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Biosafety Assessment of Human Mesenchymal Stem Cells Engineered by Hybrid Baculovirus Vectors

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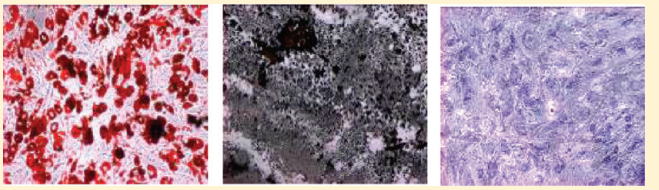
Supporting Information

ABSTRACT: Mesenchymal stem cells (MSCs) hold promise for cell therapy, and implantation of MSCs engineered with a baculovirus transiently expressing the growth factor can augment the bone repair. To prolong the baculovirus-mediated transgene expression, we developed hybrid baculovirus vectors exploiting the FLP/Frt-mediated recombination for circular episome formation. Transduction of human MSCs with the hybrid baculovirus vectors harboring the osteoinductive *bmp2* gene substantially extended the BMP2 expression and improved the cellular osteogenic differentiation. To confirm the potential in the clinical setting, the present study evaluated the biosafety profile of human MSCs engineered by the hybrid vectors. We unraveled that transduction of MSCs with the hybrid baculovirus vectors slightly impeded the cell proliferation after transduction, probably due to the perturbation of cellular gene expression and induction of innate responses. Nonetheless, the hybrid baculovirus vectors did not compromise the cell viability and cellular differentiation. No transgene integration into the host chromosome and disruption of the karyotype of the MSCs were observed. Additionally, no upregulation of proto-oncogenes or downregulation of tumor suppressor genes occurred in the MSCs transduced with the hybrid baculovirus vectors. Neither did the transduced MSCs induce tumor formation in nude mice. This study not only supported the safety of MSCs for cell therapy but also implicated the potential of the human MSCs engineered by the hybrid baculovirus vectors for their applications in clinical scenarios necessitating sustained transgene expression.



KEYWORDS: baculovirus, mesenchymal stem cell, biosafety, sustained expression, hybrid vector

INTRODUCTION

Mesenchymal stem cells (MSCs) are immunoprivileged and immunosuppressive and are capable of self-renewal and multilineage differentiation into various cell types including adipocytes, chondrocytes and osteoblasts.[1] Thanks to these attributes, MSCs are considered a promising cell source for regenerative medicine, and MSC-based cell therapy products have advanced to various phases of clinical trials.[2] One product, Prochymal (Osiris, Columbia, MD), is allogeneic MSC made from adult bone marrow donors and is in phase 3 trials to treat graft versus host disease and Crohn's disease.[3] Furthermore, MSCs can be genetically engineered to express therapeutic genes and serve as a cell-based gene delivery vehicle for tissue regeneration, cancer therapy and treatment of renal and cardiovascular diseases.[4−6] Common gene delivery vectors include adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus and plasmid. However, these vectors possess respective drawbacks.[7]

In contrast to the aforementioned vectors, baculovirus is an insect virus that is nonpathogenic to humans. However, baculovirus efficiently transduces a broad range of mammalian cells with minimal cytotoxicity and possesses a number of distinct advantages (for review see refs 8 and 9). Consequently, baculovirus has captured growing interest as a vector for in vitro and in vivo gene delivery, development of cell-based assays, surface display of eukaryotic proteins, delivery of vaccine immunogens, cancer therapy and production of virus vectors.[10−12] Aside from these applications, baculovirus can transduce MSCs at efficiencies higher than 95% and a recombinant baculovirus (Bac-CB) transiently expressing osteoinductive BMP2 (bone morphogenetic protein 2) can promote in vitro osteogenesis of MSCs.[13] Coimplantation of the MSCs transduced with Bac-CB or with

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another baculovirus expressing vascular endothelial growth factor (VEGF) into the femoral bone defects in New Zealand White (NZW) rabbits not only stimulated the angiogenesis but also augmented the bone healing in vivo.[14] Despite the wide spectrum of applications, baculovirus does not replicate in mammalian cells. As a result, the viral genome is degraded within the cells over time and the transgene expression typically extinguishes in 2 weeks,[15] which impedes its applications in conditions necessitating sustained expression.

To prolong the transgene expression, we developed a hybrid baculovirus system in which one baculovirus expressed FLP while the other harbored an Frt-flanking gene cassette composed of the transgene and *oriP*/EBNA1.[13] FLP is a recombinase that recognizes the Frt sequences for site-specific recombination, while EBNA1 (Epstein−Barr virus nuclear antigen 1) can bind to the *oriP* sequence and coordinate the replication and segregation of episomes. We demonstrated that, within the cells cotransduced with the hybrid baculoviruses, FLP catalyzed the recombination of the Frt-flanking cassette, resulting in the cassette excision off the baculovirus genome and subsequent formation of the episomal circle. The FLP-mediated recombination efficiency was as high as 75−85% in several mammalian cell lines and approached 48% in human MSCs. Thanks to the episome replication imparted by *oriP*/EBNA1, the transgene expression persisted for ≈48 days without selection whereas the baculovirus genome was rapidly degraded after unloading the cargo. When the reporter gene *egfp* was substituted by *bmp2*, the BMP2 expression was prolonged, leading to enhanced osteogenic differentiation of MSCs.[13]

To further confirm the clinical potential of the human MSCs engineered by this system, the present study sought to evaluate whether the hybrid baculovirus transduction altered essential characteristics of MSCs and increased the risks of cell transformation and tumorigenesis. Toward this end, we cotransduced the human bone marrow-derived MSCs with BacFLP (expressing FLP) and BacCON-CE (harboring the cassette comprised of *oriP*/EBNA1 and *egfp* (enhanced green fluorescent protein)) and assessed the viability, proliferation, differentiation, gene integration, karyotype, tumorigenesis-associated genes and in vivo tumorigenesis.

