The Short Stature Homeobox 2 (Shox2)-bone Morphogenetic Protein (BMP) Pathway Regulates Dorsal Mesenchymal Protrusion Development and Its Temporary Function as a Pacemaker during Cardiogenesis*

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The atrioventricular (AV) junction plays a critical role in chamber septation and transmission of cardiac conduction pulses. It consists of structures that develop from embryonic dorsal mesenchymal protrusion (DMP) and the embryonic AV canal. Despite extensive studies on AV junction development, the genetic regulation of DMP development remains poorly understood. In this study we present evidence that Shox2 is expressed in the developing DMP. Intriguingly, this Shox2-expressing domain possesses a pacemaker-specific genetic profile including Hcn4 and Tbx3. This genetic profile leads to nodal-like electrophysiological properties, which is gradually silenced as the AV node becomes matured. Phenotypic analyses of Shox2−/− mice revealed a hypoplastic and defectively differentiated DMP, likely attributed to increased apoptosis, accompanied by dramatically reduced expression of Bmp4 and Hcn4, ectopic activation of Cx40, and an aberrant pattern of action potentials. Interestingly, conditional deletion of Bmp4 or inhibition of BMP signaling by overexpression of Noggin using a Shox2-Cre allele led to a similar DMP hypoplasia and down-regulation of Hcn4, whereas activation of a transgenic Bmp4 allele in Shox2−/− background attenuated DMP defects. Moreover, the lack of Hcn4 expression in the DMP of mice carrying Smad4 conditional deletion and direct binding of pSmad1/5/8 to the Hcn4 regulatory region further confirm the Shox2-BMP genetic cascade in the regulation of DMP development. Our results reveal that Shox2 regulates DMP fate and development by controlling BMP signaling through the Smad-dependent pathway to drive tissue growth and to induce Hcn4 expression and suggest a temporal pacemaking function for the DMP during early cardiogenesis.

The mammalian embryonic heart develops from a tubular structure with a single circulation to a four-chambered structure with a dual circulation. Simultaneously, its beating pattern undergoes transformation from a peristaltic motion to a sophisticated synchronous contraction as a result of maturation of the cardiac conduction system (CCS) that consists of the sinoatrial node (SAN) and atrioventricular node (AVN) as well as His-Purkinje tracts (1). Myocytes of the SAN and AVN have unique electrophysiological features characterized by the slower upstroke and smaller action potential (AP) amplitude and presence of the diastolic depolarization compared with that of working myocardium of the atrium and the ventricle. The AV junction that lies at the merging point of cardiac chambers is critically involved in both septum and CCS developmental processes (2). It comprises the dorsally positioned second heart field-derived dorsal mesenchymal protrusion (DMP) that gives rise to the future vestibular spine (2) and ventrally positioned endocardial cushion tissue as well as AV canal-derived myocardium including AVN precursors (3, 4). The outgrowth and fusion of DMP and endocardial cushion tissue are essential for proper formation of AV septation (2, 5). During cardiogenesis in mice, in the initial simple heart tube at embryonic day 8.5 (E8.5), the ring-like AV canal, which connects single atrium and ventricle, functions to generate the delay between the atrial and ventricular contraction (6). However, the definitive AVN becomes morphologically distinguishable only at late gestation stage (3, 6). The AV conduction network, particularly the AVN in adults, receives propagation initiated by the SAN and gives a

* The abbreviations used are: CCS, cardiac conduction system; SAN, sinoatrial node; AVN, atrioventricular (AV) node; DMP, dorsal mesenchymal protrusion; BMP, bone morphogenetic protein; SBE, Smad binding element; Hcn4, hyperpolarization-activated, cyclic nucleotide-gated 4.

**This work was supported, in whole or in part, by National Institutes of Health Grant R01 DE17792 (to Y. C.).

†Supported by an American Heart Association Predoctoral Fellowship 13PRE13750003.

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Shox2 and DMP Development

We further present evidence demonstrating that BMP signaling acts through a Smad-dependent pathway to regulate Hcn4 expression and an early fate of the DMP.

EXPERIMENTAL PROCEDURES

Animals—The generation of Shox2<sup>+/−</sup>, Shox2-LacZ, Shox2-Cre, pMes-Noggin, and pMes-Bmp4 mice has been described previously (13, 16, 27, 28). Smad4<sup>−/−</sup> mice were purchased from The Jackson Laboratory. Bmp4 floxed mice (29) were kindly provided by Dr. Bridig Hogan. All described mouse lines were crossed onto CD-1 background. Wild-type CD-1 mice were obtained from Charles River. To harvest Shox2 null embryos at E13.5 or older, pregnant females were administered with the β-adrenergic receptor agonist from day 7.5 of gestation by supplementing drinking water with 200 μg/ml isoproterenol. Approval from the Institutional Animal Care and Use Committee of Tulane University was acquired for all the procedures used in this study.

Histology, X-Gal staining, in Situ Hybridization, and Immunohistochemistry—Standard hematoxylin/eosin staining was performed on 10-μm sections from paraffin-embedded samples. X-gal staining on cryosections or whole embryo was performed following standard procedures. For in situ hybridization, embryos were harvested in ice-cold diethyl pyrocarbonate-treated PBS and fixed in 4% paraformaldehyde, PBS overnight at 4 °C. After dehydration through graded ethanol and parafin embedding, samples were sectioned at 10 μm and subjected to an in situ hybridization procedure as described previously (13). Immunohistochemistry was conducted on frozen-sectioned or paraffin-sectioned samples. For cryostat sectioning, embryos were fixed in Z-fix (Anatech) at room temperature for 2–3 h. After rinsing through 15 and 30% sucrose, PBS, and OCT embedding, samples were cryosectioned at 8 μm. A standard immunofluorescence procedure without antigen recovery was conducted. For paraffin sections, embryos were fixed in Z-fix at 4 °C for 24 h. After dehydration through graded ethanol and parafin embedding, samples were sectioned at 8 μm. A standard immunofluorescence procedure with antigen recovery was carried out. The following antibodies were used: anti-β galactosidase (1:1000 dilution) (Abcam), anti-Hcn4 (1:800 dilution) (Abcam), anti-cleaved Caspase3 (1:200 dilution) (Cell Signaling), anti-Ki67 (1:400 dilution) (Cell signaling), anti-Cx40 (1:200 dilution) (Santa Cruz), anti-phosphorylated Smad1/5/8 (1:800 dilution) (Abcam), anti-GFP (1:1300 dilution) (Novus). The following secondary antibodies, all from Invitrogen, were used for visualization: Alexa Fluor 594 goat anti-rabbit antibody (1:500 dilution), Alexa Fluor 488 goat anti-chicken antibody (1:500 dilution), Alexa Fluor 594 goat anti-rabbit antibody (1:500 dilution), Alexa Fluor 488 Donkey anti-goat antibody (1:500 dilution).

Isolation of Embryonic Cardiac Myocytes and Electrophysiology—The SAN, atrium, and ventricle of embryonic hearts from Shox2<sup>lacZ/+</sup> or Shox2<sup>lacZ/lacZ</sup> mice at designated ages were dissected in prewarmed Tyrode’s solution that contained 140 mM NaCl, 5.0 mM Hepes, 5.5 mM glucose, 5.4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, with pH adjusted to 7.4 with NaOH, according to the method described previously (30–33). The isolation of the DMP followed the method reported previously for the dissection of AV junction from neonatal heart.
tate chromatin fragments containing the Smad binding element (SBE) sequence, with normal rabbit immunoglobulin G that came with the kit used as a control. A set of primers, 5′-CTGGTGATGGGATGTAGT-3′ and 5′-TAGAGCAGGGGAGTGA3′, covers the described SBE in the first intron of the Hcn4 locus amplified a 291-bp fragment, and a control set of control primers, 5′-AGCAACCTTGTGAGGAGT-3′ and 5′-CTGCTATCACCCTCTAGCA-3′, covers a 259-bp DNA sequence 5 kb downstream from the SBE site. Amplified DNA products were confirmed by correct size on 3% agarose gel and sequencing.

