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Characterization of the essential role of bone morphogenetic protein 9 (BMP9) in osteogenic differentiation of mesenchymal stem cells (MSCs) through RNA interference

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Recombinant adenovirus;
siRNA

Abstract Mesenchymal stem cells (MSCs) are multipotent stem cells and capable of differentiating into multiple cell types including osteoblastic, chondrogenic and adipogenic lineages. We previously identified BMP9 as one of the most potent BMPs that induce osteoblastic differentiation of MSCs although exact molecular mechanism through which BMP9 regulates osteogenic differentiation remains to be fully understood. Here, we seek to develop a recombinant adenovirus system to optimally silence mouse BMP9 and then characterize the important role of BMP9 in osteogenic differentiation of MSCs. Using two different siRNA bioinformatic prediction programs, we design five siRNAs targeting mouse BMP9 (or simB9), which are expressed under the control of the converging H1 and U6 promoters in recombinant adenovirus vectors. We demonstrate that two of the five siRNAs, simB9-4 and simB9-7, exhibit the highest efficiency on silencing exogenous mouse BMP9 in MSCs. Furthermore, simB9-4 and simB9-7 act synergistically in inhibiting BMP9-induced expression of osteogenic markers, matrix mineralization and ectopic bone formation from MSCs. Thus, our findings demonstrate the important role of BMP9 in osteogenic differentiation of MSCs. The characterized simB9 siRNAs may be used as an important tool to investigate the molecular mechanism behind BMP9 osteogenic signaling. Our results also indicate that recombinant adenovirus-mediated expression of siRNAs is efficient and sustained, and thus may be used as an effective delivery vehicle of siRNA therapeutics.

Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells with self-renewal and capability of differentiating into multiple cell types, such as osteoblastic, chondrogenic, and adipogenic lineages.[1–8] MSCs have attracted profound attentions in the arena of stem cell biology and regenerative medicine.[4,7–12] Osteogenic differentiation from MSCs is a tightly regulated process of sequential events that recapitulate most of the molecular processes occurring during bone and skeletal development.[7,8,13] While osteogenic differentiation is regulated by numerous pathways, such as Wnt, Insulin-like growth factors (IGFs), Fibroblast growth factor (FGFs) and Notch,[3,7,8,14–23] bone morphogenetic proteins (BMPs) are considered as the only group of osteoinductive factors that can induce de novo bone formation from MSCs.[24,25]

BMPs are members of the TGF-β superfamily and play critical roles in skeletal development, bone formation and stem cell differentiation.[3,24–26] At least 14 types of BMPs have been identified in humans and rodents.[24,25,27] We conducted a comprehensive analysis of the osteogenic activities of the 14 types of BMPs, and found that BMP9 (also known as growth differentiation factor 2, or GDF2) is one of the most potent BMPs that induce osteoblastic differentiation of MSCs.[24,28–31] However, BMP9 is one of the least studied BMPs, and the exact molecular mechanism through which BMP9 regulates osteogenic differentiation remains to be fully understood.

Originally discovered in *C. elegans* and subsequently demonstrated in diverse eukaryotes, such as insects, plants, fungi and vertebrates, RNA interference (RNAi) is a cellular process of sequence-specific, post-transcriptional gene silencing initiated by short double-stranded RNAs (dsRNA), or short interfering RNAs (siRNAs), which are homologous to the gene being suppressed through the RNA-induced silencing complex (RISC).[32–39] Since its discovery, RNAi has become a valuable and powerful tool to analyze loss-of-function phenotypes,[33–38] as well as offers unprecedented opportunities for developing novel and effective therapeutics for human diseases.[40–45] Nonetheless, the silencing efficiency of siRNAs is highly empirical and varies drastically, depending on many parameters, such as mRNA secondary structures, target availability, status of matching and intrinsic characteristics of mRNA and siRNA.[33,46–49]

In this study, we developed a recombinant adenovirus system to express the optimal siRNAs targeting mouse BMP9 and characterized the important role of BMP9 in osteogenic differentiation of MSCs. Using different siRNA bioinformatic prediction programs, we designed five siRNAs targeting mouse BMP9 (or simB9 siRNAs), which were expressed under the control of converging H1 and U6 promoters in recombinant adenovirus vectors. We demonstrated that two of the five siRNAs, simB9-4 and simB9-7, exhibited the highest efficiency on silencing exogenous mouse BMP9 in MSCs. Furthermore, we demonstrated that simB9-4 and simB9-7 acted synergistically in impairing BMP9-induced expression of osteogenic markers, matrix mineralization and ectopic bone formation from MSCs. Thus, our findings demonstrate the important role of BMP9 in osteogenic differentiation of MSCs. The characterized simB9 siRNAs may be used as an important tool to investigate the molecular mechanism behind BMP9 osteogenic signaling. Lastly, our results indicate that recombinant adenovirus-mediated

expression of siRNAs is efficient and sustained, and thus may be used as a potential delivery vehicle of siRNA therapeutics.

Materials and methods

Cell culture chemicals

HEK-293 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). HEK-293 derivatives 293pTP and RAPA were previously characterized.[50,51] The immortalized mouse adipose-derived multipotent (iMAD) cells are mouse mesenchymal stem cells as previously characterized.[52] All cell lines were maintained in the completed Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 100 units of penicillin and 100 μg streptomycin, cultured at 37 °C in 5% CO_2 incubator as described.[53–56] Unless indicated otherwise, all reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Thermos Fisher Scientific (Waltham, MA, USA).

