

Retroviral-mediated overexpression of human bone morphogenetic protein 2 affects human mesenchymal stem cells during monolayer proliferation: A cautionary note

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Abstract

Background: Retroviral vectors are commonly used for gene transfer applications and they represent an effective way to provide a sustained delivery of a bioactive factor in basic research and tissue engineering applications. Cells that have been transduced with retroviral vectors *ex vivo* are usually amplified on tissue culture plastic, for a prolonged period of time, in order to obtain sufficient cell numbers prior to the experiment of interest. However, the effect of the transgene product on the transduced cells, during this period of time, is rarely, if ever, investigated. The current study investigated if transduction with a VSG.G pseudotyped retroviral vector expressing human bone morphogenetic protein 2 (Rv.BMP-2) influences the gene expression profile of human bone marrow-derived mesenchymal stem cells (hMSCs) during monolayer proliferation. hMSCs that have been transduced with a VSG.G pseudotyped retroviral vector expressing enhanced green fluorescent protein (Rv.eGFP) served as controls.

Results: It was confirmed that Rv.BMP-2 transduced hMSCs produce detectable amounts of bone morphogenetic protein 2 (BMP-2). Gene expression analysis revealed that the hypertrophic marker collagen X was down-regulated by approximately 50% and the chondrogenic marker Aggrecan was elevated almost 9-fold in Rv.BMP-2 transduced hMSCs if compared to Rv.eGFP transduced control cells. Interestingly, the same changes in gene expression were detected when hMSCs were exposed to 100 ng/ml of recombinant human BMP-2 and their gene expression profile was compared to control hMSC. Again, collagen X message was down-regulated and Aggrecan message was up-regulated.

Conclusion: These results indicate that, when using integrating vectors and then expanding the cells after transduction, controls need to be carefully planned to ensure the results obtained during the 3D experiments are not due to artefacts created in response to the different cell proliferation conditions employed.

Keywords: constitutive expression, controls, gene transfer, monolayer expansion, retrovirus.

INTRODUCTION

Retroviral vectors are frequently used for gene transfer in basic research and represent an effective way to provide the sustained delivery of bioactive factors (Hu and Pathak, 2000). The maximum packaging capacity of these vectors is 8 kilobases (Thomas et al. 2003). For basic research applications, cells that have been transduced with retroviral vectors *ex vivo* are usually further amplified in order to obtain sufficient cell numbers for the given experimental set-up. For tissue engineering studies involving the production of three dimensional constructs, this period of time can be extensive due to the large number of cells required.

Retrovirus is produced by transfecting a packaging cell line with the vector of interest and then collecting the conditioned supernatant into which the newly packaged virus has been shed. This results in a low titre preparation which is generally only suitable for transducing a low initial number of cells. As the vector is integrating, this is not considered to be a problem as the cells are then expanded post-infection prior to use. The gene of interest generally encodes for a bioactive factor and control cells are usually transduced with a retroviral vector encoding for a non-bioactive factor, such as β -Galactosidase or enhanced green fluorescent protein (Kuroda et al. 2006; Salzman et al. 2009). As most retroviral vectors are constitutively active, the cells are exposed to the transgene product during the monolayer proliferation step. However, its effect on the cells during this step is rarely, if ever, investigated. It is natural to assume that bioactive transgene products will influence the cells during proliferation. Further, stem/progenitor cells are particularly sensitive to bioactive stimuli. Taking a typical experimental set-up where cells would be transduced with a vector, the cells are expanded and then seeded into a 3D scaffold for further tissue engineering; Day 0 is generally considered to be the day of seeding the scaffold. Therefore, the aim of this short technical note was to investigate whether transduction with a VSG.G pseudotyped retroviral vector expressing human bone morphogenetic protein 2 (BMP-2) (Rv.BMP-2) influences the gene expression profile of human bone marrow-derived mesenchymal stem cells (hMSCs) during monolayer proliferation. A VSG.G pseudotyped retroviral vector expressing enhanced green fluorescent protein (Rv.eGFP) served as control vector. Although BMP-2 is often used to induce differentiation of MSCs in three dimensional culture, that was not the aim of this study.

MATERIALS AND METHODS

MSC growth medium

α -MEM (Gibco, Carlsbad, CA) was supplemented with 10% human MSC tested foetal bovine serum (hMSC FBS; Gibco, Carlsbad, CA) and 5 ng/ml basic fibroblast growth factor (recombinant human, *E. coli*-derived; Peprotech, Rocky Hill, NY). The hMSC FBS was carefully pretested to ensure optimal properties for enhancing hMSC proliferation.

