotency markers. (B) Immunofluorescence labeling for Nanog and Sox2. (C) mRNA levels of endoderm, ectoderm, and mesoderm markers. (D) H&E staining of the teratomas derived from iPSCs. iPSCs were transduced with a BV carrying no transgene cassette (MOI of 100), and mock-transduced cells served as the controls. The mRNA expression levels were measured by qRT-PCR and normalized to those of mock transduction at 24 hpt. For confocal microscopy, the cells were counterstained by DAPI. For teratoma formation, the transduced cells were injected into the backs of nude mice, and the teratomas were sectioned for H&E staining. Respiratory (endoderm), neural epithelium (ectoderm), and muscle (mesoderm) tissues are shown. *, $P > 0.05$.

TABLE 1 Significantly changed genes associated with TLR signaling pathway by BV transduction

Gene group	Changes in gene expression in the indicated cell type ^a			
	iPSCs		MSCs ^b	
	Gene name	Fold change	Gene name	Fold change
TLRs	<i>TLR3</i>	5.3	<i>TLR3</i>	1,157.1
	<i>TLR4</i>	4.7		
Cytokines and chemokines	<i>IL-10</i>	2.7	<i>CXCL10</i>	465.1
	<i>CXCL10</i>	2.2	<i>IL1B</i>	95.4
	<i>IL-6</i>	2.0	<i>IL-6</i>	27.0
	<i>IFNβ1</i>	2.0	<i>IL-8</i>	26.4
Other genes associated with TLR	<i>Ikbkb</i>	2.5	<i>PTGS2</i>	19.3
	<i>PTGS2</i>	2.2	<i>IRAK2</i>	10.2
	<i>MyD88</i>	2.2	<i>NFKBIA</i>	9.4
	<i>IRF3</i>	2.1	<i>MyD88</i>	5.8
	<i>Csf2</i>	2.1	<i>TRIF</i>	3.4
	<i>Lta</i>	0.4	<i>TRAF6</i>	1.8

^a The data represent the average values of three independent culture experiments and are sorted based on the category and magnitude of induction.

^b Adapted from reference 9.

0.05). IP-10 expression (expressed from *CXCL10*) increased with increasing BV doses as determined by ELISA (Fig. 3B), but iPSCs did not significantly secrete IP-10 ($P > 0.05$) when treated with the agonists for TLR3 (pIC; 10 μ g/ml) and TLR2/TLR4 (LPS; 10 ng/ml) for 24 h (Fig. 3C). Likewise, ELISAs revealed no IFN- β secretion by the iPSCs transduced with BV or treated with pIC or LPS (see Table S3 in the supplemental material). Increasing the pIC and LPS concentrations up to 50 μ g/ml and 50 ng/ml, respectively, which are sufficient to activate TLR pathways in other cells (30, 45), still failed to provoke IP-10 expression (Fig. 3D).

Effects of BV transduction on the TLR and RNA/DNA sensing pathways. BV transduction of MSCs activates the TLR3 pathway and hence triggers the production of IL-6 and IL-8 (9), yet a 20-plex cytokine immunoassay and ELISA data (Fig. 3) suggested negligible induction of cytokines downstream of the TLR pathways (e.g., IL-6 and TNF- α). To elucidate the disparity, we reexamined the expression of TLR1 to TLR9 in iPSCs with or without BV transduction, using a murine TLR RT-Primer Set array. In parallel, the macrophage cell line RAW 264.7 was analyzed as the control. As shown in Fig. 4A, iPSCs expressed no discernible levels of *TLR1*, *TLR6*, *TLR8*, and *TLR9* but expressed *TLR2*, *TLR3*, *TLR4*, *TLR5*, and *TLR7* to different degrees. In agreement with the PCR array data (Table 1), *TLR3* and *TLR4* were slightly upregulated by BV transduction, whereas *TLR5* and *TLR7* transcription remained unchanged (Fig. 4A). Additionally, RT-PCR showed that TLR2 was slightly upregulated. To verify TLR stimulation at

