RUNX3 Maintains the Mesenchymal Phenotype after Termination of the Notch Signal

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Notch is a critical mediator of endothelial-to-mesenchymal transition (EndMT) during cardiac cushion development. Slug, a transcriptional repressor that is a Notch target, is an important Notch effector of EndMT in the cardiac cushion. Here, we report that the runt-related transcription factor RUNX3 is a novel direct Notch target in the endothelium. Ectopic expression of RUNX3 in endothelium induces Slug expression and EndMT independent of Notch activation. Interestingly, RUNX3 physically interacts with CSL, the Notch-interacting partner in the nucleus, and induces Slug in a CSL-dependent, but Notch-independent manner. Although RUNX3 may not be required for the initial induction of Slug and EndMT by Notch, because RUNX3 has a much longer half-life than Slug, it sustains the expression of Slug thereby maintaining the mesenchymal phenotype. CSL binds to the Runx3 promoter in the atrioventricular canal in vivo, and inhibition of Notch reduces RUNX3 expression in the cardiac cushion of embryonic hearts. Taken together, our results suggest that induction of RUNX3 may be a mechanism to maintain Notch-transformed mesenchymal cells during heart development.

Endothelial-to-mesenchymal transition (EndMT) is not only a critical process during heart development (1) but also plays a role in some pathologic conditions such as cancer and cardiac fibrosis (2, 3). Notch signaling, which determines cell fate in multiple species (4), is one of the signaling pathways that regulates EndMT (5, 6) and plays a critical role in heart development (7). Notch signaling is initiated by the binding of ligands on signaling cells with Notch receptors on adjacent cells. In mammals, four Notch receptors (Notch1 to Notch4) and five ligands (Dll (Delta-like)1, Dll3, Dll4, Jagged1, and Jagged2) have been identified (8). Upon ligand binding, the transmembrane Notch receptors undergo a series of proteolytic cleavages, which result in release and nuclear translocation of the Notch intracellular domain (NICD).

In the nucleus, NICD associates with the DNA binding protein, CSL, which facilitates the recruitment of coactivators, such as MAML (Mastermind-like) and turns CSL from a transcriptional repressor into a transcriptional activator (9, 10). Primary target genes of Notch signaling regulated by the Notch-CSL complex in mammals include HES (Hairy and enhancer of split) and Hey (HES-related repressor protein) families (11), which act as Notch signaling effectors and negatively regulate the expression of downstream target genes. Hey family members, which include Hey1, Hey2, and HeyL, have been shown to play a critical role in Notch-induced EndMT (12–14). Previous work has also demonstrated that Slug and Snail transcriptional repressors are mediators of Notch-initiated EndMT during heart development (6, 15). However, it is clear that Notch is only activated in the cardiac endothelium overlying the cushions but not in the cushion mesenchyme (16). Nevertheless, Slug continues to be expressed in the cushion mesenchyme, but the mechanism for this is not clear (15). Indeed, very little is known about how cardiac cushion cells retain their mesenchymal features after they have undergone EndMT.

The RUNX family of transcription factors, which comprises three members (RUNX1–3), are essential regulators of cell fate in development and implicated in various human diseases (17). Gene targeting studies in the mouse have shown that Runx1, Runx2, and Runx3 play critical roles in hematopoietic, osteoblastic, and neuronal development, respectively (18). In addition, Runx3-null mice develop hyperplasia of the gastric epithelium due to increased resistance to TGFβ anti-proliferative activity and TGFβ-induced apoptosis (18). Deregulation of RUNX proteins is also involved in human disease (18, 19). In this regard, RUNXI is a frequent target of chromosomal translocations in various leukemia subtypes. Missense mutations of RUNX2 cause cleidocranial dysplasia, and RUNX3 can either act as a tumor suppressor or an oncogene depending on the cancer type.

