Development of a Hybrid Baculoviral Vector for Sustained Transgene Expression

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Baculovirus is a promising gene delivery vector but its widespread application is impeded as it only mediates transient transgene expression in mammalian cells. To prolong the expression, we developed a dual baculovirus system whereby one baculovirus expressed FLP recombinase while the other harbored an Frt-flanking cassette encompassing the transgene and *oriP*/EBNA1 derived from Epstein–Barr virus. After cotransduction of cells, the expressed FLP cleaved the Frt-flanking cassette off the baculovirus genome and catalyzed circular episome formation, then *oriP*/EBNA1 within the cassette enabled the self-replication of episomes. The excision/recombination efficiency was remarkably enhanced by sodium butyrate, reaching 75% in human embryonic kidney-293 (HEK293) cells, 85% in baby-hamster kidney (BHK) cells, 77% in primary chondrocytes, and 48% in mesenchymal stem cells (MSCs). The hybrid baculovirus substantially prolonged the transgene expression to ≈48 days without selection and >63 days with selection, thanks to the maintenance of replicons and transgene transcription. In contrast to the replicating episomes, the baculovirus genome was rapidly degraded. Furthermore, an osteoinductive growth factor gene was efficiently delivered into MSCs using this system, which not only prolonged the growth factor expression but also potentiated the osteogenesis of MSCs. These data collectively implicate the potential of this hybrid baculovirus system in gene therapy applications necessitating sustained transgene expression.

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INTRODUCTION

Baculovirus has emerged as a promising gene delivery vector in recent years. Despite being a DNA virus that infects insects as its natural host, baculovirus efficiently transduces numerous mammalian cells with minimal cytotoxicity and possesses a number of distinct advantages (for review see ref. 1–3). Therefore, baculovirus has captured growing attention as a novel vector for *in vitro* and *in vivo* gene delivery,[4–6] development of cell-based assays,[7] surface display of eucaryotic proteins,[8] delivery of vaccine immunogens,[9] cancer therapy,[10] and production of virus vectors.[11,12] Aside from these applications, a recombinant baculovirus expressing bone morphogenetic protein-2 (BMP-2), Bac-CB, can restore the differentiation status of de-differentiated chondrocytes and stimulate *in vitro* formation of engineered cartilages.[13] After implantation, these engineered cartilages are able to repair osteochondral defects in rabbit knees.[14] Furthermore, Bac-CB transduction accelerates *in vitro* osteogenesis of mesenchymal stem cells (MSCs).[15] These findings altogether implicate the potential of baculovirus in tissue engineering. Despite the wide spectrum of applications, one drawback associated with baculovirus is that due to its inability to replicate in mammalian cells the viral genome undergoes dilution upon cell proliferation and is subjected to degradation over time.[16] As a result, most transgene expression typically extinguishes in ≤2 weeks. The short-term baculovirus-mediated transgene expression restricts its applications in conditions necessitating sustained expression.

FLP/Frt is a system derived form *Saccharomyces cerevisiae* in which the FLP recombinase recognizes the FLP recognition target (Frt) sites and catalyzes highly efficient site-specific recombination, resulting in the re-circularization of an extrachromosomal episome.[17] To stably maintain the episome within the cells, the episome requires a proper origin of replication. Among the well-known replication origins, o*riP* derived from Epstein–Barr virus contains Epstein–Barr virus nuclear antigen-1 (EBNA1) binding sites and the binding of EBNA1 to *oriP* orchestrates the replication and segregation of the episomes to daughter cells.[18] Plasmids containing *oriP*/EBNA1 have been exploited for long-term maintenance of transgenes and confer persistent expression *in vitro*[19] and *in vivo*.[20]

Prompted by the need to extend baculovirus-mediated expression, we designed a hybrid baculovirus system that exploited the FLP/Frt-mediated recombination for circular episome formation and *oriP*/EBNA1 for the retention of episomes. The first baculovirus expressed FLP while the second baculovirus harbored an Frt-flanking transgene cassette that encompassed *oriP*/EBNA1. We hypothesized that after cotransduction the expressed FLP would cleave the transgene cassette from the baculovirus genome and catalyze intracellular episome formation, while *oriP*/EBNA1 would enable episomal self-replication. We first confirmed, and then optimized the FLP/Frt-mediated recombination and intracellular episome formation. The hybrid

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baculovirus system functioned in various mammalian cells and the resultant replicon enabled sustained transgene expression. Finally, the feasibility to employ this system in tissue engineering was explored by delivering the BMP-2 gene into MSCs and confirmed by the osteogenesis potentiated by the prolonged BMP-2 expression.

RESULTS

Design of the hybrid baculovirus vector and confirmation of episome formation

To explore the feasibility of FLP/Frt-mediated recombination in the context of baculovirus genome (≈134 kb), we constructed BacFLP expressing FLP under the control of cytomegalovirus immediate-early promoter ($P_{CMV\text{-}IE}$), and BacFC-OEE harboring genes encoding enhanced green fluorescent protein (*egfp*), *oriP*, *EBNA1*, and $P_{CMV\text{-}IE}$ (**Figure 1a**). The transgene cassette was flanked by two Frt sites for FLP recognition and formation of the episomal pFC-OEE. Note that *egfp* was not driven by any promoter in the baculovirus genome, thus only after recombination and pFC-OEE formation could *egfp* be placed downstream of PCMV-IE. As such, the EGFP expression served as an indicator of FLP/Frt-mediated recombination.

Human embryonic kidney-293 (HEK293) cells were first transduced with BacFC-OEE alone or cotransduced with BacFLP/BacFC-OEE at multiplicity of infection (MOI) 100/400. **Figure 1b** shows that at 2 days post-transduction (dpt) EGFP was expressed only in the cotransduced cells but not in the singly transduced cells, suggesting the occurrence of recombination only after cotransduction. To verify the formation of pFC-OEE, episomal DNA was extracted and subjected to PCR using primers targeting the *egfp* gene and $P_{CMV\text{-}IE}$ that flanked the newly joined Frt sites (FrtR-1 and FrtF-1, **Figure 1c**), which would amplify a 0.65 kb fragment. **Figure 1d** reveals no PCR products from the mock-transduced and BacFC-OEE-transduced cells, indicating the absence of re-circularized pFC-OEE. In contrast, a 0.65-kb fragment was amplified from the cotransduced cells, which was further sequenced to verify the identity (data not shown). These data collectively confirmed that BacFLP/BacFC-OEE cotransduction gave rise to FLP/Frt-mediated recombination and episome formation.

