Slug is a direct Notch target required for initiation of cardiac cushion cellularization

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Snail family proteins are key regulators of epithelial-mesenchymal transition, but their role in endothelial-to-mesenchymal transition (EMT) is less well studied. We show that Slug, a Snail family member, is expressed by a subset of endothelial cells as well as mesenchymal cells of the atrioventricular canal and outflow tract during cardiac cushion morphogenesis. Slug deficiency results in impaired cellularization of the cardiac cushion at embryonic day (E)–9.5 but is compensated by increased *Snail* expression at E10.5, which restores cardiac cushion EMT. We further demonstrate that *Slug*, but not *Snail*, is directly up-regulated by Notch in endothelial cells and that *Slug* expression is required for Notch-mediated repression of the *vascular endothelial cadherin* promoter and for promoting migration of transformed endothelial cells. In contrast, transforming growth factor β (TGF-β) induces *Snail* but not *Slug*. Interestingly, activation of Notch in the context of TGF-β stimulation results in synergistic up-regulation of *Snail* in endothelial cells. Collectively, our data suggest that combined expression of *Slug* and *Snail* is required for EMT in cardiac cushion morphogenesis.

Introduction

Epithelial-mesenchymal transition is the process by which epithelial cells undergo phenotypic and morphological reorganization. Epithelial-mesenchymal transition is essential during embryogenesis for the formation of many tissues, including the formation of the mesoderm, the migration of neural crest cells, and the development of the heart valves and septa (Hay, 2005). Endothelial-to-mesenchymal transition (EMT) is a specific form of epithelial-mesenchymal transition that is initiated at embryonic day (E)–9.5 in the atrioventricular (AV) canal and E10.5 in the outflow tract (OFT) cardiac cushions, the two sites of EMT in the developing heart (Camenisch et al., 2002a). This process generates cells that contribute to the connective tissue of the valves and septa of the adult heart (Eisenberg and Markwald, 1995).

Recent studies have demonstrated a critical role of the Notch signaling pathway during cardiac EMT, and disruption of this pathway has been implicated in the pathogenesis of various cardiovascular diseases (Iso et al., 2003; Niessen and Karsan, 2007).

In the mouse, targeted deletion of *Notch1* or its key nuclear partner *CSL* (*CBF1/Suppressor of Hairless/Lag-1*) results in cardiac cushion EMT defects (Oka et al., 1995; Timmerman et al., 2004). Further, targeted deletion of the downstream Notch/CSL effector *Hey2* or double-deficiency of *Hey1* and *Hey2* or *Hey1* and *HeyL* results in various congenital heart anomalies including cardiac cushion defects (Donovan et al., 2002; Fischer et al., 2004, 2007). In humans, mutations at the *Notch1* locus are associated with bicuspid aortic valve disease as well as mitral valve anomalies and tetralogy of Fallot (Garg et al., 2005). Further, patients with mutations of the Notch ligand Jagged1 develop Alagille syndrome, a polymalformative disorder which includes cardiac cushion defects (Li et al., 1997; Oda et al., 1997; Eldadah et al., 2001).

TGF-β–related pathways have also been shown to be essential for proper heart development through their role in regulating EMT (Azhar et al., 2003). Of particular interest, *BMP2* and *TGF-β2* are expressed by the AV canal cushion myocardium (Dickson et al., 1993; Zhang and Bradley, 1996). BMP2-deficient mice die before cardiac cushion development (Zhang

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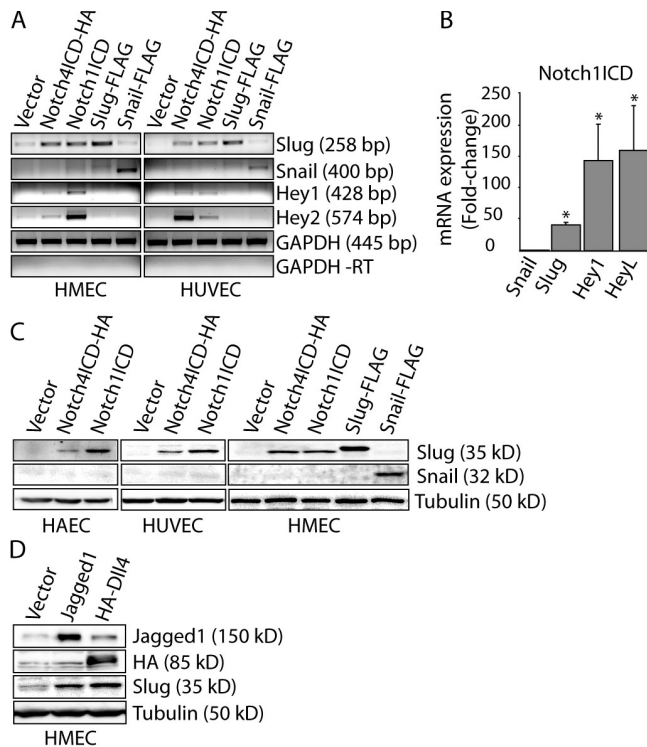www.jcb.org/cgi/doi/10.1083/jcb.200710067

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Figure 1. **Expression of *Slug*, but not *Snail*, is induced by Notch activation.** (A) Analysis of mRNA expression by semi–qRT-PCR in human mammary epithelial cells (HMEC) and human umbilical vein epithelial cells (HUVEC) expressing constitutively active Notch1 (Notch1ICD) or Notch4 (Notch4ICD). (B) Analysis of mRNA expression by qRT-PCR in HMEC expressing Notch1ICD. Results are normalized to the vector control (*n* = 3). *, P < 0.05. Error bars show SEM. (C) Immunoblots for Slug and Snail in HMEC, HUVEC, and human aortic endothelial cells (HAEC) transduced with Notch1ICD, Notch4ICD, or the empty vector. (D) HMEC expressing Jagged1 or Dll4 were cocultured with parental HMEC and immunoblotting for Slug was performed.

and Bradley, 1996); however, deficiency of the BMP2 receptor Alk2 results in AV canal EMT defects (Wang et al., 2005). *TGF-β2*–deficient mouse embryos do not display obvious cardiac cushion EMT defects, although later remodeling of the AV canal cushion is impaired (Dickson et al., 1993; Sanford et al., 1997; Bartram et al., 2001; Molin et al., 2002, 2003). However, using an ex vivo AV canal explant assay, TGF-β2–blocking antibodies or blocking antibodies for its coreceptor *TβRIII* inhibit AV canal EMT, suggesting redundancy of this pathway in vivo (Brown et al., 1999; Camenisch et al., 2002a). These data and others have established a clear role of TGF-β–related pathways during mammalian cardiac cushion development.

The Snail family members *Snail* (also known as *Snai1*) and *Slug* (also known as *Snai2*) encode zinc finger–containing transcriptional repressors that trigger EMT during embryonic development and tumor progression, in part by regulating expression of junctional proteins, most notably *E-cadherin* (Nieto, 2002). In the mouse, *Snail* has been shown to be expressed in the cardiac cushions from E9.5 onwards (Timmerman et al., 2004). Mice deficient for *Snail* die at E7.5, before cardiac development, and display defects in mesoderm formation (Carver et al., 2001). Conditional deletion of *Snail* after E8 results in lethality by E9.5, partially because of severe cardiovascular defects, but before the

initiation of cardiac cushion EMT (Murray and Gridley, 2006). In the mouse, Slug is expressed in the cardiac cushions at E13.5, and mice deficient for *Slug* are viable but are growth retarded and display defects in pigmentation and hematopoiesis (Jiang et al., 1998; Inoue et al., 2002). To date, there is no direct evidence demonstrating the requirement for any Snail family member during mammalian heart development.

