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FULL LENGTH ARTICLE

Bone morphogenetic protein-9 effectively induces osteogenic differentiation of reversibly immortalized calvarial mesenchymal progenitor cells☆

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Abstract Critical-sized craniofacial defect repair represents a significant challenge to reconstructive surgeons. Many strategies have been employed in an effort to achieve both a functionally and cosmetically acceptable outcome. Bone morphogenetic proteins (BMPs) provide a robust osteoinductive cue to stimulate bony growth and remodeling. Previous studies have suggested that the BMP-9 isoform is particularly effective in promoting osteogenic differentiation of mesenchymal progenitor cells. The aim of this study is to characterize the osteogenic capacity of BMP-9 on calvarial mesenchymal progenitor cell differentiation. Reversibly immortalized murine calvarial progenitor cells (iCALs) were infected with adenoviral vectors encoding BMP-9 or GFP and assessed for early and late stages of osteogenic differentiation *in vitro* and for osteogenic differentiation via *in vivo* stem cell implantation studies. Significant elevations in alkaline phosphatase (ALP) activity, osteocalcin (OCN) mRNA transcription, osteopontin (OPN) protein expression, and matrix mineralization were detected in BMP-treated cells compared to control. Specifically, ALP activity was elevated on days 3, 7, 9, 11, and 13 post-infection and OCN mRNA expression was elevated on days 8, 10, and 14 in treated cells. Additionally, treatment groups demonstrated increased OPN protein expression on day 10 and matrix mineralization on day 14 post-infection relative to control groups. BMP-9 also

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facilitated the formation of new bone *in vivo* as detailed by gross, microcomputed tomography, and histological analyses. Therefore, we concluded that BMP-9 significantly stimulates osteogenic differentiation in iCALs, and should be considered an effective agent for calvarial tissue regeneration.

Introduction

The repair of large, complex craniofacial defects often poses a challenge to reconstructive surgeons. The primary goals of an adequate reconstruction include the restoration of form and function. A return to "normal" or pre-morbid facial aesthetics is critical in defect repair. In the case of pediatric patients, an understanding of craniofacial growth and how the choice of and approach to surgery may impact it must also be considered. Indications for craniofacial defect repair are most often related to tumor, trauma, or congenital disease. For the majority of patients, the preferred method of reconstruction is to "replace like with like". For example, cranioplasty is ideally performed using the surrounding cranium.[1] Secondarily, iliac crest, rib and scapula are reliable options.[2–4] The major limitation, however, directly relates to the finite supply of autologous bone. Further complicating outcome is potential donor site morbidity due to infection, pain, nerve injury, and blood loss.[5]

In addition to the physical constraint of bone available for harvest, reconstructions may fail because transplanted bone is prone to resorption and reduced biomechanical strength.[6] These challenges inherent to autologous tissue-based reconstruction have encouraged both surgeons and basic scientists to seek alternative methods to facilitate bone growth and healing.[7,8] Adjunct strategies are numerous[9] and examples include the placement of biomaterials within the defect to hasten ossification[10,11]; administration of osteoinductive growth factors to augment the body's own production of new bone[12]; and the isolation, expansion, and subsequent stimulation of host osteoprogenitor cells.[13,14] These strategies, however, often fall short of the desired goals.[15,16]

One promising approach lies in the application of bone morphogenetic proteins (BMPs) to critical-sized bone defects (i.e., defects that are unable to heal spontaneously). BMPs are naturally occurring cytokines that regulate several integral functions at the molecular level including osteogenic differentiation, bone development, and fracture repair.[12] Recombinant human forms (rhBMP) of BMP-2 and BMP-7 are currently offered for abnormalities of bony healing both within and outside of the craniofacial skeleton.[17,18] A growing body of literature has demonstrated the osteoinductive effects of BMPs on craniofacial bone growth as well.[19,20] Our laboratory recently demonstrated that mesenchymal progenitor cells derived from juvenile murine calvarium preferentially differentiate toward bone in the presence of BMP-2.[18] Ironically, despite a relative paucity of research examining the effects of BMP-9 on bone formation, particularly with respect to the craniofacial skeleton, comprehensive analyses of 14 human BMP isoforms demonstrated that BMP-9 displays the greatest degree of osteogenic potential.[21–23] To elucidate the potential role of BMP-9 in craniofacial defect repair, the current study characterizes and quantifies the osteogenic effects of BMP-9 on calvarial mesenchymal progenitor cells. This study is the first to our knowledge to examine BMP-9 in the context of cranial bone engineering.

