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Original Article

Bone morphogenetic protein 9 induces osteogenic differentiation of germ cell 1 spermatogonial cells

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Abstract: Germ cell 1 spermatogonial (GC-1spg) cells are multipotent progenitors. Bone morphogenetic protein (BMP9) has previously been confirmed as one of the most osteogenic BMPs. However, whether GC-1spg cells are driven toward osteogenic differentiation under proper stimuli is uncertain and the molecular mechanism by which BMP9 promotes osteogenesis remains unclear. Our aim was to examine with evidence how BMP9 can induce osteogenic differentiation of GC-1spg cells. Using the AdEasy system, we generated recombinant adenoviruses to regulate BMP9 expression. Osteogenic markers were identified using real-time PCR and staining techniques *in vitro*. Ectopic ossification assays and histological analysis were also performed to verify the *in vivo* activity of BMP9. Finally, potential signaling pathways of BMP9 were assessed using transcriptome sequencing and KEGG enrichment analysis. We demonstrated that BMP9 upregulates osteogenic markers including Runx2, osteocalcin, osteopontin, and Sox9. BMP9 also activates alkaline phosphatase activity as well as deposition of calcium in GC-1spg cells. *In vivo* experiments showed that BMP9 overexpression in GC-1spg cells promotes ectopic bone formation and chondrogenesis. RNA-sequencing and KEGG pathway analysis demonstrated that multiple signaling pathways are involved in BMP9-mediated osteogenesis. GC-1spg cells not only maintain the capacity for spermatogenesis, but also retain the ability to form bone tissue. Therefore, BMP9 activity in GC-1spg cells may help identify signaling pathways implicated in bone formation that could be of use in regenerative medicine.

Keywords: Bone morphogenetic protein 9, spermatogonial cells, RNA-seq, KEGG pathway analysis, chondrogenesis

Introduction

Germ cell 1 spermatogonial (GC-1spg) cells are mouse spermatogonia that have been immortalized by the SV40 large T antigen [1]. They can differentiate into multiple cell types or undergo self-renewal in response to several factors [2-4]. They are largely involved in spermatogenesis and have mesenchymal stem cell (MSC) differentiation potential [5-8]; they can differentiate into different cells of the osteogenic, chondrogenic, or adipogenic lineages [9-12]. Indeed, MSCs can also essentially be isolated from the testis [5, 6]. However, several molecular events [13, 14] and factors are encountered in osteogenic differentiation of MSCs. Bone morphogenetic proteins (BMPs) are, in particular, vital for this process [15-17].

As a part of the members of the TGF-β superfamily, BMPs are essential for bone formation, stem cell differentiation, as well as male reproduction [15-20]. To date, more than 15 different BMPs have been identified. Our previous work showed that BMP9, along with those 15 or more BMPs, is the most osteogenic BMP

Table 1. Real-time PCR primers

Name	Direction	Sequence	Product size
Hum BMP9	Forward	5'-CTTCCCATTGGCTGATGACG-3'	189 bp
	Reverse	5'-GCCACACTCATCCCCTCATA-3'	
Mus GAPDH	Forward	5'-ATGGGTGTGAACCACGAGA-3'	229 bp
	Reverse	5'-CAGGGATGATGTTCTGGGCA-3'	
Mus RUNX2	Forward	5'-AGATGGGACTGTGGTTACCG-3'	203 bp
	Reverse	5'-TAGCTCTGTGGTAAGTGGCC-3'	
Mus Osteocalcin	Forward	5'-AGGACCCTCTCTCTGCTCA-3'	223 bp
	Reverse	5'-CGTCACAAGCAGGGTTAAGC-3'	
Mus Osteopontin	Forward	5'-TCCAATCGTCCCTACAGTCG-3'	249 bp
	Reverse	5'-AGCTGACTTGACTCATGGCT-3'	
Mus SOX9	Forward	5'-ATGAAGATGACCGACGAGCA-3'	196 bp
	Reverse	5'-TGCACACGGGGAACTTATCT-3'	
Mus BMP9	Forward	5'-CTTCCCATTGGCTGATGACG-3'	189 bp
	Reverse	5'-GCCACACTCATCCCCTCATA-3'	

[21-24]. BMP9, also recognized as growth differentiation factor 2 (GDF-2) [25], was isolated from the liver of fetal mice in a previous study [25, 26]. It acts through the Notch signaling pathway [14, 24, 27]. Several metabolic processes are regulated by BMP9, including glucose and lipid metabolism [28, 29], iron metabolism [30], endothelial function, and angiogenesis [31-33]. Accordingly, abnormal BMP9 expression has been implicated in multiple diseases [29, 34-36].