Regulation of RUNX1 and RUNX2 expression by Notch signaling has been reported in recent years (20, 21). Here, we report for the first time that RUNX3 is a direct target gene of...
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Notch in endothelial cells. Activation of Notch induces RUNX3 expression in a CSL- and MAML1-dependent manner. Enforced expression of RUNX3 in endothelial cells induces EndMT independent of Notch activation. Interestingly, RUNX3 physically interacts with CSL and RUNX3-induced expression of Slug, a positive regulator of EndMT, is inhibited by CSL knockdown. Our data also suggest that although RUNX3 may not be required for the initial induction of Slug and EndMT by Notch, RUNX3 may play a role in sustaining Slug expression and the mesenchymal phenotype induced by Notch. In vivo, CSL binds to the promoter of the Runx3 gene in the mouse atrioventricular canal. Furthermore, endothelial-specific inhibition of Notch by dominant-negative (dn)MAML reduces the expression of RUNX3 in the cardiac cushion of mouse embryonic hearts. Taken together, our studies indicate that RUNX3 is a direct target gene of Notch in endothelial cells and may play a role in sustaining the mesenchymal phenotype in cardiac cushion cells during heart development.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Rabbit anti-RUNX1 antibody was purchased from Active Motif. Mouse anti-RUNX3 antibody (R3–5G4) was obtained from Abcam. CSL antibodies for immunoblotting and ChIP were purchased from the Institute of Immunology (clone T6709, Tokyo, Japan) and ProteinTech Group, Inc., respectively. Mouse anti-SMA was purchased from Thermo Fisher Scientific. Rabbit anti-Slug and rabbit anti-cleaved Notch1 antibodies were purchased from Cell Signaling Technology. Mouse anti-tubulin, mouse anti-h1-calponin, and mouse anti-FLAG antibodies were from Sigma. Mouse anti-PARP was acquired from Novus Biologicals, Inc. Goat anti-VE-cadherin (C-19) and goat anti-Tie2 antibodies were obtained from Santa Cruz Biotechnology. DAPT and cycloheximide were purchased from CalBiochem.

Cell Culture and Transfection—The human microvascular endothelial cell line, retroviral packaging cell line PhoenixTM-Ampho, and 293T cells were cultured as described previously (15). pLNCx-FLAG-CSL, pMSCVpac-RUNX3, or pLNCx-FLAG-CSL, pLNCx-CSLR179H were used for retroviral infection of human microvascular endothelial cells (HMEC) or transiently transfection of 293T cells as described previously (22). TransIT-LT1 Transfection Reagent (Mirus, Madison, WI) or calcium phosphate was used for transfection of PhoenixTM-Ampho and 293T cells (22).

Preparation of Whole Cell Lysate and Cytosolic and Nuclear Fraction—Whole cell lysates and cytosolic and nuclear fractions were prepared as described previously (22). Proteins in these samples were quantified using DC protein assay (Bio-Rad) and used for immunoblotting analysis as described previously (22).

RNA Isolation and Quantitative RT-PCR (qRT-PCR)—Total RNA isolation and quantitative RT-PCR were performed as described previously (22). Total RNA was isolated using the GenEluteTM Mammalian Total RNA Miniprep Kit (Sigma), and DNase treated before cDNA was synthesized using SuperScript II reverse transcriptase reagent (Invitrogen) in the presence of RNase inhibitor. qRT-PCR was carried out using the SYBR Green method on an Applied Biosystems 7900HT (Applied Biosystems, Foster City, CA). Sequences of the primers for quantitative (q)PCR are listed in supplemental Table 1.

Immunocytochemistry—Immunocytochemistry was conducted as described previously (23). Briefly, HMEC transduced with RUNX3 or empty vector were seeded at the four-well chamber and cultured overnight. Cells were then fixed and stained with rabbit polyclonal antibodies for RUNX3, VE-cadherin, or SMA. Images were acquired with a 1350EX cooled charge-coupled device digital camera (QImaging, Burnaby, British Columbia, Canada) on a Zeiss Axioplan II Imaging inverted microscope (Carl Zeiss Canada, Ltd., Toronto, Ontario, Canada) and analyzed with Northern Eclipse image analysis software (Empix Imaging, Mississauga, Ontario, Canada).

Coimmunoprecipitation Assay—Coimmunoprecipitation assay was conducted as described previously (22). Briefly, HEK 293T cells were transiently transfected with empty vectors, pLNCx-FLAG-CSL, MSCVpac-RUNX3, or both. After 48 h, whole cell lysates were prepared, and the coimmunoprecipitation was conducted using anti-FLAG or anti-RUNX3 (R3-5G4) antibodies. The physical interaction between RUNX3 and CSL was examined by immunoblotting of the IP elute using the anti-FLAG or anti-RUNX3 antibodies.

RNA Interference—Two lentiviral-delivered shRNA constructs targeting human CSL were generated and validated as described previously (15). Knockdown of CSL in HMEC by these two shRNA constructs was confirmed by immunoblotting using CSL antibody (T6709). Two shRNA constructs (shRUNX3A and shRUNX3B) targeting human RUNX3 were generated using the same approaches as shRNAs for CSL (15). The target sequences for shRUNX3A and shRUNX3B were as follows: 5’-GGACCTTACATACCTGAGGA-3’ and 5’-GCCGTTCTCATCCCATCATCT-3’, respectively.

