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Research Article

Evaluation of BMP-2 Minicircle DNA for Enhanced Bone Engineering and Regeneration

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ABSTRACT

To date, the significant osteoinductive potential of bone morphogenetic protein 2 (BMP-2) non-viral gene therapy cannot be fully exploited therapeutically. This is mainly due to weak gene delivery and brief expression peaks restricting the therapeutic effect. The use of minicircle DNA allows prolonged expression potential. It offers notable advantages over conventional plasmid DNA. The lack of bacterial sequences and the resulting reduction in size, enable safe usage and improved performance for tissue regeneration.

In this study, we report the combination of an optimized BMP-2 cassette (in the following referred to as conventional BMP-2-Advanced plasmid) with minicircle plasmid technology, thereby attaining an improved therapeutic plasmid for osteogenic gene therapy. C2C12 cell line transfected with BMP-2-Advanced minicircle showed significantly elevated expression of osteocalcin, alkaline phosphatase (ALP) activity and BMP-2 protein amount when compared to cells transfected with conventional BMP-2-Advanced plasmid. Furthermore, the plasmids show suitability for stem cell approaches by showing significantly higher levels of ALP activity and mineralization when introduced into human bone marrow stem cells (BMSCs).

Here in, we present a highly bioactive BMP-2 minicircle plasmid with the potential to fulfill requirements for clinical translation in the field of bone regeneration.

Keywords: Osteoinductive potential; Gene therapy; BMP-2 cassette; Alkaline phosphatase (ALP)

INTRODUCTION

Bone tissue has excellent reparative capacities; however 5%-10% of fractures are associated with delayed healing or even with non-union [1]. In particular, incomplete bone healing occurs after trauma or tumor surgery causing critical size bone defects. Full regeneration may also fail due to aging, diabetes mellitus, decreased vascularity or nicotine abuse [2,3]. The most reliable way to augment bone regeneration under challenging clinical scenarios associated with poor bone stock or compromised biologic environments is autologous bone grafting (ABG) which is still today's gold standard procedure [4]. It has been demonstrated that the simultaneous use of ABG and a stable fixation accomplish successful union of the fracture site [5]. Nevertheless, this form of treatment is restricted in size and availability, associated with chronic donor site pain and complications during implantation [2]. An alternative strategy to ABG is the use of growth factors such as recombinant human bone morphogenetic protein 2 (BMP-2), which has been approved by the FDA for human spine injury [6-8]. BMP-2 is known to play a crucial role in fracture healing. Mice lacking BMP-2 expression are not capable of bone regeneration and suffer spontaneous fractures [9]. Recent studies showed that BMP-2 regulates the endogenous expression of many other BMPs, and when blocked, stem cells are incapable of differentiation into osteoblasts [10]. Accordingly, BMP-2 gene delivery to MSCs improved repair potential in large bone defects [11].

Gene therapy has attracted attention in the last few years in the field of tissue regeneration as potent osteoinductive factors such as BMP-2 can induce bone formation [12,13]. Recombinant

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BMPs have been successfully applied for the treatment of delayed healing or non-unions [14]. Nevertheless, supraphysiologic concentrations of recombinant BMP-2 have been associated with tumor formation, indicating a correlation between off-label effects of high dosages and an increased risk of developing cancer [15-17].

Gene therapy approaches offer several advantages over recombinant protein therapy. This includes lower manufacturing costs and sustained de novo production of highly bioactive therapeutic proteins by host cells during *in vivo* applications [14]. Especially non-viral vectors are regarded as safe and suitable for clinical applications in the framework of tissue [18]. Although non-viral delivery methods generally show low gene transfer efficiency when compared to viral systems, the risks in terms of immunogenicity and chromosomal integration are minimized. Several approaches exist to improve the impact of non-viral systems by increasing gene delivery to target tissues or by enhancing gene expression itself [19-26].

One method to increase efficacy of therapeutic plasmids includes the use of a minicircle plasmid system. Besides the eukaryotic expression cassette, conventional expression plasmids require additional sequences for production and purification processes in bacterial systems, including an origin of replication (ori) and a selection marker such as an antibiotic resistance gene [17,27-29]. In contrast, minicircle plasmids are cleared from all prokaryotic sequences, ending up as minimized plasmids harboring only sequence elements necessary for gene expression in eukaryotic cells (promoter, open reading frame, polyA). As unmethylated CpG motifs of bacterial origin are usually recognized by the innate immune system of the host, removal of these elements enables prolonged transgene expression [30-32]. Additionally, minicircles benefit from their smaller overall size compared to conventional plasmids. Reduced size improves tissue diffusion, crossing the cell membrane, escaping lysosomal entrapment, and overcoming the nuclear membrane [33-36]. This results in higher and prolonged bioavailability.