MATERIALS AND METHODS

Cell Culture. Human bone marrow-derived MSCs were obtained, cultured and characterized as described earlier.[16] The cells were cultured in α-modified minimal essential medium (α-MEM, Gibco, Gaithersburg, MD) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 4 ng/mL basic fibroblast growth factor (R&D Systems, Minneapolis, MN), 100 U/mL penicillin, and 100 mg/mL streptomycin. The HEK293 cell line stably expressing EGFP was generated by cotransducing the HEK293 cells with BacRep (a baculovirus expressing AAV Rep proteins) and a hybrid baculovirus incorporating the CMV-EGFP cassette flanked by AAV ITR (inverted terminal repeat), followed by G418 selection. The stable HEK293 cells continued to express EGFP and were maintained in DMEM medium containing 10% FBS (Gibco) and G418 (50 μg/mL). Ovarian cancer cell line SKOV3 was cultured in McCoy's 5A medium (Sigma, St. Louis, MO) supplemented with 15% FBS (Gibco) and $NaHCO_3$ (2.2 g/L).

Preparation of Baculoviruses and Transduction of MSCs. The wild-type baculovirus Bac-wt was supplied in the MaxBac 2.0 Expression system (Invitrogen, Carlsbad, CA). The recombinant baculovirus Bac-CE harbored *egfp* gene under the CMV promoter and mediated transient EGFP expression.[16] BacFLP carried the *flp* gene while BacCON-CE harbored an Frt-flanking cassette encompassing *egfp* and *oriP*/EBNA1, and both viruses were constructed previously.[13] The baculoviruses were amplified by infecting Sf-9 cells, harvest, stored and titrated as described.[13]

Passage 10 (P10) MSCs were transduced as described previously[13] with minor modifications. In brief, MSCs were seeded onto 6-well plates (2×10^5 cells/well) or 96-well plates (5×10^3 cells/well) and cultured for 12 h. Depending on the multiplicity of infection (MOI), a certain volume of virus supernatant was premixed with $NaHCO_3$-deficient α-MEM containing 10% FBS to adjust the final volume. Transduction was initiated by adding the virus mixture to the cells and continued by gently shaking the plates for 4 h at room temperature. After the incubation period, the virus solution was withdrawn and the cells were cultured with α-MEM containing sodium butyrate (5 mM) for 15 h, after which the medium was withdrawn and replaced with normal α-MEM.

Cell Viability and Proliferation Assays. The viabilities of transduced and mock-transduced MSCs cultured in 6-well plates (2×10^5 cells/well) were measured by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay following standard procedures when the cells reached ≈70−80% confluency. The cell viability (%) was calculated by the formula $OD_{sample}/OD_{control} \times 100\%$, where the control was the mock-transduced cells. For proliferation, MSCs cultured in 96-well plates were transduced and analyzed at 1, 3, 5, 6, 7, 9, 10, 11, 13, and 15 days post-transduction (dpt) using the BrdU cell proliferation assay kit (Millipore, Billerica, MA) as described.[17] At 6 and 10 dpt, the cells were trypsinized and reseeded to new 96-well plates (5×10^3 cells/well).

Induction and Assessment of MSCs' Differentiation. MSCs were induced toward adipogenic, osteogenic or chondrogenic pathways at 3 dpt by replacing the culture medium to respective α-MEM-based induction medium as described.[16] The adipogenic induction medium contained 1 μM dexamethasone, 5 μg/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine and 60 μM indomethacin. The chondrogenic induction medium contained 0.1 μM dexamethasone, 1 mM sodium pyruvate, 0.1 mM ascorbic acid 2-phosphate, 10 ng/mL recombinant human TGF-β1 (Perpo Tech) and 1% ITS^+ premix (6.25 μg/mL insulin, 6.25 μg/mL transferrin, 6.25 ng/mL selenous acid, 1.25 mg/mL bovine serum albumin and 5.35 μg/mL linoleic acid, BD Biosciences). The osteogenic induction medium contained 0.1 μM dexamethasone, 10 mM β-glycerol phosphate and 50 μM ascorbic acid 2-phosphate. The appearance of the adipogenic, osteogenic and chondrogenic phenotypes was revealed as described earlier,[16] by the oil droplet stained by Oil-Red O, calcium phosphate deposition stained by van Kossa and glycosaminoglycan accumulation stained by toluidine blue, respectively.

Quantitative Real-Time Reverse-Transcription PCR (qRT-PCR). The relative mRNA levels of the differentiation marker genes, oncogenes, and tumor suppressor genes were quantified by qRT-PCR. Total mRNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed to cDNA using the Omniscript RT Kit (Qiagen). The cDNA was subjected to real-time PCR reactions using the ABI PRISM 7300 (Applied Biosystems, Foster City, CA) as described previously.[16] The primers used for qRT-PCR are listed in Supplementary Table 1 in the Supporting Information.

