

# T-box 2, a mediator of Bmp-Smad signaling, induced hyaluronan synthase 2 and Tgf $\beta$ 2 expression and endocardial cushion formation

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During early heart development, *Tbx2* gene expression is initiated in the cardiac crescent and then becomes restricted to the outflow tract and the atrioventricular region. We identified a *Tbx2* regulatory region, enriched in multiple Smad sites, sufficient to reproduce *Tbx2* expression patterns overlapping *Bmp2* and *Bmp4* gene activity in the heart. The role of *Tbx2* in cardiogenesis was analyzed by using Cre-LoxP activated *Tbx2* transgenic misexpression in chamber myocardium. Ventricular *Tbx2* misexpression exhibited an abnormally narrow chamber lumen owing to the expansion of *Hyaluronan synthase 2* expression in the ECM or cardiac jelly and the appearance of the endocardial cushions (ECs). Excessive *Tbx2* also induced *Tgf $\beta$ 2*, which coincided with the outgrowth epithelial-mesenchymal transformed cells in ventricular and atrial tissues modifying cardiomyocyte identity from chamber type to non-chamber type. *Tbx2*, a central intermediary of Bmp-Smad signaling, has a central part in directing *Has2* and *Tgf $\beta$ 2* expression, facilitating EC formation.

cardiac jelly | cardiomyocyte identity | epithelial-mesenchymal transformation | extracellular matrix | misexpression

The heart develops, as a modular organ, driven by distinct transcriptional regulatory programs that control each anatomical region (1). A member of the T-box factor family, *Tbx2*, which first appears in the cardiac crescent and then later restricted to non-chamber myocardium (My) [outflow tract (OFT), atrioventricular canal (AVC), inner curvature, and inflow tract] (2, 3), is a valuable model of modular cardiac gene activity. *Tbx2* is central for endocardial cushion (EC) formation and chamber specification, and may be a transcriptional repressor (4, 5). Expression of chamber-specific myocardial genes, which include *Nppa* (encoding atrial natriuretic factor, ANF), *Gja5* (encoding connexin 40, *Cx40*), and *Gja1* (encoding connexin 43, *Cx43*), were repressed by *Tbx2* (3–5). *Tbx2* null mutant embryos exhibited small AVC and defective OFT septation (3), whereas *Tbx2* transgenic expression blocked chamber formation (4) and cell proliferation in the OFT and AVC (6).

The ECs form from localized expansion of the ECM also named cardiac jelly (7, 8) found in the cardiac OFT and AVC segments the simple heart tube into a complicated structure composed of the aortic sac, common ventricular chamber, and atrial chamber. Some endocardial cells invade into the ECM through epithelial-mesenchymal transformation (EMT) to remodel the cushion tissue into the mature valves. Several signaling pathways have been implicated in EC formation. The Bmp pathway is essential for both processes; expansion of ECM and EMT in the EC formation (9–14). Tgf $\beta$ 2 performs crucial and sequential roles in EC formation and may also be regulated by Bmp2/4 during cardiogenesis (9–11). The hyaluronan (HA) synthase 2 (*Has2*) has been recently shown to have essential role

in expansion of ECM and EMT (16). Also, *Tbx2* may be a direct target of Bmp2/4 signaling pathway during EC formation (2).

Here, we delineated an 80-bp regulatory region within the *Tbx2* 5' flanking sequences, which contain multiple Smad DNA binding sites that recapitulate expression of *Tbx2* in the AVC and OFT. Previously, myocardial-specific inactivation of *Bmp2* also inhibited the appearance of several factors, including *Tbx2*, *Tgf $\beta$ 2*, and *Has2*, and blocked cushion formation (9). To define the regulatory hierarchy shared by Bmp2/4-dependent genes, we analyzed embryos in which murine *Tbx2* was misexpressed in the developing chamber My, using a mouse genetic system based on Cre/loxP recombination. *Tbx2* altered cardiogenic lineage specification by expanding the ECM and EMT to drive EC formation via the induction of Tgf $\beta$ 2 and *Has2* gene activity in embryonic hearts.

## Results

### Smad Signaling Drives *Tbx2* Transgene Activity via a Distal Enhancer.

*Tbx2* expression was first detected in the cardiac crescent and notochordal plate (Fig. 1A). At E8.5, *Tbx2* expression was maintained only in the posterior portion of the looping heart (Fig. 1B). As the heart matured, the expression of *Tbx2* became further limited to the AVC and OFT region (Fig. 1E and F). *Tbx2* mRNA was also detected in the optic cups, otic vesicles, pharyngeal arches, and limb buds of embryonic day (E)9.5 and E10.5 mouse embryos (Fig. 1C and D). Transient F<sub>0</sub> transgenic mice harboring 4.1 kilobase (kb) of *Tbx2* 5' flanking sequences linked to the *lacZ* reporter gene (Fig. 1K) revealed  $\beta$ -galactosidase activity in the OFT, AVC, and a portion of the left atrium, whereas LacZ activity was absent from the right atrium and the ventricles (Fig. 1L). Also, *Tbx2*, a downstream target of murine Bmp2/4 signaling (2, 9), was colocalized to the AVC and OFT (Fig. 1G–J). After serial and gap deletion mutagenesis strategy, recapitulated *Tbx2* expression was delineated to a region between –3.4 and –2.6 kb in transgenic mouse embryos (Fig. 1L–O).

