Nonsynonymous Variants in the SMAD6 Gene Predispose to Congenital Cardiovascular Malformation

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ABSTRACT: Congenital cardiovascular malformation (CVM) exhibits familial predisposition, but most of the specific genetic factors involved are unknown. Postulating that rare variants in genes in critical cardiac developmental pathways predispose to CVM, we systematically surveyed three genes of the bone morphogenetic protein (BMP) signaling pathway for novel variants. Exonic, splice site, and untranslated regions of BMPR1A, BMPR2, and SMAD6 genes were sequenced in 90 unrelated sporadic cases of CVM. One nonsynonymous variant (p.C484F) with predicted functional impact was found in the MAD homology 2 domain of SMAD6, an intracellular inhibitor of BMP signaling. Sequencing this domain in an additional 346 cases of CVM yielded two further nonsynonymous variants (p.P415L and p.A325T). Functional effects of all three SMAD6 mutations were investigated using BMP signaling assays in vitro. Two SMAD6 variants (p.C484F and p.P415L) had significantly (< 0.05) lower activity than wild-type SMAD6 in inhibiting BMP signaling in a transcriptional reporter assay. In addition, the p.C484F variant had a significantly (< 0.05) lower capacity to inhibit an osteogenic response to BMP signaling. We conclude that low-frequency deleterious variants in SMAD6 predispose to CVM. This is the first report of a human disease phenotype related to genetic variation in SMAD6.

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Introduction

Cardiovascular malformation (CVM) affects around seven in 1,000 live births and is a major cause of mortality and morbidity in childhood in the western countries. In 80% of “sporadic” cases that cannot be attributed to particular malformation syndromes or teratogenic exposures, there remains a substantial excess familial risk, indicating a significant genetic contribution to disease susceptibility [Burn et al., 1998; Oyen et al., 2009]. These recurrence risks are compatible with the presence of multiple genetic variants of incomplete penetrance, likely interacting with environmental factors. The majority of genetic factors predisposing to non-Mendelian CVM remain to be identified. We therefore postulated that rare variants in genes that regulate cardiac development may predispose individuals to CVM.

Members of the bone morphogenetic protein (BMP) signaling pathway (Fig. 1) are known to be important regulators of cardiogenesis [Wang et al., 2011]. BMP signaling is required for normal heart valve and outflow tract development [Goldman et al., 2009; Jiao et al., 2008; Liu et al., 2004; Ma et al., 2005; Rivera-Feliciano and Tabin, 2006; Sugii et al., 2004; Zhang and Bradley, 1996]. BMP ligands regulate cell responses by interacting with specific serine/threonine kinase receptors on the cell surface, as summarized in Figure 1. Of the BMP signaling receptors, BMP receptor type 1A (BMPR1A, also known as ALK3) is required in endothelial cells for normal development of the cardiac cushions [Kaneko et al., 2008; Song et al., 2007], in myocardial cells for normal trabeculae and endocardial cushion formation [Gasson et al., 2003], and in neural crest for normal outflow tract septation [Stottmann et al., 2004]. BMPR2 is required for normal development of the heart valves, septa, and outflow tract [Beppu et al., 2009; Delot et al., 2003]. The level of BMP signaling is regulated by the inhibitory protein SMAD6, which is highly expressed in the cardiac valves and outflow tract of the embryonic heart and is upregulated by shear stress [Galvin et al., 2000; Topper et al., 1997]. SMAD6 inhibits BMP signaling and this negative regulation is also important during heart development, as Smad6 knockout mice have cardiac valve defects and aortic ossification [Galvin et al., 2000].

As correct functioning of the BMP signaling pathway is required for normal heart development, we hypothesized that rare functional variation in members of this pathway could predispose individuals to increased risk of CVMs. We therefore screened patients with a range of CVM phenotypes for nonsynonymous mutations in the coding region of BMPR2 (MIM# 600799), BMPR1A (MIM# 601299), and SMAD6 (MIM# 602931) genes. We identified three
nonsynonymous SMAD6 mutations, which were further investigated in laboratory assays for their effects on the ability of SMAD6 protein to regulate BMP signaling.