RESULTS

Genetic Profile and Electrophysiological Properties of Shox2-expressing DMP—To reveal comprehensive Shox2 expression patterns and to dissect genetic network in Shox2-expressing cells during cardiogenesis, we created a Shox2-LacZ allele and a Shox2-Cre allele by gene knock-in strategy (16). Shox2LacZ/+ or Shox2Cre/+ mice are indistinguishable from their wild-type littermates, but homozygous mice die in mid-gestation stage and develop phenotypes identical to that in Shox2−/− mice. Although Shox2 expression in the SANs, venous valves, and sinus horns and other developing organs was confirmed by the LacZ reporter allele, we also identified a novel Shox2 expression site that was not reported previously; that is, the developing AV junction from embryonic day 11.5 (E11.5) up to E18.5, at which time the expression became down-regulated as compared with persistent strong expression in the SAN (Fig. 1, A–C) (16). This Shox2 expression domain in the AV junction was further confirmed by in situ hybridization assays (Fig. 1D) and by X-gal staining performed on R26R-LacZ;Shox2Cre/+ mice (Fig. 1E). Because the AV junction myocardium consists of DMP and AVN, we set out to determine the identity of this Shox2-expressing tissue by cell lineage tracing using the Shox2-Cre allele compounded with the R26R-LacZ reporter and performed an immunohistochemistry assay on the expression of pacemaker markers hyperpolarization-activated, cyclic nucleotide-gated 4 (Hcn4) and Tbx3 as well as myocardium marker Cx40. At E18.5, when a definite AVN becomes distinguishable (3) and a residual Hcn4 expression was found in the LacZ-positive domain, Tbx3 expression was not detectable (Fig. 1, G–I). However, adjacent to the LacZ-positive domain on the ventral side, there was a domain positive for both Hcn4 and Tbx3, representing the maturing AVN (Fig. 1, G–I). At this stage, Cx40 expression was also absent in both domains despite its expression in the adjacent atrial septal tissue (Fig. 1J). At postnatal day 7 (P7), the expression of Hcn4 and Tbx3 was absent within the LacZ-positive domain but was persistent in the AVN (Fig. 1, K–M). At this time, consistent with the previous report (38), Cx40 expression was found in the LacZ-positive domain but was completely absent in the AVN (Fig. 1N), suggesting that the Shox2-expressing cells have differentiated into working myocardium and the AVN has become matured at this stage. These observations indicate that the Shox2-expressing cells are of DMP origin, which is further confirmed by Shox2 expression in the second heart field derivative in the AV junction (Fig. 1F). Because Hcn4 is expressed in the early developing DMP, we wondered if the Shox2-expressing DMP possesses the pacemaking genetic profile during early cardiogenesis. We conducted double immunocytochemical staining to show Shox2-expressing DMP and C12FDG substrate (Invitrogen) with a concentration of 33 μM for 40 min in culture medium or culture medium containing 10 ng/ml BMP4 (R&D Systems) at 37 °C as described previously (34). The fluorescence of labeled cells was identified using a Nikon eclipse Ti-S microscope.

A coverslip with attached myocytes was placed in the measuring chamber, and the fluorescence of β-galactosidase-positive cells was verified under an upright microscope (Olympus BX50WI). Cells were recorded in an extracellular Tyrode’s solution that contained 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 5.5 mM glucose, and 5.0 mM HEPES at pH 7.4. Whole-cell patch recordings were made using the Axon MultiClamp 700B amplifier (Axon Instruments). Glass electrodes (3–5 megohms) were pulled on a Sutter puller. Internal solution contained 120 mM potassium glutamate, 120 mM KCl, 4 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM MgATP, 14 mM phosphocreatine disodium salt, and 5 mM Tris GTP (pH 7.2). Data were digitized at a rate of 10 KHz using Clampex 10.3 and analyzed using Clampfit 10.3 (Axon Instruments). Recordings were done at 36.0 ± 0.5 °C. All reagents used in intra- and extracellular solution were purchased from Sigma. The value of the maximum rate of rise of the AP upstroke (dV/dtmax) was determined using Clampfit 10.3.

Three-dimensional Reconstruction and Volume Rendering—Twenty 10-μm X-gal-stained slices from each E12.5 embryo and 30 10-μm X-gal-stained slices from each E14.5 embryos of wild-type and Shox2 mutants were loaded into an Amira software (Version 5.4.3, Visualization Sciences Group US) to carry out three-dimensional-reconstruction and volume rendering following procedures described previously (35). Three embryos from each stage and genotype were used for three-dimensional reconstruction. Student’s t test was applied to determine the significance of difference.

Terminal Deoxynucleotidyltransferase dUTP Nick End Labeling (TUNEL) Assay—A TUNEL assay was performed to detect apoptosis using the In Situ Cell Death Detection kit from Roche Applied Science following a described method (36). Four samples of each genotype were tested.

Chromatin Immunoprecipitation—About 10–12 hearts isolated from one litter of E13.5 wild-type mouse embryos were subjected to chromatin immunoprecipitation (ChIP) assay using a kit from Invitrogen. Previously described anti-Smad1/5/8 antibodies (37) (Santa Cruz) were used to immunoprecipitate chromatin fragments containing the Smad binding element (SBE) sequence, with normal rabbit immunoglobulin G that came with the kit used as a control. A set of primers, 5′-CTGGTGATGGGATGTAGT-3′ and 5′-TAGAGCAGGGGAGTGA3′, covers the described SBE in the first intron of the Hcn4 locus amplified a 291-bp fragment, and a control set of control primers, 5′-AGCAACCTTGTGAGGAGT-3′ and 5′-CTGCTATCACCCTCTAGCA-3′, covers a 259-bp DNA sequence 5 kb downstream from the SBE site. Amplified DNA products were confirmed by correct size on 3% agarose gel and sequencing.
Shox2 and DMP Development
demonstrated that the Cx40 and Cx43 as well as the lack of endocardium marker Tie-2 expression of Tbx3, the lack of working myocardium marker genetic feature of the cardiac pacemaker at early developmental stage. To characterize the AP properties of the SAN myocytes, we used the whole-cell patch clamp technique to characterize the AP properties of the SAN myocytes, nodal-like (A), E14.5 atrial myocyte, embryonic atrial-like (B), E14.5 ventricular myocyte, embryonic ventricular-like (C), E14.5 DMP myocyte, nodal-like (D), and E18.5 DMP myocyte showing a changed firing pattern other than that of nodal-like cell (E), F, a two-dimensional diagram showing the action potential amplitude and the maximum rate of rise of the AP (dV/dtmax) for each cell type studied. In E14.5 SAN and DMP cells, action potential amplitudes were smaller than 75, and dV/dtmaxs were smaller than 4. In E14.5 atrial and ventricular cells, action potential amplitudes and dV/dtmaxs were much larger. In E18.5 DMP cells, action potential amplitudes and dV/dtmaxs were considerably increased. The red-lined region will be revisited in Fig. 4D.