Multiple studies have confirmed that GC-1spg cells have MSC characteristics [4, 7, 12, 37]; however, few have focused on the osteogenesis promoted by BMP9. We analyzed how GC-1spg cells react to BMP9 and demonstrated that it can induce bone formation in GC-1spg cells both *in vitro* as well as *in vivo* [10, 17, 38]. The GC-1spg cell transcriptome was analyzed after BMP9 treatment [27], and potential BMP9 signaling pathways were characterized by KEGG enrichment analysis.

Materials and methods

Cell culture and chemicals

GC-1spg and HEK-293 cells were supplied by the American Type Culture Collection. Cells were cultured in complete Dulbecco's modified Eagle medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone), 100 U/mL penicillin, and 100 g/mL streptomycin. The cultured cells were incubated at 37°C in 5% CO_2 [1, 39-41]. Unless otherwise specified, all chemicals were provided by Sigma-Aldrich (St. Louis, MO, USA) or Thermo Fisher Scientific (Waltham, MA, USA).

Recombinant adenovirus construction

To regulate BMP9 expression, the AdEasy system was used to generate recombinant adenoviruses [22, 39, 42, 43]. Human BMP9 DNA was amplified by high-fidelity PCR as follows: 96°C for 45 seconds; along with 18 cycles at 92°C for 20 seconds, 55°C for 30 seconds, and 70°C for 45 seconds, with a final 70°C incubation for 5 minutes. The amplified sequence was cloned into the adenoviral shuttle vector, from which recombinant adenoviruses were generated by the transformed HEK-293 cells [39]. Ad-BMP9, as the resultant adenoviruses, also expressed green fluorescent protein (GFP). Control adenoviruses expressing only GFP (Ad-GFP) were also constructed [17, 38].

RNA isolation process, reverse transcription, and real-time PCR

Total RNA from GC-1spg cells was extracted using TRIzol reagent (Aidlab, Beijing, China). Random hexamers and M-MuLV Reverse Transcriptase (Vazyme, Nanjing, China) were used for reverse transcription. Reactions were performed according to the below conditions: 25°C for 5 minutes, 50°C for 15 minutes, 85°C for 5 minutes, and 4°C for 10 minutes. The cDNA was further diluted by 10-fold and adopted as real-time PCR test templates. All PCR primers were formulated by the Primer3web software (**Table 1**) [17, 38, 44, 45]. Target sequences were incubated based on the following conditions: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds and 60°C for 30 seconds. Real-time PCR assays were conducted based upon an ABI QuantStudio 6 instrument. The reactions included diluted cDNA (4 μL), forward and reverse primers (0.4 μL each), SYBR Green Master Mix (10 μL), 50 times ROX Re-

ference Dye 2 (0.4 μL), and H_2O (4.8 μL). The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative gene expression data [46, 47]. Assays were performed with no less than three independent biological replicates.

Alkaline phosphatase (ALP) assays

The subject activity was assayed by histochemistry [17, 38, 48-50]. Log-phase GC-1spg cells were seeded in SlideFlasks (Thermo Fisher Scientific) and infected with adenoviruses expressing BMP9 or GFP, i.e. Ad-BMP9 or Ad-GFP. Under the defined timescale, ALP activity was assessed using a BCIP/NBT Chromogen assay kit (Solarbio, Beijing, China). Assays were repeated at least three times.

