Transcription factor genes *Smad4* and *Gata4* cooperatively regulate cardiac valve development

Ivan P. Moskowitz[a,1,2], Jun Wang[b], Michael A. Peterson[a], William T. Pu[c], Alexander C. Mackinnon[a], Leif Oxburgh[d], Gerald C. Chu[e], Molly Sarkar[f], Charles Berul[c], Leslie Smoot[c], Elizabeth J. Robertson[g], Robert Schwartz[b], Jonathan G. Seidman[h,1], and Christine E. Seidman[h,i,1,2]

[a]Departments of Pediatrics and Pathology, University of Chicago, Chicago, IL 60637; [b]Institute of Biosciences and Technology, Texas A&M University System Health Science Center, Houston, TX 77030; [c]Department of Cardiology, Children's Hospital Boston, Boston, MA 02115; [d]Maine Medical Center Research Institute, Scarborough, ME 04074; [e]Department of Pathology, Harvard Medical School, Boston, MA 02115; [f]Cardiology Department, Cordis Corporation, Miami Lakes, FL 33014; [g]Wellcome Trust Centre for Human Genetics, Oxford OX3 7BN, United Kingdom; [h]Department of Genetics, Harvard Medical School, Boston, MA 02115; and [i]Howard Hughes Medical Institute, Brigham and Women's Hospital, Boston, MA, 02115

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We report that the dominant human missense mutations G303E and G296S in *GATA4*, a cardiac-specific transcription factor gene, cause atrioventricular septal defects and valve abnormalities by disrupting a signaling cascade involved in endocardial cushion development. These *GATA4* missense mutations, but not a mutation causing secundum atrial septal defects (S52F), demonstrated impaired protein interactions with SMAD4, a transcription factor required for canonical bone morphogenetic protein/transforming growth factor-β (*BMP*/*TGF-β*) signaling. *Gata4* and *Smad4* genetically interact in vivo: atrioventricular septal defects result from endothelial-specific *Gata4* and *Smad4* compound haploinsufficiency. Endothelial-specific knockout of *Smad4* caused an absence of valve-forming activity: *Smad4*-deficient endocardium was associated with acellular endocardial cushions, absent epithelial-to-mesenchymal transformation, reduced endocardial proliferation, and loss of *Id2* expression in valve-forming regions. We show that Gata4 and Smad4 cooperatively activated the *Id2* promoter, that human *GATA4* mutations abrogated this activity, and that *Id2* deficiency in mice could cause atrioventricular septal defects. We suggest that one determinant of the phenotypic spectrum caused by human *GATA4* mutations is differential effects on GATA4/SMAD4 interactions required for endocardial cushion development.

The atrioventricular canal, the valve-forming region between the atria and ventricles, adopts a unique molecular and morphological program during cardiac development that requires coordinated activity of myocardial and endocardial lineages. The cardiac valve anlage is the endocardial cushion, a swelling composed of myocardial-derived extracellular matrix that becomes populated primarily by endocardial-derived mesenchymal cells (1). The development of a mature valve structure from the endocardial cushion requires multiple distinct steps (2), including activation and proliferation of endocardial cells, endothelial-to-mesenchymal transformation (EMT) of activated endocardial cells, and maturation of the cellularized cushions into functional valve leaflets.

Development of the atrioventricular endocardial cushions into the atrioventricular valves (tricuspid and mitral) and adjacent portions of the atrial and ventricular septae requires Tgf-β and Bmp signaling. At least eight Tgf-β or Bmp ligands are expressed during valve formation (3, 4), and gene ablation of individual ligands in model organisms produces phenotypes that range from pronounced valvuloseptal malformations to subtle valve maturation defects (5–18). Compound gene ablations of *Bmp5*/*Bmp7* or *Bmp6*/*Bmp7* produced more severe endocardial cushion defects than either single mutant did (16–18). These observations imply considerable redundancy of Tgf-β/Bmp signaling in the early functions of this pathway in endocardial cushion development.

Human atrioventricular septal defects (AVSDs) are defined by variable abnormalities of the mitral and/or tricuspid valves in conjunction with defects in the adjacent atrial or ventricular septae (19). The abnormalities produce substantial hemodynamic consequences that contribute to poor prognosis in affected patients and a virtually uniform requirement for surgical correction. Human genetic studies have demonstrated that mutations in the gene encoding cardiac transcription factor GATA4 cause familial atrial secundum defects and, less commonly, sporadic AVSDs (20–23).

We evaluated two families with dominant inheritance of AVSDs and identified two *GATA4* missense mutations, G303E and G296S. We investigated the canonical TGF-β/BMP pathway effector SMAD4 and showed that G303E and G296S altered the transcriptional response to TGF-β/BMP activation. Deletion of *Smad4* from the endocardium of mice caused severe maldevelopment of endocardial cushions and diminished expression of the *Id2* gene. Together these studies define a transcriptional network directing endocardial cushion formation involving *Gata4*, *Smad4*, and *Id2*.

