

## BacMam-mediated Gene Transfer into Neural Cell Types

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Modified baculoviruses containing a mammalian promoter, known as BacMam, have emerged as a key method for labeling diverse cell types, owing to its high transduction efficiencies, minimal cytopathic effects and the ability to carry DNA fragments larger than 40 KB. The transient expression without viral replication or integration into the mammalian genome makes BacMam an ideal method to label primary and adult stem cells. In the current study, we describe how BacMam enables efficient transduction and robust gene expression in neural stem cells (NSC) and progenitor cells. Utilizing the H9 human embryonic stem cell (hESC), we generated neurospheres, neural rosettes and Neural Stem Cells (NSC) and transduced them with BacMam 2.0 GFP. Commercially available human NSCs (h-NSC) and rat fetal NSCs (rNSCs) were also transduced with BacMam 2.0 GFP. Primary rat neurons and post-differentiated human NSCs were transduced with either BacMam 2.0 GFP or Plasma Membrane-GFP BacMam 2.0 and Synaptophysin-RFP BacMam 2.0 to assess the capability of gene expression in mature neural cell types. Using a GFP marker, we demonstrated that the BacMam virus can consistently transduce neural stem cells with high efficiency and low toxicity. Transduction was also enabled in differentiated NSC, and the expression in primary neurons was dependent on the age of the culture, with preferential labeling of astrocytes in older cultures. BacMam mediated gene delivery provides a superior platform for introducing diverse gene elements, creating assay-ready cells among young neuronal cells and mature astrocytes.

### INTRODUCTION

Genetic modification of primary cells and stem cells enables creation of *in vitro* models for drug discovery, a tool for dissecting basic cell biology and development of *in vivo* cell-based delivery strategies. Efficient gene transfer into primary cells and stem cells is a critical step in cellular engineering, with several viral and non-viral methods existing for stable modification of these cells, albeit with varying efficiencies. Most methods require isolation of clonal populations, followed by rigorous characterization of the clones to ensure sustained transgene expression. This approach is best suited for cell types that

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can be cultured for long periods of time such as embryonic stem cells. However, most primary and adult stem cell types are not amenable to prolonged manipulation and culture, due to their limited proliferative potential.

BacMam are modified baculoviruses (*Autographa californica multiple nucleopolyhedrovirus* or AcMNPV) with a mammalian expression cassette (Boyce and Bucher, 1996; Kost and Condreay, 1999), and have been successfully used for efficient gene delivery into several mammalian cell types (Airenne *et al.*, 2009; Ho *et al.*, 2005; Hofmann *et al.*, 1995; Hu, 2008; Huser and Hofmann, 2003; Kost and Condreay, 2002). BacMam provides an ideal solution for high efficiency and low toxicity labeling of primary cells and stem cells. Their circular double stranded DNA genome packed in a rod-shaped nucleocapsid can hold DNA fragments of approximately 40 kb, and are nonreplicating and minimally cytotoxic to mammalian cells (Kost and Condreay, 2002). Gp64 has been suggested to be the major glycoprotein on most of the traditional BacMam systems responsible for mediating transduction of mammalian cells (Oomens and Blissard, 1999). The expression of a gene delivered by

BacMam is transient and lasts for 4–5 days unless drug selection is applied on cells to select for rare random integration events (Condreay *et al.*, 1999; Huser and Hofmann, 2003; Merrihew *et al.*, 2001). Gene expression levels are largely dependent on cell type, promoter choice, and a relaxed chromatin state (Spenger *et al.*, 2004).

Several reports focus on improvised BacMam vectors for enhanced delivery and robust, prolonged expression. These include additional pseudotyping of viruses with vesicular stomatitis virus G (VSV-G) protein (Barsoum *et al.*, 1997; Kaneko *et al.*, 2006); inclusion of EBNA/Ori P elements (Shan *et al.*, 2006) from Epstein-Barr virus (EBV); woodchuck hepatitis post-transcriptional regulatory element (WPRE) (Mahonen *et al.*, 2007); and inverted terminal repeat sequences from adeno-associated virus (AAV-ITR) (Wang and Wang, 2005). Such improved baculovirus vectors with the VSV-G pseudotype and a WPRE element offer a promising means for robust expression in hard-to-transfect cell types (Zeng *et al.*, 2007). These features render the method particularly appealing for gene delivery into stem cells and progenitor cells (Ho, 2005; Ho *et al.*, 2006; Sarkis *et al.*, 2000; Zeng *et al.*,

2007; 2009), as well as primary cultured neurons. BacMam transduces mesenchymal stromal stem cells (MSCs) and progenitor cells derived from MSCs at high efficiency (Ho *et al.*, 2005), albeit with varied expression levels depending on the differentiation status and cell lineage (Ho *et al.*, 2006). Similarly, human neural primary cells have been efficiently transduced *in vitro* and direct injection into rat and mouse brains transduced neural cells *in vivo* (Sarkis *et al.*, 2000). Neurons derived from human embryonic stem cells transduced at high efficiency showed sustained expression *in vitro*, and on transplantation into mouse brain, maintained expression for several weeks *in vivo* (Zeng *et al.*, 2009). As pluripotent stem cells are derived from patient somatic cells via somatic reprogramming (Buganim *et al.*, 2013), optimal methods of gene delivery along discrete stages of differentiation and functional cell types are critical for downstream application of these cells for dissecting basic biology (Marchetto *et al.*, 2010), disease modeling (Colman and Dreesen, 2009), and in drug screening (Grskovic *et al.*, 2011). In this study, we use a BacMam GFP virus to identify high efficiency transduction conditions and their resulting short and

long-term expression patterns for undifferentiated and differentiated rat and human NSC besides rat primary hippocampal culture.

## MATERIALS AND METHODS

The materials procured from Thermo Fisher Scientific (Waltham, MA, USA) unless otherwise stated.

### Primary Neuronal Culture

Hippocampal rat cultures were prepared according to Calabrese and Halpain (2005) at a density of 300 cells/mm<sup>2</sup> and maintained in Neurobasal™ medium, supplemented with B27™ or with NeuroCult™ SM1 neuronal supplement (STEMCELL™ Technologies, Vancouver, Canada) and 0.5 mM L-glutamine (Sigma-Aldrich®, St. Louis, MO, USA).

### Neural Stem Cell Culture

Neurospheres, neural rosettes and NSC were generated from the NIH approved H9 (WA09) human embryonic stem cells (hESCs) using published protocols (Patrikoski *et al.*, 2013). Gibco™ H9-derived Human Neural Stem Cells (hNSCs) and Gibco™ Rat Fetal Neural Stem Cells (rNSCs) were propagated as adherent cultures on CELLstart™ coated

tissue culture dishes in complete StemPro™ NSC SFM media, comprised of KnockOut™ DMEM/F-12, supplemented with StemPro™ Neural Supplement, 2 mM GlutaMAX™-I, 20 ng/mL bFGF and 20 ng/mL EGF as per the manufacturer's instructions. hNSC and rNSC were passaged using StemPro™ Accutase™ cell disassociation reagent and continued to be expanded as described above for up to 5 passages.

