# Bone morphogenic protein 2 induces Runx2 and osteopontin expression in human aortic valve interstitial cells: Role of Smad1 and extracellular signal-regulated kinase 1/2

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**Objective:** Bone morphogenic protein 2 is found in calcified areas of stenotic aortic valves, and prolonged stimulation of aortic valve interstitial cells with bone morphogenic protein 2 results in increased expression of alkaline phosphatase, indicating a mechanistic role for bone morphogenic protein 2 in aortic valve calcification. The purposes of this study were to assess the effect of bone morphogenic protein 2 on the expression of the osteogenic factors Runx2 and osteopontin in human aortic valve interstitial cells and to determine the signaling mechanisms that mediate the expression of these early osteogenic factors.

**Methods:** Interstitial cells were isolated from normal and stenotic human aortic valve leaflets, and cellular bone morphogenic protein 2 levels were analyzed by means of immunoblotting. Cultured interstitial cells from normal aortic valves were stimulated with bone morphogenic protein 2 to determine its effect on cellular Runx2 and osteopontin levels.

**Results:** Interstitial cells from stenotic aortic valves express greater levels of bone morphogenic protein 2 than cells from normal valves. Stimulation of human aortic valve interstitial cells with bone morphogenic protein 2 induced marked increases in Runx2 and osteopontin levels at 48 hours. The changes in Runx2 and osteopontin levels were preceded by phosphorylation of Smad1 and extracellular signal-regulated kinase 1/2 but not p38 mitogen-activated protein kinase. Silencing Smad1 reduced Runx2 and osteopontin levels, whereas inhibition of extracellular signal-regulated kinase 1/2 reduced osteopontin expression without an influence on Runx2 expression.

**Conclusions:** Interstitial cells of stenotic human aortic valves are characterized by increased bone morphogenic protein 2 levels. A short period of exposure of human aortic valve interstitial cells to bone morphogenic protein 2 induces the expression of Runx2 and osteopontin. The extracellular signal-regulated kinase 1/2 pathway modulates bone morphogenic protein 2–induced osteopontin expression, and the Smad1 pathway plays a role in regulating the expression of both Runx2 and osteopontin induced by bone morphogenic protein 2.

Supplemental material is available online.

Calcific aortic stenosis is the third leading cardiovascular disease behind only hypertension and coronary artery disease.[1] It is found in approximately 3% of the population older than 65 years and is the most common indication for heart valve replacement surgery.[2] Long considered a disease of passive degeneration, evidence has emerged that aortic stenosis might result from an active disease process. However, the mechanisms responsible for the development of aortic stenosis remain unclear.

Insight into the pathogenesis of calcific aortic stenosis has been gained from the examination of aortic valve leaflets explanted at aortic valve replacement. Explanted calcified valve leaflets demonstrate morphologic features resembling osteogenic bone formation, as well as the expression of proteins associated with bone formation.[3] The fact that diseased aortic valve leaflets have such a histologic appearance suggests that calcific aortic stenosis might result from a process similar to that of active bone formation. This further implies that bone-forming cells (osteoblast-like cells) might be responsible for the calcification. Although the origin of such bone-forming cells is unclear, aortic valve interstitial cells (ICs) have been shown to have the ability to become osteoblast-like cells.[4]

Bone morphogenic proteins are osteogenic growth factors that belong to the transforming growth factor $\beta$ superfamily. These osteogenic growth factors are the principal inducers of osteoblast differentiation and bone formation. They act through both autocrine and paracrine mechanisms, and the

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**Abbreviations and Acronyms**
BMP-2 = bone morphogenic protein 2
ERK = extracellular signal-regulated kinase
IC = interstitial cell
MAPK = mitogen-activated protein kinase
PBS = phosphate-buffered saline
siRNA = small interfering RNA

paracrine effects are particularly important in osteogenesis.[5] Bone morphogenic protein 2 (BMP-2) has been found to play critical roles in vascular calcification.[6] This osteogenic growth factor has been identified in calcified tissue of stenotic aortic valves.[7,8] Likewise, the bone-forming protein osteopontin has been found in calcified aortic valve leaflets.[2] However, whether aortic valve ICs can be stimulated to produce osteopontin is unclear. After stimulation with BMP-2, the production of bone-forming proteins, such as osteopontin, requires the production of the transcription factor Runx2 in certain cell types. Hence in the present study the production of osteopontin and Runx2 after stimulation with BMP-2 was studied.

Although ICs appear to play an important role in aortic valve calcification,[9] it is unclear whether BMP-2 expression in ICs is altered in stenotic aortic valves. In a recent study[10] we isolated ICs from the noncalcified tissue of stenotic aortic valves and found that they are phenotypically different from ICs of normal valves. Analysis of BMP-2 expression in ICs of stenotic aortic valves might provide insights into the pathophysiology of aortic valve stenosis and calcification.