Alizarin Red-S staining

Alizarin Red-S staining was performed to identify mineralized matrix nodules or calcium precipitation [17, 38, 51]. GC-1spg cells were seeded in SlideFlasks, infected with either the two adenoviruses, Ad-BMP9 or Ad-GFP. GC-1spg cells were then maintained in DMEM containing ascorbic acid with 50 mg/mL and β-glycerophosphate with 10 mM for 7 or 9 days [51]. The culture medium was discarded, and the cells were then professionally cleansed thrice with PBS. Cells were then compounded with 1% glutaraldehyde at room temperature, around 28°C, for 10 minutes and rinsed thrice with PBS. After 30 minutes of incubation, the cells were placed for 30 minutes under the condition of approximately 28°C in 0.2% Alizarin Red-S (Solarbio) and rinsed thrice with PBS. Deposits of calcium mineral were found under the observation with microscopy in bright-field mode.

Cell implantation process and ectopic ossification assay

The research ethics committee of Hainan General Hospital approved the application of animal experiments and supervised the experiment process. Assays on ectopic bone formation were performed based on the before-mentioned process [17, 38, 52-54]. First, subconfluent GC-1spg cell samples were treated with either the two adenoviruses, Ad-BMP9 or Ad-GFP, for 24 hours. Cell samples were then harvested (5×10^6 per sample) and re-suspended in 100 μL PBS. Groups of male athymic nude mice, which are 4 weeks old, were divided into 5 animals per group. All mice were subcutaneously injected with the transformed cells. After 4 weeks, mice were euthanized, and the injection-site tissue was collected for analysis. Characteristic masses were selected, maintained in 10% buffered formalin, and a micro-CT system (Skyscan 1076, Antwerp, Belgium) was used for imaging all masses. Tissue volume, bone volume, and surface area were measured for each sample [55, 56].

Histological analysis

The tissue test samples were maintained in 10% buffered formalin overnight and embedded with paraffin. Serial sections were stained by hematoxylin and eosin (H&E), Masson's trichrome, and Alcian Blue, all from Sigma-Aldrich [17, 27, 38, 53].

Transcriptome analysis

GC-1spg cells were planted in culture dishes of 100 mm and were infected with either Ad-BMP9 or Ad-GFP. Total RNA was collected at 48 hours as the post stage of infection. At least 6×10^7 cells were used for RNA extraction, and all RNA from one group was pooled into one sample. Whole RNA-seq libraries, including lncRNA, circRNA, mRNA, and miRNA, were prepared by Novogene Bioinformatics Technology (Tianjin, China) and sequenced with an Illumina HiSeq 2000/4000 platform (San Diego, CA, USA). Potential BMP9 signaling pathways were identified using KOBAS (v.2.0) software [57, 58] to assess transcriptome enrichment for mRNA, lncRNA, circRNA, and miRNA. BMP9-related KEGG pathways were identified as described [59-61].

Statistical analysis

All relevant data were recorded and analyzed by GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA) and SAS (v.9.0; SAS Institute, Cary, NC, USA). For real-time PCR, the $2^{-\Delta\Delta Ct}$ method was applied. Significant differences between groups of Ad-BMP9 and Ad-GFP were determined by *t*-tests. A hypergeometric $P < 0.05$ was calculated to identify convincing associations in KEGG pathways.

Results

BMP9 upregulates osteogenic differentiation markers in vitro

First, the examined recombinant adenoviruses were tested to show that Ad-BMP9 upregulated BMP9 expression (**Figure 1A** and **1B**). GFP expression was also present in Ad-BMP9-infected cells (**Figure 1A** and **1B**). Since BMP9 is a potent osteogenesis inducer, we next tested its function in GC-1spg cells. Runx2, osteocalcin (OCN), osteopontin (OPN), and Sox9 [17, 38, 53], as several osteogenic differentiation markers, were upregulated by BMP9 (**Figure 1C**). We then examined the early osteogenic marker ALP [17, 62, 63] and mineral node formation. We found that BMP9 overexpression increased early osteogenesis differentiation, indicated by ALP activity in GC-1spg cells (**Figure 1D**). Similarly, Alizarin Red-S staining showed that BMP9 overexpression visibly increased mineral nodule formation *in vitro* (**Figure 1E**) as confirmed previously with similar results by PCR or ALP staining. The mentioned results demonstrate that BMP9 regulates osteogenic differentiation of GC-1spg cells.