Frozen NSCs were provided by Dr. Alysson Muotri, University of California San Diego, USA. NSCs were cultured and differentiated according to published protocols (Calabrese *et al*, 2014) at a density of  $1 \times 10^5$  cells per 500  $\mu$ l and maintained for several weeks in Neurobasal™ Electrophysiology Medium plus NeuroCult™ SM1 Neuronal supplement (STEMCELL® Technologies, Vancouver, Canada).

### Neural Stem Cell Differentiation

hNSCs and rNSCs were seeded on CELLstart™ coated dishes and incubated to 80–90% confluency in StemPro™ NSC SFM prior to initiation of differentiation. Differentiation was initiated by withdrawal of the bFGF and EGF cytokines in the culture medium.

Cells were maintained in the cytokine deprived medium for 12 days and stained to confirm mature neural phenotypes.

### BacMam

BacMam reagents used for viral transduction include BacMam 2.0 GFP Transduction control, CellLight™ Plasma Membrane-GFP, BacMam 2.0 and CellLight™ Synaptophysin-RFP, BacMam 2.0.

### BacMam Transduction

rNSCs were transduced with 1, 5, 10 and 20% (volume/volume) BacMam 2.0 GFP transduction control in complete StemPro™ NSC SFM. Transductions were performed for 60 min or overnight (16–18 h) followed by medium change. H9 NSC were transduced with 1, 2, 5, 10, and 20% (volume/volume) BacMam 2.0 GFP transduction control in complete StemPro™ NSC SFM. Transductions were performed for 60 min or overnight (16–18 h) followed by medium change.

H9 ESC derived neurospheres, adherent neural rosettes and resulting NSC were transduced with 20% (volume/volume) BacMam 2.0 GFP transduction control in complete StemPro™ NSC SFM overnight (16–18 h), followed by a media change.

Primary rat neurons and post-differentiated hNSCs were incubated for 4 h with 10–20  $\mu$ l of either CellLight™ Synaptophysin-RFP, CellLight™ plasma membrane GFP or BacMam GFP transduction control per well in a 24-well dish. The wells were rinsed once with freshly prepared media and replaced with conditioned media from cells grown for the same amount of time. Cells were fixed 16 h to 10 days post-infection.

### **Immunocytochemistry**

Neurons or NSCs were fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS) plus 120 mM sucrose for 20 minutes at 37°C. Cells were incubated in 20 mM glycine for 5 min, rinsed and permeabilized with 0.2% Triton X-100 for 5 min at room temperature, and then blocked for 30 min with 2% bovine serum albumin (BSA). Chicken anti-MAP2 antibody 1:1000 (LifeSpan Biosciences), rabbit anti-GFAP antibody 1:500 (Dako, Denmark), rabbit anti-MAP2 antibody 1:2000 (S. Halpain), rabbit anti-*nestin* (EMD Millipore) were incubated for 1 h at room temperature and rinsed with PBS, and incubated with Alexa Fluor™-conjugated secondary antibodies for 45 min at 37°C. Finally the

coverslips were washed twice with PBS and mounted using Aqua-Mount.

NSCs and differentiated cells were grown in 12-well CELLstart™ or Geltrex™ coated tissue culture dishes and labeled with BacMam GFP prior to fixation with 4% fresh Paraformaldehyde solution at room temperature for 15 min. The cells were washed with PBS, and incubated for 1 h at room temperature using a permeabilization agent-containing blocking buffer comprising 5% Normal Goat Serum, 1% BSA, and 0.1% Triton X-100 in PBS. Mouse anti-GFAP antibody 1:500 (EMD Millipore), mouse anti-MAP2 1:1000 and mouse anti-A2B5 1:200 were incubated in blocking buffer overnight (18–24 h), at 4°C. The unbound primary antibodies were washed three times using PBS and the cells were incubated with a goat anti-mouse secondary antibody conjugated to Alexa Fluor™ 594 at 1:500 in DPBS at room temperature for 1 h in the dark. Following three washes with PBS, the cells were treated with a final wash with PBS containing 1  $\mu$ g/mL DAPI. Images were collected using a Zeiss Axiovert and processed using Adobe Photoshop CS.

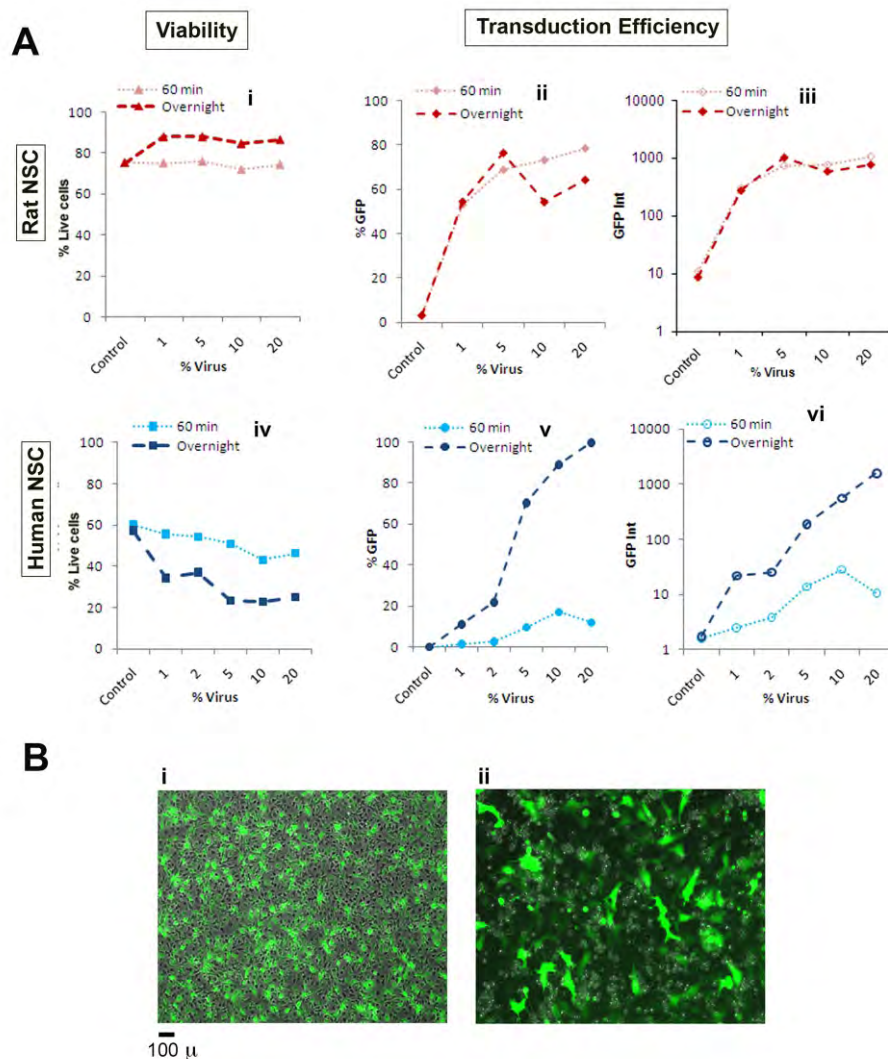
## RESULTS

Neural cells derived from pluripotent stem cells offer an appealing tool as a physiologically relevant cell model system for dissecting disease phenotypes and for use in drug screening. Efficient delivery of genes into these cells enables the creation of assay-ready cells for downstream applications. Several methods of gene delivery have been reported for neural cell types and BacMam has shown promise for efficient delivery. Here, we have optimized a protocol for efficient BacMam delivery into rat and human cells and utilized the protocol to transduce various NSC preparations during initial generation, after propagation and culturing, and at stages of differentiation.

