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Efficient *in vitro* delivery of Noggin siRNA enhances osteoblastogenesis

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Abstract

Several types of serious bone defects would not heal without invasive clinical intervention. One approach to such defects is to enhance the capacity of bone-formation cells. Exogenous bone morphogenetic proteins (BMP) have been utilized to positively regulate matrix mineralization and osteoblastogenesis, however, numerous adverse effects are associated with this approach. Noggin, a potent antagonist of BMPs, is an ideal candidate to target and decrease the need for supraphysiological doses of BMPs. In the current research we report a novel siRNA-mediated gene knock-down strategy to down-regulate Noggin. We utilized a lipid nanoparticle (LNP) delivery strategy in pre-osteoblastic rat cells. *In vitro* LNP-siRNA treatment caused inconsequential cell toxicity and transfection was achieved in over 85% of cells. Noggin siRNA treatment successfully down-regulated cellular Noggin protein levels and enhanced BMP signal activity which in turn resulted in significantly increased osteoblast differentiation and extracellular matrix mineralization evidenced by histological assessments. Gene expression analysis showed that targeting Noggin specifically in bone cells would not lead to a compensatory effect from other BMP negative regulators such as *Gremlin* and *Chordin*. The results from this study support the notion that novel therapeutics targeting Noggin have the clinically relevant potential to enhance bone formation without the need for toxic doses of exogenous BMPs. Such treatments will

undeniably provide safe and economical treatments for individuals whose poor bone repair results in permanent morbidity and disability.

Keywords: Biological sciences, Biomedical engineering, Bioengineering, Biotechnology, Cell biology, Genetics

1. Introduction

In spite of bone's natural regeneration ability following an injury, orthopaedic critical size defect (CSD), a type of serious bone defect, would not heal without invasive clinical intervention (Bosch et al., 1998; Schmitz and Hollinger, 1986; Vajgel et al., 2014). CSDs could occur following traumas (150,000 cases occur annually in the US (Cierny and Zorn, 1994)), tumor excision, developmental anomalies or infections. Currently, the most widely used surgical technique to treat CSD, is *autologous* bone grafting. In this procedure, the defect is filled with bone that has been removed from another part of the same patient's body, through an additional operation. Due to numerous morbidities associated with this technique (Myeroff and Archdeacon, 2011), there is a huge interest in ways to augment the naturally slow growth of bone to achieve fast and effective bone healing. Bone morphogenetic proteins (BMPs) are key members of the Transforming Growth Factor-β (TGF-β) superfamily that induce bone formation (Duneas et al., 1998; Simpson et al., 2006; Wu et al., 2016). Currently, two types of recombinant BMPs (hBMP-2 and hBMP-7) (Zakhary et al., 2005), are commercially available and have even been used clinically (Gautschi et al., 2007; Haidar et al., 2009a). However, extremely high doses of hBMP-2, thousands of times higher than what naturally occurs in body, would be necessary to achieve a therapeutic effect (Haidar et al., 2009a; Haidar et al., 2009b). Aside from the enormous costs, such doses cause serious safety issues such as toxicity, ectopic bone formation, ejaculation problems and tumors (Epstein, 2013; Evans, 2010; Haidar et al., 2009a; Haidar et al., 2009b).

The need for huge doses of hBMP-2 is mainly due to a cellular self-limiting negative feedback mechanism by BMP antagonists (Kloen et al., 2012; Rosen, 2006). One of the key antagonists of BMPs is a protein called Noggin (Chaturvedi et al., 2009; Davidson et al., 2007; Gerrard et al., 2005; McMahon et al., 1998; Sonntag et al., 2007; Takayama et al., 2009; Wan et al., 2007). BMP antagonists secreted in the extracellular space, such as Noggin, bind to BMP ligands and prevent them from activating BMP receptors (Basson, 2012). This phenomenon occurs by blocking the binding sites of both BMP receptors types 1 and 2 (Groppe et al., 2003). In homozygous Noggin deficient mouse models, excessive bone and cartilage and lack of joints are seen, due to unrestrained BMP signal activity. Similarly, in human, heterozygous mutations in the Noggin locus (NOG) cause joint defects (Groppe et al., 2003).

Recent developments in cell therapy and stem cell research have encouraged advanced autologous cell-based therapies for bone regeneration and treatment of fracture non-unions (Quarto et al., 2001; Undale et al., 2009). Nevertheless, finding the best type of cell with high potential for self-renewal and differentiation capacity is a challenge, particularly in the elderly population (Brockstedt et al., 1993; Stenderup et al., 2003). As such, development of a simple, safe and efficient strategy to increase the osteoblastic differentiation and bone formation potential of a small number of already available autologous precursor cells would revolutionize this approach.

One of tested approaches to decrease the expression of a protein such as Noggin is to interfere with its cellular production by small interfering RNA (siRNA). siRNA is a small sequence of nucleotides and a novel genetic tool that could be easily designed and produced against any protein-encoding gene (Lam et al., 2015).

Inhibition using siRNA is a practical approach to specifically target Noggin and decrease its mitigating effects on hBMP-2 (Kowalczewski and Saul, 2015), leading to enhanced osteoblastogenesis and bone formation (Manaka et al., 2011; Wan et al., 2007). A key limitation to successful application of siRNA as a therapeutic strategy is its delivery to the site of action. Various non-viral siRNA delivery vehicles have been developed, among them, lipid nanoparticles (LNPs) are in the most advanced stage of development. As such, in the current study we aimed to investigate the bone regenerative effects of Noggin siRNA, encapsulated in LNPs, on osteoblasts.

LNPs are presently the principal delivery systems that utilize the therapeutic potential of siRNA in cells (Davidson and McCray, 2011; Zimmermann et al., 2006). Successful and efficient delivery of siRNA has been reported by the use of LNPs (Lin et al., 2014); additionally, LNP-siRNA is able to achieve gene silencing at low doses (Basha et al., 2011; Rungta et al., 2013; Semple et al., 2010). There are various reports from clinical trials examining LNP-siRNA systems demonstrating positive results and clinical benefits (Tabernero et al., 2013). Several LNP-siRNA formulations are presently in different stages of clinical development by leading siRNA therapeutic companies; such products have demonstrated safe clinical profiles and promising activity (Alabi et al., 2012; Alnylam, 2014; Barros and Gollob, 2012; Burnett et al., 2011; Huang and Liu, 2011; Tekmira, 2014). However, LNP approaches to deliver Noggin siRNA to UMR-106 cells in order to enhance the efficiency of hBMP-2 has not been investigated previously and the effectiveness of this approach is unknown.

In this study, we hypothesized that the delivery of Noggin siRNA from LNPs would be efficient and can lead to enhanced osteoblastic differentiation and bone formation compared to control siRNA. To address our hypothesis, *in vitro* experiments were carried out to evaluate the cellular bone formation capacity in

response to siRNA-mediated Noggin inhibition. The aim of the current work was to stimulate bone formation using safe and low concentrations of hBMP-2. The proposed methodology consists in inhibiting Noggin signalling to increase the efficacy of low-dose hBMP-2 in the absence of its antagonist.