Materials & methods

Isolation and culture of calvarial mesenchymal progenitor cells

Calvariae were isolated from three-week old male CD-1 mice (Charles River, Wilmington, MA, USA) as described previously.[18] This investigation was approved by the Institutional Animal Care and Use Committee of the University of Chicago (Chicago, IL), and animal maintenance and experimental treatments were conducted in accordance with the ethical guidelines set forth by this committee. All procedures were conducted under sterile conditions.

Mice were sacrificed and calvariae were harvested by creating a mid-sagittal incision. The periosteum was incised to expose the calvarium on both sides of the midline. Soft tissue, dura and remaining periosteum were removed. The isolated calvaria were treated as previously described.[18] Cultures were incubated at 37 °C, 95% humidified air, and 5% CO_2. After approximately 7 days, cells grew to 80% confluency (% of cells covering the plate) at which point they were passaged by enzymatic digestion (0.1% Trypsin, Sigma–Aldrich) to 25 cm^2 flasks containing 8 mL DMEM with 10% FBS and 1% penicillin/streptomycin for experimentation.

Reversible immortalization of primary calvarial cells

To allow for ease of culturing and preservation of cellular growth, harvested primary calvarial cells were allowed to grow in culture for 5 weeks and then underwent immortalization using a retroviral-mediated vector as previously described.[18,24] Previous work has shown that the immortalization process does not significantly alter cell morphology and that immortalized cells maintain expression of surface antigens typically expressed by primary mesenchymal progenitor cells.[18] An added feature of this transduction technology is the ability to revert the

immortalized target back to a primary cell. All *in vitro* and *in vivo* experimentation was performed using immortalized calvarial cells (iCALs).

Recombinant adenoviral vectors

Recombinant adenoviruses were generated using AdEasy technology as previously described.[22,25–27] The coding region of human BMP-9 was PCR amplified and cloned into an adenoviral shuttle vector and subsequently used to generate recombinant adenoviruses in HEK293 cells (American Type Culture Collection, Manassas, Virginia). The resulting adenoviruses were designated as AdBMP-9. Green fluorescence protein (GFP), which served as a marker of infection efficiency, was included in the vector. Analogous adenovirus expressing only monomeric GFP (AdGFP) was used as a control. Serial titrations of the adenoviruses were performed to determine the viral dosage that optimized viral infection and cell viability. All samples were exposed to virus for up to 24 h prior to changing the medium. Immortalized cells were transduced with AdBMP-9 or AdGFP.

Alkaline phosphatase (ALP) activity

Cells were seeded into 24-well cell culture plates at a density of 2×10^4 cells/cm^2 and cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin for 3, 7, 9, 11, and 13 days with AdBMP-9 and AdGFP (control). ALP activity was assessed quantitatively with a modified assay using the Great Escape SEAP Chemiluminescence assay kit (BD Clontech, Mountain View, CA) and qualitatively with 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 described.[21,28] Each assay condition was performed in triplicate and the results were repeated in at least three independent experiments. ALP activity was normalized by total cellular protein concentrations among the samples.

Alizarin red S staining

Immortalized calvarial cells were plated into 12-well tissue culture plates at a density of 2×10^4 cells/cm^2 and cultured for 14 days with DMEM containing ascorbic acid (50 μg/mL) and β-glycerophosphate (10 mM) with AdBMP-9 and AdGFP treatments. Fresh culture medium was added to each well every 4 days and nodule formation was routinely checked by phase contrast microscopy. The presence of mineralized nodules was determined by Alizarin red staining as described.[21] Briefly, cells were washed with 1% PBS and fixed with 2.5% glutaraldehyde at room temperature for 10 min. Fixed cells were incubated with 0.4% alizarin red S (Sigma–Aldrich) for 15 min followed by washing with distilled water. Stained cells were further incubated with PBS for 5 min. Mineralization was assessed via bright field microscopy.