In this paper, we demonstrate that *Slug* is first expressed by a subset of endothelial cells as well as mesenchymal cells of the AV canal at E9.5, at the initiation of EMT. In keeping with a requirement for Slug during the initiation of cardiac EMT, the AV canal cushions show markedly reduced cellularization at E9.5, which normalizes by E10.5. Concordant with the in vivo findings, AV canal explant assays demonstrate that EMT in *Slug*-deficient embryos is impaired at E9.5 but not E10.5, as EMT in *Slug*-deficient embryos is rescued by an increase in *Snail* expression by E10.5. Accordingly, abolishing both *Slug* and *Snail* expression results in EMT defects at E10.5. In contrast to a previous study, we show that Notch signaling, through CSL, directly regulates the *Slug* promoter, resulting in the upregulation of *Slug*, but not *Snail*, in endothelial cells (Timmerman et al., 2004). We further show that *Slug* directly binds and represses the *vascular endothelial cadherin* (*VE-cadherin*) promoter. Slug also promotes increased migration toward PDGF. In contrast, TGF-β2 and BMP2 induce *Snail* expression but minimal *Slug* expression. However, Notch synergistically induces *Snail* in concert with TGF-β2. Our data demonstrate that Notch-induced expression of *Slug* plays an important role in the initiation of EMT in the heart but that increased Snail compensates for the lack of Slug in *Slug*-targeted embryos as cardiac cushion morphogenesis progresses.

Results

Activation of the Notch pathway induces *Slug* but not *Snail* in endothelial cells

It has previously been suggested that EMT initiated by Notch may proceed through the induction of Snail; however, the degenerate primers used in that study amplify both Snail and Slug (Timmerman et al., 2004). To clarify which Snail family members are regulated by Notch signaling, we activated Notch in endothelial cells by ectopically expressing the Notch ligands Jagged1 or Dll4 or the constitutively active form of Notch1 or Notch4 (Notch1ICD and Notch4ICD, respectively), all of which are expressed in the cardiac cushion (Loomes et al., 1999; Noseda et al., 2004). Activated Notch up-regulated *Slug*, but not *Snail*, in all endothelial cells tested, as demonstrated by RT-PCR (Fig. 1 A), quantitative RT-PCR (qRT-PCR; Fig. 1 B), and immunoblotting (Fig. 1, C and D; and see Fig. 6 B). As a positive control, we confirmed that the known Notch targets *Hey1*, *Hey2*, and *HeyL* were induced by NotchICD (Fig. 1, A and B). Additional experiments with NotchICD deletion constructs revealed that the Ankyrin repeats of Notch are required for induction of Slug (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200710067/DC1). These findings indicate that Notch activation induces *Slug* but not *Snail* expression in endothelial cells.

***Slug* is expressed in a subset of endothelial cells and the mesenchymal cells of the AV canal, OFT, and valves of the embryonic mouse and human heart**

It has been reported that *Slug* mRNA is not expressed at E9.5 in the cardiac cushion (Timmerman et al., 2004), which suggests that it is dispensable for cardiac EMT. Because Notch activation has been shown to be critical for EMT during cardiac cushion development (Noseda et al., 2004; Timmerman et al., 2004), we were interested in defining the expression of *Slug* during the period of cellularization of the mammalian cardiac cushions. We thus examined *Slug-lacZ* mice, which have the *lacZ* gene inserted into the *Slug* locus with concomitant deletion of the zinc finger DNA binding motifs. Expression of *lacZ* in this model has been shown to faithfully match expression of *Slug* mRNA in all tissues analyzed, as determined by in situ hybridization (Jiang et al., 1998). β-galactosidase staining of E9.5–11.5 embryos showed that *Slug*-expressing cells were abundant in the heart (Fig. 2 A) with expression within a subset of endothelial cells as well as the mesenchymal cells of the AV canal and OFT at E9.5, with increasing expression over E10.5 and 11.5 (Fig. 2 B). Detailed analysis of *Slug* expression around E9.5 revealed that *Slug* expression is induced in the AV canal at the 25–28-somite stage at the initiation of EMT (Fig. S2, available at http://www.jcb .org/cgi/content/full/jcb.200710067/DC1). Immunofluorescent staining for Slug protein at E11.5 confirmed expression in the cardiac cushion mesenchyme and a subset of endothelial cells that costained for CD31 (Fig. 2 C, arrowheads).

It has been suggested that EMT continues to take place to allow valvular remodeling later in development as well as in the adult (Armstrong and Bischoff, 2004). To confirm a role for Snail family members in the human heart, we examined expression of *Snail* and *Slug* in embryonic human hearts at various developmental stages (days 52–78 of gestation) with similar results at various stages. Fig. 2 D shows that *Snail* and *Slug* are both expressed in the tricuspid and mitral valves, the AV septum, and the interatrial septum in a 65-d human heart. This staining pattern is similar to *Slug* expression in later stages of mouse heart development (Oram et al., 2003). Higher magnification images revealed that the mesenchymal cells of the valves, as well as endothelial cells at the root of the valves, express *Snail* and *Slug* (Fig. 2 D), suggesting a role for Snail family members in human cardiac cushion development and remodeling.

***Slug* is necessary for EMT in the cardiac cushions**

To determine whether targeted disruption of the *Slug* gene has a functional effect on cardiac cushion development, the AV canal of E9.5 embryos were placed on collagen gels to monitor EMT ex vivo, as previously described (Camenisch et al., 2002a; Chang et al., 2004). Occasional endothelial cell outgrowths occur proximal to 100 μm of the AV canal explant. Therefore, only the morphologically distinct mesenchymal cells distal to 100 μm from the AV canal were quantitated to determine the degree of EMT. Homozygous *Slug-LacZ* mutants behave as *Slug*$^{-/-}$ (*Slug*-deficient) animals (Jiang et al., 1998; Inoue et al., 2002),
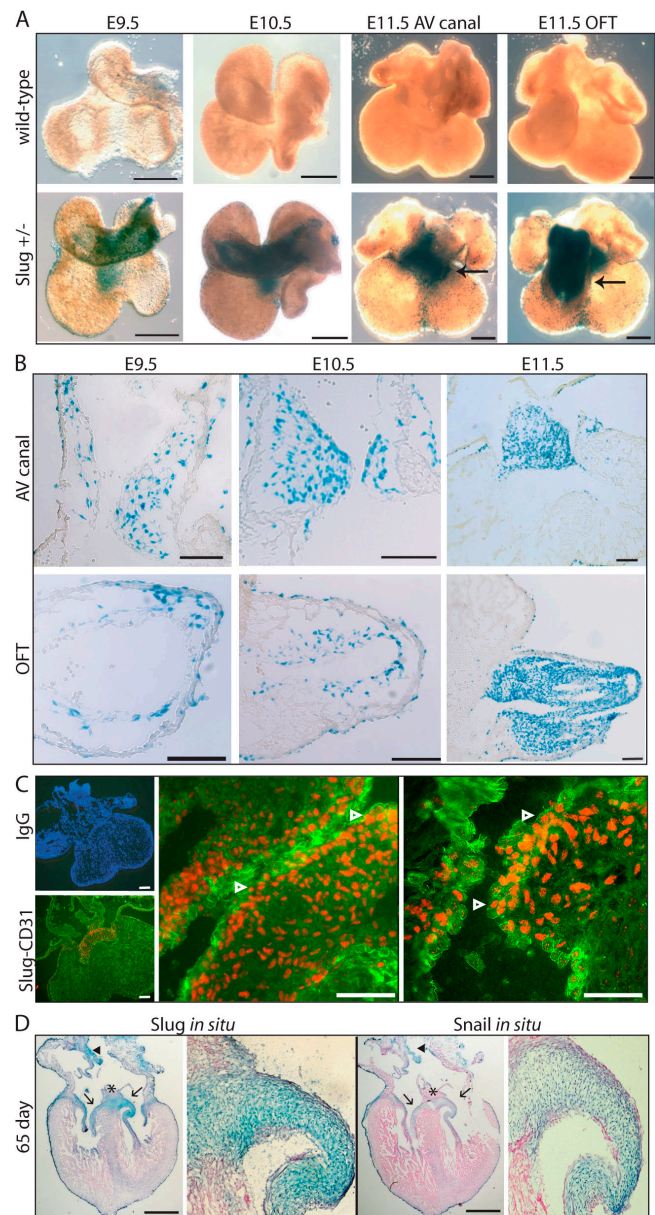

