Introduction

Mesenchymal stem cells (MSCs) are multipotent progenitors which can undergo self-renewal and give rise to multiple lineages, including osteogenic, chondrogenic, and adipogenic lineages [1-5]. While MSCs have been isolated from numerous tissues, one of the major sources in adults is the bone marrow stromal cells [4]. Several major signaling pathways, including BMPs and Wnts, play an important role in regulating MSC proliferation and lineage-specific commitments [3,6-10]. Nonetheless, molecular mechanisms governing MSC proliferation and differentiation remain to be thoroughly elucidated.

As for any sources of progenitor cells, genetic manipulations (such as transgene overexpression and/or RNAi-mediated gene expression silencing) in MSCs would require effective gene deliveries. In the case of MSCs, a great deal of attentions have been paid to their potential use in regenerative medicine, where potential therapeutic genes can be introduced into MSCs for biomaterial/tissue engineering. For example, we have found that BMP9 is one of the most potent BMPs among the 14 types of BMPs in inducing osteogenic differentiation of MSCs by...
regulating several important downstream targets [6,11–18]. It is conceivable that BMP9 can be introduced into mesenchymal progenitor cells in vivo or ex vivo for bone regeneration to treat fracture non-union and/or to facilitate spine fusion [6,19,20].

Recombinant adenoviruses are one of the most commonly-used gene transfer vehicles because they can transduce a wide variety of cells and/or tissues with relatively high efficiency [21–24]. Adenovirus infection is mediated by the coxsackievirus-adenovirus receptor (CAR) via the knob domain of the fiber protein and the major histocompatibility complex (MHC) class Iα-2 domain at the host cell surface [21–23]. After the initial attachment of the virus, the penton base interacts with αvβ3 and αvβ5 integrins, leading to internalization of the virus via receptor-mediated endocytosis [21–23]. One of the major limitations of adenoviruses is that the sensitivity of target cells to adenoviral infection correlates with cellular CAR expression. Depending on the CAR receptor levels and other cofactors, adenovirus infection efficiency varies drastically among cell lines [22,23,26]. We have found that although MSCs can be infected in vitro by adenoviruses, high virus titers have to be used to achieve high efficiency in these progenitor cells [11,12].

In this study, we investigate if the cationic polymer Polybrene can be used to enhance or potentiate adenovirus-mediated transgene delivery into MSCs, such as C2C12 cells and iMEFs. Polybrene is widely used to promote the efficiency of recombinant retrovirus or lentivirus infection [27–30]. Using the AdRFP adenovirus, we find that the AdRFP transduction efficiency is significantly increased by Polybrene in a dose-dependent fashion with a peak at 8 μg/ml in both C2C12 and iMEFs cells. Quantitative luciferase assay reveals that Polybrene significantly enhances AdFLuc-mediated luciferase activity in C2C12 and iMEFs cells at as low as 4 μg/ml and 2 μg/ml, respectively. FACS analysis indicates that Polybrene (4 μg/ml) increases the percentage of RFP-positive cells by approximately 430 folds in AdRFP-transduced iMEFs. Furthermore, we demonstrate that Polybrene can enhance AdBMP9-induced osteogenic differentiation of mesenchymal stem cells as the early osteogenic marker alkaline phosphatase (ALP) activity can be significantly increased more than 73 folds by Polybrene (4 μg/ml) in AdBMP9-transduced iMEFs cells. Cytotoxicity analysis indicates that most C2C12 and iMEFs cells are viable at the Polybrene concentrations up to 40 μg/ml, which is about 10-fold higher than the effective concentration (i.e., 4 μg/ml) required to enhance the recombinant adenovirus’ transduction efficiency in mesenchymal progenitor cells. Therefore, our results strongly suggest that Polybrene may be used as a safe, effective and inexpensive enhancer to augment adenovirus-mediated gene transfers in MSCs.

Materials and Methods

Cell culture and chemicals

Mouse preosteoblastic progenitor C2C12 cells were purchased from ATCC (Manassas, VA). The iMEFs were the immortalized mouse embryonic fibroblasts as previously described [31]. The cell lines were maintained in completed DMEM as described [32–35]. Polybrene (hexadimethrine bromide) was obtained from Sigma-Aldrich (St. Louis, MO). Unless indicated otherwise, all chemicals were at the time of infection. At 48 h post infection RFP expression was recorded under a fluorescence microscope with the same exposure time and sensitivity. Each infection/treatment condition was done in duplicate. Representative results are shown.
were purchased from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA).