2. Materials and methods

2.1. Design

This is an *in vitro* basic experimental study designed to address our hypothesis. All experimental procedures complied with national and institutional guidelines at University of McGill. Our primary outcome was to investigate whether Noggin inhibition in pre-osteoblasts could result in enhanced osteoblastic differentiation and mineralized matrix formation. Rat osteoblastic cells derived from a single source were utilized for the experiments. Independent variables consisted of the cultured cells and their medium containing different concentrations of hBMP-2, lipid nanoparticles and Noggin siRNA. The dependent variables included the *Noggin* and other osteoblast-specific gene expression, Noggin protein expression, Alp activity, cell death and mineralized matrix production by osteoblasts. All the cell types and reagents that were utilized in the experiments are listed below.

2.2. Cell culture

Rat osteosarcoma cell line, UMR-106 cells, (donation from Dr. Pierre Moffatte) were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies®, ON, Canada) at 37 °C with 5% CO2, supplemented with 10% heat inactivated fetal calf serum (FBS, Life Technologies®, ON, Canada) and 1% antibiotics (Penicillin/Streptomycin). For hBMP-2 (R&D Systems®, ON, Canada) treatment or siRNA transfection, cells were initially plated at 4×10^4/well in 24 well plates and after 24 h, culture medium was removed and cells were rinsed using 1x phosphate buffer saline (PBS) and treatment and/or transfection were carried out as described below. Quantification of cell transfection efficiency by fluorescence activated cell sorting (FACS), measurement of alkaline phosphatase activity, and Alizarin Red Assay were carried out at indicated time points. For osteoblastic differentiation experiments, the growth medium was supplemented with 10 mM β-glycerophosphate and 250 μM ascorbic acid (both from Sigma-Aldrich, Ontario, Canada)) and the culture was continued for an additional period of 10 days. The culture medium was changed every third day.

2.3. Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity was determined in cell lysates using a colorimetric Alkaline Phosphatase Assay kit (Abcam Inc., Toronto, ON) according to the manufacturer's protocol. ALP enzyme activity was normalized to the total

protein content of the cell lysates from corresponding samples, measured by a Pierce BCA Assay kit (Thermo Fisher Scientific®, Ottawa, Ontario).

2.4. Gene expression

Gene expression was examined by quantitative real-time PCR. Total RNA from D1 cell cultures was isolated using the RNeasy Plus Mini Kit (Qiagen Inc., Ontario) as described in the manufacturer's protocol. Total RNA was treated with DNase and reverse transcribed using the Maxima First Strand cDNA synthesis kit with ds DNase (Thermo Fisher Scientific®, Ottawa, Ontario). Before use, RT samples were diluted 1:5. Gene expression was determined using assays designed with the Universal Probe Library from Roche (www.universalprobelibrary.com). For each qPCR assay, a standard curve was performed to ensure that the efficiency of the assay is between 90% and 110%. qPCR reactions were performed using PERFECTA QPCR FASTMIX II (Quanta), 2 μM of each primer and 1 μM of the corresponding UPL probe. The Viia7 qPCR instrument (Life Technologies) was used to detect the amplification level and was programmed with an initial step of 20 s at 95° C, followed by 40 cycles of: 1 s at 95° C and 20 s at 60° C. Relative expression ($RQ = 2^{-\triangle\triangle CT}$) was calculated using the Expression Suite software (Life Technologies), and normalization was done against *Actb*.

The probe/primer combinations that were used in our qPCR experiments are listed in Table 1.

2.5. Cell toxicity assay

Cell toxicity was evaluated using a LIVE/DEAD® Viability/Cytotoxicity Kit, for mammalian cells according to the manufacturer's instructions. Briefly, 1×10^5 UMR cells were cultured in 12 well plates for 24 h. The cells were then washed with 1x PBS thoroughly and were treated with Lipofectamine 2000 (Thermo Fisher

Table 1. List of primers used in RT-qPCR and the reference IDs of corresponding genes.

Gene	Reference ID	Forward Sequence	Reverse Sequence
Actin	NM_031144	cccgcgagtacaaccttct	cgtcatccatggcgaact
Noggin	NM_012990.1	cctgagcaagaagctgagga	accgggcagaaggtctgt
Smad1	NM_013130.2	ccactataagcgagtggagagc	aggctgtgctgagggttgta
Smad5	NM_021692.1	ataacaagagccgcttctgc	ccaccaacgtagtatagatggaca
Runx2	NM_053470.2	catccatccattccaccac	ggtggcagtgtcatcatctg
Ibsp	NM_012587.2	gcgatagttcggaggagga	cccctcagagtcttcgttgt
Gremlin	NM_019282.2	aggatccactgaggtgacaga	cagctgctggcagtaggg
Chordin	NM_057134.1	gaaccagcgcactgtcct	tcattctgtagcagcatgtgag

Scientific®, Ottawa, Ontario), negative control siRNA or the combination of Lipofectamine 2000 and the siRNA. Treatment groups were all in triplicate. The following day, 300 ul of live/dead assay reagent was added to each well in a group of treatment at a time. The samples were then incubated at 37 °C in the staining solution for an additional 15 min followed by immediate fluorescence microscopy imaging. Images were taken from six different microscope fields randomly. ImageJ software was used to count the total number of live and dead cells in each field of the treatment groups and the final number of live or dead cells was calculated by averaging the six obtained numbers from all fields. The viability percentage was calculated according to the following equation:

Viability % = (Number of Live cells/Number of total cells) x 100%

2.6. Protein expression (ELISA)

Expression of Noggin protein in cultured cells was measured using a sandwich enzyme immunoassay ELISA kit (Cloud-Clone Corp.) according to the producer's protocol. In summary, 100 μL standard or sample was added to each measurement well and the samples were incubated for two hours at 37 °C. 100 μL of prepared detection reagent A was added to each sample and incubated for one hour at 37 °C. The samples were washed three times before 100 μL prepared detection reagent B was added. After 30 min incubation at 37 °C the samples were washed 5 times and 90 μL of substrate solution was added and incubated for 20 min at 37 °C. Finally, 50 μL of stop solution was added and the plate was read at 450 nm using a SpectraMax i3 plate reader (Molecular Devices, CA, USA). All the ELISA experiments were performed in triplicate.

2.7. Noggin siRNA screening

siRNA-mediated *Noggin* down-regulation was investigated in UMR-106 rat pre-osteoblasts. Each sample was evaluated for *Noggin* expression normalized to *Actin* in triplicate using qPCR as explained above. The Noggin siRNA inducing the most significant *Noggin* suppression was evaluated for protein suppression in UMR-106 rat pre-osteoblasts by ELISA assay and was employed in the subsequent osteoblastic differentiation experiments.

2.8. Histological analysis (Alizarin Red Assay-staining and quantification)

After 10 days of the osteoblastic differentiation culture, UMR cells were stained for calcium deposition by alizarin red S (ARS) staining (Sigma-Aldrich). UMR cells were fixed in cold 4% paraformaldehyde for 5 min, washed three times with 1x PBS and stained with 1% ARS (pH adjusted to 4.3) for 15 min at room temperature. At this point, the ARS was removed from the plates by gentle suction

and the cells were washed by 1x PBS until the PBS was clear. The stained cells were observed by microscopy and images were captured from each well using a Canon EOS 60D camera (Canon). The stained cells were de-stained with 10% acetic acid (Sigma-Aldrich). To each well of a 24 well plate, 200 µl acetic acid was added and incubated at room temperature for 30 min with moderate shaking. A 100 µl aliquot of the eluted dye from each well was transferred to a well of a 96-well plate and the absorbance was measured at 562 nm using a SpectraMax i3 plate reader (Molecular Devices).