Improvement of FLP/Frt-mediated recombination and episome formation

To improve the recombination efficiency, HEK293 cells were cotransduced with BacFLP/BacFC-OEE (MOI 100/400) and cultured in the presence of sodium butyrate (a histone deacetylase inhibitor that induces chromatin remodeling[21]). The butyrate-containing medium was replaced with fresh medium

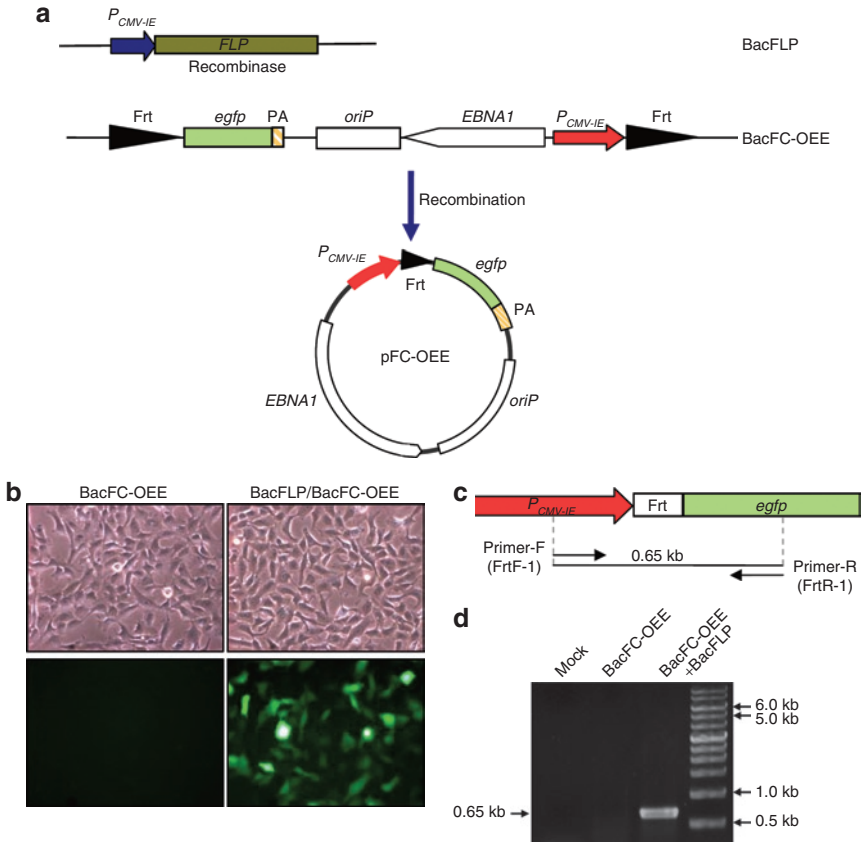

Figure 1 Baculovirus construction and confirmation of FLP/Frt-mediated recombination. (**a**) Schematic illustration of the constructs and formation of pFC-OEE. (**b**) Confirmation of recombination by EGFP expression. (**c**) Primers targeting $P_{CMV\text{-}IE}$ and *egfp* that flanked the recombined Frt sites. (**d**) Confirmation of recombination by PCR. HEK293 cells were transduced with BacFC-OEE (MOI 100) alone or cotransduced with BacFLP/BacFC-OEE (MOI 100/400), and analyzed at 2 dpt. Magnification, 200×. EGFP, enhanced green fluorescent protein; HEK293, human embryonic kidney-293; MOI, multiplicity of infection; PA, polyadenylation signal.

at 15-hours post-transduction and the cells continued to be cultured until flow cytometry analysis (**Figure 2a**) at 2 dpt. Compared with the control (0 mmol/l), 5 mmol/l sodium butyrate tremendously increased the percentage of GFP+ (%GFP+) cells and the mean fluorescence intensity (MFI). Because only the circular pFC-OEE conferred EGFP expression, the data indicated that recombination occurred in 71% cells in the presence of 5 mmol/l sodium butyrate. Raising the butyrate concentration to 10 or 15 mmol/l failed to elevate the %GFP+ cells, despite the continued increase in MFI. Thus 5 mmol/l sodium butyrate was supplemented in all subsequent experiments as described above and the resultant %GFP+ cells indicated the recombination efficiency.

We next assessed the optimal dosages for BacFLP and BacFC-OEE by cotransduction at various MOI combinations. The flow cytometry analyses performed at 2 dpt (**Figure 2b**) overtly depict the dependence of the %GFP+ cells and MFI on the MOI. Overall, the FLP/Frt-mediated recombination and episome-mediated EGFP expression culminated at MOI 50–200 for BacFLP and MOI 200–400 for BacFC-OEE, as evidence by the high %GFP+ cells (65–75%) and MFI (a.u. ≈3,175–5,471).

Episome formation in different mammalian cells

Whether this system functioned in other cells was explored by cotransducing various cell types with BacFLP/BacFC-OEE (MOI 100/400). **Figure 3a** depicts that the %GFP+ cells, and hence the recombination efficiency, exceeded 36% even for the difficult-to-transfect cell lines HepG2 and HuH-7. The recombination efficiency was the highest for baby-hamster kidney (BHK) cells (83%) and remained high for primary rabbit articular chondrocytes (77%) and human MSCs (48%). Note that baculovirus can transduce these cells at efficiencies >80%,[22–24] thus the differences in the %GFP+ cells reflected the disparities in the recombination efficiencies within these cell types.

Because our system enabled efficient episome formation, we next compared the episome delivery efficiencies via baculovirus transduction and lipid transfection. HuH-7, MSCs, and chondrocytes cultured in six-well plates (≈5 × 10^5 cells/well) were cotransduced with BacFLP/BacFC-OEE at MOI 100/400, which corresponded to the use of ≈2.0 × 10^8 plaque-forming units for BacFC-OEE. For comparison, the cells were transfected with a control plasmid (≈4 μg/well) which mimicked the recombined episome pFC-OEE in size (6.4 kb) and transgene ($P_{CMV\text{-}IE}$-Frt-*egfp*). Assuming that 1 plaque-forming unit is equivalent to 10–100 baculovirus particles,[25,26] ≈0.2–2.0 × 10^{10} copies of BacFC-OEE and ≈6.5 × 10^{11} copies of the control plasmid were used per well. Therefore, ≈33–330 times more *egfp* genes were used for transfection than for transduction. Consequently, the absolute episome copy numbers as measured by quantitative real-time PCR (Q-PCR) were tremendously higher in the transfected HuH-7, MSCs, and chondrocytes than in the baculovirus-transduced counterparts (**Figure 3b**, upper panel). However, the resultant %GFP+ cells (**Figure 3b**, lower panel) were significantly higher for the transduced cells (42, 48, and 76% for HuH-7, MSCs, and chondrocytes, respectively) than for the transfected cells, demonstrating more effective transgene expression mediated by the baculovirus-delivered episomes.