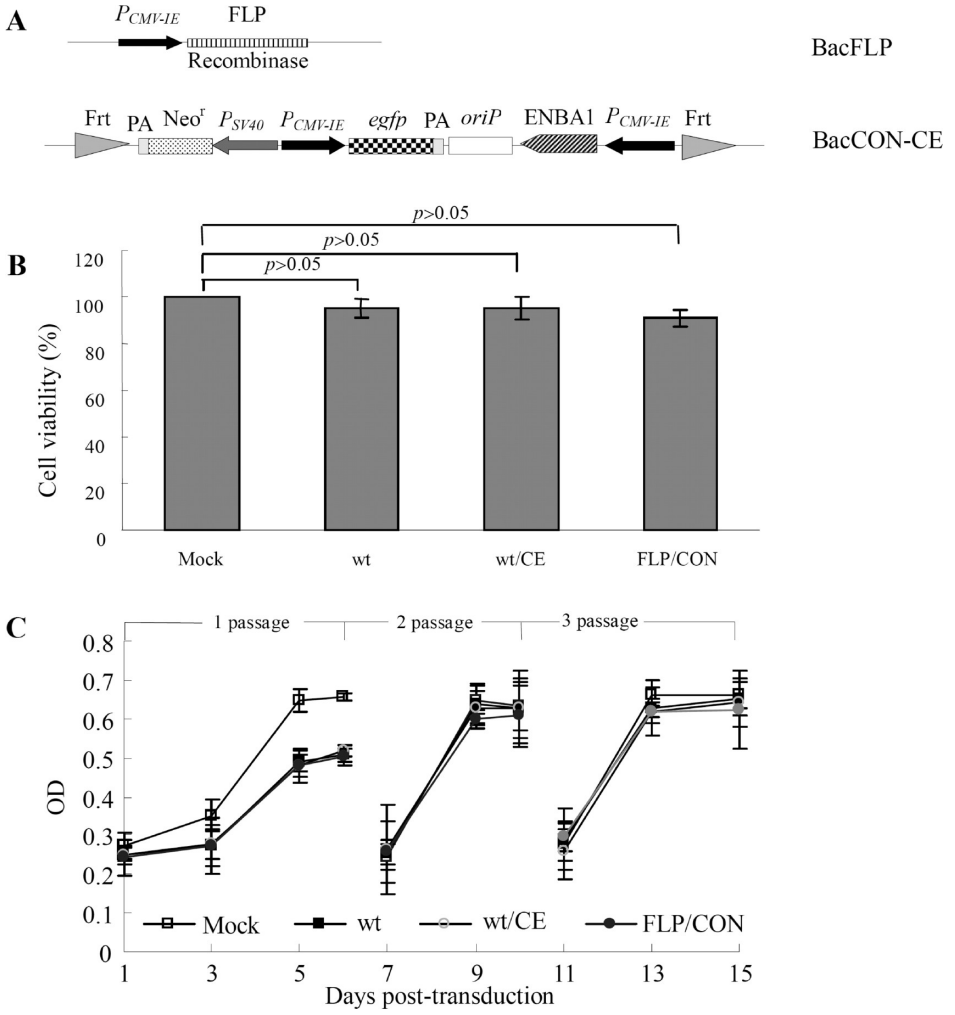

Figure 1. The hybrid baculovirus vectors and the influence on MSCs' viability and proliferation. (A) Schematic illustration of BacFLP and BacCON-CE. (B) Effects of BacFLP/BacCON-CE transduction on cell viability. (C) Effects of BacFLP/BacCON-CE transduction on cell proliferation. MSCs cultured in 6-well plates (2×10^5 cells/well) were mock-transduced, singly transduced with Bac-wt (MOI 200), cotransduced with Bac-wt (MOI 50) and Bac-CE (MOI 150) or cotransduced with BacFLP (MOI 50) and BacCON-CE (MOI 150). The viability was assessed by MTT assay at 2 dpt when the cells reached 70−80% confluency. For cell proliferation analysis, MSCs in 96-well plates (5×10^3 cells/well) were similarly transduced, cultured and passaged to new 96-well plates (5×10^3 cells/well) at days 6 and 10. The cell proliferation was assayed by BrdU labeling, and the data are expressed in optical density (OD).

Fluorescence in Situ Hybridization (FISH). The DNA fragment containing the CMV-EGFP cassette was purified from pBac-CE[18] using Gel/PCR DNA Fragments Extraction Kit (Geneaid, Taipei, Taiwan) and labeled with fluorescein dUTP for use as the probe in FISH using the Random Primed DNA Labeling Kit (Roche, Indianapolis, IN). In parallel, MSCs were seeded onto a T-25 flask (5×10^5 cells) and transduced. The mitotic MSCs were prepared at 2 dpt by colcemid (10 μg/mL, Sigma) treatment for 2 h, transferred to microfuge tubes, incubated in the hypotonic KCl solution (0.075 M) at 37 °C for 20 min, and fixed in methanol/acetic acid (3:1). After fixation, the cells were dropped onto slides and incubated at 60 °C overnight. Subsequent hybridization and DAPI staining were performed according to standard protocols.[19] The data are representative of 150 metaphases, each of which displayed 10 to 20 extrachromosomal fluorescent signals.

Karyotype Analysis. For karyotype analysis, MSCs were subjected to colcemid treatment, hypotonic treatment and methanol/acetic acid fixation as described above. The slides were incubated at 75 °C for 1 min and at 62 °C on a heater plate overnight and then incubated in the KH_2PO_4 buffer (0.025 M) at 56 °C for 10 min, followed by final incubation in PBS containing 0.05% trypsin for 1 min. The slides were dehydrated in ethanol (95%) and dried. Finally, the chromosome spreads were stained with Wright's working solution (Merck) for 3 min, and observed under the microscope.

In Vivo Tumorigenesis. The in vivo tumorigenesis was verified by subcutaneous injection of cells (SKOV3, transduced or mock-transduced MSCs, 3×10^6 cells/mouse) into the root of the right thighs of female BALB/c athymic nude mice (6−8 weeks old, National Laboratory Animal Center, Taiwan). The inoculation sites were examined and photographed to confirm tumor development from the injected cells. All experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (National Science Council, Taiwan).

Statistical Analysis. All data were analyzed using Independent Samples *t*-tests and are expressed as mean ± standard deviation (SD) or mean values of at least 3 independent experiments. *p* values less than 0.05 were considered significant.