To date, the advantage of minicircles in regenerative medicine has been demonstrated in skin, skeletal muscle, liver, heart, bone and induced pluripotent stem (iPS) cells [37-43]. Although BMP-2 minicircles have been applied previously by Keeney and collegues, comparative studies with conventional (non-minicircle) BMP-2 vector studies would be helpful to highlight the impact of the minicircle system in the field of bone regeneration. This work is mainly focused on the comparable set up of sustained gene therapeutic approaches *in vitro* in different cell types. The unique features of minicircles to enhance levels and duration of transgene expression led us to design an osteogenic minicircle system, including our previously tested optimized BMP-2 cassette and investigate its potential for bone regeneration [23]. We hypothesized that vector DNA devoid of bacterial sequences is capable of expressing persistently higher levels of BMP-2, therefore triggering the osteogenesis of target cells more efficiently compared to osteogenic induction mediated by BMP-2-Advanced enclosed in the conventional plasmid backbone [23].

The minicircle system applied in this study is based on an innovative site-specific intramolecular recombination technology that includes a ParA resolvase and ITev endonuclease. It enables the preparation of BMP-2 minicircles in a one-step procedure and provides high yields of therapeutic minicircles with maximal purity [44]. We report that a BMP-2 minicircle entails higher levels of BMP-2 expression and superior osteogenic differentiation capacity than conventional plasmids underlining the impact and advantages of this technology as a promising tool for safe and efficient treatment in bone regeneration.

MATERIAL AND METHODS

Growth factor

Recombinant human bone morphogenetic protein 2 (rhBMP-2) was purchased from Wyeth (InductOS) (USA). The lyophilised growth factor was dissolved according to manufacturers' instructions and stored at -80°C.

Plasmids

The plasmid pEF1α-BMP-2-Advanced carries an improved gene sequence, which was modified by codon optimisation and insertion of an artificial intron published previously by Hacobian et al. [23]. A truncated version of the constitutive mammalian promoter EF1 α was used to drive gene expression in cells with low CMV promoter response such as in human embryonic stem cells (hESCs) [23]. This expression cassette was cloned into pRBPS-IVR7, a novel vector system for the one-step production of minicircle [44]. The constructed pRBPS-IVR7-BMP2-Advanced plasmid was maintained in E. coli BL21 and isolated using Endofree Plasmid Maxi or Giga Kits (Qiagen, Germany) according to the manufacturers' instructions.

Cloning of PEF1α-BMP-2-advanced in PRBPS-IVR7

A PCR fragment containing the BMP-2-Advanced expression cassette with the corresponding pEF1α promoter from pEF1α-BMP-2-Advanced was generated using the sense primer (5´-ATTAGAGCTCATCTCGCTCCGGTGCCCGTCAGTG-3´) containing a SacI restriction site, and antisense primer (5´-TATTGGCCGGCCACGCCTTAAGATACATTGATGAGTTTG-3´) containing a FseI restriction site [23]. The 50 µl PCR reaction containing 2.5 µl (10 mM) primer DNA, 1 µl (10 mM) dNTPs, 20 ng template DNA, 1.5 µl DMSO, 0.5 µl Phusion polymerase in HF buffer was subjected to the following conditions: 30 sec 98°C pre-denaturation, 40 cycles: 30 sec 98°C, 20 sec 61°C, 90 sec 72°C. The PCR product was inserted into plasmid pCR-Blunt and subsequently cloned into the pRBPS-IVR7 vector via SacI restriction sites resulting in vector pRBPS-IVR7-BMP-2-Advanced (pRBPS-BMP-2Adv) (Figure 1).

