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SUSTAINED AND PROMOTER DEPENDENT BONE MORPHOGENETIC PROTEIN EXPRESSION BY RAT MESENCHYMAL STEM CELLS AFTER BMP-2 TRANSGENE ELECTROTRANSFER

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Abstract

Transplantation of mesenchymal stem cells (MSCs) with electrotransferred bone morphogenetic protein-2 (BMP-2) transgene is an attractive therapeutic modality for the treatment of large bone defects: it provides both stem cells with the ability to form bone and an effective bone inducer while avoiding viral gene transfer. The objective of the present study was to determine the influence of the promoter driving the human BMP-2 gene on the level and duration of BMP-2 expression after transgene electrotransfer into rat MSCs. Cytomegalovirus, elongation factor-1α, glyceraldehyde 3-phosphate dehydrogenase, and beta-actin promoters resulted in a BMP-2 secretion rate increase of 11-, 78-, 66- and 36-fold over respective controls, respectively. In contrast, the osteocalcin promoter had predictable weak activity in undifferentiated MSCs but induced the strongest BMP-2 secretion rates in osteoblastically-differentiated MSCs. Regardless of the promoter driving the transgene, a plateau of maximal BMP-2 secretion persisted for at least 21 d after the hBMP-2 gene electrotransfer. The present study demonstrates the feasibility of gene electrotransfer for efficient BMP-2 transgene delivery into MSCs and for a three-week sustained BMP-2 expression. It also provides the first *in vitro* evidence for a safe alternative to viral methods that permit efficient BMP-2 gene delivery and expression in MSCs but raise safety concerns that are critical when considering clinical applications.

Keywords: Bone morphogenetic proteins; mesenchymal stem cells; gene delivery; electroporation; promoter activity; marrow stromal cells.

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Introduction

Local and sustained delivery of osteoinductive growth factors, i.e., the bone morphogenetic proteins (BMPs) which have the remarkable capability of inducing the biological cascade leading to new bone formation (Urist *et al.*, 1979; Wozney *et al.*, 1988), has been used as alternatives to autologous bone grafts to promote bone regeneration. One such approach consists of delivering BMPs locally using genetically modified cells that overexpress a BMP transgene (Baltzer *et al.*, 2000; Lieberman *et al.*, 1998; Musgrave *et al.*, 2000). Mesenchymal stem cell (MSC)-mediated BMP delivery is currently a promising but not yet fully explored gene therapy approach for bone tissue repair (Blum *et al.*, 2003; Hsu *et al.*, 2007; Tsuchida *et al.*, 2003) since MSCs are both "mini-factories" for BMP secretion and a source of osteoprogenitor cells.

Because of its ease of application and efficiency, the DNA electrotransfer method has become a routine technique for introducing foreign genes into mammalian cells (Daud *et al.*, 2008; Duces *et al.*, 2008; Neumann *et al.*, 1982; Wu *et al.*, 2001). This method is particularly attractive in the context of MSC-mediated BMP delivery because it (i) leads to transient expression of the transgene, a situation that fits the bone repair process which requires expression of BMPs only until the bone is healed (i.e, a few weeks); and (ii) limits the risk of DNA integration, which is of paramount importance for patient safety in the case of bone repair, a non-lethal clinical condition.

Regardless of the method used to transfer a BMP transgene into MSCs (McMahon *et al.*, 2006; Olmsted-Davis *et al.*, 2002), the transgene expression efficiency depends not only on the transfer efficiency of the delivery system, but also on the level and duration of transgene expression, which is dependent on the promoter used to drive the transcription of the transgene. Cellular promoters such as those for the genes encoding β-actin and elongation factor-1α (EF-1α) were reported to drive higher levels of transgene expression as well as prolonged transgene expression compared to viral promoters (Gill *et al.*, 2001; Hong *et al.*, 2007; Sakurai *et al.*, 2005; Sugiyama *et al.*, 1988). Notably, the EF-1α promoter induced higher levels of transgene expression than the viral CMV promoter in

mesenchymal progenitor cells (Byun *et al.*, 2005). The efficiency of cellular promoters has been shown to be cell specific and dependent on cell activity (Doll *et al.*, 1996; Durieux *et al.*, 2005; Ramezani *et al.*, 2000; Spenger *et al.*, 2004). While studies indicated that osteoblastic specific promoters, including the promoter of the osteocalcin gene, led to increase in transgene expression over time for BMP-2 expression in MSCs (Kumar *et al.*, 2005), the effect of various cellular and tissue-specific promoters on the level and duration of BMP-2 expression in MSCs has not yet been well investigated.

For these reasons, the present study determined the influence of the promoter driving the hBMP-2 transgene on level and duration of gene expression after transgene electrotransfer into rat MSCs. The activity of the viral cytomegalovirus (CMV) promoter (the standard promoter used in most BMP transgene expression systems (Chuang *et al.*, 2007; Lieberman *et al.*, 1998; Lou *et al.*, 1999; Musgrave *et al.*, 2000)) was compared to the activity of mammalian cell promoters including those for the β-actin, EF-1α, eukaryotic initiation factor 4A1 (eIF-4A1), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the tissue-specific fibronectin and OG-2 (osteocalcin) promoters.

To this aim, plasmids encoding either the lacZ reporter gene (which directs the synthesis of the beta-galactosidase enzyme) or the human BMP-2 cDNA, both fused to one of the tested promoters, were electrotransferred into rat MSCs under previously optimised gene electrotransfer parameters (Ferreira *et al.*, 2008) (under optimal conditions for efficient gene electrotransfer into MSC, viability of MSCs was about 70 % and transfection rate was up to 30 %). Expression of beta-galactosidase and BMP-2 provided evidence that: (i) the CMV promoter was not the most efficient one in rat MSCs, and (ii) selection of the appropriate promoter could lead to modulation of BMP-2 expression as a function of the stage of MSC differentiation into an osteoblastic lineage. Most importantly, and to the authors' best knowledge, the present study is the first to demonstrate a three-week-sustained release of BMP-2 by electrotransferred MSCs.

Materials and Methods

Cell source
MSCs, obtained from the bone marrow of 4-week-old male Lewis rats, were isolated and expanded as previously described (Friedenstein *et al.*, 1970; Pittenger *et al.*, 1999). These cells were routinely cultured in alpha-minimum essential medium (αMEM, Sigma-Aldrich, Lyon, France) supplemented with 10 % foetal calf serum (PAA Laboratories, Pasching, Austria). MSCs from the fourth passage were used for the experiments.

