



FULL LENGTH ARTICLE

Argonaute (AGO) proteins play an essential role in mediating BMP9-induced osteogenic signaling in mesenchymal stem cells (MSCs)

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Abstract As multipotent progenitor cells, mesenchymal stem cells (MSCs) can renew themselves and give rise to multiple lineages including osteoblastic, chondrogenic and adipogenic lineages. It's previously shown that BMP9 is the most potent BMP and induces osteogenic and adipogenic differentiation of MSCs. However, the molecular mechanism through which BMP9 regulates MSC differentiation remains poorly understood. Emerging evidence indicates that noncoding RNAs, especially microRNAs, may play important roles in regulating MSC differentiation and bone formation. As highly conserved RNA binding proteins, Argonaute (AGO) proteins are essential components of the multi-protein RNA-induced silencing complexes (RISCs), which are critical for small RNA biogenesis. Here, we investigate possible roles of AGO proteins in BMP9-induced lineage-specific differentiation of MSCs. We first found that BMP9 up-regulated the expression of *Ago1*, *Ago2* and *Ago3* in MSCs. By engineering multiplex siRNA vectors that express multiple siRNAs targeting individual *Ago* genes or all four *Ago* genes, we found that silencing individual *Ago* expression led to a decrease in BMP9-induced early osteogenic marker alkaline phosphatase (ALP) activity in MSCs. Furthermore, we demonstrated that simultaneously silencing all four *Ago* genes significantly diminished BMP9-induced osteogenic and adipogenic differentiation of MSCs and matrix mineralization, and ectopic bone formation. Collectively, our findings strongly indicate that AGO proteins and associated small RNA biogenesis pathway play an essential role in mediating BMP9-induced osteogenic differentiation of MSCs.

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Introduction

As multipotent progenitor cells, mesenchymal stem cells (MSCs) can renew themselves and give rise to multiple lineages including chondrogenic, adipogenic, osteogenic, and myogenic lineages.^{1–5} Osteoblastic lineage-specific differentiation of MSCs is regulated by numerous major signaling pathways.^{2,6–13} Bone morphogenetic proteins (BMPs) are considered as the most potent osteoinductive factors.^{14–16} BMPs are members of the transforming growth factor β (TGF- β) superfamily,^{2,14,15} and at least 14 BMPs have been identified in humans and rodents.^{14,15,17} We conducted a comprehensive analysis of the 14 types of human BMPs, and identified the least known BMP9 as one of the most potent osteogenic BMPs in MSCs.^{14,16,18–21} We further demonstrated that, unlike other osteogenic BMPs, BMP9 is refractory to the inhibitory effect exerted by the potent naturally occurring antagonist noggin,²² and that the TGF- β /BMP type I receptors ALK1 and ALK2 are essential for BMP9 osteogenic signaling²³ and regulating downstream target genes in MSCs.^{24–31} Nonetheless, the molecular mechanisms underlying BMP9-induced osteogenic differentiation of MSCs are not fully understood.

For the past 20 years, significant progress has been in genome-wide transcriptomic studies, indicating that non-coding RNAs (ncRNAs) may play critical roles in most if not all aspects of cellular processes under physiological and/or pathologic conditions,^{32–34} since only less than 2% of the human genome encodes proteins, while eukaryotic genomes are pervasively transcribed.^{34,35} Noncoding RNAs are divided into small ncRNAs (<200 nt) and long ncRNAs or lncRNAs (>200 nt).³³ In fact, small ncRNAs, e.g., microRNAs

(miRNAs) and small interfering RNAs (siRNAs), have gained a lot of attention since their discoveries.^{36,37} It has been reported that ncRNAs, especially microRNAs, may play important roles in regulating MSC differentiation and bone formation.^{38,39}

MicroRNAs (miRNAs) are small ncRNAs of ~22 nucleotides and attenuate gene expression by guiding Argonaute (AGO) proteins to target mRNAs that are completely or partially complementary with miRNAs.^{40–42} The biogenesis of miRNAs starts with longer primary transcripts (i.e., pri-miRNAs) with a 60–120 nt RNA hairpin structure.⁴¹ The pri-miRNAs are then cleaved by Drosha and Dicer, yielding ~70 nt precursor miRNAs (i.e., pre-miRNAs) and eventually 22 nt mature miRNAs.⁴¹ Mature miRNAs usually down-regulate gene expression post-transcriptionally through binding of target mRNAs in association with the AGO-containing RNA-induced silencing complex (RISC).⁴¹ Small interfering RNAs (siRNAs) are also processed and subsequently silence target transcripts through interacting with the AGO2-containing RISC system.^{43–45} Thus, the AGO proteins are essential players in processing and biogenesis of small RNAs (smRNAs) in mammalian cells. AGOs use single-stranded small nucleic acids as guides and bind to complementary sequences in RNA targeted for modulation.⁴¹ There are at least four AGO proteins, namely, AGO1 to AGO4, in humans and mice.^{41,46}

In this study, we investigate potential roles of AGO proteins in BMP9-induced lineage-specific differentiation of MSCs. Using our recently developed FASMi technology,⁴⁷ we engineered the multiplex siRNA vectors to express multiple siRNAs targeting individual *Ago* genes or all four *Ago* genes in either recombinant adenoviral or retroviral vector

system. We demonstrated that BMP9 up-regulated the expression of *Ago1*, *Ago2* and *Ago3* in MSCs; and that silencing individual *Ago* expression led to a decrease in BMP9-induced alkaline phosphatase (ALP) activity in MSCs. We further demonstrated that simultaneous silencing of all four *Ago* genes effectively diminished BMP9-induced adipogenic and osteogenic differentiation of MSCs and matrix mineralization *in vitro*, and ectopic bone formation *in vivo*. Collectively, our findings indicate that AGO proteins and associated microRNA biogenesis pathway play an important role in mediating BMP9-induced osteogenesis in MSCs.

Material and methods

Cell culture and chemicals

HEK-293 cells were obtained from ATCC (Manassas, VA). HEK-293 derivative lines 293pTP and RAPA cells were previously described.^{48,49} Reversibly immortalized mouse bone marrow stromal cells (imBMSCs) were previously characterized.⁵⁰ All cell lines were maintained in DMEM containing 10% fetal bovine serum (FBS; Gemini Bio-Products, West Sacramento, CA), supplemented with 100U/mL penicillin and 100 µg/mL streptomycin, and incubated at 37 °C in 5% CO₂ as described.^{13,51–54} Unless indicated otherwise, all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Thermo Fisher Scientific (Waltham, MA, USA).