FIG 3 Cytokine expression by BV-transduced iPSCs. (A) Cytokine expression profile by iPSCs. (B) IP-10 expression at different MOIs. (C) IP-10 expression stimulated by BV, pIC (10 μ g/ml), and LPS (10 ng/ml) Un, untreated. (D) IP-10 expression stimulated by pIC and LPS at different concentrations. The iPSCs were mock transduced or transduced with BV (MOI of 100) as described in the legend of Fig. 2, and cytokine production was measured at 24 hpt using a 20-plex fluorescence bead immunoassay (A) or ELISA kits (B to D). *, $P > 0.05$; **, $P < 0.05$.

the protein level, iPSCs were mock transduced, transduced with BV, or treated with TLR agonists, followed by immunofluorescence labeling/flow cytometry analysis. Figure 4B illustrates that TLR2, TLR3, and TLR4 were evidently expressed in the untrans-

duced RAW 264.7 cells, whereas their expression levels in iPSCs were fairly low (green lines) and were barely disturbed by the corresponding ligands (LPS or pIC) or BV (pink lines).

NF- κ B and IRF3 are instrumental for multiple signaling path-

FIG 4 BV transduction of iPSCs did not trigger TLR activation. (A) mRNA levels of TLRs. (B) Expression of TLR2, -3, and -4. iPSCs were mock transduced or transduced with BV as described in the legend of Fig. 2, and TLR mRNA levels were measured by RT-PCR at 24 hpt. Alternatively, the cells were transduced with BV, immunostained with anti-TLR antibodies, and analyzed by flow cytometry. The green and pink lines indicate the mock-transduced and transduced cells, respectively. As controls, the cells were treated with TLR ligands (LPS and pIC) for 30 min and subjected to the same analyses. The macrophage cell line RAW 264.7 served as the positive control.

FIG 5 Nuclear translocation of NF- κ B and IRF3. iPSCs were mock transduced or transduced with BV (MOI of 100) as described in the legend of Fig. 2 and immunostained with antibodies specific for phosphorylated NF- κ B (p-NF- κ B) (A) or IRF3 (B) at 24 hpt, counterstained with DAPI, and followed by confocal microscopy. For comparison, human MSCs were transduced with BV for the same analyses.

ways (e.g., intracellular DNA detection) (6, 42); thus, we assessed whether NF- κ B and IRF3 were activated in the mock- and BV-transduced iPSCs. Immunofluorescence labeling/confocal microscopy illustrated that BV transduction of iPSCs did not trigger discernible accumulation of phosphorylated NF- κ B (Fig. 5A) and IRF3 (Fig. 5B) in the nuclei, indicating the absence of nuclear translocation and activation. In sharp contrast, pronounced accumulation of NF- κ B and IRF3 was observed in the nuclei of BV-transduced MSCs.

Since BV transduction of iPSCs stimulated IP-10 production, we wondered whether BV was recognized by other RNA or DNA sensors such as MDA5, RIG-I, and AIM2. To examine induction at the mRNA level, iPSCs were transduced with BV and subjected to RT-PCR analyses. Figure 6A shows that MDA5 gene transcription was fairly weak while RIG-I gene and AIM2 gene were highly transcribed in mock-transduced iPSCs; but their mRNA levels were not significantly upregulated by BV at either 0.5 hpt or 24 hpt. Conversely, DAI gene was not expressed in iPSCs (see Fig. S2 in the supplemental material). Concurrent with the RT-PCR data, Western blot analysis demonstrated that MDA5 expression in iPSCs was negligible, and BV transduction did not stimulate increases in MDA5, RIG-I, and AIM2 expression from 0.5 hpt to 24 hpt (Fig. 6B). These data collectively indicated that iPSCs ex-

FIG 6 BV transduction of iPSCs did not activate MDA5, RIG-I, and AIM2. iPSCs were mock transduced or transduced with BV, and the mRNA levels were analyzed by RT-PCR at 0.5 or 24 hpt (A). RAW 264.7 cells served as the controls. Alternatively, the transduced iPSCs were lysed for Western blotting using primary antibodies specific for MDA5, RIG-I, AIM2, and β -actin (B).

pressed low levels of MDA5 and DAI and relatively higher levels of RIG-I and AIM2, but BV transduction of iPSCs activated none of these sensors.