Construction and generation of recombinant adenoviruses expressing mouse BMP9, RPF, and simB9s

Recombinant adenoviruses were generated using the AdEasy system as previously described.[57–59] The coding region of mouse BMP9 was amplified and subcloned into adenoviral shuttle vector pAdTrace-TOX. The siRNAs targeting mouse Bmp9 coding region (or simB9s) were designed by using the Dharmacon's *siDESIGN* and IDT's DsiRNA design tools as described previously.[39,49] The expression of the siRNAs was driven by converging U6 and H1 promoters as described (Fig. 1).[39,49] The siRNA oligo cassettes (Fig. 1A) were subcloned into the homemade adenoviral shuttle vector pSES1 or pAdTrace-OK.[39,49] All adenoviral shuttle vectors were subjected to homologous recombination with adenoviral backbone in BJ5183/AdEasy1 cells. The resultant recombinants were used to package recombinant adenoviruses in 293pTP and/or RAPA cell lines,[50,51] yielding AdBMP9, AdsimB9-1, 2, 4, 7 and 8, respectively, all of which co-express monomeric RFP (mRFP). AdRFP was used as a mock virus control as reported.[53,60–63] For all adenovirus infection, polybrene (4–8 μg/ml) was added to improve infection efficiency as previously reported.[64]

RNA isolation and touchdown quantitative real-time PCR (TqPCR)

Subconfluent iMAD cells were infected with the indicated adenoviruses. At the indicated time points, total RNA was isolated by using NucleoZOL Reagent (Takara Bio USA, Mountain View, CA) according to the manufacturer's introduction and subjected to reverse transcription with a hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA). The resultant cDNA products were diluted 50-folds and used as templates for TqPCR. PCR primers were designed by Primer3 Plus program (Supplemental Table 1). The touchdown-quantitative PCR analysis was carried out by our optimized TqPCR protocol[65] using the 2 × SYBR Green qPCR master mix (Bimake, Houston, TX). All sample values were normalized to Gapdh expression by using the $2^{-\Delta\Delta Ct}$ method as described.[7,8]

Alkaline phosphatase (ALP) assays

Subconfluent iMAD cells were infected with the indicated adenoviruses and subjected to ALP assays at the indicated time points. ALP activity was quantitatively assessed by using the modified Great Escape SEAP chemiluminescence assay (using p-nitrophenyl phosphate as a substrate) and/or histochemical staining assay (using a mixture of 0.1 mg/ml napthol AS-MX phosphate and 0.6 mg/ml Fast Blue BB salt) as previously described.[66–70]

Matrix mineralization assay (Alizarin Red S staining)

Subconfluent iMAD cells were infected with the indicated adenoviruses and cultured in the presence of ascorbic acid (50 mg/ml) and β-glycerophosphate (10 mM). At day 7 and day 11 after infection, mineralized matrix nodules were stained for calcium precipitation by means of Alizarin Red S staining as described.[71–75] The staining of calcium mineral deposits was documented under bright field microscopy.

Subcutaneous stem cell implantation and ectopic bone formation

The use and care of animals in this study was approved by the Institutional Animal Care and Use Committee. All experimental procedures were carried out in accordance with the approved guidelines. The subcutaneous stem cell implantation was performed as previously described.[29,76–80] Briefly, subconfluent iMAD cells were infected with various adenoviruses for 36 h and collected for subcutaneous injection into the flanks of athymic nude mice (Envigo/Harlan Research Laboratories; n = 5/group, female, 5–6 week old; 5 × 10^6 cells per injection). The animals were maintained ad lib in the biosafety barrier facility. At 4 weeks after injection, animals were sacrificed for ectopic mass harvest.

Hematoxylin and eosin (H & E) staining

The retrieved specimens were fixed with 10% formalin, decalcified and embedded in paraffin. Serial sections at 5 μm of the embedded specimens were carried out, and mounted onto treated slides. Then the sections were deparaffinized and then rehydrated in a graduated fashion. H & E staining was done as described.[66,72,75]

Statistical analysis

All quantitative experiments were performed in triplicate and/or repeated three times. Data were expressed as mean ± standard deviation (SD). The one-way analysis of variance was used to analyze statistical significance.[81] A value of $p < 0.05$ was considered statistically significant.

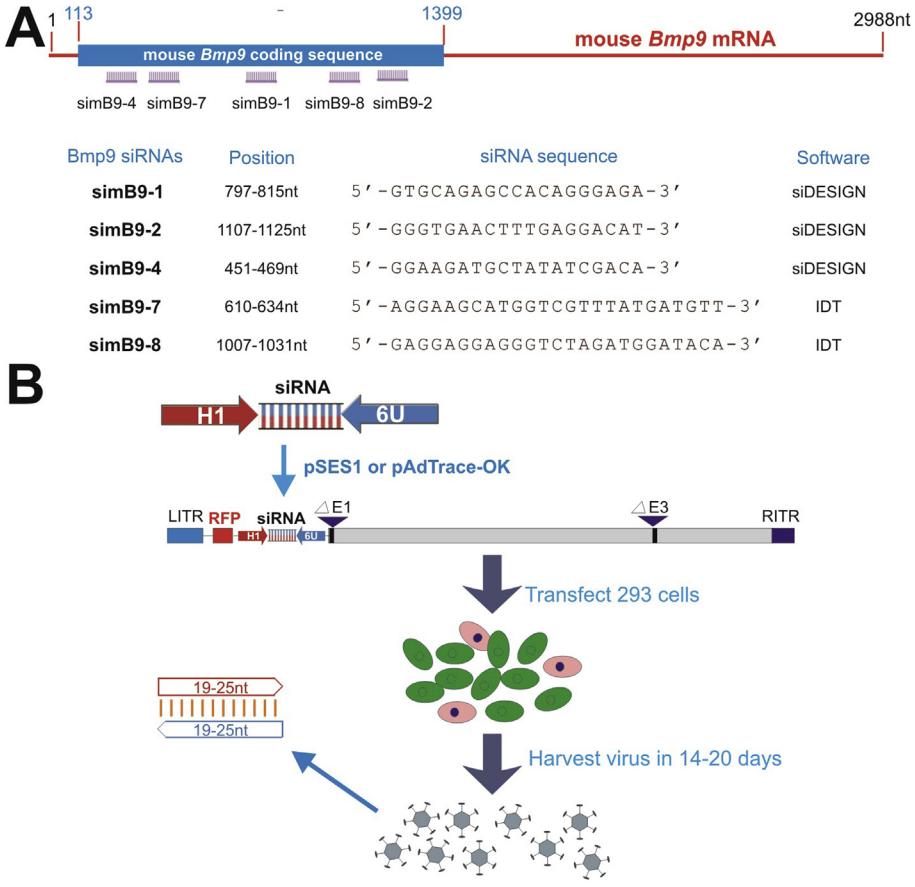

Figure 1 Selection and construction of recombinant adenoviruses expressing optimal siRNAs targeting the mouse BMP9 coding region (simB9s). (**A**) Schematic representation of mouse Bmp9 coding region and the location and sequences of the five siRNA targeting sites designed by the Dharmacon's siDESIGN and IDT's DsiRNA design tools. (**B**) Schematic depiction of the expression of siRNAs driven by the convergent H1 and U6 promoters in recombinant adenoviral vectors through the homemade shuttle vector pSES1 or pAdTrace-61, which co-expresses mRFP as a tracking marker. The resultant siRNA-expressing simB9 siRNA adenoviruses are designated as AdsimB9-1, 2, 4, 7 and 8, respectively. AdBMP9 expresses mouse BMP9 and mRFP, while AdRFP was used as a mock control virus.