### Optimal Conditions for Transduction of Rat and Human NSC

BacMam 2.0 GFP virus was utilized to optimize the virus concentration and exposure time for primary rNSC and ESC-derived hNSC. Transduction efficiency was assessed at 24 h post transduction via flow cytometry to determine the percentage of GFP positive cells and signal intensity. Viability post transduction was evaluated based on Trypan Blue exclusion. Distinct

differences in the viability and transduction efficiency were observed between rNSC and hNSC (Fig. 1). The viability of rNSC did not change significantly with the amount of time of incubation with the BacMam virus, between 1 h incubation (Fig. 1Ai, dotted line) versus overnight incubation (Fig. 1Ai, dashed line). In addition, the viability of cells also did not alter across virus concentrations with greater than 70% of the cells viable post transduction and comparable to untransduced control cells (Fig. 1Ai). In contrast, hNSC had a relatively lower basal viability of 60% with marked difference in viability of cells transduced with BacMam for 1 h (Fig. 1Aiv, dotted line) versus overnight (Fig. 1Aiv, dashed line). In addition, a further decrease in viability was observed with higher concentrations (< 5% v/v) of virus with overnight virus incubation (Fig. 1Aiv, dashed line). Further examination of transduction efficiency indicated that the rNSC percentage of GFP positive cells (Fig. 1Aii) and GFP intensity (Fig. 1Aiii) reached a saturating level at 5% v/v virus and higher viral concentrations did not improve efficiency any further. Also, no significant advantage was noticed with longer overnight incubation (dashed line)



**Figure 1: Optimal conditions for efficient transduction of NSC**

- (A) Concentration and length of exposure for maximum viability and transduction for rat NSC (i-iii) and human NSC (iv-vi) was determined following incubation of cells with GFP BacMam for 60 minutes (dotted line) or overnight (dashed line). NSCs exposed to different concentrations (v/v) of GFP BacMam were harvested after 24 hours and cells were analyzed by flow cytometry to determine viability of cells based on forward and side scatter, percent transduction based on %GFP positive cells and intensity of GFP expression in the transduced cells. Rat NSC showed no significant toxicity with BacMam virus treatment between 1-20% v/v virus either at 60 minutes or with overnight incubation of cells with the virus (i). Greater than 50% of the cells were transduced both with 60 minutes and overnight incubation of cells with 1% v/v virus with increase in percentage of GFP cells up to 80% with increasing virus concentrations (ii). The intensity of GFP in the transduced cells did not significantly increase from 5% virus to 20% treated cells for both 60 minutes and overnight incubation conditions (iii). In contrast human NSC incubated with varying concentration of BacMam showed more sensitivity to the presence of virus with 60 minutes of incubation relatively better for cell viability than overnight incubation (iv). The surviving cells however showed a linear increase in %GFP positive cells with increasing concentrations of the virus with overnight incubation resulting in higher percentages of GFP positive cells than cells transduced overnight (v). The intensity of GFP also showed a corresponding higher GFP intensities in cells treated with higher percentage virus overnight (vi).
- (B) Optimal conditions determined for rat NSC transduction (i) was 60 minutes incubation with 20% virus, and overnight incubation with 5-10% v/v virus for human NSC (ii) with the virus added directly added to adherent cells cultured in StemPro NSC media.

compared to 1 h incubation (dotted line).

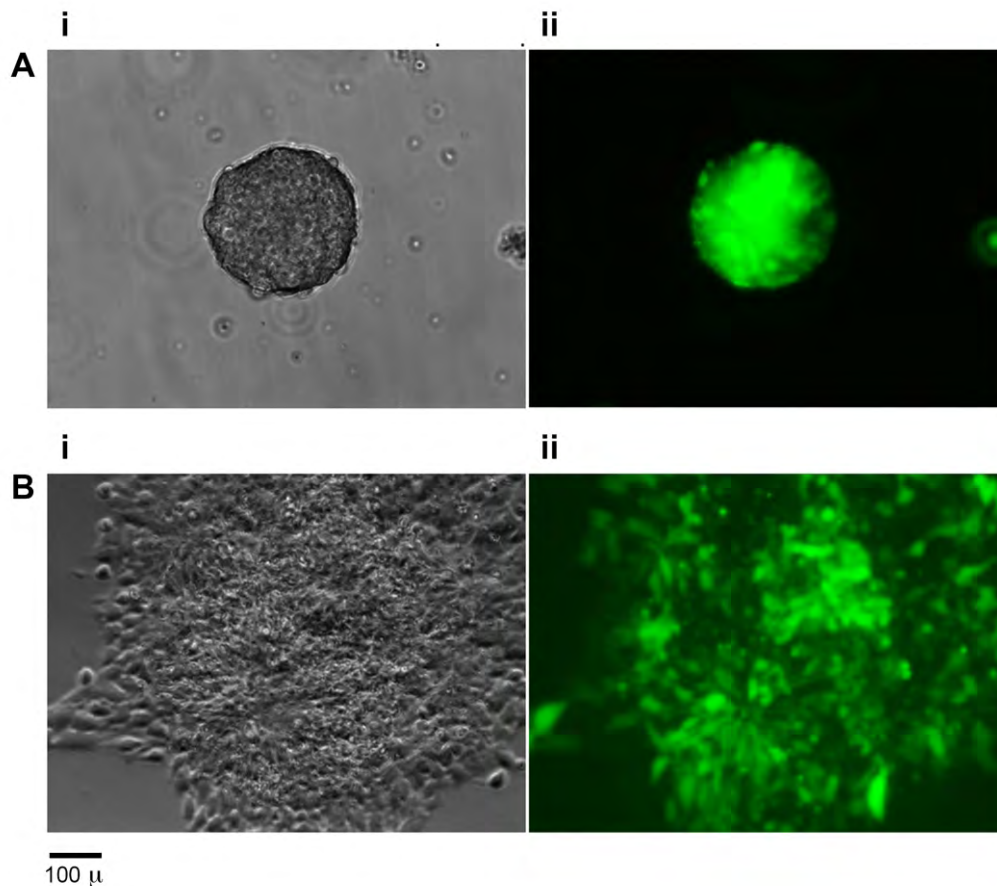
In the case of hNSC, a significant increase in the percentage of GFP positive cells (Fig. 1Av) and GFP intensity (Fig. 1Avi) was observed with increasing viral concentrations and with overnight incubation. Since the viability of cells declines at higher viral concentration ( $> 5\%$  v/v) and overnight incubation, the optimal condition for hNSC is greater than  $5\%$  v/v virus. Incubations for longer periods ranging to several hours can however be used to avoid overnight incubation that impacts cell viability.