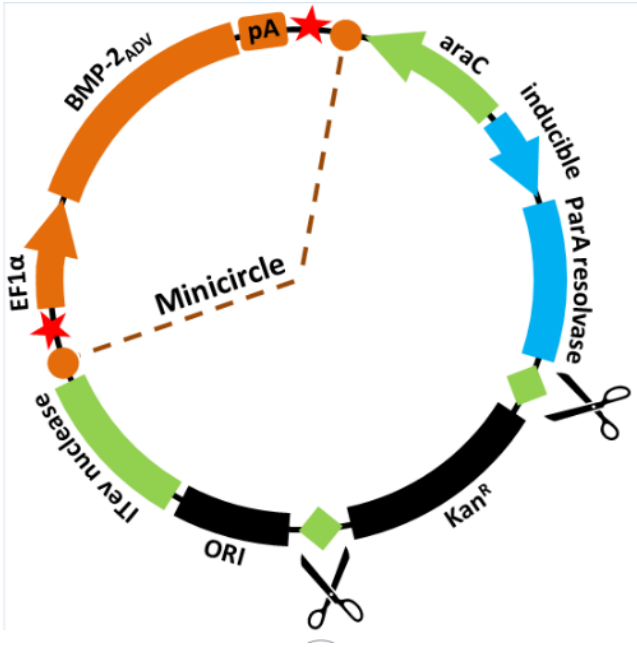

Figure 1: Schematic vector map of pRBPS-IVR7-BMP-2-Advanced (pRBPS-BMP-2Adv) EF1 α : short human elongation factor-1 alpha promoter, BMP-2Adv: improved bone morphogenetic protein 2 (BMP-2-Advanced), pA: polyadenylation signal, asterisks: transcription terminator, circles: recombination sites of ParA resolvase, ITev: intron encoded endonuclease of bacteriophage T4, scissors indicate restriction sites of ITev meganuclease, asterisks indicate flanking sites of gene expression cassette, KanR: kanamycin resistance gene, ORI: MB1 origin of replication.

Production of minicircle DNA

Minicircle production was previously described by Mayrhofer et. al. (P Mayrhofer et al., 2018). The E. coli strain BL21 was transformed with parental plasmid pRBPS-BMP-2-Advanced and pre-cultivated overnight in yeast extract medium (YE-medium) supplemented with 1% glycerol and kanamycin (50 µg/ml) at 28°C. 100 ml YE-medium (containing kanamycin 50 µg/ml) was inoculated with 1 ml overnight culture (1/100 volume). Cultivation was performed at 37°C until an OD600 of 1.9 was reached. Recombination and *in vivo* degradation process was induced by the addition of 1% of L-arabinose (stock solution: 40% L-arabinose in ddH2O; Sigma-Aldrich, USA). Cells were harvested after 3 hours of induction.

Cell culture and transfection

For *in vitro* analysis, the mouse myoblast precursor cell line C2C12 (DSMZ, Germany, #ACC565) capable of differentiating into osteogenic lineage was used. C2C12 cells were grown in Dulbecco's modified Eagle's medium high glucose (DMEM HG; Sigma-Aldrich, USA) supplemented with 5% fetal calf serum (FCS, Lonza, Switzerland) and 1% L-glutamine (Sigma-Aldrich, USA) at 37°C and 5% CO2. 20000 cells/cm2 were seeded in a 24-well plate and transfected at approximately 80% confluency using either equimolar DNA amounts of each plasmid or 1 µg total DNA with jetPEI® DNA transfection reagent (VWR, USA; 1:2 v/v) as recommended by the manufacturer. Untreated cells were used as negative control. As positive control, cells were treated with 300 ng rhBMP-2 for 6 days (one dosage in the beginning of the experiment).

Human bone marrow stem cells (hBMSCs, ATCC® PCS-500-012TM, USA) were cultivated in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA) supplemented with 10% FCS (Lonza, Switzerland), 1% L-glutamine (Sigma-Aldrich, USA), 1% Penicillin/Streptomycin and 10 ng/ml human recombinant human fibroblast growth factor (hFGF, PeproTech, USA) until passage five. For osteogenic differentiation, 2 x 105 cells per reaction were nucleofected following the instructions of the manufacturer (Human MSC Nucleofector® Kit, Lonza, Switzerland) and seeded at 5000 cells/cm2 in 24-well plates. Differentiation experiment was carried out in culture medium without addition of hFGF and medium was changed twice a week. After three weeks of osteogenic differentiation, BMSCs were stained for mineralization and used for evaluation of ALP activity.

Alkaline phosphatase assay

In vitro osteogenic differentiation after gene delivery was evaluated by alkaline phosphatase activity (ALP) assay. The enzymatic activity was measured 6 days after transfection for C2C12 cells and three weeks after transfection for BMSCs. Cells were washed with 1x PBS and lysed with buffer containing 0.25% (v/v) Triton X-100 for one hour at room temperature. Samples were incubated with p-nitrophenylphosphate (4-Nitrophenyl phosphate disodium salt hexahydrate, Sigma Aldrich, USA) for 20 min, followed by reaction stop with 0.2 M NaOH. Quantification of produced p-nitrophenol was performed at 405 nm. Depicted ALP activities are referred to nmol of p-nitrophenol liberated per minute.

Alizarin red staining

After three weeks of differentiation, hBMSC were stained with 2% Alizarin Red S (Sigma-Aldrich, USA) (pH 4.2) for 10 min. Cells were washed three times with 1x PBS and fixed with 70% ethanol at -20°C. Absorbance of the centrifuged supernatant was determined at 405 nm with a plate reader (POLARstar Omega, BMG LABTECH, Austria) (Data Analysis Software: MARS 2.30 R2).