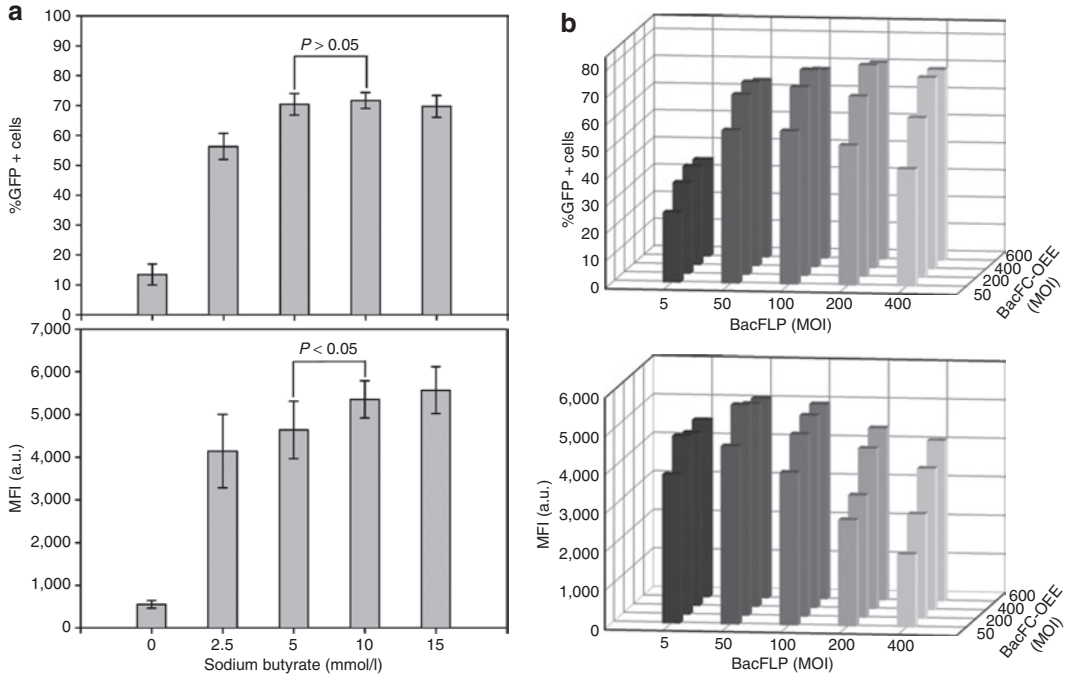

Figure 2 Improvement of FLP/Frt-mediated recombination and episome formation. (**a**) Recombination efficiency and EGFP expression at various butyrate concentrations. (**b**) Recombination efficiency and EGFP expression at various virus dosages. In all experiments, the butyrate-containing DMEM medium was replaced with fresh medium 15 hours after transduction and continued to be cultured. The cells were harvested at 2 dpt and analyzed for the %GFP+ cells and MFI by flow cytometry. All data represent the averages of three independent culture experiments. DMEM, Dulbecco's modified Eagle's medium; EGFP, enhanced green fluorescent protein; MFI, mean fluorescence intensity.

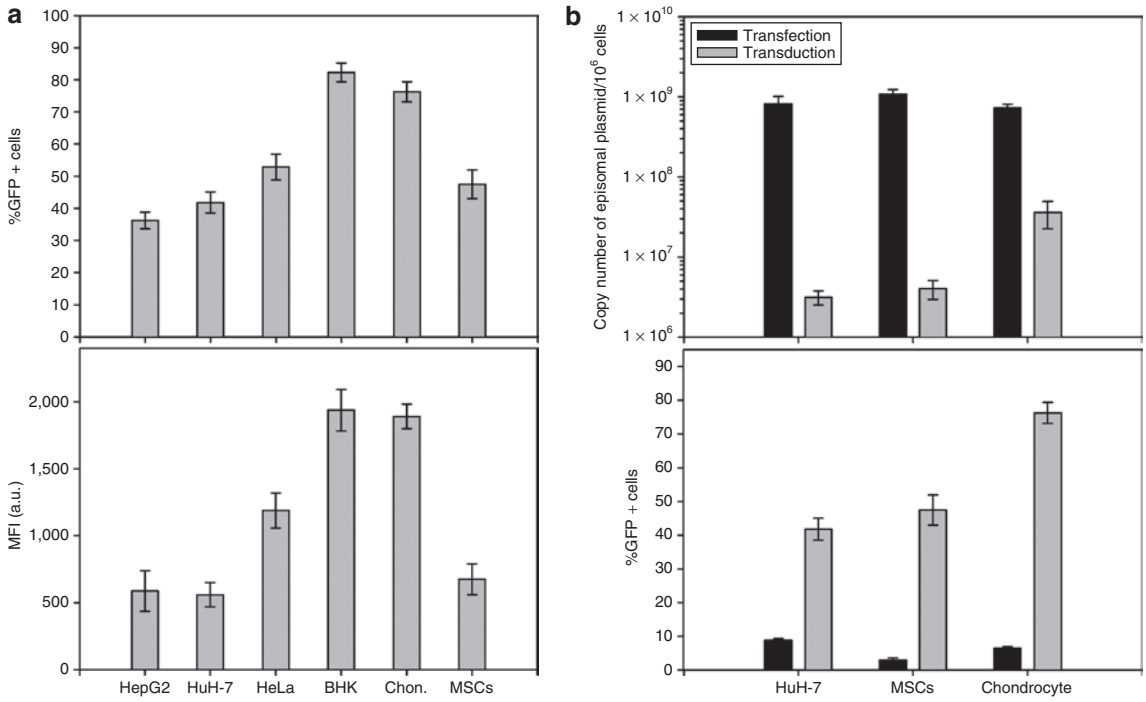

Figure 3 Episome formation in different cells. (**a**) Recombination efficiency in various cell types. (**b**) Comparison of episome delivery efficiencies. The cells cultured in 6-well plates (5×10^5 cells/well) were cotransduced with BacFLP/BacFC-OEE (MOI 100/400) or transfected with 4.0 μg/well pFC-OEE-aq plasmid, and were harvested 2 days later for analyses. The absolute episome copy numbers were quantified by Q-PCR using primers targeting the *egfp* gene and $P_{CMV\text{-}IE}$ that flanked the newly joined Frt sites. The data represent the averages of three independent culture experiments. MOI, multiplicity of infection; Q-PCR, quantitative real-time PCR.