2.9. Statistical analysis

Statistical significance was assessed using student t-test and one-way analysis of variance, followed by Tukey multiple comparisons post-test, where applicable. The GraphPad software 5.0 and statistical package (Prism®) was used to perform the statistical analysis and to generate the graphs.

3. Results

3.1. Noggin expression in response to hBMP-2 treatment

The Noggin negative feedback loop in response to hBMP-2 treatment at different time points using qPCR is shown in Fig. 1. Treatment with hBMP-2 (100 ng/ml)
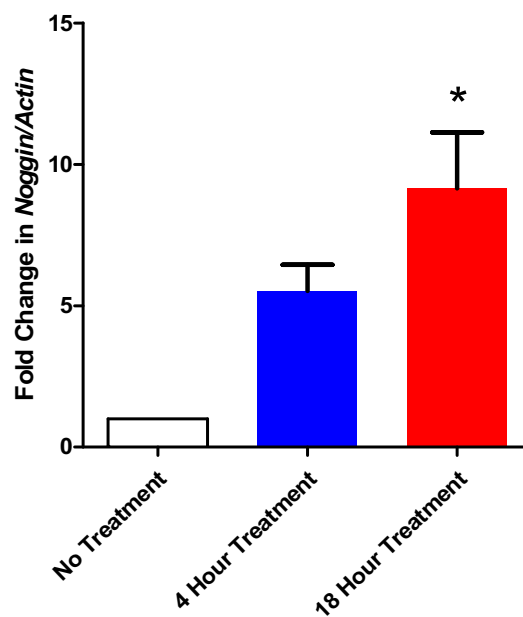

Fig. 1. hBMP2 treatment on UMR cells induces the *Noggin* response - Treatment with hBMP2 triggered the negative feedback response and resulted in a 5-fold and 9-fold increase in the expression of Noggin after 4 and 18 h, respectively. (n = 3) (Mean ± S.D. * $p < 0.05$).

led to a 5-fold and 9-fold increase in *Noggin* gene expression after 4 and 18 h, respectively (Fig. 1).

3.2. siRNA treatment and cell toxicity

Live-dead assay with cell treated with Lipofectamine-siRNA showed, on average, 20 dead cells out of 700 cells after 24-h treatment. The cell viability percentage was not statistically significantly different between three treatment groups of Lipofectamine alone, siRNA alone or the combination of Lipofectamine-siRNA (Fig. 2).
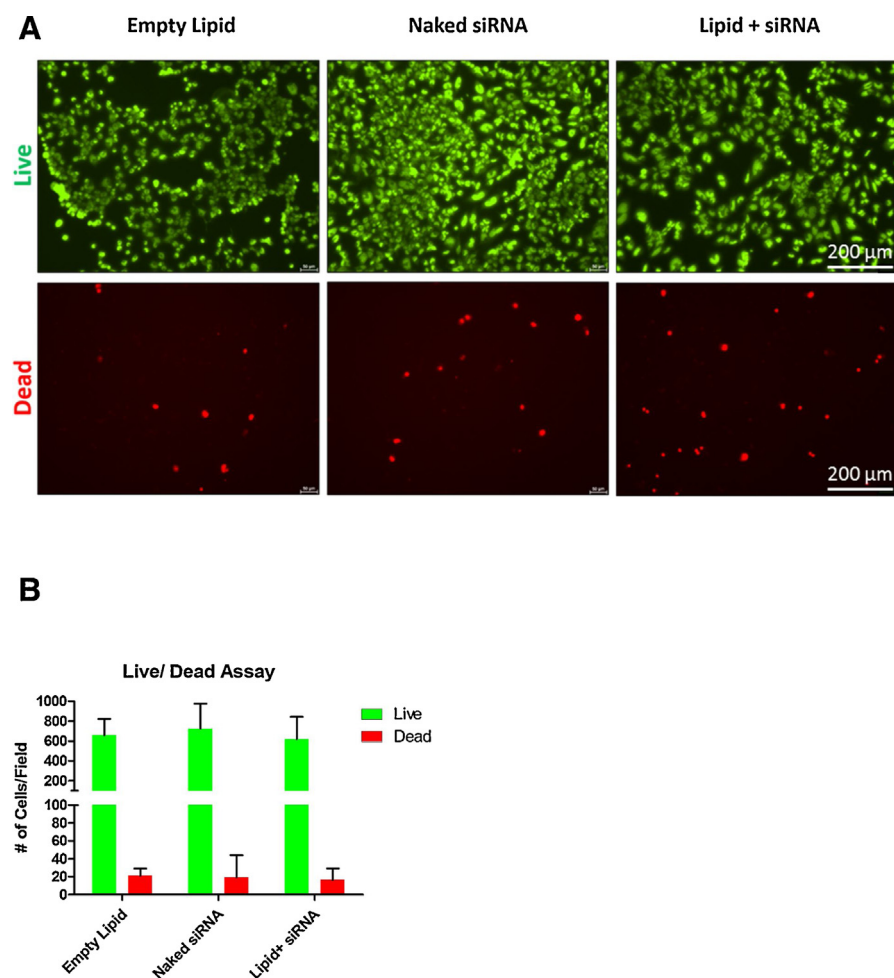

Fig. 2. Lipid nano-particle delivery of siRNA and cell toxicity - UMR cells were treated with only lipid nano particle, only siRNA or the combination of Lipid-nanoparticle and siRNA. **A.** LIVE/DEAD® Viability/Cytotoxicity assay revealed similar results in all three groups with minimal cell death (bottom panel, red) **B.** The majority of the cell population survived 24 h after the treatment in all three treatment groups. (n = 3, cells in six microscope fields were counted and averaged in each sample) (Mean ± S. D.).

3.3. siRNA treatment and transfection efficiency

The cell transfection efficiency of LNP-siRNA complex in osteoblast cells was tested by treating cultured UMR-106 cells with a Lipofectamine- fluorescently labeled negative siRNA and confocal microscopy was performed (Fig. 3A). Cell soring was performed on three groups of treatment with naked fluorescently labelled siRNA, Lipofectamine alone or the combination of Lipofectamine-fluorescently labeled siRNA. Treatment with 50 nM fluorescently labeled negative control siRNA resulted in over 85% transfection efficiency in the combination treatment group (Fig. 3B).

3.4. Noggin siRNA screening

We then screened a library of three different Noggin siRNAs −referred to as Noggin siRNA 1, 2 and 3- to find the most effective Noggin siRNA capable of down-regulating *Noggin* gene expression. UMR-106 cells initially treated with hBMP-2 were transfected with different Noggin siRNAs, then we checked the *Noggin* gene expression after 24 h. All Noggin siRNAs (1, 2 and 3) reduced the *Noggin* gene expression significantly compared to the negative control siRNA, and Noggin siRNA 1 was identified to be effective in over 60% *Noggin* gene knock-down after 24 h treatment, confirmed by RT-qPCR. This Noggin siRNA (Nog 1) was used for the rest of our experiments (Fig. 4).