Paired Smad 1/4 proteins, primary intracellular mediators of Bmp signals (12), activated transcription of the –4.1-kb reporter construct (Fig. 2A). Other Smad factor combinations did not strongly activate the *Tbx2* reporter, whereas the inhibitory Smad 6 (13, 14) blocked *Tbx2* gene activity. *Tbx2* regulatory region

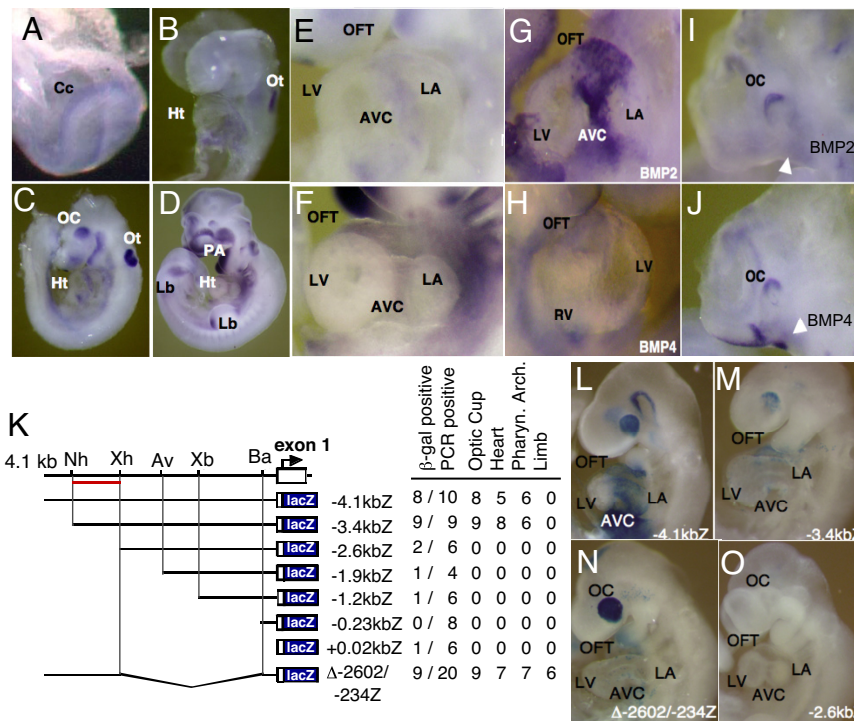
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**Fig. 1.** Mouse *Tbx2* gene activity in early cardiac development was recapitulated by distal *cis*-acting regulatory regions coincided with *Bmp2* and *Bmp4* expression. *Tbx2* expression was first detected in the cardiac crescent at E7.5 (A), and restricted to the posterior part of looping heart at E8.5 (B). The expression of *Tbx2* was also observed in the developing eye, otic cup, pharyngeal arches, and limb buds (B–D). At E9.5 and E10.5, *Tbx2* transcripts were localized in the OFT and AVC (E and F). Expression of *Bmp2* and *Bmp4* (G and H) was observed in the OFT and AVC (I and J). Schematic representation of serial deleted 5' flanking sequences of the *Tbx2* gene, which were analyzed for transgene expression patterns in E9.5–E10.5 F<sub>0</sub> founder embryos (K). An upstream regulatory region that directs *Tbx2* gene expression was indicated by a red bar. *LacZ* was expressed in the optic cup, heart, and pharyngeal arches using 4.1 kb (L), 3.4 kb (M), and  $\Delta$ -2602/–234 (N) 5' flanking fragments. No *LacZ* staining was observed in –2.6-kbZ transgenic embryos (O). Cc, cardiac crescent; Ht, heart; LA, left atrium; Lb, limb bud; LV, left ventricle; OC, optic cup; Ot, otic cup; PA, pharyngeal arch. Restriction sites shown above are: Nh, NheI; Xh, XhoI; Av, AvrII; Xb, XbaI; and Ba, BamHI.