Construction and generation of recombinant adenoviruses AdBMP9, AdGFP, AdRFP, AdR-simAgo1, AdR-simAgo2, AdR-simAgo3 and AdR-simAgo4

Recombinant adenoviruses were constructed by using the AdEasy technology as described.^{55,56} Briefly, for making the overexpression adenoviruses, the coding regions of human BMP9, eGFP and RFP were PCR amplified, subcloned into the adenoviral shuttle vector, and used to generate recombinant adenoviruses in HEK293, 293pTP, or RAPA cells as described,^{13,28,57–59} subsequently designated as AdBMP9, AdGFP and AdRFP.

The multiplex siRNA expressing adenoviruses were constructed by using our recently developed FAMSi system.⁴⁷ Briefly, three siRNAs were designed to target the coding region of each *Ago* member by using Invitrogen's BLOCK-IT RNAi Designer program. The siRNA sites were assembled into the FAMSi vector system, and subsequently cloned into a homemade adenoviral vector for generating adenoviruses as described.^{47,58,60–62} The resultant adenoviruses were designated as AdR-simAgo1, AdR-simAgo2, AdR-simAgo3, and AdR-simAgo4. All PCR amplified fragments were verified by DNA sequencing. The cloning construction details are available upon request.

It is noteworthy that AdBMP9 and AdR-simAgo viruses also express eGFP and RFP as infection tracking markers, respectively. For all adenoviral infections, AdGFP and AdRFP were used as control viruses for mock infection as described.^{22,29,30,63–69} For all adenoviral infections,

polybrene (final concentration at 5 µg/mL) was added to enhance infection efficiency as described.⁷⁰

Construction of the retroviral vector pSEB-simAgo2134 and the establishment of stable imBMSC cells expressing simAgo2134 line imBMSC-simAgo2134

To silence all four *Ago* members, a multiplex siRNA construct, pSiEB-simAgo2134, was assembled by using the FAMSi system.⁴⁷ The retroviral vector pSiEB-simAgo2134 expresses two siRNAs targeting *Ago2* and one siRNA each targeting *Ago1*, *Ago3*, and *Ago4*. The previously engineered pSEB-siControl vector was used a negative control as described.^{47,58,61} The retroviral vector pSiEB-simAgo2134 or pSiEB-siControl was co-transfected with retroviral packaging plasmids into HEK-293 cells to produce retrovirus supernatants, which were used to infect subconfluent imBMSC cells to establish imBMSC-simAgo2134 or imBMSC-siControl stable line upon blasticidin S selection (final concentration at 5 µg/mL), as previously described.^{69,71–75}

RNA extraction and touchdown quantitative PCR (TqPCR)

Total RNA isolated with the TRIZOL Reagent (Invitrogen, Carlsbad, CA) was subjected to reverse transcription reactions using hexamer and M-MuLV Reverse Transcriptase (New England Biolabs, Ipswich, MA, USA). The RT cDNA products were used as qPCR templates. The qPCR primers were designed by using the Primer3 Plus,⁷⁶ and are listed in Table S1. The quantitative PCR analysis was performed using our previously optimized TqPCR protocol.^{17,62,63,77} Briefly, SYBR Green qPCR reactions (Bimake, Houston, TX) were set up according to manufacturer's instructions. The cycling program was set up as follows: 95 °C × 3 min for 1 cycle; 95 °C × 20 s, 66 °C × 10 s per cycle, then –3 °C per cycle for 4 cycles; followed by 95 °C × 10 s, 55 °C × 15 s, and 70 °C × 1 s for 40 cycles. *Gapdh* was used as a reference gene. Relative expression for all samples was calculated by using the $2^{-\Delta\Delta Ct}$ method as described.^{58,72,77}

Alkaline phosphatase (ALP) activity assays

Exponentially growing imBMSC cells and/or their derivative lines were plated in 24-well plates and co-infected with the indicated adenoviral vectors. At days 3, 5, or 7, the infected cells were subjected to either quantitative ALP activity assessment using the modified Great Escape SEAP chemiluminescence assay kit (BD Clontech) and/or qualitative histochemical staining of ALP activity as previously described.^{47,52,57,78,79} Each assay condition was conducted in triplicate.

In vitro matrix mineralization assay

Exponentially growing imBMSCs cells were plated in 24-well plates, co-infected with the indicated adenoviruses, and cultured with 10% FBS DMEM containing ascorbic acid (50 mg/mL) and β-glycerophosphate (10 mM). At days 14