Consistent with the observation, optimal transduction conditions for rNSC was short-term incubation ( $\leq 60$  min) with  $\leq 10\%$  v/v virus in culture medium; and for hNSC, long term ( $\geq 60$  min) incubation with  $\geq 10\%$  v/v virus. Incubation period for hNSC can be reduced to a few hours to avoid higher impact on cell viability observed with overnight incubation. Fig. 1B shows representative images of robust expression of GFP in rNSC (Fig. 1Bi) and hNSC (Fig. 1Bii) both transduced with BacMam 2.0 GFP under the above-defined optimal conditions.

### **Transduction and Expression during Neural Induction of Pluripotent NSC**

Several specific differentiation methods exist to direct pluripotent stem cell types, embryonic stem cells, and induced pluripotent stem cells toward neural cell types. Traditional methods involve the formation of neurosphere followed by adherence to poly-L-lysine coated surface to generate neural rosettes. BacMam transduction of these three-dimensional differentiation intermediates was examined based on the optimized protocol above. H9 ESC induced to form neurospheres for 10 days was transduced overnight with  $20\%$  v/v BacMam 2.0 GFP and GFP expression was monitored at 24 h post transduction. The majority of neurospheres with a typical three-dimensional structure as viewed under phase contrast (Fig. 2Ai) showed robust expression of GFP (Fig. 2Aii). To determine the overall proportion of cells within the neurosphere expressing GFP, neurospheres were plated on poly-L-lysine and 24 h later the pattern of GFP expression was assessed. Phase contrast images of the adherent cells showed distinct areas with neural rosette formation (Fig. 2Bi). The majority of the





**Figure 2: Transduction of ESC-derived neuronal cells.**

Adherent H9 hESC were treated with collagenase to create embryoid bodies and cultured in NSC inducing media to achieve neurosphere differentiation.

- (A) Phase contrast (i) and GFP expression (ii) of H9 ESC differentiated into neurospheres and transduced with BacMam 2.0-GFP and imaged at 24 hours post transduction.
- (B) The neurosphere were plated down on Poly-L-Lysine coated surface and 24 hours later attached cells observed cells under phase (i) FITC fluorescence channel (ii).

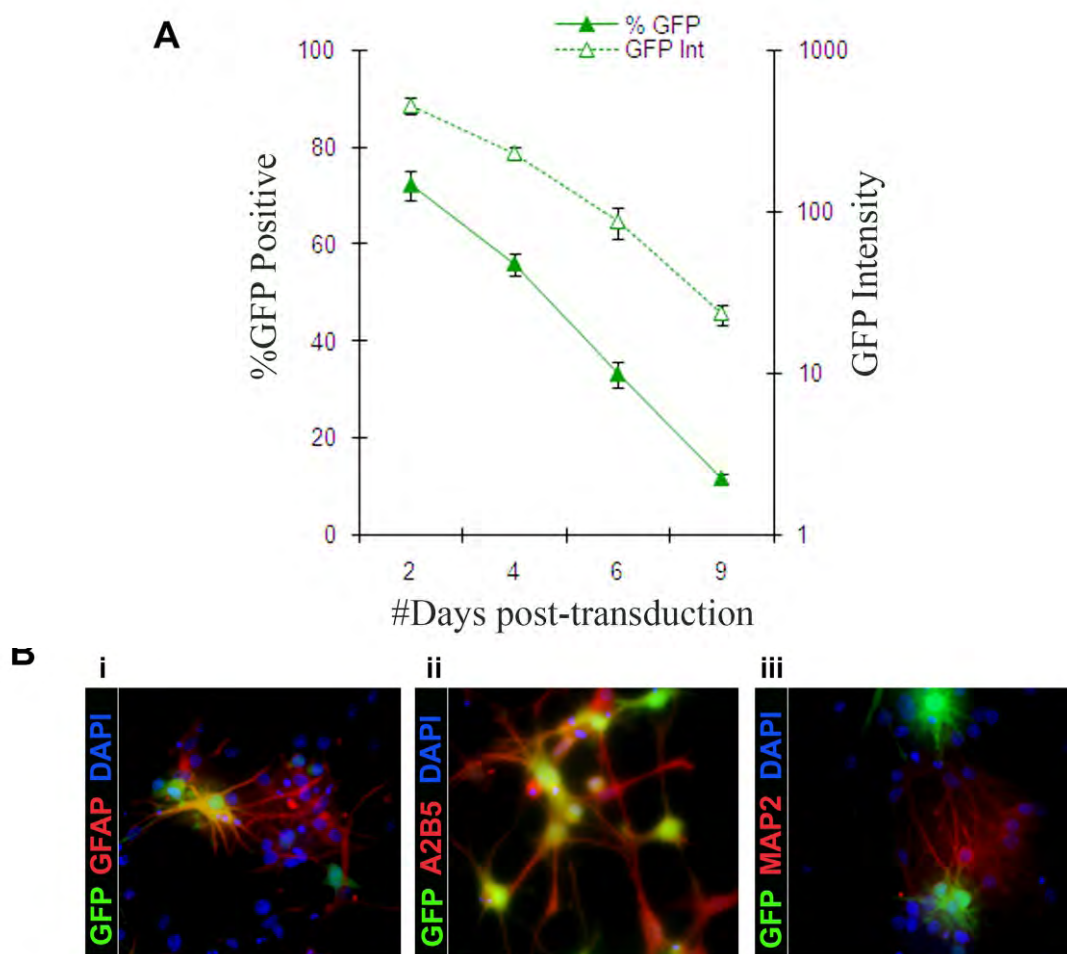
cells showed GFP expression albeit with varying intensities and distinct neural rosette structures showed robust GFP expression (Fig. 2Bii).

#### **Duration and Persistence of Expression in Transduced Undifferentiated NSC**

Transduced hNSC was further maintained in culture, without passage, to

determine the duration of BacMam-driven expression. As NSCs continued to divide, the percentage of GFP positive cells linearly dropped from greater than 70% to about 10% in 9 days (Fig. 3A, solid line). A concomitant linear decrease in GFP intensity (Fig. 3A, dotted line) was also observed.