Results

Human Mutations in GATA4 Cause Endocardial Cushion Defects. From a cohort of 103 probands with congenital heart defects, we sequenced all protein-encoding exons of candidate genes, including *GATA4*. Two *GATA4* mutations were identified in probands from unrelated families (Fig. 1) with dominantly inherited AVSDs.

Family A had six members with a spectrum of AVSDs (Fig. 1*A*). Sequence analyses of the proband revealed a heterozygous G-to-A substitution at nucleotide 4 in exon 4 of *GATA4*, which was also present in all affected Family A members (Fig. 1*A*), but absent from over 500 ethnically matched controls. This variant encodes replacement of a conserved glycine with glutamate at amino acid position 303 (denoted G303E), just distal to the carboxyl-terminal zinc finger of the transcription factor. We concluded that *GATA4* G303E caused AVSDs in Family A.

Family B had five individuals with AVSDs and four individuals with electrophysiologic abnormalities (Fig. 1*B*). Sequence analyses of the proband revealed a heterozygous G-to-A transition at nucleotide 886 of *GATA4*, which was also present in all affected members of Family B (Fig. 1*B*), but absent from over 500 ethnically matched controls. This variant is predicted to substitute a conserved glycine with serine at amino acid position 296 (denoted G296S), located directly adjacent to the nuclear localization signal and carboxyl-terminal zinc finger. *GATA4* G296S has been previously identified to cause atrial septal defects in three families (21, 24).

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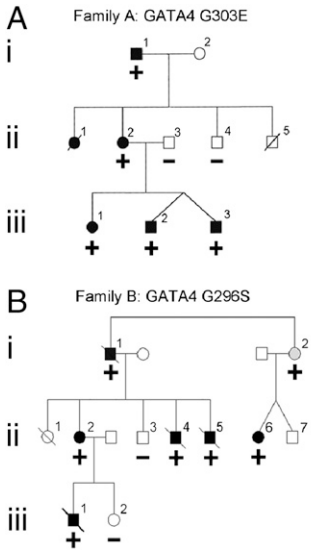
The authors declare no conflict of interest.

[1]I.P.M., J.G.S., and C.E.S. contributed equally to this study.

[2]To whom correspondence may be addressed. E-mail: imoskowitz@peds.bsd.uchicago.edu or cseidman@genetics.med.harvard.edu.



Patient	Congenital Heart Disease	Endocardial Cushion Defect	EP
I-1	VSD, inlet type	Yes	AF/PM
II-1	ASD, primum type Cleft Mitral Valve	Yes Yes	PM
II-2	ASD, primum type Cleft Mitral Valve	Yes Yes	SVT
III-1	ASD, primum type Cleft Mitral Valve	Yes Yes	EAR,LAD
III-2	ASD, secundum type Pulmonary Stenosis, valvar	Yes	SR
III-3	Atrial Aneurysm		SR

Patient	Congenital Heart Disease	Endocardial Cushion Defect	EP
I-1	ASD	?	AF
II-2	ASD, primum type Cleft Mitral Valve	Yes Yes	AF/PM
II-4	ASD, primum type Cleft Mitral Valve	Yes Yes	SR
II-5	Common Atioventricular Canal	Yes	SR
II-6	ASD, primum type Cleft Mitral Valve	Yes Yes	AF/PM
III-1	Common Atioventricular Canal	Yes	SVT/SCD

Fig. 1. *GATA4* mutations cause endocardial cushion defects. (*A*) Family A pedigree with cosegregation of *GATA4* G303E mutation (+) with congenital heart disease (*Left*). Note that five mutation carriers had endocardial cushion defects (*Right*). (*B*) Family B pedigree with cosegregation of *GATA4* G296S mutation (+) with congenital heart disease (*Left*). Note that six mutation carriers had endocardial cushion defects (*Right*). Electrophysiologic (EP) status and events of mutation carriers are indicated: AF, atrial fibrillation; EAR, ectopic atrial rhythm; LAD, left axis deviation, PM, pacemaker; SCD, sudden cardiac death; SR, sinus rhythm, SVT, supraventricular tachyarrhythmia.

***GATA4* Mutations Affect GATA4–SMAD4 Interactions.** Previous work demonstrated binding of the N-terminal portion of Smad4 to the second zinc-finger domain of Gata4 (25). This region of Gata4 (amino acids 248–335) encompasses residues 296 and 303, mutated in families A and B, respectively. Given the roles for Tgf-β/Bmp signaling in atrioventricular development, we asked whether these two GATA4 human mutations altered GATA4–SMAD4 interactions. Protein–protein interactions were analyzed by pulling down ^{35}S-labeled GATA4 protein variants with wild-type SMAD4 (Fig. 2). SMAD4 efficiently precipitated wild-type GATA4. However, SMAD4 interactions with specific GATA4 mutants were greatly reduced. SMAD4 bound GATA4 G296S with 20% efficiency (P = 0.01) and GATA4 G303E with 30% efficiency compared with wild type (P = 0.002) (Fig. 2*A*). In contrast, SMAD4 bound GATA4 S52F, a previously reported *GATA4* mutation that caused dominant secundum atrial septal defects but neither ASVDs nor valve malformations (21), with efficiency comparable to wild-type GATA4 (P = 0.15) (Fig. 2*A*). We concluded that the variable clinical consequences of *GATA4* mutations reflected differences in GATA4–SMAD4 interactions.