LacZ plasmid
A panel of pDRIVE plasmids (InvivoGen, Toulouse, France), encoding the lacZ reporter gene under the control of one of either the CMV, eIF4A1, EF1-α, β-actin, GAPDH, fibronectin or osteocalcin promoters, were prepared for electrotransfer into rat MSCs. The DNA was routinely amplified in DH5α *Escherichia coli* bacteria (Life Technologies, Cergy Pontoise, France) and purified using commercially available kits according to the manufacturer's instructions (Plasmid Midi Kit, Qiagen, Courtaboeuf, France). DNA was resuspended in molecular-biology-grade water (Eppendorf, Le Pecq, France) at a concentration of 2 μg/μL.

Construction of plasmids encoding the hBMP-2 cDNA
All constructs were generated in the pcDNA3.1(+) plasmid (Life Technologies). hBMP-2 cDNA was first amplified from the pcDEF3/hBMP-2 plasmid (a kind gift from Dr. Katagiri, Department of Biochemistry, Showa University, Tokyo, Japan) by polymerase chain reaction (PCR) using primers designed with *EcoRI* and *XbaI* restriction sites. All primers were purchased from Sigma-Aldrich, and their sequences are shown in Table 1. The hBMP-2 fragment was then sub-cloned into the *EcoRI*/*Xba*I restriction site in the multiple cloning site of pcDNA3.1(+), downstream of the human cytomegalovirus immediate-early promoter included in the vector. Subsequently, the CMV promoter sequence was excised from the pcDNA3.1(+)/hBMP-2

Table 1. Sequences of the primers used for the construction of the plasmids encoding the hBMP-2 transgene under the control of the tested promoters. The underlined portions of the primer sequences indicate introduced restriction sites. (F) stands for forward. (R) stands for reverse.

Primer name	5'- 3' sequence
(F) *EcoRI*-hBMP-2	TTGAATTCGCCGCCATGAATTCATGGTGGCCGGGACCCGCTG
(R) *XbaI*-hBMP-2	TTTCTAGAT TCTAGACTAGCGACACCCACAACCTT
(F) *HindIII*-β-actin	GGAAGCTTGTTCCATGTCCTTATATGGACTCA
(R) *SpeI*-β-actin	GGACTAGTGGTGAGCTGCGAGAATAGCC
(F) *KpnI*-EF-1α	GGGGTACCGGAGCCGAGAGTAATTCATACAAA
(R) *BamHI*-EF-1α	GGGATCCGTTGCTTTGAATTAGCGGTGG
(F) *HindIII*-eIF-4A1	GGAAGCTTTCGCTACAATATTTTCCTGAACG
(R) *SpeI*-eIF-4A1	GGACTAGTGGTC CTTAGAATCTAGGGCGG
(F) *HindIII*-fibonectin	GGAAGCTTTAACAGCTGCAAGGTCGTG
(R) *SpeI*-fibronectin	GGACTAGTGTGAGACGGTGGGGGAGA
(F) *HindIII*-GAPDH	GGAAGCTTAGTT CCCCAACTTTCCCG
(R) *SpeI*-GAPDH	GGACTAGTGTGTCTCAGCGATGTGGCTC
(F) *HindIII*-OG-2	GGAAGCTTCTAGTCACTCCCAGAGCCT
(R) *SpeI*-OG-2	GGACTAGTGTGTCTGCTAGGTGTGCACC

vector by digestion with *MluI* and *NheI* and the vector was purified, blunt-ended, and self-ligated to generate the pcDNA3.1/CMV⁻/hBMP-2 construct. Fragments of the tested promoters (Table 2) were amplified by PCR from the respective pDRIVE plasmids using primers listed in Table 1, purified and cloned upstream of the hBMP-2 sequence in the pcDNA3.1/CMV⁻/hBMP-2 plasmid. The integrity of the constructs was confirmed by enzymatic restriction analysis. DNA was routinely amplified in DH5α *Escherichia coli* bacteria (Life Technologies), purified using the Plasmid Midi Kit (Quiagen), and resuspended in molecular-biology-grade water at a concentration of 2 μg/μL.

LacZ transgene electrotransfer into MSCs

LacZ electrotransfer into rat MSCs was performed using the Cliniporator™ device (IGEA, Carpi, Italy) and established techniques (Ferreira *et al.*, 2008; Pucihar *et al.*, 2002). Briefly, cells were trypsinised, centrifuged at 500 g for 10 min, and then resuspended in Spinner's minimum essential medium electropulsation buffer (S-MEM, Life Technologies) at a concentration of 10^7 cells/mL. A 50 μL MSC suspension aliquot was electropulsed with 15×10^{11} copies of each plasmid by applying a train of eight electric pulses (100 μs) at 1500 V/cm and at a 1 Hz frequency. After electroporation, the cells were collected, suspended in 50 μL of electropulsation buffer (to prevent drying) and allowed to recover at 22 °C for 10 min. Subsequently, culture medium was added and the cells were maintained under standard cell culture conditions (37 °C, 95 % CO_2, 5 % O_2) for 48 h. All experiments were repeated at two separate times.

Quantification of beta-galactosidase production

Forty-eight hours post transfection, adherent MSCs were trypsinised, centrifuged and resuspended in 150 μL Reporter Lysis Buffer 1X (Promega, Charbonnières-les-bains, France). Quantification of β-galactosidase activity in the cell lysates was carried out as previously described (Ferreira *et al.*, 2008). Briefly, 70 μL of cell lysate was incubated with the β-galactosidase substrate, o-nitrophenyl-β-D galactopyranoside (Amsresco Inc-Interchim, Montluçon, France), at 37 °C for 30 min. The enzymatic reaction was stopped by addition of 1 M sodium carbonate, and light transmission was determined spectroscopically at 420 nm using the μQuant system (Bio-Tek Instruments Inc, Colmar, France). Beta-galactosidase activity in each sample tested was normalised to total DNA content.

hBMP-2 transgene electrotransfer into MSCs

hBMP-2 transgene electrotransfer into rat MSCs was essentially carried out as described in the section "lacZ transgene electrotransfer into MSCs". In this specific case, a 50 μL aliquot of MSC suspension (10^7 cells/mL) was electropulsed with 12×10^{11} copies of hBMP-2 plasmid. Following incubation after electropulsation, culture medium was added and the cell suspension was seeded in 12-well plates *at a density of* 20,000 cells/cm². These cells were maintained under standard cell culture conditions (37 °C, 95 % CO_2, 5 % O_2) for 1, 2, 4, 7, 10, 14 and 21 d. Twenty-four hours after gene electrotransfer,

Table 2. Origin and length of the different promoters tested.