DISCUSSION

Current iPSC research has extensively focused on how to efficiently generate iPSCs (17) and how the generation method would impact the epigenetic state (31), epigenetic memory (25), chromosomal abnormality (33), and point mutations (18) in iPSCs. Here, we demonstrated that at an MOI of 100, BV is capable of transducing iPSCs without appreciable negative effects on EB (embryonic body) formation, cell proliferation, and apoptosis (Fig. 1A to C). BV transduction neither undermined the pluripotent state (Fig. 1D and 2A and B) nor mitigated the ability to differentiate toward mesoderm, ectoderm, or endoderm (Fig. 2C and D). These data agreed with the maintenance of differentiation state and pluripotency of iPSCs after adenoviral transduction (43) and indicated the potential of BV as a vector for the genetic modification of iPSCs.

Interestingly, by the PCR array we uncovered 12 genes that were perturbed by BV transduction at statistically significant levels (Table 1). However, the magnitudes of upregulation/downregulation of these genes (e.g., *TLR3*, *CXCL10*, *IL-6*, *PTGS2*, and *MyD88*) were small in comparison with their counterparts in BV-transduced MSCs (Table 1, mesenchymal stem cells), and the disturbance of these TLR-associated genes was not detectable by microarray analysis (see Fig. S3 and Table S4 in the supplemental material). At the protein level, BV transduction of iPSCs elicited only low levels of IP-10 but triggered no other cytokines, such as IL-6 (Fig. 3A) and IFN- β (see Table S3). This phenomenon contrasted with the MSC response whereby BV engagement with TLR3 provoked robust secretion of IL-6 (9, 15), and the disparity was at least partly attributable to the distinct expression profile of TLR signaling molecules. In MSCs, TLR2, -3, -4, -6, -7, and -8 are transcribed abundantly (37), and the downstream signaling pathways are readily activated by their respective ligands (30, 37). In contrast, at the mRNA and protein levels, iPSCs expressed no

FIG 7 Overview of the cellular responses to BV transduction in MSCs and iPSCs. In MSCs, BV transduction disturbed the expression of 816 genes including *TLR3*, *IRF3*, *NF-κB* gene, *AIM2* gene, *DAI* gene, *RIG-I* gene, and *MDA5* gene, leading to IL-6 and IL-8 secretion. In contrast, iPSCs are defective in the expression of multiple TLRs (e.g., *TLR1*, -6, -8, and -9), *DAI*, and *MDA5*, despite the expression of *AIM2* and *RIG-I*. The colored molecules indicate the molecules expressed in the cells, while uncolored molecules indicate the molecules that are either not expressed or expressed at low levels.

TLR1, -6, -8, or -9 and expressed merely low levels of *TLR2*, -3, and -4 (Fig. 4A and B). Critically, *IRF3* and *NF-κB* were not activated by BV (Fig. 5A and B). *IRF3* and *NF-κB* are key mediators on which many TLR signaling pathways converge and are responsible for the induction of type I IFNs (IFN- α/β) and cytokines (23). As such, even though BV transduction of iPSCs slightly upregulated *TLR3* and *TLR4* transcription (Table 1), the signal was probably unable to relay through the downstream signaling mediators, resulting in the impaired cytokine response of iPSCs to external stimuli including BV (Fig. 3A), pIC, and LPS (Fig. 3C and D). Moreover, mouse iPSCs differed from mouse ESCs (embryonic stem cells) in terms of the TLR repertoire and response to TLR ligands. Different from iPSCs, mouse ESCs (E14 line) expressed *TLR1*, -2, -3, -5, and -6 yet expressed no *TLR4*, -7, -8, or -9 (44). Also tripalmitoyl-Cys (Pam₃Cys) stimulation of E14 cells resulted in *TLR2* activation, *NF-κB* translocation, and TNF- α and IFN- γ upregulation (44). However, these activations were not observed in BV-transduced mouse iPSCs.