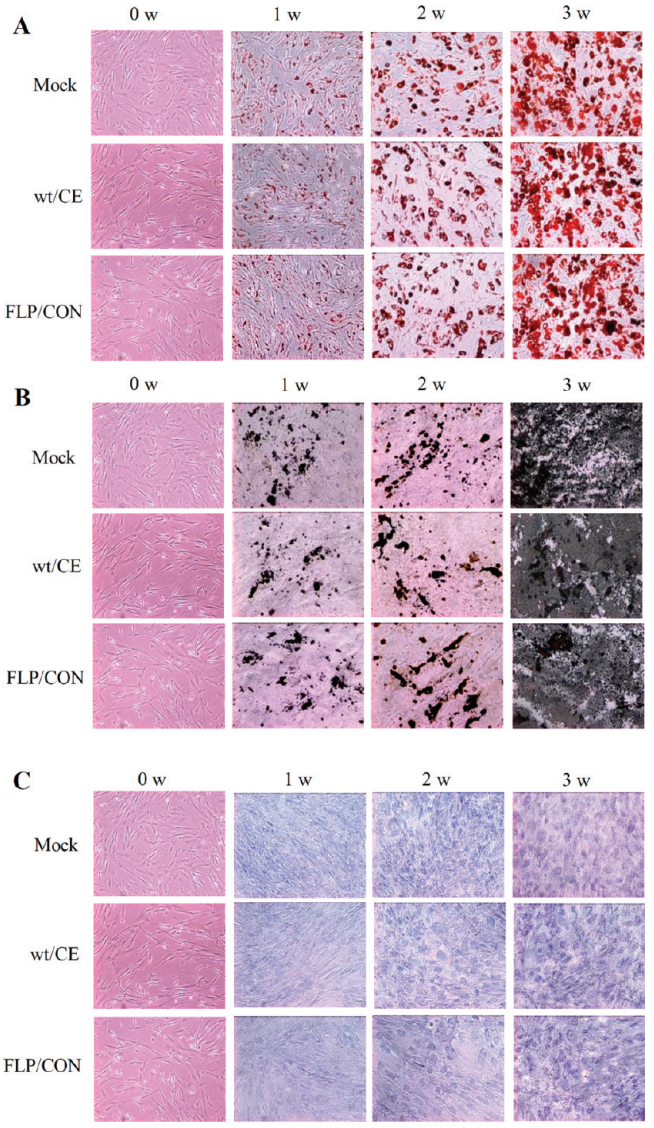

Figure 2. Effects of the hybrid baculovirus vectors on MSCs' differentiation toward (A) adipogenic, (B) osteogenic and (C) chondrogenic pathways. MSCs cultured in 6-well plates (2×10^5 cells/well) were mock-transduced, cotransduced with Bac-wt/Bac-CE or cotransduced with BacFLP/BacCON-CE as in Figure 1. The cells were induced toward various differentiation pathways at 3 dpt by culturing in the induction medium for 3 weeks. The adipogenic, osteogenic and chondrogenic differentiations were evidenced by histochemical staining with Oil-Red O, van Kossa and toluidine blue, respectively. Magnification, 100×.

RESULTS

Effects of BacFLP/BacCON-CE on MSCs' Viability and Proliferation. The dual baculoviral vectors BacFLP and BacCON-CE (Figure 1A) were constructed previously and attested to confer prolonged EGFP expression thanks to the FLP-mediated episome formation and *oriP*/EBNA1-mediated episome replication.[13] To evaluate the safety profile of MSCs transduced with BacFLP/BacCON-CE, we first examined whether the hybrid vectors influenced the cell viability and proliferation by cotransducing the MSCs with BacFLP (MOI 50) and BacCON-CE (MOI 150) for 4 h (FLP/CON group), which yielded high recombination efficiency as demonstrated previously.[13] As controls, the cells were mock-transduced (Mock group) or singly transduced with Bac-wt (wt group), a baculovirus that expressed no foreign gene, at the same total MOI 200. To compare the transient and sustained expression systems, MSCs were also cotransduced with Bac-CE (a recombinant baculovirus that transiently expressed EGFP) at MOI 150, and an additional Bac-wt (MOI 50) to adjust the total MOI to 200 (wt/CE group).

The MTT assay (Figure 1B) revealed that the viability of the wt and wt/CE group remained ≈93%, which was statistically similar ($p > 0.05$) when compared with the Mock group (≈95%). The viability of the FLP/CON group slightly decreased to ≈90%, but remained statistically similar ($p > 0.05$) to that of the Mock group. These data indicated that neither Bac-wt/Bac-CE nor BacFLP/BacCON-CE transduction impaired the MSCs' viability.

To evaluate the effects of the hybrid baculoviral vectors on MSCs' proliferation, cells were passaged onto 96-well plates, then mock-transduced or transduced as in Figure 1B. The cells continued to be cultured and passaged at 6 and 10 dpt to new 96-well plates at the same seeding density, and the cell proliferation was monitored by BrdU labeling. Figure 1C depicts that proliferation rates of the 3 transduction groups (wt, wt/CE and FLP/CON) were similar throughout the culture period. Although the transduced cells proliferated more slowly than the mock-transduced cells in the first 6 days, their proliferation rates returned to normal and approached that of the Mock group after passaging.

Effects of BacFLP/BacCON-CE on MSCs' Differentiation. MSCs are capable of adipogenic, chondrogenic and osteogenic differentiation. To assess whether BacFLP/BacCON-CE transduction hampered the cell differentiation capacity, MSCs were transduced as in Figure 1B, cultured for 3 days and induced toward various differentiation lineages by culturing in the respective induction medium for 3 weeks.

The Oil-Red O staining (Figure 2A) illustrated progressive increase of oil droplets for the FLP/CON group, whereby the cell morphology and oil droplet accumulation appeared indistinguishable from those of the Mock and wt/CE groups, suggesting that BacFLP/BacCON-CE transduction did not undermine the MSCs' differentiation into adipocytes. Likewise, the calcium deposition as stained by van Kossa (Figure 2B) and glycosaminoglycan accumulation as stained by toluidine blue (Figure 2C) unraveled similar patterns for all 3 groups, indicating that the hybrid baculovirus system mitigated neither osteogenic nor chondrogenic differentiation.

The effects of the hybrid baculovirus system on differentiation were further quantified by qRT-PCR analyses of the genes specific for adipogenic (*glut4* and *ppar-γ2*), osteogenic (*osteocalcin* and *sialoprotein*) and chondrogenic (*aggrecan* and *col II*) differentiation. Figure 3 shows the gene transcription levels that were normalized against those of the Mock control at 1 week post-transduction, and depicts that *glut4* transcription increased with time for all 3 groups without significant differences among them (Figure 3A). The *ppar-γ2* transcription levels for all 3 groups remained low in the first two weeks (Figure 3A) because *ppar-γ2* is expressed late during the differentiation;[20] nonetheless at week 3 *ppar-γ2* was considerably upregulated and no significant differences existed among the 3 groups ($p > 0.05$). Likewise, the osteogenic (Figure 3B) and chondrogenic (Figure 3C) marker gene expression levels rose with time and the differences between groups were statistically insignificant ($p > 0.05$). Figures 2 and 3 collectively confirmed that the FLP/CON group differentiated toward the adipogenic, osteogenic and chondrogenic lineages as well as the control groups.

