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 cushionmesenchymal 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.

Introduction

Cardiac valvuloseptal morphogenesis is one of the key morphogenic events during fourchambered heart formation. Defects in valvuloseptal morphogenesis are among the most common and serious of all congenital hearts 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 epithelialmesenchymal transformation (EMT) (reviews, Eisenberg and Markwald *et al*, 1995; Nakajima *et al*., 2000; Armstrong and Bishop, 2004; Person *et al*., 2005). This transformation occurs at around Hamburger and Hamilton (HH) stage 15 in the AV canal of the chick heart (Hamburger and Hamilton, 1951). 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, 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-ß superfamily proteins, and it is one among many molecules implicated in AV EMT

(Armstrong and Bishop, 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-Freitag *et al.*, 2001). Periostin is highly expressed in the maturation zone of post-EMT cardiac cushions in mice (Kruzynska-Freitag *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 TGFß-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, 1996; Oosthoek et a., 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 sec, 56˚C for 30 sec 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 reversetranscribed 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 *BMPR-1A* (**L49204**; nt 366-996) and *BMPR-1B* (**D13432**, nt 387-838).

Amplified sequences were verified by sequencing (MUSC, Biotechnology Resources Laboratory). Database sequence homology searches confirmed that the sequences for

BMPR-1A, *BMPR-1B* and *ALK*- 2 used in the ISH are not anticipated to cross-react with other family members and are highly specific.

Riboprobes were generated by DIG RNA labeling (SP6/T7, Roche). Riboprobes were purified by RNeasy column (Qiagen) and quantified using a UV spectrophotometer. ISH was performed as previously described with slight modifications (Norris, *et al*., 2004; Somi *et al*., 2004). ISH experiments were carried out on stage-24 chick embryos. A specific signal was only observed when whole hearts or sections were hybridized with the antisense riboprobe. No signal was detected when using the control sense riboprobe (Fig. 1).

Generation and preparation of retrovirus

Two mutant constructs of chick *BMPR-1B* cloned into the replication-competent avian retroviral vector RCAS- (A) (Hughes et al., 1987) were kindly provided by Dr. L. Niswander. The dominantnegative form (*dnBMPR-1B*) blocks BMP signaling pathways by a single amino acid substitution (Lys- 231 to Arg) within the adenosine triphosphate binding site in the kinase domain. Because *dnBMPR-1B* lacks the activity of an intracellular kinase domain, *dnBMPR-1B* expressed at the cell surface can bind BMPs but does not transmit signals. The constitutively active form (*caBMPR-1B*) has a single amino acid substitution (Gln-203 to Asp) within the GS activation domain. *caBMPR-1B* is activated constitutively and transmits signals without BMP binding. *dnBMPR-1B* and *caBMPR-1B* viruses were previously constructed and verified in avian embryonic tissue culture as being capable of blocking and activating BMP signaling, respectively (Zou and Niswander, 1996; Zou *et al*., 1997). Primary fibroblasts were collected from day-10 chick embryos and transfected with retroviral constructs using FuGENE 6-transfection reagent

(Roche). Supernatant from the transfected cultures was collected and concentrated by ultracentrifugation. Concentrated virus was resuspended in M199 medium (Invitrogen) and stored at −80 ˚C. The viral titer was measured with immunohistochemical detection of viral protein p19 with monoclonal antibody, AMV3c2 (Developmental Studies Hybridoma Bank) using virus-infected chick fibroblast cultures. A viral titer (2×10^9 to 5×10^{10} active viron/ml) was routinely achieved after ultracentrifugation of the viral supernatant.

AV cushion mesenchymal cell aggregate culture

AV cushion mesenchyme was dissected out from HH stage-24 chick AV cushions by carefully removing the associated myocardium. Cushion mesenchyme was dispersed by trypsinization as described previously (Sugi *et al.*, 2003). Following the procedure that was described for culturing cell aggregates from chick blastoderm (Eisenberg and Markwald, 1997), 40,000 cells were cultured as a hanging drop in 20µl of serum-free medium 199 (Invitrogen) supplemented with ITS (5µg/ml insulin, 5µg/ml transferrin and 5 ng/ml selenium, BD Sciences) and antibiotics (100units /ml penicillin and 100µg/ml streptomycin, Invitrogen) overnight. Resultant mesenchymal cell aggregates were placed on hydrated collagen gels (1mg/ml, rat tail tendon, BD Sciences) and cultured with medium 199 supplemented with 1% chick serum (Sigma), ITS and antibiotics as described above. Two hrs later, recombinant human BMP-2 (kindly provided by the Genentics Institute, Cambridge, MA) at final concentrations ranging from 20 - 200 ng/ml, recombinant human noggin (kindly provided by Regeneron Pharmaceuticals) at final concentration of 500 ng/ml or *dnBMPR1B-* or *caBMPR1B-RCAS* -virus (final concentration, 1.5×10^8 active viron/ml) was added alone or in combination with BMP-2 to the culture.

Cultures were observed daily under an inverted microscope with Hoffman optics (Olympus, IMT-2). Cell migration, expression of periostin protein and mRNA, and cell proliferation were evaluated at 72 hrs.

Cell migration assay

Seventy-two hrs after placing the AV cushion mesenchymal cell aggregates on collagen gels, cell migration was assessed under an inverted microscope with Hoffman optics (Olympus, IMT-2). Cell numbers were counted in a series of focal planes from the surface to the bottom of the collagen gels at40 μm intervals.

BrdU incorporation assay

BrdU incorporation was used as an index of cell proliferation. The BrdU incorporation assay for cushion mesenchymal cells was performed as previously described (Sugi *et al*., 2003). Briefly, BrdU was added (final concentration = 50 μM) for 2 hrs before the termination of the culture. After rinsing with phosphate buffered saline (PBS), pH. 7.4, cultured cushion mesenchymal cells were incubated in 2N HCl containing 0.5% Tween20 and processed for immunohistochemical detection of BrdU in the nuclei with anti-BrdU (BD Sciences). All nuclei were stained with propidium iodide. Stained samples were observed under a Leica TCS SP2 AOMS confocal microscope.

Immunostaining and Immunointensity analysis

Immunostaining and immunointensity analysis for cushion mesenchymal cell aggregates cultured on collagen gels were performed as described before (Sugi *et al*., 2004) with minor modifications. Briefly, cultured cushion mesenchymal cell aggregates were fixed in 100% methanol at 20˚C and processed for immunostaining with anti-phospho-Smad1/5/8 (Cell

signaling), anti-chick periostin, or an RCAS virus marker (AMV3c2, Hybridoma Bank) depending on the experimental necessity. Anti-chick periostin antibodies were produced against a peptide corresponding to amino acids 122-140 of chick periostin and characterized by Western analysis and immunohistochemistry (Kern *et al.*, 2005). Immunostained samples were observed under a confocal microscope (Leica TCS SP2 AOBS).

The immunointensity of periostin and AMV3c2 were evaluated by measuring the intensity of the immunofluorescence of the cell aggregates on photographs using computer software, Adobe Photoshop 8.0.

Quantitative RT-PCR for periostin, Id1 and Twist

Total RNA was extracted and purified using RNA STAT-60 (Tel-Test Inc) from cushion mesenchymal- cell aggregates that had been cultured for 72 hrs on the collagen gels. Obtained mRNA was further purified by PicoPure RNA Isolation kit (Arcturus). Complementary DNA was prepared using the iScriptTM cDNA synthesis kit (BIO RAD) according to the manufacturer's instruction. The following PCR primer pairs were designed to specifically amplify chick *periostin* (**AY65700**, Forward, 5'-TCGGTGGAAAACTGCTAAGAG-3'; Reverse 5'-TCTGCTGGCTTGATGATTTG - 3'), chick *Id1* (**AY040527**, Forward 5'-CTCCCAACTCCACTTTCCAG-3'; Reverse 5'AGCGGCGACCAATAGCAG-3'), chick *Twist* (**AY126449**, Forward, 5'- GGGATAGGGGTGTGTGTGTG-3'; Reverse 5'-GAGAGGAGGGACTTTTGCTG-3').

Real-time PCR reactions were performed using 50 cycles of 94°C for 30 sec, 56°C for 30

sec and 72°C for 2 min. Chick *periostin* amplification was normalized to chick *ß-actin*

amplification for each experimental treatment. QRT-PCR results represent at least three

independent experiments with reactions performed in triplicate.

Statistical analyses

Analysis of variance (ANOVA) was used to determine significant differences between

groups. When differences were found among groups, the Turkey Kramer's *t*-test was used

to determine if the difference was statistically significant. Differences were considered

significant if $p < 0.05$.

Results

mRNA expression of type I BMP receptors by HH stage-24 chick AV canal

RT-PCR analysis revealed mRNA expression of *BMPR-1A* and *BMPR-1B* in both AV

cushionmesenchyme and myocardium from HH stage-24 chick embryos (Fig. 1A). *ALK2*

was detected in theAV cushion mesenchyme; however, *ALK2* expression was not

detectable in the myocardium (Fig.1A).

By whole-mount ISH *BMPR-1A* was localized in the AV and OT cushion mesenchyme

from HHstage 24 chick embryos. *BMPR-1A* expression was also detected in the

myocardium by whole mount (Fig, 1B, cBMPR1A) and by section ISH (arrows in Fig.1C,

R1A). *ALK2* was localized in the AV and OT endocardium and cushion mesenchyme

(Fig.1 B and arrows Fig.1C, ALK2) and in the ventricular endocardium (arrows in Fig. 1B,

cALK2) but not in the myocardium. A lower level of *BMPR-1B* expression was detected

ubiquitously by the HH stage-24 whole hearts (Fig.1B, cBMPR1B) and in the sections from

HH stage-24 chick embryos (Fig. 1C, R1B). Sense probes used as negative controls for the ISH did not produce any detectable signals (sense panels in Fig1. B and C).