Promoter name	Origin	Size (bp)
CMV	viral	587
EF-1α	rat	1314
β-actin	human	1454
GAPDH	human	483
eIF4AI	human	523
fibronectin	human	787
osteocalcin	mouse	1012

the MSC culture media was supplemented with 10 μg/mL heparin (Sigma). These experiments were repeated at two separate times.

Kinetics of BMP-2 production by MSCs

BMP-2 production by electrotransferred MSCs was quantified in cell supernatants at prescribed time points after transgene electrotransfer using an ELISA kit (Quantikine, R&D Systems, Lille, France) according to the manufacturer's instructions. Briefly, at day 1, 2, 4, 7, 10, 14, 17 and 21 after transgene electrotransfer, supernatants were collected into silicon tubes. At each of the aforementioned time points, the respective adherent cells were trypsinised, counted and/or resuspended in lysis buffer for further biochemical analyses. Supernatant media samples were stored at -80 °C until ELISA analysis of BMP-2 content. The amount of protein secreted was expressed per 10^6 cells per 24 h.

BMP-2 mRNA expression

Human BMP-2 mRNA expression in MSCs was measured at days 4, 7, 10, 14 and 21 after transgene electrotransfer. Cytoplasmic RNA was extracted from cells using TRIzol reagent (Life Technologies) according to the manufacturer's protocol. RNA concentrations were quantified by measuring the optical density of each sample at 260 nm. RNA (1 μg) was transcribed onto cDNA using the ThermoScipt™ kit and oligo-dT primers (50 μM) (Life Technologies), according to the manufacturer's instructions. Real time-PCR was performed on an iCycler thermal cycler (Bio-Rad Laboratories, Marnes-la-Coquette, France) using the SYBR Green Mix kit (Bio-Rad Laboratories). The reaction mix contained 1X SYBR Green, 20-fold diluted cDNA and 0.2 μM of each primer. Specific primers used for human BMP-2 (accession number: NM001200) and rat 18S rRNA (as the endogenous reference gene, accession number: M11188) were as follows: forward hBMP-2: 5'-AACACTGTGCGCAGCTTCC-3', reverse hBMP-2: 5'-CTCCGGGTTGTTTTCCCAC-3' (74 bp), forward rat 18S rRNA: 5'-ACTCAACACGGGAAACCTCA-3', reverse 18S rRNA: 5'-AATCGCTCCACCAACTAAGA-3' (114 bp). It should be noted that the designed BMP-2 primers specifically recognise the human but not rat BMP-2 sequence. After a 10 min denaturation step at 95 °C, cDNA was amplified by performing 40 cycles of two steps: (i) at 95 °C for 15 s, and (ii) at 60 °C for 60 s. Melting curve analyses were performed at the end of each amplification step. The hBMP-2 mRNA expression levels were normalised to those of the internal standard 18S

rRNA and reported as relative values ($\Delta\Delta$CT) to those obtained from the control cell cultures. All experiments were performed in triplicate.

Evaluation of MSC osteoblastic differentiation
Alkaline phosphatase (ALP) activity of MSCs was determined at day 1, 2, 4, 7, 10, 14 and 21 after transgene electrotransfer using a commercial kit (Sigma Aldrich). Cells were washed with phosphate-buffered saline, and collected in 230 µL lysis buffer. Briefly, 15 µL of each cell lysate was incubated with the ALP substrate, p-nitrophenyl phosphate at 37 °C for 10 min and the light transmission was read at 405 nm. ALP activity was normalised to cellular DNA content. The DNA content in cell lysates was quantified using the Cyquant® Cell Proliferation Assay Kit (Life Technologies) according to the manufacturer's instructions.

Determination of the plasmid copy number
The plasmid copy number in rat MSCs was determined at days 2, 4, and 14 after hBMP-2 transgene electrotransfer using quantitative Real Time-PCR analysis. Briefly, a 50 µL aliquot of MSC suspension (1×10^7 cells/mL) was electropulsed with 12×10^{11} copies of hBMP-2 plasmid. Cells electropulsed without DNA were used as controls. Following the post-electropulsation incubation time, culture medium was added and the cell suspension was seeded in 25 cm^2 culture flasks. The cells were then maintained under standard (37 °C, 95 % CO$_2$, 5 % O$_2$) cell culture conditions for 2, 4, and 14 d. At each time point, genomic DNA was extracted from the cells using standard protocols (Bensidhoum *et al.*, 2004). Quantitative RT-PCR was performed as described in the section "BMP-2 mRNA expression" with reaction mixtures containing 1X SYBR Green, 70 ng DNA and 0.2 µM of each hBMP-2 primers. Standards made of hBMP-2 cDNA (diluted in genomic DNA isolated from untreated rat MSCs) were prepared such that the plasmid copy number ranged from 1000 to 2×10^6 in 70 ng genomic DNA (i.e., 200 to 0.1 plasmid copies per cell). A standard curve displaying a linear relationship between Ct values and the logarithm of the input plasmid copy number was generated. The number of plasmid copy in electrotransferred cells was calculated by extrapolation from the experimentally determined standard curve. These experiments were repeated on two separate occasions.

Statistical analyses
Statistical analyses of numerical data were performed using one-way ANOVA and the LSD test (for pairwise comparisons), and the Statgraphic centurion version XV software was used for these analyses. A *p* value of less than 0.05 was considered significant.

Results

Effect of the promoter driving transcription of a transgene on protein expression after transgene transfer into MSCs
A series of plasmids encoding the lacZ reporter gene and the hBMP-2 cDNA under the control of the β-actin,

EF-1α, eIF-4A1, fibronectin, GAPDH, osteocalcin or CMV promoter were prepared. First, the influence of the promoter driving the lacZ reporter gene on the β-galactosidase expression in rat MSCs was determined 48 h after the electrotransfer of an equal number of copies of plasmid DNA. Beta-galactosidase production was clearly dependent on the promoter driving lacZ gene expression (Fig. 1a). The CMV, β-actin, EF-1α, eIF-4A1 and GAPDH promoters led to increased β-galactosidase production rate of 26-, 38-, 41-, 42- and 48-fold over pertinent controls, respectively. This result suggests that the CMV promoter was less efficient than either the GAPDH (*p* < 0.05), eIF-4A1, EF-1α and β-actin promoters in undifferentiated rat MSCs. The osteocalcin promoter had a weak activity (i.e., the β-galactosidase production rate was increased 2-fold over the control); this result was predictable as the osteocalcin gene is expressed at later stages of MSC osteoblastic differentiation. In addition, cell proliferation was not affected for all promoters tested: the lacZ-electrotransferred MSCs proliferated at rates similar to the control electropulsed cells (data not shown).