3.5. Nog1 siRNA: Noggin protein knock down

Functionality of Nog 1 siRNA was tested *in vitro* by measuring the Noggin protein levels 48 h after treatment with this siRNA in culture. Protein expression analysis by sandwich enzyme immunoassay performed using a rat Noggin-specific ELISA assay (Cloud-Clone Corp®) confirmed a 70% decline in Noggin protein expression in response to Nog 1 siRNA treatment after 48 h (Fig. 5).

3.6. Other BMP signaling inhibitors

To determine whether the inhibition of Noggin would trigger a change in the activity of other BMP signaling inhibitors i.e. Gremlin and Chordin, the expression of *Gremlin* and *Chordin* in response to the treatment with Noggin siRNA (Nog 1) was investigated to explore any down-regulation or compensatory up-regulation response from these inhibitors. Pre-treatment with hBMP-2 was able to up-regulate the expression of these negative regulators of BMP signalling, however, interestingly, the same treatment with Nog-1 which led to a significant reduction in Noggin expression, did not cause any statistically significant change in the expression of either *Gremlin* or *Chordin* after 24 h of siRNA treatment (Fig. 6).
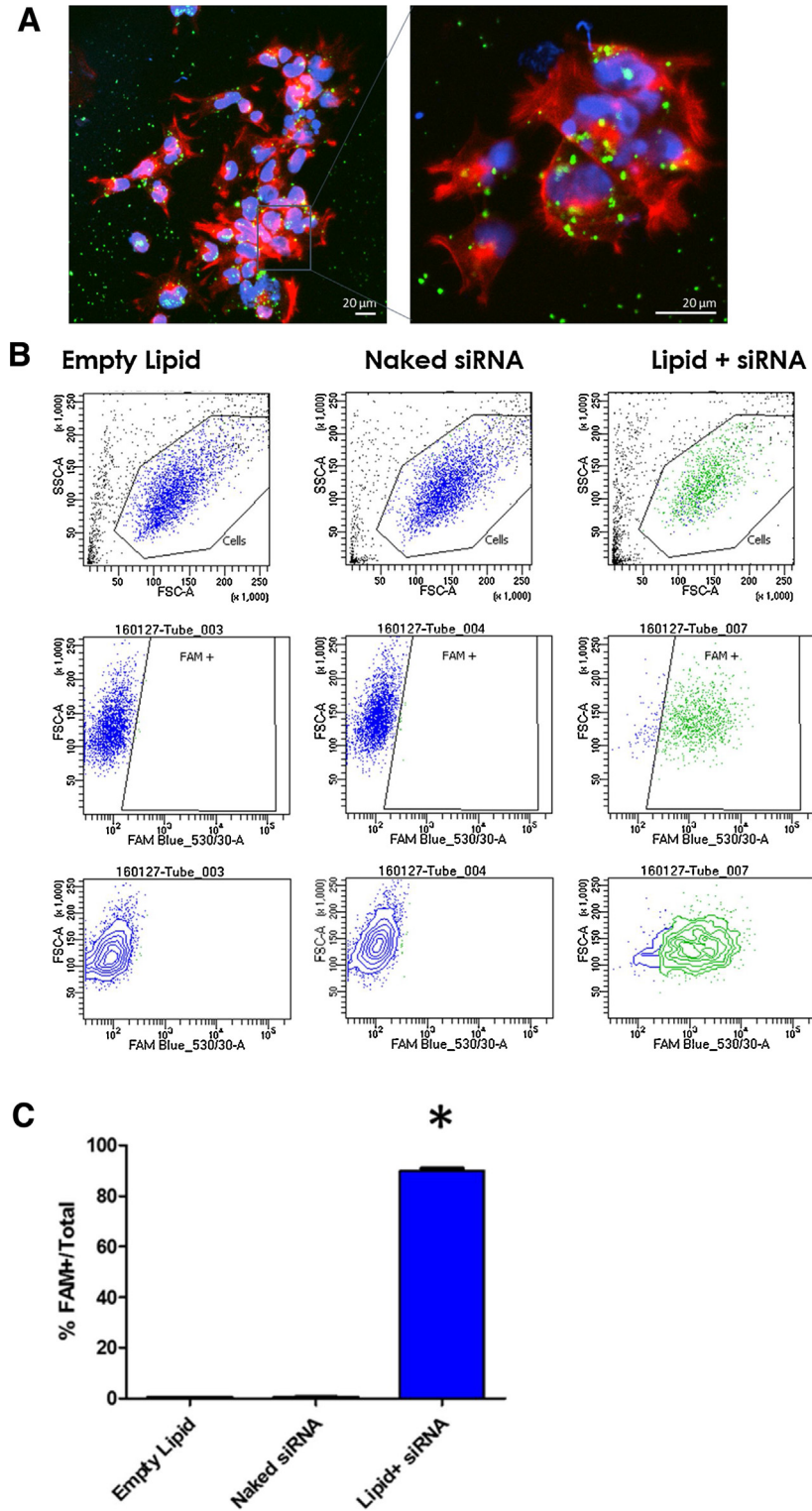
Fig. 3. Cell transfection efficiency - *A.* cultured UMR-106 cells were treated with a Lipofectamine-fluorescently labeled negative siRNA, confocal microscopy: Red: actin, Blue: nucleus, Green: siRNA. *B.*

3.7. Upregulation of downstream targets of BMP signal

The expression of direct BMP-2 downstream genes (*Smad 1&5*) and also osteoblast-specific genes after siRNA treatment were quantified to study the effects of Nog 1 siRNA on BMP downstream genes and osteoblastogenesis. Treatment with Nog 1 siRNA induced a significant increase in BMP-2 downstream targets *Smad 1* and *Smad 5* and resulted in an upward trend in the expression of *Bsp* and *Runx2*, only 24 h after treatment (Fig. 7).

3.8. Osteogenesis and matrix mineralization

To investigate the effectiveness of Nog1 siRNA in inducing the osteogenesis in culture, we designed another experiment to investigate osteoblast differentiation by measuring the activity of the specific early osteoblast differentiation marker, alkaline phosphatase (ALP) (Marom et al., 2005), and also the amount of mineralized matrix formation by the osteoblasts in culture. Cultured UMR-106 cells, treated with osteoblastic medium and Nog 1 siRNA, received low doses of hBMP-2 and the mineralized matrix formation was quantified after 10 days of culture by Alizarin Red staining assay. Treatment with Nog 1 siRNA resulted in a 2.5 fold increase in Alp activity (Fig. 8**A**) and over 3 fold increase in mineralized matrix formation, compared to the hBMP-2 treatment group, confirmed by Alizarin Red assay (Fig. 8 **B, C**).