BMP9 induces ectopic bone formation in vivo

To verify our *in vitro* results showing exogenous BMP9-induced GC-1spg cell osteogenesis, we performed ectopic ossification experiments. By applying a previous assay on stem-cell implantation [14, 17, 38, 53], we detected injection-site masses in both the experimental BMP9 and control GFP groups. The BMP9 group masses appeared insignificantly larger than the mass in the GFP group. Micro-CT analysis also showed differences in tissue volume, bone volume, and bone surface area between Ad-BMP9 and control tumors (**Figure 2A** and **2B**).

Histological analysis verified BMP9 function in GC-1spg cells. Significant differences were not observed between groups using H&E staining, but Masson's trichrome staining revealed more mature cells with increased mineralization from exogenous BMP9 expression. Analogously, Alcian Blue staining also showed accumulation of chondroid matrix in BMP9-induced cells (**Figure 2C**). Taken together, our data demonstrate that BMP9 induces bone formation in GC-1spg cells.

BMP9 activity is transduced by several cellular pathways

Several recent studies showed that for BMP9-regulated bone formation in the process of MSCs, Notch signaling is necessary [14, 27, 48, 64]. However, some have reported that BMP9 can induce MSCs to differentiate into multiple cell types [22, 65, 66]. Moreover, aberrant BMP9 expression might be involved in certain diseases [20, 29, 34]. These studies suggest that several signaling pathways are involved in BMP9-regulated cell fate or proliferation, but the specific pathways are unclear. We therefore analyzed total RNA, including lncRNA (**Figure 3A**), miRNA (**Figure 3B**), circRNA (**Figure 3C**), and mRNA (**Figure 3D**), by RNA-seq, followed by KEGG pathway analysis. Our data indicate that TGF-β, Notch, MAPK, and Ras signaling pathways mediate BMP9-induced differentiation. These data corroborate previous findings on the mechanism of BMP9-induced osteogenic differentiation [14, 27, 65-68]. Simultaneously, we also found that insulin, PPAR, glutathione metabothyroid hormone, TNF, PI3K-Akt, and several cancer pathways are involved in BMP9-mediated cellular differentiation. Thus, our findings assert that BMP9 signaling can be an attractive therapeutic target for adipogenesis, diabetes, tumors, and some chronic inflammatory diseases [17, 28, 29, 69, 70].

Discussion

BMP9, as part of the TGF-β superfamily, can induce MSCs, such as mouse embryonic fibroblasts (MEFs) or C3H10 cells, to differentiate into bone tissue [17, 22, 23, 71, 72]. The MSC-to-osteoblast transition is driven by BMP9, the compelling osteogenesis-inducing BMP signal *in vitro* and *in vivo* [21, 22, 73]. MSCs originating from the mesoderm can be isolated from multiple tissues [5, 74-77]. Indeed, pluripotent stem cells isolated from testis are similar to MSCs, as demonstrated by gene expression profiling studies [5, 77]. MSCs can be derived from the testis [5, 8, 74]. Although it is possible to differentiate bone tissue from MSCs, few studies have examined BMP9-induced osteogenesis of testis-derived MSCs.

Different statuses of type-B spermatogonia or preleptotene spermatocytes were represented by GC-1spg cells, a mouse spermatogonia-derived cell line [1, 37]. Several studies have