Second, the influence of the promoter driving the hBMP-2 transgene on BMP-2 expression was investigated by quantifying BMP-2 production using an ELISA assay, 48 h after electrotransfer into MSCs of an equal number of copies of plasmids encoding the hBMP-2 cDNA under the control of the tested promoters. LacZ electrotransferred MSCs were used as controls. BMP-2 secretion depended on the promoter driving hBMP-2 expression (Fig. 1b). The CMV, β-actin, EF-1α, eIF-4A1 and GAPDH promoters led to 0.4 (± 0.1), 1.4 (± 0.3), 3.0 (± 1.6), 0.8 (± 0.4) and 2.6 (± 0.3) ng BMP-2/48 h/10^6 cells, respectively. The CMV promoter was not the most efficient one in undifferentiated rat MSCs. The EF-1α (*p* < 0.05), GAPDH (*p* < 0.05), β-actin and eIF-4A1 promoters led to the greatest BMP-2 secretion rates (78-, 66-, 36- and 20-fold increase over pertinent controls, respectively).

Effect of the promoter driving transcription of the BMP-2 transgene on long-term BMP-2 expression after transgene transfer into MSCs
The kinetics of BMP-2 secretion as a function of the promoter driving the hBMP-2 transgene was next investigated during a 21-day time course study. The results presented in Fig. 2 provided the following evidence. First, a trend towards decreased BMP-2 secretion between day 2 and day 4 after hBMP-2 transgene electrotransfer into MSCs was observed for all, but the β-actin, promoters tested. The decrease in BMP-2 secretion was not correlated with the concomitant decrease in cell proliferation (data not shown). Second, regardless of the promoter driving the hBMP-2 transgene, an increase in BMP-2 secretion was observed from day 4 to day 14. Maximal amounts of BMP-2 were secreted at day 14 after gene electrotransfer. For example, MSCs electrotransferred with the hBMP-2 transgene under the control of the GAPDH and fibronectin promoters produced 4.8 (± 0.3) and 1.5 (± 0.2) ng BMP-2/24 h/10^6 cells, respectively. Additionally, regardless of the promoter driving the transgene, a plateau of maximal BMP-2 secretion persisted at least until day 21 after hBMP-2 gene electrotransfer. It should be noted that, given the
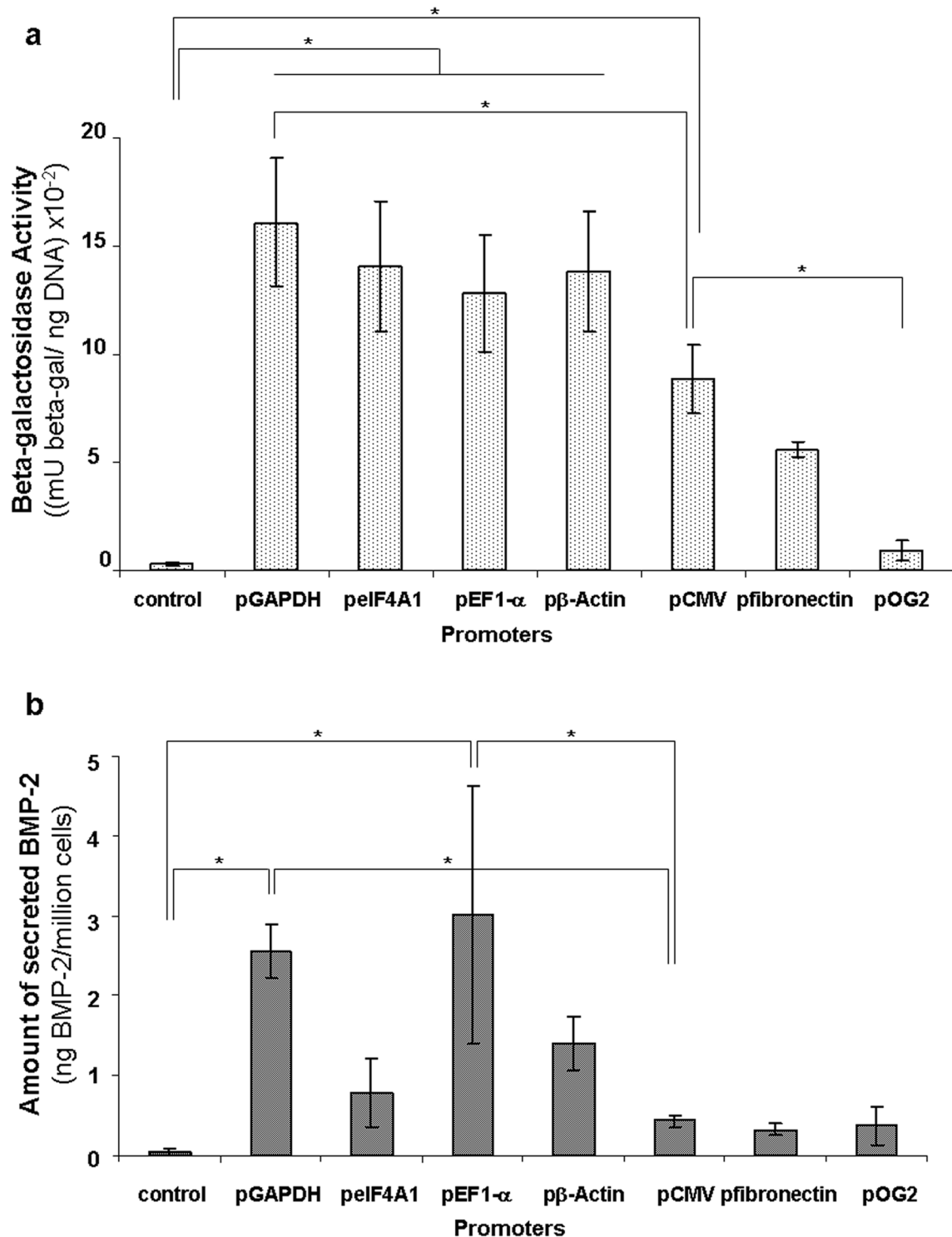
Fig.1. (A) Beta-galactosidase activity 48 h after electrotransfer into rat mesenchymal stem cells (MSCs) of an equal number of copies of plasmid encoding the lacZ gene driven by the tested promoters. Cells electropulsed without DNA were used as controls (*$p < 0.05$). **(B)** BMP-2 secretion 48 h after electrotransfer into MSCs of an equal number of copies of plasmid encoding the hBMP-2 transgene driven by the tested promoters. LacZ electropulsed cells were used as controls (*$p < 0.05$).