Mesenchymal stem cell isolation

Human bone marrow (BM) aspirates were obtained with the ethical approval (Zürich, Switzerland) and the informed written consent of patients undergoing total hip replacement. hMSCs were isolated from BM aspirates under sterile conditions as described elsewhere (Li et al. 2009). Briefly, mononuclear cells were isolated through ficoll density centrifugation. Then, hMSCs were isolated through plastic adherence.

Retroviral transduction

Passage (P) 2 hMSCs were seeded at a density of 8 888 cells/cm² onto tissue culture plastic. They were cultured in MSC growth medium under standard conditions (37°C, 5%CO₂, 90% humidity in a CO₂ incubator) for 48 hrs. Subsequently, they were transduced with 1 ml of retroviral supernatant containing 8 μ g/ml of Polybrene for 2 hrs under standard conditions. Flasks were gently rocked every 15 min to ensure that the bottom of the flasks is completely covered with medium. Afterwards, 15 ml of MSC growth medium was added and cells incubated for 24 hrs.

Human mesenchymal stem cell culture

Medium was changed 24 hrs after retroviral transduction. Next, cells were cultured under standard conditions until they reached 70-80% confluence. Now, cells were sub-cultured at a 1 to 10 ratio and again grown until 70%-80% confluent. Medium was changed twice a week. Subsequently, samples were harvested for gene expression analysis.

The experiment was independently repeated with cells from 4 donors (♀ age 81, ♀ age 55, ♂ age 56 and ♂ age 54). Additionally, the effect of recombinant human BMP-2 on monolayer expanded hMSCs, was monitored in one donor (♂ age 56). Triplicate samples were performed for each group.

For the evaluation of the effect of recombinant human BMP-2, sub-confluent, un-transduced hMSCs at P 2 were seeded at 1×10^5 cells per well into 6-well tissue culture plates. They were cultured in 3 ml of MSC growth medium with or without (control) the addition of 100 ng/ml of recombinant human BMP-2 (CHO cell-derived; R&D Systems, Minneapolis, MN). Cells were cultured for 2 weeks with medium changes twice a week under standard conditions.

BMP-2 enzyme-linked immunosorbent assay

In two of the four donors (♂ age 56 and ♂ age 54) 1 ml of medium was collected on days 2, 4, 6 and 7 after transduction with Rv.BMP-2. For the determination of BMP-2 concentration within cell culture supernatants, the Quantakine BMP-2 Immunoassay was used according to the manufacturers' protocol (R&D Systems, Minneapolis, MN).

Gene expression analysis

For gene expression analysis, 2×10^6 cells were harvested in 1 ml of TRI reagent + 5 μ l of polyacryl carrier (both Molecular Research Center Inc., Cincinnati, OH). Additionally, after 2 weeks, hMSC exposed to 100 ng/ml of recombinant human BMP-2 or left as naïve controls were harvested.

Reverse transcription, using 1 μ g of RNA, was carried out using TaqMan reagents (Applied Biosystems, Foster City, CA) and a 5700 sequence detector system (Applied Biosystems, Foster City, CA).

All oligonucleotide primers and probes, used within this study (Table 1), were previously designed and validated using the primer express oligo design software V.1.5 (Applied Biosystems, Foster City, CA). They were synthesised by Microsynth (Balgach, Switzerland). For human 18s RNA, a pre-developed kit (eukaryotic 18s rRNA endogenous control, Applied Biosystems, Foster City, CA) was used.

Table 1. Self-designed primers and probes used for RT-PCR.

Gene	Primer forward	Primer reverse	Probe (5'FAM/3'TAMRA)
human Col1A1	5'-CCC TGG AAA GAA TGG AGA TGA T-3'	5'-ACT GAA ACC TCT GTG TCC CTT CA-3'	5'-CGG GCA ATC CTC GAG CAC CCT-3'
human Col2A1	5'-GGC AAT AGC AGG TTC ACG TAC A-3'	5'-GAT AAC AGT CTT GCC CCA CTT ACC-3'	5'-CCT GAA GGA TGG CTG CAC GAA ACA TAC-3'
human Col10A1	5'-ACG CTG AAC GAT ACC AAA TG-3'	5'-TGC TAT ACC TTT ACT CTT TAT GGT GTA-3'	5'-ACT ACC CAA CAC CAA GAC ACA GTT CTT CAT TCC-3'
human Aggrecan	5'-AGT CCT CAA GCC TCC TGT ACT CA-3'	5'-CGG GAA GTG GCG GTA ACA-3'	5'-CCG GAA TGG AAA CGT GAA TCA GAA TCA ACT-3'
human Runx2 (cbaf1)	5'-AGC AAG GTT CAA CGA TCT GAG AT-3'	5'-TTT GTG AAG ACG GTT ATG GTC AA-3'	5'-TGA AAC TCT TGC CTC GTC CAC TCC G-3'