Persistence of hybrid baculovirus-mediated transgene expression

Whether this system prolonged the transgene expression was evaluated by another hybrid baculovirus BacCON-CE (**Figure 4a**) which contained Frt-flanking *oriP/EBNA1* for the retention of the re-circularized replicon pCON-CE. However, in BacCON-CE *egfp* was directly under the control of $P_{CMV\text{-}IE}$ for direct assessment of transduction efficiency and EGFP expression. Also BacCON-CE contained a neomycin-resistance gene (*Neo*^{r}) driven by SV40 promoter. HEK293 cells were cotransduced with BacFLP/BacCON-CE (MOI 100/400) and cultured with or without G418. As controls, the cells were transduced with BacCON-CE (MOI 400) alone or with a conventional baculovirus vector (Bac-CE) that transiently expressed EGFP under $P_{CMV\text{-}IE}$ (MOI 400).[16] The cells were subcultured every 3–4 days upon confluency and subjected to flow cytometry at different times (**Figure 4b**).

Under these transduction conditions, the %GFP+ cells approached 99–100% initially for all groups. However, Bac-CE-mediated EGFP expression underwent a rapid decay, with ≈16 and ≈2% cells emitting fluorescence at 14 and 21 dpt, respectively. By defining the time point at which the %GFP+ cells dropped <5% as the expression duration[16] and data extrapolation, Bac-CE led to an expression duration of ≈20 days. BacCON-CE transduction alone slightly prolonged the expression duration to ≈27 days. Without G418, BacFLP/BacCON-CE cotransduction considerably elevated the %GFP+ cells at 14 and 21 dpt to ≈66 and ≈50% with concomitant enhancement of MFI. More importantly, the cotransduction extended the expression duration to ≈48 days. Selection with G418 resulted in an even more remarkable improvement in gene expression duration and level, with the %GFP+ cells maintaining at 78 and 36% at 21 and 63 dpt, respectively. Therefore, the transgene expression was prolonged beyond 63 days.

To attest whether the persistent fluorescence truly stemmed from the long-term maintenance of pCON-CE, the experiments in **Figure 4b** were repeated and baculovirus genomic DNA and episomal pCON-CE were quantified by Q-PCR using the primers targeting endogenous baculovirus *gp64* gene and the recombined Frt sites, respectively. *egfp* mRNA was quantified by quantitative real-time reverse transcriptase PCR (qRT-PCR) using primers targeting its mature domain. All DNA and mRNA levels were normalized against those obtained at 2 dpt to yield the relative copy numbers. The time point at which the relative copy numbers dropped <0.1% was defined as the cutoff.

Without G418, baculoviral genome copy numbers (**Figure 5a**) decreased rapidly and dropped below the cutoff at 21 dpt upon Bac-CE transduction or BacFLP/BacCON-CE cotransduction. In contrast, the pCON-CE DNA decayed at a slower rate and persisted for ≈45 days. With G418 selection the cotransduction still led to a steady decrease in the baculoviral genome copy number, but nevertheless gave rise to the stable maintenance (>3%) of pCON-CE for at least 63 days.

Concurrent with the DNA decay, the *egfp* mRNA level (**Figure 5b**) in the Bac-CE-transduced cells decreased precipitously at as early as 7 dpt and vanished after ≈27 dpt. In the cotransduced cells, the relative *egfp* mRNA level declined at a remarkably slower

rate even without G418, remaining >3.5 and 0.5% at 21 and 49 dpt, respectively. G418 supplementation substantially augmented the maintenance of *egfp* transcription, whose level ranged between 5 and 10% from 35 to 63 dpt.

Prolonged baculovirus-mediated BMP-2 expression potentiated *in vitro* osteogenesis

BMP-2 is an osteoinductive factor that triggers the differentiation of MSCs into osteoblasts and then osteocytes.[15] To exploit this system for MSCs engineering and osteogenesis, we constructed BacCON-CB that resembled BacCON-CE except that *egfp* was replaced by BMP-2 gene (**Figure 6a**). Human MSCs were cotransduced with BacFLP/BacCON-CB (MOI 50/150), or transduced with a conventional baculovirus vector (Bac-CB, MOI 150) that transiently expressed BMP-2 under $P_{CMV\text{-}IE}$.[22] After transduction, the cells were cultured without passaging and subjected to medium exchange (no G418) every 3 days.

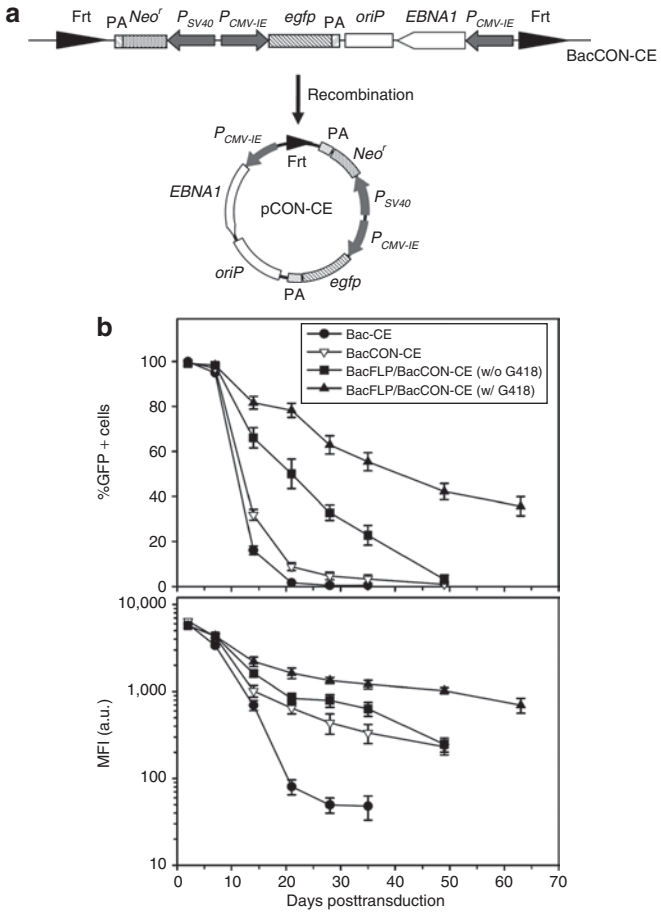

Figure 4 Persistence of transgene expression. (**a**) Schematic illustration of BacCON-CE and the formation of episomal pCON-CE. (**b**) Time-course profiles of %GFP+ cells and MFI. HEK293 cells in 12-well plates (2×10^5 cells/well) were cotransduced with BacFLP/BacCON-CE (MOI 100/400) and cultured with or without G418 (50 μg/ml). As controls, the cells were transduced with BacCON-CE (MOI 400) alone or with Bac-CE (MOI 400). All transduced cells were subcultured every 3–4 days by splitting the cells at a ratio of 1:5. The transgene expression data were measured by flow cytometry and represent the averages of 3 independent culture experiments. HEK293, human embryonic kidney-293; MFI, mean fluorescence intensity; MOI, multiplicity of infection.