Construction and generation of recombinant adenovirus
expressing RFP, FLuc, or BMP9

Recombinant adenoviruses were generated using the AdEasy
technology as described [11,12,36–38]. The coding regions of
monomeric red fluorescent protein (RFP), firefly luciferase (FLuc),
and human BMP9 were PCR amplified and cloned into
adenoviral shuttle vectors, which were subsequently used to
generate recombinant adenoviruses in HEK-293 cells as described
[36,38]. The amplified adenoviruses were titrated and stored at
–80°C.

Crystal violet cell proliferation assay

Crystal violet stain assay was carried out as previously described
[34,39–41]. Briefly, subconfluent C2C12 and iMEFs cells were
treated with different concentrations of Polybrene. At 4 days after
treatment, the cells were fixed and subjected to Crystal violet
staining. For the quantitative analysis, the stained cells were
dissolved in 10% acetic acid and subjected to the measurement of
absorbance at 570–590 nm [40,41]. Each assay condition was
done in triplicate.

Flow cytometry analysis

Subconfluent C2C12 and iMEFs cells were infected with
AdRFP and treated with different concentrations of Polybrene.
At 48 h post infection the cells were collected, washed with PBS,
and subjected to FACS analysis of RFP-positive cells using the BD
LSR II Flow Cytometer and the FlowJo software. Uninfected
cells were used as negative controls. Each assay condition was
done in triplicate.

Luciferase reporter assay

Firefly luciferase reporter assay was carried out as previously
described [17,42–45]. Briefly, subconfluent C2C12 and iMEFs
cells were infected with AdFLuc and treated with different
concentrations of Polybrene. At 24 h and 48 h post infection cells
were lysed and collected for firefly luciferase activity assay using
the Luciferase Assay Kit (Promega, Madison, WI). Each
treatment/assay condition was performed in triplicate.

Alkaline phosphatase (ALP) activity assays

ALP activity assays were conducted as described [11,12,31–33,37,44,46,47]. For the quantitative analysis, the ALP activity
was assessed with a modified assay using the Great Escape SEAP
Chemiluminescence assay kit (BD Clontech, Mountain View, CA).
For qualitative assay, the ALP activity was histochemically stained
using a mixture of 0.1 mg/ml naphthol AS-MX phosphate and
0.6 mg/ml Fast Blue BB salt. Each assay condition was performed in
triplicate, and the results were repeated in at least three
independent experiments.

Statistical analysis

The quantitative experiments were performed in triplicate and/or
repeated three times. Data were expressed as mean ± SD.
Statistical significances between groups were determined by one-
way analysis of variance and the Student’s t test. A value of p<0.05
was considered statistically significant.

Results and Discussion

Adenovirus-mediated RFP expression in mesenchymal
progenitor cells is enhanced by Polybrene in a dose-
dependent fashion

Recombinant adenoviruses are one of the most widely used
gene delivery systems for in vitro and in vivo
studies. While many
types of mammalian cells can be transduced effectively by
adenoviral vectors, the infection efficacy varies drastically among
cell types/lines, largely due to the expression levels of CAR
receptors. We have found that, while adenoviruses can infect
mesenchymal stem cells (MSCs), significantly higher titers (e.g.,
multiplicities of infection, MOI at 20–100) to achieve high
infection efficiency [11,12].

Here we analyzed if Polybrene, a commonly-used polycation for
retroviral infection, would potentiate adenovirus-mediated gene
transduction in MSCs, such as C2C12 and iMEFs. We found that
subconfluent C2C12 cells were barely transduced by AdRFP at
the MOI of 5 (Figure 1A). However, the AdRFP transduction
efficiency was significantly increased in the presence of Polybrene
and responded in a dose-dependent fashion with a peak at 8 µg/
ml (Figure 1A). In iMEFs cells, we found that the AdRFP
infectivity was extremely low at the MOI of 5, which was
significantly enhanced by Polybrene (Figure 1B). Thus, these
results strongly suggest that Polybrene may enhance adenovirus-
mediated gene transduction.

Figure 2. Polybrene enhances adenovirus-mediated firefly
luciferase activity in mesenchymal progenitor cells. Subcon-
fluent C2C12 (A) and iMEFs (B) cells were infected with AdFLuc at a
multiplicity of infection (MOI) = 5. Various concentrations of Polybrene
were added at the time of infection. At 24 h and 48 h post infection
cells were lysed and collected for firefly luciferase activity assays. Each
treatment condition was done in triplicate. ‘*’, p<0.05; ‘**’, p<0.001.
doi:10.1371/journal.pone.0092908.g002
AdFLuc-mediated firefly luciferase activity in mesenchymal progenitor cells is significantly augmented by Polybrene

