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BMP-2 induces cell migration and periostin expression during atrioventricular valvulogenesis

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Abstract

Atrioventricular (AV) endocardium transforms into the cushion mesenchyme, the primordia of the valves and membranous septa, through epithelial–mesenchymal transformation (EMT). While bone morphogenetic protein (BMP)-2 is known to be critical for AV EMT, the role of BMP-2 in post-EMT AV valvulogenesis remains to be elucidated. To find BMP signaling loops, we first localized Type I BMP receptors (BMPRs), *BMPR-1A (ALK3)*, *-1B (ALK6)* and *ALK2* in AV cushion mesenchyme in stage-24 chick embryos. Based on the BMP receptor expression pattern, we examined the functional roles of BMP-2 and BMP signaling in post-EMT valvulogenesis by using stage-24 AV cushion mesenchymal cell aggregates cultured on 3D-collagen gels. Exogenous BMP-2 or constitutively active (ca) *BMPR-1B (ALK6)*-virus treatments induced migration of the mesenchymal cells into the collagen gels, whereas noggin, an antagonist of BMPs, or dominant-negative (dn) *BMPR-1 B (ALK6)-virus treatments* reduced cell migration from the mesenchymal cell aggregates. Exogenous BMP-2 or *caBMPR-1B (ALK6)* treatments significantly promoted expression of an extracellular matrix (ECM) protein, periostin, a known valvulogenic matrix maturation mediator, at both mRNA and protein levels, whereas periostin expression was repressed by adding noggin or *dnBMPR-1B (ALK6)*-virus to the culture. Moreover, transcripts of *Twist* and *Id1*, which have been implicated in cell migration in embryogenesis and activation of the periostin promoter, were induced by BMP-2 but repressed by noggin in cushion mesenchymal cell cultures. These data provide evidence that BMP-2 and BMP signaling induce biological processes involved in early AV valvulogenesis, i.e. mesenchymal cell migration and expression of periostin, indicating critical roles for BMP signaling in post-EMT AV cushion tissue maturation and differentiation.

Keywords: Bone morphogenetic protein (BMP); Cardiac cushions; Viral gene transfer; Periostin; Extracellular matrix protein; Cell migration; Cell proliferation; Valvulogenesis; Heart; Chicken

Introduction

Cardiac valvuloseptal morphogenesis is one of the key morphogenic events during four-chambered heart formation. Defects in valvuloseptal morphogenesis are among the most common and serious of all congenital heart defects (Hoffman and Kaplan, 2002; Hoffman et al., 2004). Two segments of the endocardium–atrioventricular (AV) and outflow tract (OT) endocardium-transform into cushion mesenchyme, primordia of the valves and membranous septa through an epithelial–mesenchymal transformation (EMT) (reviews, Eisenberg and Markwald, 1995; Nakajima et al., 2000; Armstrong and Bischoff, 2004; Person et al., 2005). This transformation occurs at around Hamburger and Hamilton (HH) stage (Hamburger and Hamilton, 1951) 15 in the AV canal of the chick heart. Transformed endocardial cells subsequently migrate into the underlying extracellular matrix (ECM) referred to as "cardiac jelly" and remodel the cardiac jelly into mesenchymalized swellings, called "cardiac cushions" by HH stage 24/25. Distal outgrowth and maturation of the cardiac cushions are the initial and critical morphogenetic events during post-EMT valvulogenesis. These morphogenetic events, which begins at HH stage 26, involves i) migration of post-EMT endocardial cells into acellular cardiac jelly; ii) proliferation of post-EMT cells and iii) expression of valvulogenic molecules which include ECM proteins involved in maturation of the cushion mesenchyme (de la Cruz and Markwald, 1998; Oosthoek et al., 1998). However,

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overall mechanisms of these biological processes during distal outgrowth and maturation of post-EMT cardiac cushion mesenchyme are unknown.

Bone morphogenetic protein (BMP) is a member of the TGF-β superfamily proteins, and it is one among many molecules implicated in AV EMT (Armstrong and Bischoff, 2004; Person et al., 2005). BMP signaling was found to be essential for AV EMT in studies with explant cultures in mice (Sugi et al., 2004) and chicks (Okagawa et al., 2007), and in BMP-2 conditional knockout (CKO) experiments in mice (Ma et al., 2005; Rivera-Feliciano and Tabin, 2006). However, BMP-2 CKO at the EMT stage causes subsequent lethality, which prevents investigation of the role of BMP-2 during post-EMT valvulogenesis. BMPs exert their biological function by interacting with cell surface receptors. BMP receptors (BMPRs) consist of Type I and Type II receptors and Type II receptors transphosphorylate the glycine-serine rich domain (GS domain) of Type-I receptors and transduce signals (Hogan, 1996; Yamashita et al., 1996; de Caestecker, 2004). The Type II BMP receptor, *BMPRII* is reported to be expressed ubiquitously in the entire embryo at least up to the mid-gestation stages (Roelen et al., 1997). Therefore, to find the potential BMP signaling loops during post-EMT valvulogenesis, we first explored the expression patterns of Type I BMPRs by localizing *BMPR-1A* (*ALK3*), *BMPR-1B* (*ALK6*) and *ALK2* in the HH stage-24 post-EMT AV cushion mesenchyme in the chick. Basing our investigation on the BMP receptor expression patterns, in this work we examined whether BMP signaling regulated the biological processes necessary for distal outgrowth and maturation of post-EMT cushion mesenchyme during early valvulogenesis.