RNA isolation and semi-quantitative RT-PCR

Total RNA was isolated using TRIZOL Reagents (Invitrogen, Carlsbad, CA, USA) and cDNA was generated utilizing reverse transcription with random hexamers and Superscript II RT (Invitrogen). First strand cDNA products were further diluted 5- to 10-fold and used as PCR templates. Semi-quantitative RT-PCR was carried out using qPCR primers (~18 bps) designed by using primer sets for osteocalcin (OCN) and GAPDH. GAPDH was used as a control (approximately 150–180 bps). Primers used were as follows: OCN:sense, 5′-CCAAGCAGGAGGGCAATA-3′; antisense, 5′-TCGTCACAAGCAGGGTCA-3′; GAPDH:sense, 5′- ACCCAGA AGACTGTGGATGG-3′; antisense, 5′-CACATTGGGGGTAGGAA CAC-3′. PCR amplification was carried out with the following program: denature at 92 °C for 20 s, annealing at 55 °C, 17 cycles for GAPDH and 22 cycles for OCN. The PCR products were separated via a 1% agarose gel with 3 μL of ethidium bromide (10 mg/mL solution) for 15 min at room temperature at 80 V. Resulting bands were analyzed in a Kodak image station 440-CF using Kodak 1D 3.6 software.

Osteopontin immunohistochemical staining

To confirm the presence of osteopontin (OPN) upregulation in AdBMP-9-treated iCALs, immunohistochemical (IHC) staining on post-infection day 10 was performed as described previously.[18] Specifically, rabbit anti-OPN polyclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used.

Stem cell implantation assay

iCALs were infected with adenovirus encoding BMP-9 or GFP as indicated. At 16 h post-infection, cells were harvested, resuspended in PBS and injected (5×10^6 cells/injection) into the flanks (subcutaneous) or quadriceps (intramuscular) of athymic (nu/nu) mice (4–6 week-old males, Harlan Sprague-Dawley). At 5 weeks post-implantation, the mice were euthanized and the implantation sites were harvested and analyzed via microcomputed tomography (microCT) and histochemical staining.

microCT analysis

All retrieved specimens were fixed and imaged using the microCT component of a GE triumph (GE Healthcare) trimodality preclinical imaging systems as described.[28] All image data analyses were performed using Amira 5.3 (Visage Imaging, Inc.) and three-dimensional (3D) volumetric data were obtained as previously described.[29–33]

Histological evaluation

Retrieved tissues were fixed in 10% formalin (decalcified, if necessary) and embedded in paraffin. Serial sections of the embedded specimens were stained with hematoxylin and eosin (H&E). Paraffin-embedded sections were deparaffinized and then rehydrated in a graduated fashion. The deparaffinized samples were subjected to antigen retrieval and fixation. The sections were stained with H&E and Masson's trichrome as previously described.[21,22,26,28,34]

Statistical analysis

Statistical analysis was performed using SPSS software (IBM, Chicago, IL, USA). All values are reported as mean with associated standard error bars. Differences between two groups were compared using a student's unpaired t-test. $P < 0.05$ was considered statistically significant.

Results

Expression of adenoviral DNA

All adenovirally treated cells showed approximately equal GFP expression at 24 h post-infection, which verifies uptake of the DNA of interest via the adenoviral vectors (Fig. 1A). Control group cells that were not treated with adenovirus did not express GFP (data not shown).

Alkaline phosphatase (ALP) activity

ALP activity in cells transduced with AdBMP-9 and AdGFP was measured for up to 13 days in culture (Fig. 1B). On day 3 post-infection, AdBMP-9-treated cells demonstrated significantly higher ALP activity compared to cells treated with AdGFP. Similarly, from post-infection day 7 through post-infection day 13, ALP activity was significantly elevated in cells treated with AdBMP-9 compared to control cells.

Effect of AdBMP-9 on expression of secreted extracellular proteins

To confirm that the harvested and infected iCALs upregulated osteogenic genes and produced proteins relevant to extracellular matrix formation upon AdBMP-9 stimulation, the mRNA level of the late osteogenic marker gene osteocalcin (OCN) was assessed via RT-PCR. Levels of OCN mRNA were elevated in the AdBMP groups on days 8, 10, and 14 (Fig. 2A). Additionally, osteopontin (OPN) protein expression was elevated in the AdBMP groups by day 10 (Fig. 2B).