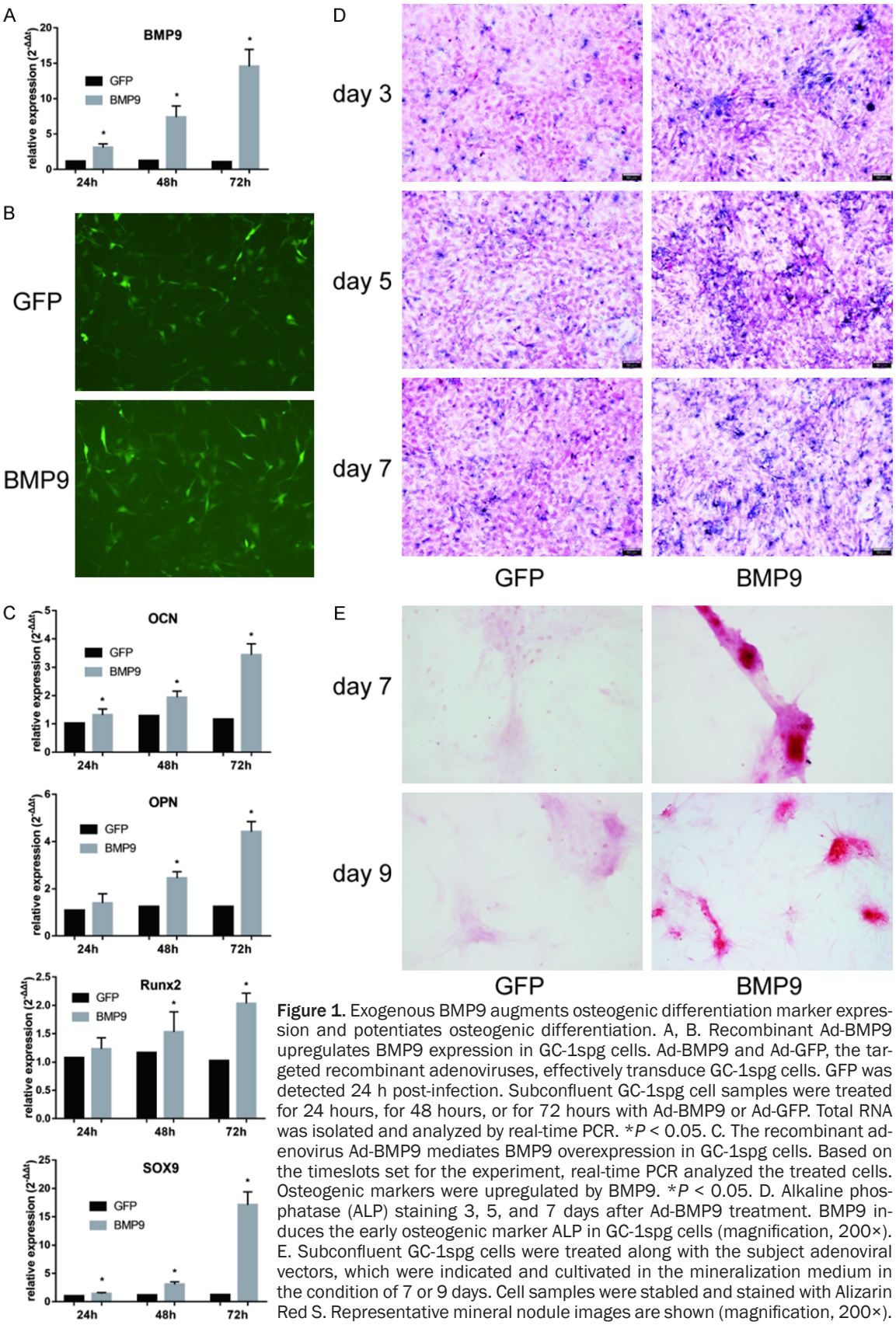

Figure 1. Exogenous BMP9 augments osteogenic differentiation marker expression and potentiates osteogenic differentiation. A, B. Recombinant Ad-BMP9 upregulates BMP9 expression in GC-1spg cells. Ad-BMP9 and Ad-GFP, the targeted recombinant adenoviruses, effectively transduce GC-1spg cells. GFP was detected 24 h post-infection. Subconfluent GC-1spg cell samples were treated for 24 hours, for 48 hours, or for 72 hours with Ad-BMP9 or Ad-GFP. Total RNA was isolated and analyzed by real-time PCR. *$P < 0.05$. C. The recombinant adenovirus Ad-BMP9 mediates BMP9 overexpression in GC-1spg cells. Based on the timeslots set for the experiment, real-time PCR analyzed the treated cells. Osteogenic markers were upregulated by BMP9. *$P < 0.05$. D. Alkaline phosphatase (ALP) staining 3, 5, and 7 days after Ad-BMP9 treatment. BMP9 induces the early osteogenic marker ALP in GC-1spg cells (magnification, 200×). E. Subconfluent GC-1spg cells were treated along with the subject adenoviral vectors, which were indicated and cultivated in the mineralization medium in the condition of 7 or 9 days. Cell samples were stabled and stained with Alizarin Red S. Representative mineral nodule images are shown (magnification, 200×).