Materials and Methods

Sample Collection

Caucasian patients of British ancestry affected by CVM were recruited at the Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle, UK. Ethics approval was granted by the Northumberland Research Ethics Committee. Fully informed written consent was obtained from all participants or their parents. Patients with chromosomal abnormalities, other multiorgan malformation syndromes, learning difficulties, known maternal exposure to significant teratogens during pregnancy, or family histories suggestive of Mendelian inheritance were excluded. DNA was extracted from peripheral blood or saliva using standard procedures.

Resequencing/Mutation Detection

The entire coding sequences, including exon–intron boundaries, of BMPR2 (NM_001204.6), BMPRIA (NM_004329.2), and SMAD6 (NM_005585.4) were resequenced in 90 CVM probands. Binomial probability indicates that if mutations in the screened sequence were present in 2.5% or greater of CVM cases, sequencing this number of probands would have 80% power of detecting at least one such mutation. Bidirectional sequencing was performed using MegaBACE 1000 sequencer (GE Healthcare). The MAD homology 2 (MH2) domain of SMAD6 encoded by exon 4 was resequenced in an additional 346 probands. Previously unreported nonsynonymous variants identified in the CVM patients were genotyped in a collection of 1,000 Caucasian controls of British ancestry free of CVM [Palomino-Doza et al., 2008], using iPLEX (Sequenom, Hamburg, Germany) or custom Taqman (Applied Biosystems, Paisley, UK). To estimate the frequency of nonsynonymous variants in the MH2 domain of SMAD6 in healthy controls, SMAD6 sequence data from 629 individuals available from the 1000 Genomes Project (http://browser.1000genomes.org/index.html) and from 200 exomes of Danish individuals (http://soap.genomics.org.cn/) were analyzed for all nucleotide changes [Durbin et al., 2010; Li et al., 2010].

Cell Culture and Transfections

Mouse myoblast C2C12 cells (American Type Culture Collection, LGC Standards, Teddington, UK) were maintained in Dulbecco’s modified Eagle’s medium in 10% fetal bovine serum with 50 units/ml penicillin and 50 μl/ml streptomycin (Invitrogen, Paisley, UK) at 37°C in 5% CO2-humidified atmosphere. Cells were transfected using FuGENE HD transfection reagent (Roche Diagnostics) according to the manufacturer’s recommendations.
Constructs

Plasmids expressing the MH2 domain, corresponding to the C-terminal region of SMAD6 protein (SMAD6C), and a constitutively active BMPR1A receptor (caBMP1A) have been previously described [Fujii et al., 1999; Hanyu et al., 2001]. SMAD6 mutations c.1244C>T (p.P415L), c.973G>A (p.A325T), and c.1451G>T (p.C484F) were introduced into the SMAD6C expression construct by site-directed mutagenesis using QuickChange kit (Stratagene-Agilent Technologies, Stockport, UK).

Luciferase Assay

Dual luciferase assays were performed using previously validated luciferase transcriptional reporter constructs [Goto et al., 2007] containing BMP/SMAD responsive elements (BRE–luc) [Korchynskyi and ten Dijke, 2001]. C2C12 cells were seeded in 12-well plates for 24 hr before being transfected with caBMPR1A; BRE–luc; and either wild-type (wt) SMAD6C, mutant SMAD6C constructs, or empty vector pcDNA3.1 (Invitrogen). A pRL–TK vector (in which Renilla luciferase is driven by a thymidine kinase promoter) was cotransfected in all samples as a control for transfection efficiency. Cells were incubated for 24 hr after transfection and luciferase activities were measured in lysates using Dual-Luciferase Reporter Assay System (Promega, Southampton, UK) following manufacturer’s protocol. Data were normalized using Renilla luciferase activity. Three independent experiments were performed (each in triplicate).

Immunoblotting

After reading the luciferase activity, NuPAGE sample reducing agent and sample buffer (Invitrogen) were added to aliquots of cell lysates. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Hybond-C extra nitrocellulose membrane (GE Healthcare, Little Chalfont, UK). Antibodies used were anti–FLAG M2-peroxidase (1:10,000; Sigma), anti-heme agglutinin-peroxidase 3F10 (1:1,000; Roche Diagnostics, Burgess Hill, UK), anti-α-tubulin DM1A (1:10,000; Sigma), and anti-nucleolin (1:10,000; Bethyl Laboratories, Montgomery, Texas). An enhanced chemiluminescent detection system (Thermo Fisher Scientific, Basingstoke, UK) was used to detect the antibodies.