Development of AV cushion mesenchymal cell aggregate culture

To study the regulation of post-EMT cushion mesenchyme maturation and differentiation, we developed a primary culture assay system, in which we were able to evaluate post-EMT mesenchymal cell migration, cell proliferation and post-EMT valvulogenic protein expression. This culture assay system was developed using dissociated HH stage-24 chick AV cushion mesenchymal cells. Cushion mesenchymal cells were cultured as a hanging drop overnight with serum-free medium. Resultant mesenchymal cell aggregates were cultured on the surface of 3D collagen gels with 1% chick serum and observed daily for 72 hrs (Fig. 2 A-C). The advantages of this culture assay system are that the mesenchymal cells are initially trypsinized and possess minimal ECM proteins before BMP or virus treatments are initiated (Fig. 2A), and that the original cell number is easily quantified for subsequent monitoring in response to BMP signaling.

BMP-2 induces AV cushion mesenchymal cell migration

AV cushion mesenchymal cells treated with BMP-2 (200ng/ml) migrated deeper into the collagen gels (Fig. 2, arrowheads in I) than the control (Fig. 2, arrowheads in H). Effects of BMP-2 were abolished when noggin (500 ng/ml) was added to the BMP-2-containing medium (Fig. 2J). Noggin alone with the Medium 199 also repressed migration of cushion mesenchymal cells (Fig. 2K). No significant differences were seen in surface migration area between treatments (data not shown). Quantitative analysis indicated the dose-dependent effects of BMP-2 in mesenchymal cell migration into collagen gels (Fig. 3). As

indicated in Fig. 3A, as little as 50 ng/ml of BMP-2 significantly promoted mesenchymal cell migration. BMP-2-promoted cell migration was abolished by adding noggin to the BMP-2-treated mesenchymal cell culture (Fig. 3B). Moreover, the endogenous level of mesenchymal cell migration was repressed in cultures treated with noggin alone compared to the control (Fig. 3B). These results indicate that BMP-2 induces cell migration by cultured AV cushion mesenchymal cells.

BMP treatment enhances phospho-Smad 1/5/8 expression in cushion mesenchymal cells

To test whether exogenous BMP-2 treatment induces intracellular BMP signaling in cultured AV cushion mesenchymal cells, activation of Smad 1/5/8 phosphorylation was examined by detecting phospho-Smad 1/5/8 expression. AV cushion mesenchymal cells treated with BMP-2 (200 ng/ml) and cultured on collagen gels showed significantly elevated phospho-Smad 1/5/8 expression (Fig. 4B and D).

Noggin treatment repressed endogenous phopho-Smad1/5/8 expression in the AV cushion mesenchymal cells compared to control culture (Fig. 4C and D). These results indicate that BMP-2 treatment activates intracellular BMP signaling in cultured AV cushion mesenchymal cells.

BMP-2 does not stimulate mesenchymal cell proliferation

To determine whether BMP-2 regulates AV cushion mesenchymal cell proliferation, we assessed BrdU incorporation as an index of cell proliferation in AV cushion mesenchymal cells. Neither exogenously added BMP-2 nor noggin treatment altered the BrdU incorporation by AV cushion mesenchymal cells compared to the control Medium 199

treatment (Supplemental Fig. 1A-H and Fig. 1I). These results indicate that BMP-2 does not alter the proliferation ratio of HH stage-24 post-EMT AV cushion mesenchymal cells.

BMP-2 treatment increases periostin expression by AV cushion mesenchymal cells

Periostin expression (mRNA and protein) was assessed in AV cushion mesenchymal cell aggregates cultured on collagen gels. Periostin protein expression was analyzed by immunostaining (Fig. 5A-F) and quantitatively evaluated by immunointensity (Fig. 5G). Periostin expression in AV cushion mesenchymal cell aggregates was elevated when treated with BMP-2 (Fig. 5B, C and D). Significant promotion of periostin protein expression was detected when AV cushion mesenchymal cell aggregates were treated with as little as 20ng/ml BMP-2 (Fig. 5G). Noggin treatments inhibited BMP-2-supported induction of periostin expression (Fig. 5E and G). Treatments with noggin alone with Medium 199 also significantly repressed endogenous periostin expression by cultured AV cushion mesenchymal cell aggregates as compared to the control treatment (Fig. 5F and G). Periostin mRNA expression was assessed with quantitative (Q) RT-PCR with AV cushion mesenchymal cell aggregates cultured on collagen gels (Fig. 5H). QRT-PCR data revealed an approximately 3-fold increase of *periostin* expression by AV cushion mesenchymal cell aggregates treated with BMP-2 over the control treatments with Medium199 (Fig. 5H). As low as 20 ng/ml of BMP-2 induced *periostin* expression (Fig. 5H). BMP-2-supported elevation of *periostin* mRNA expression was abolished when noggin was added to the culture. Noggin alone with Medium 199 also significantly repressed endogenous *periostin* mRNA expression by cultured AV cushion mesenchymal cell aggregates.

BMP-2 treatment increases Id1 and Twist expression by AV cushion mesenchymal cells

In our present work, RT-PCR analysis revealed endogenous expression of chick *Id1* and *Twist* in HH stage-24 chick AV canal. *Id1* and *Twist* were detected in the AV cushion mesenchyme but not clearly detected in the myocardium (Fig. 6A). Chick *Twist* has been localized in HH stage 22-30 chick AV cushion mesenchyme by *in situ* hybridization (Dr. R. Heimark, University of Arizona; personal communication). To test whether BMP signaling regulates expression of these transcription factors, chick *Twist* and *Id1* mRNA expression was assessed with QRT-PCR with AV cushion mesenchymal cell aggregates cultured on collagen gels (Figs.6B and C). QRT-PCR data revealed an approximately 8-fold increase of both *Twist and Id1* expression by AV cushion mesenchymal cell aggregates treated with BMP-2 over the control treatments with Medium199. BMP-2-supported elevation of *Twist* and *Id1* mRNA expression was abolished when noggin was added to the culture. Noggin alone with Medium 199 also significantly repressed endogenous *Id1* and *Twist* mRNA expression by cultured AV cushion mesenchymal cell aggregates.

Modulation of BMP signaling by dn- or caBMPR virus treatments is associated with alteration of cell migration and periostin expression by cultured AV cushion mesenchymal cells

To examine whether BMP signaling through BMP receptors which are expressed by the AV cushion mesenchymal cells regulates mesenchymal cell migration and periostin expression, *dn*-and *caBMPR-1B* -RCAS-virus were applied to AV cushion mesenchymal cell aggregates cultured on collagen gels. First, we tested whether *dn*- or *caBMPR-1B* virus infection altered intracellular BMP signaling in AV cushion mesenchymal cells. AV cushion mesenchymal cell aggregates treated with *dn* and *caBMPR-1B* virus were

examined for activation of Smad 1/5/8 phosphorylation, a down stream effector of BMP signaling, by immunohistochemical detection of phospho-Smad 1/5/8 expression and a viral marker, AMV3c2 staining. *caBMPR-1B*-infected, AMV3c2-positive, cushion mesenchymal cells showed elevated phospho-Smad 1/5/8-expression (arrowheads in Fig. 7D and H). By contrast, *dnBMPR-1B*-infected, AMV3c2-positive, cushion mesenchymal cells repressed endogenous nuclear staining of phospho-Smad 1/5/8 (arrowhead in Fig. 7B and F) as compared to the control (Fig. 7A and E). When BMP-2 was added to dnBMPR-1B infected cushion mesenchymal cells, a significant reduction of Smad 1/5/8 expression was also observed (Fig. 7C and G). These data indicate that infection of *dnBMPR-1B* or *caBMPR-1B* inhibits or enhances cytoplasmic BMP signaling respectively, in AV cushion mesenchymal cells.

AV cushion mesenchymal cells treated with *dnBMPR-1B* or *caBMPR-1B* virus were evaluated for cell migration. AV cushion mesenchymal cells treated with *caBMPR-1B* virus migrated deeper into the collagen gels than the mesenchymal cells cultured with the control Medium199 (Fig. 8). Conversely, *dnBMPR1B*-virus treatments significantly reduced AV cushion mesenchymal cell migration compared to the control culture (Fig. 8). *dnBMPR-1B*-treated culture had the same level of reduction in cell migration as observed in noggin-treated cushion mesenchymal cell culture (Fig. 3).

caBMPR1B-virus infection, as detected by a viral marker, AMB3c2 expression, significantly increased periostin protein expression (Fig. 9C and F) whereas *dnBMPR-1B*-virus infection repressed endogenous periostin protein immunostaining (Fig. 9B and E) in the cushion mesenchymal-cell aggregate culture. A higher magnification view showed that

cytoplasmic periostin expression in cushion mesenchymal cells was elevated in *caBMPR-1B*-infected cells (Fig. 9I and L), whereas periostin expression was reduced in *dnBMPR-1B*-infected cells (Fig. 9H and L). Quantitative evaluation by analyzing periostin immunointensity showed significant differences among the treatments (Fig. 9M). These data indicate that BMP signaling through the BMP receptors modulate mesenchymal cell migration and periostin expression by AV cushion mesenchymal cell cultures.

In summary, we provide the evidence that BMP-2 and BMP signaling induce cell migration and expression of a valvulogenic ECM protein, periostin but do not induce cell proliferation by post-EMT AV cushion mesenchymal cells. This evidence was demonstrated by two independent approaches of promoting BMP signaling by gain-of-function experiments: exogenous BMP-2 treatments and activation of BMP receptors by *caBMPR-1B*-virus infection in post-EMT cushion mesenchymal cells. The effect of BMPs in post-EMT valvulogenesis was further determined by two independent ways of blocking BMP signaling by loss-of-function experiments: exogenous noggin treatment and inhibition of BMP signaling by perturbing the BMP receptors with *dnBMPR-1B*–virus in post-EMT AV cushion mesenchymal cells. These results are summarized in a diagram, Fig.10.