4. Discussion

Despite the critical role of BMPs in accelerating bone formation in several orthopedic procedures, there has been growing evidence attributing various adverse events to the use of supraphysiologic doses of exogenous hBMP-2 in clinic (Epstein, 2013; Evans, 2010; Haidar et al., 2009a; Haidar et al., 2009b). For similar reasons and also lack of significant efficiency of rBMP-7 (OP-1), this product is not available in the market for regular orthopaedic use (El Bialy et al., 2017). This highlights the need for an alternative approach towards promoting physiologic bone repair. Noggin is a known target to achieve increased BMP efficacy as it is a key extracellular BMP antagonist (Canalis et al., 2003; Chen et al., 2004) and its inhibitory role in BMP-mediated osteogenesis has been extensively investigated both in animal models and rodent cells (Abe et al., 2000; Gazzerro et al., 1998). Several studies on animal models have exhibited the effectiveness of blockage of Noggin and consequent increase in BMP levels (Abe et al., 2000; Klineberg et al., 2014; Takayama et al., 2009; Wan et al., 2007). Nevertheless, previous studies utilized viral vector-mediated gene silencing

Cell sorting was performed based on the siRNA florescent tag: FAM. ***C.*** The graph shows the percentage of FAM^{+} cells over total number of cells counted.

**4 Hr BMP2 Treatment on UMR Cells (100ng/ml)
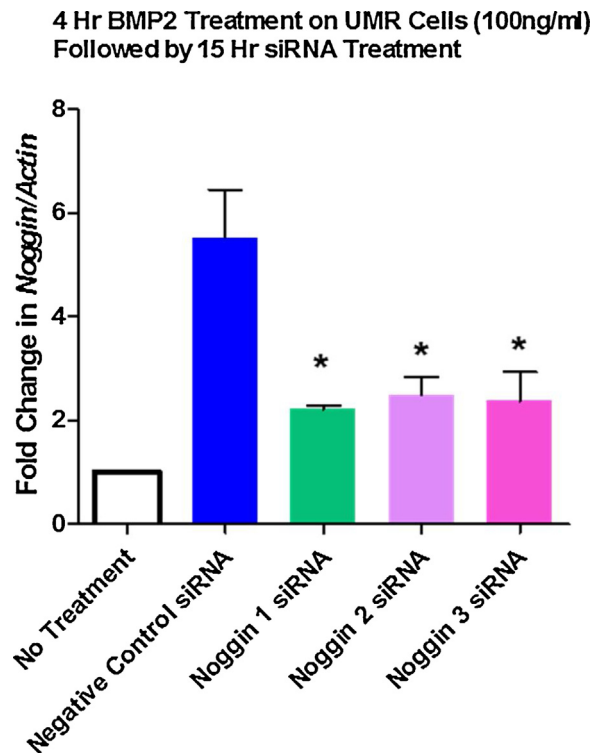Followed by 15 Hr siRNA Treatment**

Fig. 4. Noggin siRNA screening. A library of three different Noggin siRNAs was screened to find the most effective one in down-regulating the *Noggin* expression. The graph shows the results of RT-qPCR assay for *Noggin* expression. UMR-106 cells initially treated with hBMP-2 were transfected with different Noggin siRNAs, *Noggin* gene expression was quantified after 24 h and compared to the negative control siRNA. Noggin siRNA 1 was identified to be effective in over 60% *Noggin* gene knock-down after 24 h treatment. (n = 3) (Mean ± S.D.* $p < 0.05$).

methods which lacks realistic clinical translatability and raise serious safety concerns for clinical applications. In the current study, we aimed to test a natural and safe lipid-based delivery carrier for siRNA which bears the potential for actual clinical application and utility and as far as we are aware, this strategy has not been investigated previously on rat or human osteoblastic cells.

Treatment with recombinant hBMP-2 resulted in a significant up-regulation of Noggin very quickly. This is perhaps what contributes the most to low efficiency of exogenous hBMP-2 treatment in clinic, a huge negative feedback signal competing with hBMP-2 and necessitating higher doses. All of Noggin siRNAs that we tested were capable of efficient Noggin inhibition, however, we chose the most effective one and continued the experiments using that. Down-regulation of Noggin resulted in a significant increase in the expression of key downstream genes of BMP signalling and in the osteoblastic differentiation markers such as Alp. Furthermore, mineralized matrix formation by UMR cells was significantly enhanced in the Noggin siRNA treated groups. Extracellular matrix mineralization was detected by Alizarin Red staining as early as 10 days of culture in hBMP-2 and Noggin siRNA
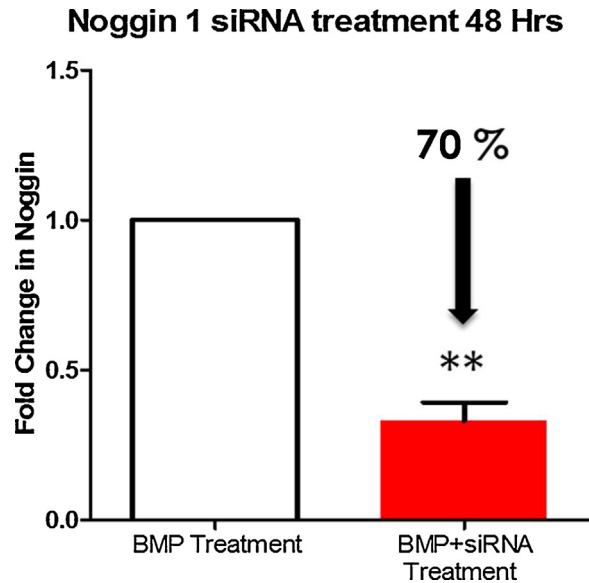
Noggin 1 siRNA treatment 48 Hrs

Fig. 5. Noggin protein knock down. Noggin protein levels were measured 48 h after treatment with Noggin 1 siRNA in culture. Sandwich enzyme immunoassay performed using a rat Noggin-specific ELISA assay (Cloud-Clone Corp.®) confirmed a 70% decline in Noggin protein expression in response to Nog 1 siRNA treatment after 48 h. (n = 3) (Mean ± S.D. * $p < 0.01$).

treated group. This confirms the combined effect of minimal doses of hBMP-2 and siRNA-mediated Noggin inhibition on bone regeneration and mineral formation.

The levels of Gremlin and Chordin were similarly unchanged in response to Noggin siRNA treatment and this suggests the specific targeting of the siRNA and no compensatory response from the other BMP signaling inhibitors. To the best of our knowledge, this aspect has never been investigated previously and the effects of Noggin inhibition on other BMP signal inhibitors was not clear. Our results revealed that not only is the Noggin inhibition via siRNA very specific, this
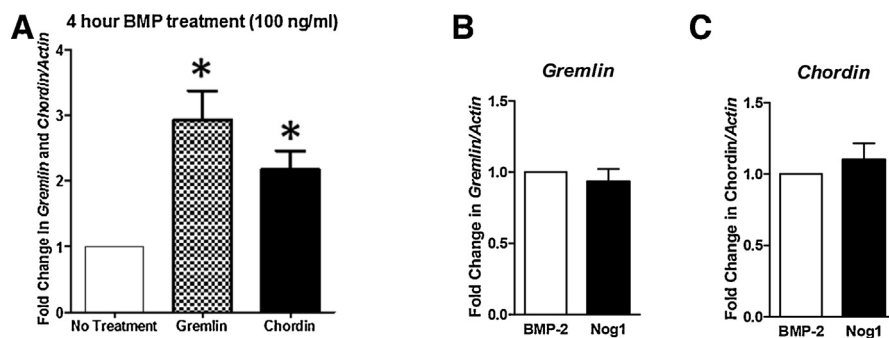

