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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