***Smad4* and *Gata4* Genetically Interact in the Endocardium.** We tested whether *Smad4* and *Gata4* interact genetically in the context of endocardial cushion development in vivo. We used *Tie2:Cre* (26) to generate endocardial-specific knockouts using floxed conditional alleles of *Smad4* (*Smad4^{fl}*) (27) and *Gata4* (*Gata4^{fl}*) (28). Atrioventricular canal morphology of mice heterozygous for endocardial *Gata4*, *Smad4*, or both was analyzed at embryonic day 14.5 (E14.5). Animals heterozygous for either *Gata4* (*Tie2:Cre^{+}; Gata4$^{fl/+}$*) (5/5) or *Smad4* (*Tie2:Cre^{+}; Smad4$^{fl/+}$*) (5/5) in the endocardium were viable and displayed normal atrioventricular canal morphology in each case (Fig. 2 *D* and *E*). In contrast, double heterozygous *Gata4*; *Smad4* embryos (*Tie2:Cre^{+}; Gata4$^{fl/+}$; Smad4$^{fl/+}$*) displayed severe atrioventricular defects

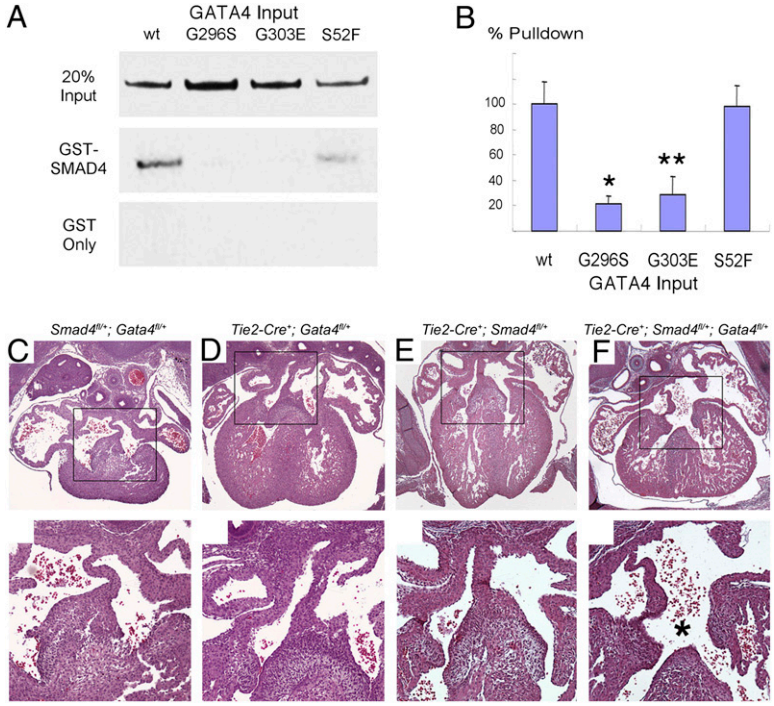


Fig. 2. *GATA4* mutations cause disrupted GATA4–SMAD4 interactions. (*A*) Autoradiograms of GST-pulldown assays showed binding of wild-type and mutant GATA4 (G296S, G303E, and S52F) to SMAD4. Twenty percent of the in vitro-translated ^{35}S methinonine-labeled mouse GATA4 protein used for binding assay (input) and the remaining GATA4 protein after precipitation by GST-SMAD4 are shown. (*B*) Quantification of the average of five independent GST pull-down assays. GATA4 protein pulled down was normalized to the relative input. *GATA4* G296S and G303E diminished the GATA4–SMAD4 interaction (*P = 0.01; **P = 0.002), whereas the GATA4 mutation S52F did not (P = 0.15). (*C–F*) Endocardial haploinsufficiency of *Gata4* and *Smad4* causes atrioventricular canal defects. Transverse sections of *Gata4$^{fl/+}$; Smad4$^{fl/+}$* (*C*), *Tie2:Cre^{+}; Gata4$^{fl/+}$* (*D*), *Tie2:Cre^{+}; Smad4$^{fl/+}$* (*E*), and *Tie2:Cre^{+}; Gata4$^{fl/+}$; Smad4$^{fl/+}$* (*F*) E14.5 hearts. (Magnification in *C–F*: 40×; *G–J*: 100×.) Atrioventricular septal defects [in *F*, boxed area (*Upper*) and asterisk (*Lower*)] can be seen in the double heterozygous *Tie2:Cre^{+}; Gata4$^{fl/+}$; Smad4$^{fl/+}$* heart. In contrast, *Tie2:Cre^{+}; Gata4$^{fl/+}$* or *Tie2:Cre^{+}; Smad4$^{fl/+}$* heterozygous animals show normal atrioventricular canal morphology (*C–E*).