We next quantitatively determined the effect of Polybrene on adenovirus-mediated transgene expression using the AdFLuc vector. When C2C12 cells were seeded at the same condition and infected with the same titer of AdFLuc, the addition of Polybrene at the time of infection significantly increased the luciferase activities in a dose-dependent fashion both at 24 h and 48 h after infection, which peaked at 4 μg/ml and maintained plateaued up to 10 μg/ml (Figure 2A). Similar results were obtained in iMEFs except that the Polybrene-enhanced luciferase activities were more pronounced at 48 h after infection (Figure 2B). In addition, it seems that Polybrene enhanced luciferase activities in iMEFs significantly at a lower concentration (2 μg/ml) than that in C2C12 cells, although peaked at a similar concentration (4 μg/ml). Taken together, these quantitative results further demonstrate that adenovirus-mediated transgene transduction can be markedly enhanced by inclusion of Polybrene at the time of infection.

FACS analysis reveals that Polybrene increases adenovirus infection efficiency in AdRFP transduced mesenchymal stem cells

In order to determine if Polybrene-enhanced luciferase activity is caused by increasing the transgene expression and/or adenovirus infection efficiency, we used FACS analysis to evaluate if Polybrene treatment increased the numbers of RFP-positive cells.

Figure 3. FACS analysis of Polybrene enhanced adenovirus-mediated RFP expression in mesenchymal stem cells. Subconfluent iMEFs cells were infected with AdRFP at a multiplicity of infection (MOI) = 5. Various concentrations of Polybrene were added at the time of infection. At 48 h post infection the cells were collected and subjected to FACS analysis of RFP-positive cells. Representative results are shown (A). Each treatment condition was done in triplicate and graphed (B).

doi:10.1371/journal.pone.0092908.g003
The iMEFs cells were infected with the same titer of AdRFP and treated with varied concentrations of Polybrene. When the cells were collected and subjected to FACS analysis, we found that the percentages of RFP-positive cells were significantly increased by Polybrene in a dose-dependent fashion (Figure 3A). Quantitatively, the average % of RFP-positive cells increased from 0.1% at 0 μg/ml to 43.6% at 4 μg/ml Polybrene, approximately 430-fold increase in the percentage of RFP-positive cells although no significant further increase at concentrations higher than 8 μg/ml (Figure 3B). These results strongly suggest that Polybrene may significantly improve adenovirus infection efficiency in addition to promoting transgene expression in mesenchymal stem cells.

AdBMP9-induced osteogenic differentiation of mesenchymal stem cells is significantly enhanced by Polybrene

We previously demonstrated that BMP9 is one of the most potent osteogenic factors to induce osteogenic differentiation in vitro and in vivo [6,11,12,18,20,48]. Here, we conducted further functional assays to determine if Polybrene can enhance AdBMP9-induced osteogenic differentiation of mesenchymal stem cells. When iMEFs were infected with the same titer of AdBMP9 and treated with different concentrations of Polybrene, we found that the early osteogenic marker alkaline phosphatase (ALP) activity increased in the presence of Polybrene in a dose-dependent fashion, which peaked at 4 μg/ml as shown by qualitative histochemical staining (Figure 4A). Quantitatively, the ALP activities were shown to increase by approximately 2.6, 3.7, 73.6, 61.9 and 65.0-fold in the presence of 1, 2, 4, 8, and 10 μg/ml of Polybrene, respectively, compared with that of the no Polybrene group (Figure 4B). Polybrene itself did not induce any detectable ALP activity (data not shown). These results demonstrate that Polybrene at as low as 4 μg/ml can significantly enhance the ability of AdBMP9 virus to transduce iMEFs and hence induce osteogenic differentiation of mesenchymal stem cells.

Mesenchymal progenitor cells can tolerate a broad range of concentrations of Polybrene

Lastly, we sought to test the cytotoxic effect of Polybrene on mesenchymal progenitor cells. When subconfluent C2C12 and iMEFs cells were treated with various concentrations of Polybrene and stained with Crystal violet, we found that most C2C12 cells and iMEFs remained viable at the Polybrene concentrations up to 40 μg/ml (Figure 5A), which is about 10-fold higher than the effective concentration (i.e., 4 μg/ml) required to enhance adenovirus transduction efficiency in mesenchymal progenitor cells. Quantitative analysis of the Crystal violet-stained cells revealed a similar trend although significant toxicities were apparent when Polybrene concentrations were at 80 μg/ml or higher (Figure 5B). These data indicate that Polybrene is well tolerated by mesenchymal progenitor cells, and most importantly, that there is a relatively large window between the effective concentration and toxicity concentration.