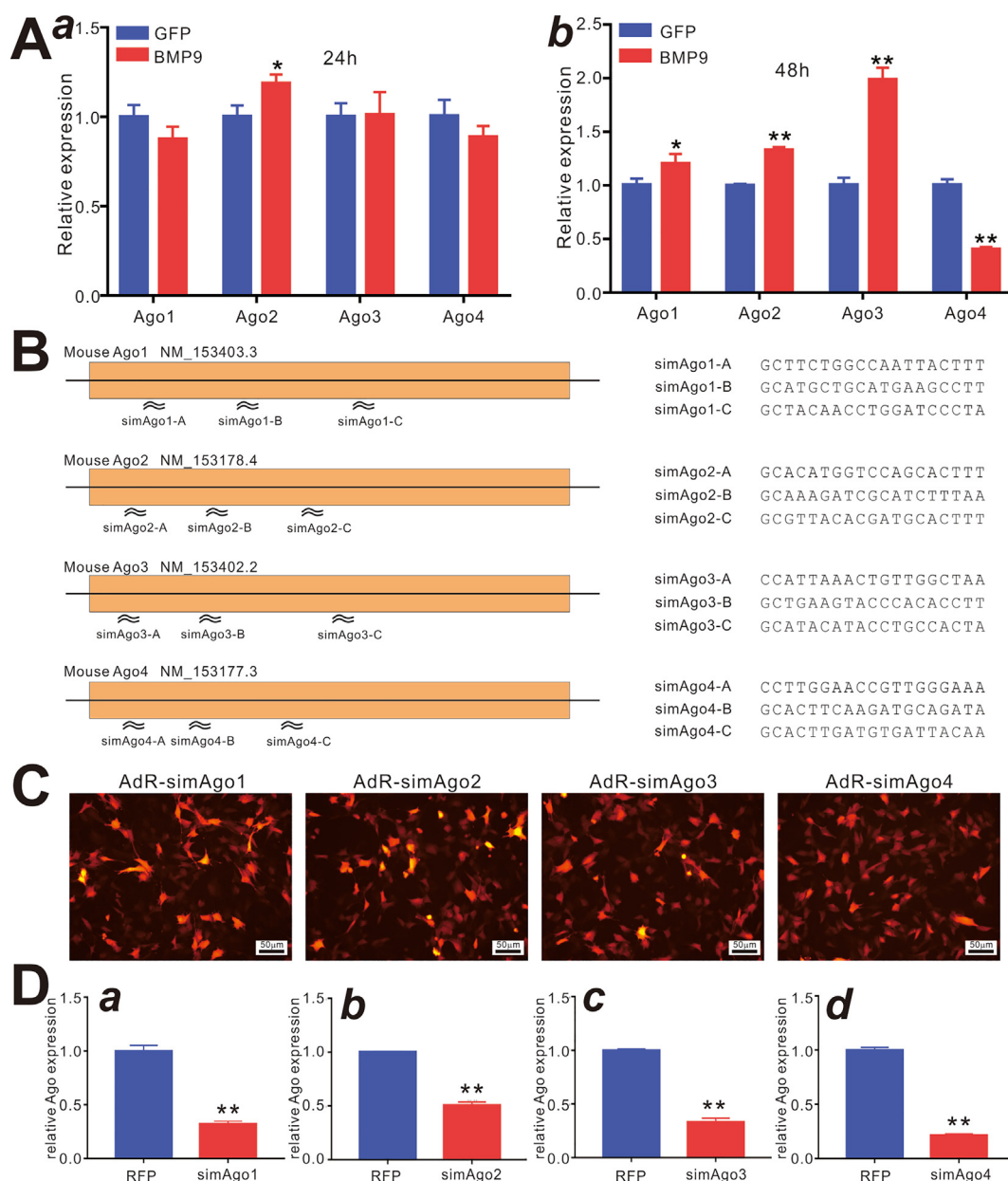


Figure 1 BMP9 impacts on the expression of Ago genes, which can be effectively silenced through adenoviral vector-mediated expression of Ago-specific siRNAs in MSCs. **(A)** Subconfluent imBMSCs were infected with Ad-BMP9 (BMP9) and AdGFP control (GFP). At 24 h **(a)** and 48 h **(b)** after infection, total RNA was isolated and subjected to TqPCR analysis of *Ago1*, *Ago2*, *Ago3* and *Ago4* expression. *Gapdh* was used as a reference gene. *, $P < 0.05$, and **, $P < 0.01$ when compared with that of the AdGFP group. Each assay was done in triplicate. **(B)** Location and sequence features of the siRNAs that target the coding regions of mouse *Ago1*, *Ago2*, *Ago3*, *Ago4*. The siRNAs targeting individual Ago gene transcript were cloned into recombinant adenoviral vectors using our recently established FAMSi technique, resulting in the adenoviruses designated as AdR-simAgo1, AdR-simAgo2, AdR-simAgo3 and AdR-simAgo4, respectively. **(C)** AdR-simAgo viruses can transduce MSCs with high efficiency. Subconfluent imBMSCs were infected with AdR-simAgo1, AdR-simAgo2, AdR-simAgo3, or AdR-simAgo4. At 48 h after infection, RFP signal was recorded. Representative images are shown. **(D)** AdR-simAgo vectors effectively silence the expression of respective Ago genes in MSCs. Subconfluent imBMSCs were infected with the indicated AdR-simAgo viruses, or AdRFP. At 48 h after infection, total RNA was isolated and subjected to TqPCR analysis of expression of mouse *Ago1* **(a)**, *Ago2* **(b)**, *Ago3* **(c)**, and *Ago4* **(d)**. *Gapdh* was used as a reference gene. Each assay was done in triplicate. **, $P < 0.01$ when compared with that of the AdGFP group.

and 21, mineralized matrix nodules were stained by Alizarin Red S staining as described.^{59,80–82} Briefly, the cells were fixed with 2.5% glutaraldehyde for 10 min, washed with PBS (pH adjusted to 4.2) twice, and then incubated

with 2% Alizarin Red S for 30 min at room temperature. The stained mineral deposits were recorded under bright field microscopy. Each assay condition was done in triplicate.