Discussion

Although accumulated data support the importance of BMPs and BMP signaling in valvuloseptal morphogenesis (Galvin *et al*., 2000; Delot *et al*., 2000; Kim *et al*., 2001; Gaussin *et al*., 2002; Jiao *et al*., 2003; Gaussin *et al*., 2005; Wang *et al*., 2005), the cell

types that are involved and the regulatory mechanisms of BMPs in post-EMT valve

formation have not been determined. Recent studies with *Nkx2.5*-cre/BMP-2 CKO mice

showed that myocardially-derived BMP-2 was essential for AV EMT (Ma *et al*., 2005;

Rivera-Feliciano and Tabin, 2006). Moreover, data from *Tie1*-cre/*ALK3* and *FLK1*-

cre/*ALK3* CKO mice indicated critical roles of ALK3 in endothelial cells for AV EMT

(Park *et al*, 2006; Song *et al*., 2007). However, subsequent lethality of the CKO embryos

after the EMT stage prevented analysis of the role of BMP signaling in post-EMT

valvulogenesis. Although myocardial expression of BMP-2 is down regulated in the AV

canal after EMT, BMP-2 is localized in AV cushion mesenchyme during post-EMT

valvulogenesis in chicks (Lincoln *et al*., 2004) and mice (Sugi *et al*., 2004), suggesting

potential autocrine signaling by BMP-2 in cushion mesenchymal cells during post-EMT

valvulogenesis. Therefore, in this work we sought to determine the roles of BMP-2 and

BMP signaling in post-EMT AV cushion mesenchymal cells by using spatio-temporal

viral-gene-transfer technique with chick AV cushion mesenchymal cell cultures *in vitro* and

whole embryo cultures *in vivo* (*ovo*). Recent work by Lincoln *et al*. (2006a) demonstrated

that BMP-2 induced *aggrecan* and *sox9*—which are characteristics of cartilage cells--in AV

cushion mesenchymal cells. This suggests the existence of active BMP signaling in cushion

mesenchymal cells. As the first step of our present study, we showed the expression of

Type I BMP receptors, *BMPR-1A*, *BMPR-1B* and *ALK2,* as supportive evidence for the

presence of BMP signaling in cushion mesenchymal cells and further demonstrated that

BMP-2 and BMP signaling through BMP receptors induce mesenchymal cell migration and

expression of periostin, both of which were crucial for maturation and differentiation of

post-EMT AV cushion mesenchyme. Therefore, our current study supports the idea that the autocrine signaling by BMPs in cushion mesenchymal cells can regulate post-EMT AV cushion mesenchymal cell differentiation. Most BMP receptors have ligand binding affinity with other TGFß superfamily proteins, i.e. TGFß and activin. However, BMPR-1A and BMPR-1B bind specifically with BMP-2 and BMP-4, and bind with BMP-7 at low affinity but do not bind with TGFßs or activin (Dijke *et al*., 1994; Raftery and Sutherland 1999; de Caestecker, 2004). In this work, we demonstrated that *dnBMPR-1B* infection as well as treatments with a BMP antagonist, noggin, significantly repressed endogenous phospho-Smad 1/5/8 expression in AV cushion mesenchymal cells, which indicated that intracellular BMP signaling in the cushion mesenchymal cells was effectively inhibited by the treatments with *dnBMPR-1B* or noggin. Conversely, BMP-2 or *caBMPR-1B-virus* treatments induced expression of phospho-Smad 1/5/8, indicating that intracellular BMP signaling was effectively enhanced by BMP-2 or *caBMPR-1B*. Although *BMPR-1B* does not appear to be the most prominent Type 1 BMP receptor expressed in the AV canal (Fig. 1), our data indicated that overexpression of *dnBMPR-B1or caBMPR-1B* evidently modulated intracellular BMP signaling in the AV cushion mesenchymal cells. For example, *dnBMPR-1B* treatments inhibited BMP-2-supported up-regulation of cell migration as well as Smad1/5/8 expression in the cushion mesenchymal cells. The effects of *dnBMPR-1B* treatments on cushion mesenchymal cell migration and psmad1/5/8 expression are as profound as noggin treatments (Figs. 7 and 8). Because noggin is expected to antagonize BMP signaling through any Type I BMP receptors, the present data strongly suggest that the excess dominant negative forms of *BMPR-1B* are potentially disrupting BMP signaling

by competing with other endogenously expressed Type I BMP receptors, i. e. BMPR-1A and/or Alk2. *dnBMPR-1B* and *caBMPR-1B* appear to function as broader reagents that can inhibit or activate BMP signaling through Type I BMP receptors in our AV cushion mesenchymal cell cultures. Therefore, our data do not resolve the specific biological function of BMPR-1B; however, the present study using AV cushion mesenchymal cells provides an example that the dominant-negative and constitutively active approach can be a powerful tool to effectively modulate BMP signaling when multiple Type I BMP receptors may cause functional redundancies.

Cell proliferation is the one of the critical biological processes in elongation and expansion of post-EMT AV cushion tissues in early valvulogenesis (de la Cruz and Markwald, 1998). Recent studies in cardiac valvulogenesis demonstrated that cell proliferation in cushion mesenchyme was high at early stages of valvulogenesis, but significantly decreased after remodeling stages in mice (Hinton *et al.*, 2006) and chicks (Lincoln *et al.*, 2004) and persisted at low levels in juvenile mice (Hinton *et al.*, 2006). Our previous work revealed that fibroblast growth factor 4 (FGF4) induced cell proliferation in HH stage-24 post-EMT AV cushion mesenchyme *in vitro* and *in vivo* (*ovo*) (Sugi *et al.*, 2003). In the present study, we found that BMP-2 and BMP signaling did not enhance mesenchymal cell proliferation in HH stage-24 AV cushion mesenchymal cell culture. BMP-2 is shown to induce Tbx20 that induces cell proliferation but represses expression of an ECM proteoglycan, *aggrecan* (Shelton and Yutzey, 2007). However, BMP-2 is also reported to induce *aggrecan* expression from cultured HH stage-25 AV cushion mesenchymal explants (Lincoln *et al.*, 2006a). Tbx20 may exert positive (Cai *et al.*, 2005) or negative feed back (Singh *et al.*,

2005) on *BMP-2* expression. Moreover, resent studies on deletion of noggin, which is an antagonist of BMPs and is expressed in both AV myocardium and cushion mesenchyme, showed that proliferation was increased in the myocardium but not in the cushion mesenchyme, suggesting tissue specific responses of BMP signaling (Choi *et al*., 2007). Therefore, precise signaling mechanisms and molecular interactions of BMP signaling governing cell proliferation in cushion mesenchyme are not fully understood. Taken together, our present study demonstrates that unlike FGF4 or Tbx20, BMP-2 is not responsible for a high proliferation ratio in endocardial cushions during early valvulogenesis but may be a key regulator in other necessary morphological processes in early valvulogenesis, i.e. production of valvulogenic ECM protein such as periostin and induction of cell migration.

Valvulogenesis is thought to be regulated by a complex coordination of growth factor signaling and ECM interactions (Camenisch *et al*., 2002; Schroeder *et al*., 2003); however, these processes have not fully elucidated. In the cardiac cushion, periostin expression is observed only after the EMT (Kern et al., 2005) and is closely correlated with the morphogenic processes associated with differentiation of cardiac cushion mesenchyme into dense, fibrous tissues and is proposed to be responsible for delamination of the AV cushion leaflets and the formation of the AV valvular suspensory apparatus (Markwald *et al*., 2004; Litvin *et al*., 2005). Periostin is also proposed to potentially regulate differentiation and recruitment of circulating adult bone marrow cells into postnatal hearts (Visconti and Markwald, 2006). Moreover, recent data in post-EMT valvulogenesis using chick primary culture assays indicated that periostin enhanced cell invasion/migration and collagen

condensation by cushion mesenchyme which highlighted periostin as a mediator of matrix maturation and remodeling (Butcher *et al.,* 2007). Therefore, periostin is proposed to promote and sustain differentiation of cushion mesenchyme into fibrous connective tissues and is a central player in cushion differentiation and maturation. Although periostin deficient mice do not show early lethality that could be attributed to serious defects in early valvulogenesis (Kii *et al*., 2006; Rios *et al*., 2006; Norris *et al*., 2007), this could be explained by molecular redundancies with a closely related molecule, bigH3, which is in the same gene family and is co-expressed with periostin in developing valvular tissues (Norris *et al*., 2004).

Our present work also demonstrates a striking increase in chick *Twist* and *Id1* expression in cushion mesenchymal cells cultured with BMP-2. Twist1, a member of the bHLH family protein, can specifically bind to the putative binding site, the Twist-box, on the periostin promoter *in vitro* in osteoblast MC3T3 cells (Oshima *et al*., 2002). It has also been determined by using Twist1 overexpressing 293T cells that Twist up-regulates mouse periostin transcription (Oshima *et al*., 2002). Id1 protein, a Class II HLH protein that lack the basics domain which is required for DNA- binding (Benezra, *et al*., 1990), preferentially dimerizes with E proteins and, hence, enhances Twist homodimer formation (Connerney et al., 2006). Critical evidence generated using in C3H10T1/2 mesenchymal cells is that overexpressing Twist1 homodimers enhances periostin expression, whereas expression of Twist 1 heterodimer inhibits it (Connerney et al., 2006). Therefore, an increase in the transcripts of the chick homologue of *Twist1* and *Id1* by BMP signaling in our experiments can be associated with activation of the periostin promoter in

AV cushion mesenchymal. Collectively, our present work provides initial evidence that BMP-2 and BMP signaling induce periostin expression in post-EMT AV cushion mesenchymal cells. Other than the inductive roles for periostin promoter activation, Id1 and Twist have been known to regulate various aspects of embryonic development including cell migration as well as tumor metastasis (Israel, 1999; Norton *et al*., 2000; Ruzinova and Benezra, 2003; Kang and Massague, 2004; Yang *et al*., 2004; Reinhold *et al*., 2006; Alexander, et al., 2006; Komaki *et al*., 2007). Particularly relevant to the present study is the recent observation that *Id1* and *Twist1* are disrupted in AV endocardium in *Nkx2.5*-cre/BMP-2 CKO mice (Ma *et al*., 2005; Rivera-Feliciano and Tabin, 2006) as well as in *FLK1*-cre/*ALK3* CKO mice (Park *et al*, 2006). These findings indicate that expression of *Id1* and *Twist1* are regulated by BMP-2 and/or BMP signaling in AV endocardial cells and suggest the roles of *Id1* and *Twist1* in AV EMT in mice. Furthermore, *Id1* is known to play a critical role in mediating BMP-induced endothelial cell migration by mouse embryonic endothelial cells *in vitro* (Valdimarsdottir *et al*., 2002). Chick homologues of Ids (Id1-Id4) were cloned and localized in early chick embryos (Martinsen and Bronner-Fraser, 1998; Kee and Bronner-Fraser, 2001a, 2001b and 2001c).