Chromatin Immunoprecipitation Assay—ChIP to examine the binding of CSL to the RUNX3 promoter in HMEC was conducted using an anti-FLAG antibody as described previously (24). Briefly, HMEC were cross-linked using 1% formaldehyde and harvested using lysis buffer. After sonication, the equal amount of chromatin was used for ChIP assay using anti-FLAG antibody with IgG as a negative control. Enrichment of the RUNX3 promoter DNA was detected by qPCR using primers flanking the CSL binding sites (22). Two shRNA constructs flanking the CSL bind sites in RUNX3 or SMA promoter, normalized again the input DNA, and expressed as the relative signal with vector/antibody samples designated as 1. ChIP assay to examine the CSL binding to the Runx3 promoter of mouse embryonic hearts was conducted using a protocol described by Wederell et al. (25). Briefly, atrioventricular canals at embryonic day 10.5 (E10.5) were dissected from C57BL/6j mouse embryos following procedures approved by the animal care Committee at the University of British Colombia. After the cross-linking and sonication, equal amount of chromatin (15 μg) was used for ChIP assay using 1 μg CSL antibody (ProteinTech Group, Inc.) or 1 μg normal rabbit IgG (Vector Laboratories, Burlingame, CA) as negative control. Enrichment of the Runx3 or the SMA promoter in the purified ChIP DNA was detected by qPCR using primers flanking the CSL binding sites on the Runx3 or the SMA promoter, normalized again the input
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FIGURE 1. Notch activation induces RUNX3 expression in human endothelial cells. Notch signaling in HMEC is activated by overexpression of NICD or coculture of parental HMEC with ligand-expressing HMEC at a 1:1 ratio (Dll4 or Jag1). A and B, the mRNA level of RUNX1–3 and two known Notch target genes, HEY2 and HEYL was examined by qRT-PCR and normalized with that of GAPDH. The relative mRNA levels were expressed as fold changes with that of vector samples designated as 1 and shown as mean ± S.E. of three independent experiments. *, significantly different (p < 0.05) from the respective vectors. C, the RUNX protein expression in the cytosolic and the nuclear fractions was examined by immunoblotting. Tubulin and PARP were used as loading controls for the cytosolic and the nuclear fraction, respectively. D–F, Dll4 or Jag1 coculture was treated with 10 μM DAPT or an equal volume of DMSO for 24 h. Expression of cleaved Notch1 and VE-cadherin in the whole cell lysates were examined by immunoblotting. Tubulin was used as a loading control (D). The mRNA level of RUNX3 was examined by qRT-PCR and normalized with that of GAPDH. The relative mRNA levels were expressed as fold changes with that of DMSO-treated vector sample designated as 1 and shown as mean ± S.E. of three independent experiments. *, significantly higher (p < 0.05) than all other three samples (E). Expression of RUNX3 and VE-cadherin in the whole cell lysates were examined by immunoblotting. Tubulin was used as a loading control (F).

and expressed as relative signal with that of IgG control designated as 1. Primer sequences for the ChIP assays for HMEC and mouse embryonic hearts are listed in supplemental Tables 2 and 3, respectively.

RUNX3 Expression in Mouse Cardiac Cushion Cells—Tet<sup>COS</sup>-dnMAML1 transgenic mice were generated in house and will be described elsewhere. <sup>6</sup> dnMAML1 mice were crossed with Tie1 (receptor tyrosine kinase 1)-tTA mice (26) (gift of D. Dumont, University of Toronto, Toronto, ON) and in the double transgenic offspring, inducible expression of dnMAML1 blocks Notch activity in an endothelial cell-specific manner. Inhibition of Notch activity by dnMAML1 expression was initiated at E9.5 by removal of tetracycline from the drinking water. RUNX3 protein expression in the cardiac cushion cells was examined at E10.5 by immunofluorescence staining using anti-RUNX3 antibody (R3-5G4). RUNX3 protein expression was quantified using ImageJ software and normalized to total cell numbers by counting the DAPI-stained nuclei in the same area.