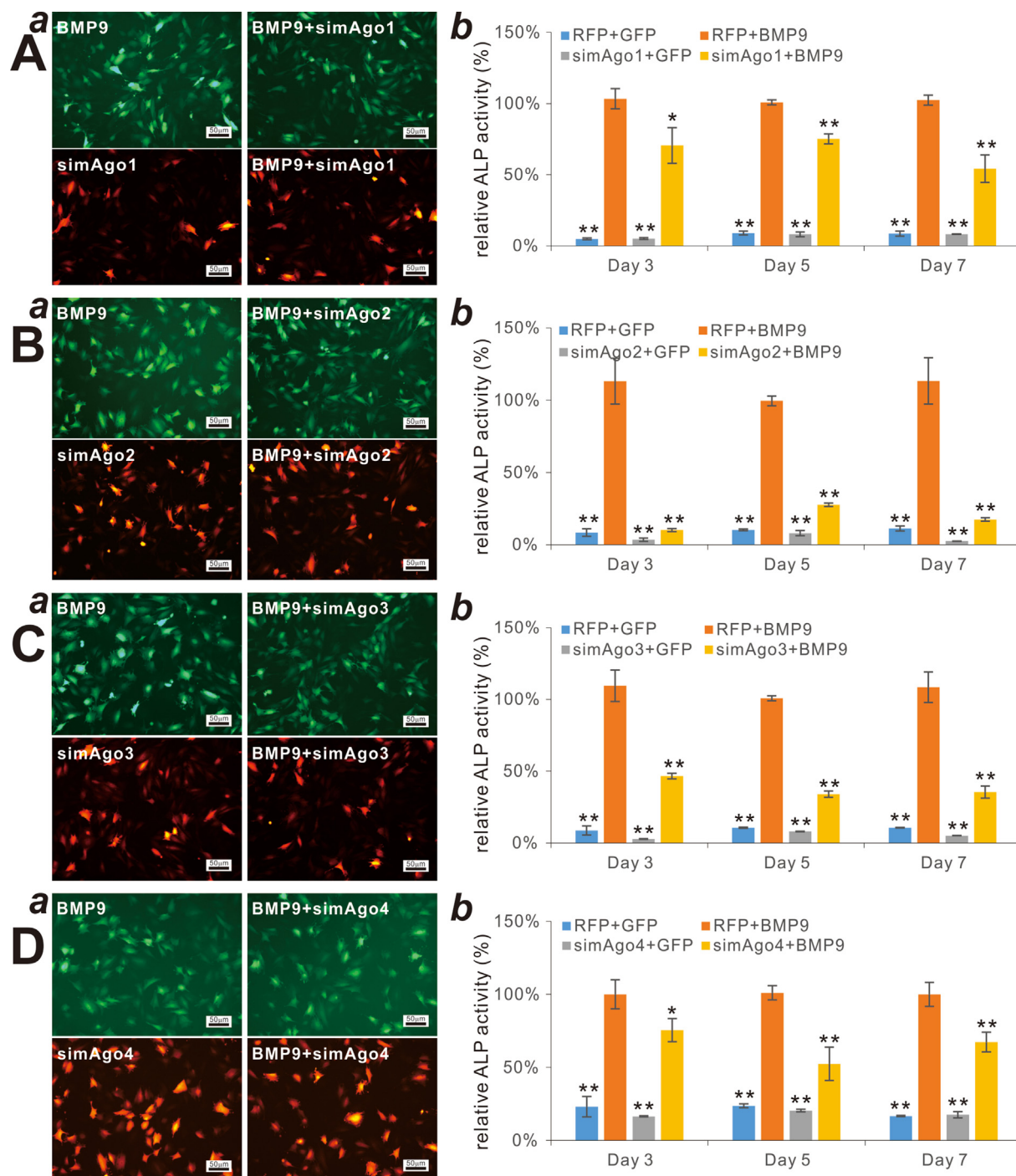


Figure 2 Silencing Ago expression leads to a decrease in BMP9-induced ALP activity in MSCs. Subconfluent imBMSCs were co-infected with Ad-RFP or Ad-BMP9 and AdGFP, AdR-simAgo1 (A), AdR-simAgo2 (B), AdR-simAgo3 (C), or AdR-simAgo4 (D). At 48 h post infection, GFP and RFP signals were recorded, and representative images are shown in (a). At the indicated time points, ALP activity was quantitatively assessed (b). Each assay condition was performed in triplicate. *, $P < 0.05$ and **, $P < 0.01$, compared with that of the RFP + BMP9 group at respective time points.

Oil Red O staining assay

Exponentially growing imBMSCs cells were seeded in 24-well cell culture plates and co-infected with the indicated adenoviruses. At day 7, the cells were subjected to Oil Red O staining as described.^{29,57,69,83–85} Briefly, the cells were

fixed with 10% formalin at room temperature for 10min, washed with PBS, and stained with freshly prepared Oil Red-O solution at room temperature for 60 min, followed by washing with distilled water. The staining of lipid droplets was recorded under a bright field microscope. Each assay condition was performed in triplicate.

Subcutaneous stem cell implantation and ectopic bone formation

All animal use and care in this study followed the approved protocol by the Institutional Animal Care and Use Committee. All experimental procedures were carried out according to the approved guidelines. Subcutaneous stem cell implantation procedure was performed as previously described.^{22,50,59,86–88} Briefly, subconfluent imBMSC-siControl and imBMSC-simAgo2134 cells were infected with AdGFP or AdBMP9 for 36 h. The infected cells were collected, resuspended in PBS, and subcutaneously injected into the flanks of athymic nude mice (Envigo/Harlan Research Laboratories; $n = 5$ /group, 6–8-week old; 2×10^6 cells in 50 μ l per injection site). At three weeks after implantation, the mice were sacrificed, and masses at implantation sites were recovered for μ CT and histologic analyses.

Micro-computed tomographic (μ CT) analysis

The retrieved bony masses were fixed in 10% PBS-buffered formalin and subjected to μ CT imaging by using the GE triumph (GE Healthcare) trimodality preclinical imaging system. All imaging data were analyzed with the Amira 6.0 (Visage Imaging, Inc.). The average bone volume was determined as previously described.^{89–92}

Hematoxylin and eosin (H & E) and Masson's trichrome staining

The retrieved samples were decalcified and paraffin embedded. Serial sections of the embedded samples were

subjected to H & E staining and Masson's trichrome staining as previously described.^{19,93–96}

Statistical analysis

The quantitative assays were performed in triplicate and/or repeated in three independent batches. Statistical differences between samples were determined by *t*-test or ANOVA. The $P < 0.05$ was defined as statistically significant.

Results

BMP9 regulates the expression of Ago family members in MSCs

We first analyzed the effect of BMP9 on the expression of Ago family members in MSCs. At 24 h of AdBMP9 infection, only Ago2 was up-regulated upon BMP9 stimulation (Fig. 1A, panel a). However, at 48 h after BMP9 stimulation, Ago1, Ago2 and Ago3 were significantly up-regulated while Ago4 was down-regulated. While the detailed regulatory mechanism remains to be fully investigated, these results indicate that Ago family members play an important role in mediating BMP9 signaling in MSCs.

To further investigate the role of the Ago members in BMP9 signaling, we designed a series of siRNAs that target four Ago genes, each of which was targeted by three siRNAs (Fig. 1B). The three siRNAs (e.g., simAgo) for each Ago gene were assembled into a recombinant adenoviral vector, and the generated adenoviruses were shown to transduce MSCs with high efficiency (Fig. 1C). Quantitative qPCR analysis