Mineralized nodule formation

To examine the mineralization capacity of the iCALs in the presence of BMP and GFP, mineral nodule formation was assessed on day 14 of culture in cells grown in the presence of AdBMP-9 or AdGFP via alizarin red S staining (Fig. 2C). Alizarin red S staining intensity of samples was higher by day 14 in AdBMP-treated groups compared to AdGFP controls.

Transduction of iCALs with AdBMP-9 enhances bone formation *in vivo*

Using the well-established stem cell implantation assay (Fig. 3A),[18] we tested the hypothesis that BMP-9 would induce a significant osseous response *in vivo*. GFP-treated cells produced miniscule bone nodule formation at implantation sites. In contrast, BMP-9-treated cells produced bone nodules that were significantly larger (Fig. 3B and C).

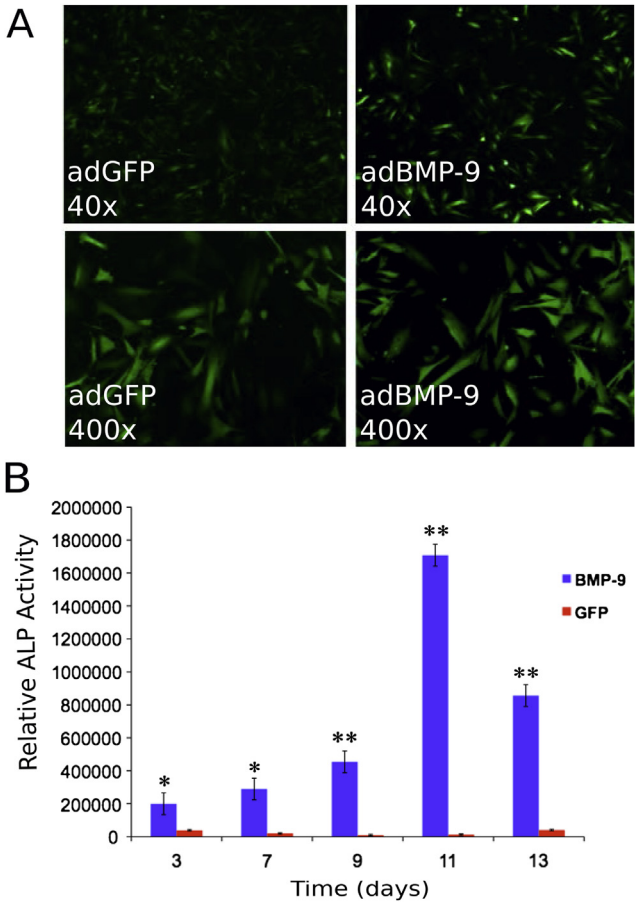

Fig. 1 Detection of early markers of osteogenic differentiation. (A) Fluorescent microscopy imaging of green fluorescent protein (GFP) expression at 24 h post-adenoviral infection of calvarial cells in GFP (control) and BMP-9 groups. (B) Alkaline phosphatase activity is a well-established marker of early osteogenic differentiation. Average relative alkaline phosphatase activity in calvarial cells on days 3, 7, 9, 11, and 13 post-adenoviral infection with BMP-9 (blue bars) or GFP (red bars) is shown. Experiments were performed in triplicate. Error bars represent standard error.*$P < 0.05$, **$P < 0.01$.

Ectopic bone derived from BMP-9 treated cells had a similar appearance and quality on microCT analysis to native bone (Fig. 3D). Histological survey of these masses revealed more mature bone and thicker trabeculae in BMP-9-treated cells compared to cells treated with GFP (Fig. 4A). Additionally, Mason's trichrome staining demonstrated matrix mineralization in the osseous nodules formed by BMP-9-stimulated cells (Fig. 4B).