Alkaline Phosphatase Assay

Alkaline phosphatase (ALP) activity was measured in C2C12 cells transfected with SMAD6 variants. Cells were transfected with caBMPR1A and either wt SMAD6C, mutant SMAD6C constructs, or empty vector pcDNA3.1, and were cultured for 3 days with one change of medium. Cells were then washed with phosphate-buffered saline, lysed with 50 mM Tris–HCl (pH 7.5) and 0.1% Triton X-100 and sonicated. ALP activity was determined using p-nitrophenyl phosphate in 100 mM glycine, 1 mM MgCl2, and 0.1 mM ZnCl2 (pH 10.5), as previously described [Katagiri et al., 1994]. Protein concentration of cell lysates was measured using a BCA kit (Thermo Scientific).

Homology Modeling

In the absence of a structure of SMAD6, a homology model was generated based on 34% sequence identity to the MH2 domain of SMAD1, the crystal structure of which is available (Protein Data Bank [PDB] ID, 1KHU). The sequence alignment between SMAD1 and SMAD6 was generated using the default settings of ClustalW2 (www.ebi.ac.uk/Tools/clustalw2/index.html) and was used as the template to thread the SMAD6 amino acid sequence onto the SMAD1 structure using CCP4 module Chainsaw [Stein, 2008]. Manual correction of the model was performed in Coot [Emsley and Cowtan, 2004] and was then conjugate gradient energy minimized for 200 cycles with crystallography and NMR system [Brunger et al., 1998]. To create the p.C484F mutant model, the cysteine at position 484 (Cys484) was replaced by the most commonly observed phenylalanine (Phe) rotamer in the Coot library and energy minimized as mentioned above. Figures were produced using the PyMOL Molecular Graphics System (Schrödinger, Camberley, UK).

Statistical Analysis

Results

Identification of Nonsynonymous Coding Region Mutations in Patients with CVM

The cardiac phenotypes of the patients are described in Supp. Table S1. We adopted a two-stage strategy consisting of a “discovery cohort” of 90 patients and a “replication cohort” of 346 patients. The first group of 90 patients was screened for novel sequence variants in the coding region of BMPR2, BMPRIA, and SMAD6 genes. A nonsynonymous variant in exon 4 of SMAD6, which corresponded to a cysteine to phenylalanine substitution at position 484 of the protein (p.C484F), was found in a patient with bicuspid aortic valve, aortic valve stenosis, and coarctation and calcification of the aorta (Supp. Table S1). No nonsynonymous variants were found in the coding region of BMPRIA or BMPR2. The p.C484F variant identified in SMAD6 was absent from the 1,000 Caucasian controls of British ancestry free of CVM [Palomino-Doza et al., 2008]. The p.C484F mutation lies in the MH2 domain of SMAD6 (Fig. 2), the function of which is critical for the protein’s interaction with other members of the BMP signaling pathway. Resequencing of the MH2 domain of SMAD6 (encoded by exon 4) in an additional 346 probands with a broad range of CVM phenotypes was therefore undertaken. This yielded two further nonsynonymous variants: an alanine to threonine substitution at position 325 of the protein (p.A325T) in a patient with congenital mitral regurgitation and a proline to leucine substitution at position 415 of the protein (p.P415L) in an infant with a bicuspid aortic valve and moderate aortic stenosis (Supp. Table S1). These three SMAD6 variants were not found in the publicly available single-nucleotide polymorphism (SNP) databases (dbSNP; www.ncbi.nlm.nih.gov/projects/SNP/). We next analyzed available sequencing data for the MH2 coding region of SMAD6 from 629 individuals in the 1000 Genomes Project and from 200 individuals in the Danish Exome Project [Durbin et al., 2010; Li et al., 2010]. There were no nonsynonymous variants. Thus, nonsynonymous variants in the MH2 domain of SMAD6 were significantly more common in patients (3/436) than in controls (0/829; Yates’ chi-squared = 3.152; degree of freedom = 1; one-tailed P value = 0.0375).
Predicted Structural Changes in SMAD6 Variants