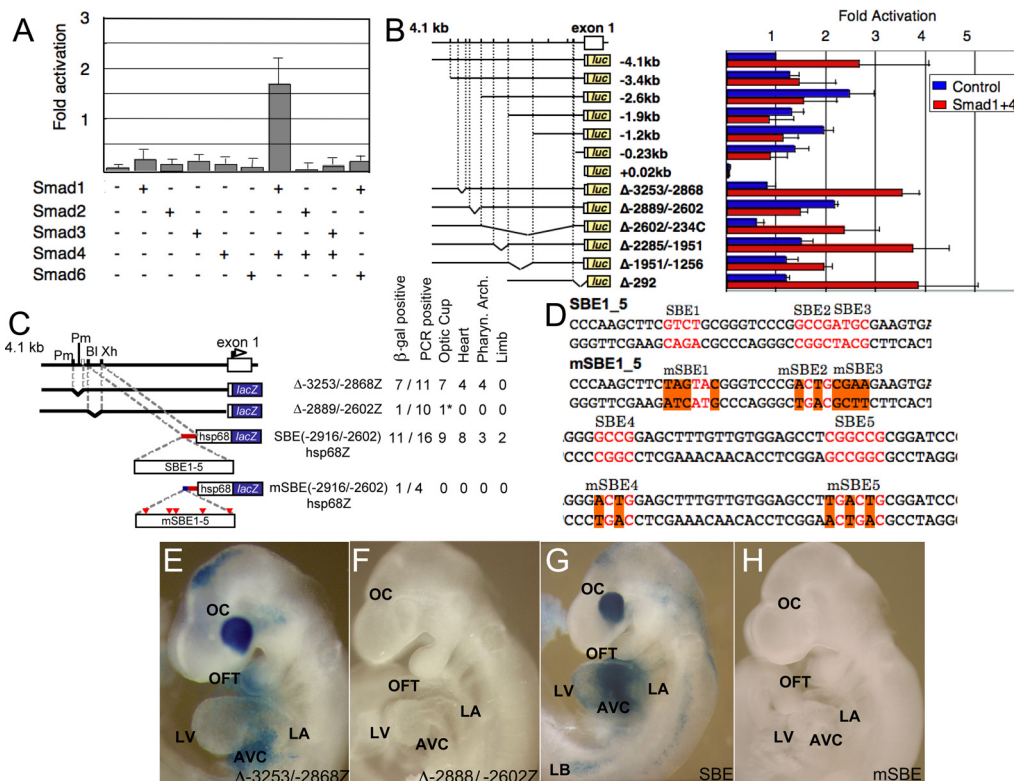
responsive to Bmp signaling was localized to a 290-bp region expression in the developing heart (Fig. 2 B and C), which contains at least five conserved Smad sites, two of which, SBE1 and SBE5 sequences, were potent Smad1/4 cofactor binding sites (Fig. S1). Schematic representation of *Tbx2* transgenes analyzed in E9.5 F<sub>0</sub> embryos and a summary of the tissue restricted expression activity is shown in Fig. 2C, whereas five SMAD sites in SBE1-5 were mutated by site directed mutagenesis as shown in mSBE1-5 (Fig. 2D). The gap deletion mutant  $\Delta$ -2899/–2602 hsp68lacZ construction, in which the five Smad sites were removed from the *Tbx2* 5' flanking sequence, showed a complete loss of *lacZ* expression activity in the hearts of F<sub>0</sub> transgenic embryos (Fig. 2 C and F). The Smad site enriched region (SBE; –2916/–2602) linked to a minimal *hsp68* promoter *lacZ* transgene, revealed robust expression in the OFT and AVC, sufficient to recapitulate the restricted *Tbx2* expression pattern in the heart (Fig. 2 C and G), whereas site directed Smad site mutations eliminated gene activity in transgenic mice (Fig. 2 C and H).

**Abnormal Deposition of ECM in *mTbx2*-Misexpressing Embryonic Hearts.** To study the role of *Tbx2* in the cardiac morphogenesis, we generated transgenic mice that conditionally misexpressed murine *Tbx2* gene in embryonic chamber-cardiomyocytes (Fig. 3 A and B; Fig. S2). Activated *Tbx2* embryos exhibited enlarged hearts, with marked myocardial hypoplasia associated with rich deposition of ECM in the compact and trabecular My (Fig. 3 C and D). Expansion of the EC (cardiac jelly) between the endocardium (En) and My (Fig. 3E) stained with alcian blue for acidic glycosaminoglycans (15) caused a narrow ventricular lumen. In control littermates, acidic glycosaminoglycans were

deposited mainly in the ECM of OFT and AVC regions at E10.5 compared with the expanded ECM induced by activated *Tbx2*.

Glycosaminoglycan HA, a major constituent of the cardiac jelly, may be required for the expansion of EC (16,17). Excessive acidic glycosaminoglycans deposited through the cardiac tube in *mTbx2*-misexpressing embryos were also revealed by deposition of HA with the biotinylated-HA binding protein (BP; Fig. 3F). The specific binding of HABP to HA was eliminated by hyaluronidase treatment (Fig. S3). HA deposition was observed between the En and My in the inner curvature and the AVC of both control littermates and *mTbx2*-misexpressed mice, and in between the En and My of the outer curvature of *mTbx2*-misexpressed ventricles (Fig. 3F). Also, cultured cardiomyocytes from quartered hearts identified by *lacZ* and or by *Myh6* induced *mTbx2* expression (Fig. 3G; Fig. S4) were classified by the amount of HA secretion (Fig. 3H). *Tbx2*-misexpressing ventricular myocytes secreted greater amount of HA compared with ventricular myocytes from control littermates.

***Has2* and *Tgfb2* are *Tbx2* Downstream Gene Targets.** Is there a hierarchical relationship between *Tbx2*, *Has2*, and *Tgfb2*? HA synthetase (*Has2*), the major enzyme responsible for HA synthesis in the AVC and atria, was strongly up-regulated in the ventricular My by *mTbx2* misexpression (Fig. 4). In comparison, other components of the ECM, including *Colla1* (encoding type I collagen), *Cspg2* (encoding chondroitin sulfate proteoglycan 2, versican), *Fnl1* (encoding fibronectin 1), and *Tnc* (encoding tenascin C) did not show obvious differences (data not shown). *Has2* is a direct *Tbx2* target, because T-box binding sites were conserved between *Has2* promoter regions, which recruited en-



**Fig. 2.** Delineation of the distal Smad factor dependent *Tbx2* enhancer. (A) Multiple Smad expression vectors transfected into CV1 cells revealed activation caused by Smad1/4 of the 4.1 kb of 5' flanking sequence of *Tbx2* fused in-frame to the luciferase. The bars represent the average of three independent transfections, and the error bars represent the SE of corrected luciferase activity relative to the pCMV5 control vector. (B) Activation by Smad1/4 required the -2889/-2602 region. (C) Schematic representation of *Tbx2* transgenes analyzed in E9.5 F<sub>0</sub> embryos and a summary of the tissue restricted expression activity. (D) Five SMAD sites in SBE1-5 were mutated by site directed mutagenesis in mSBE1-5. (E-J) *LacZ* expression patterns in E9.5 F<sub>0</sub> embryos. (E) Δ-3253/-2868Z hsp68Z transgene; a gap deletion mutant, of a region immediately upstream of the multiSmad sites. (F) Δ-2889/-2602 hsp68Z lacZ; a gap deletion mutant in which the multiple Smad sites were removed from the 5' flanking sequence. (G) SBE(-2916/-2602)hsp68Z lacZ; the region from -2916 to -2602bp, containing five Smad sites, was linked to the hsp68Z lacZ reporter gene. (H) mSBE(-2916/-2602)hsp68Z lacZ; mutations were inserted at the multiple Smad sites in the -2916/-2602 fragment. *LacZ* expression were recapitulated by the DNA fragments containing Smad binding sites (E and G), whereas gap deletion and point mutagenesis of Smad binding sites eliminated gene activity in transgenic mice (F and H). Restriction sites are: Bpl; Xh, XhoI.