short half-life of BMP-2 *in vitro* (Zhao *et al.*, 2006), these results suggested that a three-week sustained release of BMP-2 can be achieved after gene electrotransfer into rat MSCs. Finally, from day 4 to day 10 after hBMP-2 gene electrotransfer, the GAPDH, β-actin and EF-1α promoters led to significantly ($p < 0.05$) greater amounts of secreted BMP-2/24 h/10^6cells compared to CMV, eIF-4A1, fibronectin and OG2 promoters.

It should be noted that, at day 14, the GAPDH, β-actin, EF-1α and OG2 promoters led to significantly ($p < 0.05$) greater secretion of BMP-2 compared to the CMV, eIF-4A1 and fibronectin promoters. These results
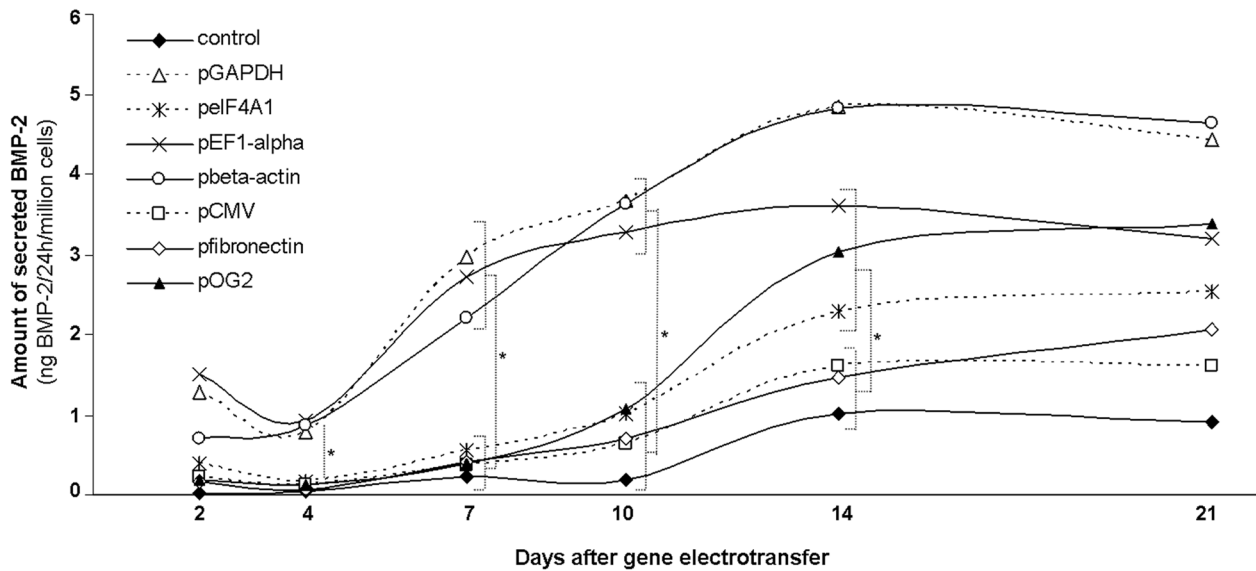
Fig. 2. Kinetics of BMP-2 secretion by MSCs at days 2, 4, 7, 10, 14 and 21 after electrotransfer of an equal number of molecules of plasmid encoding the hBMP-2 transgene under the control of the tested promoters. LacZ electropulsed cells were used as controls. Note: To facilitate reading of the results on this graph, error bars are not included (*$p < 0.05$).
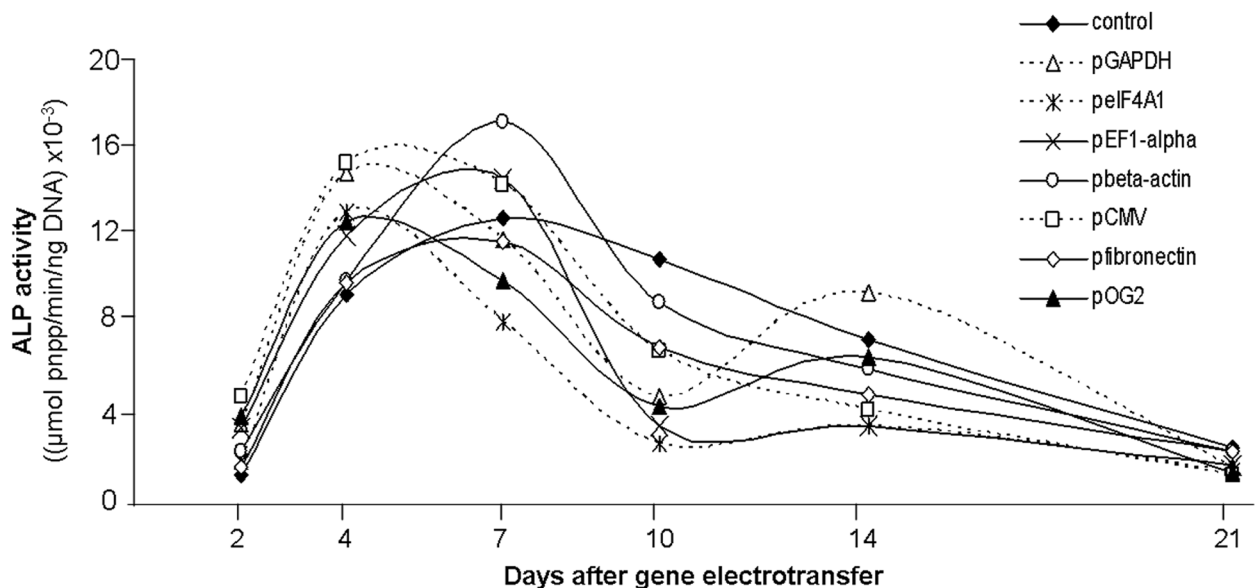