Figure 2. ***Slug* expression during cardiac cushion development.** (A) β-galactosidase staining (representing *Slug* expression) of whole-mounted hearts from *Slug-lacZ*$^{+/-}$ embryos from E9.5 to 11.5. Arrows point to the AV canal or OFT at E11.5. Bars, 250 μm. (B) Sections through the AV canal and OFT of β-galactosidase–stained *Slug-lacZ*$^{+/-}$ hearts from E9.5 to 11.5. Bars, 100 μm. (C) Immunofluorescence staining for Slug (red) and CD31 (green) in E11.5 embryonic mouse hearts. Arrowheads point to cells coexpressing Slug and CD31. Bars, 25 μm. (D) In situ hybridization for *Snail* and *Slug* in a 65-d human embryonic heart. Arrows point to the mitral and tricuspid valves, arrowheads indicate the interatrial septum, and the asterisk marks the AV septum. A higher magnification image of the heart valve is shown in the right panel of each analysis. Bars, 1 mm.

and *Slug*$^{-/-}$ AV canal explants had significantly reduced migration and invasion compared with *Slug*$^{+/-}$ or wild-type controls at E9.5 (Fig. 3, A and B). Of the few *Slug*$^{-/-}$ cells that did migrate, many had a rounded morphology, and were not able to differentiate into spindled mesenchymal cells. Analysis of β-galactosidase activity in *Slug*$^{+/-}$ AV explants revealed *Slug* expression in the migrating cells as well as the proximal

Figure 3. *Slug*$^{-/-}$ **embryos display defects in AV canal EMT at E9.5.** (A) Phase contrast (left) and DAPI (right) images of AV canal explants from wild-type and *Slug*$^{-/-}$ embryos. Bars, 250 µm. (B) Quantitative analysis of EMT in AV canal explants from E9.5 wild-type (wt), *Slug*$^{+/-}$, and *Slug*$^{-/-}$ embryos after 48 h in culture. Results represent the distance of a positive pixel (DAPI-stained nucleus) to the closest point of the AV canal normalized to the area of the AV canal tissue. *, $P < 0.05$. (C) *Slug* expression in the AV canal explant assay as visualized by β-galactosidase staining of wild-type and *Slug-lacZ*$^{+/-}$ AV explants. The rounded morphology of most of the LacZ$^+$ cells is shown on the right. The black square indicates the region of higher magnification shown to the right. Bars, 50 µm. (D) Representative sections of wild-type and *Slug*$^{-/-}$ hearts counterstained with Nuclear Fast Red used for analysis in E. Dotted blue lines highlight the superior and inferior AV cushions. Bars, 50 µm. (E) Quantitation of the cellularity of the superior and inferior cushions in E9.5 wild-type (wt; $n = 3$) and *Slug*$^{-/-}$ ($n = 3$) embryos. Error bars show SEM. (F) BrdU analysis on the percentage of proliferating cells in wild-type ($n = 4$) and *Slug*$^{-/-}$ ($n = 6$) AV canal cardiac cushions (total), the AV canal endocardium (Endo), and AV canal mesenchymal cells (Mesen; 10–15 sections per embryo). Error bars show SEM. (G) Vector- or Slug-transduced HMEC were subjected to an endothelial wounding assay. Bars represent the distance migrated after 24 h ($n = 4$). *, $P < 0.05$. Error bars show SD. (H) Vector- or Slug-transduced HMEC were evaluated in a modified Boyden chamber assay with 20 ng/ml PDGF-BB present in the lower chamber. Bars represent the total number of cells migrated after 4 h ($n = 6$). *, $P < 0.05$. Error bars show SD.
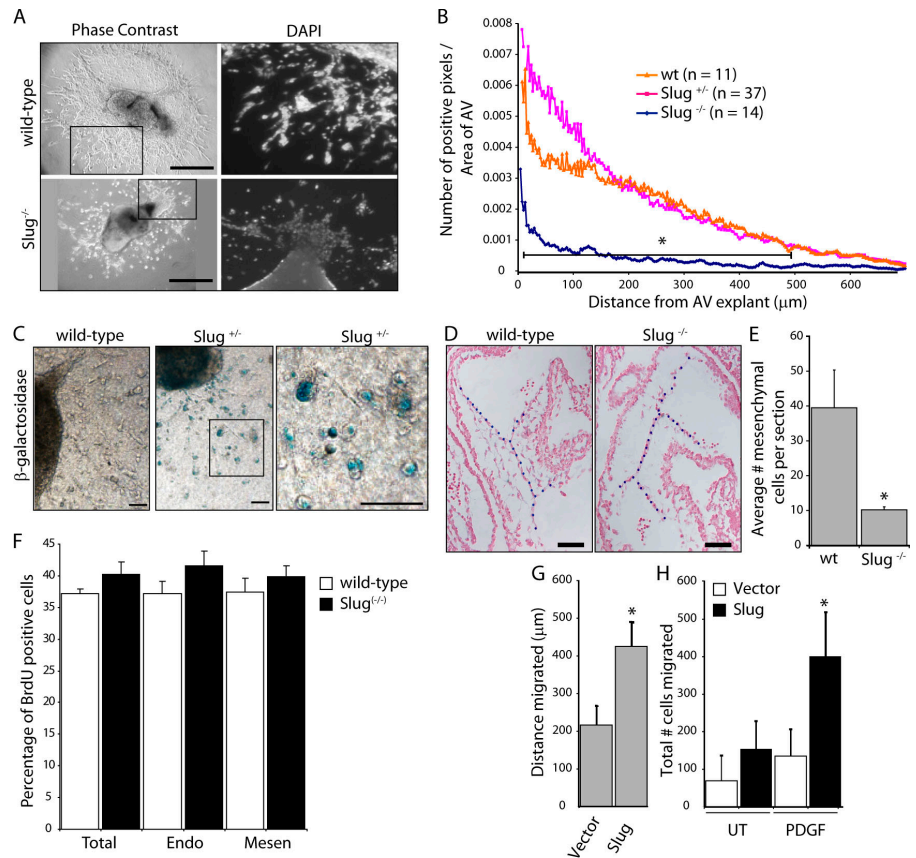
cardiac endothelial cells (Fig. 3 C). Interestingly, the majority of β-galactosidase staining was seen in rounded cells, which is consistent with morphology of cells that are intermediate between endothelial and mesenchymal phenotype, as previously described (Camenisch et al., 2002b).