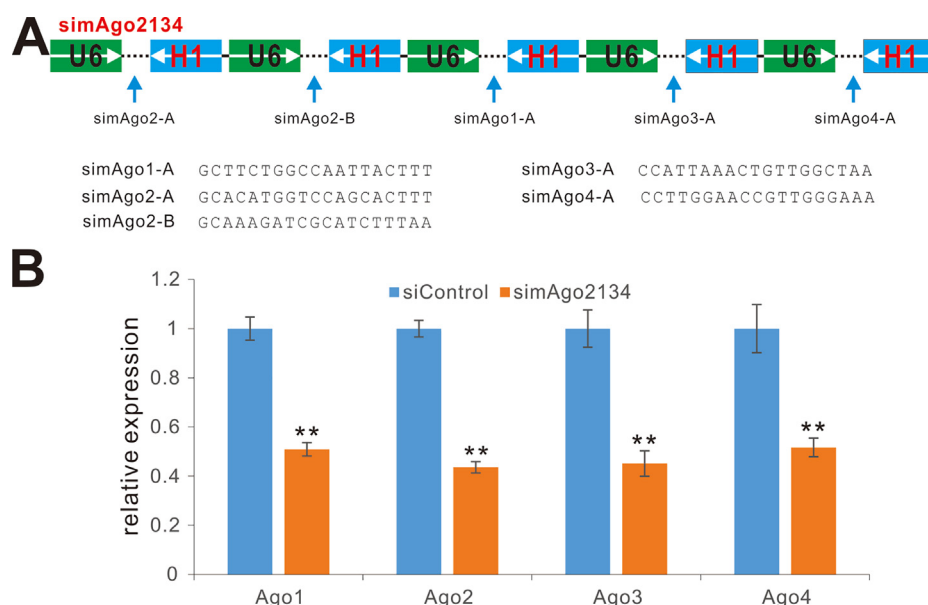


Figure 3 Characterization of a retroviral vector that simultaneously silences the expression of all four Ago family members in MSCs. **(A)** Schematic depiction of the five-siRNA-containing multiplex retroviral vector pSEB-simAgo2134 and the selected siRNA sequences for individual Ago genes. The multiplex siRNA construct was accomplished by using our recently developed FAMSi system **(B)** Multiplex siRNA vector simAgo2134 effectively silences the expression of four Ago genes in MSCs. Retroviral stable lines imBMSC-simAgo2134 and imBMSC-siControl cells were first established. Total RNA was isolated from subconfluent imBMSC-siControl and imBMSC-simAgo2134 cells and subjected to TqPCR analysis of the expression of mouse Ago1, Ago2, Ago3, and Ago4. Each assay condition was done in triplicate. *Gapdh* was used as a reference gene. **, $P < 0.01$, compared with that of the siControl group.

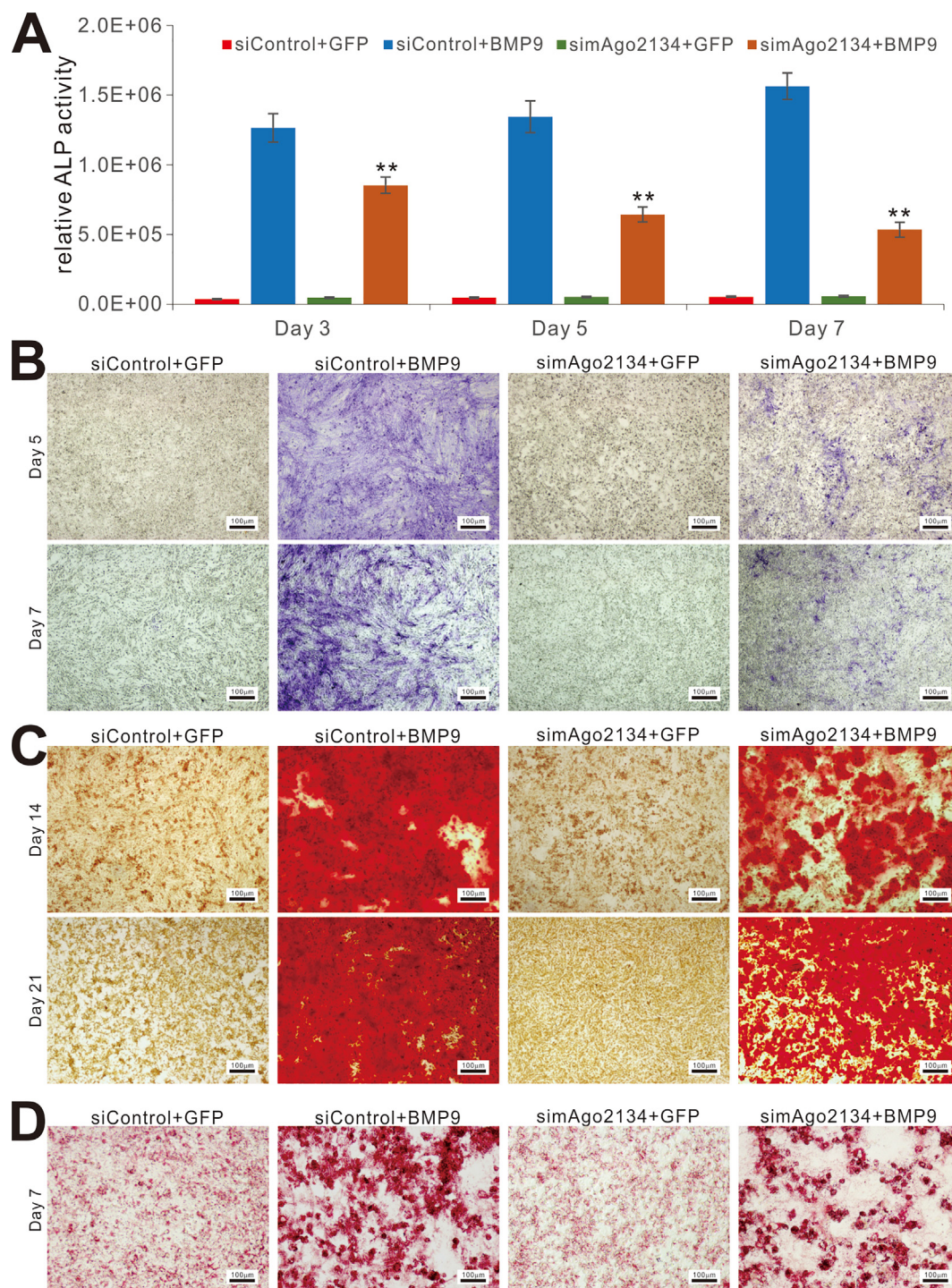


Figure 4 Silencing the expression of the Ago family members diminishes BMP9-induced osteogenic and adipogenic differentiation of MSCs. (A–C) Silencing the expression of the Ago family members diminishes BMP9-induced ALP activity and matrix mineralization in MSCs. Subconfluent imBMSC-siControl and imBMSC-Ago2134 cells were infected with AdGFP or AdBMP9. At 3, 5, and 7 days after infection, ALP activity was determined quantitatively (A) and histochemically (B). At 14, 21 days after infection, the cells were fixed and subjected to Alizarin Red S staining (C). Each assay condition was done in triplicate. Representative images are shown. **, $P < 0.01$, compared with that of the siControl + BMP9 group (D) Silencing Ago family members reduces BMP9-induced adipogenic differentiation of MSCs. Subconfluent imBMSC-siControl and imBMSC-Ago2134 cells were infected with AdGFP or AdBMP9. At 7 days after infection, the cells were fixed and subjected to Oil Red O staining. Representative images are shown. Each assay condition was done in triplicate.

demonstrated that individual *simAgo* adenoviruses effectively silenced the expression of respective *Ago* genes (Fig. 1D, panels a-d).

