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Osteogenesis on nanoparticulate mineralized collagen scaffolds via autogenous activation of the canonical BMP receptor signaling pathway

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ABSTRACT

Skeletal regenerative medicine frequently incorporates deliverable growth factors to stimulate osteogenesis. However, the cost and side effects secondary to supraphysiologic dosages of growth factors warrant investigation of alternative methods of stimulating osteogenesis for clinical utilization. In this work, we describe growth factor independent osteogenic induction of human mesenchymal stem cells (hMSCs) on a novel nanoparticulate mineralized collagen glycosaminoglycan scaffold (MC-GAG). hMSCs demonstrated elevated osteogenic gene expression and mineralization on MC-GAG with minimal to no effect upon addition of BMP-2 when compared to non-mineralized scaffolds (Col-GAG). To investigate the intracellular pathways responsible for the increase in osteogenesis, we examined the canonical and non-canonical pathways downstream from BMP receptor activation. Constitutive Smad1/5 phosphorylation with nuclear translocation occurred on MC-GAG independent of BMP-2, whereas Smad1/5 phosphorylation depended on BMP-2 stimulation on Col-GAG. When non-canonical BMPR signaling molecules were examined, ERK1/2 phosphorylation was found to be decreased in MC-GAG but elevated in Col-GAG. No differences in Smad2/3 or p38 activation were detected. Collectively, these results demonstrated that MC-GAG scaffolds induce osteogenesis without exogenous BMP-2 addition via endogenous activation of the canonical BMP receptor signaling pathway.

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1. Introduction

Skeletal regenerative medicine emerged as a field of investigation to address the current limitations for treating large osseous defects secondary to congenital, traumatic, and post-oncologic conditions. Although there is little debate that the optimal method of bone replacement is using completely autologous vascularized or non-vascularized bone, significant donor site morbidity occurs from harvesting bone [1–3].

Current methods for bone tissue engineering incorporate three elements: cells capable of undergoing osteogenic differentiation, growth factors, and scaffolding material [4,5]. The cellular component is frequently utilized to both initiate osteogenesis on the scaffold as well as to induce migration of osteogenic and angiogenic cells of the host environment. Growth factors are added to stimulate osteogenesis and potentially induce host site cells to differentiate into osteogenic cells. One of the most common family of growth factors used to induce osteogenesis is the bone morphogenetic protein (BMP) family [6]. To date, over 15 molecules of the BMP and growth and differentiation factor (GDF) subfamily have been identified and two have been approved for use in clinical medicine [7]. However, both cost and complications such ectopic bone formation, resorption, and decreased maxillary growth suggest that alternative clinical methods of inducing bone regeneration are warranted [8–10].

BMPs are first synthesized as precursor proteins that dimerize intracellularly. Upon dimerization, precursor proteins are cleaved at

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the consensus Arg-x-x-Arg site, yielding carboxy-terminal mature dimers that are secreted. Following secretion from cells, BMP dimers activate intracellular processes by binding to BMP receptor (BMPR) complexes [11]. Depending on the method of BMPR oligomerization, activation of the canonical or non-canonical pathways may occur (Fig. 1). In the canonical BMPR pathway, the receptor Smads (Smad1/5/8) are recruited and phosphorylated. Phosphorylated receptor Smads associate with co-Smad (Smad4) and translocate to the nucleus to activate transcription of various genes. In the non-canonical pathway, activation of ERK and p38 MAPK pathways occur. Both ERK and p38 MAPK have the capabilities to target receptor Smads for proteasomal degradation [12]. In addition, BMP receptors can also activate the Smad2/3 pathway as an additional non-canonical pathway. Although Smad2/3 is traditionally thought to be downstream of TGF-β receptor signaling, activation of the Smad2/3 pathway via BMP receptors has been reported in development and cancer [13,14].

Differences in scaffolding material have differential osteogenic properties depending on the material, porosity, and ability to mimic the organic and inorganic components of the normal extracellular matrix of bone [4]. Without the organic component, inorganic scaffolds based on calcium phosphate or calcium sulfate are osteoconductive but can be limited by variable resorption rates or brittle mechanical properties [15]. Without the inorganic component, collagen scaffolds lack structural strength and demonstrate significant contraction during mineralization [16–19]. The combination of collagen and mineral content has been evaluated previously and found to have promise in osteoconduction and bone healing, although the mechanism remains unknown and the superiority of such scaffolds in comparison to other types of scaffolds are unclear [20–23]. We have found that combining both the organic and inorganic components of the ECM in the form of a novel nanoparticulate mineralized collagen glycosaminoglycan (MC-GAG) scaffold results in a highly osteogenic and structurally stable scaffold for both primary rabbit bone marrow stromal cells and primary human mesenchymal stem cells [18,19,24,25]. In this work, we investigate osteogenic differentiation of human mesenchymal stem cells on MC-GAG scaffolds in conjunction with BMP-2 stimulation.