Following the identification of three SMAD6 variants within the MH2 domain (Fig. 2), the crystal structure of the SMAD1 homotrimeric complex (PDB entry, 1KHU), which is homologous to the MH2 domain of SMAD6, was utilized as a basis for modeling the structure of wt and variant SMAD6 MH2 domains. The change of cysteine to phenylalanine in the p.C484F variant resulted in significant changes in the structure that was limited to the immediate vicinity of residue 484. In the wt model, Cys484 is buried from solvent by the “L3 loop” of the MH2 domain and the thiol packs, in particular, against the indole ring of Trp472. To accommodate the extra bulk of a phenylalanine side chain, the structure in the immediate vicinity of the L3 loop adopts an altered conformation (Fig. 3). Glycines 471 and 473, which immediately flank the tryptophan, provide the potential for necessary flexibility in this part of the structure of the L3 loop during receptor binding, and the p.C484F variant may therefore affect receptor binding.

Similar comparative structural modeling of the p.P415L variant suggests that the α-helix H2, which, unusually, is N-capped by P415, is destabilized by mutation to leucine. Residue 415 is solvent accessible and is on the same face of the MH2 domain as the L3
SMAD6 Mutants Show Impaired Inhibition of BMP Signaling In Vitro

The MH2 domain is a critical region for SMAD6 protein function, which includes the "L3 loop," a region required for interaction with the BMP type I receptors; the SMAD6 MH2 domain alone is sufficient to inhibit type I receptor function [Hanyu et al., 2001]. The inhibitory effects of the MH2 domains of the variant SMAD6 proteins were therefore compared with that of the wt protein on BMP-responsive transcriptional activity using a BRE–luc transcriptional reporter. SMAD6 has been reported to preferentially inhibit BMPR1A signaling [Goto et al., 2007]; therefore, caBMPR1A was selected as the signal transducer in the cell system. The MH2 domain of p.A325T mutant SMAD6 functioned in the same way as the wt MH2 domain of SMAD6 in this assay and appeared to have normal SMAD6 activity. However, the MH2 domain of p.P415L and p.C484F mutant SMAD6 proteins inhibited BMP signaling less efficiently than the equivalent domain of wt SMAD6, with relative luciferase readings sevenfold (p.P415L) and 24-fold (p.C484F) higher than the wt (Fig. 4). The p.P415L mutant appeared to be hypomorphic ($P < 0.05$ for difference with wt), whereas the p.C484F mutant appeared to be a null allele ($P < 0.05$ for difference with wt) as there was almost no inhibitory effect of the p.C484F mutant SMAD6 MH2 domain on caBMPR1A activity in this assay (Fig. 4).

SMAD6 p.C484F Shows Impaired Inhibition of BMP-Induced ALP Activity

To determine whether the MH2 domains of mutant SMAD6 proteins also had a less marked inhibitory effect on osteogenic potential than wt, C2C12 cells were transiently cotransfected with caBMPR1A and either wt SMAD6, p.P415L, or p.C484F expression constructs. Osteogenic potential was assessed by induction of ALP activity, a commonly used early marker of osteoblast differentiation. Quantitative analysis revealed that the p.C484F mutant showed reduced capacity to inhibit ALP activity ($P < 0.05$; Fig. 5), suggesting that mutant protein had lower efficacy in preventing tissue calcification. The p.P415L mutant, which was suggested by homology modeling and luciferase assay to be less deleterious to SMAD6 function than the p.C484F mutant, inhibited ALP activity to the same extent as the wt SMAD6C protein.

Discussion

This is the first description of a human disease associated with mutations in SMAD6, and it suggests that genetic variants in the MH2 domain of SMAD6 contribute to increased risk of CVM. In a cohort of 436 CVM patients, we found three heterozygous nonsynonymous variants in the MH2 domain of SMAD6 that were absent in 1,000 Caucasian controls of British ancestry free of CVM [Palomino-Doza et al., 2008]. Furthermore, no nonsynonymous variants in the SMAD6 MH2 domain were detected in two recent high-throughput sequencing projects involving a total of 829 individuals [Durbin et al., 2010; Li et al., 2010].