To identify the physiological properties of the Shox2-expressing DMP myocytes, we used the whole-cell patch clamp technique to characterize the AP properties of the Shox2-positive cells in the DMP from the embryonic hearts. First, recordings were performed on the myocytes from the SA node, the atrium, and the ventricle of E14.5 embryos to establish distinct AP patterns. The typical firing pattern of each measured cell group is shown in Fig. 2. These correspond to similar characteristics described in numerous previous studies (39–41). In brief, the nodal-like APs of SAN cells show the slow upstroke (dV/dtmax), the small AP amplitude, and the presence of the diastolic depolarization (Fig. 2A). However, the APs of contracting atrial and ventricular myocardium show fast upstroke, large AP amplitude, and weak or no diastolic depolarization.

staining assay on β-Gal and Hcn4 in the Shox2lacZ/+ DMP. Indeed, Hcn4 expression overlapped with that of β-Gal in the AV junction (Fig. 1O and see Fig. 5, A–C). In addition, the expression of Tbx3, the lack of working myocardium marker Cx40 and Cx43 as well as the lack of endocardium marker Tie-2 demonstrated that the Shox2-expressing DMP possesses the genetic feature of the cardiac pacemaker at early developmental stage (Fig. 1, P–R; and data not shown).

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FIGURE 1. Expression of Shox2 and pacemaker markers in the developing DMP. A–C, Shox2 expression in the developing AV junction was revealed by X-gal staining at E11.5 (A), E12.5 (B), and E18.5 (C) when the expression became down-regulated as compared with the strong expression in the SAN (C and inset). D, in situ hybridization confirmed Shox2 expression the AV junction. Arrowheads in B and D point to the absent Shox2 expression in the lateral AV canal tissue. E, X-gal staining shows localization of Shox2-expressing cells in the AV junction an E12.5 R26R-LacZ/Shox2lacZ/+ mice. F, an E16.5 Mef2C-Cre;R26R embryo exhibits Shox2 expression in the DMP, revealed by immunohistochemistry, which is part of second heart field-derived tissues labeled by EGFP. G–J, lineage tracing by Shox2-Cre allele shows that Shox2-expressing cells (asterisk) do not contribute to the forming AVN, as assessed by Hcn4 and Tbx3 expression. Although Cx40 expression is seen in the adjacent atrial septal tissue (white arrowhead), it is absent in both Shox2-expressing domain, and the AVN (U). The inset in H shows strong Hcn4 expression in the SAN at the same stage as positive control. K–N, immunohistochemistry shows the Hcn4+/Tbx3+/Cx40+ AVN and the LacZ-positive domain (asterisk) that is Hcn4+/Tbx3+/Cx40+ in the AV junction of P7 R26R;Shox2lacZ/+ mice. Note that LacZ-positive cells are also found near the orifice of the pulmonary vein (white arrowheads in R) at this stage. O–R, immunohistochemistry reveals that Hcn4 (dotted line in O) and Tbx3 (asterisk in P) but not Cx40 (asterisk in Q) and Cx43 (asterisk in R) are expressed within the Shox2-expressing DMP at E12.5. By contrast, Cx40 is expressed in the wall of coronary sinus (white arrow in Q) and Cx43 (red arrow in R) is expressed in the wall of LSH. The DMP domain is demarcated by dotted lines based on the expression domain of β-Gal and the AVN domain, also outlined by dotted lines, is defined by its expression of Hcn4 and Tbx3. CT, cushion tissue; LA, left atrium; LV, left ventricle; RA, right atrium; VS, ventricular septum; VV, venous valve; AVB, atrioventricular bundle; LSH, left sinus horn. Scale bar = 40 μm.

FIGURE 2. Multiple types of action potentials. A–E, action potential patterns of E14.5 SAN myocyte, nodal-like (A), E14.5 atrial myocyte, embryonic atrial-like (B), E14.5 ventricular myocyte, embryonic ventricular-like (C), E14.5 DMP myocyte, nodal-like (D), and E18.5 DMP myocyte showing a changed firing pattern other than that of nodal-like cell (E). F, a two-dimensional diagram showing the action potential amplitude and the maximum rate of rise of the AP (dV/dtmax) for each cell type studied. In E14.5 SAN and DMP cells, action potential amplitudes were smaller than 75, and dV/dtmaxs were smaller than 4. In E14.5 atrial and ventricular cells, action potential amplitudes and dV/dtmaxs were much larger. In E18.5 DMP cells, action potential amplitudes and dV/dtmaxs were considerably increased. The red-lined region will be revisited in Fig. 4D.
Enhanced Apoptosis Underlies Hypoplastic DMP in Shox2 Mutants—The detection of Shox2 expression in the developing DMP prompted us to examine potential phenotype in Shox2-deficient mice. Because the majority of Shox2 mutants died around E12.5, to harvest mutant embryos at relatively late developmental stages we administered the β-adrenergic receptor agonist isoproterenol, which stimulates cardiac beating, to pregnant Shox2 heterozygous females to prevent earlier embryonic lethality of Shox2−/− mice. Histological analyses revealed a hypoplastic AV junction in Shox2 mutants beginning at E12.5 as compared with littermate controls (Fig. 3, A, B, F, and G). To quantify the volume reduction in Shox2 mutant DMP, three-dimensional rendering was conducted based on X-gal staining of both Shox2LacZ/+ and Shox2lacZ/lacZ mice. We identified a 34.4% volume decrease at E12.5 and 64.5% volume decrease at E14.5 in the Shox2-expressing tissue on the right side of the AV junction in Shox2lacZ/lacZ animals compared with heterozygous animals (Fig. 3, C–E and H–I).

To reveal the potential cellular defects underlying the hypoplastic DMP in Shox2 mutants, we examined cell proliferation rate and apoptosis in the AV junction at E12.5. Although the ratio of Ki67-positive cell in the DMP of Shox2 mutants (13.38%) was slightly lower as compared with wild-type controls (14.7%), statistical analysis determined a lack of significance between the controls and mutants (p = 0.38) (Fig. 3, K–M). However, a TUNEL assay revealed an 8-fold elevation of cell apoptosis in the developing DMPs of Shox2 mutants compared with littermate controls (Fig. 3, N–P). This observation was further confirmed by detection of ectopic activation of cleavage Caspase-3 in the mutant AV junction (Fig. 3, Q and R), indicating the contribution of elevated apoptosis to the reduced DMP tissue volume in Shox2 mutants.

Shox2 Deficiency Leads to Defective DMP Differentiation and Aberrant Electrophysiological Properties—Because ablation of Shox2 causes defective cell differentiation of the SAN (11), particularly in the sinoatrial junction domain,5 and reduced Shox2 expression level is accompanied by down-regulation of Hcn4 in the DMP at late gestation stage (Fig. 1), we sought to determine if Shox2 regulates Hcn4 expression in the developing DMP within the AV junction by immunohistochemistry. At E11.5, the level of Hcn4 within the Shox2-expressing domain appeared comparable between Shox2lacZ/lacZ and Shox2lacZ/lacZ embryos (Fig. 4, A–F). However, at E12.5, along with the hypoplastic phenotype described above, Hcn4 expression domain and intensity were reduced significantly in the mutant DMP (Fig. 4, G–L). In addition, in the E12.5 mutant DMP, we detected strong ectopic expression of Cx40 as compared with controls (Fig. 4, M–R). Similar reduced Hcn4 and ectopic Cx40 expression patterns were also observed in the mutant DMP at E14.5 as compared with controls (Fig. 5, A–H). However, the expression of podoplanin, which was shown to be required for SAN development (42), remained unaltered (data not shown). These observations suggest that the fate of DMP as potential temporary pacemaking cells in Shox2 mutants is deviated and likely adopts a working myocardial fate precociously. Indeed, the APs of the DMP cells from E14.5 Shox2 mutant demonstrated significant differences compared with the wild-type counterpart, characterized by increased maximum rate of rise of the AP and AP amplitude, and it was further illustrated by a changed firing pattern (Fig. 6, A–D, and Table 1). On the other hand, the AP properties of mutant atrial and ventricular myocytes were relatively unchanged (Fig. 6, E–H, and Table 1).