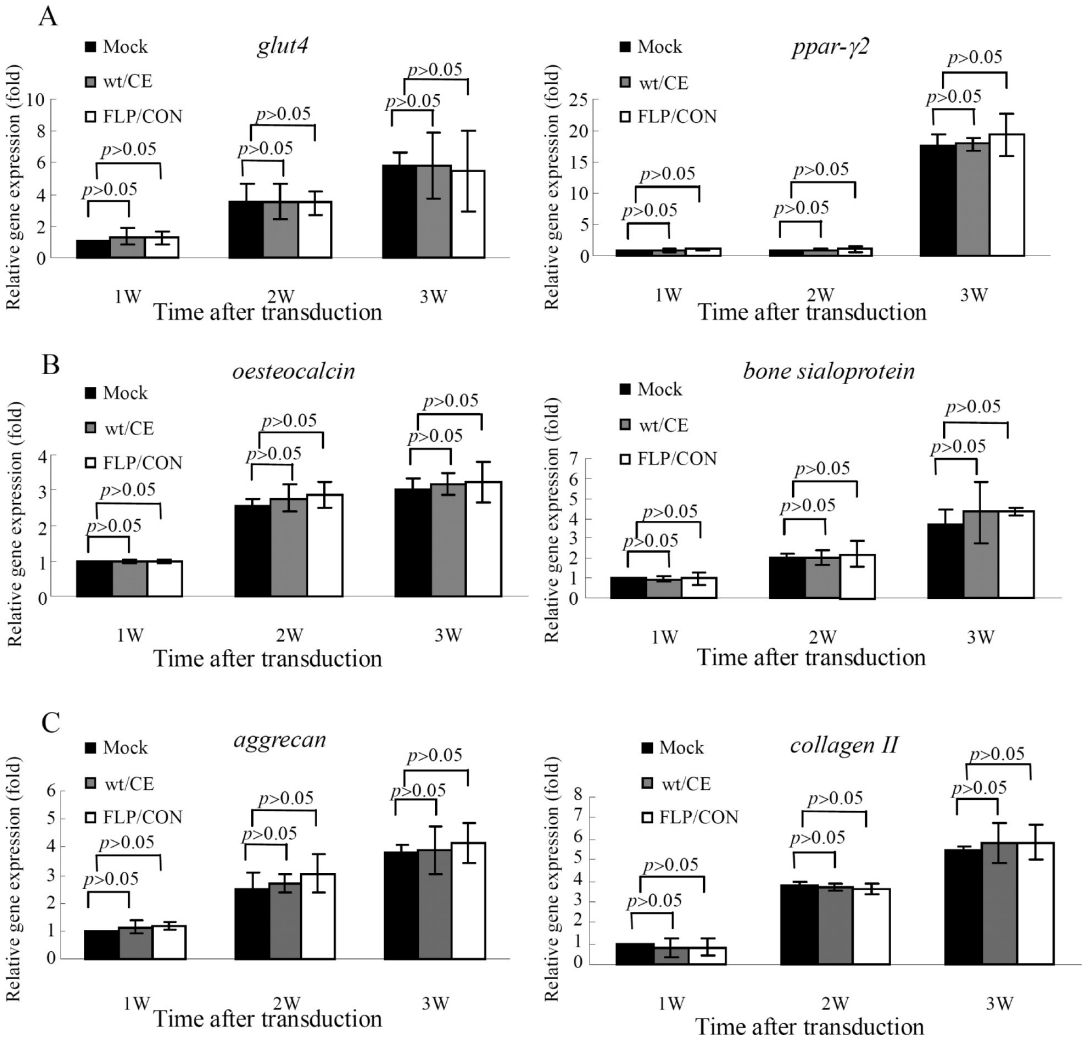

Figure 3. Effects of the hybrid baculovirus vectors on differentiation marker gene expression: (A) adipogenic genes *glut4* and *ppar-γ2*; (B) osteogenic genes *osteocalcin* and *sialoprotein*; (C) chondrogenic genes *aggrecan* and *col II*. The gene transcription levels in the cells transduced as in Figure 2 were quantified by qRT-PCR and were normalized against those of the Mock control at 1 week post-transduction.

BacFLP/BacCON-CE Caused Neither Transgene Integration nor Chromosome Aberration. Whether BacFLP/BacCON-CE transduction led to inadvertent transgene integration into the chromosomes was explored by transducing the MSCs with BacFLP/BacCON-CE as in Figure 1B and subsequent FISH analyses using the DNA probe specific for *egfp*. In parallel, stable HEK293 cells that contained integrated *egfp* genes (see Materials and Methods) were subjected to FISH as a positive control. Figure 4A illustrates the fluorescence emission (upper panel) and bright fluorescent spots colocalizing with the chromosomes (lower panel) in the stable HEK293 cells, indicating the success of FISH experiments. In contrast, although the BacFLP/BacCON-CE-transduced MSCs emitted fluorescence (upper panel, Figure 4B), the fluorescent spots (i.e., *egfp* gene) were surrounding the chromosomes without physical engagement (lower panel, Figure 4B), attesting that *egfp* remained episomal in the transduced MSCs.

Additionally, chromosomal abnormalities are thought to be critical in tumor formation. The karyotype analysis of 40 metaphase cells revealed no chromosome deletion or aberration (Figure 5), indicating that BacFLP/BacCON-CE transduction did not result in chromosomal abnormality in MSCs.

BacFLP/BacCON-CE Did Not Increase the Risk of Tumorigenesis. Whether the risk of tumorigenesis increased due to the hybrid baculoviral vectors was assessed by mock- or BacFLP/BacCON-CE transduction, and subsequent qRT-PCR analyses of the proto-oncogenes (*c-myc*, *N-ras*, *K-ras* and *H-ras*) and tumor suppressor genes (*p53* and *p16*) at 2 and 7 dpt. In parallel, the expression of these genes in the ovarian cancer cell line SKOV3 was analyzed as positive controls. All gene expression data were normalized against the corresponding data of the Mock group at 2 dpt.

