Integration of a Notch-dependent mesenchymal gene program and Bmp2-driven cell invasiveness regulates murine cardiac valve formation

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Cardiac valve formation is crucial for embryonic and adult heart function. Valve malformations constitute the most common congenital cardiac defect, but little is known about the molecular mechanisms regulating valve formation and homeostasis. Here, we show that endocardial Notch1 and myocardial Bmp2 signal integration establish a valve-forming field between 2 chamber developmental domains. Patterning occurs through the activation of endocardial epithelial-to-mesenchymal transition (EMT) exclusively in prospective valve territories. Mice with constitutive endocardial Notch1 activity ectopically express Hey1 and Heyl. They also display an activated mesenchymal gene program in ventricles and a partial (noninvasive) EMT in vitro that becomes invasive upon BMP2 treatment. Snail1, TGF-β2, or Notch1 inhibition reduces BMP2-induced ventricular transformation and invasion, whereas BMP2 treatment inhibits endothelial Gsk3β, stabilizing Snail1 and promoting invasiveness. Integration of Notch and Bmp2 signals is consistent with Notch1 signaling being attenuated after myocardial Bmp2 deletion. Notch1 activation in myocardium extends Hey1 expression to nonchamber myocardium, represses Bmp2, and impairs EMT. In contrast, Notch deletion abrogates endocardial Hey gene transcription and extends Bmp2 expression to the ventricular endocardium. This embryonic Notch1-Bmp2-Snail1 relationship may be relevant in adult valve disease, in which decreased NOTCH signaling causes valve mesenchyme cell formation, fibrosis, and calcification.

Introduction
Congenital cardiac valvuloseptal defects are the most frequently diagnosed developmental malformations during the first year of human life (1), and several valvular defects manifest in adult disease (2). Evidence from animal models has linked many genes to targeted mutant mice suggest they affect functionally interconnected signaling pathways (5).

Cardiac valve formation (CVF) in mice begins at approximately E9.5. In the developing atrioventricular canal (AVC) and outflow tract (OFT), myocardial signals induce adjacent endocardial cells (ENCs) to undergo epithelial-to-mesenchymal transition (EMT) (5). These cells invade the underlying matrix (cardiac jelly) to generate the heart valve primordia (endocardial cushions). Cardiac EMT begins with ENC activation, in which ENCs swell due to the enlargement of the Golgi and rough endoplasmic reticulum. Transformation follows, in which ENCs lose cell-cell contacts, acquire the ability to move into the plane of the monolayer, polarize their Golgi, and form migratory appendages. EMT is completed once the transformed ENCs invade the cardiac jelly to form the valvuloseptal mesenchyme (6). ENC transformation is dependent on the initiation of EMT-specific transcriptional machinery, the physical detachment of epithelial cells, and the activation and stabilization of a migratory and invasive mesenchymal phenotype.

Defining the primary signal or signals inducing cardiac EMT has been problematic due to the difficulties of extrapolating results from chick and mouse (5) and because the exact extent of spatiotemporal interaction between myocardium and endocardium cannot be fully reproduced in vitro. Data from collagen gel assays with chicken tissue suggested TGF-β2 or TGF-β3 as the main myocardial EMT inducers (7), however, Tgfb2- or Tgfb3-deficient mice show no endocardial EMT defect, suggesting induction of mammalian EMT by other signals (8). Indeed, in mice, Bmp2 is critical for EMT induction in vitro (9) and in vivo (10) and Bmp2 alone can specify a field of cardiac progenitors as a heart valve-inducing region (11).

Within the endocardium, EMT is regulated by Notch (12), an ancient local cell signaling system in which both ligands (Delta and Jagged) and receptors (Notch) are membrane bound (13). Ligand-receptor binding initiates 2 consecutive receptor cleavage steps, the last one involving γ-secretase (13), to generate the biologically active Notch intracellular domain (NICD). In the nucleus, NICD binds the RB/PJK/CSL transcription factor and regulates target gene expression and thus cell fate specification (13). Notch1 activity delineates AVC and OFT endocardium in the mouse E9.5 heart (14), and the AVC of RBPJK- and Notch1-targeted mutants almost completely lacks mesenchymal cushion cells (12). Although
This correlates with reduced transcription of the cadherin repres-  
Snail1 protein stabilization mediated by Bmp2 via Gsk3  
maintain adherens junctions, and fail to invade the cardiac jelly.  
WT mouse AVC explants and zebrafish embryos (12). Moreover,  
venting pathways. Snail1 expression induced by Notch1 and Bmp2 and  
protein stabilization mediated by Bmp2 via Gsk3β inhibition  
suggest that Notch1 and Bmp2 signals converge to promote ENC  
inasiveness. We propose a model in which the interplay between  
myocardial Bmp2 and endocardial Notch1 restricts EMT to pro-  
spective valve territory. In this setting, the prevalvar endocardium  
is not a passive tissue, simply responding to inductive myocardial  
signals, but plays an active role in promoting EMT.

Results

Endocardial Notch1 activation causes heart dysmorphogenesis and ecto-  
pic target gene expression. To investigate whether ectopic Notch1  
activation in the heart was sufficient to drive EMT, we activated  
Notch1 in the endocardium, myocardium, or both tissues. We used  
the conditional N1ICD transgenic line R26N1ICD, which  
upon Cre activation expresses the Notch1 intracellular domain (ama-  
ino acids 1749–2293) and nuclear-localized enhanced GFP  
(20). R26N1ICD mice (from now on, N1ICD) were crossed with  
the endothelium/endocardium-specific Tie2-Cre driver line (ref.  
21 and Supplemental Figure 1A; supplemental material available  
online with this article; doi:10.1172/JCI42666DS1). At E9.5,  
Tie2-Cre;N1ICD embryos were smaller than WT littermates and  
showed pericardial distension and enlarged LV and atrium (Fig-  
ure 1A). EGFP expression delineated the endocardium, indicating  
n1ICD expression (Figure 1B). Scanning electron microscopy  
revealed AVC mesenchymal cells invading the cushion region in  
E9.5 WT embryos (Figure 1, C and F). Transgenic littermates had  
relatively fewer mesenchymal cells in AVC cushion tissue (Figure  
1, D and F), probably due to a slight developmental delay. H&E-  
stained sections were consistent with these findings (Supplemen-  
tal Figure 1, B–E) and revealed poorly formed trabeculae in E9.5  
transgenic embryos (Supplemental Figure 1, F and G).