Discussion

The search for methods to facilitate bony healing within the craniofacial skeleton has led to the development of many novel therapies. Synthetic materials including ceramics, titanium, porous polyethylene, and demineralized bone matrix have demonstrated efficacy but are associated with infection, the inability to expand with the growing

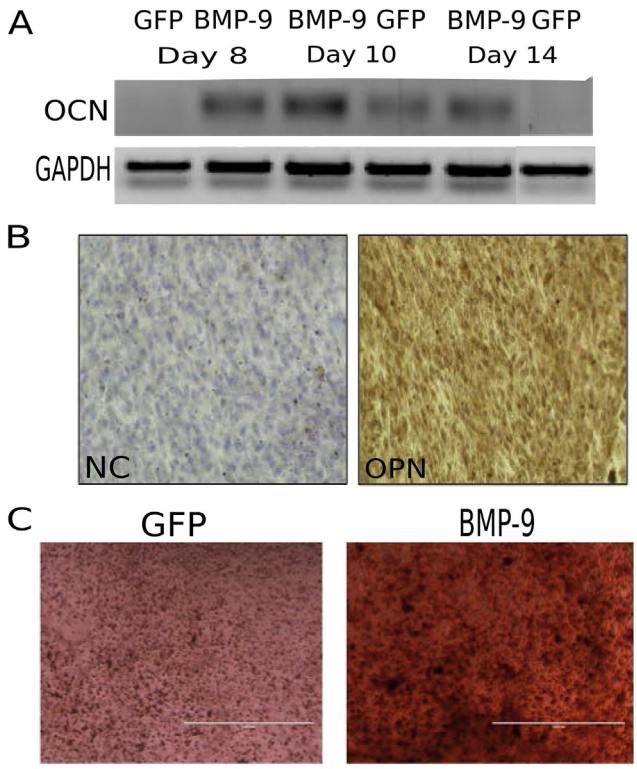

Fig. 2 Detection of late markers of osteogenic differentiation. (A) mRNA expression levels of osteocalcin were measured in calvarial cells on days 8, 10, and 14 post-adenoviral infection with BMP-9 or GFP. Internal control with GAPDH is also shown. (B) Immunohistochemical staining for osteopontin expression in AdBMP-9 and negative control (NC) groups at 10 days post infection. (C) Matrix mineralization (Alizarin red S staining) on post-infection day 14 in cells treated with AdGFP and AdBMP-9.

craniofacial skeleton, and failure over time.[5] For large composite defects that are deficient in skin, bone, and/or dura, a more complex reconstructive approach in the form of chimeric free flaps may be required.[35] More recently, the concept of tissue engineering has become a promising strategy for defect repair and/or reconstruction. Tissue engineering encompasses a broad array of therapies that include cell-based, growth factor-based, and/or scaffold-based approaches. The current study investigates the viability of cell- and growth factor-based strategies for bone formation.

The delivery of signaling molecules (mitogens, morphogens, and growth factors) to the site of intended repair describes growth factor-mediated tissue regeneration.[36] Many authors have studied the use of BMPs for growth factor-based craniofacial repair, predominantly focusing on BMP-2 and BMP-7.[12,37] Preliminary work from our laboratory demonstrated that direct transfer of AdBMP-2 into murine cranial defects results in improved but incomplete healing compared to AdGFP controls.[38] More recently, AdBMP-2 infection of calvarial-derived osteoprogenitor cells revealed successful formation of bone in both *in vitro* and *in vivo*.[18] Recombinant forms of BMP (rhBMP-2 and rhBMP-7) have even been used in off-label fashion for particularly complex reconstructive challenges of the human craniofacial skeleton with limited success.[39,40]

Despite abundant research of their osteoinductive properties and FDA-approval for clinical use, neither BMP-2 nor -7 appears to be the most osteogenic of the BMP subtypes. Several experiments from our laboratory have elucidated BMP-9 as a potent inducer of osteogenic differentiation of mesenchymal stem cells (MSCs), demonstrating greater osteogenicity than both BMP-2 and -7.[41] However, BMP-9 has been one of the least studied isoforms and has never been examined in the context of cranial defect repair. Therefore, we analyzed the osteogenic effects of BMP-9 on calvarial-derived osteoprogenitor cells. ALP activity was measured to assess early osteogenic differentiation.[42] Compared to control cells, by day 3 post-infection BMP-9-treated cells demonstrated a significant elevation in ALP activity. This pattern persisted at all time points, suggesting that BMP-9 stimulates a more pronounced early osteogenic response than control.