Figure 4. Polybrene enhances AdBMP9 transduction and osteogenic differentiation in MSCs. Subconfluent iMEFs cells were infected with AdBMP9 (or AdRFP, not shown) at a multiplicity of infection (MOI) = 5. Various concentrations of Polybrene were added at the time of infection. At 5 days post infection the cells were subjected to qualitative histochemical staining (A) or quantitative chemiluminescence assay (B). Each treatment condition was done in triplicate. Representative results are shown. ’’, p<0.05; ’’’, p<0.001. doi:10.1371/journal.pone.0092908.g004
Polybrene is a safe, effective and inexpensive cationic polymer that can be used to augment adenovirus-mediated gene transfer. As a cationic polymer Polybrene is widely used to increase the efficiency of retrovirus infection of certain cells in vitro [27–30]. It has been postulated that Polybrene acts by neutralizing the charge repulsion between virions and sialic acid on the cell surface [49]. Several studies showed that Polybrene, together with dimethyl sulfoxide, can be used to mediate DNA transfection in many types of cells, including CHO cells [50–54]. Our fluorescence microscopic results and FACS analysis strongly suggest that Polybrene may augment the adenovirus-mediated gene transfer by enhancing both infection efficiency and transgene expression. Nonetheless, the exact mechanisms through which Polybrene enhances adenovirus-mediated transgene expression remain to be fully understood.

An earlier study examined the use of polycations, such as Polybrene, protamine, DEAE-dextran, and poly-L-lysine, to enhance adenovirus type 2 vector-mediated gene delivery in primary cultures of human airway, Madin-Darby canine kidney cells, an immortalized cystic fibrosis airway epithelial cell line, and primary cultures of sheep pulmonary artery endothelium and found that these polycations increased the percentage of Ad2-transduced cells [55]. It was also shown that in human and rat epithelial cell lines and keratinocytes the addition of Polybrene during adenoviral transduction of Adβ-gal resulted in a marked increase of β-gal positive cells and β-gal protein expression in dose-dependent manner [56]. A recent study reported that Polybrene can improve transduction efficacy of recombinant adenovirus in cutaneous cells and burned skin models [57]. These findings in epithelial and endothelial cells are consistent with our results obtained from mesenchymal stem cells. Thus, these findings strongly suggest that Polybrene may be used an universal enhancer.

![Figure 5. Cytotoxicity of Polybrene in mesenchymal progenitor cells.](image-url)
for recombinant adenovirus infection of mammalian cells. Interestingly, the polyanion heparin did not significantly affect adenovirus-mediated gene transfer efficiency, but completely abrogated the effects of polyacations [55], suggesting that negatively charged moieties on the cell surface reduce the efficiency of adenovirus-mediated gene transfer, and that alteration of the charge interaction between adenoviruses and the cell surface may improve adenovirus infection efficiency.

In summary, we investigate if Polybrene can enhance adenovirus-mediated transgene delivery into MSCs lines C2C12 and iMEFs. We find that AdRFP transduction efficiency is significantly increased by Polybrene in a dose-dependent fashion with a peak at as low as 4 μg/ml in C2C12 and iMEFs cells. AdFluc-mediated luciferase activity is enhanced by Polybrene in C2C12 and iMEFs at as low as 4 μg/ml and 2 μg/ml, respectively. FACS analysis indicates that Polybrene (4 μg/ml) increases the percentage of RFP-positive cells by approximately 430 folds in AdRFP-transduced iMEFs. Furthermore, Polybrene can enhance AdBMP9-induced osteogenic differentiation of MSCs as the early osteogenic marker ALP activity can be significantly increased more than 73 folds by Polybrene (4 μg/ml) in AdBMP9-transduced iMEFs cells. Cytotoxicity analysis indicates that most C2C12 and iMEFs cells are viable at the Polybrene concentrations up to 40 μg/ml, which is about 10-fold higher than the effective titers. Therefore, 4 μg/ml required to enhance adenovirus transduction efficiency in MSCs. Therefore, our results strongly suggest that Polybrene may be routinely used as an effective, safe, and cost-effective enhancer to augment adenovirus-mediated gene transfers in MSCs and other mammalian cells.

Author Contributions

Conceived and designed the experiments: CZ N. Wu FD TCH WS RCH IHHL. Performed the experiments: N. Wu CZ FD HZ XG SW WZ JZ N. Wang. Analyzed the data: N. Wu GZ C. TCH. Contributed reagents/materials/analysis tools: LY ZL ZZ QZ ZY WL DW JY YD N. Wang. Wrote the paper: TCH N. Wu GZ RCH WS.

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