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BMP-9 dependent pathways required for the chondrogenic differentiation of pluripotent stem cells

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ABSTRACT

Current cartilage repair therapies focus on the delivery of chondrocytes differentiated from mesenchymal stem cells, and thus understanding the factors that promote chondrogenesis may lead to improved therapies. Several bone morphogenetic proteins (BMPs) have been implicated in chondrogenic differentiation and/or chondrocyte function. Although the signaling pathways downstream of BMPs have been studied in other systems, their role in chondrogenesis is less well characterized. Here, we investigated the effects of BMP-9 in chondroprogenitor cells. Compared to BMP-2 and BMP-6, we showed that BMP-9 was significantly more potent in inducing chondrogenic differentiation in mouse C3H10T1/2 and ATDC5 cells. Moreover, we demonstrated that BMP-9 induces the phosphorylation of SMAD1/5 in a dose and time dependent manner. Confocal immunofluorescence microscopy further demonstrated an accumulation of phosphorylated SMAD1/5 in the nuclei of BMP-9 treated cells. Consistent with activation of the SMAD signaling pathway, we also observed an up-regulation of *Id1* and *PAI-I* expression. Importantly, we demonstrated that the simultaneous knockdown of SMAD1 and SMAD5 was able to inhibit chondrogenesis. Additionally, we also observed activation of p38 by BMP-9, and pharmacological inhibition of this pathway blocked chondrogenesis. In contrast, inhibition of p44/42 ERK had no effect. Finally, we tested the ability of Noggin to block the actions of BMP-9. While Noggin potently inhibited the ability of BMP-2 to mediate differentiation, it had no significant effect on BMP-9. Our findings provide a clearer understanding of the cellular pathways utilized by BMP-9 for chondrogenesis that may help improve current therapies for regenerative cartilage repair.

1. Introduction

Osteoarthritis (OA) is a degenerative joint disease, associated with the breakdown of cartilage that causes pain and dysfunction, affecting more people than any other joint disease (Buckwalter and Martin, 2006; Zhang and Jordan, 2010). OA often results in total joint replacement. More than 20 million Americans are estimated to have OA, and approximately 20% of adults will have developed OA in North America by the year 2030. It is expected that OA will continue to have a rising impact on health care in the future.

Chondrogenesis describes the process of cartilage formation (Michigami, 2014). In healthy individuals, chondrocytes are the majority of cells found in cartilage, and arise from the differentiation of mesenchymal stem cells (MSCs). Prior to cartilage formation, MSCs undergo proliferation and formation of dense cell–cell contacts, a stage known as “mesenchymal condensation”. This is followed by differentiation into chondrocytes, which can then form cartilage via extracellular matrix remodeling involving expression of cartilage-specific collagens (e.g. collagens II, IX, and XI), and through the actions of matrix metalloproteinases (MMPs) (Sekiya et al., 2002).

Despite the occurrence of chondrogenesis, damaged cartilage has a limited ability to repair itself. Chondrocytes are affixed in cavities, and are thus unable to migrate to damaged areas. Furthermore, since cartilage lacks a blood supply, the deposition of new matrix occurs at a very slow pace. As a result, damaged cartilage is often replaced by fibrocartilage scar tissue (Schminke and Miosge, 2014). Although fibrocartilage is strong and can provide support, it is not as ideal for facilitating the movement of joints.

A major strategy for cartilage repair is based on the use of autologous chondrocytes. Although successful, several significant limitations exist, including the restricted number of cells available and their limited proliferative capacity. Therefore, new techniques being developed focus on the delivery of MSCs within an appropriate carrier system to repair and regenerate the damaged cartilage (Bernardo et al., 2007). As such, understanding the

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mechanisms that control the differentiation of MSCs into chondrocytes will improve the effectiveness of this strategy.

Bone morphogenetic proteins (BMPs) are a group of molecules that regulate a multitude of developmental processes, including bone and cartilage formation (Brazil et al., 2015). At least 20 BMPs have been identified to date. Amongst these, BMP-2 has been extensively investigated for its role in osteogenic and chondrogenic differentiation. Although BMP-2 deficient mice are embryonic lethal, tissue specific targeting has demonstrated a role for BMP-2 in chondrocyte proliferation and maturation (Shu et al., 2011), as well as in enamel formation (Feng et al., 2011; Guo et al., 2015). Furthermore, BMP-2 has also been shown to promote chondrogenesis in human MSCs (Majumdar et al., 2001). Indeed, the Unites States Food and Drug Administration had approved BMP-2 for specific clinical uses in bone and cartilage regenerative repair (Bessa et al., 2008).