Statistical analysis

Statistical analysis was conducted with the SPSS software (SPSS 19, IBM, NY). Normality of each group was tested with independent samples Kruskal-Wallis test. Levene's Test of equality of error variances was conducted to test for equal variances between groups. The significance of differences between the groups was determined with an independent samples t-test. All descriptive results are displayed as mean \pm standard deviation.

RESULTS and DISCUSSION

BMP-2 production starts after 4 days in Rv.BMP-2 transduced human mesenchymal stem cells

First, it was confirmed that Rv.BMP-2 transduced hMSCs produce detectable amounts of BMP-2 during monolayer proliferation. Therefore, 1 ml of medium was collected at day 2, 4, 6 and 7 after Rv.BMP-2 transduction and the concentration of BMP-2 was quantified by ELISA. Cells from two different donors were used for this analysis (♂ age 56 and ♂ age 54).

During the first four days of culture, little BMP-2 (below 0.05 ng/ml) was detectable in the cell culture medium. After day 4, the concentration of the transgene product began to rise. It peaked at 3.19 ng/ml (♂ age 56 on day 6) respectively 4.28 ng/ml (♂ age 54 on day 7) (data not shown).

The gene expression profile of Rv.BMP-2 transduced human mesenchymal stem cells is altered during monolayer proliferation

Next, it was determined if Rv.BMP-2 transduction alters the gene expression profile of hMSCs during monolayer proliferation. Therefore, the gene expression of Rv.eGFP and Rv.BMP-2 transduced hMSCs, after monolayer proliferation, was compared. Gene expression was monitored using RT-PCR and the comparative $\Delta\Delta C_t$ method. 18s RNA was used as an endogenous control. The gene expression of Rv.BMP-2 transduced cells was normalised to gene expression of Rv.eGFP transduced cells. N-fold changes in gene expression between groups were calculated for the genes Col I (fibroblastic marker), Col X (hypertrophic marker), Aggrecan (chondrogenic marker) and Runx2 (osteogenic marker) (Figure 1a). Col 2 message was not detected and is therefore not included in Figure 1a.

For genes Col I and Runx2, there was no distinct difference in gene expression between Rv.eGFP and Rv.BMP-2 transduced hMSCs. Col I expression was almost identical in both cell populations (94.1% \pm 37.9% of Rv.eGFP transduced cells). Runx2 message was only very slightly elevated (121.9% \pm 59.7%) in the Rv.BMP-2 transduced hMSCs. The expression of the hypertrophic marker Col X was diminished in Rv.BMP-2 transduced cells. It reached only 45.98% \pm 29.43% if compared to Rv.eGFP transduced hMSCs. On the other hand, the chondrogenic marker gene Aggrecan was significantly ($p = 0.004$) up-regulated in Rv.BMP-2 transduced cells. Its expression level was elevated almost 9-fold (864% \pm 368%) if compared to Rv.eGFP transduced cells.

Exposure to 100 ng/ml of recombinant human BMP-2 alters the gene expression profile of human mesenchymal stem cells during monolayer proliferation

Finally, the effect of exposure to 100 ng/ml of exogenous recombinant human BMP-2 on the gene expression profile of hMSCs during monolayer proliferation was examined. hMSCs were cultured for two weeks in MSC growth medium (control) or exposed to 100 ng/ml of recombinant BMP-2. After 2 weeks, cells were harvested in TRI reagent and gene expression analysis was conducted as described above (Figure 1b). Col 2 message was not detected and is therefore not included in Figure 1b. Col I (2.46 \pm 0.73 fold) and Runx2 (2.16 \pm 0.73 fold) message were approximately 2-fold up-regulated in hMSCs which have been exposed to 100 ng/ml recombinant human BMP-2, when compared to control cells. On the other hand, the gene Col X was significantly down-regulated in these cells ($p = 0.001$). Its expression level reached only 41% \pm 2.5% of control cells. By contrast, Aggrecan gene expression was markedly increased in hMSCs exposed to 100 ng/ml recombinant human BMP-2 when compared to control cells ($p = 0.001$). An up-regulation of over 1000-fold (1029 \pm 43 fold) was detected.