Whole-mount in situ hybridization (WISH) and RT-PCR in Tie2-  
Cre;N1ICD embryos showed that endocardial expression of N1ICD  
and its targets Hey1 and Hey2 (22), Hey1l (17), Delta-like 4 (23), Nrarp  
(24), and c-myc (25) was increased and expanded from the AVC to  
the ventricles (Supplemental Figure 1, H–T), indicating increased  
Notch activity throughout the endocardium. CD31/PECAM and  
MLC2v expression, indicative of early endocardial and myocardial  
differentiation, were normal (Supplemental Figure 2 and data not  
shown). The expression domains of Anf (26) and Chisel (27) in the  
AVC were less well defined in Tie2-Cre;N1ICD embryos compared  
with WT animals (Supplemental Figure 2, A and B), though their  
expression levels were unaffected (Supplemental Figure 2K and  
data not shown). Irx5, normally expressed in ventricular endocar-  
dium (ref. 28 and Supplemental Figure 2C), was strongly reduced  
in Tie2-Cre;N1ICD embryos (Supplemental Figure 2, D and K), sug-  
gest ing loss of chamber identity.

The expression of endothelial identity markers was examined,  
given that the Tie2-Cre driver is active in the vascular endothelium as  
well (21). Tie2-Cre;N1ICD embryos ectopically expressed the Dhh4  
and Efnb2 markers in veins (Supplemental Figure 3, A–D), while  
expression of the venous marker Epbh4 was reduced (Supplemen-  
tal Figure 3, E and F), suggesting that the vascular endothelium of  
transgenic embryos had lost its venous identity. Tie2-Cre;N1ICD  
embryos died at E11.0, probably as a consequence of defective  
vascular and hematopoietic development (P. Melgar and J.L. de  
la Pompa, unpublished observations).

Activation of a mesenchyme gene expression program characteristic of  
AVC in the ventricular endocardium of Tie2-Cre;N1ICD embryos. Bmp2  
is expressed in AVC myocardium at E9.5 and triggers the EMT of  
adjacent ENCs (9). Bmp2 expression was slightly weaker at E9.5  
and was unaffected in E10.5 Tie2-Cre;N1ICD embryos (Supple-
Figure 2

Tie2-Cre;N1ICD ventricular explants undergo Tgf-β2– and Snail1-mediated ectopic EMT. (A, E, and I) Details of WT explants. (B, F, J, M, and N) Details of Tie2-Cre;N1ICD explants. Full lateral views of explants are shown below panels A–N. Schematic of a full lateral view of explant is shown at the bottom of panel A. e., endocardium (red); m., myocardium (green). All explants were stained with phalloidin-FITC (green), anti-α-SMA-Cy3 (red), and DAPI (blue). Arrows mark ENCs. (C, G, K, and Q) Quantitative analysis of 2D and 3D TI. (D, H, L, and P) RT-PCR of explant endocardium. (A) WT. Arrow. ENCs growing as a monolayer. The lateral section shows ENC outgrowth on the collagen surface. (B) Tie2-Cre;N1ICD. Arrows. scattered ENCs that have undergone partial EMT. (C) 2D TI is increased in Tie2-Cre;N1ICD explants (P = 3.7 x 10^-4). (D) Snail1, Snail2, Tgfβ2, Vimentin, and Periostin expression is upregulated; Has2 is slightly increased and Twist1 appears unaffected. (E, F, and G) ENCs scatter without invading the collagen in Tgf-β2–treated WT explants, and 2D TI is increased with respect to untreated WT ones (C; P = 1.8 x 10^-7) and Tie2-Cre;N1ICD explants. (H) Increased Snail1, Snail2, and Tgfβ2 expression. (I and K) ENCs of WT explants cultured with Tgf-β2 and anti-TGF-β2 antibody grow as a monolayer and show reduced 2D TI with respect to Tgf-β2–treated WT (G; P = 8.1 x 10^-4). (J and K) Anti-TGF-β2 reduces ENC migration in Tie2-Cre;N1ICD explants, reflected in a reduced 2D TI (P = 2.3 x 10^-4) and attenuated Snail1 expression (L). (M–O) Lentiviral-mediated shRNA Snail1 downregulation in transgenic explants reduces ENC migration with respect to GFP-transduced control explants (P = 3.3 x 10^-6). LVI, lentivirus. (P) Expression of Snail2 and Tgfβ2 is reduced. Scale bar: 50 μm. Results are expressed as mean ± SD. ***P < 0.001.

mental Figure 2, E, F, and K). Tgf-β2, which acts downstream of Bmp2 (9) is transcribed in AVC myocardium at E9.5 and at lower levels in AVC endocardium and mesenchyme (ref. 8, Figure II, and Supplemental Figure 5A). Tgfβ2 showed slightly weaker expression in Tie2-Cre;N1ICD AVC and was ectopically expressed in ventricular endocardium (Figure IJ and Supplemental Figure 5B). RT-PCR revealed normal Tgfβ2 expression, probably because of ectopic expression in the ventricles (Figure 1G). Snail1, expressed in response to Notch (12) and TGF-β2 (29), was detected in AVC endocardium and mesenchymal cells of WT embryos (Figure 1K and Supplemental Figure 5C). In transgenic embryos, Snail1 transcription was extended to the ventricular endocardium (Figure 1L and Supplemental Figure 5D) and increased (Figure 1G). Ectopic Snail1 expression in ventricles was confirmed by Western blot (Figure 1H). Immunostaining showed Snail1 expression in the nuclei of WT and Tie2-Cre;N1ICD AVC endocardial and mesenchyme cells (Figure 1, Q–T). Transgenic embryos displayed ectopic nuclear Snail1 expression in ventricular ENCs (Figure 1, U and V). Sox9, a Tgfb2 target in mesenchymal cells implicated in endocardial cushion cell differentiation (30), was transcribed in AVC endocardium and mesenchyme (Supplemental Figure 4A) and its expression was increased and expanded to the ventricles in transgenic embryos (Supplemental Figure 4, B and I).