To examine if the GFP signal persisted through differentiation, NSC transduced



**Figure 3: Length of expression in transduced rat SC and with differentiation and efficiency of transduction in differentiated NSC.**

- (A) Human NSC transduced overnight with 10%v/v BacMam 2.0 GFP, in replicates, were monitored for persistence of GFP expression. Percent GFP expression (Solid line) and GFP intensity (dotted line) was measured via flow cytometry at 2, 4, 6 and 9- days post transduction.
- (B) NSC transduced with GFP-BacMam allowed to spontaneously differentiate for 6 days by withdrawal of cytokines and stained with (i) GFAP, (ii) A2B5, and (iii) MAP2, lineage specific markers for astrocyte, oligodendrocyte, and neurons, respectively. Green: GFP, Blue: DAPI, Red: Lineage specific primary antibody and Alexa Fluor® 594 secondary antibody.

in the undifferentiated condition were spontaneously differentiated via withdrawal of growth factors and, at the end of 7 days, cells were stained with antibodies used for detecting specific neural lineages: oligodendrocytic marker A2B5, astrocytic marker GFAP and neuronal marker MAP2 (Fig. 3B, red).

Results indicated a high degree of co-localization of GFP with GFAP (Fig 3Bi) and A2B5 (Fig 3Bii). In contrast MAP2 positive cells did not appear to overlap with GFP expression, although several GFP positive cells were observed surrounding areas that were immunopositive for MAP2 (Fig. 3Biii).

This lack of co-localization between MAP2 positive cells and GFP suggested poor BacMam-mediated expression of the transgene in neurons

These results indicated that sustained expression of a transgene may be suppressed in neurons, while astrocytes and oligodendrocytes displayed robust expression that persisted for several days.

#### ***Transduction and expression in neurons differentiating from NSC***

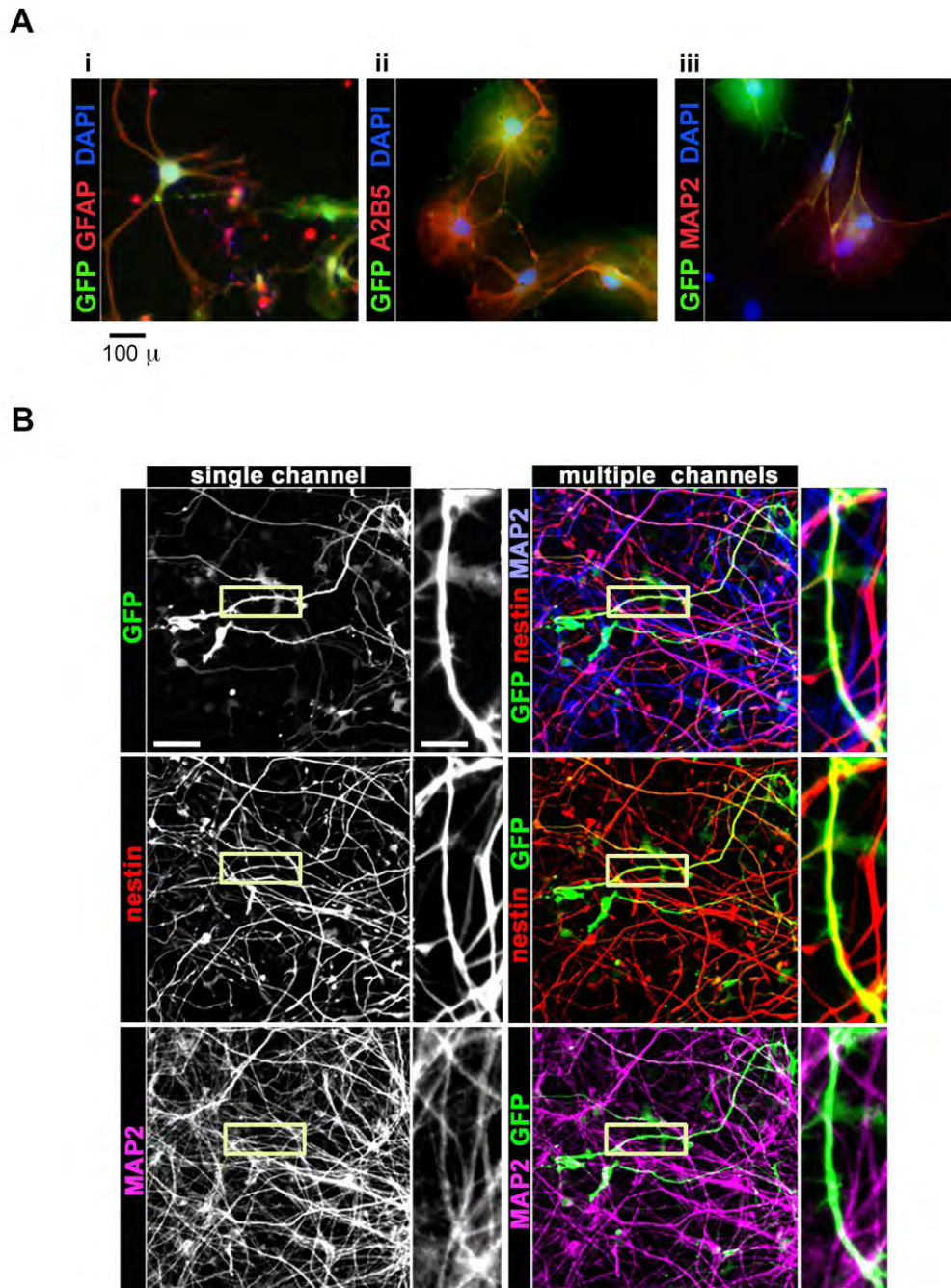
We tested whether we could directly transduce NSC previously differentiated for 7 days, using BacMam 2.0 GFP virus, under similar conditions as for transduction of undifferentiated NSC. The majority of the differentiated cells showed expression of GFP and robust localization was observed with GFAP (Fig. 4Ai) and A2B5 (Fig. 4Aii), but again MAP2-positive cells showed very weak or no expression of GFP.

NSC differentiated for 12 days were infected with BacMam 2.0 GFP and expression of GFP was monitored 4 days post transduction. Multichannel detection of GFP and immunoreactivity for nestin (a marker of undifferentiated NSC) and MAP2 (a marker of differentiated neurons) (Fig. 4B, left panels) indicated that while the majority of the cells were

positive for one or both neural markers (the presence of both markers likely indicates cells in transition to becoming neurons), only a small portion of cells were GFP positive. This indicated that BacMam typically transfected only a small fraction of neural cells in the differentiation condition. Further examination of the co-localization of GFP with nestin and MAP2 showed that GFP positive cells were positive for nestin but MAP2 staining was also faintly detected in a portion of dendrite regions (Fig. 4B, right panels).