At different times the medium was withdrawn for enzyme-linked immunosorbent assay.

Figure 6b reveals that BMP-2 expression by the mock-transduced MSCs was undetectable at all times. Bac-CB transduction led to the maximum BMP-2 expression at 3 dpt (14.0 ng/5×10^5 cells/72 hours), which then precipitously dropped below 1 ng/5×10^5 cells/72 hours at 9 dpt. BacFLP/BacCON-CB cotransduction not only augmented the maximum BMP-2 expression (≈27.8 ng/5×10^5 cells/72 hours) at 6 dpt, but also sustained the expression level higher than 1.0 ng/5×10^5 cells/72 hours for 15 days. qRT-PCR analyses (**Figure 6c,d**) attested that BacFLP/BacCON-CB cotransduction enhanced the transcription levels of the early (alkaline phosphatase) and late (osteocalcin) osteogenic differentiation markers when compared with Bac-CB transduction. The MSCs differentiation into osteocytes was also compared by calcium deposition as stained by Alizarin red at weeks 2 and 4. **Figure 6e** illustrates that the mock-transduced MSCs deposited virtually no calcium spots, indicating minimal spontaneous MSCs differentiation without BMP-2 stimulation. Bac-CB transduction triggered progressive calcium deposition with time, but calcium spots were still absent in a large portion of cells at week 4. In marked contrast, BacFLP/BacCON-CB cotransduction increased the density of calcium spots at weeks 2 and 4. **Figure 6** confirmed that the hybrid baculovirus system prolonged BMP-2 expression in MSCs and potentiated the osteogenic differentiation into osteocytes.

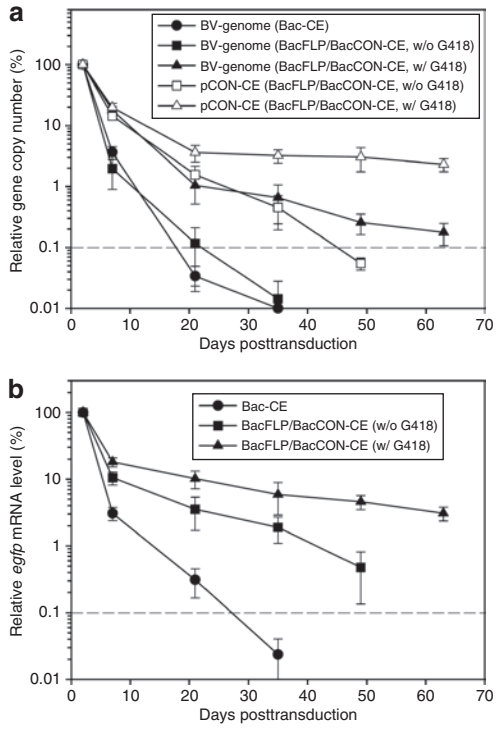

Figure 5 Time-course profiles of DNA and *egfp* mRNA. The copy numbers of (**a**) viral and episomal DNA and (**b**) *egfp* mRNA were measured by Q-PCR and qRT-PCR, respectively. All data were normalized against those obtained at 2 dpt to yield the relative copy numbers. The data represent the averages of three independent culture experiments. The dotted line indicates the cutoff. Q-PCR, quantitative real-time PCR; qRT-PCR, quantitative real-time reverse transcription PCR.