Results and discussion

Selection of optimal siRNAs that effectively silence the expression of mouse BMP9 in mesenchymal stem cells (MSCs)

We previously developed a fluorescence-based optimal siRNA selection system and demonstrated its utility for evaluating the siRNA silencing efficiency.[49] We and others have since used this siRNA expression system and demonstrated effective gene silencing in numerous studies.[7,8,39,71,72,82,83] In order to effectively knock down mouse BMP9 expression in mesenchymal stem cells (MSCs), we designed five siRNAs targeting the mouse BMP9 coding region, namely simB9-1, simB9-2, simB9-4, simB9-7 and simB9-8 (Fig. 1A). The expression of these siRNAs was driven by the converging H1 and U6 promoters as described,[39,49] followed by generation of recombinant adenoviruses to ensure high transduction efficiency (Fig. 1B).

We first examined whether exogenously expressed mouse BMP9 could be effectively silenced by adenovirus-mediated transduction of simB9 siRNAs in MSCs. As expected, the recombinant adenoviruses transduced the iMAD cells with high efficiency (Fig. 2A). Through qPCR analysis, we found that the endogenous BMP9 expression was fairly low in iMADs cells (Fig. 2B). However, the exogenously expressed mouse BMP9 was significantly silenced by the five simB9 siRNAs to various degrees, with simB9-4 and simB9-7 being the most effective (Fig. 2B). It is noteworthy that we constructed two scrambled siRNAs as negative controls and obtained similar results to that of the GFP or RFP control group (data not shown). To simplify the experimental design we thus used Ad-GFP or Ad-RFP as a negative control.

Exogenous mouse BMP9-induced osteogenic differentiation can be attenuated by simB9 siRNAs in MSCs

We previously demonstrated that BMP9 is one of the most potent osteogenic factors and yet least studied.[24,28–30,76] Using these simB9 siRNA adenoviral vectors, we examined

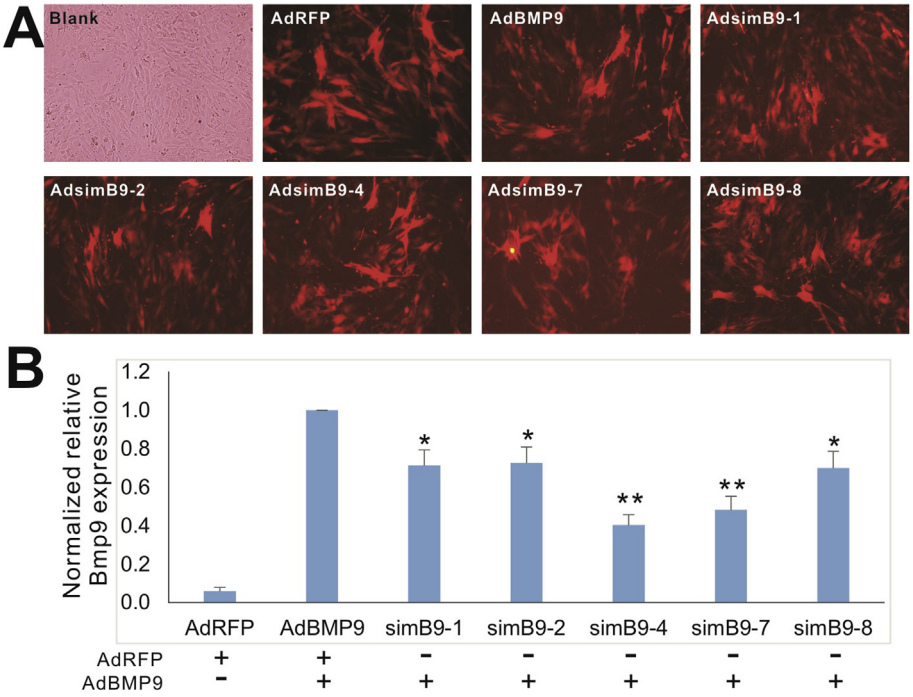

Figure 2 Characterization of the silencing efficiency of the selected five simB9 siRNAs targeting exogenously expressed mouse BMP9. (**A**) Efficient transduction of subconfluent iMAD MSCs by recombinant adenoviruses expressing siRNAs targeting mouse Bmp9. RFP signal was recorded at 48 h after infection. Representative results are shown. (**B**) siRNA-mediated knockdown of exogenously expressed mouse BMP9 in iMADs cells. Subconfluent iMADs were infected with the equal titer of the indicated adenoviruses. At 48 h after infection, total RNA was isolated and subjected to reverse transcription and qPCR analysis of the indicated mouse genes. All samples were normalized with Gapdh. Each assay condition was done in triplicate. "*" $p < 0.05$, "**" $p < 0.01$ compared with the AdBMP9-infected group.

whether BMP9-induced osteogenic differentiation would be effectively hampered by BMP9-specific siRNAs in MSCs. We found that co-expression of mouse BMP9 and its simB9 siRNAs resulted in decreases in early osteogenic marker ALP activity to various degrees both qualitatively (Fig. 3A) and quantitatively (Fig. 3B). Consistent with qPCR findings from Fig. 2B, simB9-4 and simB9-7 were shown to exhibit the highest inhibitory effects on BMP9-induced ALP activity, at as early as day 3 and day 5 (Fig. 3A—a vs 3A-b; and Fig. 3B). Interestingly, simB9-4 and simB9-7 target sites are located more proximal to the 5′-end of the mouse BMP9 coding region than that of the other three siRNA target sites. Nonetheless, the above results suggest that the adenovirus-mediated expression of simBP9 siRNAs, especially simB9-4 and simB9-7, can effectively silence BMP9 expression and hence BMP9-induced osteogenic differentiation in MSCs.