Silencing individual *Ago* family members diminishes BMP9-induced early osteogenic marker ALP in MSCs

Using the AdR-*simAgo* adenoviruses constructed above, we analyzed the effect of individual *Ago* members on BMP9-induced osteogenic differentiation of MSCs. When imBMSC cells were effectively co-infected with AdR-*simAgo* or AdRFP, and AdBMP9 or AdGFP (Fig. 2A–D, panel a), BMP9-induced ALP activities were significantly decreased in the AdR-*simAgo* infection groups at the tested time points, compared with that of the AdRFP groups (Fig. 2A–D, panel b). Among the four *Ago* genes, silencing *Ago2*, and to a lesser extent, *Ago3*, led to the most significant reduction of BMP9-induced ALP activity in imBMSC cells, while smaller decreases in ALP activity were observed in *Ago1* and *Ago4* knockdown groups (Fig. 2B–b and 2C-b vs. 2A-b and 2D-b). Collectively, these findings reveal that *Ago2* and *Ago3* play an important role in mediating BMP9-induced osteogenic signaling in MSCs.

Simultaneously silencing the expression of all four *Ago* family members significantly diminishes BMP9-induced osteogenic and adipogenic differentiation in MSCs

To further investigate whether the *Ago* expression was required for BMP9-induced lineage-specific differentiation of MSCs, we sought to construct a retroviral vector expressing five siRNAs, e.g., *simAgo2134*, two of which target *Ago2* and one each for *Ago1*, *Ago3* and *Ago4*, respectively (Fig. 3A). This retroviral vector was used to

establish the stable MSC line imBMSC-*simAgo2134*, whereas imBMSC-siControl cells were established as a control line. Quantitative qPCR analysis indicates that the expression of all four *Ago* members was effectively silenced in the imBMSC-*simAgo2134* cells, compared with that in the control imBMSC-siControl cells (Fig. 3B).

When these stable lines were infected with AdBMP9, both quantitative and qualitative measurements revealed that the ALP activity significantly decreased in the imBMSC-*simAgo2134* cells, compared with that of the control imBMSC-siControl cells (Fig. 4A and B). Furthermore, silencing *Ago* expression was shown to significantly decrease BMP9-induced *in vitro* matrix mineralization in the imBMSC-*simAgo2134* cells, compared with that of the control imBMSC-siControl cells (Fig. 4C), indicating that AGO proteins are important for both early and late stage of BMP9-induced osteogenic differentiation of MSCs. As BMP9 also induces adipogenic differentiation,⁸³ we also examined whether BMP9-induced adipogenic differentiation would be impacted when the expression of the *Ago* genes was silenced. As shown in Fig. 4D, silencing *Ago* expression decreased BMP9-induced adipogenic differentiation of MSCs as revealed by Oil Red O staining.

We further analyzed the impact of *Ago* silencing on the expression of the key BMP9-regulated lineage-specific transcriptional factors Runx2 (osteogenic), Sox9 (chondrogenic) and Ppar γ (adipogenic) in MSCs.^{24,25,83} As expected, while BMP9 stimulation significantly up-regulated the expression of Runx2, Sox9 and Ppar γ in the imBMSC-siControl cells, compared with that of the GFP groups, silencing *Ago* expression effectively decreased the expression of the Runx2, Sox9 and Ppar γ in the imBMSC-*simAgo2134* cells, compared with that of the imBMSC-siControl cells (Fig. 5A). We further analyzed the effect of *Ago* silencing on the expression of common osteogenic markers and found that the BMP9-induced expression of