**TABLE 1**

| Characteristics of action potentials in multiple types of myocytes | E14.5 SAN | E14.5 DMP+ | E14.5 DMP− | E14.5 rescue | E18.5 DMP+ | E18.5 Atrial+ | E18.5 Atrial− | E14.5 Ventricular+ | E14.5 Ventricular− |
|---|---|---|---|---|---|---|---|---|---|
| Number of cells | 3 | 7 | 7 | 4 | 3 | 9 | 5 | 8 | 4 |
| APA, mV | 63.46 ± 3.66 | 63.76 ± 1.90 | 82.28 ± 5.70 | 84.30 ± 6.97 | 90.15 ± 3.04 | 96.11 ± 3.14 | 106.61 ± 2.65 | 108.24 ± 2.40 | 107.58 ± 2.45 |
| dV/dtmax, V/S | 3.59 ± 0.01 | 3.278 ± 0.11 | 19.92 ± 9.78 | 6.10 ± 0.74 | 26.26 ± 10.41 | 79.63 ± 10.45 | 105.96 ± 12.49 | 78.21 ± 13.16 | 71.53 ± 10.53 |
| MDP, mV | −53.77 ± 1.51 | −50.22 ± 1.68 | −54.29 ± 2.16 | −60.50 ± 1.46 | −50.65 ± 7.16 | −62.87 ± 1.71 | −60.84 ± 1.11 | −65.90 ± 0.94 | −67.51 ± 1.61 |
| Peak, mV | 9.69 ± 4.89 | 13.54 ± 2.92 | 23.99 ± 3.78 | 23.80 ± 6.13 | 29.84 ± 6.38 | 33.23 ± 3.12 | 45.77 ± 2.12 | 42.35 ± 1.78 | 40.05 ± 4.69 |

5 W. Ye and Y. Chen, unpublished data.
FIGURE 3. Hypoplasia and altered cellular processes in the developing DMP of Shox2 mutant. A–J, Shox2 null embryos display hypoplasia in the developing DMP, as shown by X-gal staining and three-dimensional reconstructions from X-gal-stained images at E12.5 (A–E) and E14.5 (F–J). DMP tissue volume reduction is shown by statistical results of volume comparison in the same region between Shox2LacZ/H11001 embryos and Shox2LacZ/LacZ embryos at E12.5 (E) and E14.5 (J). K–M, Ki67 immunofluorescence on representative sections of E12.5 wild-type (K) and Shox2 null (L) embryos shows no significant alteration of cell proliferation in the DMP between control and mutant animals, as determined by statistical analysis (M). NS, not significant. N–P, TUNEL assay on representative sections of E12.5 wild-type (N) and Shox2 null (O) embryos shows significantly increased cell apoptosis (black arrowheads) in the DMP region, as determined by statistical analysis (P). However, a comparable level of cell apoptosis (red arrowheads in N and O) in the adjacent cushion tissue of both control and Shox2 mutant was observed. Q and R, enhanced cell apoptosis was further confirmed by immunofluorescence of cleaved Caspase-3 (c-Caspase-3) (white arrowheads) in the DMP (asterisk) of E12.5 Shox2 null embryos (R) as compared with control (Q). Red arrowheads point to cleaved Caspase-3 positive cells in the adjacent cushion tissue. Dotted lines demarcate the DMP based on expression of β-Gal (A, B, F, and G). The DMP region in K, L, N, and O is defined based on β-gal expression at comparable stage. *, p < 0.05; ***, p < 0.0001. CT, cushion tissue; LV, left ventricle; RA, right atrium; VV, venus valve; LSH, left sinus horn. Scale bar = 40 μm.
the control DMP but was dramatically reduced in Shox2 mutants (Fig. 7, E–H). Accompanied with this down-regulation of Bmp2 and Bmp4 was a reduction of pSmad1/5/8 in the Shox2 mutant AV junction (Fig. 7, I and J), indicating disrupted BMP signaling.

**Disruption of BMP Signaling in the Developing DMP Recapitulates Shox2 Mutant Phenotype**—Although it has been reported that BMP signaling plays a critical role in DMP development (5), we wondered if disrupted BMP signaling attributes to the fate change of the developing DMP in Shox2 mutants. We...
ablated Bmp4 from Shox2 expression domains using a Shox2-Cre allele and floxed Bmp4 mice (16, 29). Histological analyses showed that mice carrying Shox2-Cre;Bmp4/F/F compound alleles manifest a hypoplastic DMP at E12.5 (Fig. 8, A and B). Although the phenotypes appeared less compromised as compared with that in Shox2 mutants, the results nevertheless demonstrate an essential role of Bmp4 in the maintenance of the proper fate of the DMP.

Because Bmp2 is expressed in the adjacent AV canal at E11.5 and become localized to the developing DMP at E12.5, a functional compensation from Bmp2 may account for the relatively milder DMP defects in Shox2-Cre;Bmp4/F/F mice. To further establish a role for BMP signaling in DMP development and its fate maintenance, we overexpressed Noggin, a potent BMP antagonist preferentially binding to BMP2, BMP4, and BMP7 in Shox2-expressing cells by crossing Shox2-Cre mice to mice carrying a conditional Noggin transgenic allele (pMes-Nog). Taking the advantage of the co-expressed EGFP along with transgenic Noggin by the pMes-Nog allele (28), we were able to define the DMP domain where Shox2-Cre is expressed and trace molecular changes in Shox2-Cre;pMes-Nog transgenic animals. Not surprisingly, the phosphorylation of Smad1/5/8 was jeopardized severely in the AV junction (Fig. 8, E–H). As we expected, immunohistochemical analyses showed that Hcn4 expression was almost completely abolished accompanied by ectopic expression of Cx40 and aberrant activation of Caspase3 within the hypoplastic DMP region of Shox2-Cre;pMes-Nog mice (Fig. 8, I–X), resembling that observed in Shox2 mutants.

Smad-mediated BMP Signaling Regulates Hcn4 Expression—Although extensive studies have pinpointed the critical function of Hcn4 in CCS, the upstream regulators of Hcn4 expression have yet to be identified. Because phosphorylation of Smad1/5/8 is compromised along with the down-regulation of Hcn4 in the DMP of Shox2 mutants or mice carrying disrupted BMP signaling, we wondered if Smad-mediated BMP signaling acts as a positive upstream regulator of Hcn4 expression in the developing DMP. We conducted a loss-of-function study by creating Shox2Cre/H11001;Smad4F/F mice. In Smad4 conditional knock-out animals, again we found consistently deprived Hcn4 expression in the DMP at E12.5 and E14.5, establishing a Smad-Hcn4 regulatory cascade (Fig. 9, A and B; and data not shown).