Enzyme-linked immunosorbent assay (ELISA)

Supernatants of BMP-2 expressing cells were collected 6 days post transfection and subjected to protein quantification by ELISA (R&D Systems, USA). The protocol was carried out according to the manufacturer's instructions.

Cell viability assay

Cytotoxicity was evaluated using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (Promega, Madison, USA). Cells at a density of 0.25 × 106/cm^2 suspended in 1 ml culture medium were seeded in 24-well plates and treated as indicated. Negative control (Neg) was performed by incubating with 0.9%

Triton X-100. After 24 h, cells were washed with 1X PBS and 5 mg/ml MTS reagent in 1X PBS (50 µl/well) and 100 µl fresh medium were added. After 1 h of incubation at 37°C, the absorbance at 490 nm was determined with a plate reader (POLARstar Omega, BMG LABTECH, Austria). The data was analysed using the data analysis software MARS 2.30 R2.

Gene expression analysis

Total RNA was extracted from C2C12 cells using peqGOLD TriFast (VWR, USA) according to manufacturer's instruction. 1 µg RNA was reverse transcribed to cDNA using EasyScript™ cDNA Synthesis Kit (Thermo Fisher Scientific, USA Abm, Onescript). Subsequently, 40 ng cDNA per reaction was used as sample for quantitative real-time PCR (total volume 20 µl) in the SYBR Green PCR Master Mix (KAPA SYBR FAST universal; VWR, USA) performed according to the manufacturer's instructions. The optimal conditions (Biorad CFX96 real-time PCR cycler) were defined as follows: 39 cycles at 95°C for 3 min, at 95°C for 10 s, at Ta for 30 s, at 72°C for 10 s, followed by 1 cycle at 95°C for 10 s, at Ta for 30 s, at 95°C for 15s, and finally at 95°C for 5 s. Following primer pairs were used: osteocalcin, sense 5´-CCTAGCAGACACCATGAG-3´ antisense 5´-CTTGGACATGAAGGCTTT-3´; bone sialoprotein (BSP), sense 5´-GACCCATCTCAGAAGCAGAATCT-3´antisense 5´-CCATGTGGTCATGGCTTTCATT-3´; myogenin, sense 5´-GGTCCCAACCCAGGAGATCAT-3´, antisense 5´-ACGTAAGGGAGTGCAGATTG-3´; Id1, sense 5´-AGTGGGTAGAGGGTTTGA-3´, antisense 5´-TCCGAGAAGCACGAAATG-3´. The relative mRNA expression was calculated with the 2^-ΔCT-method and adjusted to the expression of hypoxanthine guanine phosphoribosyl transferase (HPRT, sense 5´-GCAAGTCTTTCAGTCCTGTCC-3´, antisense 5´-GCAGCGTTTCTGACCCAT-3´).

Statistical analysis

Data were analysed using Prism v5.0c (GraphPad Software, La Jolla, CA). One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test has been applied for multiple comparison. For single comparison, two-tailed Student's t-test was used, except when stated otherwise. Results are presented as means ± standard deviation (means ± SD). $p<0.05$ was considered statistically significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

RESULTS

Increased osteogenic response induced by BMP-2 minicircles in C2C12 and BMSCs. When using the same total amount of DNA (1 µg/ml), higher levels of BMP-2 protein were quantified in the minicircle group compared to cells transfected with the conventional plasmid. The expression level was significantly increased around 4-fold after 6 days (Figure 2). The strong osteoinductive capacity of the minicircle DNA was also confirmed by measurement of ALP activity (Figure 2a).