Synergistic silencing effect between simB9-4 and simB9-7 leads to an effective inhibition of exogenous BMP9-induced osteogenic differentiation of MSCs *in vitro*

The above results indicate that simB9-4 and simB9-7 may represent two most potent siRNAs targeting mouse BMP9. We further examined whether these two siRNAs would exhibit a synergistic effect on inhibiting BMP9-induced osteogenic differentiation in MSCs. We showed that when the iMADs cells were effectively co-infected with AdBMP9, AdsimB9-4 and/or AdsimB9-7 (Fig. 4A—a) the co-expression of simB9-4 and simB9-7 led to greater reduction of exogenous BMP9 expression than either simB9-4 or simB9-7 siRNA alone (Fig. 4A—b).

We next analyzed the effect of co-targeting of BMP9 by simB9-4 and simB9-7 on BMP9-induced expression of osteogenic regulators Runx2 and Osterix (Osx), and found that exogenous BMP9-induced expression of Runx2 and Osx was most significantly inhibited by the co-expression of simB9-4 and simB9-7 siRNAs while either simB9-4 or simB9-7 exerted certain inhibitory effects (Fig. 4B). Furthermore, the three late stage osteogenic markers induced by exogenous BMP9, such as Opn, Ocn and Bsp, exhibited the lowest level of expression in the presence of both simB9-4 and simB9-7, compared with that of either simB9-4 or simB9-7 alone (Fig. 4B).

We also assessed the effect of co-targeting of BMP9 by simB9-4 and simB9-7 on BMP9-induced ALP activity and matrix mineralization in MSCs. While adenovirus-mediated expression of either simB9-4 or simB9-7 alone exhibited inhibitory effects on BMP9-induced ALP activities to varied degrees, a co-expression of simB9-4 and simB9-7 exerted highest inhibitory effects on BMP9-induced ALP activities, as assessed by either histochemical staining (Fig. 5A) or quantitative bioluminescence assay (Fig. 5B). Accordingly, the co-expression of simB9-4 and simB9-7 exerted highest inhibitory effects on BMP9-induced matrix mineralization in

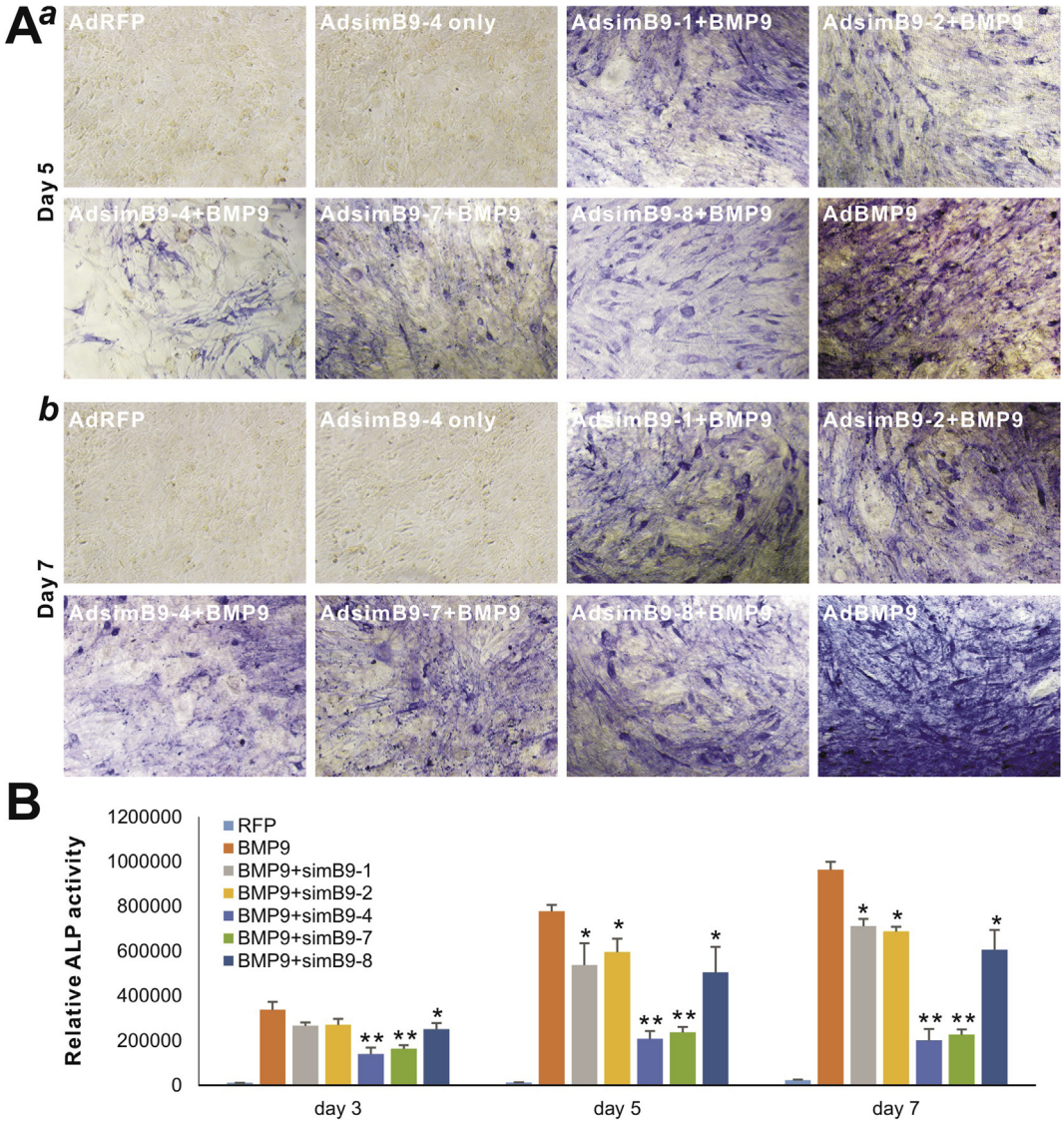

Figure 3 Adenovirus-mediated expression of simB9 siRNAs significantly diminishes exogenous BMP9-induced ALP activity in MSCs. (**A**) Qualitative ALP assay. Subconfluent iMADs were infected with the indicated adenoviruses. At day 5 (***a***) and day 7 (***b***), the infected cells were subjected to histochemical staining of ALP activity. Each assay condition was done in duplicate. Representative results are shown. (**B**) Quantitative ALP assay. Subconfluent iMADs were infected with the indicated adenoviruses. At days 3, 5, and 7, the infected cells were subjected to bioluminescence assay of ALP activity. Each assay was done in triplicate. "*" $p < 0.05$, "**" $p < 0.01$ compared with the AdBMP9-infected iMADs group.