Two of the nonsynonymous variants in SMAD6 protein (p.C484F and p.P415L) led to amino acid changes in evolutionarily conserved residues and were predicted to affect the protein structure. Furthermore, both mutations were clearly deleterious to function in BMP signaling assays. The patient heterozygous for the p.C484F SMAD6
variant allele was found to have a bicuspid aortic valve with mild aortic stenosis and aortic coarctation at the age of 30 years in the course of investigation for hypertension, and the coarctation was repaired. He subsequently developed significant aortic stenosis and underwent aortic valve replacement and reréparation of the aortic arch. At his second operation, it was noted that the transverse aortic arch, proximal to and distant from the previous conduit, was heavily calcified. It is possible that this is a consequence of the reduced efficiency of the p.C484F mutant SMAD6 in inhibiting osteogenic potential (Fig. 5). There was no evidence of inappropriate calcification in noncardiovascular tissues.

The other functionally significant SMAD6 variant we discovered (p.F415L) was present in a patient who presented with a heart murmur at 18 months and was found to have a bicuspid aortic valve with moderate aortic stenosis. There was no evidence of coarctation. Both patients carrying functionally significant SMAD6 variants had bicuspid aortic valves, the commonest cardiovascular malformation, occurring in approximately 1% of the adult population. There is a phenotypic spectrum in this condition dependent on the degree of valvular malformation, ranging from severe aortic stenosis in the neonatal period to the usual presentation either as an asymptomatic murmur or established aortic stenosis in adult life. As this cohort of CVM cases was mainly recruited through a pediatric cardiology service, the numbers with bicuspid aortic valve were relatively small (24/436). It will be interesting to test whether SMAD6 mutations are over-represented in a larger cohort of CVM patients with bicuspid aortic valves, the commonest cardiovascular malformation, occurring in approximately 1% of the adult population. There is a phenotypic spectrum in this condition dependent on the degree of valvular malformation, ranging from severe aortic stenosis in the neonatal period to the usual presentation either as an asymptomatic murmur or established aortic stenosis in adult life. As this cohort of CVM cases was mainly recruited through a pediatric cardiology service, the numbers with bicuspid aortic valve were relatively small (24/436). It will be interesting to test whether SMAD6 mutations are over-represented in a larger cohort of CVM patients with bicuspid aortic valves, the commonest cardiovascular malformation, occurring in approximately 1% of the adult population.

In the Smad6 knockout mouse originally described by Galvin et al. (2000), multiple cardiovascular developmental abnormalities, including hyperplastic thickening of the cardiac valves and aortic ossification, are present. The association of SMAD6 mutations with an aortic stenosis phenotype in the patients described in this study is entirely consistent with those observations and with a similar role for SMAD6 in human cardiovascular development. Our findings are also consistent with the expression of SMAD6 in the cardiac valves and outflow tract, which continues into adult life in mouse [Galvin et al., 2000], and with a recent clinical phenotypic study showing that reduced SMAD6 expression was associated with calcification of the aortic valve [Ankeny et al., 2011].

Exon-focused sequencing of two other genes (BMPR2 and BMPRIA) in the BMP signaling pathway revealed no nonsynonymous variants. We therefore conclude that such variants are uncommon in these genes in CVM patients; however, sequencing of much larger number of cases would be required to exclude prevalences of 1–2%. On the basis of information from mouse models, there is a significant involvement of other BMP- and transforming growth factor β (TGFβ)-related genes in cardiac development that also warrants further investigation in congenital heart disease [Arthur and Bamforth, 2011; Wang et al., 2011]. Variants in some of these genes have already been shown to be associated with cardiovascular abnormalities in human studies, for example, Nodal, GDF1, TGFβ3, and BMP2 [Beffagna et al., 2005; Karkera et al., 2007; Mohapatra et al., 2009; Roberts et al., 2004; Roessler et al., 2009]. In one interesting case, a dominant-negative form of the BMP receptor ALK2 was found in a patient with endocardial cushion defects [Smith et al., 2009].