Our present work with stage-24 chick cardiac cushion mesenchymal cells revealed that *Id1* and *Twist* expression was induced by BMP-2 in post-EMT cushion mesenchymal cells. Because Id1 regulates embryonic vascular endothelial cell migration (Valdimarsdottir *et al*., 2002), it will be of interest to learn interactions between BMP signaling and these transcription factors and roles of their signaling pathways in cushion mesenchymal cells during post-EMT valvulogenesis.

In conclusion, our current work provides strong evidence that BMP-2 and BMP signaling regulate cushion mesenchymal cell migration and expression of a valvulogenic ECM protein, periostin, indicating that BMP-2 and BMP signaling play a critical role in post-EMT AV cushion mesenchyme differentiation and maturation. Because many molecules are known to be expressed in the post-EMT cushion mesenchyme and involved in valvulogenesis (Reviews, Armstrong and Bischoff, 2004; Schroeder *et al.*, 2003; Person *et al.*, 2006; Lincoln *et al.*, 2006b), further studies are warranted to better characterize the interaction of BMPs and/or BMP signaling with other valvulogenic regulatory molecules and their molecular cascades in post-EMT valvulogenesis.

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Figure Legends

Fig. 1. A. RT-PCR analysis revealed mRNA expression of type I BMP receptors in HH stage-24 chick AV canal. BMPR-1A and BMPR-1B were detected in AV cushion mesenchyme and myocardium. ALK2 was detected in the AV cushion mesenchyme; however, little ALK2 expression was seen in the AV myocardium. B. Whole-mount in situ hybridization revealed mRNA expression of Type I BMP receptors in HHstage-24 chick AV hearts. Abundant BMPR1A expression was found in the AV and OT cushion mesenchyme and in the myocardium. ALK2 was localized in the AV and OT cushion mesenchyme and ventricular endocardium but not in the myocardium (arrows show endocardium). Lower levels of BMPR-1B expression were detected in HH stage-24 whole hearts. C. Section in situ hybridization revealed BMPR-1A expression in the AV and OT endocardium and cushion mesenchyme. BMPR-1A expression was also detected in the myocardium (arrows) in the HH stage-24 chick heart. Intense expression of ALK2 was detected in the AV and OT endocardium and cushion mesenchyme but not in the myocardium. Low levels of BMPR-1B expression were detected ubiquitously in stage-24 whole hearts. No specific binding was detected with the sense probes.

Fig. 2. A-C. Representative pictures from cultured AV cushion mesenchymal cell aggregates. These pictures show how cushion mesenchymal cell aggregates grow on collagen gels with control M199 medium. AV cushion mesenchymal cells from HH stage-24 chick embryos were cultured as a hanging drop overnight. Resultant mesenchymal cell aggregates were placed on collagen gels and cultured for an additional 72 hrs. D-K. Higher

magnification view of AV cushion mesenchymal cell aggregate culure. Upper panels show the surface view, and lower panels show a deeper view in collagen gels (240μm from the surface). Mesenchymal cells treated with BMP-2 (200 ng/ml) migrated deeper into the collagen gels (arrowheads in I) than the control culture (arrow heads in H). BMP-2-promoted mesenchymal cell migration was abolished when noggin (500 ng/ml) was added to the medium (F and J). Noggin treatments reduced mesenchymal cell migration below the level of control Medium 199 treatments (G and K).

Fig. 3. Quantitative evaluation of cell migration by HH stage-24 AV cushion mesenchymal cell aggregate culture. A. BMP-2 (as low as 50 ng/ml) promoted cell migration (*p<0.001). B. BMP-2- promoted cell migration was abolished by adding noggin (500 ng/ml) to the mesenchymal cell-aggregtes culture (*p<0.001). Mesenchymal cell migration was reduced in cultures treated with noggin alone or noggin with BMP-2 compared to the control (**p<0.05).

Fig. 4. A-C. Phospho-Smad 1/5/8 expression was detected in cultured AV cushion mesenchymal cells. BMP-2 treated mesenchymal cells showed more phopho-Smad1/5/8 immunostaining (B) than control Medium 199 treated cushion mesenchymal cells (A). Noggin treatments reduced phospho-Smad 1/5/8 staining in the cells (C). D. Quantitative evaluation of phospho-smad1/5/8 immunostaining revealed that cushion mesenchymal cells treated with BMP-2 expressed elevated phopho-Smad1/5/8 staining (p<0.01), while noggin treatments significantly reduced phospho-Smad1/5/8 staining (D).

Fig. 5. A-F. Periostin immunostaining in AV cushion mesenchymal cell aggregate culture. The mesenchymal cells were cultured with control medium 199 (A), BMP-2, 20ng/ml (B), BMP-2, 50ng/ml (C), BMP-2, 200 ng/ml (D), BMP-2 200 ng/ml plus noggin, 500 ng/ml (E) or noggin, 500 ng/ml (F). as low as 20 ng/ml of BMP-2 promoted periostin immunostaining by cultured cushion mesenchymal cell aggregates. G, Quantitative evaluation of periostin immunostaining in AV cushion mesenchymal cell aggregate culture. Immunointensity of the mesenchymal cell aggregates treated with as little as 20 ng/ml of BMP-2 was significantly higher than the control (*p<0.01). Conversely, immunointensity in mesenchymal cell aggregates cultured with noggin was significantly lower than the control (**p<0.05). H, Quantitative evaluation of periostin mRNA expression in AV cushions mesenchymal cell aggregate culture. Real-time PCR data revealed an approximately 3-fold increase of periostin expression in AV cushion mesenchymal cell aggregate culture treated with BMP-2 over the culture treated with control Medium199 (*p<0.01). BMP-2-supported elevation of periostin expression was abolished when noggin was added to the culture (**p<0.05).

Fig. 6. A. RT-PCR analysis revealed mRNA expression of chick Id1 and Twist in HH stage-24 chick AV canal. Id1 and Twist were detected in AV cushion mesenchyme but not detected in myocardium. B and C. Quantitative evaluation of chick Id1 and Twist mRNA expression in AV cushions mesenchymal cell aggregate culture. Real-time PCR data revealed an approximately 8-fold increase in expression of both Twist (A) and Id1 (B) in

AV cushion mesenchymal cell aggregate culture treated with BMP-2 over the culture treated with control Medium199 (*p<0.01). BMP-2-supported elevation of Twist and Id1 expression was abolished when noggin was added to the culture (*p<0.01).

Fig. 7. Phospho.Smad 1/5/8 immunostaining. Mesenchymal cells infected with dnBMPR-1B-virus, as seen as AMV3c2 positive cells (B and F) showed less phospho-Smad1/5/8 staining (an arrowhead in F) than the control cells (A and E). Mesenchymal cells infected with dnBMPR-1B-virus and treated with BMP-2 (200 ng/ml) (C and G) also showed less phospho-Smad1/5/8 staining (arrowheads in G) than the control cells (A and E). Mesenchymal cells treated with caBMPR-1B-virus (D and H) showed more intense phopho-Smad1/5/8 staining (arrowheads in H). Panels A-D were stained for, a viral marker, AMV3c2 (green). Panels E-H were stained for phopho-Smad1/5/8 (red).

Fig. 8. Quantitative evaluation of cell migration by AV cushion mesenchymal cells treated with dnBMPR-1B-virus or caBMPR-1B-virus. Mesenchymal cells treated with caBMPR-1B-virus migrated significantly deeper into the gels than the control (*p<0.01) similar to the level seen in BMP-2-treated mesenchymal cells. Conversely, BMP-2-supported up-regulation of cell migration was significantly reduced by dnBMPR-1B treatments (**p<0.05).

Fig. 9. A-F. Periostin Immunostaining in AV cushion mesenchymal cell aggregate culture.

Mesenchymal cell aggregates treated with dnBMPR-1B-virus (B and E) resulted in a significant decrease in periostin immunostaining compared to the no-virus control (A and D). Infection with caBMPR-1Bvirus significantly increased periostin expression in AV mesenchymal cell culture (C and F). Upper panel (A-C), a viral marker, AMV3c2 immunostaining (green). Lower panel (D-F), periostin immunostaining (red). G-L. Higher magnification view of RCAS-virus-infected AV cushion mesenchymal cells. Cells infected with dnBMPR-1B-virus showed less periostin staining (an arrow in K) than no-virus infected cells (arrow in J). Cells infected with caBMPR-1B showed more intense periostin staining (arrows in L) than no-virus control (an arrow in J). Upper panel (G-I), viral marker AMV3c2 immunostaining (green). Lower panel (J-L), periostin immunostaining (red). M. Quantitative evaluation of periostin immunostaining in AV cushion mesenchymal cell aggregates treated with dn-and caBMPR-1B-virus. Immunointensity of the mesenchymal cell aggregates treated with caBMPR-1B was significantly higher than the control (*p<0.01). Conversely, immunointensity in mesenchymal cell aggregates infected with dnBMPR-1B was significantly lower than the control (*p<0.01).

Fig. 10. Summary diagram illustrating the results from bioassays to assess the role of BMP-2 and BMP signaling using AV cushion mesenchymal cell aggregates cultured on 3D-collagen gels. Results were evaluated 72hrs after placing the aggregates on the collagen gels. Exogenous BMP-2 or caBMPR-1B treatments induced mesenchymal cell migration and expression of periostin, Twist and Id1, while a BMP antagonist, noggin, or dnBMPR-

1B treatment inhibited BMP-2 promoted cell migration and expression of periostin, Twist and Id1. Phospho-Smad 1/5/8 expression was induced by BMP-2 or caBMPR1-B treatments but reduced by noggin or dnBMPR-1B treatments. There was no significant difference in proliferation among treatments.