To test if Smad complexes regulate Hcn4 expression directly, we took a bioinformatic approach to search for SBEs in about 17 kb covering the Hcn4 locus among placental mammals (43, 44). Although four potential SBEs were identified, one was highly conserved among the placental mammals, which is located within the first intron (Fig. 9D). Because the first intron of Hcn4

FIGURE 5. Altered gene expression in the Shox2 mutant DMP at E14.5. A–F, immunohistochemistry reveals down-regulation of Hcn4 in the β-Gal labeled hypoplastic DMP (dashed line) of an E14.5 Shox2LacZ/LacZ embryo (D–F) as compared with controls (A–C). G and H, immunohistochemistry shows the absence of Cx40 expression in the DMP (asterisk) of an E14.5 wild-type embryo but ectopic Cx40 expression (dashed line) in the DMP of an E14.5 Shox2LacZ/LacZ embryo. CT, cushion tissue; LV, left ventricle; RA, right atrium; VV, venus valve; RBC, red blood cell. Scale = 40 μm.
FIGURE 6. Aberrant APs of Shox2-deficient DMP myocardium. A and B, typical APs of Shox2\textsuperscript{LacZ/+} (A) and Shox2\textsuperscript{LacZ/LacZ} (B) DMP cells of E14.5 mice. C, overlaying of a typical AP of Shox2\textsuperscript{LacZ/+} DMP cells with that of Shox2\textsuperscript{LacZ/LacZ} DMP cells demonstrates the increased action potential amplitude and the rate of upstroke in Shox2 mutant DMP cells. D, a two-dimensional diagram showing the action potential amplitude and the maximum rate of rise of the AP (dV/dtmax) for each wild-type SAN, Shox2\textsuperscript{LacZ/+} DMP, and Shox2\textsuperscript{LacZ/LacZ} DMP cell studied. Please note the red lined region is identical to that mentioned in Fig. 2F. E–H, by contrast, in both mice, the atrial and ventricular myocytes show the similar action potential amplitude and the maximum rate of rise of the AP (dV/dtmax).
Shox2 and DMP Development

**Shox2\textsuperscript{LacZ/+}**

**Shox2\textsuperscript{LacZ/LacZ}**

|   | Bmp2 |   |
|---|------|---|
| A | RA   | LSH |
|   | CT   |    |

|   | Bmp4 |   |
|---|------|---|
| C | RA   | LSH |
|   | CT   | *  |

|   | Bmp2 |   |
|---|------|---|
| D | RA   | LV |
|   | CT   |    |

|   | Bmp4 |   |
|---|------|---|
| F | RA   | LSH |
|   | CT   |    |

|   | Bmp4 |   |
|---|------|---|
| H | RA   | LV |
|   | CT   |    |

|   | p-Smad1/5/8 |   |
|---|--------------|---|
| I | RA           | LSH |
|   | CT           |    |

|   | p-Smad1/5/8 |   |
|---|--------------|---|
| J | RA           | AVC |
|   | CT           | LV  |
has been demonstrated to harbor regulatory elements that are required for Hcn4 expression in cardiac myocytes (45, 46) as well as for its faithful recapitulation of Hcn4 expression in the AV conduction axis of transgenic animals (47), we therefore focused on this conserved Smad binding within the first intron. Subsequent in vivo ChIP assay using E13.5 heart extract and anti-Smad1/5/8 antibodies confirmed binding of the Smad1/5/8 to the vestibular spine, indicating its DMP origin, and does not contribute to the adjacent AVN. Although the mature AVN does not consist of Shox2-Cre lineage cells, a gene expression assay demonstrated that the Shox2-expressing DMP has the strongest combined expression of pacemaker maker Hcn4 and Tbx3 as well as nodal-like electrophysiological properties in the AV junction before the emergence of an identifiable AVN at late gestation stage. Thus, it appears that the embryonic DMP possesses the pacemaker feature and probably acts as a temporary pacemaker before the emergence of a mature AVN to ensure the proper sequence of conducting cardiac electrical pulses in the AV junction. The developing DMP appears to lose its pacemaking function, which may be gradually taken over by the maturing AVN at late gestation and early post-natal stages.

Shox2 Is Essential for DMP Development—Similar to the SAN defects observed in Shox2 null mutants, the Shox2 mutant DMP also displayed hypoplasia and defective differentiation. However, unlike the Shox2−/− SAN in which reduced cell proliferation rate was thought to contribute to the hypoplastic phenotype, the developing DMP in Shox2 mutants exhibited a slightly reduced cell proliferation rate but a significantly elevated cell apoptosis, suggesting that Shox2 regulates the development of SAN and DMP by controlling different cellular event. It was reported previously that some adjacent AV canal cells undergo apoptosis to facilitate the formation of AV structures in the four chambered heart (49). It appears that Shox2 functions to shield DMP cells from apoptotic fate, and the DMP cells in Shox2 mutants adopt similar cellular properties of neighboring working myocardium. The aberrant electrophysiological character and the loss of the expression of CCS-specific gene Hcn4 and ectopic activation of the working myocardial marker gene Cx40 within the DMP domain of Shox2 mutants, similar to the phenotype observed in the SAN, indicate a deviation of DMP cell fate and precocious adoption of working myocardial fate. These observations suggest an essential role for Shox2 not only in the regulation of tissue size but also in fate determination of both the SAN and DMP. It was reported previously that the defective SAN in Shox2 mutant embryos is the causative of bradycardia, which possibly contributes to embryonic lethality (10, 11, 48). However, the sinus bradycardia or even loss of sinus function normally is not a direct cause of death within a short period of time, because the AV junction is able to compensate the conducting firing rate (50). Thus, the severely slowed heart rate in Shox2 mutant animals is likely due to the disrupted functions in both the SAN and AV junction.

**DISCUSSION**

**Shox2-expressing DMP May Function as a Temporary Pacemaker during Embryogenesis**—We and others have shown previously that Shox2 is expressed in the SAN to promote cell proliferation and to function as a repressor of working myocardium genes and an inducer of CCS markers (10, 48). In our current study we revisited the expression pattern of Shox2 in the developing heart using a Shox2-LacZ mouse line and revealed a novel Shox2 expression domain in the developing AV junction. By lineage tracing, we were able to pinpoint that the Shox2-expressing population in the AV junction gives rise to the vestibular spine, indicating its DMP origin, and does not contribute to the adjacent AVN. Although the mature AVN does not consist of Shox2-Cre lineage cells, a gene expression assay demonstrated that the Shox2-expressing DMP has the strongest combined expression of pacemaker maker Hcn4 and Tbx3 as well as nodal-like electrophysiological properties in the AV junction before the emergence of an identifiable AVN at late gestation stage. Thus, it appears that the embryonic DMP possesses the pacemaker feature and probably acts as a temporary pacemaker before the emergence of a mature AVN to ensure the proper sequence of conducting cardiac electrical pulses in the AV junction. The developing DMP appears to lose its pacemaking function, which may be gradually taken over by the maturing AVN at late gestation and early post-natal stages.