iMADs, as assessed by Alizarin Red S staining (Fig. 6AB). Collectively, these results provide strong evidence to demonstrate that simB9-4 and simB9-7 may effectively silence BMP9 expression in MSCs, and subsequently inhibit BMP9-induced osteogenic signaling.

Synergistic silencing of BMP9 with simB9-4 and simB9-7 effectively diminishes BMP9-induced ectopic bone formation *in vivo*

To further confirm the knockdown efficiency of BMP9 through the adenovirus-mediated transient co-expression of simB9-4 and simB9-7 *in vivo*, we also conducted ectopic bone formation assays in athymic nude mice. When the iMADs cells were transduced with AdBMP9 or AdRFP and AdsimB9-4 and/or AdsimB9-7 and injected into the flanks of athymic nude mice, at four weeks after injection apparent bony masses were formed in the BMP9-treated group. The animals injected with iMADs co-infected with AdBMP9, AdsimB9-4 and AdsimB9-7 formed smallest masses, while the masses formed in the animals injected with iMADs co-infected with AdBMP9 and AdsimB9-4 or AdsimB9-7 were smaller than that of AdBMP9 only group (data not shown). Consistent with our earlier studies,[52,63,69] the iMADs cells transduced with AdRFP, AdsimB9-4 or AdsimB9-7 alone failed to form any detectable masses under the same condition (data not shown).

Histologic evaluation indicated that masses retrieved from BMP9 treatment group exhibited active and robust

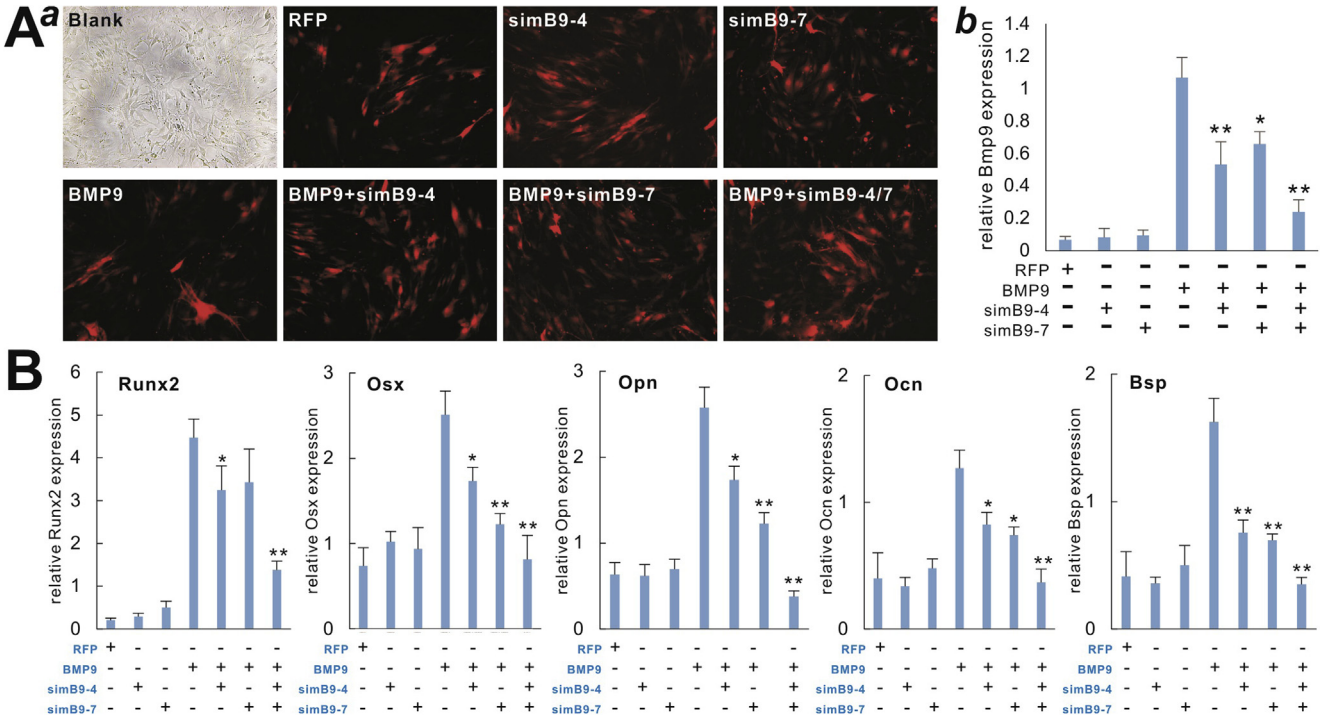

Figure 4 BMP9-induced expression of osteogenic regulators and biomarkers is effectively inhibited by the co-expression of simB9-4 and simB9-7 siRNAs in MSCs. (A) Efficient knockdown of exogenous BMP9 by co-expression of simB9-4 and simB9-7. Subconfluent iMADs were infected with the indicated adenoviruses for 48 h (***a***) and total RNA was isolated from the cells and subjected to qPCR analysis of mouse Bmp9 expression (***b***). All samples were normalized with Gapdh. Each assay condition was done in triplicate. "*" $p < 0.05$, "**" $p < 0.01$ compared with the AdBMP9-infected group. (**B**) BMP9-induced expression of osteogenic regulators and biomarkers is effectively inhibited by simB9-4 and simB9-7 siRNAs. Subconfluent iMADs were infected with the indicated adenoviruses. Total RNA was isolated from the cells at day 3 (for Runx2 and Osx) and day 5 (for Opn, Ocn and Bsp), and subjected to qPCR analysis of mouse gene expression. All samples were normalized with Gapdh. Each assay condition was done in triplicate. "*" $p < 0.05$, "**" $p < 0.01$ compared with the AdBMP9-infected group.