Fig. 6. Other BMP signaling inhibitors. *A.* Pre-treatment with 100 ng/ml hBMP-2 was performed. *B & C.* The expression of *Gremlin* and *Chordin* in response to the treatment with Noggin siRNA (Nog 1) was investigated after 24 h to explore any down-regulation or compensatory up-regulation response from these inhibitors. (n = 3) (Mean ± S.D. * $p < 0.05$).
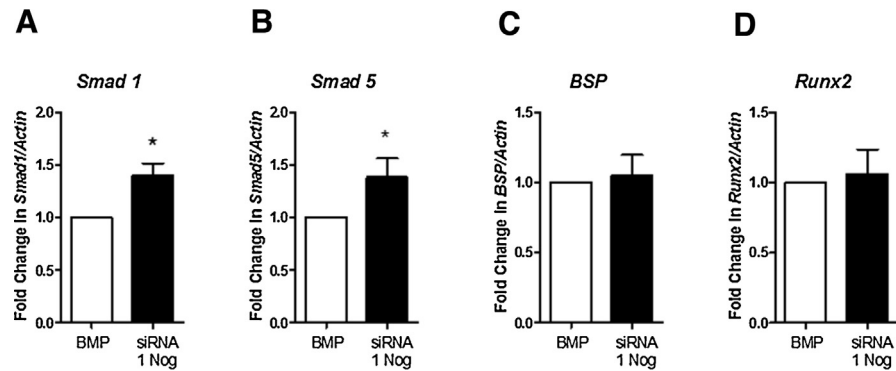
A **B** **C** **D**

Fig. 7. Upregulation of downstream targets of BMP signal. **A&B.** The expression of direct hBMP-2 downstream genes (*Smad 1&5*) and **C&D.** osteoblast-specific genes after siRNA treatment were quantified using RT-qPCR assay to study the effects of Nog 1 siRNA on BMP downstream genes and osteoblastogenesis. (n = 3) (Mean ± S.D. * $p < 0.05$).

inhibition does not trigger any compensatory inhibition by other BMP inhibitors. Nonetheless, these results provide basic evidence of the interaction between Noggin, Chordin and Gremlin pathways and further research is needed to elucidate these interactions in animal models of bone regeneration.

Cell toxicity assays showed very limited harmful effects of LNPs on the cultured cells. The development of new lipid delivery vehicles is an ever-changing area of research that constantly introduces novel carriers for drug delivery. Recent advancements in this area have led to the development of interesting LNPs which enhance the drug stability, improve bioavailability and minimize drug degradation (Li et al., 2008). One type of novel LNPs that has gained substantial attention by clinicians and proves to be suitable for orthopaedic applications is the layer-by-layer LNP consisting of natural biodegradable polymers. Our group and others have developed and characterized various LNPs with a lipid core and alginate-chitosan layers (Choi et al., 2013; Douglas and Tabrizian, 2005; Ghadakzadeh et al., 2016; Nayef et al., 2017). Such formulations possess interesting features which can encapsulate multiple therapeutic agents. Utilizing these nanoparticles would allow the slow release of Noggin siRNA and small nontoxic amounts of hBMP-2 simultaneously or chronologically over the course of bone repair. Treatment of large bone defects and particularly CSD is still a challenging orthopaedic procedure which would benefit the most from novel therapeutics, as such, this is certainly an area to be explored in future research. Additionally, it is of great importance to evaluate the pharmacokinetic profile of these delivery systems to safeguard localization of the siRNA and hBMP-2 cargo at the desired site of bone regeneration to prevent off-target effects.
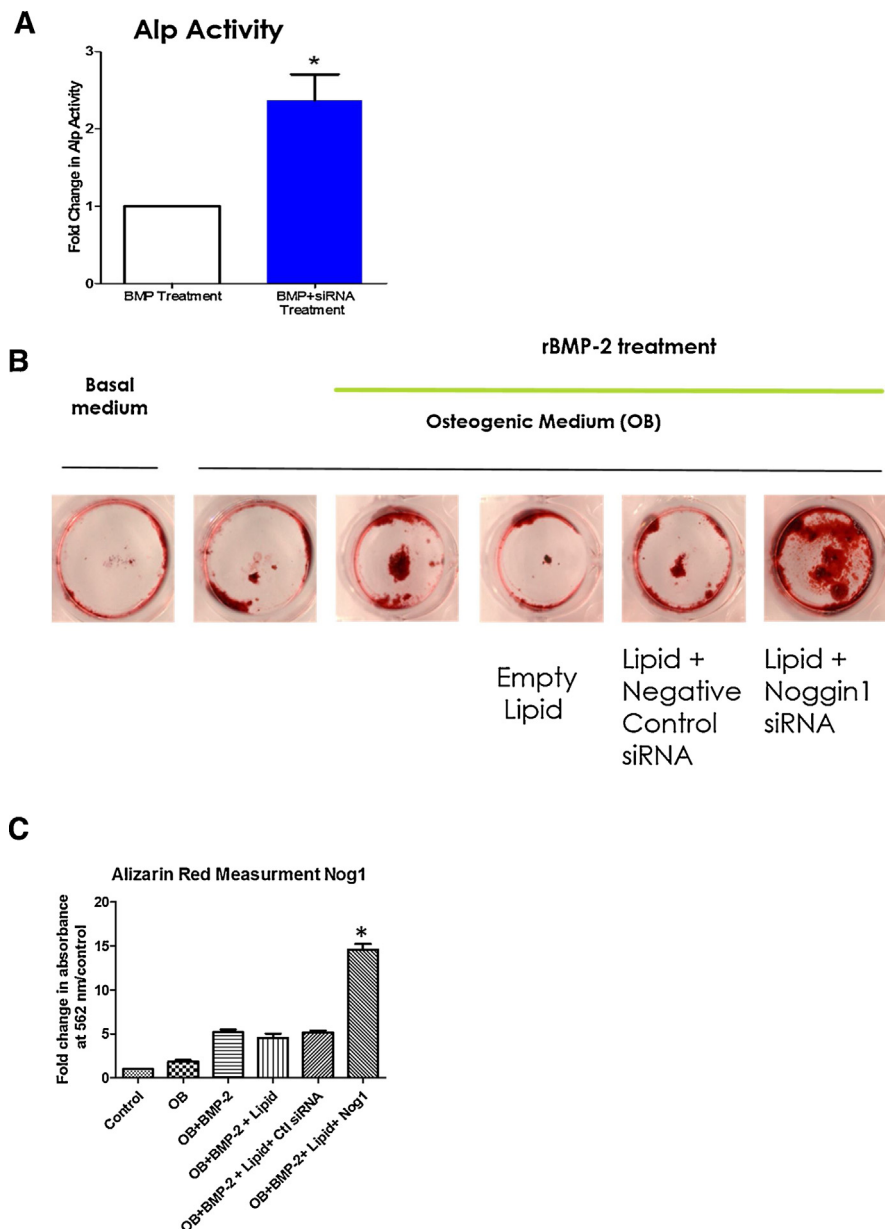
A

Alp Activity

B

rBMP-2 treatment

Basal medium

Osteogenic Medium (OB)

Empty Lipid

Lipid + Negative Control siRNA

Lipid + Noggin1 siRNA

C

Alizarin Red Measurment Nog1

Fig. 8. Osteogenesis and matrix mineralization. *A.* ALP activity measured by an ALP-specific enzymatic test on UMR cells treated with hBMP-2 and Noggin siRNA 1 for 7 days (n = 3) (mean ± S. D.* *p* < 0.05) *B.* Alizarin Red staining of cultured UMR cells with different treatments. *C.* Quantification of Alizarin Red stain. Treatment with Noggin siRNA, significantly enhances hBMP-2 efficiency and increases osteoblast mineral formation.