For all 4 proto-oncogenes (Figure 6A), the expression levels in the Mock and FLP/CON groups were statistically similar ($p > 0.05$), either at 2 or 7 dpt, and were significantly lower than the expressions of their counterparts in SKOV3. Likewise, the expression levels of the tumor suppressor genes *p53* and *p16* were similar in the Mock and FLP/CON groups at 2 dpt (Figure 6B). The expression levels for both groups at 7 dpt were elevated when compared with those at 2 dpt, and were considerably higher ($p < 0.05$) than those in SKOV3.

Furthermore, the MSCs transduced with BacFLP/BacCON-CE were cultured for 2 or 7 days and implanted subcutaneously into the nude mice separately. As controls, mock-transduced MSCs and SKOV3 cells were also implanted into the mice, respectively.

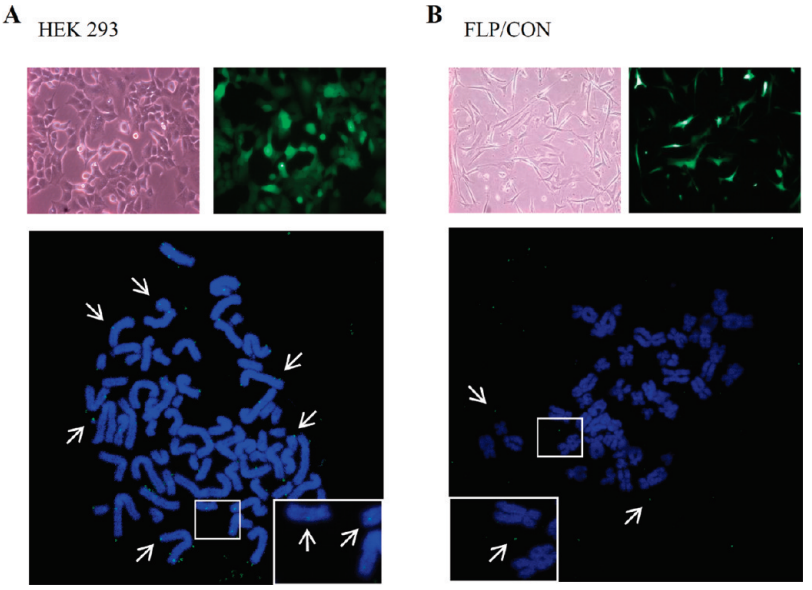

Figure 4. The hybrid baculovirus vectors did not lead to transgene integration: (A) HEK293; (B) MSCs. The HEK293 cells were engineered to carry integrated *egfp* gene and examined by FISH as the positive control. The MSCs were transduced with BacFLP/BacCON-CE and subjected to FISH analysis at 2 dpt using the *egfp*-specific DNA probes. The fluorescence micrographs of the cells are shown in the upper panel. The images are representative of 40 metaphases and reveal normal chromosome pattern of 46, XY.

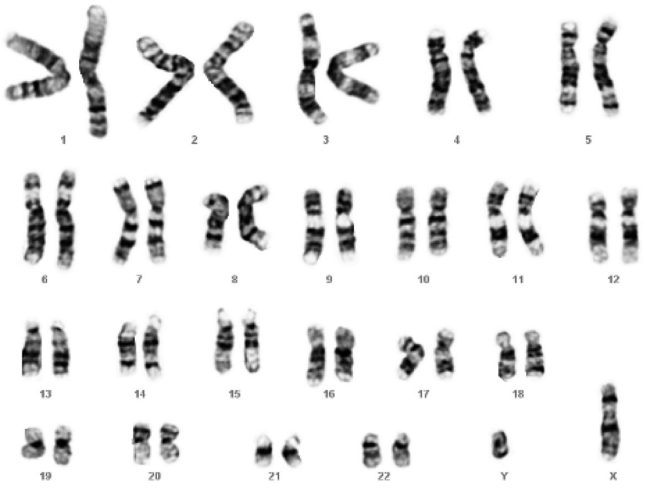

Figure 5. The hybrid baculovirus vectors did not result in chromosome aberration. The cells transduced with BacFLP/BacCON-CE were subjected to karyotype analyses at 2 dpt. The images are representative of 40 metaphases.

Figure 6C demonstrates that the mock-transduced MSCs did not give rise to tumor formation 4 months after implantation, while the cancer cell SKOV3 resulted in apparent tumor development in 1 month. Similar to the mock-transduction group, at 4 month postimplantation no tumor developed in the mice implanted with the transduced MSCs cultured for 2 or 7 days. Figure 6 altogether indicates that BacFLP/BacCON-CE transduction of MSCs neither altered the expression of genes associated with oncogenesis and tumor suppression nor induced tumorigenesis in vivo, even after cell subculture.

DISCUSSION

The overriding objective of the present study was to evaluate the characteristics and biosafety profile of human MSCs engineered by the hybrid baculovirus system. Our data unraveled that BacFLP/BacCON-CE transduction of MSCs did not compromise the cell viability (Figure 1B), but somewhat hampered the cell proliferation after transduction (Figure 1C). Since transduction of the cells with Bac-CE (expressing EGFP transiently) and Bac-wt (expressing no foreign protein) also impeded the cell proliferation, the slightly lower growth rate was not attributed to the intrinsic cytotoxicity or extra metabolic burden as a result of the FLP or EGFP expression. Instead, the proliferation might be decelerated by baculovirus itself. Increasing lines of evidence have proven that baculovirus transduction of mammalian cells can transiently perturb the global gene expression profiles[21−23] and trigger innate immune responses (e.g., elicitation of type I interferons, proinflammatory cytokines and activation of natural killer cells) in vitro and in vivo at least partly due to the activation of toll-like receptor (TLR) 9 pathway.[8,24−27] Baculovirus transduction of human MSCs also perturbs 816 genes associated with antigen processing/presentation, apoptosis and TLR3 pathway that leads to the production of IL-6 and IL-8.[28] Nonetheless, the transduced MSCs only elicited transient, mild macrophage responses without provoking systemic induction of monocytes and $CD8^+$ T cells after transplantation into allogeneic animals, and the cells remained tolerant.[29] Although no direct evidence unveils that baculovirus alters the expression of genes governing the MSCs' proliferation and/or cell cycle, the crosstalk between the gene regulatory circuits and additional metabolic load resulting from IL-6 and IL-8 production might have contributed to the impeded proliferation. Nonetheless, baculovirus only induces transient responses that plummet after 1 dpt,[26,28] which might explain the restoration of proliferation rate to normal after passaging (Figure 1C).