**Ectopic Bmp4 Expression Rescues DMP Defect in Shox2 Mutants**—The evidence that BMP signaling is compromised in the developing DMP of Shox2 mutants and disruption of BMP signaling produces DMP defects similar to that in Shox2 mutants strongly suggests that BMP signaling is a major player to mediate Shox2 function during DMP development. To test this hypothesis, we conducted genetic rescue experiments using a conditional transgenic Bmp4 allele (pMesBmp4) compounded with Shox2-Cre and Shox2-LacZ alleles (Shox2Cre/LacZ). In such compound mice (pMes-Bmp4; Shox2Cre/LacZ), transgenic Bmp4 was activated in Shox2-expressing cells including the DMP under the Shox2 mutant background. In the developing DMP of pMes-Bmp4;Shox2Cre/LacZ mice, the pSmad1/5/8 level was resumed, and its expression domain was expanded as compared with wild-type controls and mutants (Fig. 10, A–C). Similarly, Hcn4 expression within the DMP region was also resumed (Fig. 10, D–F). Interestingly, although the majority of pMes-Bmp4;Shox2Cre/LacZ mice died perinatally, we were able to harvest one survived mutant mouse at the birth. Furthermore, the addition of exogenous BMP4 to isolated Shox2LacZ/LacZ DMP cells in cell culture for 18 h rescued the phenotype of electrophysiological properties, particularly the maximum rate of rise of the AP upstroke (Fig. 10, G–I; Table 1). These observations indicate that the Shox2 function in the regulation of DMP cell fate decision and development is mediated, at least partially, by BMP signaling.

**FIGURE 7. Disrupted BMP signaling in the Shox2 mutant DMP.** A and B, in situ hybridization assay reveals Bmp2 expression in the AV canal tissue (red arrowheads) but not in the DMP of both E11.5 Shox2LacZ−/− (A) and Shox2LacZ/LacZ (B) embryos. C and D, Bmp4 expression (arrowheads), as assayed by in situ hybridization, was observed in the DMP and the wall of the left sinus horn of an E11.5 Shox2LacZ−/− embryo (C) but was down-regulated in Shox2LacZ−/− embryo at the same stage (D). E–H, at E12.5, expressions of Bmp2 (E) and Bmp4 (G) is found in the developing DMP of wild-type embryos (E and G) but was dramatically reduced in Shox2 mutants (F and H). I and J, immunohistochmistry reveals a significantly reduced level of phosphorylated Smad1/5/8 in the DMP of Shox2 mutant (J) as compared with control (I). Asterisks indicate the DMP. CT, cushion tissue; LV, left ventricle; RA, right atrium; VV, venous valve; AVC, atrioventricular canal; LSH, left sinus horn. Scale bar = 40 μm.
cardiovascular development and embryonic lethality at early gestation stage (51, 52). Although Bmp2 and Bmp4 are expressed in the embryonic AV canal (21, 22), their roles in CCS development have not been addressed. The similar AV septal defect found in mice carrying cardiac-specific ablation of either Bmp4, Alk3, or Shox2 (5, 21) (this study) and the fact that Shox2 ablation led to deprived Bmp4 expression in the embryonic SAN (15) suggest the existence of a Shox2-Bmp4 regulatory cascade in the developing DMP. The down-regulation of Bmp4 expression within the Shox2-expressing DMP in the AV junction supports this notion, consistent with the observation found in the Shox2−/− SAN (15). Furthermore, Bmp2 expression was also reduced dramatically in the Shox2−/− DMP. Consistent with the abrogation of BMP ligands, phosphorylation of

FIGURE 8. Altered gene expression in the DMP of mice carrying disrupted BMP signaling. A–D, an E12.5 Shox2-Cre;Smad4f/f embryo exhibits absent Hcn4 expression (white arrowhead) in the DMP (B) as compared with control (A). C and D, ChIP assay and schematic diagrams of genomic regions of the Hcn4 locus show the position of the conserved Smad binding site highlighted in the red rectangle and conserved binding sequence highlighted in background among several placental mammals (D) and binding of pSmad1/5/8 to conserved Smad binding site (C). E and F, immunohistochemistry reveals an elevated pSmad1/5/8 level in the forming AV node but a significantly reduced pSmad1/5/8 level in the DMP (asterisk) derived from Shox2-expressing lineage cells. Dotted lines demarcate the DMP, defined by the expression domains of Hcn4 (A and B) and β-Gal (E and F). CT, cushion tissue; RA, right atrium; VV, venous valve; VS, ventricular septum; LSH, left sinus horn. Scale bars = 40 μm.

FIGURE 9. Smad4-mediated signaling is essential for Hcn4 expression in the DMP. A and B, an E12.5 Shox2-Cre;Smad4f/f embryo exhibits absent Hcn4 expression (white arrowhead) in the DMP (B) as compared with control (A). C and D, ChIP assay and schematic diagrams of genomic regions of the Hcn4 locus show the position of the conserved Smad binding site highlighted in the red rectangle and conserved binding sequence highlighted in background among several placental mammals (D) and binding of pSmad1/5/8 to conserved Smad binding site (C). E and F, immunohistochemistry reveals an elevated pSmad1/5/8 level in the forming AV node but a significantly reduced pSmad1/5/8 level in the DMP (asterisk) derived from Shox2-expressing lineage cells. Dotted lines demarcate the DMP, defined by the expression domains of Hcn4 (A and B) and β-Gal (E and F). CT, cushion tissue; RA, right atrium; VV, venous valve; VS, ventricular septum; LSH, left sinus horn. Scale bars = 40 μm.
Smad1/5/8 was almost abolished. It should be pointed out that at E11.5, Bmp2 expression is largely not overlapped with Shox2 in the AV junction. The strong Bmp2 expression in the surrounding AV canal tissue likely compensates for the loss of Bmp4 expression, which explains why Shox2 null mice did not display obvious DMP defect at this stage. Although the importance of BMP signaling in DMP development has been illustrated in mice carrying Alk3 deletion in the second heart field (5), in our current study we found that mice bearing Bmp4 deletion (Shox2-Cre;Bmp4^{f/f}) or Noggin overexpression (Shox2-Cre;Mes-Nog) in Shox2-expressing cells exhibit not only hypoplastic but also defectively DMP differentiation that is precocious deviation of its pacemaking-like fate to working myocardium. However, the relatively milder DMP phenotype seen in Shox2-Cre;Bmp4^{f/f} mice as compared with that in Shox2-null and Shox2-Cre;Mes-Nog mice further supports a functional redundant role of Bmp2 and Bmp4 during DMP development. A piece of additional compelling evidence to support the critical role of BMP signaling in DMP development came from the rescue experiments in which activation of a transgenic Bmp4 allele in the Shox2-expressing domain under the Shox2 mutant background resumed Hcn4 expression and rescued, if not fully, the hypoplastic phenotype of DMP. In addition, the AP pattern of Shox2^{−/−} DMP cells could also be rescued by exogenous BMP4. Taken together, our results demonstrate that Shox2 functions through Bmp2/Bmp4 to regulate DMP development and differentiation, and BMP signaling is both necessary and sufficient to induce Hcn4 expression in the developing DMP.

**Smad-dependent Pathway Regulates Hcn4 Expression**—The hyperpolarization-activated, cyclic nucleotide-gated 4 (Hcn4) is regarded as a molecular marker for CCS and functions to generate If (funny/pacemaker) current that is essential for pacemaking activity. Indeed, deprivation of Hcn4 results in rhythm disorders and embryonic lethality (24, 25). Several transcription factors including Tbx3, MEF2, NRSF, and SP1 have been reported to regulate Hcn4 transcription, but these factors were either shown to bind to the Hcn4 regulatory elements by in vitro assays or characterized as an inducer for ectopic Hcn4 expression in chamber myocardium at a late developmental stage (45, 46, 53). BMP signaling is known to be mediated by Smad-dependent canonical and Smad-independent non-canonical pathways (18, 19). Although it has been reported that Bmp2-mediated Smad signaling functions through Tbx2 as a
Shox2 and DMP Development

repressor of chamber-specific genes such as Cx40, Cx43, and ANF in the developing AV conduction axis (23), little is known about the direct role of BMP/Smad in promoting conduction cell differentiation. In our study we present evidence that Smad-mediated signaling is essential for DMP development and for Hcn4 expression. Our in vivo ChIP assay confirmed direct binding of pSmad1/5/8 to the conserved sequence within the first intron of Hcn4 that has been shown to be essential for Hcn4 expression in cell cultures and for faithful recapitulation of Hcn4 expression in transgenic animals (45–47), suggesting a direct regulation of Hcn4 transcription by Smad complex. Nevertheless, our studies reveal an essential function for Shox2 in DMP development and differentiation and establish a Shox2-BMP/Smad signaling axis in the regulation of Hcn4 expression in the developing DMP, which likely functions as a temporary pacemaking tissue in the AV junction during cardiogenesis.