bone formation, consisting of ample trabecular bone structure and well-mineralized matrices (Fig. 7A). Overexpression of AdsimB9-4 or AdsimB9-7 inhibited BMP9-induced ectopic bone formation and matrix mineralization in iMADs cells (Fig. 7BC). Accordingly, co-expression of AdsimB9-4 and AdsimB9-7 significantly impaired BMP9-induced bone formation and matrix mineralization (Fig. 7D). These *in vivo* results further demonstrate that co-expression of simB9-4 and simB9-7 may effectively silence BMP9 expression and drastically inhibit exogenous BMP9-induced osteogenic signaling in MSCs.

Discussion

As a transforming growth factor β member, BMP9 was originally identified in developing mouse liver.[30,84] In addition to its role in inducing osteogenic differentiation, BMP9 plays important roles in inducing and maintaining embryonic basal forebrain cholinergic neurons, inhibiting hepatic glucose production and inducing the expression of key enzymes of lipid metabolism, stimulating hepcidin 1 expression, and regulating angiogenesis.[30] We also found that BMP9 is resistant to the naturally occurring antagonist noggin,[85] and demonstrated that TGFβ/BMP type I receptors ALK1 and ALK2 are essential for BMP9-induced osteogenic signaling in MSCs.[78] As BMP9 is one of the least studied BMP, we performed mechanism-based studies and identified several early downstream targets,[30,31,70,82,86–89] and demonstrated that BMP9 signaling has extensive cross-talks with other signaling pathways, especially Wnt and Notch signaling.[7,8,31,71–73,77,79,80,90,91] Nonetheless, many mechanistic aspects of BMP9 in regulating lineage-specific differentiation of BMP9 remain to be fully understood. In this study, we have developed a highly effective tool to silence mouse Bmp9 expression, which should be useful for delineating the molecular mechanisms underlying BMP9 osteogenic signaling.

RNAi-mediated gene silencing has become a valuable tool for functional studies, reverse genomics, and drug discoveries.[33–38,40–45] One major challenge of using RNAi is to identify the most effective short interfering RNAs (siRNAs) sites of a given gene. Despite several published bioinformatic prediction models, the process to select and validate optimal siRNA sites for a given gene is empirical and laborious.[39,49] In this study, we analyzed five siRNAs targeting mouse Bmp9 and found that simB9-4 and simB9-7 exhibited highest silencing efficiency, while other three simB9 siRNAs also were able to silence Bmp9 expression to varied extents. Interestingly, both simB9-4 and simB9-7 are located at the 5′-end of Bmp9 coding region, compared with other three simB9 siRNAs, suggesting siRNAs located at 5′-