Fig. 3. Kinetics of the ALP activity displayed by MSCs at days 2, 4, 7, 10, 14 and 21 after electrotransfer of an equal number of molecules of plasmid encoding the hBMP-2 transgene under the control of the tested promoters. LacZ electropulsed cells were used as controls.

suggest that the activity of the osteocalcin promoter was enhanced during the time-course of the present study. To address this observation, the osteoblastic differentiation of the electrotransferred MSCs was examined. The kinetics of alkaline phosphatase (ALP) activity (an early marker of osteoblastic differentiation) were investigated. Between day 4 and day 7, MSCs displayed maximal ALP activity, suggesting that cells had initiated osteoblastic differentiation (Fig. 3). In contrast, from day 7 to day 21, ALP activity decreased to basal levels, suggesting that the cells had completed their differentiation to a mature osteoblastic phenotype (Malaval *et al.*, 1994). These results

suggest that the osteocalcin promoter may have been activated during the time-course of the study (i.e., during osteoblastic MSC differentiation) and that the activation of the osteocalcin promoter further up-regulated expression of the BMP-2 transgene.

In addition, the lacZ electrotransferred MSCs used as controls displayed increased basal levels of secreted BMP-2 over the time of the study, albeit at statistically lower ($p < 0.05$) levels than the hBMP-2 electrotransferred cells. Under the conditions of these experiments, lacZ electrotransferred cells also displayed similar kinetics of ALP activity compared to hBMP-2-electrotransferred
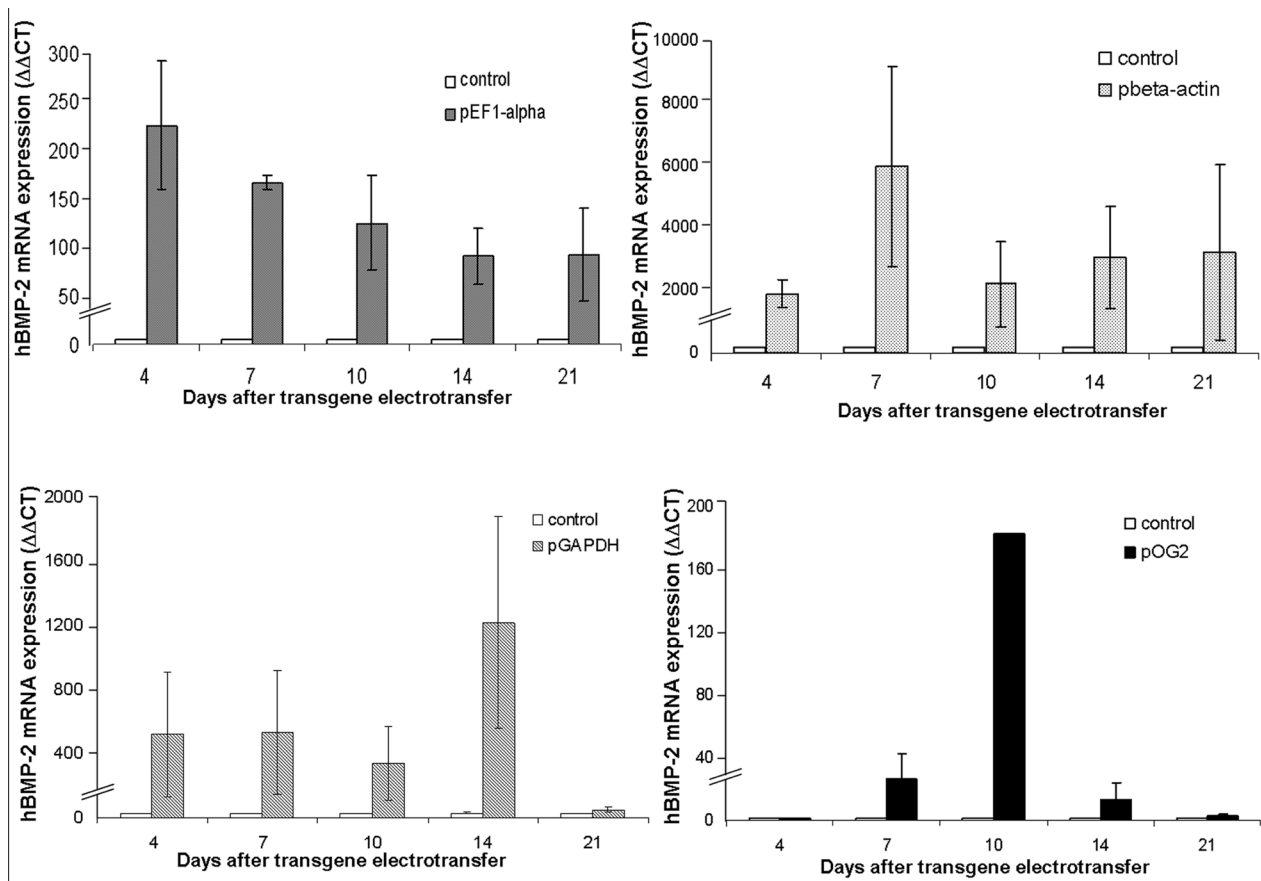
Fig. 4. Kinetics of the hBMP-2 expression by MSCs at days 4, 7, 10, 14 and 21 after electrotransfer of an equal number of molecules of plasmid encoding the hBMP-2 transgene driven by the EF-1α, β-actin, GAPDH and OG2 promoters. hBMP-2 mRNA levels were normalised first to that of the respective 18S RNA (internal standard), and then to hBMP-2 mRNA levels of lacZ electrotransferred cells (negative control).

MSCs. These results suggested that control rat MSCs might have increased expression of endogenous BMP-2 during osteoblastic differentiation. It should be noted that rat BMP-2 cannot be distinguished from human BMP-2 by the ELISA assay used in the present study. However, using primers specific for hBMP-2, we could distinguish expression of transgene from endogenous (i.e., rat) BMP-2 at the transcript level using RT-PCR.