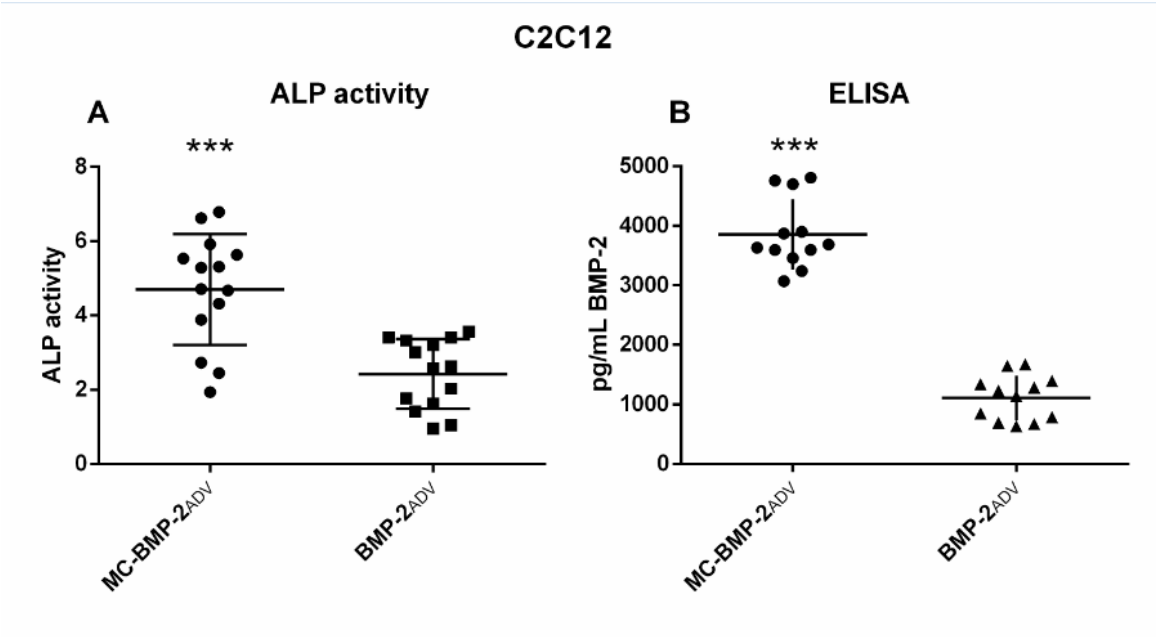

Figure 2: Efficiency of BMP-2 minicircle in cell lines C2C12 cells were transfected with 1 µg of either conventional BMP-2-Adv plasmid (BMP-2adv) or minicircle BMP-2-Adv plasmid (MC-BMP-2adv). A: Differentiation of C2C12 cell line induced after plasmid transfection. Evaluation was performed by quantification of alkaline phosphatase activity 6 days post-transfection. B: BMP-2 expression quantified by ELISA 6 days post-transfection. Data is presented as means ± SD. n=14 (ALP), n=12 (ELISA), *** $p<0.001$.

The minimal expression cassette showed significantly elevated ALP activity when compared to conventional plasmid DNA. Additionally, gene expression analyses using quantitative reverse transcription PCR (qRT-PCR) indicate overall superiority of minicircle DNA (Figure 3).

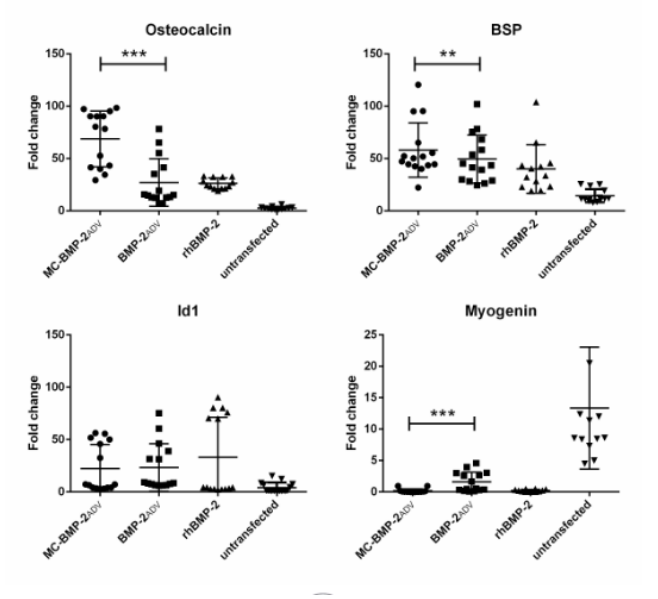

Figure 3: Comparison of expression level of osteogenic markers qRT-PCR analysis at day 4 of the osteogenic markers osteocalcin, bone sialoprotein (BSP), inhibitor of DNA-binding protein (Id1) and the myogenic marker myogenin in C2C12 cells transfected with either pEF1 α -BMP-2adv or BMP-2adv minicircle, or treated with recombinant human BMP-2 (fold change relative to untransfected cells). Values are normalized to HPRT gene expression. Data is presented as means ± SD. n>13 (3 independent experiments). ** p<0.01, *** p<0.001.

Endogenous expression of osteocalcin and bone sialoprotein was significantly upregulated. Concomitantly, myogenin expression as negative control was decreased. Interestingly, the levels of Id1 were comparable with the conventional DNA vector.