Affymetrix microarray analysis to identify additional genes differentially expressed in Tie2-Cre;N1ICD E9.5 hearts uncovered 516 genes, 66 of which were downregulated and 450 of which were upregulated (Supplemental Excel file). Many of the upregulated genes were associated with EMT initiation, mesenchymal phenotype maintenance, cell migration, invasion, adhesion, and collagen deposition (Supplemental Table 1). These included Snail2, Twist2, Bmp6, and the Bmp receptor genes Alk3 and Alk6 (Supplemental Table 1), all of which are essential for EMT in different cellular contexts (31–33). Snail2, like Snail1, is required for Notch-induced EMT (31), and its expression was expanded in Tie2-Cre;N1ICD embryos from AVC endocardium and mesenchyme to the ventricular endocardium (Figure 1, M and N, and Supplemental Figure 5, E and F) and increased (Figure 1G). Twist1 is required downstream of Bmp2 during AVC EMT (10), but Twist1 expression was not altered in the microarray (Supplemental Excel file) or by WISH or RT-PCR (Supplemental Figure 2, G, H, and K), suggesting that Bmp2-dependent signaling in Tie2-Cre;N1ICD embryos was unaltered. In contrast, Twist2 expression was expanded from AVC to ventricles (Figure 1, O and P, and Supplemental Figure 5, G and H) and increased (Figure 1G), but the late EMT marker Has2 (34) was unaltered (Supplemental Figure 2, I–K). Similarly, Bmp6, normally expressed in AVC endocardium (Supplemental Figure 4E) and required for cushion morphogenesis (33), was ectopically expressed in the ventricle of transgenic embryos (Supplemental Figure 4, F and I). Expression of the chemokine receptor gene Cxcr4, involved in cell migration and metastasis (35), was increased in transgenic embryos (Supplemental Table 1 and Supplemental Figure 4I) and expanded from AVC endocardium to ventricular endocardium (Supplemental Figure 4, G and H). A further 12 genes involved in EMT, cell migration, and invasion were significantly increased in transgenic embryos (Supplemental Table I), and upregulation of the Notch pathway genes detected by WISH and RT-PCR (Supplemental Figure 1, H–T) was also confirmed. Thus, Tie2-Cre;N1ICD embryos show the expansion of mesenchymal gene expression from AVC to ventricles. Moreover, expression of the chamber marker Hand2 was strongly reduced (Supplemental Table I), confirming the loss of ventricular chamber identity in Tie2-Cre;N1ICD embryos.

Tie2-Cre;N1ICD ventricular explants undergo Tgf-β2 and Snail1-dependent EMT. Culture of AVC explants on a type I collagen gel lattice is an excellent functional assay to evaluate EMT (36). To quantify differences between WT and transgenic explants, we determined the “transformation index” (TI) in 2 and 3 dimensions. 2D TI is the fraction of ENCs able to detach and migrate on the gel surface without invading the collagen matrix. 3D TI is the fraction able to invade the gel. After 72 hours, E9.5 WT AVC explants produced a halo of mesenchymal cells around the myocardium (Supplemental Figure 6A), with mesenchymal cells invading the collagen depths (Supplemental Figure 6A and Supplemental Video 1). E9.5 Tie2-Cre;N1ICD explants also produced invasive mesenchymal cells (Supplemental Figure 6B and Supplemental Video 1), but while 3D TI was similar for both genotypes (3D = 0.1), 2D TI was 3-fold higher in transgenic explants (0.35 vs. 0.15; Supplemental Figure 6C). Expression of EMT-inducing molecules in WT and transgenic ENCs was similar. Similar to the situation in vivo, Hey1 was ectopically and upregulated in transgenic ENCs, together with Hey2, Hey4, and the mesenchymal marker Vimentin (Supplemental Figure 6D).

To determine whether ectopic Notch1 activity conferred transforming ability on ventricular tissue, we performed explant cultures with the distal tip of the LV. WT explants generated an endocardial monolayer surrounding the myocardium (Figure 2A), and ENCs grew as a coherent epithelium on the collagen surface (Figure 2A and Supplemental Video 2). In contrast, in Tie2-Cre;N1ICD explants, ENCs migrated over the gel and had a mesenchymal phenotype revealed by α-SMA staining (Figure 2B). These transformed cells did not invade the collagen matrix (Figure 2B and Supplemental Video 2), but their migratory ability was reflected in a sharply increased 2D TI (0.9 vs. 0.1; Figure 2C). Unlike WT cells,
ENCs of transgenic explants expressed the mesenchymal markers Snail1, Snail2, Tgfβ2, Vimentin, Periostin (37), and very low levels of Has2 (ref. 34 and Figure 2D) and Collagen. Twist1 was unaffected, consistent with the in vivo data (Figure 2D).

The transformed ventricular ENC of Tie2-Cre;N1ICD explants showed increased expression of Tgfβ2 (Figure 2D), a key EMT inducer in AVC cells (8). Addition of TGF-β2 to WT ventricular explants mimicked the results with Tie2-Cre;N1ICD explants, inducing surface migration of ENCs but not invasion (Figure 2E). TGF-β2-treated WT ventricular explants had a 2D TI similar to untreated transgenic explants (0.75 vs. 0.9; Figure 2G) and expressed Snail1, Snail2, and Vimentin (Figure 2H). TGF-β2 had no additive effect on Tie2-Cre;N1ICD explants (Figure 2, F and G). A neutralizing anti–TGF-β2 antibody reverted the phenotype of Tie2-Cre;N1ICD and TGF-β2–treated WT ventricular explants to the endocardial monolayer seen in untreated WT explants (Figure 2, I–K), accompanied by reduced mesenchymal marker expression (Figure 2L).