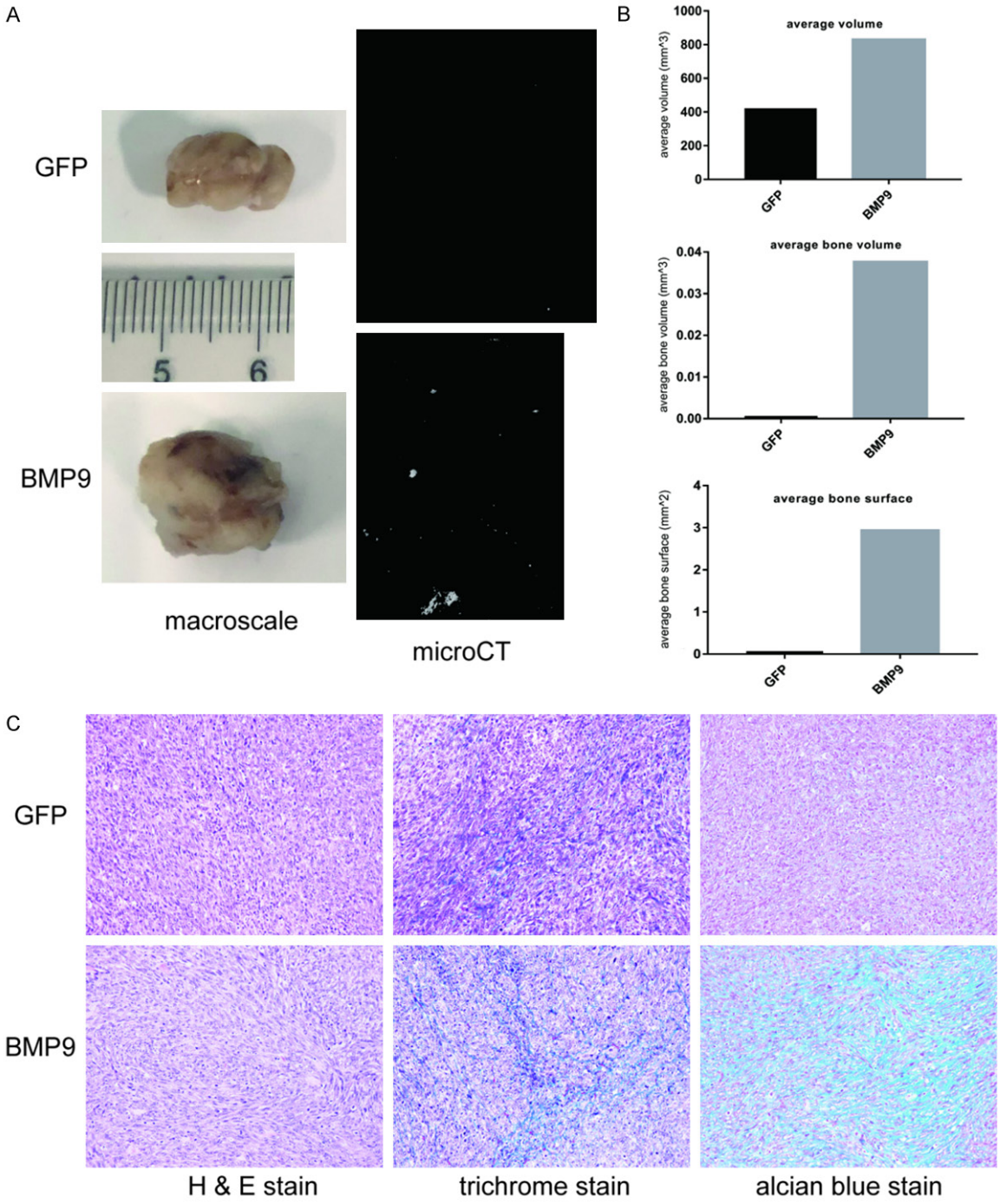

Figure 2. BMP9 induces mineralization and maturation of ectopic bone formation. A. Sub-confluent GC-1spg cell samples were treated in the period of 16 hours with Ad-BMP9 or Ad-GFP. Athymic nude mice were injected with the collected treated cells for subcutaneous injections. Cells were allowed to grow for 4 weeks. Characteristic masses were retrieved and examined by micro-CT. B. Injection-site products in the group with BMP9 were larger than the ones in the group with GFP. Ossification structure volume and surface area of the masses in the group with BMP9 group were, moreover, higher than the ones in the group with GFP. C. Tumor samples were fixed, decalcified, and embedded with paraffin and stained with H&E, Masson's trichrome, or Alcian Blue. Representative images are shown. The Ad-GFP group exhibited less mineralized, immature chondroid matrix compared to the Ad-BMP9 group.