Furthermore, to evaluate effects of BMP-2 minicircle in therapeutically relevant cells, alkaline phosphatase activity and cell mineralization were quantified in human bone marrow stem cells after 3 weeks. Significantly higher ALP activity was measured in BMSCs transfected with minicircle DNA (Figure 4a). These findings were further supported by increased mineralization in the Alizarin Red S stained minicircle group (Figure 4b). Taken together, the results show that nucleofection of a BMP-2 minicircle is capable of pushing BMSCs into the osteogenic lineage and achieves stronger induction of BMSCs osteogenic differentiation than a conventional plasmid with the same BMP-2 expression cassette.

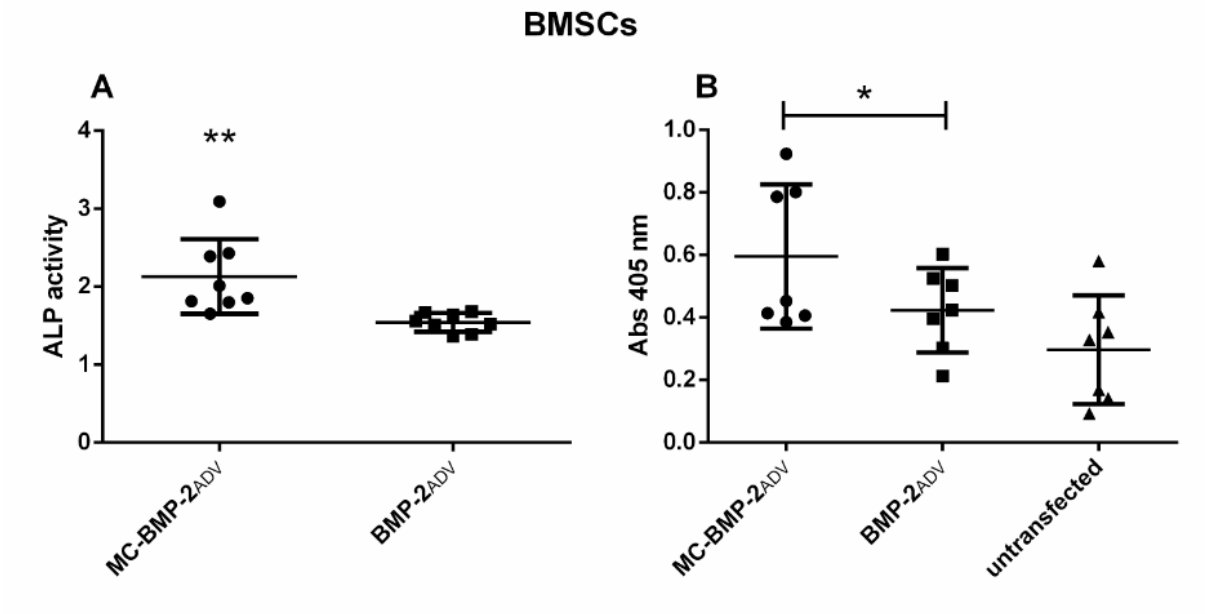

Figure 4: Efficiency of BMP-2 minicircle in bone marrow derived stem cells BMSCs were transfected with 1 µg of either conventional BMP-2-Adv plasmid (BMP-2adv) or minicircle BMP-2-Adv plasmid (MC-BMP-2adv). A: Osteogenic differentiation of BMSCs induced after plasmid transfection. Evaluation was performed by quantification of alkaline phosphatase activity 3 weeks post-transfection. B: Mineralization of BMSCs 3 weeks post-transfection quantified by Alizarin red staining. Data is presented as means ± SD. n=8 (ALP), n=7 (Alizarin red), * p<0.05, ** p<0.01.

Equimolar transfections show similar osteogenic effect with lower amounts of DNA and transfection reagent.

To determine the efficiency of BMP-2 minicircles to induce osteogenic differentiation, C2C12 and bone marrow derived

stem cells were transfected with equimolar amounts of conventional and minicircle BMP-2 advanced plasmid. It was shown that less amount of minicircle-BMP-2 was necessary to achieve similar ALP activity and mineralization in both cell types (Figure 5). The results highlight the capability of minimizing cytotoxic effects of plasmid or transfection agents by the application of similar biological outcome whilst applying less amounts of biological agents.