Despite the temporary retardation of cell growth, the histochemical staining (Figure 2) and gene expression analyses (Figure 3) demonstrated that the cells transduced with BacFLP/BacCON-CE differentiated toward adipogenic, osteogenic and chondrogenic lineages at rates indistinguishable from those of

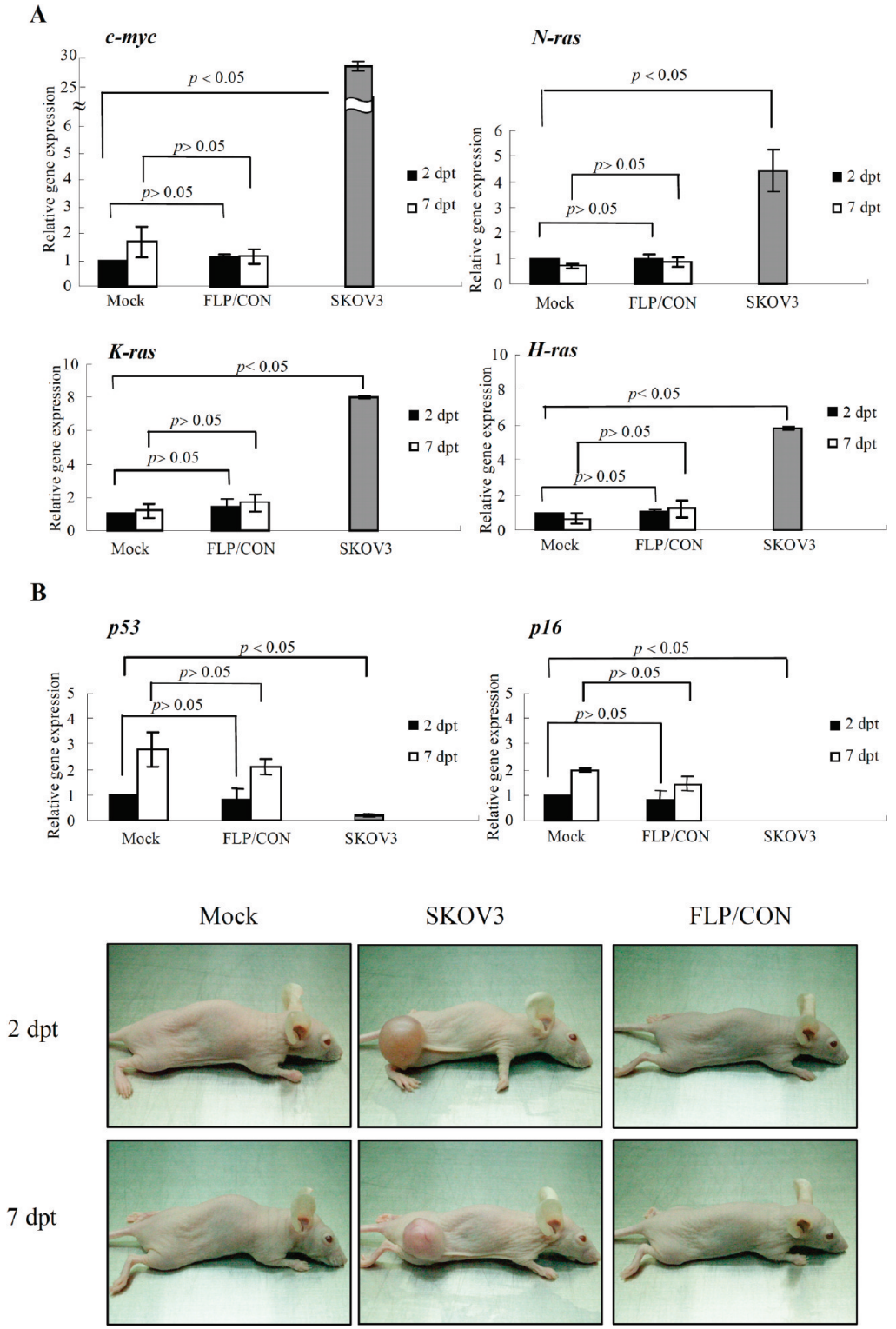

Figure 6. The hybrid baculovirus vectors did not elevate the risk of tumorigenesis. (A) Transcription levels of 4 proto-oncogenes (*c-myc*, *N-ras*, *K-ras* and *H-ras*). (B) Transcription levels of tumor suppressor genes (*p53* and *p16*). (C) Tumor development after cell injection into nude mice. The cells were mock transduced or transduced with BacFLP/BacCON-CE and subjected to qRT-PCR analyses at 2 and 7 dpt. The expression profiles in the ovarian cancer cell line SKOV3 was analyzed as controls. All gene expression data were normalized against the corresponding gene transcription levels of the Mock group at 2 dpt. The tumorigenesis was further examined by subcutaneously injecting SKOV3 (positive control), mock-transduced (negative control) and transduced MSCs into the right thighs of nude mice (3×10^6 cells/mouse). The tumor formation was examined and photographed 1 (for SKOV3) or 4 (for MSCs) months after injection.

mock-transduced and Bac-wt-transduced cells. In accord with these data, MSCs transduced with a conventional baculovirus vector neither appreciably change major surface marker expression nor alter the immunological characteristics;[29] also MSCs

engineered by other nonintegrating vectors (e.g., adenovirus and plasmid) remain capable of differentiation toward the 3 pathways[30] and retain the regenerative properties to repair defects in vivo.[31,32] Furthermore, human embryonic stem (hES) cells genetically manipulated with an EGFP-expressing baculovirus retain the pluripotency.[33] As such, it appears that stem cells such as hES cells and MSCs are fairly tolerant of genetic modifications using the common gene delivery vectors. Our data further support the assertion that the hybrid baculovirus transduction and the expression of FLP and EBNA1 do not impair the plasticity of MSCs.