RESULTS

Activation of Notch Induces RUNX3 in Endothelial Cells—A functional Notch-RUNX axis has been suggested in hematopoiesis in zebrafish and mouse (20, 27). To investigate whether RUNX proteins are downstream targets of Notch in endothelial cells, we activated Notch signaling in HMEC by overexpression of constitutively-active Notch (NICD) or by coculture of parental HMEC with Notch ligand Dll4- or Jagged1-expressing HMEC at a 1:1 ratio (Dll4 or Jag1 coculture) (15, 22). We selected HMEC for this study because these cells have proved to be a good in vitro model to investigate EndMT (5, 15, 24). qRT-PCR revealed that Notch activation significantly increased RUNX3 mRNA expression, but did not affect the expression of RUNX1 (Fig. 1, A and B). RUNX2 mRNA expression was inhibited by NICD, but increased by Dll4 coculture (Fig. 1, A and B). As a positive control, we included the induction of HEY2 and HEYL, two known direct Notch target genes, by NICD and Dll4...
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**FIGURE 2. RUNX3 is a direct target gene of Notch in human endothelial cells and mouse embryonic hearts.** A, Dll4 or Jag1 coculture was performed using HMEC transduced with FLAG-CSL-R179H or vector. Expression of RUNX3 and CSL-R179H in the whole cell lysates was examined by immunoblotting. Tubulin was used as a loading control. B, HMEC were transduced with dnMAML, NICD, or both. Expression of RUNX3 in the whole cell lysates was examined by immunoblotting. Tubulin was used as a loading control. C, HMEC were transduced with FLAG-CSL or vector. Expression of FLAG-CSL and endogenous CSL in whole cell lysates were examined by immunoblotting using anti-FLAG and anti-CSL antibodies, respectively. Tubulin was used as a loading control. D, CSL occupancy on CSL binding sites in the RUNX3 or SMA promoter was examined by ChIP using anti-FLAG antibody with IgG as negative control. ChIP-qPCR was conducted using primers flanking CSL sites in RUNX3 and SMA promoters. The occupancy of CSL on these sites were calculated as percentage of the respective input DNA concentration and expressed as relative signal after normalized against the vector antibody samples (designated as 1). Values are shown as mean ± S.E. of four independent experiments. *, significantly higher (p < 0.05) than all other three samples. E, CSL occupancy on CSL binding sites in the Runx3 and SMA promoters in mouse embryonic hearts was examined by ChIP using anti-CSL antibody with IgG as negative control. ChIP-qPCR was conducted using primers flanking CSL sites on the mouse Runx3 and SMA promoters. The occupancy of CSL on these sites were calculated as percentage of the respective input DNA concentration and expressed as relative signal after normalized against the IgG samples (designated as 1). Values are shown as mean ± S.E. of four independent experiments. *, significantly higher (p < 0.05) than the respective IgG samples.

coculture (Fig. 1, A and B). We thus focused on RUNX3, because RUNX3 expression was consistently induced by both NICD and Dll4 coculture. Immunoblotting of the nuclear fraction of HMEC showed that HMEC express a low level of the endogenous RUNX3 protein, which was greatly induced by NICD and Dll4 or Jag1 coculture (Fig. 1C). In contrast, RUNX1 was only slightly increased by Jag1 coculture or NICD (Fig. 1C). Thus, our data demonstrate that RUNX3 is induced by Notch activation in endothelial cells.

**Notch Induces RUNX3 Expression in a CSL- and MAML1-dependent Manner**—To confirm that Dll4 or Jagged1-induced RUNX3 expression is mediated by Notch activation, we treated Dll4 or Jag1 cocultures with DAPT, a γ-secretase inhibitor that blocks the cleavage of Notch receptors and the consequent activation of Notch. Immunoblotting showed that DAPT effectively blocked Dll4-induced Notch activation as indicated by inhibition of Notch1 cleavage and restoration of the repressed VE-cadherin expression in Dll4 cocultures (Fig. 1D). In keeping with the blockade of Notch cleavage, induction of RUNX3 by Dll4 or Jagged1 was also inhibited by DAPT at both the mRNA (Fig. 1E) and protein level (Fig. 1F), indicating that Dll4 or Jagged1-induced RUNX3 expression indeed depends on Notch activation.

To determine whether Notch-induced RUNX3 expression was CSL-dependent, we cocultured Dll4 or Jag1 HMEC with HMEC transduced with a dominant-negative mutant of CSL (CSL-R179H) or an empty vector control. Because CSL-R179H does not bind DNA but retains the ability to interact with NICD, it inhibits CSL-dependent Notch signaling by sequestering NICD away from endogenous CSL at the promoters of target genes (28). As shown in Fig. 2A, Dll4 or Jagged1-induced RUNX3 protein expression was inhibited by overexpression of CSL-R179H, suggesting that Notch-induced RUNX3 is CSL-dependent. In the nucleus, the association of NICD with CSL recruits the coactivator MAML1 to form a ternary complex to activate gene expression (9, 10). A dominant-negative MAML1 (the first 13–74 amino acids of N terminus of MAML1) interrupts the transactivation activity of Notch (29). As shown in Fig. 2B, NICD-induced RUNX3 protein expression was abrogated by the expression of dnMAML. Therefore, our data suggest that Notch-induced RUNX3 expression requires the intact and functional NICD-CSL-MAML1 ternary complex.