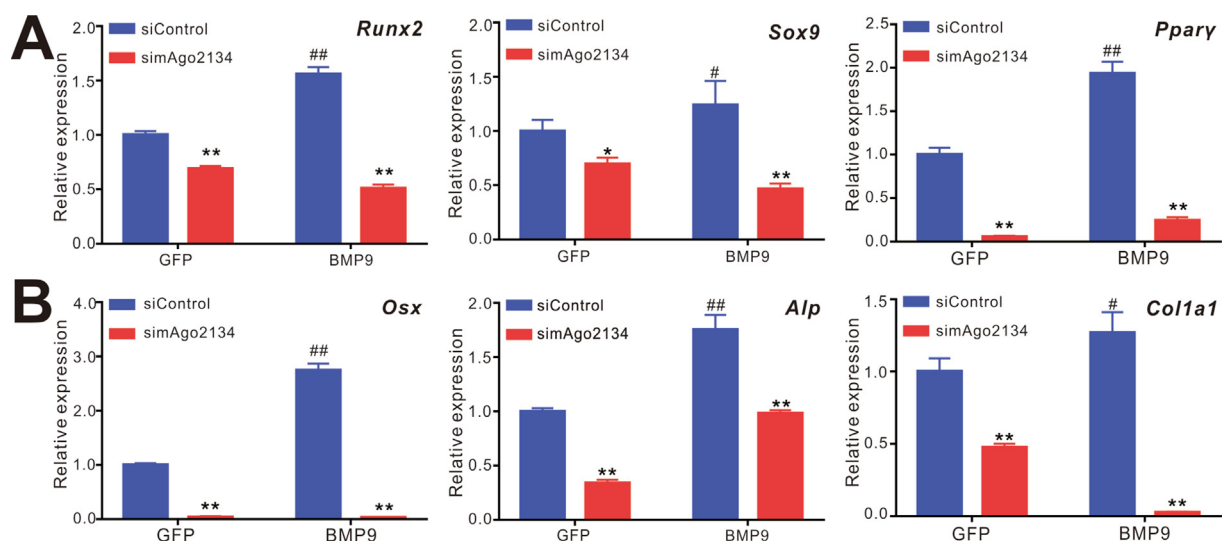


Figure 5 Silencing *Ago* family members diminishes BMP9-regulated expression of osteogenic and adipogenic differentiation related genes in MSCs. Subconfluent imBMSC-siControl and imBMSC-*simAgo2134* cells were infected with AdBMP9 or AdGFP. At 72 h post infection, cells were lysed and total RNA was isolated and subjected to TqPCR analysis of the expression of MSC lineage-specific regulators including *Runx2*, *Sox9* and *Pparγ* (A) and osteogenic markers including *Osx*, *Alp* and *Col1a1* (B) *Gapdh* was used as a reference gene. Each assay condition was done in triplicate. *, $P < 0.05$ and **, $P < 0.01$, compared with that of the siControl group. #, $P < 0.05$ and ##, $P < 0.01$, compared with that of the GFP group.

Osx, *Alp* and *Col1a1* was significantly reduced in the imBMSC-siAgo2134 cells, compared with that of the imBMSC-siControl cells (Fig. 5B). Collectively, these results demonstrate that silencing Ago significantly diminishes BMP9-induced lineage-specific, especially osteogenic and adipogenic differentiation of MSCs.

Silencing Ago family members inhibits BMP9-induced ectopic bone formation *in vivo*

Lastly, we analyzed whether silencing Ago expression would impact BMP9-induced bone formation *in vivo*. Using the MSC implantation assay, we found that, upon BMP stimulation, the ectopic bony masses were slightly smaller and much softer in the imBMSC-siAgo2134 injection group, than that

of the imBMSC-siControl injection group (Fig. 6A, panel a). MicroCT imaging analysis indicated that silencing Ago expression led to a significant decrease in bone mineral density and average bone volume in the imBMSC-siAgo2134 injection group, compared with that of the imBMSC-siControl injection group (Fig. 6A, panel a and b). Histologic evaluation revealed that silencing Ago expression significantly decreased mature bone formation in the imBMSC-siAgo2134 injection group, compared with that of the imBMSC-siControl injection group (Fig. 6B, panel a), which was further confirmed by Masson's Trichrome staining (Fig. 6B, panel b). Collectively, these *in vivo* results were supportive of the *in vitro* findings, strongly indicating that Ago-mediated microRNA biogenesis plays an essential role in BMP9-induced osteogenic differentiation of MSCs.

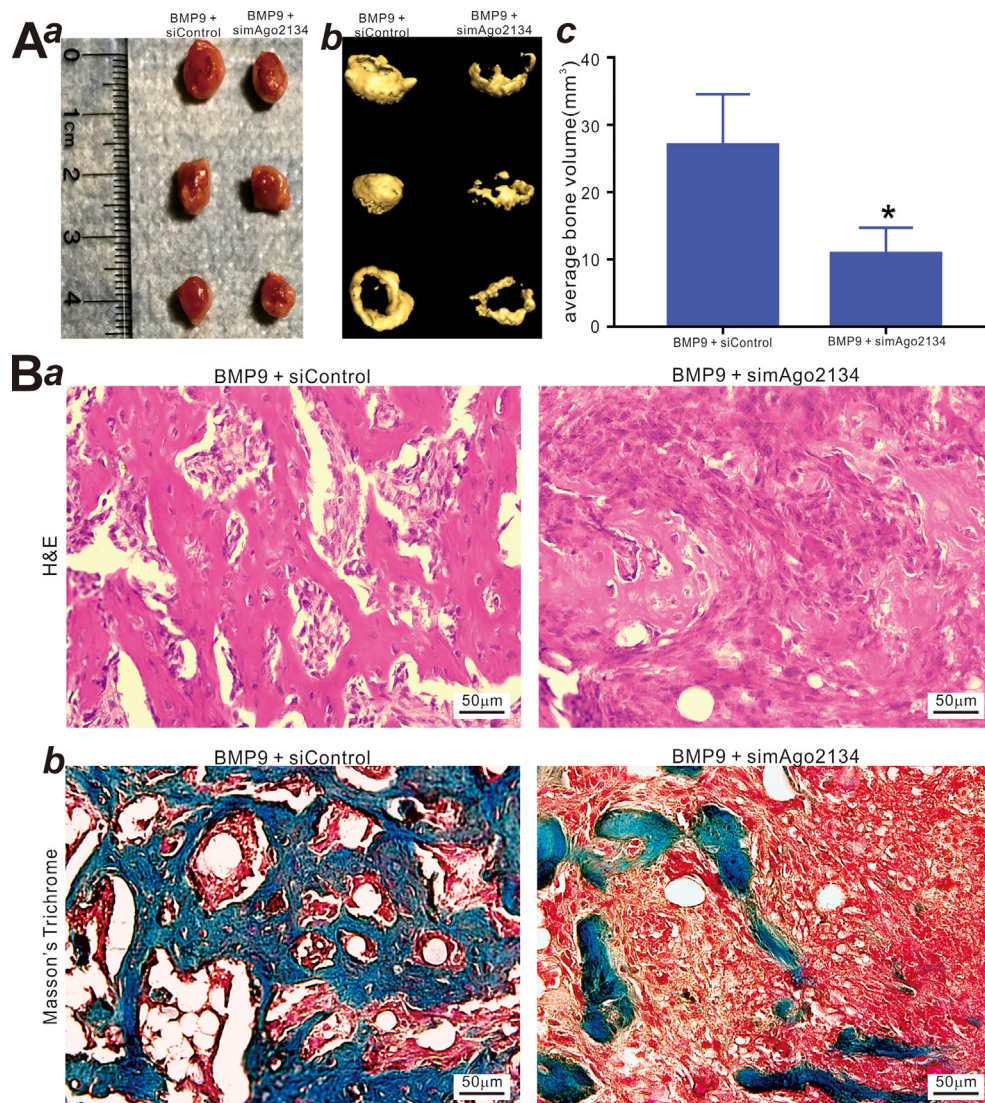


Figure 6 Silencing Ago family members attenuates BMP9-induced ectopic bone formation *in vivo*. (A) Subconfluent imBMSC-siControl and imBMSC-siAgo2134 cells were infected with AdBMP9 or AdGFP for 36 h and collected for subcutaneous injection into the flanks of athymic nude mice. At 3 weeks after implantation, the mice were sacrificed and ectopic bone masses were retrieved. No retrievable masses were found in the AdGFP group. Representative gross images (a) and micro-CT iso-surface 3D reconstruction (b) are shown. The average bone volumes were quantitatively analyzed by using the Amira software (c). *, $P < 0.05$, compared with that of the siControl + BMP9 group. (B) H & E and Masson's Trichrome staining. The retrieved masses were fixed, decalcified and subjected to H & E staining (a) and Masson's trichrome staining (b). Representative images are shown.