To confirm that cushion cellularization was impaired in Slug-deficient embryos in vivo, E9.5 hearts were serially sectioned (between 7 and 20 sections for each heart) and the number of cushion cells was quantitated in every section. At E9.5, *Slug*$^{-/-}$ embryos had significantly fewer mesenchymal cells in the cardiac cushions compared with wild-type controls (Fig. 3, D and E). However, by E10.5 there was no difference in cellularity of the cardiac cushions, and a defect in AV canal EMT ex vivo was not evident (Fig. S2). These findings implicate *Slug* in the early activation and migration of endothelial cells during cushion EMT with potential compensation by other factors later in cardiac cushion development. To investigate the reason for the normalization of cardiac cushion cellularization by E10.5, the degree of cardiac cushion apoptosis and proliferation was evaluated. AV canal endocardial and mesenchymal cell proliferation was measured by BrdU incorporation and no difference in S-phase entry was noted at E9.5 (unpublished data). However, analysis at E10.25 revealed that there is an increase in BrdU incorporation in both the endocardium

and mesenchyme in *Slug*$^{-/-}$ embryos (Fig. 3 F). Although the increase in BrdU incorporation was small, a greater pool of endocardial cells able to undergo EMT combined with increased mesenchymal proliferation may be sufficient to normalize cushion cellularity by E10.5. In contrast, quantitation of active caspase-3 to enumerate the numbers of cells undergoing apoptosis did not reveal much cell death (< 1%) in either wild-type or *Slug*$^{-/-}$ AV canals, with no difference between the two groups (unpublished data).

To determine whether *Slug* is sufficient to promote a motile phenotype in endothelial cells, endothelial cells were transduced with vector or Slug, and an in vitro wound healing (scratch) assay was performed. The scratch assay revealed increased migration of Slug-expressing endothelial cells as early as 4 h and up to 24 h after wounding of the endothelial monolayer, resulting in Slug-expressing cells migrating almost twice as far after 24 h (Fig. 3 G). PDGF has been shown to be expressed in the cardiac cushions during EMT (Van Den Akker et al., 2005). Using a modified Boyden chamber assay with 20 ng/ml PDGF-BB present in the lower chamber, we found that Slug-expressing endothelial cells showed significantly increased directed migration toward PDGF-BB (Fig. 3 H). Thus, Slug expression in the endothelium appears to be sufficient for endothelial motility and directed migration.

Slug represses endothelial phenotype

Given our findings demonstrating the requirement of *Slug* in cardiac EMT, we sought to examine the role that *Slug* plays in modulating the endothelial phenotype. Enforced expression of Slug repressed expression of key endothelial genes such as *VE-cadherin*, *CD31*, and *Tie2* as determined by qRT-PCR, immunoblotting, and immunofluorescence (Fig. 4, A and B; and Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200710067/DC1). However, in contrast to activated Notch, Slug did not induce the mesenchymal markers smooth muscle α-actin and h1-calponin (Fig. 4 B). These findings suggest that Slug expression promotes the initial phases of EMT associated with the loss of endothelial phenotype but is not sufficient to complete the transition into a mesenchymal cell that is mediated by Notch activation.

VE-cadherin is a key endothelial adherens junction protein that is required for maintenance of endothelial homeostasis and that must be down-regulated before endothelial remodeling (Crosby et al., 2005). We thus determined whether Slug was capable of directly repressing *VE-cadherin*. Promoter analysis of human *VE-cadherin* identified two putative Slug binding E2-box (CACCTG) motifs 5′ to the transcriptional start site (TSS), located at −306 to −311 and −379 to −384 (Prandini et al., 2005). As demonstrated by electrophoretic mobility shift assays (EMSA), Slug was capable of binding both E2-box motifs, but was unable to bind a CAGCTG E-box element located at −97 to −102 in the human *VE-cadherin* promoter (Fig. 4 C). Of the three cis elements tested in the human VE-cadherin promoter, only the −379 to −384 E2-box and the −97 to −102 E-box motifs are conserved in the mouse *VE-cadherin* promoter. Consistent with the EMSA result, mutation of the E2-box, but not the E-box motif, significantly reduced the ability of Slug to repress the mouse *VE-cadherin* promoter as measured by luciferase assays (Fig. 4 D). Thus, *VE-cadherin* transcription is directly repressed by Slug binding to the E2-box promoter elements in endothelial cells.

Notch acts through CSL to induce *Slug* and repress the endothelial phenotype

We next determined whether Notch induces *Slug* through the canonical CSL-dependent pathway or the less well-defined CSL-independent route (Ramain et al., 2001). Dll4-mediated induction of *Slug* mRNA and protein was dramatically reduced when *CSL* was knocked down using either of two lentiviral-delivered short hairpin RNA (shRNA) constructs, which target distinct regions of *CSL* (Fig. 5, A–C). As expected, induction of the Notch target *HeyL* was also abolished by *CSL* knockdown (Fig. 5 B). In addition, the ability of Notch activation to down-regulate the endothelial markers VE-cadherin and CD31 (Fig. 5 C) was abrogated when *CSL* was knocked down. We also targeted *Slug* using two distinct lentiviral-delivered shRNAs and found that the ability of Dll4-activated Notch to down-regulate VE-cadherin and CD31 was also reversed by Slug knockdown (Fig. 5 C and Fig. S3), thus demonstrating the requirement of CSL-mediated induction of *Slug* for Notch-mediated EMT. Furthermore, activation of CSL using a constitutively active CSL mutant (CSL-VP16; MacKenzie et al., 2004) demonstrated that CSL activation alone was sufficient to up-regulate *Slug* expression as well as the Notch target
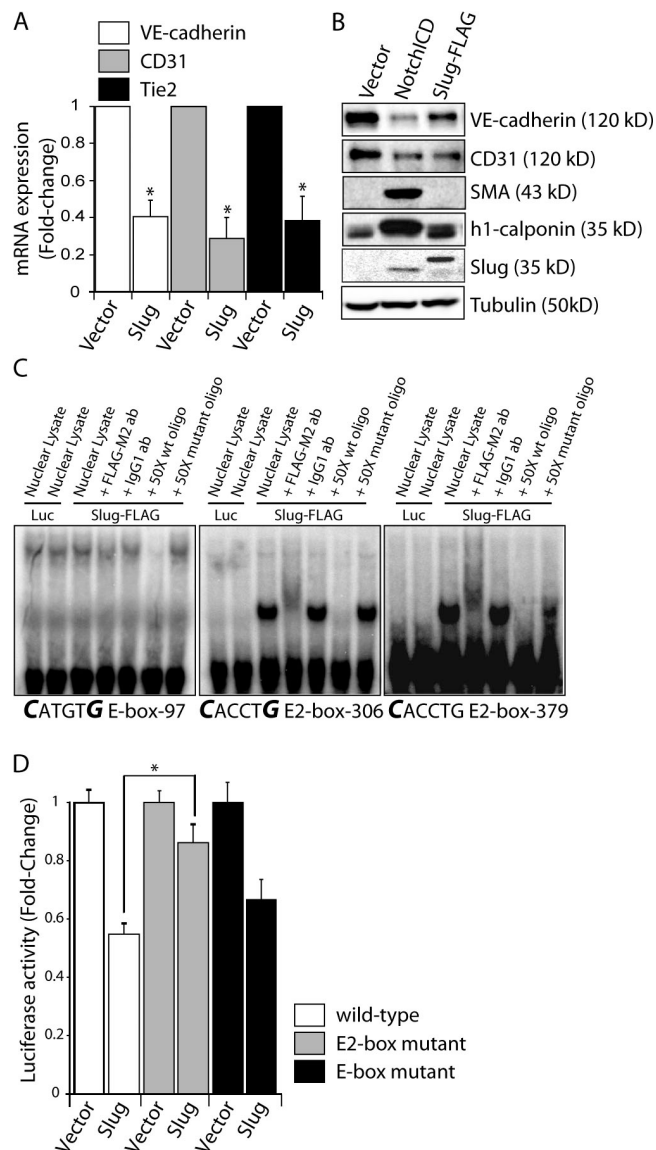