With the exception of BMP-1, all other BMPs belong to the TGF-β family of proteins (Poniatowski et al., 2015). These BMPs signal through a heteromeric complex of transmembrane receptor serine kinases consisting at least two Type I and two Type II receptors (BMPRI and BMPRII). Upon ligand binding, the receptors oligomerize and the BMPRII phosphorylates the BMPRI. Subsequently, this activates the BMPRI that can then activate downstream signaling events. The canonical pathway utilized by BMPs involves a family of proteins called SMADs (Heldin and Moustakas, 2012). The activated BMPRI/BMPRII complex phosphorylates a set of SMAD proteins called receptor SMADs (R-SMAD; SMADs 1, 2, 3, 5, 8 and 9); these R-SMADs then bind to the common mediator SMAD (Co-SMAD; SMAD4) and accumulates in the nucleus, to mediate BMP-dependent gene transcription of target genes. An additional class of SMADs called the inhibitory SMADs (I-SMAD; SMADs 6 and 7) act as antagonistic signals to attenuate the function of R-SMADs and Co-SMADs.

Numerous studies link SMAD proteins to chondrogenesis. For example, tissue specific targeting of SMAD1 or SMAD5 haploinsufficiency has demonstrated that the SMAD1/5 complex is important for chondrocyte function (Keller et al., 2011). Furthermore, while overexpression of SMAD7 in vivo inhibited proper chondrogenesis (Iwai et al., 2008), its loss leads to a disruption in the proper maturation of chondrocytes. Together, this suggests that the SMAD signaling pathway is tightly regulated at multiple steps to control chondrogenesis and/or chondrocyte function (Estrada et al., 2013).

In addition to BMP-2, several other BMPs (such as BMP-4-7, 9, 12-14) are implicated in chondrocyte differentiation and/or function. Along with BMP-2, BMP-9 was also demonstrated to promote chondrogenic differentiation of human MSCs over a decade ago (Majumdar et al., 2001). Despite this, the molecular mechanisms involved downstream of BMP-9 have not been thoroughly investigated. Therefore, we were interested in addressing this question by identifying downstream signaling pathways of BMP-9 in chondroprogenitor cells. Our current studies revealed that compared to BMP-2 and BMP-6, BMP-9 is significantly more potent in promoting chondrogenesis. We also identified the pathways activated or induced by BMP-9 involving TGF-β and SMAD proteins. Finally, using siRNA knockdown and inhibitors, we identify key proteins involved in BMP-9 mediated chondrogenesis. These data provide new insight into the process of chondrogenic differentiation that may enhance current cartilage repair therapies.

2. Materials and methods

2.1. Chemicals and antibodies

Antibodies in this study were purchased from Cell Signaling Technology (Danvers, MA). The inhibitors SB431542, PD98059, SB202190 and SB203580 were obtained from Sigma (St. Louis, MO). Bone morphogenetic proteins, TGFβ1, and Noggin were purchased from R&D Systems (Minneapolis, MN).

2.2. Cell culture

C3H10T1/2 cells (Taylor and Jones, 1979) were obtained from the American Type Culture Collection (Manassas, VA). ATDC5 cells (Atsumi et al., 1990) were purchased from Abgent (San Diego, CA). Human bone marrow derived stem cells were obtained from Lonza (Allendale, NJ). All cells were grow according to supplier's instructions, in a humidified chamber with an environment of 5% CO_2.

To induce chondrogenesis, we prepared cells in micromass cultures as previously described (Denker et al., 1999) with minor modifications. In brief, cells were trypsinized and spun down in 15 ml Falcon tubes at 100*g* for 5 min. Media was aspirated and the cell pellet was resuspended in medium at a concentration of 15 million cells/ml. Cells in 10 μl medium (150,000 cells) were then added to the center of a 24-well plate. Cells were allowed to attach for two hours before adding more media to the well. BMPs were added to induce differentiation for four to six days followed by collection for analyses.

2.3. Transfection of siRNAs

Silencer Select siRNAs targeting *Smad1* or *Smad5* were purchased from ThermoFisher (Grand Island, NY). For transfections, 150,000 cells were seeded in 60 mm dishes and then transfected with 60 pmol siRNA using RNAiMax (Thermofisher) according to the manufacturer's instructions. Experiments were scaled upwards depending on the number of cells needed. The next day, cells were trypsinized, counted, and then re-plated for micromass culture. Two hours after attachment, cells were then treated with BMPs for either 4 days (for gene expression and western blots) or 6 days (for alcian blue staining).

2.4. Alcian blue staining

In some cases, chondrogenesis was evaluated using alcian blue that stains the acidic glycosaminoglycans found in bone and cartilage. Cells were first fixed with 0.1% glutaraldehyde in PBS at room temperature for 20 min. After three washes in PBS, cells were stained overnight in 1% alcian blue in 0.1 M HCl. Cells were then washed in PBS and then kept in water before obtaining photographs. To measure the staining, alcian blue was extracted overnight with 6 M guanidine hydrochloride and absorbance read at 595 nm using a spectrophotometer.

2.5. Preparation of cell lysates and immunoblotting

For isolation of lysates, cells were washed in ice-cold phosphate buffered saline (PBS) and then directly lysed in SDS sample buffer. Samples were run on SDS PAGE using Novex 4–20% gradient gels (Invitrogen). Gels were transferred to nitrocellulose, and membranes were processed for western blotting with the indicated antibodies. Protein bands were detected by enhanced chemiluminescence with film (Supplemental Fig. 2) or with the LICOR Odyssey Imaging System.