(5/5) and died by E12.5 (Fig. 2*F*). From these results we deduced that *Gata4* and *Smad4* genetically cooperate in the endocardium during atrioventricular valve formation.

Endocardial *Smad4* Is Required for Endocardial Cushion Development. To further explore the consequences of endocardial *Smad4* deficiency on atrioventricular valve formation, we generated endocardial *Smad4* knockout embryos (*Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$). The developing atrioventricular endocardial cushions of *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ mutant embryos were entirely acellular at E10.5 (Fig. 3 *B* and *D*). In contrast, the developing atrioventricular endocardial cushions of wild-type embryos were densely populated with mesenchymal cells (Fig. 3 *A* and *C*). The outflow tract cushions of *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ embryos were also acellular (Fig. 3 *F* and *H*), in contrast to the well-populated outflow tract cushions of wild-type embryos at E10.5 (Fig. 3 *E* and *G*). The endocardial cushion structures of the atrioventricular canal and outflow tract were of normal size in both wild-type and *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ embryos (Fig. 3 *A–H*). Furthermore, the myocardium of the atrioventricular canal and the cardiac chambers appeared morphologically normal in both wild-type and *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ embryos (Fig. 3 *A–H*). We concluded that endocardial expression of *Smad4* is required to promote normal cellularity in the developing endocardial cushion.

We asked whether loss of *Smad4* from the atrioventricular endocardium altered the expression of molecular regulators implicated in valve morphogenesis, which included *Gata4*, *Notch1*, and *Tbx2* (34, 40, 43–45). In situ hybridization of atrioventricular canal sections from wild-type and *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ embryos showed comparable pattern and intensity of expression for *Gata4* (Fig. 3 *I* vs. *J*), *Notch1* (Fig. 3 *K* vs. *L*), and *Tbx2* (Fig. 3 *M* vs. *N*) at E10.5.

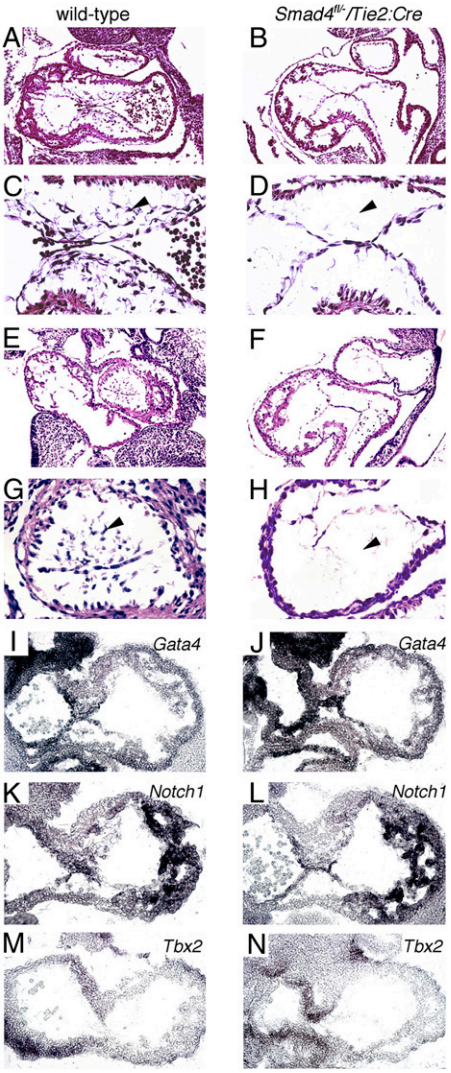


Fig. 3. Endocardial loss of *Smad4* causes acellular endocardial cushions. Parasagital heart sections showing the atrioventricular canal (*A–D*) and outflow tract (*E–H*) endocardial cushions from wild-type (*A*, *C*, *E*, and *G*) and *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ (*B*, *D*, *F*, and *H*) embryos at E10.5. *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ atrioventricular and outflow endocardial cushions are acellular (black arrowheads in *D* and *H*) compared with wild type (black arrowheads in *C* and *G*). In situ hybridization of sections from wild-type (*A*, *C*, *E*, *G*, *I*, and *K*) and *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ (*B*, *D*, *F*, *H*, *J*, and *L*) embryos at E10.5. The patterns of expression for *Gata4* (*I* and *J*), *Notch1* (*K* and *L*), and *Tbx2* (*M* and *N*) were comparable in wild-type and *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ mutant embryos. (Magnification in *A*, *B*, *E*, *F*, *I*, *J*, *K*, *L*, *M*, and *N*: 40×; *C*, *D*, *G*, and *H*: 100×.)