Within this technical note, the question if transduction with Rv.BMP-2 changes the gene expression profile of hMSCs during the initial monolayer proliferation step was addressed. hMSCs were transduced with Rv.eGFP or Rv.BMP-2. Then, they were sub-cultured and allowed to grow until 70%-80% confluent. Afterwards, gene expression analysis was conducted using the comparative $\Delta\Delta C_t$ method with 18s RNA as internal control. The gene expression of Rv.BMP-2 transduced cells was normalised to the Rv.eGFP transduced cells. Thereby, any differences in gene expression, of the monitored genes (Col I, Col X, Aggrecan and Runx2), were detected. These genes were analysed as they are commonly investigated in studies involving osteo- or chondrogenesis. Additionally, it was

confirmed that Rv.BMP-2 transduced hMSCs started to produce detectable amounts of BMP-2 after day 4 of monolayer culture.

As expected, the gene expression profile of both cell populations was different following monolayer proliferation. The expression level of the fibroblastic marker Col I and the osteoblastic marker Runx2 were comparable between both cell populations. However, for the gene Col X and the gene Aggrecan differences were detected. The hypertrophic marker Col X was down-regulated by approximately 50% (not significant) and the gene expression of the chondrogenic marker gene Aggrecan was elevated almost 9-fold ($p = 0.004$).