Figure 4. **Slug represses the endothelial phenotype and directly regulates the *VE-cadherin* promoter.** (A) Analysis of endothelial marker expression by qRT-PCR in Slug-transduced HMEC ($n = 3$). *, $P < 0.05$. (B) Immunoblots for endothelial and mesenchymal markers in empty vector–, NotchICD-, and Slug-expressing HMEC. (C) EMSA using in vitro–translated luciferase (Luc) or Slug-FLAG protein and ^{32}P-labeled double-stranded oligonucleotides for an E-box cis element (−97) or two putative *Slug* E2-box motifs (−306 and −379) in the human *VE-cadherin* promoter. Supershift assays with anti–FLAG-M2 or IgG control antibodies and competition assays with 50× wild-type (wt) or mutant probes are also shown. (D) Promoter activity in endothelial cells cotransfected with vector or Slug plasmids and wild-type, E2-box mutant, or E-box mutant mouse *VE-cadherin* promoter-luciferase constructs ($n = 4$; each experiment performed in triplicate). *, $P < 0.05$. Error bars show SEM.

HeyL (Fig. 5 D). However, enforced expression of the Notch targets Hey1 or Hey2, which have been implicated in cardiac EMT, did not up-regulate Slug or repress VE-cadherin (Fig. S3). Together, these findings indicate that Notch, via CSL, directly up-regulates *Slug* expression and that *Slug* is the Notch target responsible for repressing VE-cadherin expression.

Analysis of the human and mouse *Slug* promoters (−2,000 to +100 relative to the TSS) identified six putative CSL binding
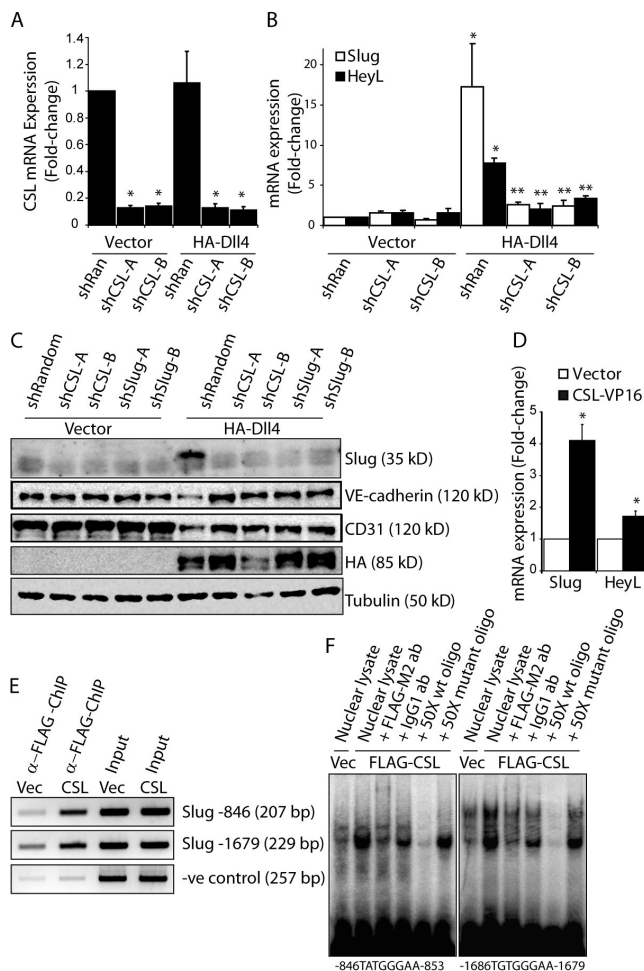
Figure 5. **Notch signaling regulates *Slug* expression through a CSL-dependent pathway.** (A) qRT-PCR analysis demonstrating efficient knockdown of *CSL* in HMEC with two different shRNAs targeting *CSL* (shCSL) compared with a random control sequence (shRan). (B) qRT-PCR of *Slug* and *HeyL* in vector- or Dll4-activated HMEC transduced with shCSL constructs (*n* = 3). *, P < 0.05 vector shRandom versus HA-Dll4 shRandom; **, P < 0.05 HA-Dll4 shRandom versus HA-Dll4 shCSL-A or shCSL-B. (C) Immunoblotting for Slug, VE-cadherin, and CD31 in vector- or Dll4-activated HMEC transduced with shCSL or shSlug constructs. (D) qRT-PCR of vector- or CSL-VP16–expressing HMEC for *Slug* and *HeyL* (*n* = 3). *, P < 0.05. (E) PCR after ChIP with anti–FLAG-M2 antibody on HMEC-expressing vector (vec) or FLAG-CSL (CSL) to demonstrate CSL binding to the human *Slug* promoter. The negative (-ve) control represents PCR of the *ZNF3* promoter after ChIP using FLAG-M2. (F) EMSA using nuclear lysates collected from vector- or FLAG-CSL–expressing HMEC and ^{32}P-labeled double-stranded oligonucleotides spanning each of the two CSL binding sites in the human *Slug* promoter. Supershift assays with anti–FLAG-M2 or IgG control antibodies, and competition assays with 50× wild-type (wt) or mutant probes are also shown. Error bars show SEM.

sites ((C/T)(A/G)TG(A/G/T)GA(A/G/T)) in the human and two putative CSL binding sites in the mouse. Of the six putative binding sites in the human *Slug* promoter, two were further investigated based on conservation of the *CSL* binding sites in the mouse *Slug* promoter. The first binding site (TATGGGAA) is located at −846 to −853, whereas the second binding site (TGTGGGAA) is located at −1,679 to −1,686 upstream of the TSS. Using chromatin immunoprecipitation (ChIP) followed by PCR with primers flanking the CSL elements, we found that CSL was capable of binding both CSL consensus motifs in the

Slug promoter (Fig. 5 E). In contrast, PCR of the same ChIP DNA did not enrich the *ZNF3* promoter, which lacks a putative CSL binding site. EMSA of nuclear lysates harvested from FLAG-CSL–expressing endothelial cells confirmed that CSL is capable of binding both consensus elements present in the human *Slug* promoter (Fig. 5 F). Collectively, these data demonstrate that *Slug* is a direct target of Notch through a CSL-dependent pathway and that Slug induction is required for Notch-mediated repression of the endothelial phenotype.

Notch and TGF-β act synergistically to induce *Snail*

Components of the TGF-β pathway have been shown to be required for EMT and for the regulation of Snail family genes during heart development (Romano and Runyan, 2000; Camenisch et al., 2002a; Wang et al., 2005). Additionally, the Notch and TGF-β pathways have been shown to coregulate target gene expression in various cell types (Blokzijl et al., 2003; Zavadil et al., 2004). To investigate the relationship between the Notch and TGF-β pathways and Snail family member expression, endothelial cells cocultured with vector- or Dll4-transduced cells were treated with 2.5 ng/ml TGF-β2 or 20 or 50 ng/ml BMP2. TGF-β2 stimulation induced maximal induction of *Snail* mRNA and protein expression after 2 h of treatment in vector-transduced cells, followed by rapid down-regulation (Fig. 6, A and B). Although Dll4 stimulation alone did not induce *Snail*, combined activation of the Notch and TGF-β pathways resulted in a synergistic increase of *Snail* mRNA levels and maintenance of expression for at least 8 h after stimulation with TGF-β2 (Fig. 6 A). Protein expression of Snail peaked slightly later (4 h) and was sustained at a much higher level in the context of Dll4 and TGF-β2 costimulation compared with TGF-β2 stimulation alone (Fig. 6 B). In contrast, there was minimal induction of *Slug* by TGF-β2, whereas Notch activation alone dramatically up-regulated *Slug* (Fig. 6, B and C). Costimulation by Dll4 and TGF-β2 did not increase the level of *Slug* induction over that seen with Dll4 alone (Fig. 6, B and C).