Supplemental Fig. 1. A- H. BrdU incorporation assay. Exogenously added BMP-2 did not stimulate BrdU incorporation by AV cushion mesenchymal cells. Note that incidence of BrdU-positive (green) nuclei appears to be the same in all cultures treated with control Medium 199 (E), BMP-2 (F), BMP-2 plus noggin (G) or noggin (H). Upper panels (A-D) show all nuclei stained with propidium iodide (red). I. Quantitative analysis of exogenous BMP-2 treatments in AV cushion mesenchymal cell. BrdUpositive and.negative nuclei in the cultured mesenchymal cells were counted to determine the percentage of cells in cell-cycle transit. A total 1,000 nuclei in each culture was evaluated in 4 random fields. There was no significant difference among the treatments.
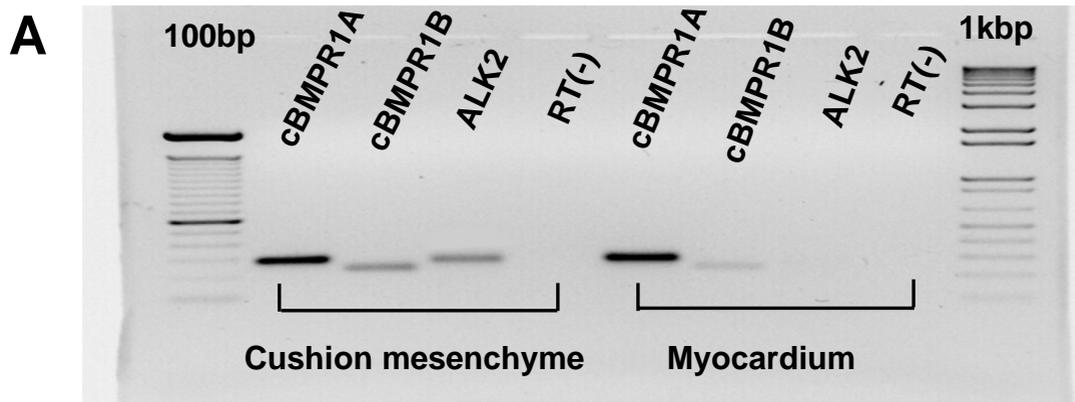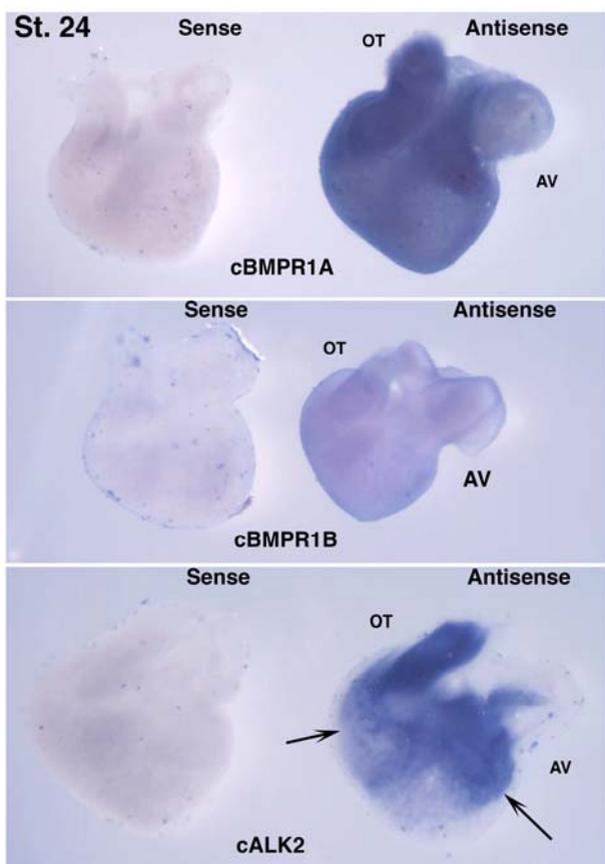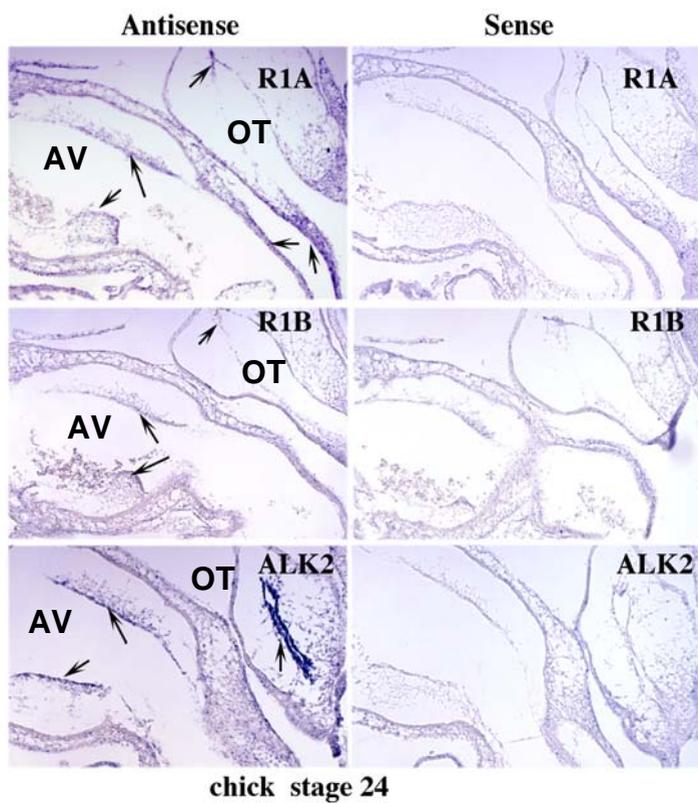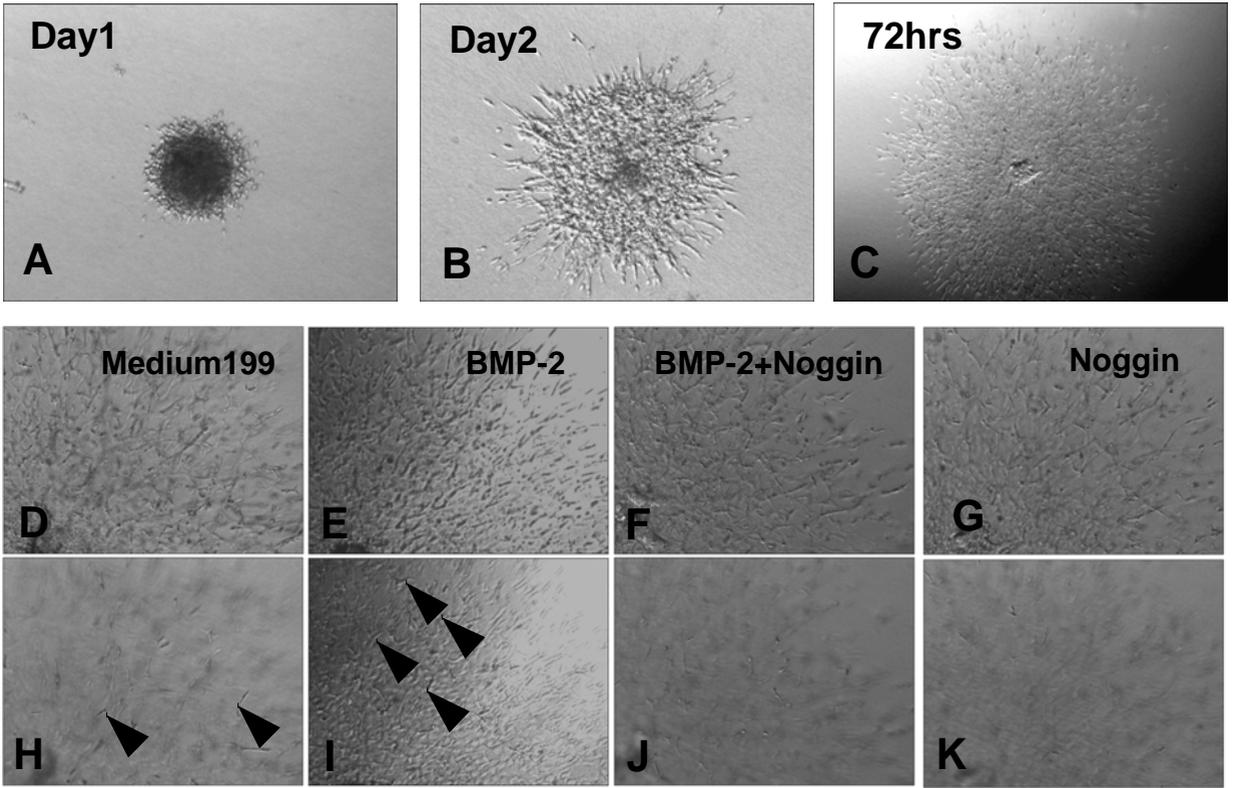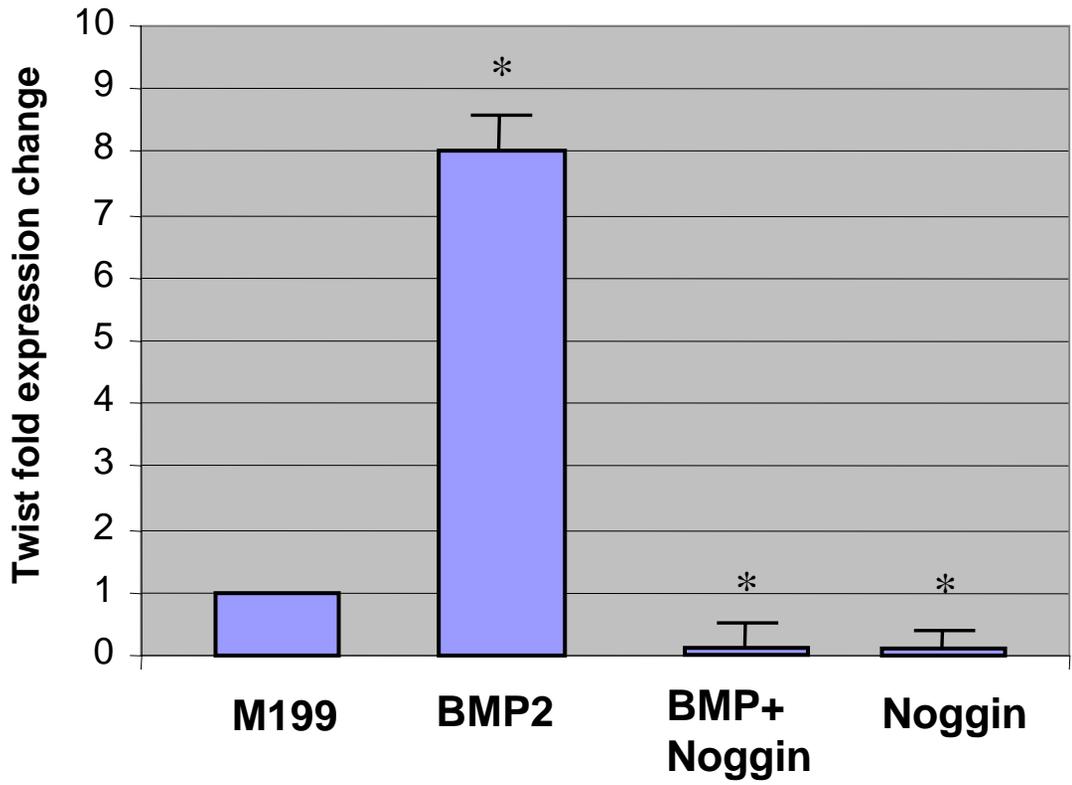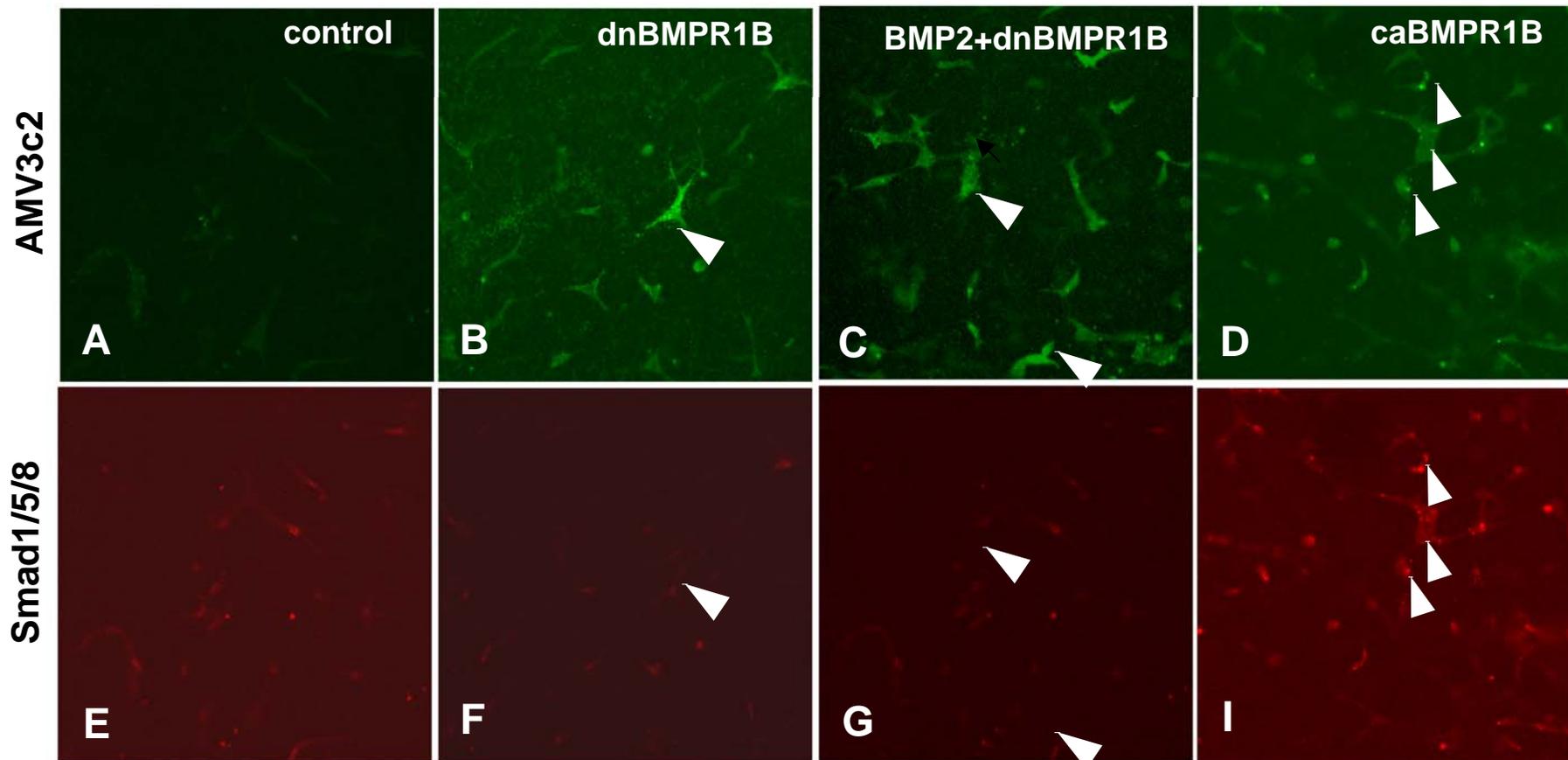
Fig.1