This work was performed *in vitro* and it is the main limitation of the study. Further preclinical research in animal models deems necessary to provide us with a more in-depth understanding of the future clinical applications of such therapeutics.

5. Conclusion

Our findings add to the growing body of basic evidence suggesting that Noggin inhibition can enhance the efficacy of hBMP-2 on osteoblastogenesis and bone formation. With technological advances in the field of nanotechnology and biomedical engineering, several lipid based nanoparticles are now available. We showed here that the delivery of siRNA to hard-to-transfect osteoblasts is done very efficiently by LNP. The outcomes of this research will undoubtedly assist safe and economical treatment of individuals whose poor bone repair results in permanent morbidity and disability.

Declarations

Author contribution statement

Saber Ghadakzadeh: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Reggie Charles Hamdy: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Maryam Tabrizian: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

References

Abe, E., Yamamoto, M., Taguchi, Y., Lecka-Czernik, B., O'Brien, C.A., Economides, A.N., Manolagas, S.C., 2000. Essential requirement of BMPs-2/4 for both osteoblast and osteoclast formation in murine bone marrow cultures from adult mice: antagonism by noggin. J. Bone Miner. Res. 15 (4), 663–673.

Alabi, C., Vegas, A., Anderson, D., 2012. Attacking the genome: emerging siRNA nanocarriers from concept to clinic. Curr. Opin. Pharmacol. 12 (4), 427–433.

Alnylam, 2014. Alnylam 5 × 15. http://www.alnylam.com/product-pipeline/alnylam-5(15/.

Barros, S.A., Gollob, J.A., 2012. Safety profile of RNAi nanomedicines. Adv. Drug Deliv. Rev. 64 (15), 1730–1737.

Basha, G., Novobrantseva, T.I., Rosin, N., Tam, Y.Y., Hafez, I.M., Wong, M.K., Cullis, P.R., 2011. Influence of cationic lipid composition on gene silencing properties of lipid nanoparticle formulations of siRNA in antigen-presenting cells. Mol. Ther. 19 (12), 2186–2200.

Basson, M.A., 2012. Signaling in cell differentiation and morphogenesis. Cold Spring Harb. Perspect. Biol 4 (6).

Bosch, C., Melsen, B., Vargervik, K., 1998. Importance of the critical-size bone defect in testing bone-regenerating materials. J. Craniofac. Surg. 9 (4), 310–316.

Brockstedt, H., Kassem, M., Eriksen, E.F., Mosekilde, L., Melsen, F., 1993. Age- and sex-related changes in iliac cortical bone mass and remodeling. Bone 14 (4), 681–691.

Burnett, J.C., Rossi, J.J., Tiemann, K., 2011. Current progress of siRNA/shRNA therapeutics in clinical trials. Biotechnol. J. 6 (9), 1130–1146.

Canalis, E., Economides, A.N., Gazzerro, E., 2003. Bone morphogenetic proteins, their antagonists, and the skeleton. Endocr. Rev. 24 (2), 218–235.

Chaturvedi, G., Simone, P.D., Ain, R., Soares, M.J., Wolfe, M.W., 2009. Noggin maintains pluripotency of human embryonic stem cells grown on Matrigel. Cell Prolif. 42 (4), 425–433.

Chen, D., Zhao, M., Mundy, G.R., 2004. Bone morphogenetic proteins. Growth Factors 22 (4), 233–241.

Choi, A.Y., Kim, C.T., Park, H.Y., Kim, H.O., Lee, N.R., Lee, K.E., Gwak, H.S., 2013. Pharmacokinetic characteristics of capsaicin-loaded nanoemulsions fabricated with alginate and chitosan. J. Agric. Food Chem. 61 (9), 2096–2102.

Cierny III, G., Zorn, K.E., 1994. Segmental tibial defects. Comparing conventional and Ilizarov methodologies. Clin. Orthop. Relat. Res. 301, 118–123.

Davidson, B.L., McCray Jr., P.B., 2011. Current prospects for RNA interference-based therapies. Nat. Rev. Genet. 12 (5), 329–340.

Davidson, K.C., Jamshidi, P., Daly, R., Hearn, M.T., Pera, M.F., Dottori, M., 2007. Wnt3a regulates survival, expansion, and maintenance of neural progenitors derived from human embryonic stem cells. Mol. Cell Neurosci. 36 (3), 408–415.

Douglas, K.L., Tabrizian, M., 2005. Effect of experimental parameters on the formation of alginate-chitosan nanoparticles and evaluation of their potential application as DNA carrier. J. Biomater. Sci. Polym. Ed. 16 (1), 43–56.

Duneas, N., Crooks, J., Ripamonti, U., 1998. Transforming growth factor-beta 1: induction of bone morphogenetic protein genes expression during endochondral bone formation in the baboon, and synergistic interaction with osteogenic protein-1 (BMP-7). Growth Factors 15 (4), 259–277.

El Bialy, I., Jiskoot, W., Reza Nejadnik, M., 2017. Formulation, Delivery and Stability of Bone Morphogenetic Proteins for Effective Bone Regeneration. Pharm. Res. 34 (6), 1152–1170.

Epstein, N.E., 2013. Complications due to the use of BMP/INFUSE in spine surgery: The evidence continues to mount. Surg. Neurol. Int. 4 (Suppl 5), S343–352.

Evans, C.H., 2010. Gene therapy for bone healing. Expert Rev. Mol. Med. 12, e18.

Gautschi, O.P., Frey, S.P., Zellweger, R., 2007. Bone morphogenetic proteins in clinical applications. ANZ J. Surg. 77 (8), 626–631.

Gazzerro, E., Gangji, V., Canalis, E., 1998. Bone morphogenetic proteins induce the expression of noggin, which limits their activity in cultured rat osteoblasts. J. Clin. Invest. 102 (12), 2106–2114.

Gerrard, L., Rodgers, L., Cui, W., 2005. Differentiation of human embryonic stem cells to neural lineages in adherent culture by blocking bone morphogenetic protein signaling. Stem Cells 23 (9), 1234–1241.

Ghadakzadeh, S., Mekhail, M., Aoude, A., Tabrizian, M., Hamdy, R.C., 2016. Small Players Ruling the Hard Game: siRNA in Bone Regeneration. J. Bone Miner. Res. 31 (7).

Groppe, J., Greenwald, J., Wiater, E., Rodriguez-Leon, J., Economides, A.N., Kwiatkowski, W., Choe, S., 2003. Structural basis of BMP signaling inhibition by Noggin, a novel twelve-membered cystine knot protein. J. Bone Joint Surg. Am. 85-A (Suppl 3), 52–58.