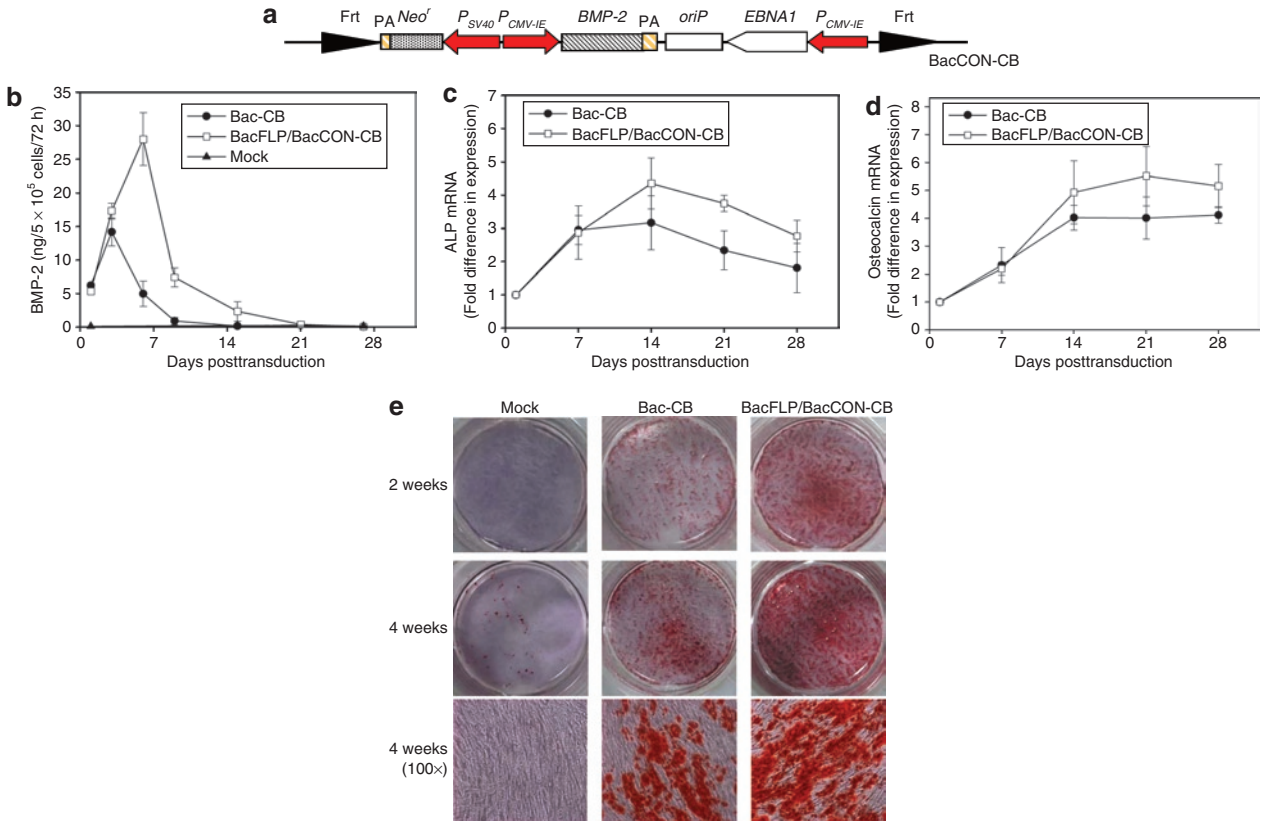

Figure 6 The hybrid baculovirus system prolonged BMP-2 expression and potentiated osteogenesis. (**a**) Schematic illustration of BacCON-CB. (**b**) Time-course profile of BMP-2 expression. (**c**) Relative ALP mRNA levels. (**d**) Relative osteocalcin mRNA levels. (**e**) Calcium deposition. Human MSCs were mock-transduced, singly transduced with Bac-CB (MOI 150) or cotransduced with BacFLP/BacCON-CB (MOI 50/150). Extracellular BMP-2 concentrations were measured by enzyme-linked immunosorbent assay at 1, 3, 6, 9, 15, 21, and 27 dpt to calculate the expression levels. The mRNA levels were measured by qRT-PCR and normalized against those from the mock-transduced MSCs at 1 dpt. The calcium deposition was stained by Alizarin red at weeks 2 and 4. The photographs indicating the wells stained at weeks 2 and 4, and the micrographs indicating the cells stained at week 4 (100×) are shown. All data are representative of three independent culture experiments. ALP, alkaline phosphatase; BMP-2, bone morphogenetic protein-2; MSC, mesenchymal stem cells; MOI, multiplicity of infection; qRT-PCR, quantitative real-time reverse transcriptase PCR.

DISCUSSION

One major hurdle to the widespread application of baculovirus as a gene therapy vector is its nonreplicative nature in mammalian cells, which leads to transient expression. To address this issue, a baculovirus-adeno-associated virus (AAV) hybrid vector containing a gene cassette flanked by the AAV inverted terminal repeats was developed.[27] Transduction of 293 cells with this hybrid vector expressing additional *rep* gene results in specific integration of the inverted terminal repeat-flanking cassette into AAVS1 site. Similar baculovirus-AAV hybrid vectors incorporating inverted terminal repeat-flanking cassette also extend transgene expression in the rat brain[28] and human embryonic stem cells.[29] However, this system relies on gene integration thus safety concerns may arise. Besides, this hybrid system fails to extend *in vitro* transgene expression in BHK cells (Y.-C. Hu unpublished results), as such it is probably cell-type specific.

To prolong the transgene expression and circumvent the safety concerns regarding gene integration, we developed a hybrid baculovirus vector that exploited FLP/Frt-mediated recombination and Epstein–Barr virus *oriP*/EBNA1 for sustained maintenance of the episomal replicon. Upon cotransduction with BacFLP, our hybrid baculovirus efficiently delivered the transgene cassette into cells and allowed for ensuing transgene excision from the baculovirus genome and episome formation (**Figure 1**). The excision/recombination was remarkably ameliorated by the histone deacetylase inhibitor sodium butyrate (**Figure 2a**), which concurred with the finding that sodium butyrate augments the excision of AAV inverted terminal repeat-flanking transgene cassette from baculovirus.[12] For HEK293 cells, MOI 50–200 for BacFLP and MOI 200–400 for BacFC-OEE gave rise to fairly high recombination efficiencies (65–75%) and MFI (3,175–5,471 a.u.). This system was also applicable to mammalian cell lines and primary cells, attaining recombination efficiencies up to 85% in BHK cells, and 77% in difficult-to-transfect primary chondrocytes (**Figure 3a**). Consequently, our hybrid vector resulted in significantly more efficient episome delivery and episome-mediated gene expression when compared with transfection (**Figure 3b**).

More importantly, the split cassette and *oriP*/EBNA1 substantially prolonged the transgene expression duration to at least 63 days in the presence of selection (**Figure 4b**), potentially rendering this system attractive for continuous production of biologically active proteins while obviating the need to generate stable cell lines. Without selection the transgene expression

persisted for ≈48 days, which was considerably prolonged as compared with ≈20 days when using the conventional vector Bac-CE (**Figure 4b**). Such sustained expression was attributed to the prolonged maintenance of the replicon (**Figure 5a**) and hence the transgene transcription (**Figure 5b**). Because it has been shown that the EBNA1 expression level is essential for the maintenance of oriP-containing genome,[30] future optimization of the EBNA1 expression may further prolong the expression period. In sharp contrast to the persistent episomes, after unloading the payload the baculovirus genome was rapidly degraded (**Figure 5a**), thereby easing the concerns about the residual viral DNA residing in the transduced cells. These attributes may benefit the development of baculovirus as an appealing system alternative to transfection for replicon delivery into difficult-to-transfect primary cells.

Of note, BacCON-CE transduction alone led to rapid extinction of EGFP expression (**Figure 4b**), suggesting that the baculovirus carrying the *oriP*/EBNA1 cassette itself failed to extend the transgene expression. This observation contradicted the data reported previously whereby transduction of cells with a single baculovirus harboring the *oriP*/EBNA1 cassette was sufficient to support presistent expression.[30] The exact reason accounting for the discrepancy remains to be investigated, but we believe that the whole baculovirus genome (≈134 kb), as compared with the split episome, is more susceptible to nuclease attack, which contributes to the more rapid degradation as observed in this study.

Although the transgene expression was gradually lost in the absence of selection, it is observed in various gene delivery systems exploiting *oriP*/EBNA1[19,31,32] and can be ascribed to: (i) the cells that did not harbor the recombined replicons predominated in the cell population after serial passaging; (ii) the *oriP*/EBNA1 system was insufficient to support stable retention of the replicon. The first problem may be tackled by improving the recombination efficiency so that the majority of cells, if not all, harbor the replicons. Because FLP exerts optimal activity at 30 °C,[33] engineering the FLP to shift its optimal activity to 37 °C may help augment the recombination efficiency. The second problem might be alleviated by using a new replication origin. Because vectors incorporating the scaffold matrix attachment region (S/MAR) can be retained episomally for >100 generations in the absence of selection[34] and are effective for persistent retention of transgenes *in vivo*,[35] future extension of the expression may be achieved by replacing *oriP*/EBNA1 with S/MAR.