ogenous *Tbx2* from cardiac nuclear chromatin extracts and was transactivated by *Tbx2* (SI Materials and Methods and Fig. S5).

*Tgfb2* is normally expressed in the myocardial cells in the OFT and AVC, but was induced by *Myh6-Cre* activated *mTbx2* in ventricular and atrial My (Fig. 5A and B). Also, phosphorylated Smad2 appeared in the endocardial and myocardial cells of *mTbx2*-activated ventricles and atria (Fig. 5C), which coincided with robust in vitro EMT assays (Fig. 5D and E); thus, suggesting that the *Tgfb2* signaling pathway was activated by *mTbx2*. *Tgfb2* may also be a direct target of *Tbx2*, because the *Tgfb2* promoter has conserved T-box binding sites, bound endogenous *Tbx2*, and was activated with a *Tbx2*-expression vector (SI Materials and Methods and Fig. S6).

**Discussion**

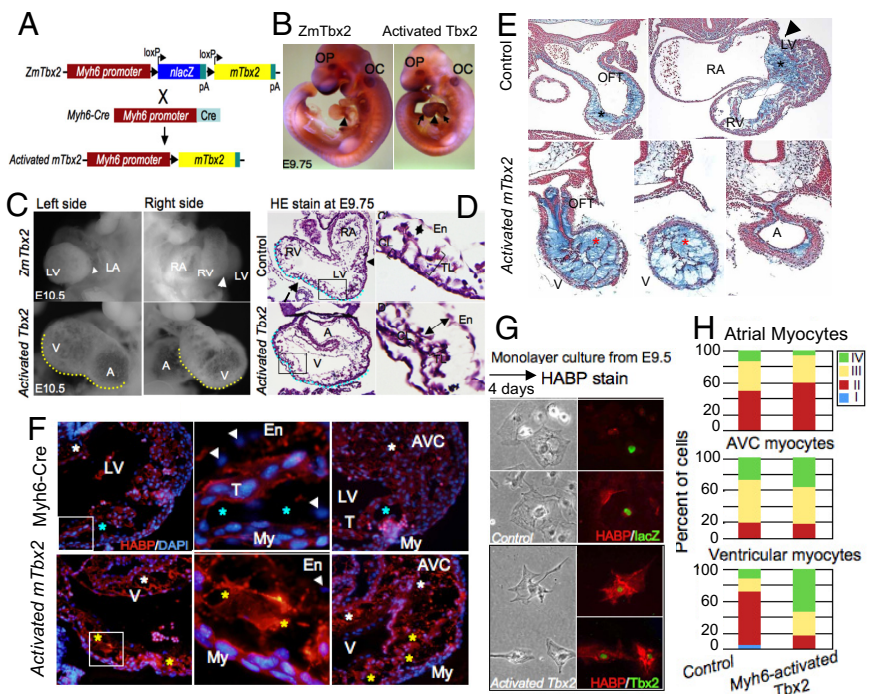
**Smad Factor Signaling Directed *Tbx2* Expression Through a Distal Enhancer During Early Murine Cardiogenesis.** *Tbx2* transcripts first appear in the cardiac crescent and then later became restricted to the OFT and AVC during early avian (2) and mouse embryogenesis (Fig. 1). *Tbx2* is also expressed in the embryonic limb, optic cup, otic cup, and pharyngeal arches. Approximately 4 kb of 5' flanking sequence of the murine *Tbx2* gene was sufficient to reproduce much of these mouse embryonic expression patterns, as a *lacZ* reporter transgene. *LacZ* expression patterns closely overlapped with those of *Bmp2* and *Bmp4* in the heart and eye, and was delineated within the *Tbx2* 5' flanking sequences, which contained Bmp directed multiple high-affinity binding

sites for Smad transcription factors. Removal of these multiple Smad sites by gap deletion mutagenesis from the 4-kb flanking sequences blocked reporter gene activity in early embryos. In contrast, this short enhancer region linked to a minimal *hsp68* promoter was sufficient for steering the restricted expression of *lacZ* in a pattern, highly similar to the endogenous *Tbx2* gene activity. Previously, application of *Bmp2* selectively induced *cTbx2* expression in noncardiogenic embryonic tissue, and the *Bmp* antagonist *Noggin* down-regulated *cTbx2* activity (2). Also, the appearance of murine *Tbx2* was blocked in *Bmp2* null mouse embryos (2). Thus, *Tbx2* expression depended on *Bmp* signaling through Smad factors, a regulatory paradigm that also guides other modular-restricted genes in the developing heart.