Aside from TLRs, iPSCs expressed negligible levels of *MDA5* and *DAI* despite evident expression of *RIG-I* and *AIM2*. Notably, BV did not elicit appreciable induction of these sensors, as judged by RT-PCR (Fig. 6A; see also Fig. S2 in the supplemental material), Western blotting (Fig. 6B), and microarray (see Table S5) data. These cytoplasmic sensors were recently discovered to be crucial detectors of various DNA and RNA viruses (22, 42). For instance, double-stranded DNA (dsDNA) recognition by *DAI* leads to *IRF3* activation and, hence, IFN- α/β expression (42), and intracellular DNA recognition may initiate a protease cascade through the assembly of the *AIM2* inflammasome, leading to IL-1 β secretion (6). BV is a dsDNA virus and can induce antiviral responses in multiple somatic mammalian cells due to the recognition of CpG

motifs in viral DNA and ensuing activation of *TLR9*-dependent and -independent pathways (1–3). The absence of *TLR9* (Fig. 4A) and *DAI* expression in iPSCs hence partly accounted for the escape of BV recognition. Although *AIM2* expression was detected in iPSCs, BV transduction neither upregulated *AIM2* nor stimulated IL-1 β secretion; as such, the BV DNA was not detected by the *AIM2*-associated inflammasome.

Conversely, *MDA5* and *RIG-I* can recognize the genomic RNA of dsRNA viruses or the replication intermediates of ssRNA viruses (e.g., influenza virus and Sendai virus), leading to IFN- β expression (42). Although BV gene transcription can occur in certain transduced mammalian cell lines (49), the negligible induction of the sensors for viral ssRNA (Table 1, *TLR7*, and Fig. 4A) and dsRNA (*MDA5* and *RIG-I*) suggested the silencing of BV genes in iPSCs. Furthermore, the signaling pathways initiated by *RIG-I*, *MDA5*, and *DAI* all converged to activate *NF-κB* and *IRF3* (6). The lack of *NF-κB* and *IRF3* activation by BV also contributed to the missing IFN- β expression and impaired signaling via these pathways.

In light of the aforementioned data, we conclude that iPSCs are distinct from other cells such as MSCs in terms of their antiviral responses. In MSCs, BV transduction disturbed the expression of 816 genes, including *TLR3*, *IRF3*, *NF-κB* gene, *AIM2* gene, *DAI* gene, *RIG-I* gene, and *MDA5* gene, leading to IL-6 and IL-8 secretion (9) (Fig. 7; see also Table S5 in the supplemental material). In contrast, iPSCs are defective in the expression of multiple TLRs (e.g., *TLR1*, -6, -8, and -9) and certain sensors for DNA (*DAI*) and RNA (*MDA5*), despite the expression of *AIM2* and *RIG-I*. Critically, BV transduction activated none of the aforementioned sensors, *NF-κB* and *IRF3*. The diminished sensor expression and abrogated signaling molecule activation collectively accounted for

the attenuated antiviral responses (Fig. 7). A similar attenuated antiviral response in human ESCs was also recently reported and was ascribed to diminished expression of MDA5 and TLR3 and abolished induction of RIG-I (13). Because the attenuated antiviral response in human ESCs was restored after cellular differentiation (13), the impaired antiviral response of iPSCs likely stemmed from the pluripotent state.

One intriguing question is why BV transduction of iPSCs led to IP-10 secretion, which also occurred in BV-transduced neuronal cells (5) and MSCs treated with pIC and LPS (45). IP-10 (IFN- γ -inducible protein 10) is a chemokine that stimulates leukocyte migration and regulates T cell maturation and can be induced by IFN- γ and virus infection (32). It is suggested that IRF3 is critical for the activation of IP-10 expression (32) and that NF- κ B is instrumental for the synergistic induction of IP-10 (46). Since NF- κ B and IRF3 were not activated by BV transduction, it is likely that iPSCs mount the IP-10 secretion via an unidentified pathway.

In summary, this study for the first time explored the responses of iPSCs to BV vector transduction and determined that iPSCs are defective in their antiviral response to BV vector due to the down-regulation of key genes involved in sensing and signaling. These findings together justify the genetic modification of iPSCs using BV vectors.

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