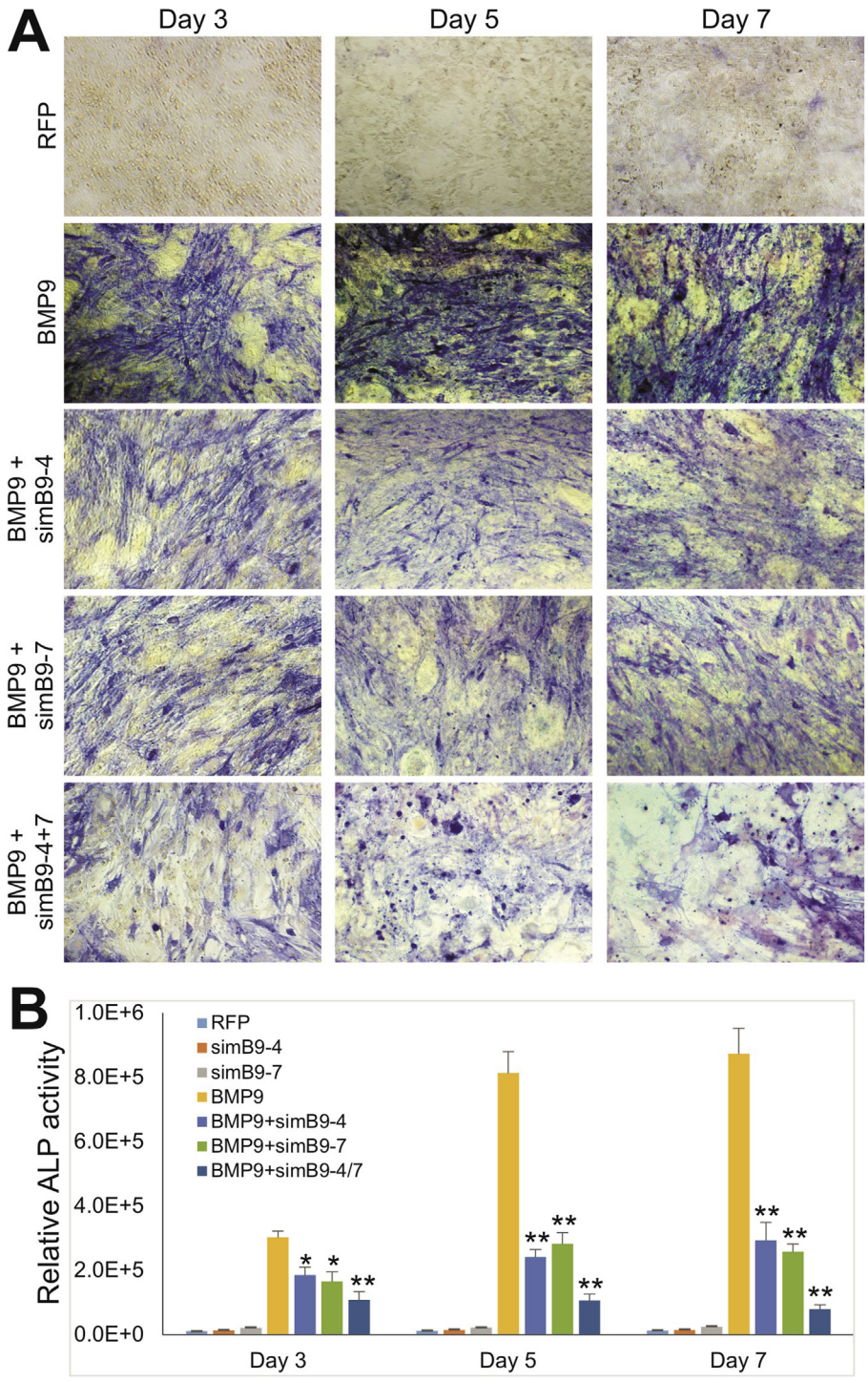

Figure 5 BMP9-induced ALP activity is effectively inhibited by the co-expression of simB9-4 and simB9-7 siRNAs in MSCs. Subconfluent iMADs were infected with the indicated adenoviruses. At days 3, 5, and 7, the infected cells were subjected to ALP activity assays by either histochemical staining (**A**), or quantitative bioluminescence assay (**B**). Each assay was done in triplicate. Representative staining results are shown. "*" $p < 0.05$, "**" $p < 0.01$ compared with the AdBMP9-infected iMADs group.

end of coding regions may silence target genes more efficiently. Furthermore, we have demonstrated that simB9-4 and simB9-7 can act synergistically in terms of silencing Bmp9 expression and hence its biological activities, indicating that it is highly beneficial to combine multiple siRNAs in order to accomplish high knockdown efficiency.

Efficient introduction and sustained expression of siRNAs in mammalian cells are important perquisites for effective target gene silencing.[40—45,92] One common-used approach is to transfect mammalian cells with synthetic siRNAs, However, this approach is limited by transfection efficiency and short-term transient knockdown, and can be only used for

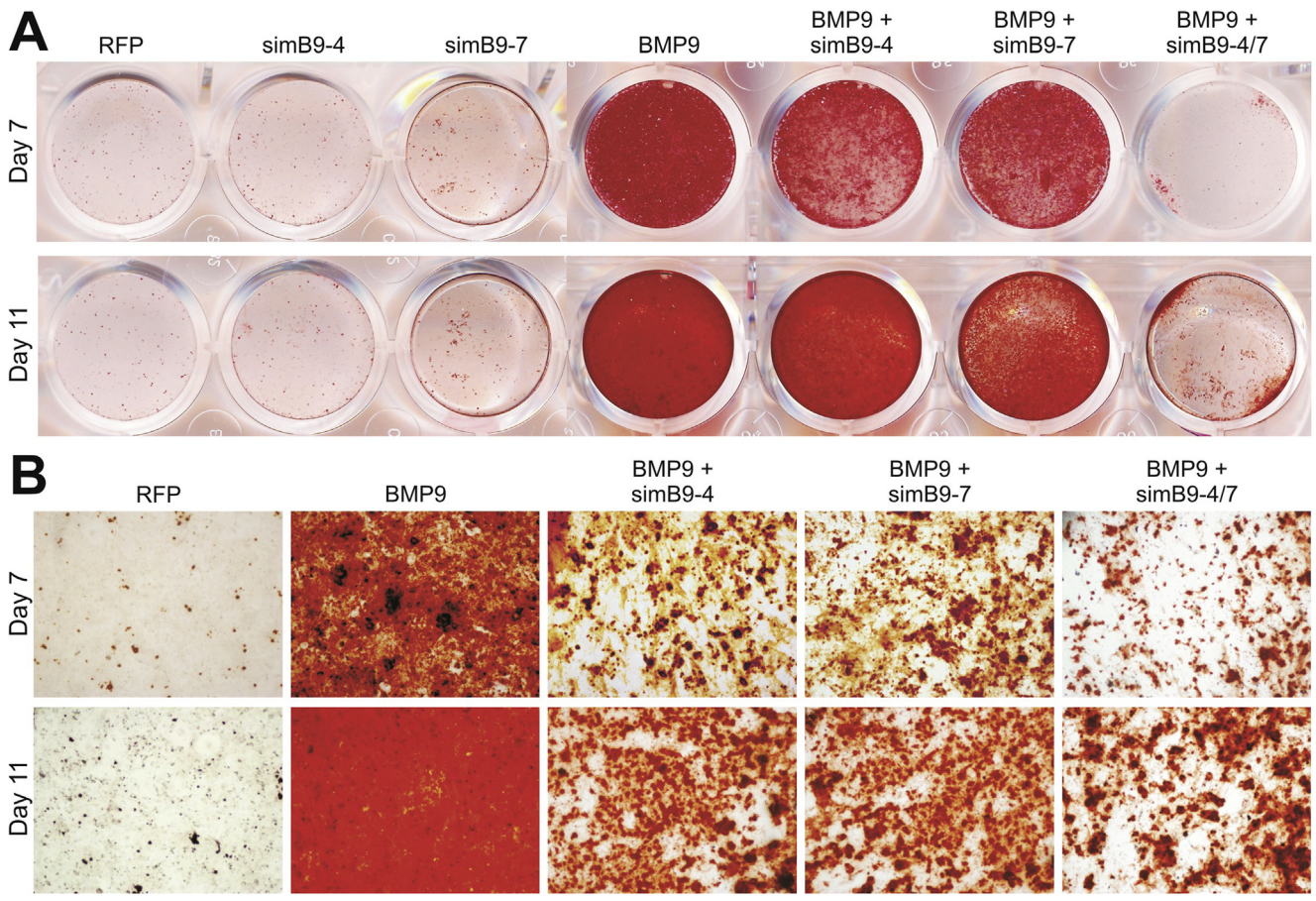

Figure 6 BMP9-induced matrix mineralization is effectively inhibited by the co-expression of simB9-4 and simB9-7 siRNAs in MSCs. Subconfluent iMADs were infected with the indicated adenoviruses. At days 7 and 11, the infected cells were subjected to Alizarin Red S staining. Staining results were recorded macrographically (**A**) or under a low power microscope (**B**). Each assay condition was done in duplicate. Representative staining results are shown.