Periostin is a 90-kDa secreted ECM protein, related to the midline fasciclin-1 gene in Drosophila (Horiuchi et al., 1999). It has also been identified as a heart-enriched gene in embryonic day (ED) 10.5 mouse embryos by a subtractive cDNA microarray approach (Kruzynska-Frejtag et al., 2001). Periostin is highly expressed in the maturation zone of post-EMT cardiac cushions in mice (Kruzynska-Frejtag et al., 2001; Norris et al., 2005) and in chicks (Norris et al., 2004; Kern et al., 2005). Periostin is found to regulate collagen I fibrillogenesis and contribute to the biomechanical properties of connective tissues in skin and AV valves (Norris et al., 2007). Recent data using chick primary culture assays demonstrated that periostin enhanced cell invasion/migration and collagen condensation by AV cushion mesenchyme, indicating that periostin mediates matrix maturation, a crucial process in early valvulogenesis (Butcher et al. 2007). Regarding the regulation of periostin expression, periostin is known to be induced by BMP-2 in MC3T3 cells (Ji et al., 2000) and by TGFβ-2 in primary osteoblast cells (Horiuchi et al., 1999). BMP inducible helix–loop–helix (HLH) proteins, Twist1 and Id1 (Valdimarsdottir et al., 2002; Komaki et al., 2007), are implicated in regulating the activation of the periostin promoter in osteogenesis (Oshima et al., 2002; Connerney et al., 2006). However, little is known about regulation of periostin expression in cardiac cushion mesenchymal cells during post-EMT valvulogenesis. Therefore, we have chosen periostin as a post-EMT differentiation and maturation target protein whose expression can be regulated by BMP signaling during early AV valvulogenesis.

To study the regulation of post-EMT cushion mesenchyme differentiation and maturation, which begins at HH stage 26 (de la Cruz and Markwald, 1998; Oosthoek et al., 1998) as mentioned above, we developed and established a primary culture assay system using HH stage-24 chick AV cushion mesenchymal cell aggregates with three-dimensional (3D) collagen gels in which we are able to evaluate i) post-EMT mesenchymal cell migration, ii) post-EMT mesenchymal cell proliferation and iii) post-EMT valvulogenic protein expression. Using this culture assay system, we show that BMP-2 and BMP signaling through BMP receptors induce mesenchymal cell migration and periostin expression at both the mRNA and protein levels as well as *Id1* and *Twist* mRNA expression but do not induce proliferation of post-EMT AV cushion mesenchymal cells.

Materials and methods

Chick embryos

Viral-free fertilized eggs of White Leghorn (*Gallus gallus domesticus*) chicken were purchased from Spafas Inc. (Norwich, CT) and incubated in a humid atmosphere at 37 °C. Stages of embryonic development were determined by using the criteria of Hamburger and Hamilton (HH) (1951).

Detection of the Type I BMP receptor expression from HH stage-24 chick AV canal by reverse transcription-polymerase chain reaction (RT-PCR)

HH stage-24 AV cushion mesenchyme was carefully separated from the associated myocardium. RT-PCR analysis of *BMPR-1A*, *BMPR-1B* and *ALK2* was performed as described previously (Sugi and Markwald, 2003; Okagawa et al., 2007) with minor modifications. Briefly, total RNA was extracted and purified from HH stage-24 AV cushion mesenchyme or myocardium using RNA STAT-60 (Tel-Test Inc). Complementary DNA was prepared with an iScript cDNA synthesis kit (BIO RAD) according to the manufacturer's instructions. PCR primer pairs were designed for *BMPR-1A* (L49204, forward, 5′-CTGAGAGTTGAGCGATTG-3′, reverse, 5′-CAGCCAGAAGCAAGTGTTGG-3′), *BMPR-1B* (D13432, forward, 5′-GGAGAGCAGAAAAGAAGATAGTGAGG-3′, reverse, 5′-TGGTGTGGAATAGGAGTGTCC-3′) and *ALK2* (U38622, forward, 5′-CTGTGTTGGGGTCACTG-3′, reverse, 5′-TGGAAGCAGCCTTTCTGG-3′). PCR was performed with these primer pairs and Taq polymerase on a iCycler iQ Real-Time PCR machine (BIO-RAD) using 30 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 2 min. That the PCR products were not amplified from genomic DNA was verified by treating samples with RNase-free DNase-1 (Stratagene) before RT. As a negative control, the RT step was omitted. The PCR products were verified via the thermal cycle sequencing using TagDNA polymerase and fluorescent dye-labeled termination (Medical University of South Carolina (MUSC), Biotechnology Resources Laboratory).

Whole-mount and section in situ hybridization (ISH) for BMP receptors

HH stage-25 chick heart RNA was isolated using RNeasy Column (Qiagen) and reverse-transcribed into cDNA (Stratagene) (Norris et al., 2004). For whole-mount ISH, a 200–250 bp sequence was used for better penetration and rinsing of the probes to reduce the background binding. The following sequences were amplified for type I BMP receptors using HH stage-25 chick heart cDNA: *BMPR-1A* (L49204; nt 351–640), *BMPR-1B* (D13432; nt 387–720) and *ALK2* (U38622; nt 21–299) for the whole mount ISH probes. For section ISH, longer sequences were used for the better detection of the mRNA expression for