Acknowledgment—We thank Dr. Jeffrey Tasker for fruitful discussions and technical support.

REFERENCES

1. Mangoni, M. E., and Nargeot, J. (2008) Genesis and regulation of the heart autonomic. Physiol. Rev. 88, 919–982
2. Wessels, A., Markman, M. W., Vermeulen, J. L., Anderson, R. H., Moorman, A. F., and Lamers, W. H. (1996) The development of the atrioventricular junction in the human heart. Circ. Res. 78, 110–117
3. Christoféls, V. M., Smits, G. J., Kispert, A., and Moorman, A. F. (2010) Development of the pacemaker tissues of the heart. Circ. Res. 106, 240–254
4. Munshi, N. V. (2012) Gene regulatory networks in cardiac conduction system development. Circ. Res. 110, 1525–1537
5. Briggs, L. E., Phelps, A. L., Brown, E., Kakarla, J., Anderson, R. H., van den Hoff, M. J., and Wessels, A. (2013) Expression of the BMP receptor Alk3 in the second heart field is essential for development of the dorsal mesenchymal protrusion and atrioventricular septation. Circ. Res. 112, 1420–1432
6. Argüello, C., Alanís, J., Pantoja, O., and Valenzuela, B. (1986) Electrophysiological and ultrastructural study of the atrioventricular canal during the development of the chick embryo. J. Mol. Cell Cardiol. 18, 499–510
7. Blaschke, R. J., Monaghan, A. P., Schiller, S., Scheckinger, B., Rao, E., Padilla-Nash, H., Ried, T., and Rappold, G. A. (1998) SHOT, a SHOX-related homeobox gene, is implicated in craniofacial, brain, heart, and limb development. Proc. Natl. Acad. Sci. U.S.A. 95, 2406–2411
8. Semina, E. V., Reiter, R. S., and Murray, J. C. (1998) A new human homeobox gene OGI2X is a member of the most conserved homeobox gene family and is expressed during heart development in mouse. Hum. Mol. Genet. 7, 415–422
9. Binder, G. (2011) Short stature due to SHOX deficiency: genotype, phenotype, and therapy. Hormn. Res. Paediatr. 75, 81–89
10. Blaschke, R. J., Hahurij, N. D., Kuijper, S., Just, S., Wisse, L. J., Deissler, K., Bruneau, B. G., Blaschke, R. J., Steinbeisser, H., and Rappold, G. (2010) Shox2 mediates Tbx5 activity by regulating Bmp4 in the pacemaker region of the developing heart. Hum. Mol. Genet. 19, 4625–4633
11. Sun, C., Zhang, T., Liu, C., Gu, S., and Chen, Y. (2013) Generation of Shox2-Cre allele for tissue specific manipulation of genes in the developing heart, palate, and limb. Genesis 51, 515–522
12. Wozney, J. M., Rosen, V., Celeste, A. J., Miltosk, L. L., Whitters, M. J., Kritz, R. W., Hewick, R. M., and Wang, E. A. (1988) Novel regulators of bone formation: molecular clones and activities. Science 242, 1528–1534
13. Yu, L., Gu, S., Alappat, S., Song, Y., Yan, M., Zhang, X., Zhang, G., Jiang, Y., Zhang, Z., Zhang, Y., and Chen, Y. (2005) Shox2-deficient mice exhibit a rare type of incomplete clefing of the secondary palate. Development 132, 4397–4406
14. Cobb, J., Dierich, A., Huss-Garcia, Y., and Duboule, D. (2006) A mouse model for human short-stature syndromes identifies Shox2 as an upstream regulator of Runx2 during long-bone development. Proc. Natl. Acad. Sci. U.S.A. 103, 4511–4515
15. Puskár, S., Schmitteckert, S., Mori, A. D., Glaser, A., Schneider, K. U., Kriz, R. W., Hewick, R. M., and Wang, E. A. (2003) Notch-2 regulates bone formation: molecular Clones and activities. Science 242, 1528–1534
16. Sun, C., Zhang, T., Liu, C., Gu, S., and Chen, Y. (2013) Generation of Shox2-Cre allele for tissue specific manipulation of genes in the developing heart, palate, and limb. Genes Dev. 51, 515–522
17. Wozney, J. M., Rosen, V., Celeste, A. J., Miltosk, L. L., Whitters, M. J., Kritz, R. W., Hewick, R. M., and Wang, E. A. (1988) Novel regulators of bone formation: molecular Clones and activities. Science 242, 1528–1534
18. Massague, J., and Chen, Y. G. (2000) Controlling TGF-β signaling. Genes Dev. 14, 627–644
19. Zhang, Y. E. (2009) Non-Smad pathways in TGF-β signaling. Cell Res. 19, 128–139
20. Wang, J., Greene, S. B., and Martin, J. F. (2011) BMP signaling in congenital heart disease: new developments and future directions. Birth Defects Res. A Clin. Mol. Teratol. 91, 441–448
21. Inoue, K., Kurella, H., Tompkins, K., Zhou, Y., Batts, L., Baldwin, H. S., and Hogan, B. L. (2003) An essential role of Bmp4 in the atrioventricular septation of the mouse heart. Genes Dev. 17, 2362–2367
22. Ma, L., Lu, M. F., Schwartz, R. J., and Martin, J. F. (2005) Bmp2 is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning. Development 132, 5601–5611
23. Shirai, M., Imanaka-Yoshida, K., Schneider, M. D., Schwartz, R. J., and Morisaki, T. (2009) T-box 2, a mediator of Bmp-Smad signaling, induced hyaluronan synthase 2 and Tgfβ2 expression and endocardial cushion formation. Proc. Natl. Acad. Sci. U.S.A. 106, 18604–18609
24. Stieber, J., Herrmann, S., Feil, S., Löster, J., Feil, R., Biel, M., Hofmann, F., and Ludwig, A. (2003) The hyperpolarization-activated channel HCN4 is required for the generation of pacemaker action potentials in the embryonic heart. Proc. Natl. Acad. Sci. U.S.A. 100, 15235–15240
25. Baruscotti, M., Bucchi, A., Viscomi, C., Mandelli, G., Consolati, G., Gnecci-Rusconi, T., Montano, N., Casali, K. R., Micheli, S., Barbuti, A., and DiFrancesco, D. (2011) Deep bradycardia and heart block caused by inducible cardiac-specific knockout of the pacemaker channel gene Hcn4. Proc. Natl. Acad. Sci. U.S.A. 108, 1705–1710
26. Frank, D. U., Carter, K. L., Thomas, K. R., Burr, R. M., Bakker, M. L., Coetzee, A. W., Tristani-Firouzi, M., Bamshad, M. J., Christoffels, V. M., and Moon, A. M. (2012) Lethal arrhythmias in Tbx3-deficient mice reveal extreme dosage sensitivity of cardiac conduction system function and homeostasis. Proc. Natl. Acad. Sci. U.S.A. 109, E154–E163
27. He, F., Hu, X., Xiong, W., Li, L., Lin, L., Shen, B., Yang, L., Gu, S., Zhang, Y., and Chen, Y. (2014) Directed Bmp4 expression in neural crest cells generates a genetic model for the rare human bony synosthia birth defect. Dev. Biol. 391, 170–181
28. He, F., Xiong, W., Wang, Y., Matsui, M., Yu, X., Chai, Y., Klingen smith, J., and Chen, Y. (2010) Modulation of BMP signaling by Noggin is required for the maintenance of palatal epithelial integrity during palatogenesis. Dev. Biol. 347, 109–121
29. Kurella, H., and Hogan, B. L. (2002) Generation of a loxP flankedbmp4loxP-lacZ allele marked by conditional lacZ expression. Genesis 32, 66–68
30. Mangoni, M. E., and Nargeot, J. (2001) Properties of the hyperpolarization-activated current (If) in isolated mouse sino-atrial cells. Cardiovasc. Res. 52, 51–64
31. Hancock, J. C., Levi, A. J., Lee, C. O., and Heap, P. (1993) A method for isolating rabbit atrioventricular node myocytes which retain normal morphology and function. Am. J. Physiol. 265, H755–H766
32. Chen, B., Wu, Y., Mohler, P. J., Anderson, M. E., and Song, L. S. (2009) Local control of Ca2+-induced Ca2+ release in mouse sinoatrial node cells.
J. Mol. Cell Cardiol. 47, 706–715