***Smad4* Is Necessary for Endocardial Cushion EMT.** We next studied the transformation of endothelial cells to mesenchymal cells (EMT) in the endocardial cushions during valve morphogenesis using a collagen gel culture system (29). Wild-type endocardial cells from atrioventricular canal explants became motile and invaded the collagen matrix (Fig. 4*A*). *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$en-

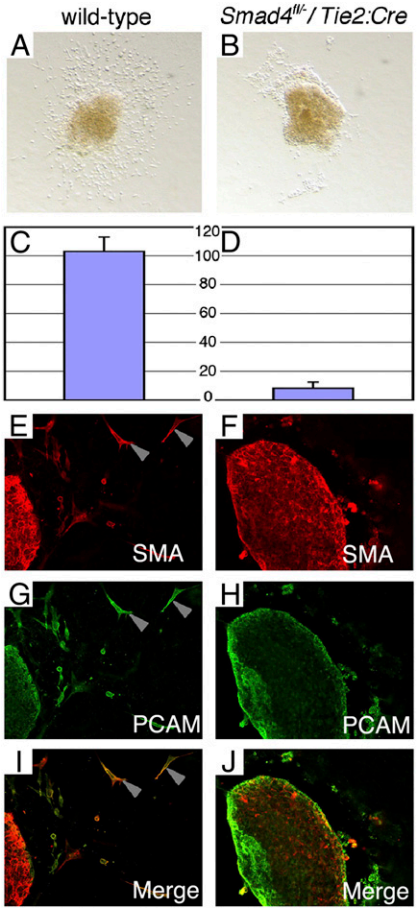


Fig. 4. Endocardial *Smad4* is required for EMT in the endocardial cushion. (*A–J*) Atrioventricular cushion explants from wild-type (*A*, *C*, *E*, *G*, and *I*) and *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ (*B*, *D*, *F*, *H*, and *J*) embryos. Mesenchymal cells were abundant in wild-type explants (*A*) but rare in mutant explants (*B*). Average numbers of invasive mesenchymal cells in wild-type explants (*C*: 102.8 ± 10.2 cells) was significantly greater ($P = 3.8 \times 10^{-7}$) than in mutant explants (*D*: 8.2 ± 4.4)($n = 5$ each). Mesenchymal cells from control explants expressed Pecam (*G* and *I*: gray arrowheads), consistent with endocardial origin, and Sma (*E* and *I*; gray arrowheads), indicative of a mesenchymal fate. Cells from mutant explants did not express Sma (*F* and *J*).

docardial cells from atrioventricular canal explants showed markedly reduced migration and invasion (Fig. 4*B*). The numbers of mutant cells entering the collagen matrix (8.2 ± 4.4) were markedly less than in wild-type atrioventricular explants (102.8 ± 10.2; $P = 3.8 \times 10^{-7}$) (Fig. 4 *C* vs. *D*).

We further characterized EMT in mutant and wild-type explants using immunohistochemistry. Endocardial cells express the surface cell adhesion protein Pecam, whereas mesenchymal cells express smooth muscle actin (Sma). Coexpression of both Pecam and Sma in endocardial cells is indicative of EMT. Cells surrounding the atrioventricular explants from both wild-type and *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ embryos demonstrated Pecam expression, consistent with an intact endothelium in vivo (Fig. 4 *G* and *H*). Whereas abundant Sma-positive cells were found in wild-type atrioventricular canal explants (Fig. 4*E*), few if any Sma-positive cells were found in explants from *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ embryos (Fig. 4*F*). Cells that coexpressed Sma and Pecam were abundant in the wild-type explants (Fig. 4*I*) but absent from *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ explants (Fig. 4*J*). This result demonstrated failed EMT in *Smad4*-deficient endocardial cells.

Quiescent Endocardial Cushion Endocardium in *Smad4* Mutant Embryos. To assess for early morphological correlates of endocardial cell activation and EMT, endocardial *Smad4*-deficient and wild-type endocardial cells were evaluated by transmission electron microscopy. At E9.5, many wild-type endocardial cells were rounded, demonstrated filopodia and pseudopodia, and had lost cell–cell contacts (tight junctions) (Fig. 5*B*). In contrast, *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ endocardial cells showed intact tight junctions without filopodia or pseudopodia (Fig. 5*D*). The morphology of *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ endocardial cells was similar to the ventricular chamber endocardial cells from wild-type embryos (Fig. 5 *D* vs. *A*), which do not undergo EMT or participate in valve formation.

***Smad4* Is Required for Proliferation of Endocardial Cushion Endocardium.** The quiescent cellular morphology of endocardial cells in *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ embryos implied an early defect in the EMT sequence. To determine whether *Smad4* was required for endocardial proliferation, we labeled embryos with BrdU at E9.5 and stained surface endocardium with anti-BrdU antibodies at E10.5 to identify proliferating cells. Actively proliferating cells at the surface of the endocardium were counted from four sections per embryo; proliferating cells undergoing EMT, present only in wild-type embryos, were specifically excluded. Surface atrioventricular canal endocardial cells of wild-type embryos had significantly more BrdU-labeled nuclei (3.24 ± 1.4; Fig. 6 *A*, *C*, and *E*) than those of *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ embryos (1.04 ± 0.7, $P = 1.8 \times 10^{-5}$; Fig. 6 *B*, *D*, and *F*). We concluded that endocardial expression of *Smad4* is required to promote proliferation during the activation process of the pre-EMT endocardial cell.