#### ***Differential targeting in younger versus older rat hippocampal cultures***

To further examine transduction of neurons in heterogeneous rat hippocampal cultures, BacMam 2.0 GFP was used to transduce short term and long-term cultures. Young primary rat hippocampal neuronal cultures (6-14 days *in vitro*) were infected at 11 DIV with BacMam 2.0, to express GFP prior to fixation and immunostaining at 21 DIV. GFP expression was detectable and persisted for at least 10 days, both in astrocytes and neurons in culture. Fig. 5A shows GFP transduced MAP2-positive neurons (Fig. 5A, red asterisks) with neighboring untransduced GFAP-positive



**Figure 4: GFP-BacMam transduction of differentiated NSCs.**

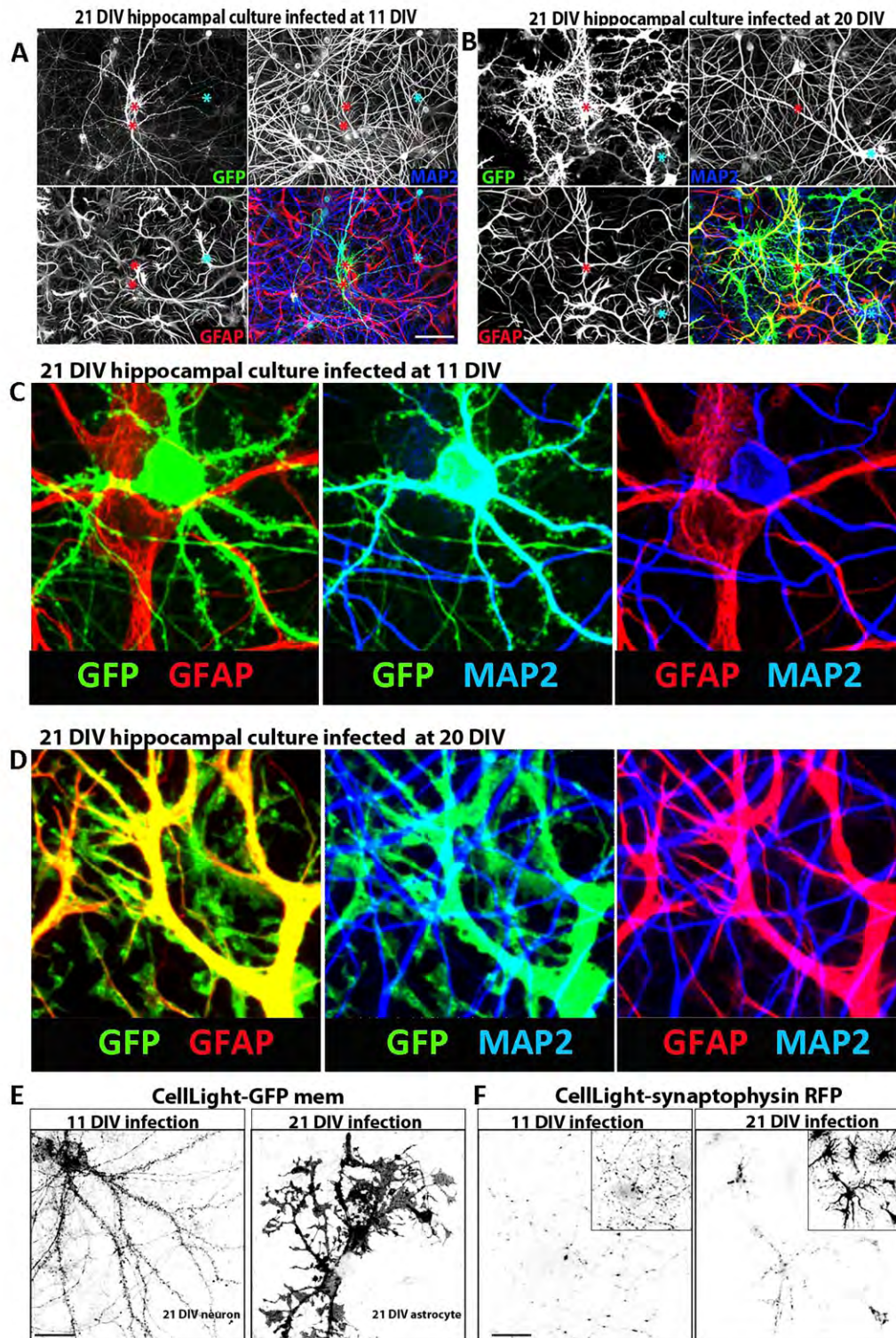
(A) NSC spontaneously differentiated for 6 days and then transduced overnight with 10% v/v BacMam 2.0 GFP. At day 7 the transduced cells were stained with astrocyte marker GFAP (i), oligodendrocyte marker A2B5 (ii) and neuronal marker MAP2 (iii).

Green: GFP, Blue: DAPI, Red: Lineage specific primary antibody and Alexa Fluor® 594 secondary antibody

(B) NSCs differentiated into neurons for 12 days and transduced with BacMam 2.0 GFP and visualized 4 days post transduction. Scale bar: 36  $\mu\text{m}$ . Inserts shown to the right of each panel are enlarged from the boxed region (yellow rectangle). Scale bar: 9  $\mu\text{m}$ .

*Left Panel:* GFP, Nestin and MAP2 channels individually shown for the same field of view.

*Right Panel:* Merged images of the GFP, Nestin and MAP2 channels to better distinguish the identity of the infected cell types.



**Figure 5: BacMam targets both astrocytes and neurons in younger rat hippocampal cultures but almost exclusively astrocytes in older cultures and is independent of the DNA expressed.**

Early primary rat hippocampal cultures (6-10 days in vitro, 11 DIV) and later stages of maturation (20 DIV) transduced with BacMam 2.0 GFP.

- A) Selected field of a hippocampal culture infected at 11 DIV with BacMam 2.0 to express GFP prior to fixation and immunostaining at 21 DIV. Cell identity was assessed by double labeling for the neuron-specific marker MAP2

- (*blue*) and the astrocytic-specific marker glial fibrillar acidic protein (GFAP) (*red*). The *red asterisks* indicate infected neurons; the *cyan asterisk* indicates a neighboring uninfected astrocyte. Scale bar: 62  $\mu$ M.
- B) Selected field of a hippocampal culture infected at 20 DIV with BacMam 2.0 to express GFP prior to fixation and immunostaining at 21 DIV. Cells were fixed and immunolabeled as in **A**. Note that many astrocytes in the field are GFP-positive, but no neurons, which was typical when infection was carried out in such older cultures. The *red asterisk* indicates an infected astrocyte; the *cyan asterisk* indicates a neighboring uninfected neuron.
- C) Higher magnification of a GFP-expressing spiny glutamatergic neuron with the Green GFP signal colocalizing with the neuronal specific markers MAP2 (*in blue*). Image width: 75  $\mu$ M
- D) Higher magnification of a GFP-expressing astrocyte and the astrocytic specific marker GFAP (*in red*) Image width: 75  $\mu$ M
- E) Membrane targeted CellLight™ GFP plasma membrane infected at 11 DIV and at 21 DIV. Scale bars: 20  $\mu$ M
- F) Cell Light™ Synaptophysin RFP infected at 11 DIV and at 21 DIV. The Same images are shown with a more saturated display level (*insets*). Scale bars: 20  $\mu$ M.