During AVC EMT, Snail1, and Snail2 are key downstream effectors of Notch (12, 31) and TGF-β2 (38). To test the role of the Snail genes in the acquisition of the mesenchymal phenotype by ventricular ENC, we transduced ventricular explant
cells with Snail1 shRNAs. Two Snail1 shRNAs reduced the 2D TI of Tie2-Cre;N1ICD ventricular explants effectively (Figure 2, M–O). RT-PCR confirmed reduced Snail1 expression, though the reduction was greater for Snail2 (Figure 2P), consistent with the reported requirement of Snail1 for Snail2 expression (39). Tgfb2 expression was also reduced by Snail1 shRNAs (Figure 2P). This is consistent with the dose-dependent activation by Snail1 of a TGF-β2 reporter (data not shown), suggesting a functional link between Snail1 and TGF-β2.

Ectopic endocardial N1ICD expression was examined in a stage-dependent manner using the tamoxifen-inducible line Cdh5(PAC)-CreERT2 (40). Recombination was induced from E9.5 to E10.5 and at E11.5, Cdh5(PAC)-CreERT2;N1ICD embryos showed hemorrhage in the heart and trunk region (Supplemental Figure 7, A and B) and increased N1ICD and Snail1 cardiac expression (Supplemental Figure 7C). Importantly, ENCs in ventricular trabeculae showed a fibroblastic, star-like morphology, contrasting with the flat ENCs in WT trabeculae (Supplemental Figure 7, D–I). Cdh5(PAC)-CreERT2;N1ICD ENCs transformed and migrated in ventricular explant assays (Supplemental Figure 7, J–L) but like Tie2-Cre;N1ICD ENCs were not invasive.

**BMP2 confers invasivity on ventricular ENCs.** Functional studies have shown that Bmp2 is a critical inducer of AVC EMT upstream of TGF-β2 (9, 10). Bmp2 expression was unaffected in Tie2-Cre;N1ICD embryos (Supplemental Figure 2, E, F, and K), suggesting that Notch acts either downstream or independently of Bmp2 during cardiac EMT. BMP2 treatment of WT and Tie2-Cre;N1ICD ventricular explants produced invasive mesenchymal cells, significantly increasing 2D and 3D TI in WT explants and 3D TI in transgenic explants (Figure 3, A, B, D, and E). Thus, BMP2 confers invasive ability to transformed ventricular ENCs. Expression of Snail1, Has2, and Periostin (but not Twist1) was upregulated in WT ventricular cells treated with BMP2 (Figure 3F), indicating that BMP2-treated WT ventricular explants acquire full mesenchymal features.
Anti–TGF-β2 antibody inhibited the effect of BMP2 on WT explants (Figure 3, C and D), indicating that, as in AVC EMT, TGF-β2 acts downstream of Bmp2 during ventricular EMT. Although Bmp6 was ectopically expressed in Tie2-Cre;N1ICD ventricular endocardium (Supplemental Table 1 and Supplemental Figure 4, E, F, and I), Bmp6 did not induce EMT in WT ventricular explants (not shown).

To examine the requirement for Notch in BMP2-induced ventricular EMT, we cultured WT ventricular explants with BMP2 plus the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) (41). DAPT reduced invasion by RT-PCR (Figure 4P). Myocardial Tgf-β2 transcription in AVC myocardium (Figure 4G) was strongly increased in Nkx2.5-Cre;N1ICD embryos (Supplemental Figure 8B). At E9.5, Nkx2.5-Cre;N1ICD embryos showed pericardial distension and heart dilation, with poorly defined boundaries between chamber and nonchamber tissue, an AVC region devoid of transformed cells, and a thin ventricular myocardium with underdeveloped trabeculae (Supplemental Figure 8, C–G). Hey1 was expressed throughout the myocardium and endocardium, including the AVC (Figure 4, A and B). The expanded Hey1 domain was reflected in increased transcription by RT-PCR (Figure 4P). Myocardial Hey2 and Hey1 expression was unaltered (not shown), but was increased overall (Figure 4P and data not shown) due to stronger endocardial expression.

The ectopic Hey1 expression was accompanied by reduced Bmp2 in Nkx2.5-Cre;N1ICD AVC myocardium (Figure 4, D, E, and P). Tgfb2 transcription in AVC myocardium (Figure 4G) was strongly reduced in transgenic embryos (Figure 4, H and P). Expression of the myocardial chamber marker Anf was expanded to the AVC (Figure 4, J, K, and P), suggesting that AVC identity was lost in myocardial cells. Snail1 was exclusively expressed in AVC and OFT endocardium and mesenchyme of WT embryos (Figure 4M), while Snail1 transcript and protein were ectopically expressed throughout the endocardium and myocardium of Nkx2.5-Cre;N1ICD embryos (Figure 4N and data not shown).

To substantiate these findings, Notch1 was activated exclusively in the myocardium by breeding into the cTnT-Cre line, which is active in every heart segment from E7.5 onwards (45). Supplemental Figure 8H shows myocardial Cre reporter activity at E9.5.