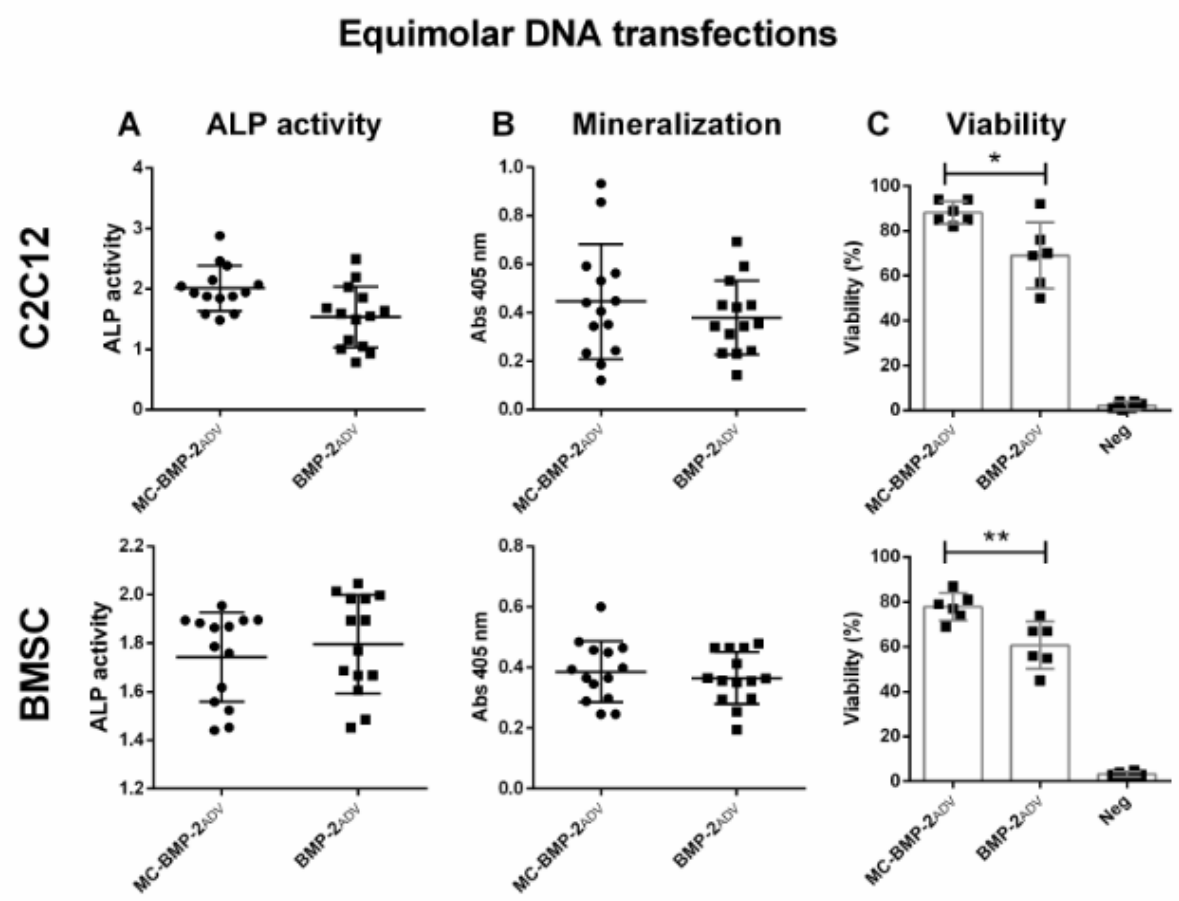

Figure 5: Transfection of C2C12 and BMSCs with equimolar DNA amounts. Cells were transfected with equimolar concentrations of the conventional BMP-2-Adv plasmid (BMP-2adv) and minicircle BMP-2-Adv plasmid (MC-BMP-2adv). A: Cell differentiation induced after plasmid transfection. Evaluation was performed by quantification of alkaline phosphatase activity 6 days post-transfection. B: Mineralization 3 weeks post-transfection quantified by Alizarin red staining. C: Cell viability, 24 hours post-transfection Data is presented as means ± SD. n=14 (ALP), n=14 (Alizarin red), n=6 (MTS), * $p<0.05$, ** $p<0.01$.

DISCUSSION

C2C12 cell line transfected with minicircle DNA produced significantly more BMP-2 protein compared to cells transfected with conventional plasmid carrying the same expression cassette (Figure 2b). Several studies provided evidence that the lack of prokaryotic plasmid backbone elements results in improved performance of minicircle plasmids. Antibiotic resistance genes and bacterial ORI segments can lead to a decrease in levels and duration of gene expression *in vivo* [45,46]. This can be explained by repressive heterochromatin formation on the bacterial backbone sequence which spreads and silences the transgene [47]. In this regard, Maniar et al. reported differences in DNA methylation between plasmid and minicircle vectors. The prolonged expression of minicircle DNA at a transcriptional level could be attributed to active chromatin structure [48].