***Tbx2* Directs EC Formation.** *Tbx2* is generally considered to be a transcriptional repressor. For example, *Tbx2*-null embryos have expanded chamber-specific gene expression into the AVC My (3). Also, *Tbx2*-overexpressing transgenic mice and *Tbx20*-null mice, in which *Tbx2* is up-regulated, exhibited decreased expression of several chamber-specific genes and caused hypoplasia of ventricular chamber (4-6, 18, 19). However, *Tbx2* also contains an activation domain (20), and is important for EC formation, because *Tbx2* null mutant embryos exhibited small AVC and defective OFT septation (3). As shown here, *Tbx2* enhanced ECM synthesis from myocardial cells and EMT from endocardial cells. *Tbx2* directly bound *Has2* and *Tgfb2* promoters and increased their transcriptional activities. *Tgfb* signaling is crucial

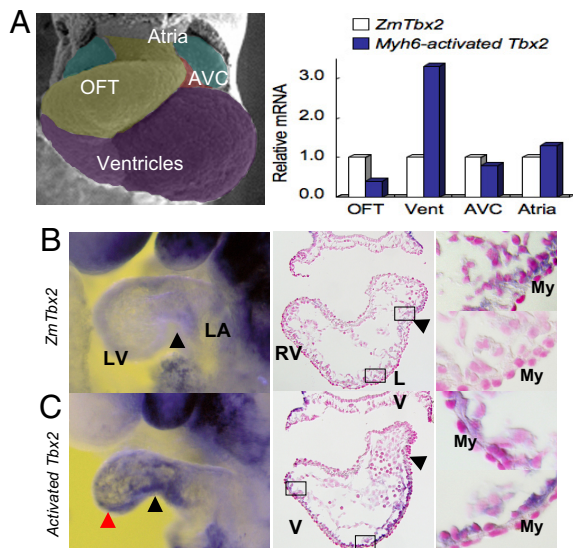


**Fig. 3.** Abnormal cardiac morphogenesis induced by activated *mTbx2* coincided with excessive HA deposition. (A) Schematic representation of *Myh6-Cre* induced activation of *mTbx2* by breeding *ZmTbx2* mice to the Cre deleter mouse line, *Myh6-Cre*. (B) Cardiomyocyte-specific activated *mTbx2* was detected by immunoperoxidase staining in the atria and ventricles at E9.75. (C) Enlarged images highlight embryonic hearts at E10.5. Relative to control littermates, activated *Tbx2* embryos exhibited an enlarged heart, which appeared as swollen single ventricular and atrial chambers, and a dilated AVC (yellow dotted line in C) at E10.5. (D) Histological sections of activated *mTbx2* in embryonic hearts at E9.75 stained with HE staining showed rounded ventricular chamber (blue dotted line) and an abnormally narrow ventricular lumen due to expansion of EC like structure between the En and My (two-headed arrow in D), and arrow indicate intraventricular sulcus (IVS) and arrowheads indicate AVC. (E) Histological sections of embryonic hearts stained at E10.5 with alcian blue revealed acidic glycosaminoglycans (blue stain) not only deposited in the OFT and AVC regions of control littermates (black asterisks), but throughout the chambers of activated *Tbx2* hearts (red asterisks). (F) Visualization of HA on sections of E9.75 embryos with HABP (red). HA was deposited between En and My in the inner curvature and AVC of activated *mTbx2* embryos and control littermates (white asterisks) and not in the outer curvature of control littermates. Extra deposition of HA was observed between En and My in the outer curvature of *mTbx2*-misexpressing ventricles (yellow asterisks). DAPI stain was used to visualize nuclei (blue). (G) Visualization of HA on cultured ventricular myocytes from embryos of E9.5 or E9.75 with HABP (red). Immunocytochemical detection of lacZ and Tbx2 (green). (H) Percentage of classified cardiomyocytes with HA secretion. Type I cardiomyocytes, no secretion of HA; Type II, little amount of HA around the cells; Type III, much HA is secreted around the cells and deposited in the cells (Fig. S4). Majority of ventricular cardiomyocytes secreted excessive HA in the activated *mTbx2* embryos. T, trabeculae; CL, compact layer; and TL, trabecular layer.



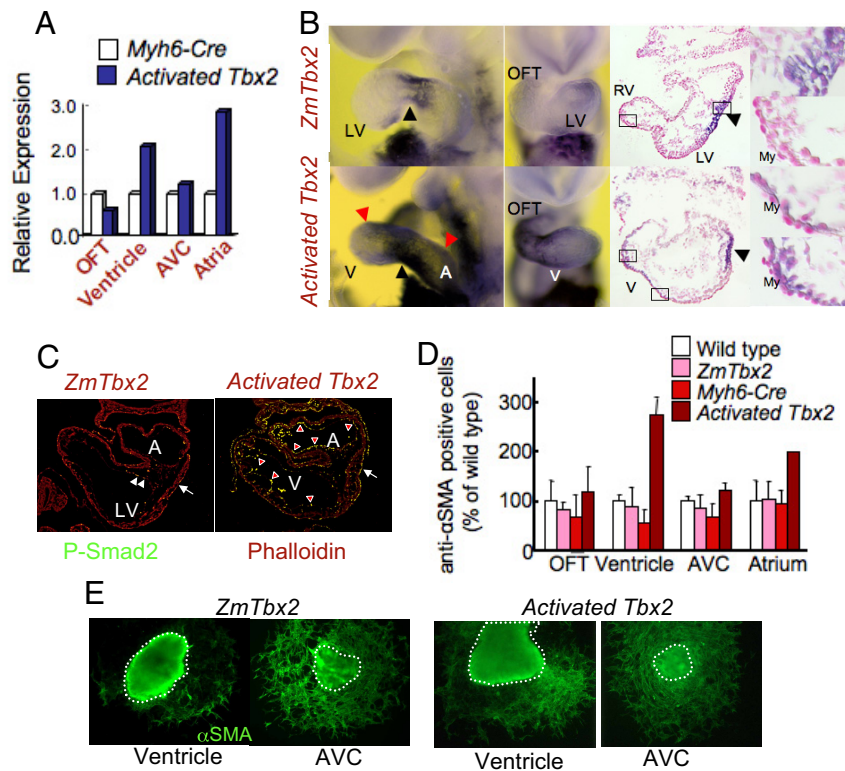
for EMT in the AV cushions (20–22). Accelerated appearance of EMT from the ventricular and atrial tissues, in vitro EMT assays and increased Smad2 phosphorylation in *mTbx2*-activated embryos supports activation of Tgfβ2 pathway by Tbx2.