We also determined the osteogenic effect of BMP-9 using established markers of late osteogenic differentiation.[43] Upregulation of OCN mRNA was observed in AdBMP-treated groups compared to control cells. Similarly, expression of OPN protein was elevated in treatment groups compared to control on day 10. This evidence further supports that BMP-9 is capable of inducing osteogenic differentiation *in vitro*. Future experiments are needed to characterize upregulation of other proteins requisite for BMP signaling (e.g., Runx2 and osterix) in the presence of AdBMP-9 stimulation.

To assess the final stages of osteogenesis, alizarin red S staining was used to detect formation of calcium nodules.[44] By day 14, treatment cells demonstrated positive staining, indicating that cells were undergoing the terminal steps of osteogenic differentiation. Taken together, these results indicate that BMP-9 stimulation of calvarial mesenchymal progenitor cells results in significantly greater *in vitro* bone production than control.

In contrast to a growth factor-based approach to tissue engineering, cell-based strategies generally rely on the direct injection of cells into the site of interest. Cells may serve as biologic agents to stimulate tissue repair, as biological vehicles for gene delivery, or a combination thereof.[45,46] In the current study, iCALs provided a vehicle to deliver BMP to our animal model as well as a rich source of osteoprogenitor cells capable of forming new bone. Prior *in vivo* examination of iCAL behavior has demonstrated its ability to undergo osteogenic differentiation leading to bone formation upon BMP-2 stimulation.[18] The present study similarly demonstrated that BMP-9 infection of iCALs led to greater *in vivo* bone formation compared to GFP. These results suggest that BMP-9 is capable of stimulating bone formation of mesenchymal progenitor cells both *in vitro* and *in vivo* to a degree significantly greater than control.

At present, there is very limited research examining the effects of BMP-9 on the craniofacial skeleton. Using direct adenoviral-mediated BMP therapy, Alden and colleagues found that mandibular defects treated with AdBMP-9 displayed enhanced bony healing compared to controls.[47] Here, we reveal that BMP-9 can effectively induce osteogenic differentiation of mesenchymal progenitor cells