Previous studies by our group and others have found that stimulation of human aortic valve ICs for 2 or 3 weeks with BMP-2 upregulates the expression of alkaline phosphatase.[4,10] In C2C12 cells, BMP-2 activates the Runx2 pathway through Smad and p38 mitogen-activated protein kinase (MAPK).[11-13] The roles of the Smad and p38 MAPK pathways in mediating the effect of BMP-2 on human aortic valve ICs remain unclear. Further determination of the effect of BMP-2 on human aortic valve IC osteogenic response and the underlying signaling mechanisms is important to elucidate the role of BMP-2 in the pathogenesis of aortic valve calcification and stenosis.

In the present study we sought to determine (1) whether ICs of stenotic human aortic valves express higher levels of BMP-2, (2) the effect of BMP-2 on the expression of the early osteogenic factors Runx2 and osteopontin in human aortic valve ICs, and (3) the signaling mechanisms that mediate the effect of BMP-2 on the expression of these early osteogenic factors.

The results of the present study offer mechanistic insight into the pathogenesis of calcific aortic stenosis. The ICs of stenotic human aortic valves were found to be characterized by increased BMP-2 levels. Exposure of normal human aortic valve ICs to BMP-2 induces the expression of Runx2 and osteopontin. The extracellular signal-regulated kinase (ERK) 1/2 pathway modulates BMP-2–induced osteopontin expression, and the Smad1 pathway plays a role in regulating the expression of both Runx2 and osteopontin induced by BMP-2.

## MATERIALS AND METHODS

### Chemicals and Reagents

M199 medium and human serum albumin were purchased from Lonza (Walkersville, Md). Rabbit polyclonal antibody against human BMP-2 was obtained from ProSci (Poway, Calif). Rabbit polyclonal antibody against Runx2 was purchased from Novus Biologicals (Littleton, Colo). Rabbit polyclonal antibody against osteopontin was purchased from Abcam (Cambridge, Mass). Antibodies against phosphorylated and nonphosphorylated Smad1, ERK1/2, and p38 MAPK were purchased from Cell Signaling Technology (Danvers, Mass). Recombinant human BMP-2 was obtained from R&D Systems (Minneapolis, Minn). PD98059 was purchased from Calbiochem (San Diego, Calif). Smad1 small interfering RNA (siRNA) and DharmaFECT1 transfection reagent were purchased from Dharmacon (Lafayette, Colo). Opti-MEM I medium was purchased from Invitrogen (Carlsbad, Calif). Collagenase and other reagents were purchased from Sigma (St Louis, Mo).

### Cell Isolation and Culture

Normal aortic valves were collected from 5 explanted hearts of heart transplant recipients with cardiomyopathy (3 male and 2 female patients 49–65 years old), and stenotic aortic valves were collected from 5 patients who underwent valve replacement surgery for calcific aortic stenosis (2 male and 3 female patients 53–72 years old). All valves were tricuspid. This study was approved by the Colorado Multiple Institutional Review Board, and all patients provided written informed consent. On gross examination, normal valve leaflets were thin, and their surfaces were smooth. Microscopic examination of hematoxylin and eosin–stained cryosections confirmed that no leukocytes had infiltrated the leaflets. All stenotic valves were thick and exhibited overt calcification. Noncalcific tissue was used for cell isolation.

Small portions of each valve were either processed into tissue homogenate, frozen in OCT for tissue staining, or used for IC isolation. ICs were isolated by means of collagenase digestion, as previously described,[10,14] and cultured in growth medium (M199 medium with penicillin G, streptomycin, amphotericin B, and 10% fetal bovine serum) in an incubator supplied with 5% $CO_2$. Cells of passage 1 were used for analysis of cellular BMP-2 levels. Cells of passages 3 to 5 were grown to approximately 90% confluence and treated with recombinant human BMP-2 (100 ng/mL) for a varied period of time to examine the osteogenic response.

### Immunohistochemistry

Immunohistochemical detection of BMP-2 protein in aortic valve leaflets was performed by using the biotin-linked peroxidase technique. Cryosections (5 $\mu$m) were prepared and dried at room temperature for 2 hours. Sections were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 minutes and then washed with PBS. Unless indicated, all incubations were performed at room temperature. Endogenous peroxidase activities were quenched by incubating sections with 1% $H_2O_2$ in methanol for 10 minutes. Sections were incubated for 30 minutes with 10% goat serum in PBS to block nonspecific binding sites. Sections were then incubated for 90 minutes with rabbit