*Tbx2* also induced *Has2* myocardial expression and increased HA deposition. HA is known as an essential factor for EC formation (8, 16, 23), and interacts with other molecules such as versican and fibrillin, which expands cardiac jelly providing extracellular space for cell migration (8). In addition to organizing extracellular environment, HA stimulates EMT of several types of epithelial cells (24) and endocardial cells dependent on Ras-activation (15) via ErbB2 receptor (23). In *Tbx2*-misexpressing embryos, extradeposition of HA was observed in the dilated chamber My; thus, *Tbx2* has an important role in EC formation by increasing synthesis of HA. During normal heart development, chamber-cardiomyocytes undergo a critical maturation step that is manifested by a transition from production to degradation of ECM between E8.0 and E9.5 (25). Whereas HA is required for cushion formation, excess HA deposition may cause hemodynamic alteration and prevent cardiomyocyte differentiation necessary for chamber maturation.



**Fig. 4.** HA synthetase, *Has2*, activated by *Tbx2* in cardiac chamber myocytes. (A) Real-time PCR analysis of dissected embryonic hearts at E9.5 or E9.75. *Has2* transcripts were tripled in the ventricles of activated *mTbx2* embryos. (B) Whole-mount in situ hybridization analysis for *Has2* expression was detected in the AVC (black arrowheads) of control littermates whereas expanded to ventricles (red arrowheads) of activated *Tbx2* embryos. The middle and right columns show sections after WISH. *Has2* expression was activated in ventricular myocardial cells of activated *Tbx2* embryos (right column). Nuclear were stained by nuclear fast red.

**Hierarchical Relationship Between *Bmp2/4*, *Tbx2*, *Has2*, and *TGFβ2*.** We propose a model in which Bmp-Smad responsive *Tbx2* is stimulated to perform a central role in promoting EC formation by inducing expansion of ECM and EMT by directing *Has2* and Tgfβ2 gene activity (Fig. 6). Several signaling pathways have been implicated in EC formation. The Bmp pathway is essential for expansion of ECM and EMT in EC formation. Myocardial-specific inactivation of *Bmp2*, *Bmp4*, and a Bmp type I receptor gene, *Alk3*, respectively, failed to form the EC (9, 10, 26). Sugi et al. (11) demonstrated that Bmp2 could substitute for the My to induce EMT. Also, noggin treatment of explants efficiently inhibited EMT. In both chicken and mouse EMT assays, Tgfβ2 is able to replace the overlying My to activate EMT in Tgfβ2 null mice (11, 16, 21, 22). Analysis of Tgfβ2-deficient mice also indicated that Tgfβ2 is important for valvulogenesis (27). Recent studies have shown that several factors, including *Tbx2*, *Tgfβ2*, and *Has2*, are downstream targets of Bmp2/4 pathway (9, 10). In



**Fig. 5.** Activated *mTbx2* induced *Tgfβ2* and enhanced EMT. (A) Real-time PCR analysis of quartered embryonic hearts at E9.5. *Tgfβ2* was increased in the ventricles and atria of activated *mTbx2* heart. (B) Whole-mount in situ hybridization analysis detected *Tgfβ2* expression in the AVC (black arrowheads in controls) and OFT of control littermates which expanded to ventricles and atria of activated *Tbx2* hearts (red arrowheads in misexpressed *Tbx2*). The middle and right columns show sections after WISH. *Tgfβ2* expression was activated in ventricular myocardial cells of activated *Tbx2* embryos (right column). Nuclear were stained by nuclear fast red. (C) Immunohistochemical detection of phospho-Smad2, an effector of *Tgfβ* signaling in the AVC (white arrows) En of control littermates (white arrowheads), which was expanded to the En of the ventricles and atria in activated *mTbx2* hearts (red arrowheads). (D and E) Immunohistochemical detection of  $\alpha$ -smooth muscle actin (SMA) showed increased EMT in in vitro collagen gel assays of ventricular and atrial explants. The percentage of anti- $\alpha$ SMA positive cells formed in explants from *mTbx2*-misexpressing ventricles and atria was approximately doubled compared with controls.