Compared with HEK293 cells, in human MSCs the recombination efficiency (≈48%, **Figure 3a**) was lower and the expression period was shorter (**Figure 6b**), concurring with the notion that the prolonged expression is cell-type dependent.[30] Nevertheless, BacFLP/BacCON-CB cotransduction augmented the BMP-2 expression (**Figure 6b**) and extended the expression to at least 15 days in the absence of selection, which outlasted the duration (≈8 days) resulting from the conventional baculovirus vector (Bac-CB) and potentiated the osteogenesis (**Figure 6b–e**). The elevated expression can be attributed to the interaction of o*riP* and EBNA1 which enhances the expression of neighboring genes in the context of baculovirus genome.[36] Although short-term BMP-2 expression is sufficient to promote ectopic bone formation in the nude mice model,[15] a longer-term expression might be beneficial and required for the repair of massive bone defects (*e.g.*, massive segmental bone fracture). Furthermore, it is proposed that MSCs should be differentiated into a specific lineage prior to implantation into patients owing to concerns that clinical use of undifferentiated stem cells may result in uncontrolled proliferation and hence tumor formation.[37] As such, it may be preferable to culture the genetically engineered MSCs and initiate lineage-specific differentiation *in vitro* before implantation. In this regard, prolonged growth factor expression with this vector can not only potentiate *in vitro* differentiation, but also ensure sustained secretion of the growth factor after implantation, which exerts autocrine and paracrine effects (*e.g.*, recruitment of other progenitor cells) *in vivo* to promote the tissue regeneration.

In summary, we developed a novel baculovirus vector that confers sustained expression in various mammalian cells including MSCs, and demonstrated its potential in tissue engineering. This system may also be used for continuous recombinant protein production in mammalian cells or for the treatment of other indications (*e.g.*, cancer) requiring sustained expression. Although long-term expression can be achieved by other viral vectors such as retrovirus, lentivirus, and AAV, these vectors are fairly labor-intensive to use and have limited cloning capacity. Additionally, retrovirus and lentivirus are prone to integration into coding or regulatory regions of transcriptionally active genes,[38,39] raising concerns about gene silencing and insertional mutagenesis. AAV-mediated integration is also associated with hepatocellular carcinoma in neonatal mice.[40] Alternatively, stable expression can be mediated by transposons such as *Sleeping Beauty*[41] and *PiggyBac*[42], or by phage integrase such as φC31.[43] However, they still may impose position effects and more experiments are required to scrutinize the possibility of insertional mutagenesis. In contrast to these integrating vectors, our hybrid baculovirus is based on extrachromosomal maintenance of the transgene cassette, which is less likely to cause gene silencing and insertional mutagenesis.[18] Although such *oriP*/EBNA1-based replicon can be delivered via HSV-1 (herpes simplex virus type 1)[44] and adenoviral vector,[45] HSV-1 is immunogenic and cytotoxic to non-neuronal cells while adenovirus also mounts strong immune responses. Despite the advent of gutless adenoviral vectors to minimize the immune responses, the production and purification of gutless vectors are cumbersome. In contrast to these vectors, baculovirus is not pathogenic to humans and has a cloning capacity as large as 38 kb.[46] Furthermore, recombinant baculovirus can be easily constructed and produced to high titers simply by infecting insect cells in Biosafety Level 1 laboratories. All these features support the use of this hybrid baculovirus vector for gene therapy.

MATERIALS AND METHODS

Cell culture. Human cell lines HEK293, HepG2, HuH-7, HeLa, and BHK cell line were cultured using Dulbecco's modified Eagle's medium (Sigma, St Louis, MO) containing 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD) and 1% penicillin/streptomycin solution (Gibco). Primary articular chondrocytes were isolated from New Zealand White rabbits and cultured using Dulbecco's modified Eagle's medium.[22] Human bone marrow-derived MSCs were obtained and cultured as described,[23] using α-modified Eagle's medium (Hyclone, Ogden, UT) containing 20% FBS, 4 ng/ml basic fibroblast growth factor (R&D Systems, Minneapolis, MN), 100 U/ml penicillin, and 100 mg/ml streptomycin.

Donor plasmids. All baculovirus donor plasmids were constructed using pFastBac-DUAL plasmid (Invitrogen, Carlsbad, CA) as the backbone. The gene cassette encoding FLP under the control of cytomegalovirus immediate early ($P_{CMV\text{-}IE}$) promoter was digested by *Sal*I/*Pvu*II from pOG44 (Invitrogen) and subcloned into pFastBac-DUAL to yield pBacFLP.

To generate the hybrid donor plasmids, a transfer plasmid was first constructed. The *egfp* gene (0.7 kb) was cleaved from pEGFP-N1 (Clontech, Mountain View, CA) and subcloned into pRep4 plasmid containing *oriP*/*EBNA1* (Invitrogen) by *Sal*I/*Xba*I treatment. The cassette comprised of *egfp* at the upstream of *oriP*/*EBNA1* (6.0 kb) was then subcloned into pET-30a(+) (Novagen, San Diego, CA) by *Eco*RI/*Sal*I digestion to yield pET-EGFP-*oriP*/EBNA1.

pFC-OEE was constructed in three stages. First, a DNA fragment composed of a multiple cloning site flanked by two Frt sites in a parallel orientation was PCR-amplified from pLOI2226[47] using two primers (5′-TGCCGGTACCATCGATGAATTGATCCGAA-3′ and 5′-TAGCC TGCAGACCAATTCGAAGTTCCTA-3′). The amplicon (0.25 kb) was subcloned into pFastBac-DUAL by *Kpn*I/*Pst*I digestion to yield pBac-Frt. Second, $P_{CMV\text{-}IE}$ (0.6 kb) was PCR-amplified from pcDNA3.1(+) (Invitrogen) using two primers (5′-TCAGGGATCCGCGTTGACATTGATTATTG-3′ and 5′-TGGACCCGGGAGTTAGCCAGAGAGCTCTG-3′) and subcloned into pBac-Frt by *Bam*HI/*Sma*I treatment. The resultant pBac-Frt-RCMV thus contained $P_{CMV\text{-}IE}$ which was in an orientation opposite to the Frt direction. Third, the *egfp-oriP*/EBNA1 cassette in pET-EGFP-oriP/EBNA1 was digested with *Sal*I/*Bgl*II and subcloned into pBac-Frt-RCMV with *Sal*I/*BamH*I treatment to yield pFC-OEE (see **Figure 1a**).

pBacCON-CE was constructed in four stages. First, $P_{CMV\text{-}IE}$ promoter was amplified by PCR from pcDNA3.1(+) using two primers (5′-TCAG CCCGGGGCGTTGACATTGATTATTG-3′ and 5′-TGGAGGATCCAG TTAGCCAGAGAGCTCTG-3′) and the amplicon was subcloned into pBac-Frt by *Sma*I/*Bam*HI digestion to yield pBac-Frt-LCMV. Second, the neomycin-resistance gene under the simian virus 40 (SV40) promoter was PCR-amplified from pcDNA3.1(+) and subcloned into pBac-Frt-LCMV to form pBac-Frt-Neo^r-LCMV. Third, the *egfp* gene under $P_{CMV\text{-}IE}$ was PCR-amplified from pBac-CE[48] using two primers (5′-TCC GGTCGACTCATACCGTCCCACCATC-3′ and 5′-TGGATCTAGATTT CACTTATCTGGTTC-3′) and the amplicon was subcloned into pET-EGFP-*oriP*/EBNA1 by *Sal*I/*Xba*I digestion to replace the promoterless *egfp*. Fourth, the whole cassette was cleaved with *Sal*I/*Bgl*II and subcloned into pBacFrt-Neo^r-LCMV by *Sal*I/*Bam*HI treatment to yield pBacCON-CE (**Figure 4a**).

pBacCON-CB was constructed in a way similar to pBacCON-CE construction except that BMP-2 gene was cloned in lieu of *egfp*. The BMP-2 gene under the control of $P_{CMV\text{-}IE}$ was PCR-amplified from pBac-CB[22] using two primers (5′-TCAGGTCGACGCGTTGACATTGATT ATTG-3′ and 5′-GGACTCTAGATATAGTTCTAGTGGT TGGC-3′).