***Id2* Is Required for Atrioventricular Valve Morphogenesis.** *Id2*, a member of a gene family encoding helix–loop–helix-containing transcriptional repressors (*Id1–Id4*), is a target of Tgf-β/Bmp signaling (30) and has been implicated in endocardial cushion development (31). Although no structural cardiovascular phenotype has been reported in *Id2* knockout mice, we observed over 20% perinatal lethality of *Id2*$^{-/-}$ mutant newborn mice in the C57BL/6 genetic background (8 newborns with perinatal lethality among 36 sequential live-born *Id2*$^{-/-}$ pups). We found AVSDs and membranous ventricular septal defects in each *Id2*$^{-/-}$ perinatal lethal ($n = 5$) (Fig. 7*B*). In contrast, normal cardiac morphology with intact atrioventricular septation was observed in littermate-surviving *Id2*$^{-/-}$ mice ($n = 8$; $P = 0.02$; Fig. 7*A*) and in wild-type littermate controls ($n = 12$; $P = 0.01$). Immunohistochemistry at E14.5 demonstrated Id2 protein expression in the developing endocardial cushions, but not in the myocardium (Fig. 7*C*). *Id2* expression was also detected by in situ hybridization in surface endocardium of the developing endocardial cushions at E10.5 (Fig. 7*E*). In contrast, expression of *Id2* was substantially reduced in *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ embryos on the surface endocardium of the atrioventricular canal (Fig. 7*F*).

Smad4 and Gata4 Cooperatively Activate the *Id2* Promoter. We evaluated transcriptional regulation of *Id2* by *Gata4* and *Smad4*.

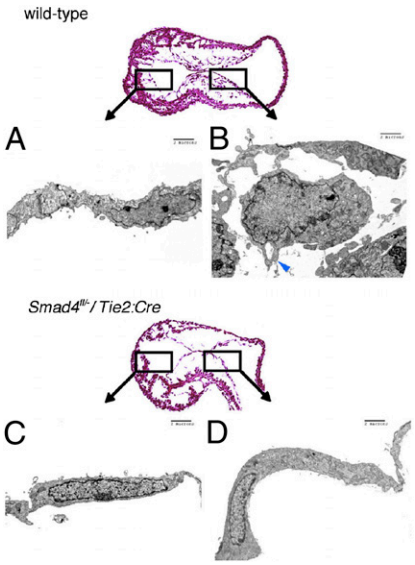


Fig. 5. Quiescent endocardium in endocardial-deficient *Smad4* embryos. Transmission electron microscopy of E9.5 wild-type (*A* and *B*) or *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ (*C* and *D*) endocardium. Wild-type atrioventricular canal endocardial cells (*B*) were rounded with pseudopodia and filopodia (*B*: blue arrowhead). *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ atrioventricular canal endocardial cells (*D*) demonstrated a thin cellular profile, maintenance of cellular junctions, and absence of pseudopodia and filipodia, similar to wild-type unactivated ventricular endocardium (*A* and *C*).

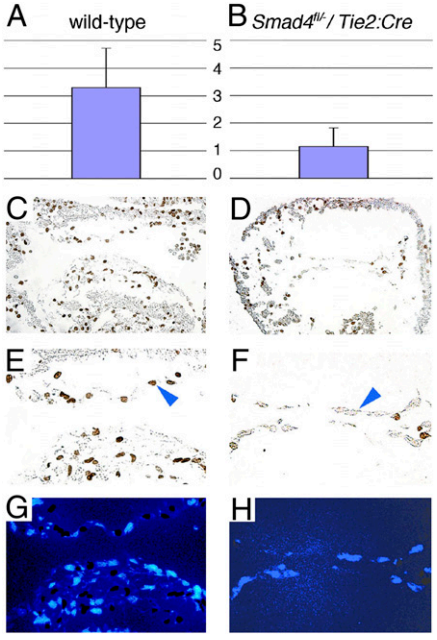


Fig. 6. Endocardial proliferation defect in endocardial-deficient *Smad4* embryos. (*A* and *B*) Significantly more surface endocardial cell proliferation in wild type (*A*: 3.24 ± 1.4) than in *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ (*B*: 1.04 ± 0.7) embryos at E10.5 ($P = 1.8 \times 10^{-5}$). (*C–H*) Parasagital heart sections from wild-type (*C*, *E*, and *G*) and *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ (*D*, *F*, and *H*) embryos treated with BrdU at E9.5 and stained for BrdU uptake (*C–F*) or DAPI (*G* and *H*) at E10.5.