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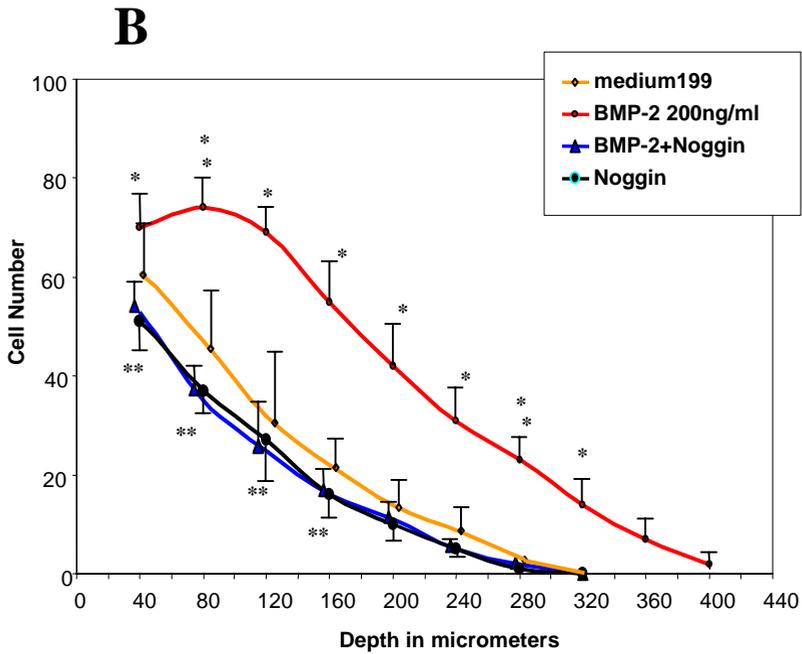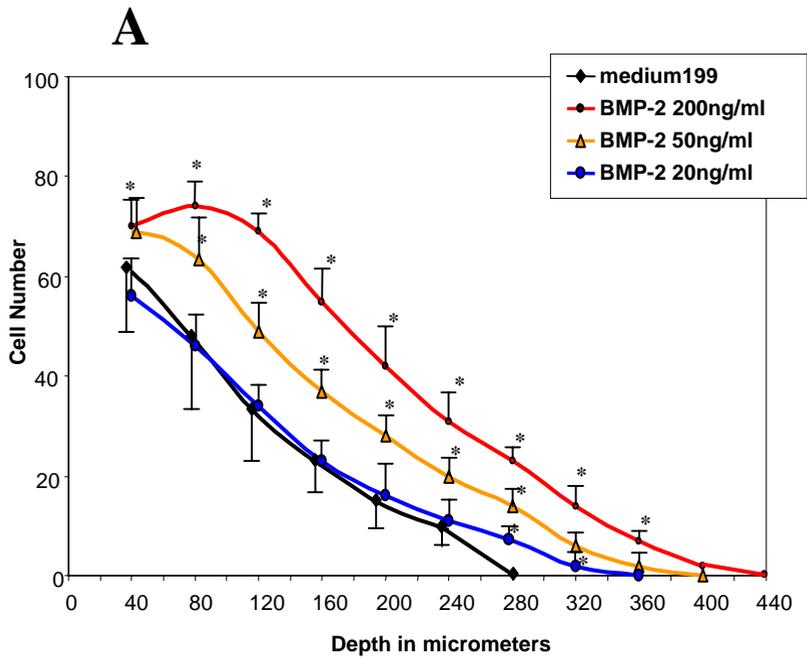
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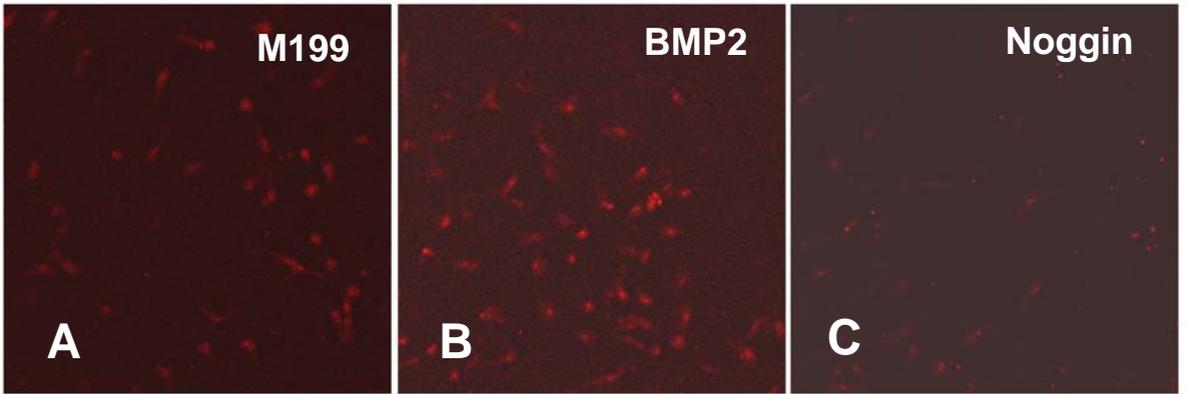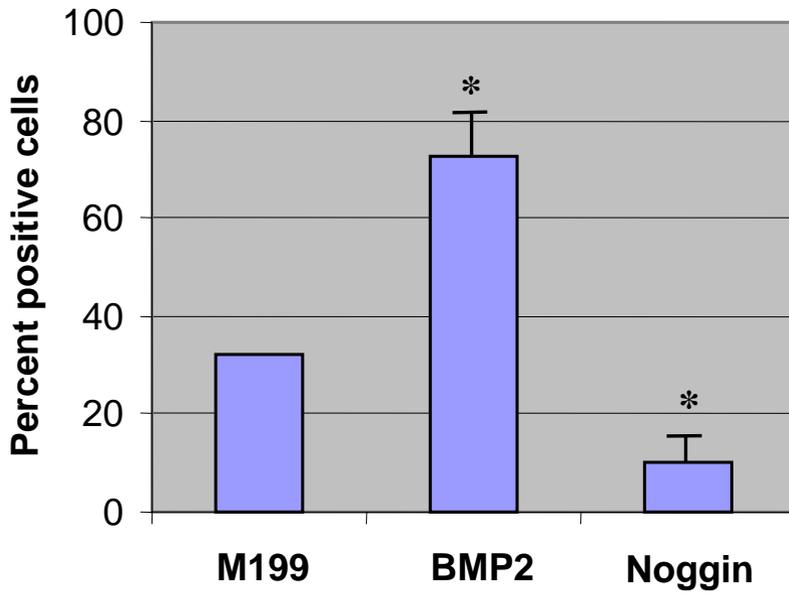
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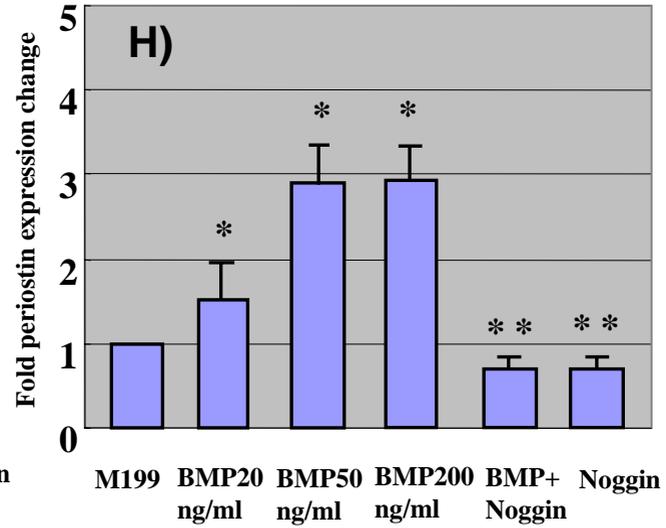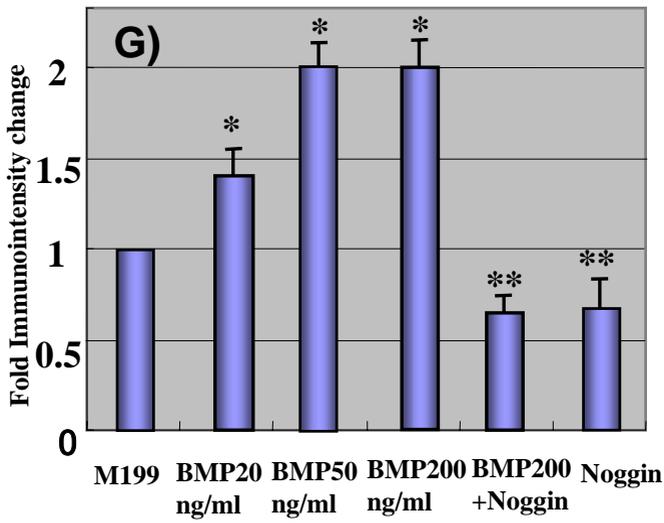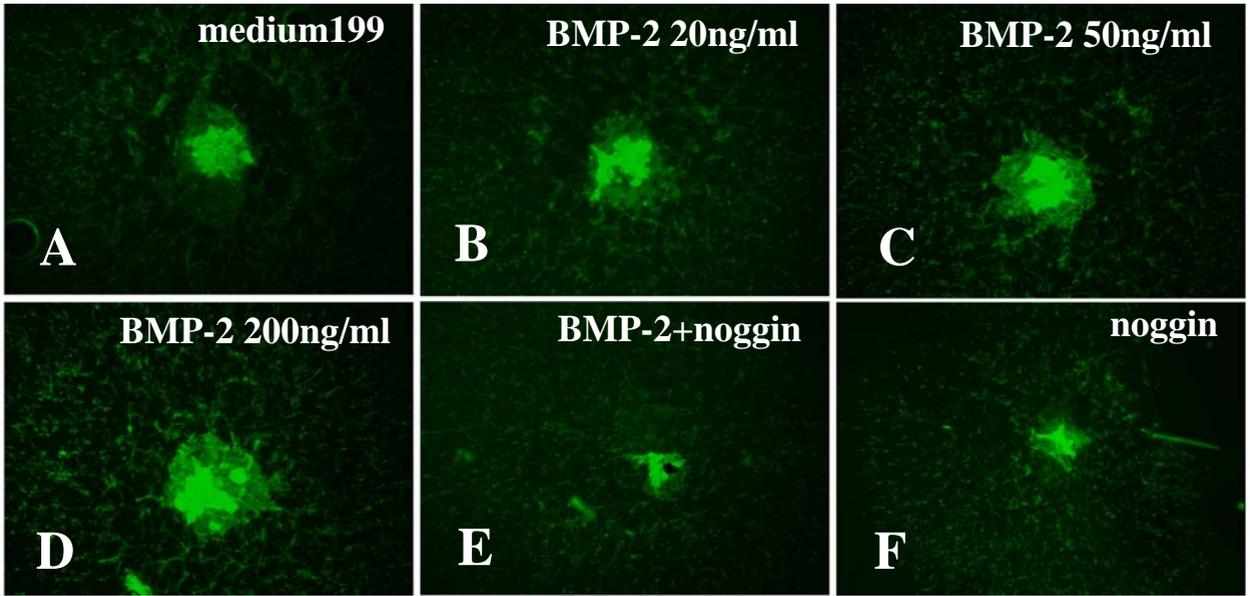
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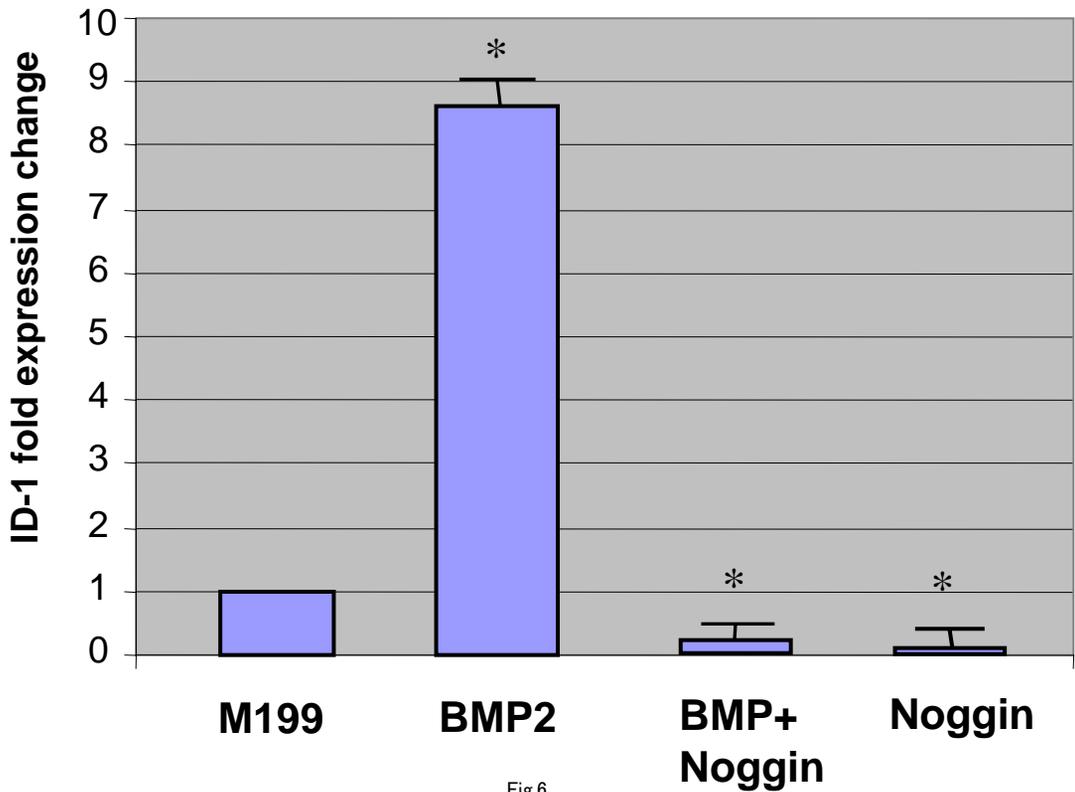