To investigate the role of Notch activation in TGF-β2–mediated induction of *Snail*, we used the γ-secretase inhibitor DAPT to block ligand-activated Notch signaling. TGF-β2 treatment dramatically up-regulated the expression of *Snail*, but the addition of DAPT did not affect the ability of TGF-β2 to induce *Snail*, which is consistent with Notch-independent induction (Fig. 6 C). In the context of combined Notch and TGF-β2 activation, the synergistic up-regulation of *Snail* expression was reduced by DAPT to the level seen by TGF-β2 stimulation alone (Fig. 6 C). TGF-β2 had no effect on *Slug* levels, and the addition of DAPT abrogated *Slug* induction by Dll4, suggesting complete dependence on Notch activation for *Slug* up-regulation (Fig. 6 C). Similar results were observed with TGF-β1 treatment (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200710067/DC1). As expected, stimulation of endothelial cells with TGF-β2 induced expression of *Smad7*, a TGF-β target gene, to similar levels in control and Notch-activated cells (Fig. 6 C). Addition of DAPT appeared to block the ability of TGF-β2 to induce *Smad7*, but the results were variable and did not reach statistical significance for TGF-β2 or TGF-β1 (Fig. 6 C
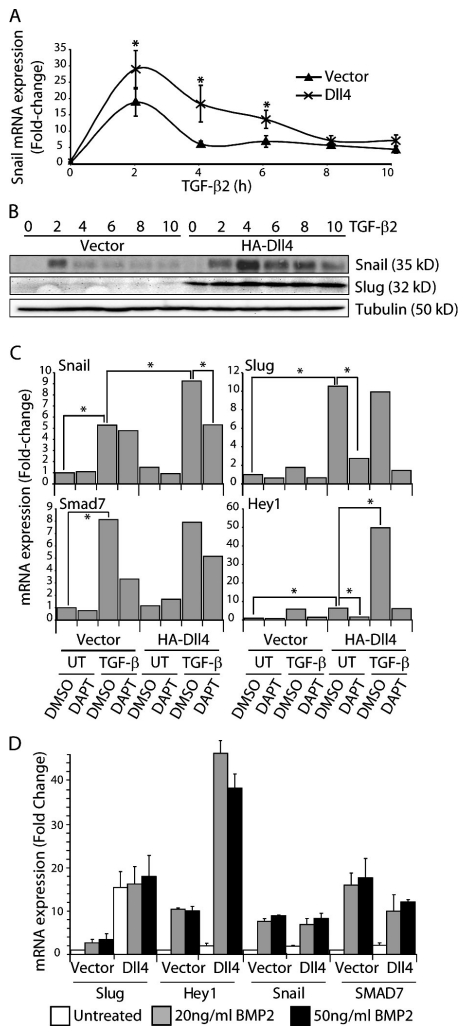
Figure 6. **Induction of *Snail* by TGF-β2 is synergistically enhanced in Dll4-activated endothelial cells.** (A) qRT-PCR for *Snail* mRNA in vector- or Dll4-activated HMEC treated with 2.5 ng/ml TGF-β2 for the indicated times (*n* = 3). *, P < 0.05. (B) Immunoblots for Snail and *Slug* in vector- or Dll4-activated HMEC treated with 2.5 ng/ml TGF-β2. (C) qRT-PCR for *Snail*, *Slug*, *Hey1*, and *Smad7* mRNA in vector- or Dll4-activated HMEC treated with 10 µM DMSO or DAPT for 16 h followed by treatment with 2.5 ng/ml TGF-β2 for 3 h (*n* = 3). *, P < 0.05. (D) qRT-PCR for *Snail*, *Slug*, *Hey1*, and *Smad7* mRNA in vector- or Dll4-activated HMEC treated with 20 or 50 ng/ml BMP2 (*n* = 3). Error bars show SEM.

and Fig. S4), suggesting a minimal role for Notch activation in TGF-β–induced *Smad7* induction. *Hey1* expression was induced by Notch signaling and TGF-β2 and was dependent on active Notch signaling (Fig. 6 C). Similar to what has been described for BMP4/6, *Hey1* was also synergistically induced to very high levels by TGF-β2 and Dll4 (Fig. 6 C; Dahlqvist et al., 2003; Itoh et al., 2004).

Similar to TGF-β2, when endothelial cells were stimulated with BMP2 there was dramatic up-regulation of *Snail* expression, minimal up-regulation of *Slug*, and synergistic activation of *Hey1* in Notch activated cells (Fig. 6 D). However, unlike TGF-β2, combined activation of the Notch pathway and BMP2 stimulation did not synergistically up-regulate *Snail* expression (Fig. 6 D). This suggests that a Smad3-dependent process is involved in the synergistic activation of *Snail* expression by the Notch and

TGF-β pathways. These findings clearly confirm that *Slug* is a direct target of Notch and that *Snail* is not but that *Snail* is synergistically induced when Notch activation is superimposed on TGF-β stimulation.

### *Snail* and *Slug* cooperatively induce cardiac EMT

Given that the cardiac EMT defect seen in *Slug*$^{-/-}$ mice at E9.5 was reduced by E10.5 (Fig. 3, A and B; and Fig. S2), we sought to determine whether *Snail* was compensating for the absence of *Slug* in vivo. qRT-PCR analysis of wild-type and *Slug*$^{-/-}$ hearts was conducted for *Snail* and *Slug*. *Slug* expression increased from E9.5 to 11.5 in the wild-type heart and its expression was abolished in the *Slug*$^{-/-}$ hearts. In contrast, *Snail* expression did not increase from E9.5 to 11.5 in the wild-type hearts. However, in the *Slug*$^{-/-}$ hearts Snail was up-regulated 3.6-fold by E11.5 (Fig. 7 A). In situ hybridization at E10.5 and 11.5 revealed *Snail* expression in the AV canal and OFT in both wild-type and *Slug*$^{-/-}$ hearts, with increased expression in the *Slug*$^{-/-}$ embryos (Fig. 7 B), suggesting that the region of *Snail* expression is not expanded but, rather, that the cells normally expressing *Snail* do so at a higher level in the *Slug*$^{-/-}$ hearts.

We next determined whether the TGF-β pathway, potentially through *Snail*, could compensate for *Slug* deficiency at E9.5. Treatment of E9.5 AV canal explants with 5 ng/ml TGF-β2 completely rescued the EMT defect seen in E9.5 *Slug*$^{-/-}$ embryos (Fig. 7 C). Given the increase in *Snail* expression noted at E10.5 and 11.5 in *Slug*$^{-/-}$ hearts (Fig. 7, A and B), the ability of *Snail* to compensate for the absence of *Slug* after E9.5 was directly assessed using a lentiviral-delivered shRNA to knock down *Snail* expression in *Slug*$^{-/-}$ E10.5 AV canal explants. Knockdown of *Snail* in wild-type or heterozygote *Slug* AV explants did not result in a decrease in the number or distance of migrating cells at E10.5 (Fig. 7 D). In contrast, knockdown of *Snail* in *Slug*$^{-/-}$ AV explants resulted in a significant reduction in the number of migrating/invading cells (Fig. 7 D). The degree of EMT is reduced in E10.5 AV canals compared with E9.5 AV canals, which is consistent with previous data (Dor et al., 2001; Chang et al., 2004). These data support the redundancy of *Slug* and *Snail* during the later stages of EMT in the cardiac cushions and suggest that parallel activation by the Notch and TGF-β–BMP pathways is required to maintain the appropriate level of expression of Snail family members in order for cushion development to proceed.