demonstrated GC-1spg cell plasticity [1, 4, 78, 79]. In our study, we drove BMP9 expression using a recombinant adenovirus. Real-time PCR results confirmed that our adenovirus

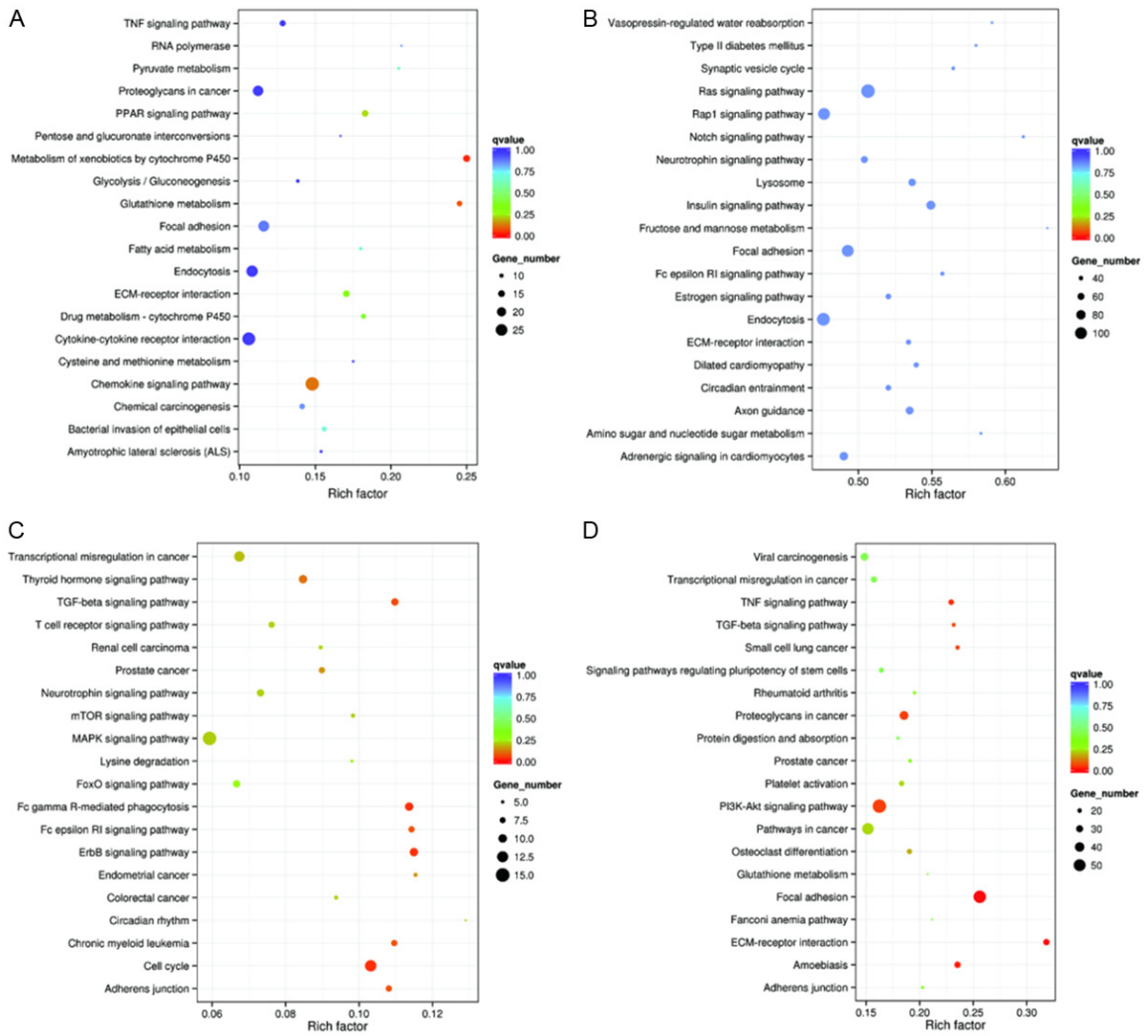

Figure 3. GC-1spg cell signaling pathways induced by BMP9. The top 20 KEGG pathways with differentially expressed. (A) lncRNA; (B) miRNA; (C) circRNA; and (D) mRNA are shown.

stably overexpressed BMP9 in GC-1spg cells. Secondly, the expressions of OPN, OCN, Sox9, and Runx2 [17, 38, 62, 65, 71, 80], which are osteogenic markers, are enhanced by BMP9 in GC-1spg cells. Furthermore, ALP and mineralized calcium nodules, early osteogenesis markers, are increased by BMP9 overexpression. Therefore, our *in vitro* results demonstrate that exogenous BMP9 expression promotes osteogenic differentiation and mineralization in GC-1spg cells. To verify our *in vitro* data, we implanted BMP9-overexpressing cells in mice to induce ectopic ossification. Bony mass formation is augmented by exogenous BMP9 expression. Masson's trichrome staining also showed that BMP9 increased trabeculae thickness, and Alcian Blue staining demonstrated that elevated BMP9 intensified cartilaginous matrix accumulation. Combined with Sox9 expression, we posit that chondrogenic signaling for BMP9-enhanced differentiation of terminal osteogenic is important.

As one of the most significant osteogenic BMPs, *in vitro* and *in vivo* [21, 22, 25], BMP9 plays a crucial role in several diseases [29, 35, 69, 81-83]. Signaling by BMP9 is a synergistic factor regulating the proliferation and migration of specific cell types and stem cell differentiation through extensive crosstalk with several signaling pathways. Though numerous signaling pathways are intended to involve BMP9-mediated cellular differentiation and proliferation, the cellular transduction mechanism of