**Figure 5**

BMP2 treatment rescues EMT in Notch1-expressing myocardium. Heart explants triple stained as in Figure 2. (A–I) Details of explants. Full lateral views are shown below. (A) WT ventricular explants show an endocardial monolayer. (B and C) WT AVC explants ± BMP2 undergo EMT. (D) In Nkx2.5-Cre;N1ICD ventricular explants, ENCs migrate across the collagen surface, but are not invasive. (E) Nkx2.5-Cre;N1ICD AVC explants produce migratory mesenchymal cells that show reduced invasion, which is rescued by BMP2 (F). (G) In cTnT-Cre;N1ICD ventricular explants, most ENCs grow as a monolayer and are noninvasive. (H) Mesenchymal cells in cTnT-Cre;N1ICD AVC explants migrate but show reduced invasion, which is rescued by BMP2 (I). Arrows, ENCs; arrowheads, invasive mesenchymal cells. (J) TI analysis of ventricular explants. Most ENCs in Nkx2.5-Cre;N1ICD explants (Nkx) migrate over the gel surface (P = 2.2 × 10^-7 for 2D TI versus WT explants), whereas few cells in cTnT-Cre;N1ICD explants (abbreviated cTnT) migrate in 2D. 3D TI is very low for both genotypes. (K) TI analysis of AVC explants. BMP2 treatment of WT explants significantly increases 2D and 3D TI (P = 3.7 × 10^-2 and 6.1 × 10^-3). Nkx2.5-Cre;N1ICD explants (Nkx) show increased 2D TI (P = 3.5 × 10^-4) but a markedly reduced 3D TI compared with WT (P = 5.7 × 10^-3). BMP2 treatment increases Nkx2.5-Cre;N1ICD 3D TI (P = 6.7 × 10^-4) and slightly reduced 2D TI. BMP2 treatment sharply increases the 3D TI of cTnT-Cre;N1ICD explants (cTnT; P = 1.9 × 10^-2). m, myocardium. Scale bar: 50 μm. Results are expressed as mean ± SD. *P < 0.005; **P < 0.01; ***P < 0.001.
cTnT-Cre;N1ICD embryos expressed N1ICD-GFP throughout the myocardium (Supplemental Figure 8I). E10.5 transgenic embryos were smaller than WT and showed pericardial distension, poorly defined cardiac regions, and a lack of mesenchymal cells in the AVC (Supplemental Figure 8, J–M). Trabeculae were apparent (Supplemental Figure 8, L and N) but slightly delayed compared with WT embryos (Supplemental Figure 1, B and F). Hey1 was expressed throughout the myocardium of cTnT-Cre;N1ICD embryos, including the AVC (Figure 4C and data not shown), and was increased (Figure 4Q). Bmp2 and Tgfb2 expression were reduced (Figure 4, F, I, and Q), and as in Nkx2.5-Cre;N1ICD embryos, Anf expression was extended to AVC myocardium (Figure 4, L and Q), suggesting loss of AVC identity. Likewise, Snail1 was abnormally expressed in the myocardium (Figure 4O) and increased (Figure 4Q).

The aberrant Hey1 and Bmp2 expression in the myocardium of Nkx2.5-Cre;N1ICD and cTnT-Cre;N1ICD embryos supports the idea that N1ICD-driven ectopic Hey1 expression represses Bmp2 in AVC myocardium. Hey1 and Bmp2 expression were examined in Notch loss of function (LOF) (Supplemental Figure 9). The expression of Hey1 and Hey2 was detected in chamber myocardium (Supplemental Figure 9, A and C). Hey1 was also found in atrial endocardium (Supplemental Figure 9A), Hey2 throughout the endocardium (Supplemental Figure 9C), and Hey1 in AVC endocardium (Supplemental Figure 9E). In RBPJk mutants, myocardial Hey1 and Hey2 expression were unaffected (Supplemental Figure 9, B and D), whereas endocardial Hey gene expression was reduced (Supplemental Figure 9, B, D, F, and I). In RBPJk mutants (Supplemental Figure 9, G and H), Bmp2 expression was not.
affected in the myocardium but was upregulated in the endocardium (Supplemental Figure 9, H and I). These data suggest that Hey2 acts downstream of Notch in repressing Bmp2 expression in the endocardium.

Ectopic myocardial Notch1 activity impairs EMT in a Bmp2-dependent fashion. To study the effect of deregulated Bmp2 and Snail1 expression on EMT, we performed cardiac explant experiments with E9.5 Nkx2.5-Cre;N1ICD and cTnT-Cre;N1ICD embryos. As expected, WT ventricular endocardium grew as a monolayer (Figure 5A) with a few scattered ENCs at the explant edge and almost no invasion (Figure 5J). WT AVC explants showed ENC outgrowth and several invading mesenchymal cells (Figure 5B), with 2D and 3D TI between 0.1 and 0.15 (Figure 5K). Addition of BMP2 significantly increased surface migration and invasion (Figure 5C and Supplemental Video 3), reflected in 2D and 3D TI values of greater than 0.3 and greater than 0.2 (Figure 5K).

Ventricular explants of Nkx2.5-Cre;N1ICD embryos generated scattered, noninvasive ENCs (Figure 5D) with a 2D TI (0.9) significantly higher than that of WT explants (Figure 5J). Nkx2.5-Cre;N1ICD AVC explants (Figure 5E and Supplemental Video 4) showed relatively high surface migration (2D TI = 0.4; Figure 5K) but severely reduced invasion (3D TI < 0.1; Figure 5K). Addition of BMP2 restored the WT phenotype (3D TI = 0.2; Figure 5, F and K, and Supplemental Video 4).

cTnT-Cre;N1ICD ventricular explants behaved similarly to WT ventricular explants, with no endocardial transformation (Figure 5, G and J), consistent with the lack of ectopically activated endocardial Notch1 in this model. cTnT-Cre;N1ICD AVC explants (Figure 5H) transformed very poorly (3D TI < 0.1; Figure 5K), but addition of BMP2 restored invasiveness (3D TI = 0.3; Figure 5, I and K).

These data indicate that Notch1 activation in endocardium endows chamber ENC with transforming ability. Notch1 activation in myocardium leads to Bmp2 and Tgfβ2 downregulation by ectopic Hey1 expression.

Bmp2 promotes Snail1 expression and stability, and its inactivation impairs Notch1 signaling in AVC. During EMT, Snail1 is regulated by 2 sequential GSK3β-mediated phosphorylation events. The first Snail1 phosphorylation event induces nuclear export; the second, proteosomal degradation (46). In endothelial cells, BMP2 induces ERK1/2 phosphorylation (activation), which itself phosphorylates (inactives) GSK3β (47). We tested the putative link among Bmp2 signaling, Gsk3β inhibition, and Snail1 stabilization in ECs. Immunoblotting of bovine aortic endothelial cells (BAEcs) incubated with the proteasome inhibitor MG132 (46) detected a 31-kDa Snail1 band, while the levels of p-Gsk3β and p-Erk1/2 were low (Figure 6A). Addition of BMP2 markedly increased Snail1 expression as well as the levels of p-Gsk3β and p-Erk1/2, while the total amount of Gsk3β was unaffected (Figure 6A). Immunofluorescence of unstimulated BAEcs detected weak nuclear Snail1 expression (Figure 6, B and C) and faint, predominantly perinuclear staining of p-Gsk3β (Figure 6, D and E). In BMP2-treated BAEcs, Snail1 nuclear staining was increased (120% increase) and perinuclear p-Gsk3β staining was also stronger (80%; Figure 6, G–J).