Although a few families have been described in which CVM segregates in a Mendelian fashion, for example, due to mutations in cardiac transcription factors [Garg et al., 2003; Schott et al., 1998], these families are exceptional and usually the inheritance pattern is less obvious; indeed, in the majority of cases, there is a single affected individual in a family. On analysis of pedigrees, however, the risk is clearly increased in the relatives of affected individuals, indicating a significant genetic contribution [Burn et al., 1998; Oyen et al., 2009]. These recurrence risks are compatible with the presence of multiple genetic risk variants of incomplete penetrance, likely interacting with environmental factors. We, and others, have already shown that incompletely penetrant alleles in key genes can predispose to CVM [Goldmuntz et al., 2001; Griffin et al., 2010; McElhinney et al., 2003; Sperling et al., 2005]. Future studies utilizing the rapidly increasing power of genome sequencing technologies to interrogate a much wider range of candidate genes, and eventually the whole exome, for rare variants that predispose to CVM will be of great interest. In the context of SMAD6, our results clearly demonstrate
statistically significant differences between some of the mutant and wt SMA6D proteins with respect to inhibitory activity in the in vitro assays, but the quantitative effects of different levels of mutant constructs remain to be explored, as do the downstream effects of BMP ligand treatments in the mutant cells in vivo. Nevertheless, results such as those described here could have important implications for clinical practice. If a number of genes with contributions of similar magnitude as SMA6D to the risk of aortic abnormalities could be identified, screening of such genes to provide individual-specific counseling about recurrence risks to offspring would be a valuable addition to the current counseling.

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References

Ankeny RF, Thouzani VH, Weiss D, Vega JD, Taylor WR, Neren RM, Jo H. 2011. Preferential activation of SMAD1/5/8 on the fibroblast endothelium in calcified human aortic valves—association with low BMP antagonists and SMA6D. PLoS One 6(4):e19269.

Arthur HM, Bamforth SD. 2011. TGFbeta signaling and congenital heart disease: insights from mouse studies. Birth Defects Res A Clin Mol Teratol 91:423–434.

Bai S, Shi X, Yang X, Cao X. 2000. Smad6 as a transcriptional corepressor. J Biol Chem 275:8267–8270.

Beppu H, Malhotra R, Beppu Y, Lepore J, Parmaekes MS, Bloch KD. 2009. BMP type II receptor regulates positioning of outflow tract and remodeling of atrioventricular cushion during cardiovascular development. Dev Biol 331:167–175.

Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang B, Bai S, Shi X, Yang X, Cao X. 1998. Crystallography & NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr D Biol Crystallogr 54:905–921.

Burn J, Brennan P, Little J, Holloway S, Coffey R, Somerville J, Dennis NR, Allan L, Arnold R, Deanfield J, Godman H, Moistan A, Keeton B, Oakley C, Scott O, Silove E, Wilkinson J, Pembrey M, Hunter AS. 1998. Recurrence risks in offspring of adults with major heart defects: results from first cohort of British collaborative study. Lancet 351:311–316.

Delot EC, Bahamonde ME, Zhao M, Lyons KM. 2003. BMP signaling is required for septation of the outflow tract of the mammalian heart. Development 130:209–220.

Durbin RM, Abecasis GR, Altshuler DL, Auton A, Brooks LD, Durbin RM, Gibbs RA, Hurles ME, McVean GA. 2010. A map of human genome variation from population-scale sequencing. Nature 467:1061–1073.

Edsley P, Cowtan K. 2004. Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr 60:2126–2132.

Fujii M, Takeda K, Imamura T, Aoki H, Sampath TK, Enomoto S, Kawabata M, Kato M, Ichijo H, Miyazono K. 1997. Smad6 in development and homeostasis of the cardiovascular system. Nat Genet 12:186–197.

Goldman DC, Donnelly N, Christian JL. 2009. Genetic interaction between Bmp2 and Bmp4 reveals shared functions during multiple aspects of mouse organogenesis. Mech Dev 126:117–127.

Goldmuntz E, Geiger E, Benson DW. 2001. Nkx2.5 mutations in patients with tetralogy of fallot. Circulation 104:2565–2568.

Goto K, Kamiya T, Imamura T, Miyazono K, Miyazawa K. 2007. Selective inhibitory effects of Smad6 on bone morphogenetic protein type I receptors. J Biol Chem 282:20669–20673.