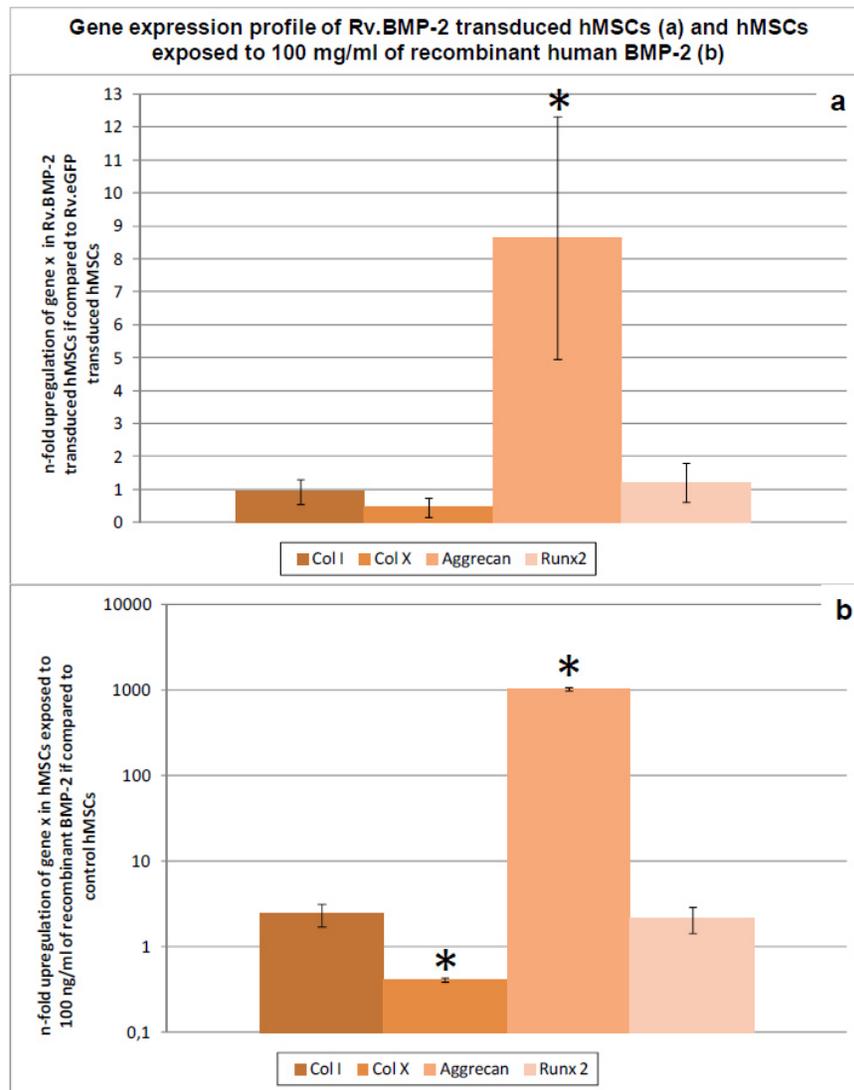


Fig. 1 (a) Relative gene expression of Rv.BMP-2 transduced hMSCs after monolayer proliferation. Gene expression analysis was conducted, using the comparative $\Delta\Delta C_t$ method, for genes Col I, Col X, Aggrecan and Runx2. 18s RNA was used as endogenous control. Rv.eGFP transduced hMSCs were used as calibrator. Results are displayed as average \pm standard deviation. The experiment was independently repeated with cell from four donors with an n of 3 per donor. * Significantly different from Rv.eGFP transduced hMSCs ($p \leq 0.004$). **(b) Relative gene expression of hMSCs which have been exposed to 100 ng/ml of recombinant human BMP-2 during monolayer proliferation.** Gene expression analysis was conducted, using the comparative $\Delta\Delta C_t$ method, for genes Col I, Col X, Aggrecan and Runx2. 18s RNA was used as endogenous control. Control cells, cultured in hMSCs growth medium without exogenous BMP-2, were used as calibrator. Results are displayed as average \pm standard deviation. The experiment was conducted with cells from one donor with an n of 3. * Significantly different from control hMSCs ($p \leq 0.001$).

Finally, it was examined how hMSCs respond to exposure to 100 ng/ml of recombinant human BMP-2 during monolayer proliferation. Control cells were grown in regular hMSCs growth medium. Treated cells were exposed to 100 ng/ml of exogenous recombinant human BMP-2, a concentration commonly used in studies applying BMP-2 exogenously (Majumdar et al. 2001; Meinel et al. 2006). After 2 weeks, cells were harvested and gene expression analysis was conducted. The gene expression of BMP-2 treated cells was normalised to the gene expression of the control cells. Interestingly, again, the genes Col 1 and Runx2 were almost unresponsive towards exposure to exogenous BMP-2, even at the higher dose. Their message was only elevated around 2-fold, when compared to control cells. The effect on the chondrogenic marker gene Aggrecan was large, in this experimental setup. Aggrecan message was elevated over 1,000-fold ($p = 0.001$) if compared to the control cells. Interestingly, the decrease in hypertrophic marker expression (Col X) was almost identical between both experimental setups. Again, its message was approximately halved when compared to control cells.

The combined results of both experiments make it tempting to speculate that the Aggrecan response is dose dependent, while the Col X response appears to be threshold dependant. This assumption is based on the observation that exposure to 25-times higher concentrations of BMP-2 (100 ng/ml vs. 3-4 ng/ml) resulted in a massive up-regulation of the gene Aggrecan. Yet, the gene Col X responded similarly to both concentrations of the bioactive factor. Summarised, the PCR data show that exposure to human BMP-2 influences the hMSCs population during the initial monolayer proliferation step. This is not surprising as BMP-2 is a known bioactive factor, with several distinct biological functions. Among other things, it is used for chondrogenic or osteogenic induction of stem/progenitor cells (Majumdar et al. 2001; Schmitt et al. 2003; Meinel et al. 2006). While there were differences observed in gene expression, the exact genes and the later effect on the differentiation of human MSCs was not the main consideration or motivation of this study. With the data presented, we aim to highlight the modified behaviour of the cells during monolayer expansion in order to increase awareness so that these considerations can be implemented during experimental planning. Although studies use a control retrovirus, such as GFP, it cannot be assumed that the control cells are comparable to cells expressing a soluble growth factor on the day of preparing a three dimensional construct. Thus, control samples should be harvested immediately prior to transduction as well as immediately prior to seeding in 3D constructs in order to gain a full picture of which changes occurred during expansion and which occurred in the 3D experimental set-up.

It, therefore, indicates that when using integrating vectors and further expanding the cells after transduction, controls need to be carefully planned to ensure the results obtained during the 3D experiments are not due to artefacts created in response to the different cell proliferation conditions employed. This suggests that the use of inducible systems, such as tet-on, may be more appropriate vectors for complex, multistep experimental procedures that are often employed for tissue engineering. This would allow for expansion under identical conditions without induced expression, and then later induction in the required 3D group.

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