Discussion

The Notch signaling pathway has been found to be a key regulator of cardiac cushion EMT and has been implicated in the pathogenesis of various cardiovascular diseases (Niessen and Karsan, 2007). TGF-β–related pathways have also been shown to be essential for proper heart development through their role in regulating EMT (Azhar et al., 2003). Thus, there are clear requirements for both the Notch and TGF-β–related pathways during mammalian cardiac cushion development. However, there is limited detail of how these pathways function and interact with each other during cardiac development. Our findings suggest

Figure 7. **Increased *Snail* expression compensates for *Slug* deficiency.** (A) qRT-PCR analysis for *Snail* and *Slug* of whole hearts isolated from E9.5, 10.5, and 11.5 wild-type (wt) and *Slug*$^{-/-}$ embryos (*n* = 3). *, P < 0.05. Error bars show SEM. (B) In situ hybridization for *Snail* in E10.5 and 11.5 wild-type (wt) and *Slug*$^{-/-}$ hearts (*n* = 3; five embryos in each replicate). Bars, 250 µm. (C) Quantitation of EMT in AV canal explants from E9.5 wild-type (wt) or *Slug*$^{+/-}$ and *Slug*$^{-/-}$ embryos treated with 5 ng/ml TGF-β2 or vehicle (UT). (D) Quantitation of EMT in AV canal explants from E10.5 wild-type (wt) or *Slug*$^{+/-}$ and *Slug*$^{-/-}$ embryos transduced with shRandom or shSnail constructs.
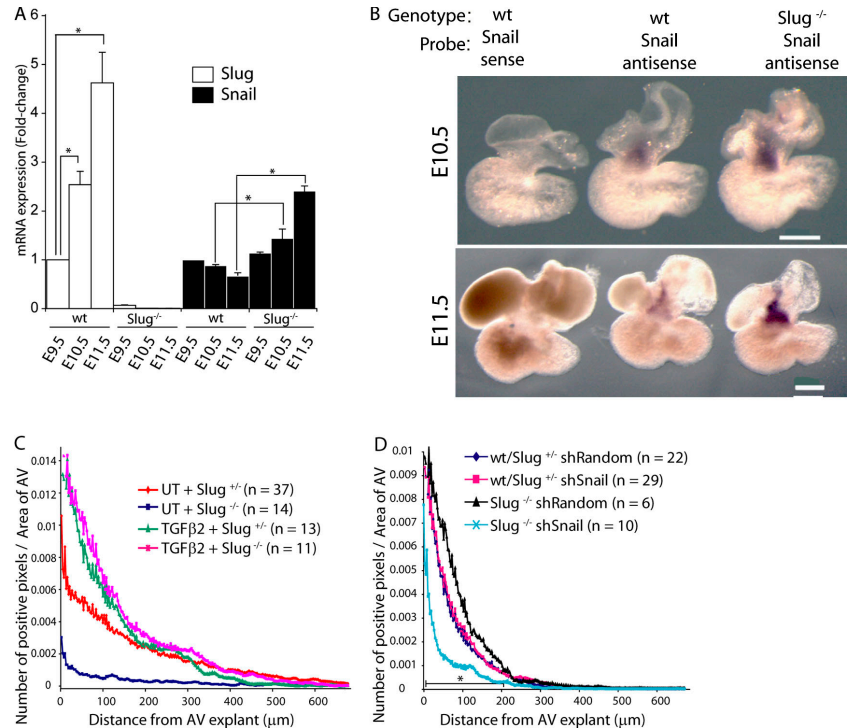
cooperation between the Notch and TGF-β–BMP pathways during cardiac EMT, through the coordinate regulation of a group of genes such as the Snail family of transcription factors. In contrast to a previous study, we demonstrate that the transcriptional repressor *Slug*, but not *Snail*, is a direct target of the Notch pathway (Timmerman et al., 2004). Conversely, activation of the TGF-β pathway dramatically up-regulates the expression of *Snail* but not *Slug*, and *Slug* is not required for TGF-β–mediated EMT. Importantly we reveal synergistic up-regulation of *Snail* expression by the Notch and TGF-β pathways, despite the fact that *Snail* is not a direct target of Notch. This synergistic activation of TGF-β–induced *Snail* by Notch is consistent with the decrease in *Snail* expression seen in *CSL*-deficient embryos (Timmerman et al., 2004).

Interestingly, endothelial-specific gene targeting of the BMP receptor *Alk2* results in cardiac cushion defects that are associated with a decrease in the expression of *Snail*, but not *Hey2* or *Slug*, in the AV canal (Wang et al., 2005). In contrast, Notch-mediated EMT is cell autonomous and TGF-β independent (Noseda et al., 2004). Collectively, these findings support the data presented herein that *Slug* is a direct target of the Notch pathway and that *Snail* is a target of TGF-β–related pathways.

We show for the first time that *Slug* is expressed in a subset of cardiac endothelial cells and the mesenchyme of the AV canal and OFT from the onset of EMT at E9.5 and is essential for initiating cardiac cushion cellularization. We further demonstrate that Slug binds and represses the *VE-cadherin* promoter, inducing a motile phenotype. Taken with the defect in AV canal EMT at E9.5, the ability of Slug to bind and repress the *VE-cadherin* promoter and induce migration suggests that the activation phase of EMT in the endocardium is impaired by loss of *Slug*.

Using an AV canal explant model, we demonstrate that *Slug*-deficient hearts have a specific defect in cardiac cushion EMT at E9.5 but not at E10.5. In *Slug*-deficient hearts at E10.5, cardiac EMT is compensated for by a relative increase in *Snail* expression. Accordingly, inducing *Snail* expression by treatment with TGF-β2 at E9.5 rescues the EMT defect in *Slug*-deficient mice. Conversely, abolishing both *Slug* and *Snail* expression results in EMT defects at E10.5. Consistent with a requirement for both *Snail* and *Slug* during cardiac EMT, both members are expressed during mouse and human heart development with similar localization in a subset of endothelial cells and the mesenchymal cells of the AV canal and OFT. It is of interest that deletion of *Slug* results in up-regulation of *Snail*. This finding suggests that *Slug* may act to repress *Snail*, either by directly targeting the *Snail* promoter or through the repression of elements of the TGF-β–related or Notch pathways. Consistent with the latter hypothesis, we have seen that both *Hey2* and *Smad7* are up-regulated in *Slug*-deficient hearts at E11.5 (unpublished data). Additionally, it has been demonstrated that Slug does not affect *Snail*-promoter activity (Peiro et al., 2006), which we have verified (unpublished data). Our findings are concordant with a recent study showing that Snail heterozygosity increases the penetrance of palate defects in Slug-deficient mice, suggesting that Snail also compensates for Slug deficiency during palate development (Murray et al., 2007). In addition, the finding that there is increased *Slug* expression in the developing palate in *Snail*-deficient embryos (Murray et al., 2007) suggests reciprocal regulation of gene expression between *Slug* and *Snail*.