Prokaryotic selection markers can cause integration into host cell genome or into human bacterial flora by horizontal gene transfer [49,50]. Exclusion of these sequences is beneficial for effective transgene expression and biosafety. Furthermore, it reduces the minicircle backbone size significantly. In our study, BMP-2 minicircle size is 54% smaller compared to conventional BMP-2-Advanced plasmid. An inverse correlation between vector size and gene delivery efficiency in human mesenchymal stem cells has been reported [51]. The same correlation was observed by Yin et al., when they compared transgene expression of different sized reporter plasmids. This phenomenon can be explained by the restricted free diffusion of larger DNA constructs in the cytoplasm and into the nucleus as opposed to smaller DNA molecules [52].

The robust and persistent transgene expression of minicircle DNA has been shown in many studies *in vitro* and *in vivo* [53-56]. We hypothesized that minicircle DNA may be an attractive

platform for bone regeneration, since stable and sustained levels of BMP-2 are crucial to enable bone regeneration, especially after large bone defects. Under physiological conditions, genomic BMP-2 expression can be observed over a longer time period during fracture healing in animal models [57]. Therefore, prolonged expression of transgenic BMP-2 is expected to be beneficial especially in large bone defects. To confirm our hypothesis, minicircle based induction of differentiation was investigated by constitutive expression of a previously described potent BMP-2 expression cassette under the control of the efficient EF1 α promote and compared to the activity of a conventional plasmid with the same expression cassette [23].

The minicircle construct did not only outperform the BMP-2 vector with the conventional plasmid backbone when transfecting the same total amount of DNA, but was also able to achieve comparable ALP activity and yield the same amount of protein when using equimolar amounts of transgenic BMP-2 (Figure 5). Hence, less transfection agent is needed to reach similar osteogenic effects. To date, most commercially available transfection reagents are still toxic (e.g. liver accumulation, tissue damage). Thus, reducing the amounts needed for clinical applications would be highly beneficial for the patient. Interestingly, for the minicircle DNA we expected better delivery efficiency and prolonged expression than the conventional plasmid, resulting into a better osteogenic effect than the traditional BMP-2 plasmid. However, when using equimolar of the BMP-2 minicircle and the conventional BMP-2 plasmid, BMP-2 minicircle did not outperform the conventional BMP-2 plasmid. Therefore, in this study, with the same molecule numbers, the BMP-2 minicircle did not exhibit better effects than the conventional BMP-2 plasmid, indicating no difference in gene delivery and duration of gene expression.

When comparing expression of different osteogenic marker genes, transfection with minicircle BMP-2-Advanced resulted in either higher or similar mRNA amount as conventional BMP-2-Advanced plasmid. Osteocalcin – a late marker for osteogenic differentiation – was significantly up-regulated relative to both C2C12 transfected with conventional BMP-2-Advanced plasmid and cells treated with recombinant BMP-2. Down-regulation of myogenin – a marker gene specific for differentiation into the myogenic lineage – was stronger in the minicircle group. Cells treated with conventional BMP-2-Advanced plasmid might partially have differentiated into the myogenic lineage, emphasizing the enhanced efficiency of BMP-2-Advanced minicircle. Furthermore, transfection with conventional BMP-2-Advanced plasmid resulted in lower bone sialoprotein mRNA levels – a late marker for osteogenic differentiation. Taking in account the outperformance of BMP-2-Advanced minicircle in regard to other markers examined in the course of this study, its superiority over conventional BMP-2-Advanced plasmid is clearly shown.

This study confirms that minicircle-induced osteogenic differentiation can also be applied to human stem cells. When introduced into human BMSCs, BMP-2-Advanced minicircle achieved significantly higher ALP activity and augmented matrix mineralization. The pro-osteogenic effect of exogenous BMP-2 introduced into BMSCs is corroborated by various studies [58-60]. Further development of this therapeutic strategy for bone regeneration is advantageous particularly for clinical therapy.

CONCLUSION

We developed a BMP-2 minicircle and investigated its potential as a non-viral gene therapeutic agent for osteogenic induction. We demonstrated the enhanced suitability of minicircle-DNA compared to conventional plasmid DNA at equal DNA dosage. Based on the reduced cell toxicity demonstrated by transfection experiments performed with equimolar DNA amounts, the results strongly suggest an advantage of delivering minicircles coding for BMP-2 in bone regeneration because they are safe, efficient and enable the sustained and prolonged delivery of host cell produced therapeutic protein. The combination of the optimized BMP-2 cassette and the minicircle system was able to further enhance the potential for osteogenic differentiation. The increased activity of osteogenic markers could be observed both in murine C2C12 and human BMSCs, showing the suitabilty of BMP-2 minicircles, particularly for stem cell-based approaches. Hence, safe application of gene therapy in autologous stem cell approaches presents a powerful tool to improve the field of bone regenerationin the near future. Further investigations, especially in *in vivo* settings will be necessary to confirm this hypothesis.

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