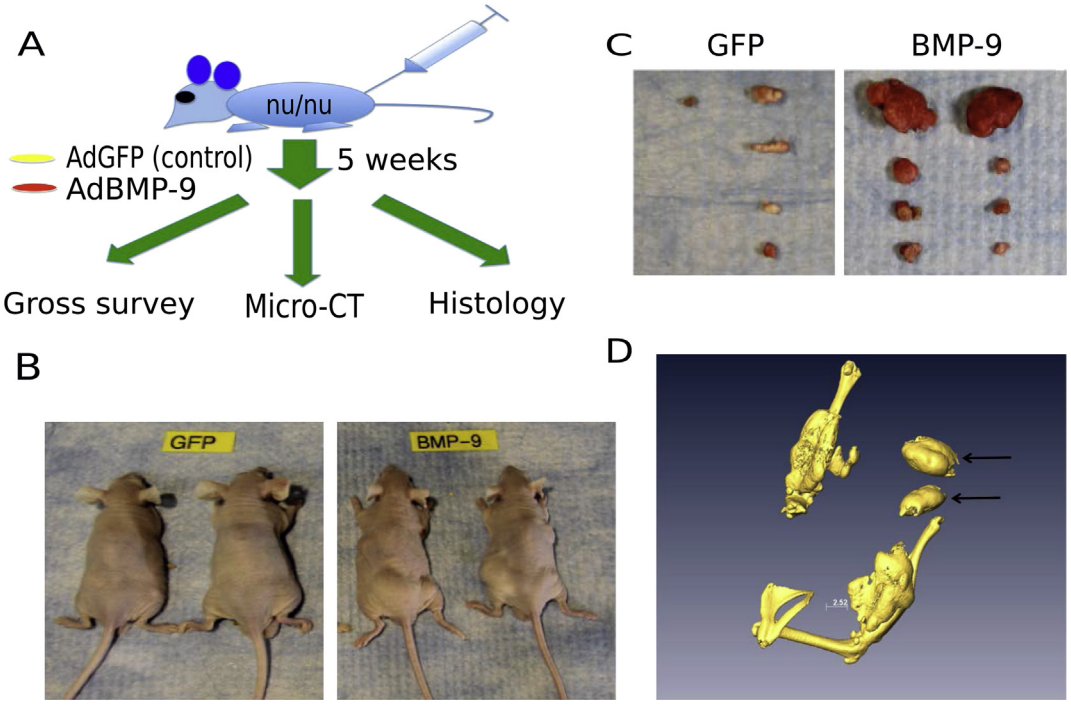

Fig. 3 Stem cell implantation assay to assess *in vivo* effects of AdBMP-9 infection. (A) Schematic diagram of experimentation. (B) Digital photography of representative mice after sacrifice. (C) Representative gross specimens after retrieval. (D) Using microCT imaging analysis, ectopic osseous nodules (denoted by arrow) derived from BMP-9-treated cells that were injected into mice are similar in character and appearance to native bone harvested after sacrifice of animals.

derived from the calvarium. Future studies should address whether the combined cell- and growth factor-based approach to bone engineering used in the current study is sufficient to heal critical-sized osseous defects. In addition, in an effort to increase the clinical applicability of our results, it would be beneficial to test the osteogenic effect of BMP-9 on mesenchymal progenitor cells isolated from the human craniofacial skeleton.

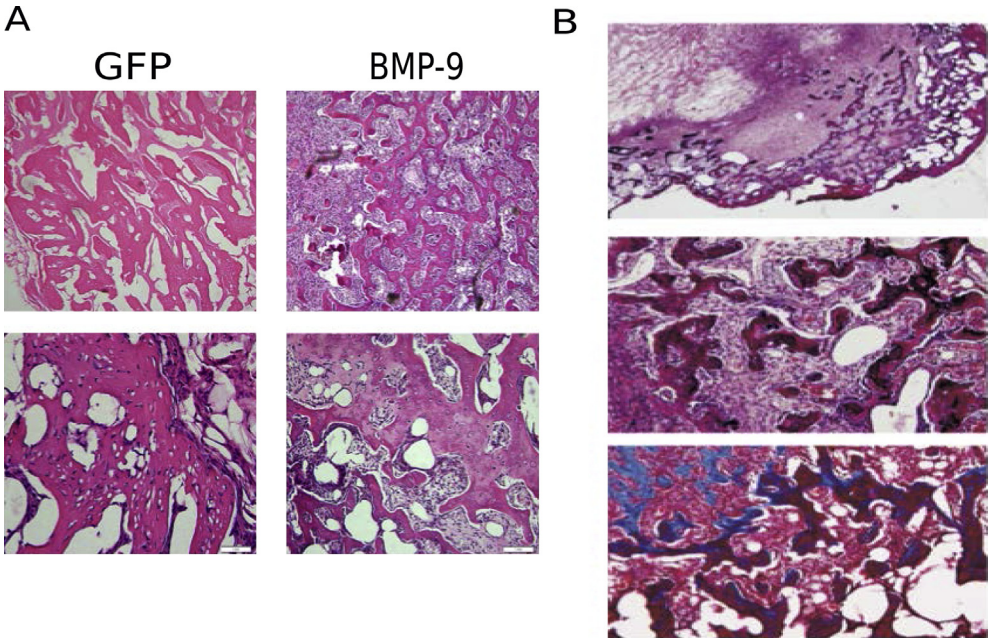

Fig. 4 iCALs treated with BMP-9 form more mature ectopic bone than iCALs treated with GFP. (A) Representative hematoxylin and eosin staining of 0.1 mm cross sections of harvested specimens reveal dense trabecular bone in experimental groups versus fibrous cell reaction present in the control group (GFP). Top panel, lower power; bottom panel, higher power. (B) Representative trichrome staining of 0.1 mm cross sections of harvested specimens reveal matrix mineralization in the osseous nodules produced from iCALs treated with BMP-9. From top to bottom, low to high power.

Conclusions

We have demonstrated for the first time that BMP-9 effectively stimulates osteogenic differentiation and subsequent bone formation of mesenchymal progenitor cells isolated from murine calvariae. Further research detailing the effects and therapeutic application of BMP-9 on human calvarial mesenchymal progenitor cells should be performed.

Conflicts of interest

All authors have none to declare.

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