In epithelial cells, Gsk3β has also been shown to regulate Snail1 at the transcriptional level, together with Gsk3α, via NF-κB inhibition (48). We tested to determine whether this mechanism contributes to the effect of BMP2 in BAEcs. Figure 6K shows that adding BMP2 to BAEcs markedly increased Snail1 mRNA while BMP2 plus an NF-κB inhibitor markedly reduced it. We tested the effect of NF-κB inhibition on the BMP2-driven EMT of ventricular explants. WT ventricular explants cultured with BMP2 underwent invasive EMT (Supplemental Figure 10, A and C), which was notably reduced when an NF-κB inhibitor was added to the culture medium (Supplemental Figure 10, B and C). This effect was also reflected by the marked reduction of Snail1 mRNA upon addition of the NF-κB inhibitor (Supplemental Figure 10D). Together these observations suggest that BMP2 treatment of endothelial cells augments Snail1 mRNA in an NF-κB–dependent manner and stabilizes Snail1 protein by Gsk3β phosphorylation/ inactivation.

Our data show that Bmp2 and Notch1 cooperate to induce invasive EMT in ventricular explants. This process is Notch dependent, and the noninvasive phenotype of AVC explants ectopically expressing Notch1 in the myocardium is rescued by exogenous BMP2. To explore the Bmp2-Notch1 relationship in vivo, we crossed a conditional Bmp2 mutant (49) with the myocardial-specific cTnT-Cre driver line. E10.5 cTnT-Cre;Bmp2flox embryos lacked defined AVC and EC tissue (Supplemental Figure 11, A–F), similar to the phenotype obtained with the Nkx2.5-Cre driver (10, 11). Cardiac expression of Notch1 (and Snail1) was markedly reduced (Figure 6L), which, given the exclusive endocardial Notch1 expression at this stage, suggests that myocardial Bmp2 deletion impairs endocardial Notch1 expression. In E10 WT embryos, N1ICD is localized in most ECs in the AVC and to ECs at the base of trabecular in the ventricles (Figure 6, M–O, and ref. 14). In cTnT-Cre;Bmp2flox mutants, N1ICD staining was strongly reduced in AVC endocardium (Figure 6, P and Q) but ventricular staining was unaffected (Figure 6, P and R). These data suggest that Bmp2 deletion in the myocardium specifically impairs Notch1 expression and activity in AVC endocardium.

Discussion
We previously showed that Notch is a key signal during CVF that via Snail1 activation, promotes the EMT of ECs to form the valve primordium (12). Notch/RBPJK directly regulate Snail1 transcription (12, 50), and Notch inactivation leads to a downregulation of Snail1 expression in AVC endocardium (12). This earlier report did not, however, provide information about the role of Notch targets in EMT and neither did it define the domain-specific (chamber vs. nonchamber) and tissue-specific (endocardium vs. myocardium) patterning of Notch function or determine the extent of Notch interaction with myocardial signals that trigger EMT (e.g., Tgfβ2 and Bmp2). The main goal of the present report was to shed light on the integration of EMT-driving signals and the establishment of valve developmental domains in the embryonic heart.

Transgenic mice constitutively expressing N1ICD throughout the endocardium, myocardium, or both have allowed us to establish Notch as a key endocardial signal that acts in concert with myocardial Bmp2 to promote a valve-forming field in the developing heart. We propose that Snail1 is a critical point of convergence between Notch1 and Bmp2 signaling based on the following experimental evidence: (a) ectopic N1ICD expression in either endocardium or myocardium leads to Snail1 upregulation in these tissues; (b) the noninvasive EMT of Tie2-CRE;N1ICD ECs in ventricular explants is mediated by Snail1, and the complete EMT driven by Bmp2 is also Snail1-dependent; (c) targeted Bmp2 inactivation in the myocardium leads to reduced Snail1 expression in AVC endocardium (ref. 10 and this report); and (d) BMP2 stimulation increases Snail1 expression in BAEcs. An important issue raised by these findings is how these signals are restricted to specific tissues and their activities coordinated.

3502 The Journal of Clinical Investigation http://www.jci.org Volume 120 Number 10 October 2010
Two reports have examined the effect of ectopic Notch1 activation on cardiovascular development. In the first study, Notch1 was activated in cardiogenic mesoderm using the Mesp1-Cre driver line, demonstrating that transgenic mice show defective AVC myocardial differentiation and ventricular maturation (19). Similarly to WT embryos, the AVC of transgenic embryos contained mesenchymal cells, but also showed ectopic myocardial masses of uncertain origin. Since N1ICD activation occurred in both endocardium and myocardium, these authors did not study the specific effect of Notch1 activation in either of these layers (19). The second study used the Tie2-Cre driver line to express N1ICD in the endocardium and vascular endothelium (51). These authors reported defective trabeculation, thinner myocardium, and delayed endocardial cushion formation, but the cellular and molecular bases of these defects were not examined.

Our results provide the first demonstration of the expansion of the characteristic AVC-restricted transcription of Notch target genes (Hey1, Hey2, Dll4, c-myc, and Nrarp) to the ventricular endocardium after N1ICD overexpression in this tissue (Tie2-Cre;N1ICD). In light of earlier observations that Hey1 and Dll4 are downregulated in RBPs/Jk and Notch1 mutants (12), we suggest that Notch target gene expression is regulated by a positive feedback loop and that Notch signals in the endocardium by lateral induction, with AVC ENCs behaving as a developmental field. We find a set of genes involved in EMT, cell migration, metastasis, and invasion to be upregulated and ectopically expressed in the ventricular endocardium of Tie2-Cre;N1ICD embryos (Supplemental Table 1). These data indicate that WT Notch1 activity in AVC ENCs promotes EMT by driving, in a cell-autonomous manner, a mesenchyme gene program that can be experimentally extended to the ventricles by endocardial NICD overexpression. This notion is consistent with the downregulation of the endothelial chamber marker Inx5, suggesting that ventricular ENCs of Tie2-Cre;N1ICD embryos have lost their chamber identity.