Effect of the promoter driving the hBMP-2 transgene on its transcriptional activity after transgene transfer into MSCs

The effect of the promoter on the transcriptional activity of the transgene was further investigated at the mRNA level using primers specific for hBMP-2. The EF-1α, GAPDH and β-actin promoters were selected because they led to the greatest BMP-2 protein secretion rates while the OG-2 promoter was chosen because it modulated BMP-2 protein secretion rates as a function of MSC osteoblastic differentiation. hBMP-2 mRNA expression was measured by semi-quantitative real-time-PCR at days 4, 7, 10, 14 and 21 after transgene electrotransfer into MSCs. LacZ electrotransferred MSCs were used as controls. hBMP-2 mRNA expression was detected at days 4, 7 and 14 after

transgene electrotransfer regardless of the promoter driving the transgene (Fig. 4). hBMP-2 mRNA expression driven by the EF-1α, GAPDH or β-actin promoter was maintained at least until day 21 after transgene electrotransfer. These results provided important evidence that long-term (up to 21 d) expression of BMP-2 can be achieved after gene electrotransfer into rat MSCs. In addition, the expressed hBMP-2 mRNA levels depended on the promoter driving the transgene. For example, at 4 d after transgene electrotransfer, the EF-1α, GAPDH, β-actin and OG-2 promoters led to approximately a 190-, 500-, 1500- and 2-fold increase of BMP-2 expression over the controls, respectively. Moreover, stable hBMP-2 mRNA expression levels were observed upon electrotransfer of the hBMP-2 transgene driven by either EF-1α, GAPDH or β-actin promoter over the time course analysis, suggesting that the promoter activity was not affected during the differentiation of the cells. In contrast, electrotransfer of the hBMP-2 transgene driven by the OG-2 promoter led to a respective 2-, 28-, 164-, 21- and 4-fold increase of hBMP-2 expression over the respective controls at days 4, 7, 10, 14 and 21 after transgene electrotransfer. These results support the hypothesis that the osteocalcin promoter was activated during MSC osteoblastic differentiation
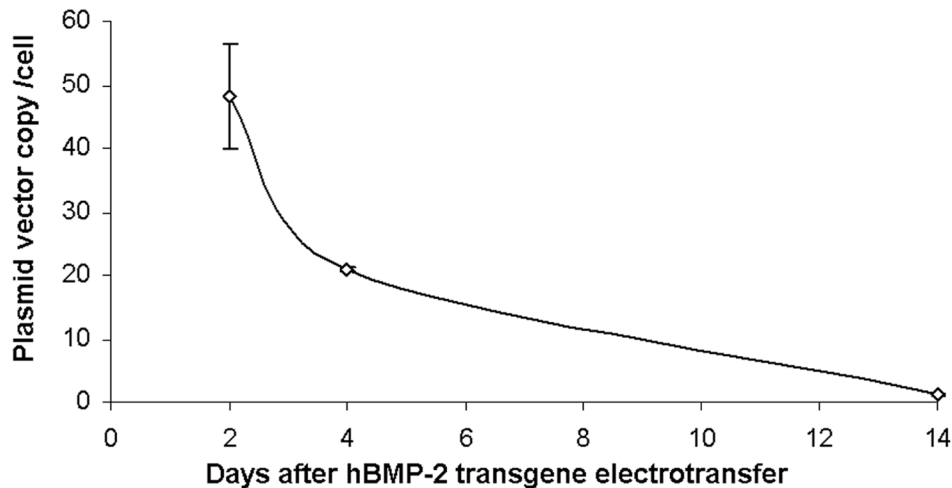
Fig. 5. Kinetics of plasmid copy number at days 2, 4 and 14 after electrotransfer of hBMP-2 plasmid into MSCs.

Analysis of the plasmid copy number after hBMP-2 plasmid electrotransfer into MSCs

Quantitative real-time PCR analysis of the kinetics of hBMP-2 plasmid copy number after electrotransfer into rat MSCs showed 48 ($\pm$ 8.00), 21 ($\pm$ 0.20) and 1 ($\pm$ 0.16) copies of hBMP-2 plasmid per cell at days 2, 4 and 14 after transgene electrotransfer, respectively (Fig. 5). This decrease in plasmid copy number per cell correlated with cell division and proliferation (data not shown), since gene electrotransfer led to episomal plasmid maintenance.

Discussion

Transplantation of MSCs genetically modified with BMP-2 transgene is an attractive potential option for the repair of large bone defects because this approach provides essential components for adequate bone repair. In the present study, using rat MSCs overexpressing hBMP-2 after transgene electrotransfer, a method which leads to episomal location of the transferred DNA and limits the risk of DNA integration, we have attempted to optimise BMP-2 transgene expression by investigating the role of the promoter driving the human BMP-2 gene on the level and duration of electrotransferred transgene expression in MSCs.

First, the type of the promoter driving either lacZ or BMP-2 transgene clearly influenced β-galactosidase and BMP-2 levels expressed by transgene electrotransferred MSCs. The EF-1α, β-actin, GAPDH and eIF-4A1 promoters led to significantly (p < 0.05) greater β-galactosidase and BMP-2 production compared to the CMV promoter. This finding is consistent with results that the mammalian EF-1α and ubiquitin C promoters led to transgene expression levels 98- and 20-fold higher, respectively, than those driven by the CMV promoter in mouse MSCs (Byun *et*

al., 2005). In addition, the murine leukaemia virus long terminal repeat promoter led to higher levels of BMP-2 expression in rat MSCs compared to the CMV promoter (Sugiyama *et al.*, 2005). Taken together, these results suggest that the CMV promoter may not be the most effective one in driving maximal BMP-2 expression levels in MSCs.

The promoters of EF-1α, β-actin and eIF-4A1 (which are ubiquitously expressed in eukaryotic cells) induced higher levels of transgene expression than viral promoters in haematopoietic and mesenchymal stem cells (Byun *et al.*, 2005; Sakurai *et al.*, 2005), fibroblasts (Sugiyama *et al.*, 1988) and macrophages (Quinn *et al.*, 1999), respectively. Expression of GAPDH, an enzyme involved in glycolysis, can be upregulated by physiological components in certain cell types; for example, by insulin in adipocytes (Ercolani *et al.*, 1988) and by hypoxia in endothelial cells (Graven *et al.*, 1999). Further investigation is needed to elucidate the molecular mechanisms sustaining high levels of transgene expression driven by the eIF-4A1 and GAPDH promoters in MSCs.