Insertional mutagenesis remains a major concern for integrating vectors such as retrovirus and lentivirus, especially after the report of malignant cell expansion in recent clinical trials.[34] Although in general baculovirus does not spontaneously integrate the viral DNA into the mammalian chromosomes[11,35] and it degrades over time in the cells,[13,15] baculovirus DNA integration into the host genome does occur after antibiotic selection[36,37] and take place spontaneously at high frequency in the ES cells derived from medaka fish.[38] These findings call for more stringent scrutiny on the safety of baculovirus-engineered stem cells for cell therapy, especially this hybrid baculovirus system enables persistent maintenance of the transgene cassette in the cells.[13] Fortunately, the hybrid baculovirus vector transduction did not integrate the transgene into the host (Figure 4), nor did it disrupt the karyotype of the MSCs (Figure 5). These data concurred with the findings that stable transgene expression mediated by a hybrid baculovirus vector in human ES cells does not lead to abnormal karyotype.[33]

Tumorigenesis is another critical issue related to the safe application of MSCs. In particular, recent papers unraveling the spontaneous transformation of MSCs in culture[39,40] and development of sarcomas in mice implanted with MSCs[41] cast shadows over the clinical trials with MSCs being contemplated or in progress.[42] Contradictory to these reports, our data revealed no upregulation of proto-oncogenes (Figure 6A) or downregulation of tumor suppressor genes (Figure 6B) in the MSCs, regardless of mock-transduction or BacFLP/BacCON-CE transduction. Neither did the MSCs, transduced or mock-transduced, induce tumor formation in nude mice after 4 months (Figure 6C). Furthermore, rabbit MSCs transduced with our hybrid baculovirus vectors resulted in no signs of osteosarcoma or other tumors at 3 months postimplantation into NZW rabbits (Lin and Hu, unpublished data). Our data agree with the general notion that MSCs are safe but contradict the literature reporting the tumorigenesis.[39−41,43] The disparity might arise from the differences in species because only murine, but not human MSCs, generated osteosarcoma-like lesions in the lungs of mice.[43] More likely, the tumorigenesis reported recently is an experimental artifact[42] as one of the laboratories reports that the transformation stems from the contamination of MSC cultures with malignant cells.[44]

Taken together, this study not only echoed the safety of MSCs for cell therapy but also validated the safety of the hybrid baculovirus vectors for MSC engineering. These results are critical because insertional mutagenesis/transformation or tumorigenesis arising from integrating (e.g., retrovirus[45] and lentivirus[46]) and nonintegrating (e.g., AAV[47]) vectors have been documented. The hybrid baculovirus system exploiting the FLP/Frt-mediated recombination prolongs the transgene expression based on episomal maintenance of the cassette, which eases the concerns regarding the insertional mutagenesis as seen in integrating vectors. Compared with other nonintegrating vectors such as plasmids, baculovirus can mediate comparable or superior gene delivery into MSCs.[13] Therefore, this hybrid vector holds great promise in the scenarios requiring sustained expression. For instance, adipose-derived MSCs can be isolated in abundance more readily than the bone marrow-derived MSCs, and represent an amenable alternative cell source for bone repair. However, adipose-derived MSCs have less capacity for osteogenic differentiation and bone healing. Therefore, adipose-derived MSCs may be engineered by the hybrid baculoviruses for persistent growth factor expression to potentiate the MSCs' differentiation. Indeed, the hybrid baculovirus vectors harboring the *bmp2* gene extended the BMP2 expression in MSCs to >21 days, as opposed to <12 days conferred by the conventional baculovirus Bac-CB (without the FLP/Frt-mediated recombination) (Supplementary Figure S1 in the Supporting Information). Implantation of the engineered adipose-derived MSCs into NZW rabbits healed the critical-sized bone defects in 12 weeks, whereas the Bac-CB-engineered MSCs failed to repair the defects (Supplementary Figure S2 in the Supporting Information). Therefore the MSCs engineered by the hybrid baculovirus vectors effectively ameliorate the bone healing.

Besides, MSCs are a potential cell source for cartilage regeneration, but their chondrogenic differentiation capacity is less pronounced,[48] making their in vitro chondrogenic differentiation more difficult and time-consuming. Consequently, MSCs transduced with a baculovirus vector mediating transient expression of chondrogenic growth factors (e.g., TGF-β1 or TGF-β3) may not be sufficient to stimulate chondrogenesis and cartilage repair in vivo. In this regard, MSCs can be engineered by the hybrid baculoviruses for prolonged expression of TGF-β1 or TGF-β3 to substantiate and accelerate the chondrogenic differentiation of MSCs. Moreover, genetically modified MSCs have been implicated in cancer therapy[4,49] and MSCs transduced with a baculovirus transiently expressing herpes simplex virus thymidine kinase are shown to inhibit glioma growth in vivo after ganciclovir prodrug injection.[50] Although the MSC-based approach holds promise, cancer therapy necessitates sustained expression of the therapeutic genes. Therefore, MSCs may be transduced with our hybrid baculovirus stably expressing antitumor genes and implanted into tumor-bearing animals or individuals for cancer treatment. The demonstration of safety profiles in this study altogether justifies the applications of the hybrid baculovirus system in these settings.

■ ASSOCIATED CONTENT

Supporting Information. Table of primers used in qRT-PCR and figures depicting prolonged BMP2 expression conferred by the hybrid baculovirus system and segmental bone repair mediated by the MSCs engineered by the hybrid baculovirus vectors. This material is available free of charge via the Internet at http://pubs.acs.org.

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