Discussion

We have previously demonstrated that BMP9 is not only the most potent osteogenic factor but also induces adipogenic and chondrogenic differentiation in MSCs.^{14,16,18,19,21,83} Our subsequent studies revealed that BMP9 binds to ALK1/2 type I receptors and regulates a panel of downstream target genes, as well as cross-talking with several major signaling pathways in MSCs.^{12,15,24–30,64,66,88,97–99} However, the exact mechanism through which BMP9 regulates lineage-specific differentiation of MSCs remains to be elucidated. In this study, through a comprehensive multiplex siRNA-based silencing expression analysis of both individual *Ago* genes, or all four *Ago* genes, we have demonstrated that AGO proteins are important mediators of BMP9-induced osteogenic and adipogenic differentiation in MSCs, indicating that noncoding RNAs, especially microRNAs participate in BMP9-regulated lineage commitment in MSCs.

Noncoding RNAs, particularly miRNAs, have been implicated in bone formation and/or skeletal homeostasis.^{38,39,100–105} However, how noncoding RNAs or miRNAs exert their impact on osteogenesis remains to be fully investigated. In this study, we found that BMP9 up-regulated *Ago1*, *Ago2* and *Ago3* in MSCs at the immediate early stage, indicating that regulating *Ago* gene expression provides at least a plausible mechanism to govern miRNA biogenesis and thus regulate miRNA functions in osteogenic differentiation. Alternatively, BMP9 may regulate miRNAs that target other lncRNAs and/or signaling pathways, thus indirectly mediating BMP9 osteogenic signaling. In fact, we have recently found that upon BMP9 stimulation lncRNA H19 was upregulated in MSCs at the early stage and then decreased rapidly, followed by a gradual return to basal expression. Notably, H19 overexpression or H19 expression knockdown in MSCs interrupted BMP9-induced osteogenesis, which can be rescued by activated Notch signaling.⁶⁵ We further revealed that H19 might modulate Notch pathway-targeting miRNAs and then indirectly impact BMP9 signaling in MSCs.^{64,65} It has been reported that several miRNAs, including miR-133, miR-135, miR-206, miR-141 and miR-200a, miR-208, miR-125b, miR-204, miR-26a, miR-29, miR-27 and miR-2861, regulate the BMP-mediated induction of osteoblastic differentiation.¹⁰² Furthermore, miRNAs may regulate osteoblast and osteoclast lineage-specific transcription factors, and orchestrate feedforward and reverse loops of signaling pathways and regulatory networks, leading to control of normal bone formation and turnover. Thus, miRNA deregulation may impact the progression of bone-related disorders including osteoporosis.^{38,39}

AGOs are conserved and specialized smRNA-binding proteins for miRNAs, siRNAs or PIWI-associated RNAs (i.e., piRNAs), leading to silencing target mRNAs by interacting with other factors.^{46,106,107} Thus, AGO proteins serve as a major component of the RISCs with small RNAs and other proteins such as Dicer, GW182 proteins, and Hsp70/Hsp90.¹⁰⁶ Four AGO proteins (AGO1–4) in humans and mice are conserved at the amino acid level with 99% for AGO2, 3, and 4 and 100% for AGO1 between mouse and human.¹⁰⁷ The *Ago2* knockout mice were lethal during early development at post-implantation stages, whereas *Ago1*, 3, and 4

knockout mice are viable.¹⁰⁷ Nonetheless, the phenotype of the *Ago2*-deficient mice compared to other RNAi-deficient mice, e.g., *Dicer*- or *Drosha*-deficient mice, is not identical, indicating distinct regulatory roles for the RNAi or RISC effectors during mouse development.¹⁰⁷ In fact, emerging evidence indicates that the biological functions of AGO-associated smRNAs expand beyond the post-transcriptional regulation by miRNAs, and are implicated in transcriptional gene silencing or activation, alternative splicing, antiviral defense, genome integrity control, DNA repair, and epigenetic modifications.¹⁰⁷ An early *in vitro* study showed that silencing *DICER* or *DROSHA* in human MSCs inhibited osteogenic differentiation.¹⁰⁸ Thus, it is conceivable AGO proteins and the associated smRNAs, especially miRNAs, may play an essential role in modulating lineage commitments and tissue-specific terminal differentiation of MSCs, and bone homeostasis, although many detailed mechanisms have yet to be uncovered.

In summary, we examined the potential roles of AGO proteins in BMP9-induced lineage-specific differentiation of MSCs. Using multiplex siRNA vectors to express multiple siRNAs silencing individual *Ago* genes or all four *Ago* genes, we demonstrated that the expression of *Ago1*, *Ago2* and *Ago3* was up-regulated by BMP9 in MSCs and that silencing individual *Ago* expression decreased BMP9-induced ALP activity in MSCs. We further demonstrated that simultaneous silencing of all four *Ago* genes effectively diminished osteogenic and adipogenic differentiation in MSCs, *in vitro* matrix mineralization, and *in vivo* ectopic bone formation induced by BMP9. Taken together, our results strongly suggest that AGO proteins and associated small RNA biogenesis pathway may be essential for BMP9-induced osteogenic differentiation of MSCs.

Authors contribution

HHL, TCH, LC, JY, LS and YM conceived and designed the study. YM, NN, LH, JF, HW and FH performed the experiments and collected data. QL, DS, KF, MP, WW, ABT and CC participated in molecular cloning experiments; provided essential experimental materials; and assisted in histological preparations and staining, and qPCR data analysis and interpretations. YM, LC, RRR, HHL, RCH, SHH, MJL, JY, LS and TCH drafted and revised the manuscript. All authors read, reviewed and approved the final manuscript.

Conflict of Interests

The authors declare no competing conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gendis.2021.04.004>.

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