astrocyte (Fig. 5A, cyan asterisk). In contrast, infection at later stages of maturation (20 DIV) resulted in little or no detectable transgene expression in identified neurons, but numerous transgene-positive astrocytes. Using infection at 20 DIV, 80–90% of astrocytes were infected, but no neurons expressed GFP. In contrast, infection at 10 DIV, about 5% of astrocytes and 10–20% of neurons were infected. Fig. 5B shows many GFP transduced GFAP-positive astrocytes (red asterisks) but no GFP expression in the neighboring MAP2-positive neurons (cyan asterisk). Higher magnification images further illustrate the distinct morphological details of a GFP-expressing spiny glutamatergic neuron that is positive for the neuronal specific marker MAP2 (Fig. 5C) and of a GFP-expressing astrocyte with expression of the astrocytic specific marker GFAP (Fig. 5D). This cell type-

specific targeting and expression was confirmed using two additional targets delivered via BacMam, membrane targeted GFP (CellLight-GFP mem, Fig. 5E) and CellLight-synaptophysin RFP (Fig. 5F). In both the cases, expression was seen in neurons or just neuronal presynaptic boutons along axons when the cells were infected at 11 days in vitro (DIV). In contrast, at 21 DIV, both synaptophysin RFP and membrane targeted GFP are expressed within primary rat hippocampal astrocytes. This is more obvious when the same images are shown with a more saturated display level with inverted gray scale versions of the fluorescent channels for easier viewing (Fig. 5F, *see insets*). In summary, we observed that primary heterogeneous cultures are differentially transduced depending on the age of the cultures, with early cultures showing GFP expression in both neural and

astrocytic cells, while expression in late cultures was primarily limited to astrocytic cells.

## DISCUSSION

Traditional non-viral and viral delivery methods of gene delivery into neurons that are post mitotic have represented a major challenge. Although varying delivery efficiencies can be achieved, toxicity to cells remains an issue (Davidson and Breakefield, 2003; Slack and Miller, 1996; Washbourne and McAllister, 2002). There have been several studies reporting the use of BacMam for transduction of neural cells (Li *et al.*, 2005; Sarkis *et al.*, 2000). High efficiencies of transduction were reported in two neuroblastoma cell lines and three human primary neural cultures. Transduction in the presence of the histone deacetylase inhibitor, Butyrate, was required for optimal transduction efficiency (Sarkis *et al.*, 2000). Newer generation pseudotyped BacMam with VSV-G are known to transduce a wider variety of mammalian cells (Barsoum *et al.*, 1997) and has enabled *in vitro* and *in vivo* delivery of genes into mammalian astrocytes and oligodendrocytes (Kobayashi *et al.*, 2006). Inclusion of

WPRE has also been demonstrated to increase the length of transgene expression in ESC-derived neurons (Zeng *et al.*, 2009). In this study utilizing a BacMam 2.0 GFP virus that includes both these elements, we carried out a systematic approach to optimize transduction conditions for rat and human NSC. Rat NSC were more tolerant to higher viral doses and longer incubation with virus compared to human cells. Human NSC required higher viral concentration and longer incubation times for optimal transduction, although these conditions resulted in decreased cell viability. Reducing the incubation time from overnight to a range of 3–6 h alleviated these effects. The optimal method for BacMam transduction of rat and human cells hence offers a guideline on transducing neural subtypes and intermediates.

Different optimal methods for rat and human cells were used to transduce NSC during derivation, expansion and differentiation to monitor the pattern and persistence of expression in the various neural cell subtypes. Differentiation of GFP transduced NSC and transduction of differentiated NSC both indicated higher

colocalization of GFP expression with oligodendrocytic and astrocytic markers, than with neuronal markers. This is indicative of suppression of transgene expression in neurons.

In addition, preferential BacMam transduction of astrocytes in long-term rat hippocampal cultures and neurons in younger cultures was observed, however the molecular phenomenon behind this is unclear. Preferential labeling of astrocytes in the older cultures is not due to a decrease in the ratio of neurons to astrocytes over time in culture, since the fraction of neurons does not change significantly between 10 DIV and 24 DIV (fraction of neurons at 10 DIV =  $39 \pm 1.5\%$ ; fraction of neurons at 24 DIV =  $37 \pm 2.5\%$ ). Moreover, the average surface area occupied by astrocytes and neurons per field was not significantly different between 10 DIV and 21 DIV (10 DIV GFAP area/21 DIV GFAP area = 0.86; 10DIVMAP2 area/21DIV MAP2 area = 0.88). It therefore seems more likely that endosome mediated uptake of virus may increase over time in the actively dividing astrocytes, and decrease over time in the post-mitotic, maturing neurons. Evidence suggests that phagocytic activity increases with

astrocyte differentiation, affecting astrocytic response to viral infection (Iacono and Berria, 1999). In addition, over time the pH of conditioned media in which the cells grow becomes more acidic (Calabrese, unpublished observations). The efficiency of Gp64 mediated viral tropism reportedly improved at lower pH ( $\text{pH} \leq 5$ ) in insect cells (Blissard and Wenz, 1992). These factors may explain why the efficiency of infection reaches nearly 100% for the astroglial population, but fails to fully explain why neurons appear to lose their infectability over time in culture. Macropinocytic activity might decrease in neurons as they mature. Further optimization of culture conditions may potentially overcome this deficiency for older neurons. Nevertheless, the preferential transduction of astrocytic culture in mature long-term cultures may be harnessed to either selectively enrich or deplete astrocytes in heterogeneous cultures of neurons and astrocytes.

The use of pluripotent stem cell-derived neural stem cells for functional screening assays is promising for use in drug discovery for neurodegenerative diseases. The current challenge in generating homogeneous population of



cells in large quantities is being overcome with detailed protocols with confirmed gene expression patterns that can be labeled with live stains for further downstream viability and functional analysis (Efthymiou *et al.*, 2013). Besides the ability to utilize BacMam for delivery of varied gene content into neural cell subtypes provides a powerful tool for overexpression or knockdown of genes along different developmental stages.