cultured cells *in vitro*. Thus, a more commonly-used approach is to express short hairpin RNAs (shRNAs) or siRNAs in cells.[43,44,92] In this case, the shRNA or siRNA expression of siRNAs is driven by Pol III promoters such U6 and H1[92]. In our studies, the siRNAs are driven by converging U6 and H1 promoters, yielding functional siRNAs without the requirement of additional processing by RISC machinery, which is significantly advantageous over shRNA processing.[39,49] Another formidable technical challenge is to effectively deliver RNAi expression vectors into mammalian cells in order to knock down target genes with high efficiency. Among many exogenous gene delivery methods, recombinant adenovirus-based delivery system remains as one of the most favorable strategies because of its high efficiency *in vitro* and *in vivo*.[59,62,93] In this study, we have demonstrated that adenovirus-mediated expression of siRNAs can achieve effective gene silencing both *in vitro* and *in vivo*. Thus, adenoviral vectors should be useful for effective delivery of siRNAs into mammalian cells *in vitro* and/or animal tissues *in vivo*.

It should point out that the clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9), or CRISPR/Cas9-based genome editing technology has recently become a popular tool for gene manipulations.[54,94–102] CRISPR/Cas9 is a recently developed and naturally occurred genome editing tool, which is used by bacteria for immune defense.[94–96] CRISPR RNA-guided Cas9 nucleases have been shown to work in a wide range of model organisms and mediate genome editing by introducing a double-stranded break (DSB) in a target DNA sequence, which in turn can lead to the efficient generation of insertions or deletion mutations (indels) through nonhomologous end-joining repair, or in the presence of an appropriately designed homologous donor DNA template, precise DNA alterations can be made through homology-directed repair of the DSB.[54,94–102] While CRISPR/Cas9 and related Cas nuclease systems will continue to evolve and undoubtedly change the landscape of genome editing and gene manipulations, the gene editing efficiency in cultured cells is limited by the technical challenges in expressing multiple components including Cas9 nuclease and small guide-RNAs, and/or delivering homologous donor DNA templates. While it is conceivable that the BMP9 gene can be deleted at genome level, the technical challenges will prevent accomplishing such feat effectively in cultured MSCs. Thus, siRNA-mediated knockdown of Bmp9 expression in MSCs provides a viable alternative to investigate the loss of function effect on BMP9-induced osteogenic differentiation of mesenchymal stem cells.

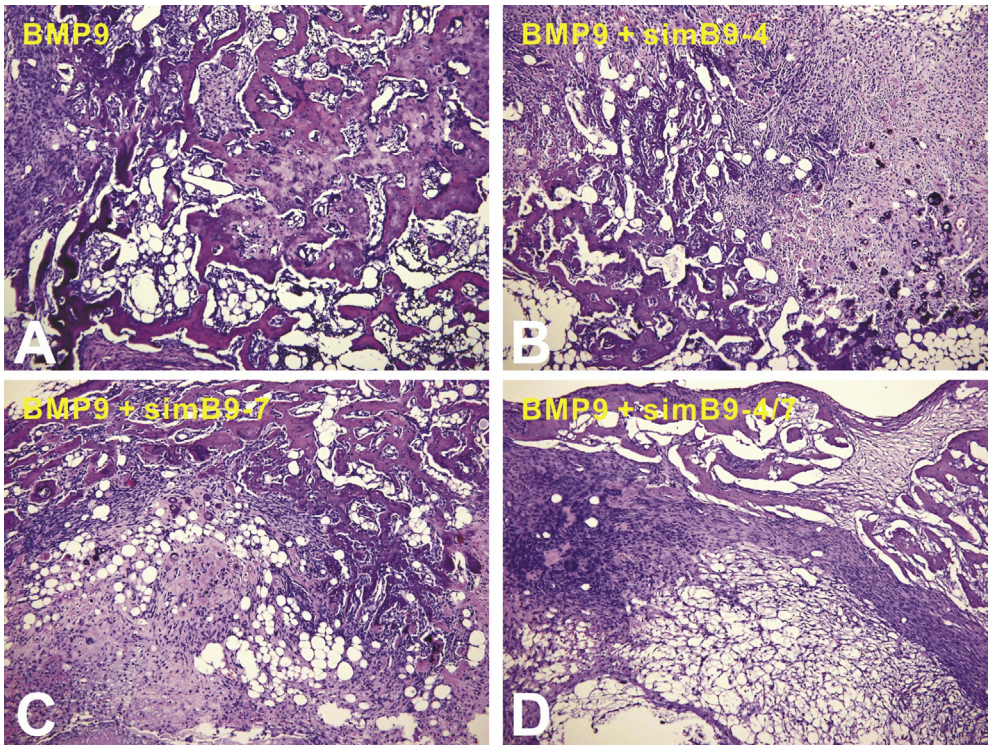

Figure 7 BMP9-induced ectopic bone formation is significantly inhibited by the co-expression of simB9-4 and simB9-7 siRNAs in MSCs. Subconfluent iMADs were infected with the indicated adenoviruses for 36 h, and then collected for subcutaneous injection into the flanks of athymic nude mice (n = 5/group). Ectopic bony masses were harvested at 4 weeks after injection and subjected to H & E staining. No masses were retrieved from the groups injected with AdRFP, AdsimB9-4 and AdsimB9-7 infected iMADs. Representative histologic images are shown.

In summary, using different siRNA bioinformatic prediction programs, we designed five simB9 siRNAs, which were expressed under the control of converging H1 and U6 promoters in recombinant adenovirus vectors. We demonstrated that simB9-4 and simB9-7 exhibited the highest efficiency on silencing exogenous mouse BMP9 in MSCs individually. Furthermore, simB9-4 and simB9-7 acted synergistically in inhibiting exogenous BMP9-induced expression of osteogenic markers, matrix mineralization and ectopic bone formation from MSCs. Thus, the characterized simB9 siRNAs may be used as an important tool to investigate the molecular mechanism behind BMP9 osteogenic signaling. Furthermore, our results indicate that recombinant adenovirus-mediated expression of siRNAs is efficient and sustained, and thus may be used as a potential delivery vehicle of siRNA therapeutics.

Conflicts of interests

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.gendis.2018.04.006.

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