Ye Sheng, X., Qu, Y., Dan, P., Lin, E., Korthout, L., Bradford, A., Hovemadsen, L., Sandani, T., and Tibbits, G. F. (2011) Isolation and characterization of atrioventricular nodal cells from neonate rabbit heart. Circ. Arrhythm. Electrophysiol. 4, 936–946

Laugwitz, K. L., Moretti, A., Lam, J., Gruber, P., Chen, Y., Woodard, S., Lin, L. Z., Cai, C. L., Lu, M. M., Reth, M., Platoshyn, O., Yuan, J. X., Evans, S., and Chien, K. R. (2005) Postnatal islet+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433, 647–653

Soufan, A. T., Ruijter, J. M., van den Hoff, M. J., de Boer, P. A., Hagoort, J., Gier-de Vries, C., Anderson, R. H., Kispert, A., Moorman, A. F., and Moorman, A. F. (2003) Three-dimensional reconstruction of gene expression patterns during cardiac development. Physiol. Genomics 13, 187–195

Zhang, Z., Song, Y., Zhao, X., Zhang, X., Fermin, C., and Chen, Y. (2002) Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development 129, 4135–4146

Wang, J., Greene, S. B., Bonilla-Claudio, M., Tao, Y., Zhang, J., Bai, Y., Huang, Z., Black, B. L., Wang, F., and Martin, J. F. (2010) Bmp signaling regulates myocardial differentiation from cardiac progenitors through a microRNA-mediated mechanism. Dev. Cell 19, 903–912

Aanhaanen, W. T., Mommersteeg, M. T., Norden, I., Wakker, V., de Gier-de Vries, C., Anderson, R. H., Kispert, A., Moorman, A. F., and Christoffers, V. M. (2010) Developmental origin, growth, and three-dimensional architecture of the atrioventricular conduction axis of the mouse heart. Circ. Res. 107, 728–736

McGuire, M. A., de Bakker, J. M., Vermeulen, J. T., Moorman, A. F., Loh, P., Thibault, B., Vermeulen, J. L., Becker, A. E., and Janse, M. J. (1996) Atrioventricular junctional tissue. Discrepancy between histological and electrophysiological characteristics. Circulation 94, 571–577

Zhang, Y. M., Hartzell, C., Narlow, M., and Dudley, S. C., Jr. (2002) Stem cell-derived cardiomyocytes demonstrate arrhythmic potential. Circulation 106, 1294–1299

Zipes, D., and Jalife, J. (2009) Cardiac Electrophysiology: From Cell to Bedside, 2009 Ed., Elsevier, Philadelphia, PA

Mahtab, E. A., Vicente-Steijn, R., Hahurij, N. D., Jongbloed, M. R., Wisse, L. J., DeRuiter, M. C., Uhrin, P., Zaujec, J., Binder, B. R., Schalji, M. J., Poelmann, R. E., and Gittenberger-de Groot, A. C. (2009) Podoplanin deficient mice show a Rhoad-related hypoplasia of the sinus venous myocardium including the sinoatrial node. Dev. Dyn. 238, 183–193

Karaulanov, E., Knöchel, W., and Niehrs, C. (2004) Transcriptional regulation of BMP4 synexpression in transgenic Xenopus. EMBO J. 23, 844–856

Karolchik, D., Barber, G. P., Casper, J., Clawson, H., Cline, M. S., Diekhans, M., Deser, T. R., Fujita, P. A., Gurudadoo, L., Haeussler, M., Harte, R. A., Heitner, S., Hinichs, A. S., Learned, K., Lee, B. T., Li, C. H., Raney, B. J., Rhead, B., Rosenbloom, K. R., Sloan, C. A., Speir, M. L., Zweig, A. S., Haussler, D., Kuhn, R. M., and Kent, W. J. (2014) The UCSC Genome Browser database: 2014 update. Nucleic Acids Res. 42, D764–D770

Kuratomi, S., Kuratomi, A., Kuwahara, K., Ishii, T. M., Nakao, K., Saito, Y., and Takano, M. (2007) NRSF regulates the developmental and hypertrophic changes of HCN4 transcription in rat cardiac myocytes. Biochem. Biophys. Res. Commun. 353, 67–73

Kuratomi, S., Ohmori, Y., Ito, M., Shimazaki, K., Muramatsu, S., Mizukami, H., Usaki, H., Yamashita, J. K., Arai, Y., Kuwahara, K., and Takano, M. (2009) The cardiac pacemaker-specific channel Hcn4 is a direct transcriptional target of MEF2. Cardiovasc. Res. 83, 682–687

Vedantham, V., Evangelista, M., Huang, Y., and Srivastava, D. (2013) Spatiotemporal regulation of an Hcn4 enhancer defines a role for Mef2c and HDACs in cardiac electrical patterning. Dev. Biol. 373, 149–162

Espinoza-Lewis, R. A., Liu, H., Sun, C., Chen, C., Jiao, K., and Chen, Y. (2011) Ectopic expression of Ntx2.5 suppresses the formation of the sinoatrial node in mice. Dev. Biol. 356, 359–369

Hahurij, N. D., Gittenberger-de Groot, A. C., Kolditz, D. P., Bökenkamp, R., Schalji, M. J., Poelmann, R. E., and Blom, N. A. (2008) Accessory atrioventricular myocardial connections in the developing human heart: relevance for perinatal supraventricular tachycardias. Circulation 117, 2850–2858

Hall, J. (2011) Guyton and Hall Textbook of Medical Physiology, Elsevier, Philadelphia, PA

Winner, G., Blessing, M., Labosky, P. A., and Hogan, B. L. (1995) Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev. 9, 2105–2116

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

Lin, H., Xiao, J., Luo, X., Chen, G., and Wang, Z. (2009) Transcriptional control of pacemaker channel genes HCN2 and HCN4 by Sp1 and implications in re-expression of these genes in hypertrophied myocytes. Cell Physiol. Biochem. 23, 317–326