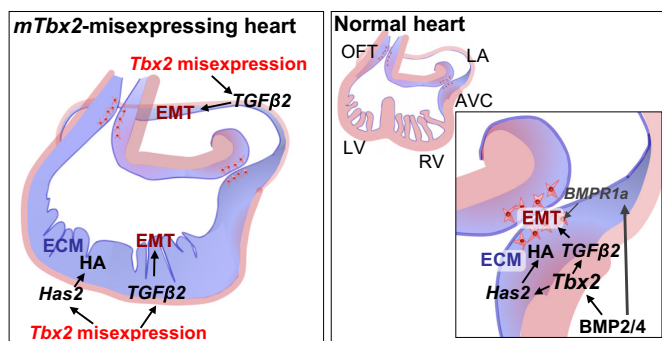
addition to myocardial-derived Bmp function, Bmp signals directly to the cushion En through the *Bmpr1a* to induce EMT (9). In our experiments, *Bmp2/4* were not up-regulated in *Tbx2*-misexpressing embryos (Fig. S7). Recently, Singh et al. (28) showed that *Tbx20* directly interfered with Bmp/Smad signaling to suppress *Tbx2* expression in the chambers; thereby, confining

*Tbx2* expression to the prospective AVC region. They also confirmed our observation that *Tbx2* distal enhancer directs *Tbx2* expression to the AVC and OFT. Here, we showed Bmp-Smad signaling dependent *Tbx2* expression directed *Has2* and *Tgfβ2* gene activity to coordinately regulate EC formation.

### Experimental Procedures

**Generation of *mTbx2* Reporter Gene Constructions.** A genomic fragment that contained the *Tbx2* locus was isolated from a 129SVJ mouse genomic library. A 4.5-kb *NotI* fragment flanking the 5' transcription start site and overlapping the first coding exon was cloned into the *NotI* site of pBluescript-KS for sequencing. The *mTbx2* reporter construct were generated from 4.1 kb of *mTbx2* flanking sequence 5' was linked in-frame in front of the *lacZ* and luciferase cDNA from pPD46.21 and pGL3-Basic (Promega). Deletion constructs were generated by restriction endonuclease digestion. The region from -2916 to -2602bp, containing multiple Smad sites, was linked to the hsp68lacZ reporter gene (29). Mutations at multiple Smad sites in -2916/-2602 fragment were inserted using In-Fusion PCR cloning kit (Clontech) (Fig. 2D; Fig. S1C).

**Whole-Mount in Situ Hybridization.** Staged mouse embryos were obtained after timed mating of mice with the morning of the copulation plug being E0.5. Embryos were fixed in MEMFA (0.1 M Mops/2 mM EGTA/1 mM MgSO<sub>4</sub>/3.7% formaldehyde) and stored in 90% methanol at -20 °C until use for hybridization. Whole-mount in situ hybridization was performed as described by Yamada et al. (2), except that polyvinyl alcohol was included to increase signal intensity. A full-length of *mTbx2* cDNA (kindly provided by Roni Bollag) was cloned into the *EcoRI* site of pBluescript-KS to synthesize digoxigenin-labeled RNA probes. After restriction endonuclease digestion with *SacI*, anti-sense probes were transcribed with RNA labeling kit (Stratagene).



**Fig. 6.** Model of *Tbx2* function for the EC formation in the AVC. The *mTbx2* misexpression induces expression of *Has2*; thereby, driving the synthesis and deposition of HA and/or cardiac jelly through the heart. Misexpressed *Tbx2* signals also promoted expression of *Tgfβ2* gene that supports the induction of EMT in the ventricles and atria. In the normal heart development, *Tbx2* works as one of the important factor to induce expansion of ECM and EMT under the *Bmp2/4*-Smad signaling pathway. Bmp signals also directly to the En through the *Bmpr1a*.



**Transient Transfection Assays.** Monkey CV-1 fibroblasts were grown in DMEM with 10% FBS. Cells were plated at  $1 \times 10^5$  cells per well in a 24-well plate and transfected 24 h later with DNA mixture containing a total of 2  $\mu$ g of total DNA, which included 500 ng of luciferase reporter vector, 500 ng of  $\beta$ -galactosidase vector, and a total of 1  $\mu$ g of pCMV5-derived vectors. Transfections were performed using Lipofectamine (Invitrogen) as described (30). Luciferase activity were measured using a luminometer to detect activates substrates, then normalized by  $\beta$ -galactosidase activity (29).

**Histology.** Embryos were dissected in Dulbecco's PBS and fixed overnight at 4 °C in 4% PFA in PBS for histological analysis. After fixation, embryos were rinsed in PBS, then dehydrated through graded ethanol or methanol and embedded in paraffin wax. Sections were cut and stained with hematoxylin-eosin or alcian blue according to standard methods. The HA was detected with 2  $\mu$ g/mL biotinylated HABP isolated from bovine nasal cartilage (Seikagaku). The HABP was detected using streptavidin-conjugated AlexaFluor (Invitrogen). For immunohistochemistry, sections were incubated with rabbit anti-Tbx2 IgG (Upstate Biotechnology) or rabbit anti- $\beta$ -galactosidase (Biogenesis). Primary an-

tibody was detected with goat anti-rabbit IgG labeled with Alexa Fluor (Invitrogen).

**Real-Time PCR.** Embryonic hearts at E9.5–E9.75 were divided to 4-parts, OFT, ventricles, AVC, atria at the posterior boundary of EC in OFT, anterior and posterior boundaries of EC. Dissected tissues were immediately frozen in liquid nitrogen and stored at  $-80$  °C until embryo and yolk sac DNA was genotyped. Total RNA isolation and first strand cDNA synthesis were performed with TRIzol reagent, SuperScript III (Invitrogen) and random primer, as per the manufacturer's instructions.

**Primary Culture of Embryonic Cardiac Cells and Classification of Cardiomyocytes with HA Synthesis.** Quartered embryonic cardiac tissues were separated as described above. After trypsinization, isolated cardiac cells were cultured on gelatinized dishes by using culture medium containing DMEM (D5796; Sigma-Aldrich), 10% heat-inactivated FCS (HyClone), 0.1 g/mL penicillin, and 0.1 mg/mL streptomycin. After 4 days culture, cells were fixed in 4% PFA in PBS and detected HA, Tbx2 and  $\beta$ -galactosidase as described above. Classification of cardiomyocytes with HA secretion was followed as described in Fig. S4.