AVC explants of Tie2-Cre;N1ICD embryos undergo EMT, with ENCs invading the collagen matrix (3D EMT) and showing increased migratory capacity on the gel surface (2D EMT) with respect to WT AVC. In contrast to the characteristic EMT-refractory ventricular endocardium of WT mice, ENCs of Tie2-Cre;N1ICD ventricular explants (or those from Cdh5[PAC1-CreERT2];N1ICD mice) generate mesenchymal cells expressing Snail1, Snail2, and Tgfβ2 that lose contact and scatter over the gel surface, but are unable to invade the gel matrix. Addition of TGF-β2 can mimic this effect in WT ventricular endocardium, whereas lentiviral- or antibody-mediated inhibition of Snail1 and Tgfβ2 inhibits transformation of Tie2-Cre;N1ICD explants, indicating that these molecules lie downstream of Notch in EMT. Thus, Notch alone is able to promote noninvasive EMT in a Snail1 and Tgfβ2-dependent manner, but cannot trigger a fully invasive EMT. WT ventricular ENCs become fully invasive after Bmp2 addition and inhibition of Notch, Tgfβ2, or Snail1 severely attenuates Bmp2-induced invasion, indicating that Notch and Bmp2 are critical signals acting in concert during EMT.

 Constitutive Notch1 activation in the myocardium (Nkx2.5-Cre;N1ICD or CtnT-Cre;N1ICD) caused cardiac dysmorphogenesis. The expansion of myocardial chamber markers Anf and Chisel to the AVC and the Hey1 upregulation and concomitant Bmp2 downregulation in AVC myocardium suggested that myocardial cells have lost their AVC identity. Thus, ectopic myocardial Notch activity—via the activation of Hey1—prevents Bmp2 expression in chamber myocardium, supporting the idea that Hey proteins regulate Bmp2 expression and thus AVC patterning (42, 43). Loss of Bmp2 expression is consistent with the poor ENC invasion observed in AVC explants from CtnT-Cre;N1ICD embryos. Remarkably, Snail1 expression is upregulated in both the endocardium and myocardium of Nkx2.5-Cre;N1ICD embryos, whereas Bmp2 is downregulated in the AVC (presumably via ectopic Hey1 activity), in agreement with the very poor transforming ability of AVC explants from these embryos. The rescue of invasiveness by Bmp2 indicates that this molecule is critical for the acquisition of invasiveness by ENCs and that Notch promotes the transformed mesenchymal phenotype in AVC or OFT endocardium. Thus, Notch is likely to cooperate with Bmp2 to promote full deployment of invasive properties by transformed ENCs.

What is the role of Notch, Bmp2, and Hey in cardiac patterning? Supplemental Figure 12 summarizes the data from studies of Notch (this report and ref. 19) and Hey1 and Hey2 (42, 43) gain of function (GOF) and LOF, in terms of their effect on the Bmp2 expression domain and cardiac patterning. In the myocardium, Bmp2 expression is confined to the AVC by Hey1 and Hey2, which are expressed in the chambers (refs. 42 and 43, and Supplemental Figure 12A). In the endocardium, Notch1-dependent Hey1, Hey2, and Heyl are differentially expressed in the AVC and chambers and repress Bmp2 (Supplemental Figure 9 and Supplemental Figure 12A). Ectopic N1ICD expression in endocardium expands AVC patterning to ventricular endocardium but does not affect Hey1, Hey2, or Bmp2 myocardial expression or patterning (Supplemental Figure 12B). Ectopic N1ICD expression in the myocardium leads to expansion of Hey1 and loss of Bmp2 expression, and chamber markers expand to the AVC (Figure 4, K and L, and Supplemental Figure 12C). In both N1ICD GOF models, Bmp2 is repressed in the endocardium, similarly to the WT situation. Systemic abrogation of Notch signaling leads to downregulation of Hey genes and ectopic endocardial Bmp2 expression, indicating that Notch represses Bmp2 in the endocardium via Hey proteins (Supplemental Figure 9 and Supplemental Figure 12D) and suggesting that Notch might normally prevent ENCs from acquiring primitive cardiomyocyte features, such as those of the AVC. Notch inactivation impairs endocardial patterning of the AVC and chambers (refs. 12 and 52, and Supplemental Figure 12D), but does not affect myocardial patterning or the myocardial expression domains of Hey1, Hey2, or Bmp2 (Supplemental Figure 9 and Supplemental Figure 12D).

Our findings also shed light on how Notch1 and Bmp2 signals are integrated to promote EMT. Our data establish that Bmp2 triggers full EMT in WT and Tie2-Cre;N1ICD ventricular explants and that cell invasion in WT explants can be markedly reduced by inhibiting Tgfβ2, Notch, or Snail1. This supports the idea that these molecules are required for Bmp2-driven invasive EMT and indicate that Bmp2 and Notch1 signaling converge during AVC (and OFT) differentiation and the establishment of EMT competence in ENCs. At least 2 lines of evidence suggest that Bmp2 acts upstream of endocardial Notch activity during cardiac EMT: (a) impaired Notch1-Snail1–dependent signaling in AVC endocardium of Bmp2 myocardial mutants (CtnT-Cre;Bmp2flox) indicates that Bmp2 is required for Notch activity in AVC territory; (b) Notch deficiency, while blocking transformation in vivo and in vitro by loss of Snail1 expression (12), does not affect Bmp2 expression in AVC myocardium (Supplemental Figure 9H). Moreover, ectopic Notch activity in endocardium upregu-
lates and expands Snail1-dependent expression of Twist2, but not that of Bmp2-dependent Twist1 (10), suggesting that Bmp2 and Notch1 elicit overlapping but different mesenchyme gene expression programs in the AVC endocardium.