The interaction between the Notch and TGF-β pathways likely occurs at multiple levels and may be context-dependent. Targeting of *CSL* results in reduced *TGF-β2* and its receptor *TBRII* in the mouse heart (Timmerman et al., 2004). In contrast,

the Notch-ligand *Jagged1* has been shown to be induced by TGF-β at the onset of EMT in epithelial cells (Zavadil et al., 2004). Despite our evidence showing cooperation of the TGF-β and Notch pathways in cardiac cushion development, several studies have suggested that constitutively active NotchICD inhibits TGF-β signaling through the sequestration of Smad3 or the co-activator p300 (Masuda et al., 2005; Sun et al., 2005). However, overexpression of NotchICD may have resulted in artifactual sequestering of TGF-β signaling components. Alternatively, the outcome of Notch–TGF-β cross talk may be dependent on the context. Indeed, in mouse embryonic endothelial cells, BMP signaling synergizes with NotchICD through a ternary interaction between Smad5, NotchICD, and p/CAF (Itoh et al., 2004).

Combining the findings in this paper with published data, one can propose a model where endothelial Notch activation induces *Slug* and release of TGF-β and BMPs from the cushion myocardium activates the cardiac endothelium to up-regulate *Snail*, which is enhanced in Notch-activated cells. Based on the *Slug*-deficient hearts and RNAi studies in the AV explants, a minimal total dose of *Snail/Slug* is required in order for EMT to be initiated at E9.5, with a diminishing requirement at E10.5 as cushion development proceeds.

Materials and methods

Reagents
The mouse monoclonal antibody against the FLAG epitope (M2), mouse anti–h1-calponin, and mouse anti-tubulin were purchased from Sigma-Aldrich. Goat anti–VE-cadherin (C-19), goat anti-CD31 (C-20), and goat anti-Slug (G-18) were obtained from Santa Cruz Biotechnology Inc. Rabbit anti–Snail antibody was obtained from Abcam. Mouse anti–VE-cadherin (TEA1/31) was obtained from Beckman Coulter. Rabbit anti–α–smooth muscle actin antibody was obtained from Thermo Fisher Scientific.

Cell culture and gene transfer
The HMEC-1 human microvascular endothelial cell line, HUVEC, and human aortic endothelial cells were obtained and cultured as previously described (Noseda et al., 2004). Endothelial cells were transduced using the retroviral vectors pLNCX, pLNC-*Slug*-FLAG, pLNC-FLAG-*CSL*, MIY, MIY-*Slug*-FLAG, MIY-*Notch4IC*-HA, MIY-*Notch1IC*, and MIY-*CSL-VP16* as previously described (Karsan et al., 1996). pcDNA3-*Slug*-FLAG cDNA was a gift from E.R. Fearon (The University of Michigan Heath Systems, Ann Arbor, MI).

RNA collection and RT-PCR
RNA was isolated and cDNA was made as previously described (Noseda et al., 2004). PCR was performed on a PCR cycler (PTC-200 [Bio-Rad Laboratories] or 7900HT [Applied Biosystems]) with primers listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200710067/DC1).

Luciferase reporter assay
8×10^4 HMEC was plated 24 h before transfection in 24-well plates. HMEC were transfected using SuperFect (QIAGEN) reagent, with 0.3125 µg of total plasmid DNA as per the manufacturer's recommendations. Each well was transfected with 0.3 µg of the VE-cadherin promoter plasmid or mutant VE-cadherin promoter constructs, 5 ng pcDNA3 or pcDNA3-*Slug*-FLAG, and 7.5 ng pRL-CMV (Promega). Luminescence was measured on a Lumat LB 9507 (EG&G Berthold) 24 h after transfection using dual luciferase reporter assays according to the manufacturer's recommendations (Promega).

EMSA
For Slug EMSA assays, in vitro–translated (TNT; Promega) Slug-FLAG or control luciferase protein was preincubated with FLAG-M2 antibody overnight at 4°C in 12 mM Hepes, pH 7.9, 4 mM Tris, pH 7.9, 133 mM KCl, 10% Glycerol, and 2 µg PolydI-dC binding buffer. 50-fold excess nonradioactive duplex oligos were preincubated for 15 min on ice, and then 150,000-cpm ^{32}P-labeled double-stranded oligo nucleotides were added and incubated for 30 min at room temperature. Binding reactions were run on 5% Tris-borate EDTA gels and exposed to a phosphorimager plate for 12–16 h. For CSL EMSA assays, nuclear lysates were collected from FLAG-CSL–overexpressing HMEC cells. Binding reaction and detection were the same as used for *Slug*-FLAG EMSA assays.

ChIP
HMEC were transduced with pLNCX or pLNC-FLAG-*CSL*, and ChIP assay was performed as previously described (Noseda et al., 2006). ChIP DNA was amplified for the *ZNF3* promoter or the two CSL binding sites in the human *Slug* promoter using primers listed in Table S1.

Mice and AV explant assay analysis
Slug-lacZ mice were provided by T. Gridley (Jackson Laboratories, Bar Harbor, ME). *Slug-lacZ+/−* mice were crossed to C57BL/6J mice for embryo collection. Embryos were assayed for β-galactosidase activity in situ using published protocols (Nagy et al., 2003). AV canal explants were performed as previously described (Camenisch et al., 2002a). Explants were cultured for 48 h and analyzed for the number and distance of migrating cells.

RNA interference
shRNAs targeting human *CSL*, *Snail*, and *Slug* were cloned into the HpaI–XhoI sites of the pLentilox3.7 vector (gift from L. Van Parijs, Massachusetts Institute of Technology, Cambridge, MA; Table S1). Constructs were sequence verified and validated for efficient knockdown.

Collection of human tissues
Human embryonic hearts were collected, after institutionally approved protocols and informed consent, at the Children's and Women's Health Sciences Centre (Vancouver, Canada). Tissue was fixed in 4% PFA overnight, embedded in OCT, and cryosectioned.

In situ hybridization
In situ hybridization was performed as previously described (Wilkinson, 1992). Mouse *Snail* probe (−55 to +454) was cloned into pBluescript. Human *Snail* and human *Slug* probes were comprised of the entire ORF cloned into pCDNA3.

BrdU analysis
Slug-lacZ+/− male and female mice were crossed and pregnant females were injected with 1,500 mg/ml BrdU (Sigma-Aldrich) 2 h before killing. Embryos were collected, paraffin embedded, and sectioned (6 µm) onto Histobond slides (Marienfeld Laboratory Glassware). Slides were boiled for 30 min in 0.1M citrate buffer, rinsed in water, and then denatured in 2N HCl for 45 min at 37°C. Slides were then rinsed in PBS, and BrdU staining was performed using mouse anti-BrdU (BU33; Sigma-Aldrich) and goat anti–mouse Alexa 488 (Invitrogen).

Online supplemental material
Fig. S1 shows that the ankyrin repeats of NotchICD are required for induction of Slug expression. Fig. S2 shows the Slug-lacZ expression in the 18 to 29 somite stage heart and that the AV canal EMT defect observed in Slug-deficient embryos at E9.5 is no longer present at E10.5. Fig. S3 shows that Slug represses endothelial cell phenotype in HMEC and HUVEC, that the knockdown of Slug expression in Notch-activated cells restores VE-cadherin and CD31 expression, and that the ectopic expression of Hey1 or Hey2 does not induce Slug expression or repress VE-cadherin expression. Fig. S4 shows that the induction of Snail by TGF-β1 is synergistically enhanced in Notch-activated endothelial cells. Table S1 is a list of primers used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200710067/DC1.

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