Griffin HR, Topf A, Glen E, Zwieier C, Stuart G, Parsons J, Peart I, Deanfield J, O’Sullivan J, Rauch A, Scambler P, Burn J, Cordell HJ, Keavney BD, Goodship JA. 2010. Systematic survey of variants in Tbx5 in non-syndromic tetralogy of Fallot identifies a novel 37 base pair deletion that reduces transcriptional activity but finds no evidence for association with common variants. Heart 96:1651–1655.

Hanley A, Ishidou Y, Ehsawia T, Shimizu T, Imamura T, Miyazono K. 2001. The N domain of Smad2 is essential for specific inhibition of transforming growth factor-beta signaling. J Cell Biol 155:1017–1027.

Hata A, Lagna G, Massague J, Hemmati-Brivanlou A. 1998. Smad6 inhibits BMP/Smad1 signaling by specifically competing with the Smad3 tumor suppressor. Genes Dev 12:186–197.

Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, Kawabata M, Miyazono K. 1997. Smad6 inhibits signaling by the TGF-beta superfamily. Nature 389:622–626.

Jiao K, Kulesha H, Tompkins K, Zhou Y, Bats I, Baldwin HS, Hogan BL. 2003. An essential role of Bmp6 in the atrioventricular septation of the mouse heart. Genes Dev 17:2539–2543.

Kaneko M, Li X, Zhang X, Lamberti JJ, Jamieson SW, Thistlethwaite PA. 2008. Endothelial expression of bone morphogenetic protein receptor type 1a is required for atrioventricular valve formation. Ann Thorac Surg 85:2090–2098.

Karkera JD, Lee JS, Roessler E, Barnerje-Basu S, Oupsenskaia MV, Mez J, Goldmuntz E, Bowers P, Tovbin J, Belmont JW, Baxevanis AD, Schir AF, Muenke M. 2007. Loss-of-function mutations in growth differentiation factor-1 (GDF1) are associated with congenital heart defects in humans. Am J Hum Genet 81:987–994.

Katagiri T, Yamaguchi A, Komaki M, Abe E, Takashashi N, Ikeda T, Rozen V, Wozney JM, Fujisawa-Sehara A, Suda T. 1994. Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. J Cell Biol 127:1755–1766.

Kerchovsky O, ten Dijke P. 2001. Identification and functional characterization of distinct critically important bone morphogenetic protein-specific response elements in the Id1 promoter. Journal Biol Chem 276:4883–4891.

Li Y, Vinkenbosch N, Tian G, Huerta-Sanchez E, Jiang T, Jiang H, Albrechtsen A, Andersen G, Cao H, Korneliusen T, Garparu N, Guo Y, Hellman I, Jin X, Li Q, Liu X, Sparso T, Tang M, Wu H, Wu R, Yu C, Zheng H, Astrup A, Bolund L, Holmkvist J, Jorgensen T, Kristiansen K, Schmitz O, Schwartz TW, Zhang X, Li R, Yang H, Wang J, Hansen T, Pedersen O, Nielsen R, Wang J. 2010. Resequencing of 200 human exomes identifies an excess of low-frequency non-synonymous coding variants. Nat Genet 42:969–972.

Liu W, Selever J, Wang D, Lu MF, Moses KA, Schwartz RJ, Martin JF. 2004. Bmp4 signaling is required for outflow-tract septation and branchial-arch artery remodeling. Proc Natl Acad Sci USA 101:4489–4494.

Ma L, Lu M, Schwartz RJ, Martin JF. 2005. Bmp2 is essential for cardiac cushion epithelial–mesenchymal transition and myocardial patterning. Development 132:5601–5611.

McElhinney DB, Geiger E, Blinder J, Benson DW, Goldmuntz E. 2003. Nkx2.5 mutations in patients with congenital heart disease. J Am Coll Cardiol 42:1650–1655.

Mohapatra B, Casey B, Li H, Ho-Dawson T, Smith L, Fernbach SD, Molinari L, Niesh SR, Jefferies JL, Craigin WJ, Tovbin JA, Belmont JW, Ware SM. 2009. Identification and functional characterization of NODAL rare variants in heterozygous and isolated cardiovascular malformations. Hum Mol Genet 18:861–871.