## REFERENCES

- Airenne KJ, Laitinen OH, Mahonen AJ, Yla-Herttuala S. Transduction of vertebrate cells with recombinant baculovirus. *Cold Spring Harbor protocols*. 2009;2009:pdb prot5182.
- Barsoum J, Brown R, McKee M, Boyce FM. Efficient transduction of mammalian cells by a recombinant baculovirus having the vesicular stomatitis virus G glycoprotein. *Human Gene Ther* 1997;8:2011–2018.
- Blissard GW, Wenz JR. Baculovirus gp64 envelope glycoprotein is sufficient to mediate pH-dependent membrane fusion. *J Virol* 1992; 66:6829–6835.
- Boyce FM, Bucher NL. Baculovirus-mediated gene transfer into mammalian cells. *Proc Natl Acad Sci USA* 1996;93:2348–2352.
- Buganim Y, Faddah DA, Jaenisch R. Mechanisms and models of somatic cell reprogramming. *Nat Rev Genet* 2013;14:427–439.
- Calabrese B, Halpain S. Essential role for the PKC target MARCKS in maintaining dendritic spine morphology. *Neuron* 2005;48: 77–90.
- Calabrese B, Dehmelt L, Halpain S. Morphometric assay for neural differentiation and synaptic development of human iPSC-derived NSC. *In: Vemuri MC, ed. Stem Cell Assays* 2014; John Wiley and Sons, Ch. 21.
- Colman A, Dreesen O. Pluripotent stem cells and disease modeling. *Cell Stem Cell* 2009;5:244–247.
- Condrey JP, Witherspoon SM, Clay WC, Kost TA. Transient and stable gene expression in mammalian cells transduced with a recombinant baculovirus vector. *Proc Natl Acad Sci USA* 1999;96:127–132.
- Davidson BL, Breakefield XO. Viral vectors for gene delivery to the nervous system. *Nat Rev Neurosci* 2003;4:353–364.
- Efthymiou A, Shaltouki A, Steiner JP, Jha B, Heman-Ackah SM, Swistowski A, *et al.* Functional screening assays with neurons

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## CONFLICT OF INTEREST

The authors claim no conflict of interest.

- generated from pluripotent stem cell-derived neural stem cells. *J Biomol Screen* 2013; 1087057113501869.
- Fontes A, Quintanilla R, Lakshmipathy U. Novel platforms to create labeled stem cells. In: Gholamrezanezhad A, ed. *Stem Cells in Clinic and Research* 2011; InTech pp. 791–804.
- Grskovic M, Javaherian A, Strulovici B, Daley GQ. Induced pluripotent stem cells – opportunities for disease modelling and drug discovery. *Nat Rev Drug Discov* 2011;10: 915–929.
- Ho YC, Chung YC, Hwang SM, Wang KC, Hu YC. Transgene expression and differentiation of baculovirus-transduced human mesenchymal stem cells. *J Gene Med* 2005;7: 860–868.
- Hofmann C, Sandig V, Jennings G, Rudolph M, Schlag P, Strauss M. Efficient gene transfer into human hepatocytes by baculovirus vectors. *Proc Natl Acad Sci USA* 1995;92: 10099–10103.
- Hu YC. Baculoviral vectors for gene delivery: a review. *Curr Gene Ther* 2008;8:54–65.
- Ho YC, Lee HP, Hwang SM, Lo WH, Chen HC, Chung CK, Hu YC. Baculovirus transduction of human mesenchymal stem cell-derived progenitor cells: variation of transgene expression with cellular differentiation states. *Gene Ther* 2006;13:1471–1479.
- Huser A, Hofmann C. Baculovirus vectors: novel mammalian cell gene-delivery vehicles and their applications. *Am J Pharmacogenomics* 2003;3:53–63.
- Kaneko H, Suzuki H, Abe T, Miyano-Kurosaki N, Takaku H. Inhibition of HIV-1 replication by vesicular stomatitis virus envelope glycoprotein pseudotyped baculovirus vector-transduced ribozyme in mammalian cells. *Biochem Biophys Res Commun* 2006;349: 1220–1227.
- Kobayashi H, Watabe K, Isuka S, Tani H, Matsuura Y, Barsoum J, *et al.* Successful transduction of mammalian astrocytes and olifodendrocytes by "pseudotyped" baculovirus vector *in vitro* and *in vivo*. *Jikeikai Med J* 2006;53:55–62.
- Kost TA, Condreay JP. Recombinant baculoviruses as expression vectors for insect and mammalian cells. *Curr Opin Biotechnol* 1999;10:428–433.
- Kost TA, Condreay JP. Recombinant baculoviruses as mammalian cell gene-delivery vectors. *Trends Biotechnol* 2002;20: 173–180.
- Li Y, Yang Y, Wang S. Neuronal gene transfer by baculovirus-derived vectors accommodating a neurone-specific promoter. *Exp Physiol* 2005; 90:39–44.
- Oomens AG, Blissard GW. Requirement for GP64 to drive efficient budding of Autographa californica multicapsid nucleopolyhedrovirus. *Virology* 1999;254:297–314.
- Mahonen AJ, Airene KJ, Puroola S, Peltomaa E, Kaikkonen MU, Riekkinen MS, *et al.* Post-transcriptional regulatory element boosts baculovirus-mediated gene expression in vertebrate cells. *J Biotechnol* 2007;131:1–8.
- Marchetto MC, Winner B, Gage FH. Pluripotent stem cells in neurodegenerative and neurodevelopmental diseases. *Human Mol Genet* 2010;19:R71–76.
- Merrihew RV, Clay WC, Condreay JP, Witherspoon SM, Dallas WS, Kost TA, *et al.*

- Chromosomal integration of transduced recombinant baculovirus DNA in mammalian cells. *J Virol* 2001;75:903–909.
- Patrikoski M, Juntunen M, Boucher S, Campbell A, Vemuri MC, Mannerstrom B, Miettinen S. Development of fully defined xeno-free culture system for the preparation and propagation of cell therapy-compliant human adipose stem cells. *Stem Cell Res Ther* 2013; 4:27.
- Sarkis C, Serguera C, Petres S, Buchet D, Ridet JL, Edelman L, Mallet J. Efficient transduction of neural cells in vitro and in vivo by a baculovirus-derived vector. *Proc Natl Acad Sci USA* 2000;97:14638–14643.
- Shan L, Wang L, Yin J, Zhong P, Zhong J. An OriP/EBNA-1-based baculovirus vector with prolonged and enhanced transgene expression. *J Gene Med* 2006;8:1400–1406.
- Slack RS, Miller FD. Viral vectors for modulating gene expression in neurons. *Curr Opin Neurobiol* 1996;6:576–583.
- Spenger A, Ernst W, Condreay JP, Kost TA, Grabherr R. Influence of promoter choice and trichostatin A treatment on expression of baculovirus delivered genes in mammalian cells. *Protein Expr Purif* 2004;38:17–23.
- Washbourne P, McAllister AK. Techniques for gene transfer into neurons. *Curr Opin Neurobiol* 2002;12:566–573.
- Wang CY, Wang S. Adeno-associated virus inverted terminal repeats improve neuronal transgene expression mediated by baculoviral vectors in rat brain. *Human Gene Ther* 2005; 16:1219–1226.
- Zeng J, Du J, Lin J, Bak XY, Wu C, Wang S. High-efficiency transient transduction of human embryonic stem cell-derived neurons with baculoviral vectors. *Mol Ther* 2009;17: 1585–1593.
- Zeng J, Du J, Zhao Y, Palanisamy N, Wang S. Baculoviral vector-mediated transient and stable transgene expression in human embryonic stem cells. *Stem Cells* 2007;25: 1055–1061.