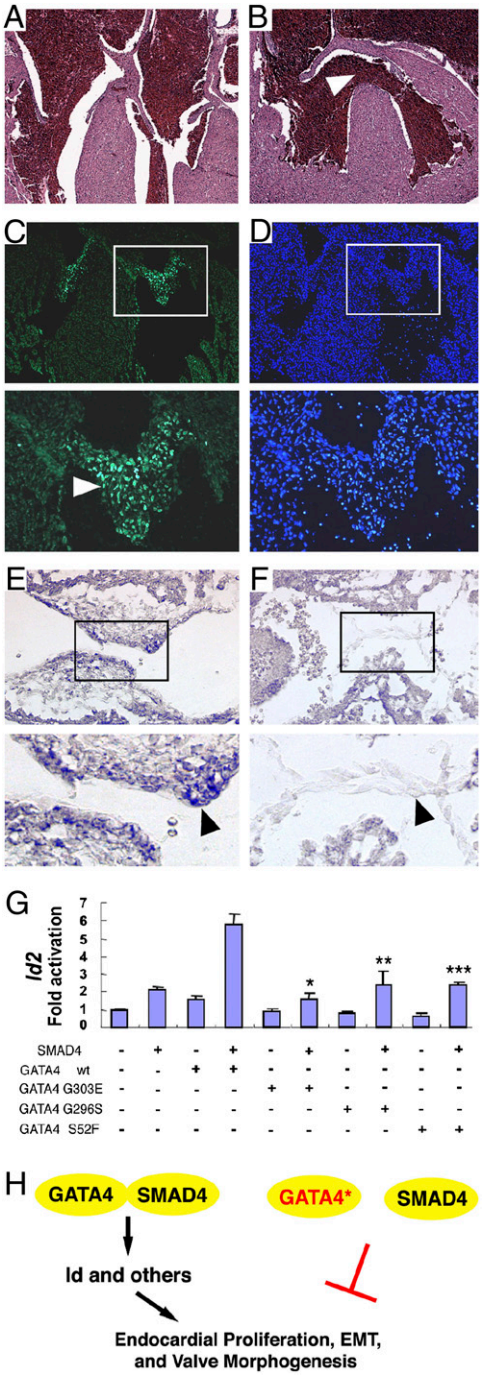


Fig. 7. *Id2* is a target of *Smad4* and *Gata4* required for atrioventricular septation. (*A* and *B*) *Id2*$^{-/-}$ mice with perinatal lethality had atrioventricular septal defects (*B*: membranous ventricular septal defects) in contrast to surviving *Id2*$^{-/-}$ mice (*A*: normal atrioventricular septation). (Magnification: 40×.) (*C* and *D*) Id2 immunohistochemistry demonstrated expression in the endocardial cushion endocardium and mesenchyme (*C*: white arrowhead) at E14.5. (*Right* panels in *C* and *D*) DAPI staining. (Magnification in *C* and *D*: *Upper* panels—40×; *Lower* panels—100×.) (*E* and *F*) *Id2* expression is decreased in *Tie2:Cre*$^{+}$*; Smad4*$^{fl/-}$ (*F*: black arrowhead) compared with wild-type endocardium (*E*: black arrowhead) at E10.5. (Magnification in *E* and *F*: *Upper* panels—40×; *Lower* panels—100×. (*G*) Cooperative activation of a 1052-bp fragment of the *Id2* promoter by *Smad4* and *Gata4*. Luciferase constructs were transfected alone or with *Smad4* or *Gata4* expression vectors, singly and in combination. Mutant *Gata4* constructs abrogated cooperative luciferase activity of *Smad4* and *Gata4*. Compared with wild-type *Smad4-Gata4*: **Smad4-Gata4*G303E, $P = 0.001$; ***Smad4-Gata4*G296S, $P = 0.02$; ****Smad4-Gata4*S52F, $P = 0.002$. (*H*) GATA4–SMAD4 interaction is required for *Id2* expression and cardiac valve development. Congenital heart disease-causing GATA4 mutations abrogate interaction with SMAD4, resulting in failed valve development.

We assessed expression of the firefly *luciferase* reporter gene under control of a 1052-bp fragment from the *Id2* promoter (from nucleotide +1584 to +532) containing three putative *Smad4*-binding sites (nucleotide positions 630–633, 977–980, and 1133–1136) and a putative *Gata4*-binding site (nucleotide positions 838–841). Compared with empty vector, transfection of CV-1 cells with either wild-type *Smad4* or wild-type *Gata4* expression constructs produced twofold activation of the *Id2* promoter fragment (Fig. 7*G*). In contrast, cotransfection with both *Smad4* and *Gata4* produced sixfold cooperative transcriptional activation of the *Id2* promoter fragment (Fig. 7*G*). Cotransfection with *Smad4* and mutant *Gata4* eliminated the cooperative activation (compared with wild-type *Gata4-Smad4*: *Gata4*G303E-*Smad4*, $P = 0.001$; *Gata4*G296S-*Smad4*, $P = 0.02$; *Gata4*S52F-*Smad4*, $P = 0.002$). These findings demonstrate that Gata4 mutations Gata4 G296S, Gata4 G303E, or GataS52F cause a loss of *Smad4*-dependent cooperative activation of the *Id2* promoter.