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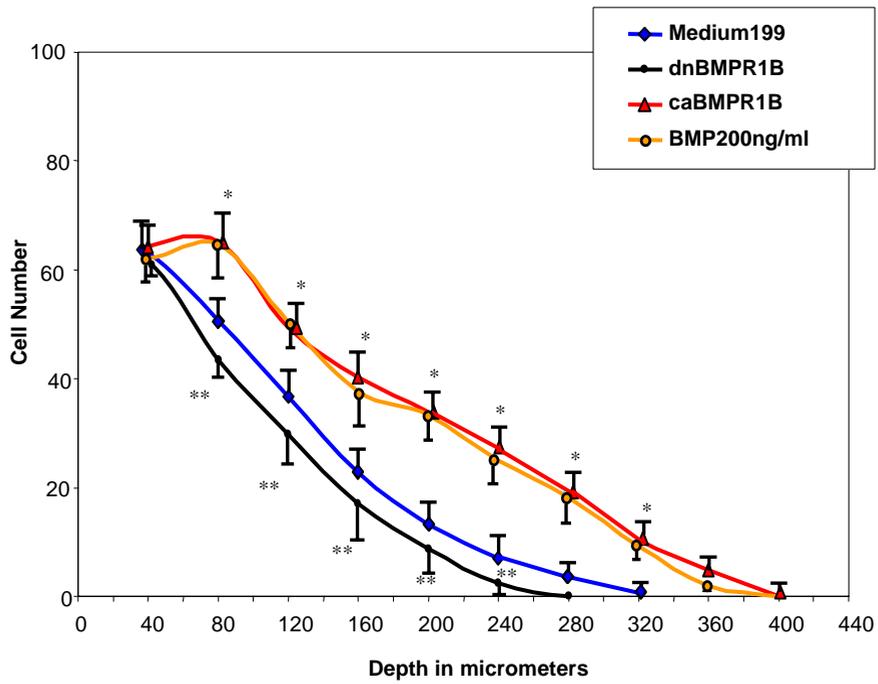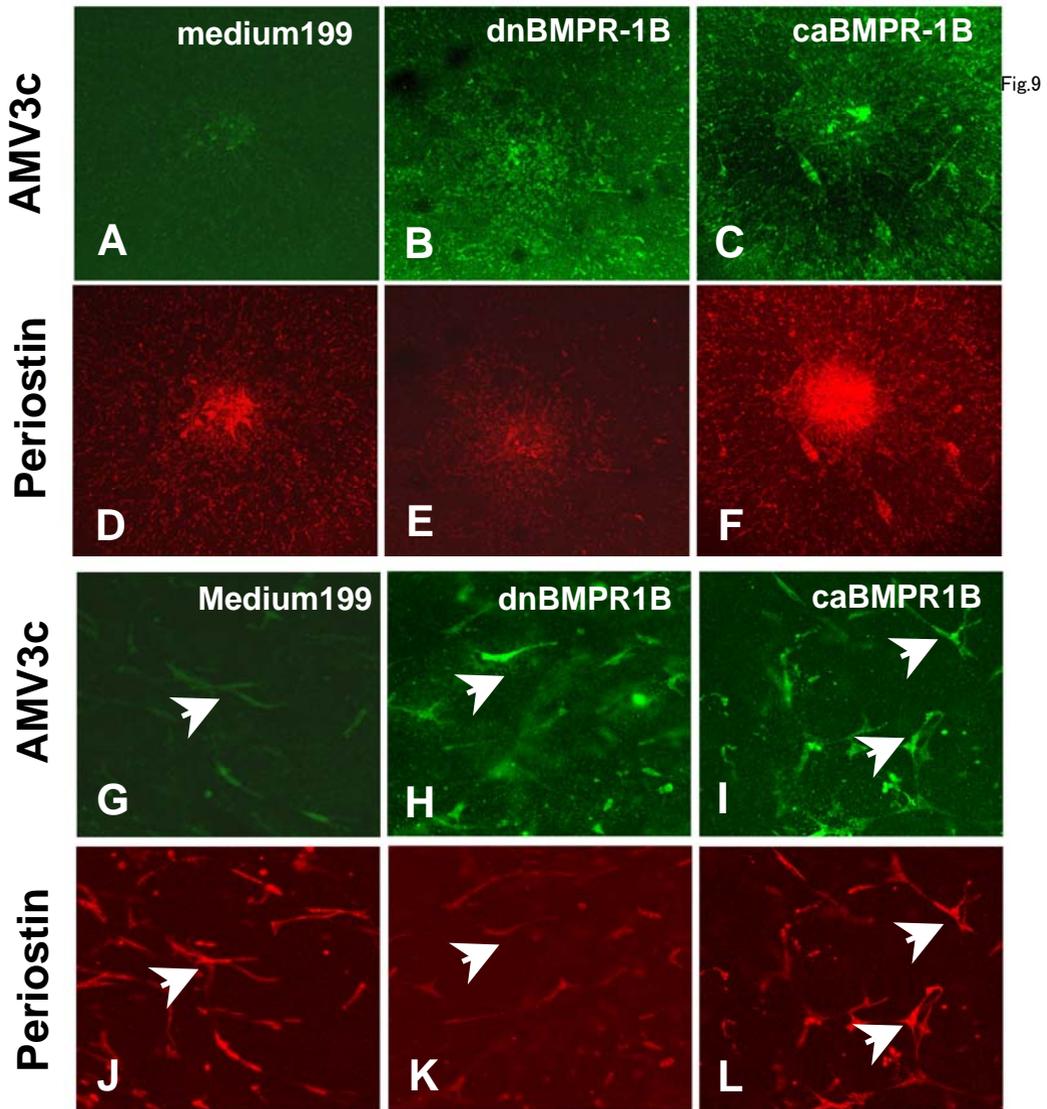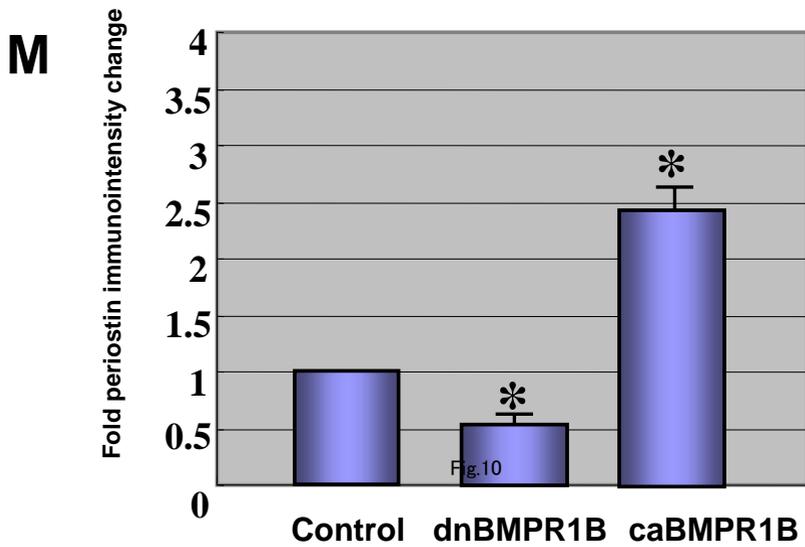
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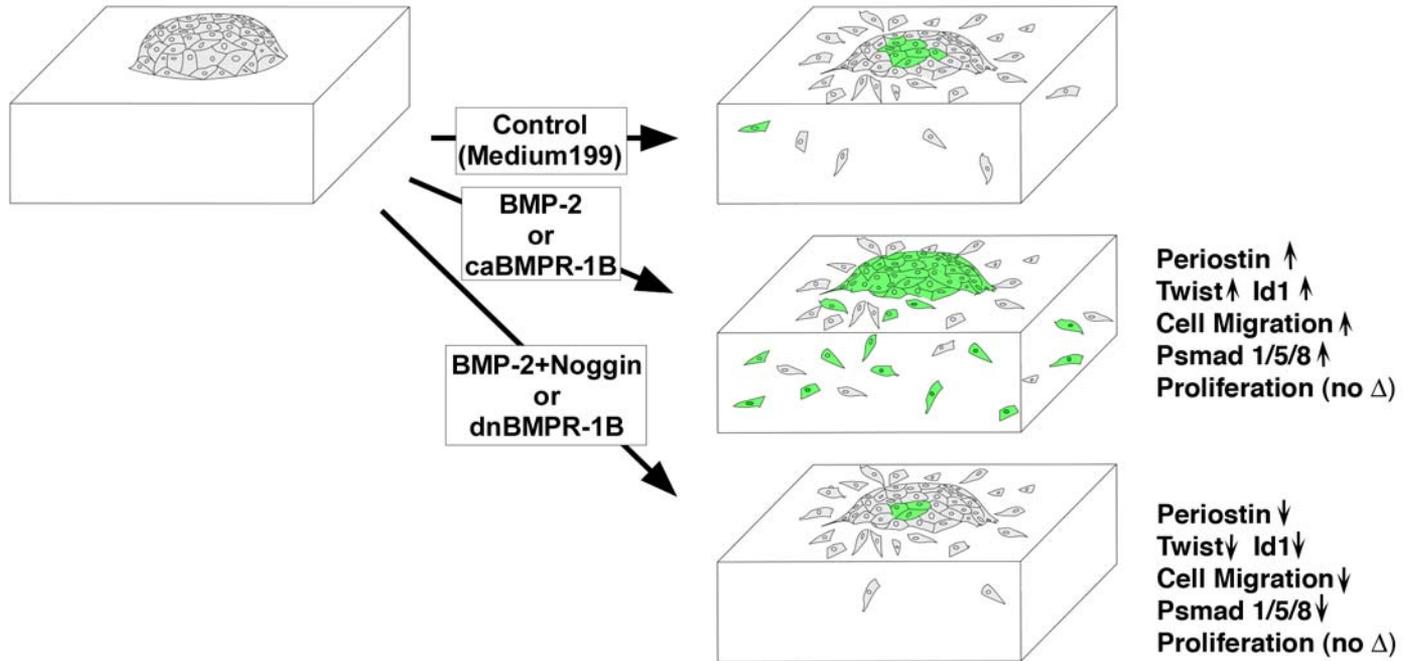AV cushion mesenchymal cell-aggregates
from HH stage 24 chick hearts

72 hrs

Control
(Medium199)

BMP-2
or
caBMPR-1B

BMP-2+Noggin
or
dnBMPR-1B

Periostin ↑
Twist↑ Id1 ↑
Cell Migration ↑
Psmad 1/5/8 ↑
Proliferation (no Δ)

Periostin ↓
Twist↓ Id1↓
Cell Migration ↓
Psmad 1/5/8 ↓
Proliferation (no Δ)

Fig.10