Haidar, Z.S., Hamdy, R.C., Tabrizian, M., 2009a. Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair. Part A: Current challenges in BMP delivery. Biotechnol. Lett. 31 (12), 1817–1824.

Haidar, Z.S., Hamdy, R.C., Tabrizian, M., 2009b. Delivery of recombinant bone morphogenetic proteins for bone regeneration and repair. Part B: Delivery systems for BMPs in orthopaedic and craniofacial tissue engineering. Biotechnol. Lett. 31 (12), 1825–1835.

Huang, L., Liu, Y., 2011. In vivo delivery of RNAi with lipid-based nanoparticles. Annu. Rev. Biomed Eng 13, 507–530.

Klineberg, E., Haudenschild, D.R., Snow, K.D., Garitty, S., Christiansen, B.A., Acharya, C., Gupta, M.C., 2014. The effect of noggin interference in a rabbit posterolateral spinal fusion model. Eur. Spine J.

Kloen, P., Lauzier, D., Hamdy, R.C., 2012. Co-expression of BMPs and BMP-inhibitors in human fractures and non-unions. Bone 51 (1), 59–68.

Kowalczewski, C.J., Saul, J.M., 2015. Surface-mediated delivery of siRNA from fibrin hydrogels for knockdown of the BMP-2 binding antagonist noggin. Acta Biomater. 25, 109–120.

Lam, J.K., Chow, M.Y., Zhang, Y., Leung, S.W., 2015. siRNA Versus miRNA as Therapeutics for Gene Silencing. Mol. Ther. Nucleic Acids 4, e252.

Li, P., Dai, Y.N., Zhang, J.P., Wang, A.Q., Wei, Q., 2008. Chitosan-alginate nanoparticles as a novel drug delivery system for nifedipine. Int. J. Biomed. Sci. 4 (3), 221–228.

Lin, Q., Chen, J., Zhang, Z., Zheng, G., 2014. Lipid-based nanoparticles in the systemic delivery of siRNA. Nanomedicine (Lond) 9 (1), 105–120.

Manaka, T., Suzuki, A., Takayama, K., Imai, Y., Nakamura, H., Takaoka, K., 2011. Local delivery of siRNA using a biodegradable polymer application to enhance BMP-induced bone formation. Biomaterials 32 (36), 9642–9648.

Marom, R., Shur, I., Solomon, R., Benayahu, D., 2005. Characterization of adhesion and differentiation markers of osteogenic marrow stromal cells. J. Cell Physiol. 202 (1), 41–48.

McMahon, J.A., Takada, S., Zimmerman, L.B., Fan, C.M., Harland, R.M., McMahon, A.P., 1998. Noggin-mediated antagonism of BMP signaling is required for growth and patterning of the neural tube and somite. Genes Dev. 12 (10), 1438–1452.

Myeroff, C., Archdeacon, M., 2011. Autogenous bone graft: donor sites and techniques. J. Bone Joint Surg. Am. 93 (23), 2227–2236.

Nayef, L., Castiello, R., Tabrizian, M., 2017. Washless Method Enables Multilayer Coating of an Aggregation-Prone Nanoparticulate Drug Delivery System with Enhanced Yield, Colloidal Stability, and Scalability. Macromol. Biosci.

Quarto, R., Mastrogiacomo, M., Cancedda, R., Kutepov, S.M., Mukhachev, V., Lavroukov, A., Marcacci, M., 2001. Repair of large bone defects with the use of autologous bone marrow stromal cells. N. Engl. J. Med. 344 (5), 385–386.

Rosen, V., 2006. BMP and BMP inhibitors in bone. Ann. N. Y. Acad. Sci. 1068, 19–25.

Rungta, R.L., Choi, H.B., Lin, P.J., Ko, R.W., Ashby, D., Nair, J., Macvicar, B.A., 2013. Lipid Nanoparticle Delivery of siRNA to Silence Neuronal Gene Expression in the Brain. Mol. Ther. Nucleic Acids 2, e136.

Schmitz, J.P., Hollinger, J.O., 1986. The critical size defect as an experimental model for craniomandibulofacial nonunions. Clin. Orthop. Relat. Res. 205, 299–308.

Semple, S.C., Akinc, A., Chen, J., Sandhu, A.P., Mui, B.L., Cho, C.K., Hope, M.J., 2010. Rational design of cationic lipids for siRNA delivery. Nat. Biotechnol. 28 (2), 172–176.

Simpson, A.H., Mills, L., Noble, B., 2006. The role of growth factors and related agents in accelerating fracture healing. J. Bone Joint Surg. Br. 88 (6), 701–705.

Sonntag, K.C., Pruszak, J., Yoshizaki, T., van Arensbergen, J., Sanchez-Pernaute, R., Isacson, O., 2007. Enhanced yield of neuroepithelial precursors and midbrain-like dopaminergic neurons from human embryonic stem cells using the bone morphogenic protein antagonist noggin. Stem Cells 25 (2), 411–418.

Stenderup, K., Justesen, J., Clausen, C., Kassem, M., 2003. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. Bone 33 (6), 919–926.

Tabernero, J., Shapiro, G.I., LoRusso, P.M., Cervantes, A., Schwartz, G.K., Weiss, G.J., Burris III, H.A., 2013. First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement. Cancer Discov. 3 (4), 406–417.

Takayama, K., Suzuki, A., Manaka, T., Taguchi, S., Hashimoto, Y., Imai, Y., Takaoka, K., 2009. RNA interference for noggin enhances the biological activity of bone morphogenetic proteins in vivo and in vitro. J. Bone Miner. Metab. 27 (4), 402–411.

Tekmira, 2014. Pipeline Products. http://www.tekmira.com/pipeline/pipeline-products.php.

Undale, A.H., Westendorf, J.J., Yaszemski, M.J., Khosla, S., 2009. Mesenchymal stem cells for bone repair and metabolic bone diseases. Mayo Clin. Proc. 84 (10), 893–902.

Vajgel, A., Mardas, N., Farias, B.C., Petrie, A., Cimoes, R., Donos, N., 2014. A systematic review on the critical size defect model. Clin. Oral Implants Res. 25 (8), 879–893.

Wan, D.C., Pomerantz, J.H., Brunet, L.J., Kim, J.B., Chou, Y.F., Wu, B.M., Longaker, M.T., 2007. Noggin suppression enhances in vitro osteogenesis and accelerates in vivo bone formation. J. Biol. Chem. 282 (36), 26450–26459.

Wu, M., Chen, G., Li, Y.P., 2016. TGF-beta and BMP signaling in osteoblast, skeletal development, and bone formation, homeostasis and disease. Bone Res. 4, 16009.

Zakhary, K., Motakis, D., Hamdy, R.H., Campisi, P., Amar, Y., Lessard, M.L., 2005. Effect of recombinant human bone morphogenetic protein 7 on bone density during distraction osteogenesis of the rabbit mandible. J. Otolaryngol. 34 (6), 407–414.

Zimmermann, T.S., Lee, A.C., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M. N., MacLachlan, I., 2006. RNAi-mediated gene silencing in non-human primates. Nature 441 (7089), 111–114.