- Schwartz RJ, Olson EN (1999) Building the heart piece by piece: Modularity of *cis*-elements regulating to Nkx2-5 transcription. *Development* 126:4187–4192.
- Yamada M, et al. (2000) Expression Of Chick Tbx-2, Tbx-3 and Tbx-5 genes during early heart development: Evidence for BMP2 induction of Tbx2. *Dev Biol* 228:95–105.
- Harrelson Z, et al. (2004) Tbx2 is essential for patterning the atrioventricular canal and for morphogenesis of the outflow tract during heart development. *Development* 131:5041–5052.
- Christoffels VM, et al. (2004) T-box transcription factor Tbx2 represses differentiation and formation of the cardiac chambers. *Dev Dyn* 229:763–770.
- Habets PE, et al. (2002) Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: Implications for cardiac chamber formation. *Genes Dev* 16:1234–1246.
- Cai CL, et al. (2005) T-box genes coordinate regional rates of proliferation and regional specification during cardiogenesis. *Development* 132:2475–2487.
- Pierpont ME, Markwald RR, Lin AE (2001) Genetic aspects of atrioventricular septal defects. *Am J Med Genet* 297:289–296.
- Schroeder JA, Jackson LF, Lee DC, Camenisch TD (2003) Form and function of developing heart valves: Coordination by extracellular matrix and growth factor signaling. *J Mol Med* 81:392–403.
- Ma L, Lu MF, Schwartz RJ, Martin JF (2005) Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. *Development* 132:5601–5611.
- Gaussin V, et al. (2002) Endocardial cushion and myocardial defects after cardiac myocyte-specific conditional deletion of the bone morphogenetic protein receptor ALK3. *Proc Natl Acad Sci USA* 99:2878–2883.
- Sugi Y, Yamamura H, Okagawa 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.
- Itoh S, Itoh F, Goumans MJ, Ten Dijke P (2000) Signaling of transforming growth factor-beta family members through Smad proteins. *Eur J Biochem* 267:6954–6967.
- Galvin KM, et al. (2000) A role for smad6 in development and homeostasis of the cardiovascular system. *Nat Genet* 24:171–174.
- Yamada M, et al. (1999) Evidence for a role of Smad6 in chick cardiac development. *Dev Biol* 215:48–61.
- Whiteman P (1973) The quantitative measurement of Alcian Blue glycosaminoglycan complexes. *Biochem J* 131:343–350.
- Camenisch TD, et al. (2000) Disruption of hyaluronan synthase-2 abrogates normal cardiac morphogenesis and hyaluronan-mediated transformation of epithelium to mesenchyme. *J Clin Invest* 106:349–260.
- Baldwin HS, Lloyd TR, Solursh M (1994) Hyaluronate degradation affects ventricular function of the early postlooped embryonic rat heart in situ. *Circ Res* 74:244–252.
- Stennard FA, Harvey RP (2005) T-box transcription factors and their roles in regulatory hierarchies in the developing heart. *Development* 132:4897–4910.
- Takeuchi JK, et al. (2005) Tbx20 dose-dependently regulates transcription factor networks required for mouse heart and motoneuron development. *Development* 132:2463–2474.
- Paxton C, Zhao H, Chin Y, Langner K, Reecy J (2002) Murine Tbx2 contains domains that activate and repress gene transcription. *Gene* 283:117–124.
- Brown CB, Boyer AS, Runyan RB, Barnett JV (1999) Requirement of type III TGF-beta receptor for endocardial cell transformation in the heart. *Science* 283:2080–2082.
- Nakajima Y, Yamagishi T, Hokari S, Nakamura H (2000) Mechanisms involved in valvuloseptal endocardial cushion formation in early cardiogenesis: Roles of transforming growth factor (TGF)-beta and bone morphogenetic protein (BMP). *Anat Rec* 258:119–127.
- Camenisch TD, Schroeder JA, Bradley J, Klewer SE, McDonald JA (2002) Heart-valve mesenchyme formation is dependent on hyaluronan-augmented activation of ErbB2-ErbB3 receptors. *Nat Med* 8:850–855.
- Zoltan-Jones A, Huang L, Ghatak S, Toole BP (2003) Elevated hyaluronan production induces mesenchymal and transformed properties in epithelial cells. *J Biol Chem* 278:45801–45810.
- Bernanke DH, Orkin RW (1984) Hyaluronidase activity in embryonic chick heart muscle and cushion tissue and cells. *Dev Biology* 106:351–359.
- Jiao K, et al. (2003) An essential role of Bmp4 in the atrioventricular septation of the mouse heart. *Genes Dev* 17:2362–2367.
- Bartram U, et al. (2001) Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGF-beta (2)-knockout mice. *Circulation* 103:2745–2752.
- Singh R, et al. (2009) Tbx20 interacts with smads to confine tbx2 expression to the atrioventricular canal. *Circ Res* 105:442–452.
- Chi X, et al. (2005) Complex cardiac Nkx2-5 gene expression activated by noggin-sensitive enhancers followed by chamber-specific modules. *Proc Natl Acad Sci USA* 102:13490–13495.
- Brown CO, III, et al. (2004) The cardiac determination factor, Nkx2-5, is activated by mutual cofactors GATA-4 and Smad1/4 via a novel upstream enhancer. *J Biol Chem* 279:10659–10669.