Baculovirus preparation and transduction. The recombinant baculoviruses (BacFLP, BacFC-OEE, BacCON-CE, BacCON-CB) were constructed using the corresponding donor plasmids (pBacFLP, pFC-OEE, pBac-CON-CE, pBacCON-CB) following the instructions of Bac-To-Bac system (Invitrogen). The recombinant baculovirus that transiently expressed EGFP (Bac-CE) or BMP-2 (Bac-CB) was constructed previously.[22,48] All viruses were propagated and titered as described.[22,48]

The cells were transduced according to the protocol developed previously[16,49] with minor modifications. Depending on the MOI, a certain volume of virus supernatant was pre-mixed with $NaHCO_3$-deficient Dulbecco's modified Eagle's medium containing 10% FBS to adjust the final volume to 500 µl (per well). Transduction was initiated by adding the virus mixture to the cells in 6-well plates (5×10^5 cells/well) and continued by gently shaking the plates for 6 hours (4 hours for MSCs) at room temperature. After the incubation period, the virus solution was withdrawn and the cells continued to be cultured. To enhance the FLP-mediated recombination efficiency (see Results), the cells were cultured with butyrate-containing medium for 15 hours, after which the medium was withdrawn and cells were cultured with normal medium.

For long-term expression experiments, HEK293 cells in 12-well plates (2×10^5 cells/well) were cotransduced with BacFLP/BacCON-CE and cultured in the presence or absence of 50 µg/ml G418 after 2 dpt. The cells were passaged at a 1:5 split ratio upon confluence. For MSCs, the cells in 6-well plates (5×10^5 cells/well) were cotransduced with BacFLP/BacCON-CB as described above. The cells were cultured with medium exchange every 3 days, but were not subcultured.

Detection of recombination and episome formation. The recombination and episome formation were confirmed by fluorescence microscopy and PCR at 2 dpt. The cells were observed by a fluorescence microscope and the DNA was extracted with Blood & Tissue Extraction Mini Kit (Viogen, Taipei, Taiwan) as the PCR template. The PCR primers FrtF-1 (5′-CATAGTAACGCCAATAGGGAC-3′) and FrtR-1 (5′-CAGATGAACTTCAGGGTCAGC-3′) were designed to probe the $P_{CMV\text{-}IE}$ promoter and *egfp* which flanked the newly re-joined Frt sites (**Figure 1c**). The PCR products were subjected to 1% agarose gel electrophoresis.

Analysis of transgene expression. The percentage of GFP+ cells (%GFP+ cells) and MFI were measured by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).[16,23] The MFI is expressed in arbitrary unit (a.u.). The BMP-2 concentration in the culture medium was measured using an enzyme-linked immunosorbent assay kit (R&D Systems).

Plasmid transfection. The cells in 6-well plates (5×10^5 cells/well) were transfected with ≈4.0 µg/well control plasmid pFC-OEE-aq which mimicked the recombined episome pFC-OEE in size and transgene by lipofectamine 2000 (Invitrogen).

Q-PCR and qRT-PCR. The absolute episome copy number was quantified by Q-PCR using primers (forward: 5′-GGATCCATGATAAATTTAATT ATTGATG-3′, reverse: 5′-GGTACCTTATTTAGTATATTTTAAGTG-3′) targeting the *egfp* gene and $P_{CMV\text{-}IE}$ that flanked the newly re-joined Frt sites in the episome. Total DNA was extracted using Blood & Tissue Extraction Mini Kit. Real-time PCR reactions were performed using ABI PRISM 7300 (Applied Biosystems, Foster City, CA) under the conditions as described previously.[23] For each PCR, a no-template reaction was included as negative control. Copy number quantification was based on the external standard curve created using known amounts of pFC-OEE-aq.

The relative copy numbers of the baculoviral genome and the replicon were also quantified by Q-PCR. The baculoviral genome was probed using primers that targeted *gp64* (forward: 5′-CGCCTTCAGCCATGGAAGT-3′, reverse: 5′-CCACCATGGAGAACACCAAGTT-3′). The replicon DNA was probed using primers that targeted the newly recombined Frt site (forward: 5′-ATCAATGTCAACGCGTATATCTG-3′, reverse: 5′-CATGT CTGTATACCCTCGACCA-3′). The housekeeping gene *gapdh* (internal control) was probed using another set of primers (forward: 5′-CTGG TCATCAATGGGAAAC-3′, reverse: 5′-CAAAGTTGTCATGGATGA-3′). Real-time PCR reactions were performed as described above.

The relative *egfp* transcription levels were quantified by qRT-PCR. Total mRNA was extracted using RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed to cDNA using Omniscript RT kit (Qiagen). The cDNA was subjected to Q-PCR using primers targeting *egfp* (forward: 5′-TATATCATGGCCGACAACA-3′, reverse: 5′-TGTTCTGCTGGTAG TGGTCG-3′) and *gapdh* (forward: 5′-CCACCCATGGCAAATTCC-3′, reverse: 5′-TGGGATTTCCATTGATGACAA-3′). For relative quantification, the threshold cycle values were normalized against that of *gapdh*. All data were normalized against those obtained at 2 dpt.

The relative transcription levels of alkaline phosphatase and osteocalcin were quantified by qRT-PCR following the aforementioned procedures, except that the primers were specific for alkaline phosphatase (forward: 5′-TGCGGAAGAACCCCAAAG-3′, reverse: 5′-ATGGTGCCCGTGGTCAAT-3′) and osteocalcin (forward: 5′-AGGAGGGCAGCGAGGTAG-3′; reverse: 5′-GAAAGCCGATGTGGTCAGC-3′).

Alizarin red staining. The mineralization was confirmed by calcium phosphate deposition as stained by Alizarin red.[15]

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