Moreover, in the present study, promoter activity depended on the stage of osteoblastic MSC differentiation. Analysis of mRNA levels showed that hBMP-2 expression driven by the EF-1α, GAPDH and β-actin promoters was stable during the time course (up to 21 d) of the present study. In contrast, the levels of hBMP-2 expression driven by the osteocalcin promoter markedly increased over the same time period and correlated to the observed increased BMP-2 production rates; in fact, the production rates driven by the osteocalcin promoter were statistically equivalent to those driven by the strongest promoters (specifically, the EF-1α, GAPDH and β-actin promoters) at day 14 after transgene electrotransfer into MSCs. Transgene expression driven by the promoter of osteocalcin (a protein specifically expressed by osteoblasts) was evident only from days 7 to 21 after induction of transgenic MSC differentiation into the osteoblastic lineage (Kumar *et al.*, 2005). The data of the present study suggest that, whereas the activity

and that this osteocalcin-promoter activation led to the observed hBMP-2 up-regulation.

of the osteocalcin promoter was predictably weak in undifferentiated MSCs, it might have been boosted during osteoblastic MSC differentiation, resulting in the observed up-regulation in BMP-2 expression.

In contrast, under the conditions in the present study, the fibronectin promoter led to the lowest beta-galactosidase and BMP-2 production rates in undifferentiated rat MSCs after transgene electrotransfer; the activity of the fibronectin promoter was not modulated during MSC osteoblastic differentiation. Fibronectin, a key protein in facilitating wound healing, is generally expressed at low levels in undifferentiated cells. In contrast, its expression is drastically increased after cell differentiation (Choi *et al.*, 1996; Suzuki *et al.*, 1998).

Considering *in vivo* delivery of BMP-2 by transgenic MSCs for applications in bone repair, the use of a promoter, such as the osteocalcin one, may be inappropriate as it may lead to low levels of BMP-2 expression in undifferentiated cells and to high levels of BMP-2 expression in bone-differentiated cells. However, considering therapeutic gene delivery for either bone disorders or skeleton tumour metastasis treatment, the osteocalcin promoter may provide an efficacious approach to confine gene expression to bone tissue (Hou *et al.*, 1999). Additional *in vivo* studies are required to investigate whether the fibronectin promoter could be efficacious in driving high levels of BMP-2 expression for wound healing applications.

One limitation to our model is that the level of BMP-2 expression immediately after transfection (which varies depending on the promoter used) might have influenced MSC proliferation rate and, thereby, downstream levels of BMP-2 expression. However, the electrotransferred MSCs were seeded at a high density and reached confluence within a few days. Such culture conditions might have limited the effects of early BMP-2 expression on cell proliferation and, therefore, the long term levels of BMP-2 expression.

Notably, the present study provided the first evidence that sustained (up to 21 d) BMP-2 delivery by MSCs can be achieved after transgene electrotransfer, a reproducible non-viral technique useful for transient expression of therapeutic transgenes (Birkholz *et al.*, 2009; Parham *et al.*, 1998). Kinetics analysis of plasmid copy number showed decreased plasmid copy numbers per cell over the time course of the study; specifically, one copy of plasmid remaining per cell at day 14 after hBMP-2 transgene electrotransfer. Because the kinetics analysis was determined in the total electropulsed cell population which includes hBMP-2 electrotransferred and non-electrotransferred cells, the results of the present study may not reflect the kinetics of plasmid copy number in the hBMP-2 electrotransferred MSC population. All in all, a strong plasmid dilution correlated with cell division and proliferation was expected to lead to drastic decrease in the hBMP-2 expression levels. In contrast, sustained BMP-2 release for at least 3 weeks was observed in the present study. Independently of the promoter driving the transgene, BMP-2 production rates increased from days 4 to 14 and sustained protein secretion was maintained until at least day 21 after transgene electrotransfer into MSCs. In the past, BMP-2 production by transgenic rat

MSCs was maintained 3 weeks after transgene transfer using recombinant adenoviruses (Feeley *et al.*, 2006). The present study demonstrates the feasibility of gene electrotransfer for efficient BMP-2 transgene delivery into MSCs and for sustained BMP-2 expression, and provides the first *in vitro* evidence for an alternative to viral methods that permit efficient BMP-2 gene delivery and expression in MSCs but raise safety concerns that are critical when considering clinical applications.

In addition, the present study showed that BMP-2 production correlated with sustained (up to 21 d) hBMP-2 mRNA expression. Specifically, a hBMP-2 mRNA increase of 1500-, 1700-, 2380- and 2430-fold over controls was observed at days 4, 10, 14 and 21, respectively, in cells electrotransferred with the hBMP-2 transgene driven by the β-actin promoter (Fig. 4). In previous studies, mRNA level analysis of hBMP-2 expression in MSCs, transfected with a hBMP-2 plasmid using the electroporation-based technology called "Nucleofector®", demonstrated that hBMP-2 expression was maximal on day 1 and maintained at significantly elevated levels until day 10, but then decreased to baseline levels (Aslan *et al.*, 2006); this outcome has been a major drawback of most non-viral techniques developed to facilitate the transplantation of *in vitro* BMP-2 genetically modified MSCs.

Additional *in vivo* studies are required to investigate whether the levels of BMP-2 expressed by electrotransferred MSCs are physiologically relevant for inducing bone formation. In previous studies, implantation of BMP-2-transfected MSCs led to enhanced ectopic bone formation in mouse models (Aslan *et al.*, 2006; Lu *et al.*, 2012; Wegman *et al.,* 2011). Interestingly, the low *in vitro* levels of BMP-2 expressed by these systems were comparable to the ones observed in our study (e.g., 6-12 ng BMP-2/24 h/ million BMP-2 nucleofected MSCs).

In conclusion, the present study demonstrates that: (i) the EF-1α, β-actin and GAPDH promoters are more efficient than the CMV promoter in driving gene expression in rat MSCs; (ii) BMP-2 secretion by genetically modified MSCs was modulated by the promoter driving the transgene, and (iii) sustained (up to 21 d) BMP-2 delivery by MSCs was achieved after transgene electrotransfer.

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Discussion with Reviewer

Reviewer I: The rationale for the experiments is apparently to find the promoter that gives the highest level and longest duration of expression of BMP-2. However, it is unclear whether higher and/or longer levels of expression of BMP-2 are needed *in vivo*. It also is not clear if MSCs are the best cell type in which to express BMP-2 for bone formation.
Authors: We agree that, from the literature, it is unclear whether long term delivery of high levels of BMP-2 is required for bone formation *in vivo*. The best delivery profile may, in fact, depend on the particular clinical case (e.g., fracture *versus* segmental defect). While it is also unclear whether MSCs are the best cells for BMP-2 delivery in the context of bone formation, we chose to focus on these cells for our promoter comparison study because of their distinct advantages (e.g., extensive expansion capacity; osteogenic potential; engraftment ability).