The concerted activities of Notch1 and Bmp2 in valve formation are reflected in the activation of Snail1 expression in chamber endocardium in response to ectopic Notch1 activity and in the reduced Snail1 expression after myocardial Bmp2 deletion. Moreover, inhibition of Gsk3β in Bmp2-stimulated BAECs suggests that whereas Notch1 promotes Snail1 expression, Bmp2 stimulates both Snail1 expression and protein stabilization by inhibiting Gsk3β activity. The interplay between Notch and Bmp2 during the acquisition of the invasive phenotype by AVC ENCs involves Notch1- and Bmp2-induced Snail1 expression and Bmp2-mediated Snail1 nuclear accumulation, leading to sustained expression of mesenchymal genes. This provides what we believe is a novel mechanism for the integration of Notch1 and Bmp2 activity in EMT.

For simplicity, we use Tieg2-Cre;N1ICD to refer to Tieg2-Cre/+;R26N1ICD/+ mice, Nkx2.5-Cre;N1ICD for Nkx2.5-Cre/+;R26N1ICD/+ mice, Tieg2-Cre;N1ICD for Tieg2-Cre/+;R26N1ICD/+ mice, and Bmp2-Cre;N1ICD for Bmp2-Cre/+;R26N1ICD/+ mice. Nkx2.5-Cre mice were provided by D.A. Melton (Harvard University, Cambridge, Massachusetts, USA), and Nkx2.5-Cre mice were provided by R.P. Harvey (Victor Chang Cardiac Research Institute, Darlinghurst, Australia). (Ctnf-Cre driver lines were provided by S. Evans (University of California San Diego, La Jolla, California, USA). Bmp2 conditional lines were provided by V. Rosen (Harvard University School of Dental Medicine, Boston, Massachusetts, USA). Details are provided in Supplemental Data and Supplemental Table 2.

All experimental procedures and protocols were approved by the Ethics Committee and Animal Welfare Committee of the CNIC and conformed to European Union guidelines for the care and use of laboratory animals. 

AVC and LV explants. A solution (1.5 mg/ml) of rat-tail collagen type I (BD Biosciences) was dispensed into 4-well microculture dishes and allowed to solidify inside a 37°C, 5% CO2 incubator. Collagen gels were washed several times with DMEM containing 8% FBS, 0.1% insulin-transferrin-selenium (ITS, Gibco; Invitrogen), and antibiotics, and drained. AVCs were harvested in sterile PBS from E9.5 or E11.5 embryos. LV were carefully dissected, avoiding contamination from AVC tissue. Explants were placed with the endocardium face down and allowed to attach (16 hours, 37°C, 5% CO2). Medium (100 μl/well in AVC explants or 50 μl/well in LV) was added and explants cultured for up to 3 days (AVC) or 4 days (LV). Explants were fixed and stained with phalloidin-FITC (1:100; Sigma-Aldrich) to reveal mesenchymal cells as described (34). Explants were mounted on excavated slides in Vectashield medium containing DAPI. For explant treatments, the medium was supplemented with human TGF-β2 (30 ng/ml; Peprotech), human BMP2 (200 ng/ml; R&D Biosciences), anti-human TGF-β2 antibody (1 μg/ml; R&D Biosciences), or NF-kB SN50 inhibitor (18 μM; Calbiochem). For treatments with DAPT (γ-secretase inhibitor IX; 565770; Calbiochem), collagen gels were conditioned in explant media containing 50 μM DAPT or vehicle (ethanol). Medium was replaced every 24 hours.

Lentivirus production and Snail shRNA interference. Pseudotyped lentivectors were produced according to ref. 56. Subconfluent HEK293T cells cultured in 10-cm plates with DMEM-Glutamax (Invitrogen), 10% FBS, and antibiotics were transiently cotransfected using Lipofectamine Plus (Invitrogen) with the following plasmids: 5 μg LV-eGFP control vector or lentiviral Snail-shRNA vectors TRCN0000096260 and TRCN0000096262 (mission shRNA library; Sigma-Aldrich), 5 μg packaging plasmid pCMVΔR8.74, and 2 μg vesicular stomatitis virus G envelope protein plasmid pMD2G (plasmid 12259; Addgene). Supernatants were collected after 2 days and added together with polybrene (10 μg/ml) to ventricular explants on collagen gels. Snail mAb was provided by I. Virtanen (University of Helsinki, Helsinki, Finland).


Confocal imaging. See Supplemental Data.

Statistics. The TI of AVC and ventricular explants is the ratio of the number of scattered cells (2D migration) or the number of cells invading the collagen gel (3D migration) to the total number of cells (2D cells + 3D cells + nonmigrating ENCs) as follows: 2D TI = 2D/(2D + 3D + nonmigrating ENCs); 3D TI = 3D/(2D + 3D + nonmigrating ENCs).

Cell numbers were determined by counting DAPI-stained nuclei in the Z stack, distinguishing between 2D migrating cells, 3D migrating cells,
and nonmigrating ENCs. At least 15 explants were assayed per genotype and condition. Results are expressed as mean ± SD. An unpaired 2-tailed Student’s t test was performed to assess differences among groups. P < 0.05 was considered significant.

RNA isolation and semiquantitative RT-PCR. See Supplemental Data. For primers and conditions, see Supplemental Table 3. Microarray hybridization and scanning. See Supplemental Data.

Acknowledgments

We thank D.A. Melton for the B26N1CD mice; R.P. Harvey for the Nkx2.5-Cre mice; S. Evans for the cTnt-Cre driver lines; V. Rosen for the Bmp2 conditional lines; I. Virtanen for the snail mAb; A. García-Herreros, F.J. Díaz-Benjumea and D. MacGrogan for helpful discussions; M. Manzanares for critical reading of the manuscript; and S. Bartlett for English editing. L. Luna-Zurita is supported by a fellowship linked to grant LSHM-CT-2005-018630 (HeartRepair, European Union FP6) and the Spanish Society of Cardiology. The CNIC is supported by the Spanish Ministry of Science and Innovation and the Pro-CNIC Foundation. This work was funded by grants SAF2007-62445 (Spanish Ministry of Science and Innovation), P-2006/BIO-194 (Regional Government of Madrid), RD06/0014/038 and RD06/0010/1013 (Spanish Ministry of Health), and LSHM-CT-2005-018630 (HeartRepair, European Union FP6) to J.L. de la Pompa.

Received for publication February 14, 2010, and accepted in revised form August 4, 2010.

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