Murakami G, Watabe T, Takaoaka K, Miyazono K, Imamura T. 2003. Cooperative inhibition of bone morphogenetic protein signaling by Smurf1 and inhibitory Smad6. Mol Biol Cell 14:2809–2817.

Nakagawa Y, Ishidou Y, Ehsawia T, Shimizu T, Imamura T, Miyazono K. 2001. The N domain of Smad2 is essential for specific inhibition of transforming growth factor-beta signaling. J Cell Biol 155:1017–1027.

Owen N, Poulsen G, Boyd HA, Wohlfahrt J, Jensen PK, Melbye M. 2009. Recurrence of congenital heart defects in families. Circulation 120:295–301.

Palomino-Deza J, Rahman TJ, Avery PJ, Mayosi BM, Farrall M, Watkins H, Edwards CR, Keavney B. 2008. Ambulatory blood pressure is associated with polyvaricomial polymorphism in P2X2 receptor genes. Hypertension 52:980–985.

Rivera-Feliciano J, Rahman TJ, Avery PJ, Mayosi BM, Farrall M, Watkins H, Edwards CR, Keavney B. 2008. Ambulatory blood pressure is associated with polyvaricomial polymorphism in P2X2 receptor genes. Hypertension 52:980–985.

Roper E, Pei W, Oupsenskaia MV, Karkera JD, Veler JJ, Barnerje-Basu S, Gibney G, Lupin PJ, Mitchell LE, Tovbin JA Bowers P, Belmont JW, Goldmuntz E, Baxevanis AD, Feldman B, Muenke M. 2009. Cumulative ligand activity of NODAL mutations and modifiers are linked to human heart defects and holoprosencephaly. Mol Genet Metab 98:225–234.
Schott JJ, Benson DW, Basson CT, Pease W, Silberbach GM, Moak JP, Maron BJ, Seidman CE, Seidman JG. 1998. Congenital heart disease caused by mutations in the transcription factor NKX2-5. Science 281:108–111.

Smith KA, Joziasse IC, Chocron S, van Dinther M, Guryev V, Verhoeven MC, Rehmann H, van der Smagt JJ, Doevendans PA, Cuppen E, Mulder BJ, Ten Dijke P, Bakkers J. 2009. Dominant-negative ALK2 allele associates with congenital heart defects. Circulation 119:3062–3069.

Song L, Fassler R, Mishina Y, Jiao K, Baldwin HS. 2007. Essential functions of Alk3 during AV cushion morphogenesis in mouse embryonic hearts. Dev Biol 301:276–286.

Sperling S, Grimm CH, Dunkel I, Mebus S, Sperling HP, Ebner A, Galli R, Lehrach H, Fuch C, Berger F, Hammer S. 2005. Identification and functional analysis of CITED2 mutations in patients with congenital heart defects. Hum Mutat 26:575–582.

Stein N. 2008. CHAINSAW: a program for mutating pdb files used as templates in molecular replacement. J Appl Crystallogr 41:641–643.

Stottmann RW, Choi M, Mishina Y, Meyers EN, Klingensmith J. 2004. BMP receptor IA is required in mammalian neural crest cells for development of the cardiac outflow tract and ventricular myocardium. Development 131:2205–2218.

Sugi Y, Yamamura H, Okamura H, Markwald RR. 2004. Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice. Dev Biol 269:505–518.

Topper JN, Cai J, Qu Y, Anderson KR, Xu YY, Deeds JD, Feeley R, Gimerone CI, Woolf EA, Taylor O, Mays GG, Sampson BA, Schoen FJ, Gimbrone MA, Jr., Falb D. 1997. Vascular MAD: two novel MAD-related genes selectively inducible by flow in human vascular endothelium. Proc Natl Acad Sci USA 94:9314–9319.

Wang J, Greene SB, Martin JF. 2011. BMP signaling in congenital heart disease: new developments and future directions. Birth Defects Res A Clin Mol Teratol 91:441–448.

Zhang H, Bradley A. 1996. Mice deficient for BMP2 are nonviable and have defects in amnion/chorion and cardiac development. Development 122:2977–2986.