Discussion

Role of *Smad4* in Atrioventricular Canal Development. Endocardial cushion development requires a series of events within endothelial cells to enable EMT, including proliferation and the acquisition of morphological and molecular features of mesenchymal cells (1). Removal of *Smad4* from the endocardium resulted in defects in each aspect of the endocardial activation sequence required for EMT. *Smad4*-deficient endocardial cells failed to proliferate (Fig. 6) or undergo EMT (Fig. 4), retained morphological features of unactivated ventricular endocardium (Fig. 5), and showed decreased *Id2* gene expression (Fig. 7). Furthermore, loss of *Id2* alone was sufficient to cause AVSDs in some cases (Fig. 7). Gata4 and Smad4 cooperatively activated the *Id2* promoter in vitro, and *Gata4* mutations abrogated this cooperated activation (Fig. 7). Collectively, these data define a signaling network comprising endocardial Gata4, Smad4, and Id2 that regulates cardiac cushion development and is disrupted by human *GATA4* mutations.

GATA4 and Human Congenital Heart Disease. Gata4 associates with a variety of other transcriptional regulators, including Smad4, Nkx2-5, Tbx5, and Fog2 (21, 25, 32), implying that lineage-specific activity may be contextual and achieved through formation of specific combinatorial transcriptional complexes. Our data indicating that Smad4-dependent signaling in the endocardium was modulated by *Gata4–Smad4* interactions supports this model. Smad4 may preferentially recruit Gata4 to endocardial enhancers, given its selective requirement in this lineage during atrioventricular canal development, and the differential clinical expression of GATA4 mutations provides a biological context for the importance of Gata4 and Smad4 as a transcriptional complex. *GATA4* mutations produce an array of cardiovascular malformations that range from secundum ASDs (21, 33) to profound defects in endocardial cushion derivatives, including complete atrioventricular canal and electrophysiologic abnormalities (Fig. 1). Our data suggest that the lineage-specific impact of mutations on GATA4 function contributes to phenotype heterogeneity.

A role for *Gata4* in transcriptional control of the conduction system is emerging (34). Given the electrophysiologic abnormalities observed in patients with *GATA4* mutations G303E and G296S but not with other reported *GATA4* mutations (24), we hypothesize that there are lineage-specific effects by Gata4 in conducting myocytes. Further studies will reveal whether Gata4 interacts with Tbx5 and Nkx2-5 (33) to regulate conduction system-specific transcription, including activation of *Id2*, participating in a described network that promotes normal structure and function of the cardiac conduction system (35). Because *GATA4* mutations G303E and G296S but not S52F produced electrophysiologic abnormalities, we speculate that *GATA4* may

have SMAD-dependent functions in the cardiac conduction system as well as in the endocardial cushions.

The G303E and G296S mutations, in contrast to S52F, not only reduced *GATA4* promoter activation but also attenuated SMAD4 interactions (Fig. 2*A*). Within the endocardial cushion, loss of GATA4–SMAD4 interactions due to specific mutations could reduce the responses to TGF-β/BMP or other developmental cues, altering differentiation and/or proliferation (36, 37). Given the requirement of endothelial-SMAD4 signaling for atrioventricular valve formation, we hypothesize that GATA4 mutations produced either a restricted constellation of congenital heart defects, predominantly secundum ASDs or more profound AVSDs with electrophysiologic abnormalities, depending on whether endocardial SMAD4-GATA4 signaling is preserved during heart development.

Materials and Methods

Human Subjects. Studies were performed in accordance with institutional guidelines for human research and written informed consent was obtained from all participants.

Animal Handling. Mice were analyzed as 129 SV inbred and mixed 129 SV/C57BL/6 genetic backgrounds. All protocols conformed to the Association for the Assessment and Accreditation of Laboratory Animal Care and the Harvard Medical School Animal Care and Use Committee.

Gene Expression Studies. Mouse heart in situ hybridizations were performed as previously described (34). Id1, Id2, and Id3 probe templates were made from plasmid accession numbers BE945568, AI843393, and AI839283, respectively, from the Brain Molecular Anatomy Project library (38).

Plasmid Constructs. The constructions of Nkx2-5 promoter-driven luciferase reporter and Id2 promoter-driven luciferase reporter, respectively, were described (35, 39, 40). Bacterial and mammalian SMAD4 expression vectors were detailed previously (25). All GATA mutants S52F, G296S, and G303E were generated by a two-step PCR mutagenesis protocol as described (40, 41).

GST-Pulldown Assays. GST pulldown assay was performed as described (38, 39).

Cell Culture and Transfection. Cultured CV-1 cells were maintained in DMEM plus 10% FBS. Transient transfections of plasmid-based expression vectors were performed using Lipofectamine 2000 based on the protocol provided by the manufacturer. Reporter assays were performed in 12-well per plate containers with reporter construct of 200 ng per well and expression vectors as noted and using empty expression vectors to maintain the total amount of DNA constant per well. Promoter activity was expressed as the ratio of luciferase activity induced by the presence of specific factor(s) in comparison with the control group usually in the presence of empty vector or as otherwise stated. Data are expressed as the mean ± SD from at least two independent assays performed in duplicate.

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