BMP9 remains unclear. To identify the signaling pathways activated by BMP9, we analyzed total RNA, including lncRNA, mRNA, circRNA, and miRNA, by RNA-seq and KEGG pathway analysis. Mechanistically, the TGF-β type I receptors, i.e. ALK1 and ALK2, are required for BMP9-enhanced differentiation [67]. These receptors mediate nuclear signaling via Smad phosphorylation, suggesting that Smad or TGF-β signaling pathways mediate BMP9-induced osteogenesis. Related pathways, including TNF [84, 85], MAPK [86, 87], Wnt [68], and Notch [14, 27, 53], may also play important roles. In our study, KEGG pathway analysis, expressed lncRNA, mRNA, circRNA, and miRNA differentially, showed that BMP9 treatment alters the expression of cell cycle, focal adhesion, chemokine signaling, and ErbB signaling pathways genes. These pathways are related to the MAPK signaling pathway [88], differentiation and angiogenesis [89], as well as the PPAR signaling pathway, which plays an essential role in lipid metabolism and adipocyte differentiation [90].

Certain limitations have been identified in our study. First, MEFs and C3H10 are frequently used in osteogenesis studies; however, GC-1spg cells are rarely used in BMP9 research. This may explain why our results differ from previous findings on BMP9 activity. Indeed, BMP9-related signaling pathways have been identified in MEFs, but not in GC-1spg cells. Second, we used adenovirus transfection to regulate BMP9 expression. It is possible that the signaling pathways identified in our study could have been induced by the adenovirus and not by BMP9 itself.

Collectively, our results indicate that the potent osteogenic signal BMP9 induces GC-1spg cells toward osteogenic differentiation both *in vitro* and *in vivo*. In addition, high Sox9 expression and chondroid matrix accumulation in bony masses suggest that chondrogenesis might have a dominant status in BMP9-induced bone formation. However, many signaling pathways other than MAPK and Notch are involved in BMP9-stimulated GC-1spg cells. Future studies investigating BMP9-mediated bone formation are warranted.

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Disclosure of conflict of interest

None.

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