2. Materials and methods

2.1. Fabrication of non-mineralized and mineralized collagen scaffolds

Collagen-GAG scaffolds were prepared using the lyophilization process described previously [18,25,26]. Briefly, a suspension of collagen and GAGs or collagen-glycosaminoglycan-calcium phosphate (CGCaP) were produced by combining microfibrillar, type I collagen (Collagen Matrix, Oakland, NJ) and chondroitin-6-sulfate (Sigma–Aldrich, St. Louis, MO) in a solution of 0.05 M acetic acid (pH 3.2) or with calcium salts (calcium nitrate hydrate: $Ca(NO_3)_2{\cdot}4H_2O$; calcium hydroxide: $Ca(OH)_2$, Sigma–Aldrich) in a solution of phosphoric acid, respectively. The suspension was frozen using a constant cooling rate technique (1 °C/min) from room temperature to a final freezing temperature of −10 °C using a freeze dryer (Genesis, VirTis). The ice phase was sublimated under vacuum (<200 mTorr, 0 °C). Disks 5.8 mm in height and 8 mm in diameter were prepared using punch biopsies for cultures. Scaffold porosity was 85 ± 3% [27], pore size was 156 ± 6 μm [26,27], and morphology consisted of isotropic pores with a transverse:longitudinal pore aspect ratio of 0.95 ± 0.01 [26] as we previously reported. All scaffolds were sterilized via ethylene oxide.

2.2. Chemical crosslinking of mineralized and non-mineralized collagen scaffolds

Non-mineralized scaffolds (Col-GAG) and mineralized scaffolds (MC-GAG) were weighed before chemical crosslinking. These were placed into 100% ethanol under a laminar flow hood and left overnight. The scaffolds were then placed in serial dilutions of ethanol and phosphate buffered saline (PBS, Sigma Aldrich) every 2 h, to a

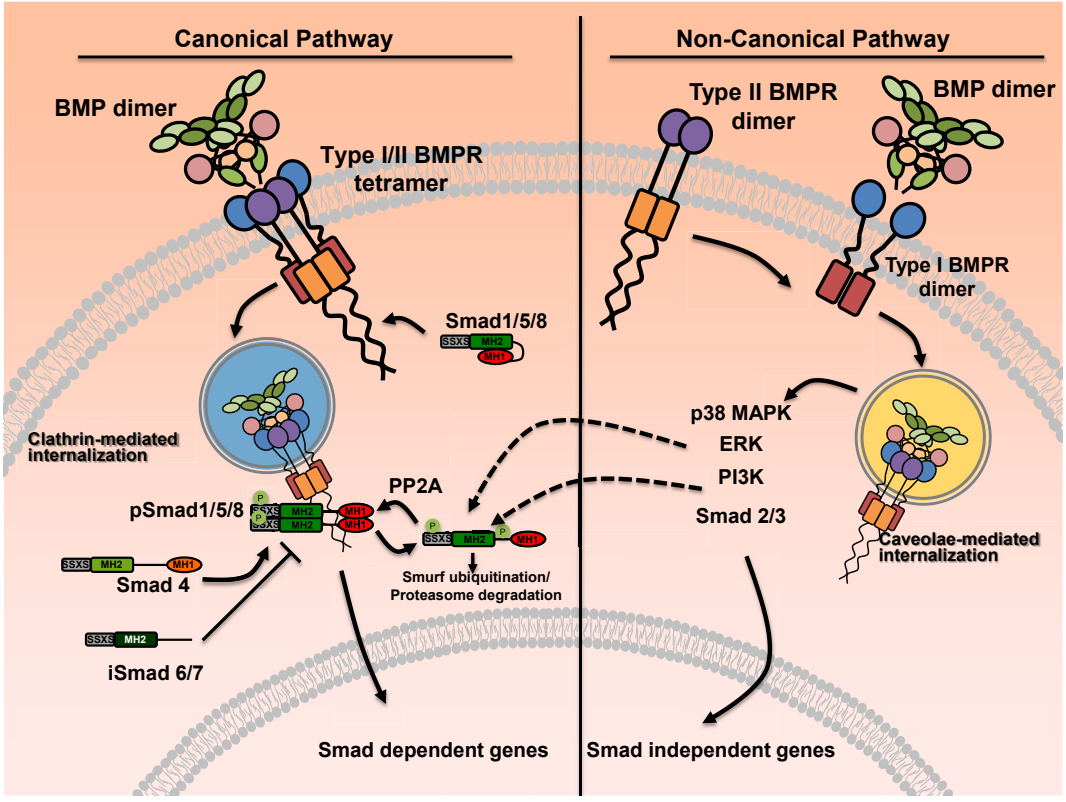

Fig. 1. BMP receptor mediated pathways. The canonical BMP receptor mediated pathway is activated upon ligand binding with the BMPR tetramer composed of type I and type II dimers. Upon binding, the BMP receptor Smads (Smad1/5/8) are recruited to the receptor complex and phosphorylated. Phosphorylated Smad1/5/8 bind to Smad4 (the co-Smad) resulting in nuclear translocation and transactivation of osteogenic genes. In the non-canonical pathway, BMP binds to the type I BMPR dimer thereby recruiting type II BMPR dimers. The assembled receptor/ligand complex is then internalized and various intracellular signaling molecules are activated including the p38 MAPK, ERK1/2, PI3K/Akt, and the Smad2/3.