Next, we examined whether CSL directly binds the RUNX3 promoter. Because we were not able to find a CSL antibody that pulled down endogenous human CSL in a ChIP assay, we transduced HMEC with a FLAG-tagged CSL or empty vector and performed a ChIP assay using anti-FLAG antibody (15, 24). The increased relative expression of the exogenous FLAG-CSL permits competition for CSL binding sites over endogenous CSL (Fig. 2C). The enrichment of the RUNX3 proximal promoter in the ChIP DNA was analyzed by qPCR using a pair of primers flanking a putative CSL consensus sequence in the human RUNX3 promoter (TGGGAAA, −151 to −145, relative to the transcriptional start site), which is conserved in the mouse. ChIP-qPCR showed that CSL bound the RUNX3 promoter to a similar extent as the SMA promoter (Fig. 2D), which we have previously shown (24). To examine whether Runx3 is a direct target gene of Notch in vivo, we conducted a ChIP assay using a CSL antibody that can pull down the endogenous mouse CSL in a ChIP assay. As shown in Fig. 2E, Runx3 promoter DNA was
enriched by CSL ChIP of atrioventricular canals of E10.5 mouse hearts. Taken together, our data demonstrate that RUNX3 is a direct target gene of Notch in endothelial cells and embryonic hearts.

**Overexpression of RUNX3 Induces an EndMT**—Our finding that RUNX3 is a direct Notch target gene in endothelial cells prompted us to examine whether RUNX3 plays a role in EndMT as a downstream effector of Notch (5, 6). To this end, we overexpressed RUNX3 in HMEC (Fig. 3A) and found that overexpression of RUNX3 down-regulated the mRNA expression of endothelial markers (Fig. 3B) and up-regulated the expression of two positive regulators of EndMT, **SLUG** and **SNAIL**, and the mesenchymal markers (**SMA**, **TAGLN**, **CALPONIN**, and **DESMIN**) (Fig. 3C). Interestingly, RUNX3 also induced the classic Notch target genes **HEY1**, **HEY2**, and **HEYL** (Fig. 3C), albeit the induction was much less pronounced than that by Notch (22). Down-regulation of endothelial markers and up-regulation of mesenchymal markers were further confirmed by immunoblotting and immunocytochemistry (Fig. 3, D and E). Thus, our data demonstrated that, similar to Notch, overexpression of RUNX3 in endothelial cells induces EndMT.

**RUNX3-induced EndMT Is Independent of Notch Activation**—Because RUNX3 induces EndMT similar to Notch (Fig. 3, B–E) (5), we examined whether RUNX3 induces EndMT by up-regulating Notch signaling through a positive feedback mechanism. First, we examined the expression of Notch ligands and receptors in RUNX3-expressing HMEC and found that overexpression of RUNX3 decreased the components of Notch signaling examined except for Notch3, which in contrast, was increased (Fig. 4A). Because Notch3 is predominantly expressed in vascular smooth muscle cells (30), the increased expression of Notch3 by RUNX3 is likely a consequence of EndMT. Thus, our data suggest RUNX3-induced EndMT is unlikely to be mediated by up-regulating Notch ligands or receptors. To determine whether RUNX3-induced EndMT requires the activation of Notch, we blocked Notch signaling in

![FIGURE 3. Overexpression of RUNX3 induces EndMT.](image-url)
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**A**

![Graph showing mRNA level (Fold) for Jag1, Dll4, Notch1, Notch2, Notch3, and Notch4](image)

**B**

![Graph showing mRNA level (Fold) for VE-cadherin, Slug, and SMA](image)

**C**

![Graph showing protein expression for Tie2, VE-cadherin, Slug, SMA, Calponin, and Tubulin](image)

**FIGURE 4. RUNX3-induced EndMT is independent of Notch activation.**

A, HMEC were transduced with RUNX3 or vector. The mRNA level of Jag1, Dll4, and Notch1–4 in these cells was examined by qRT-PCR and normalized with that of GAPDH. The relative mRNA levels were expressed as fold changes with that of vector samples designated as 1 and shown as mean ± S.E. of three independent experiments. *, significantly different (p<0.05) from the respective vectors. B, HMEC transduced with RUNX3 or vector were treated with 10 μM DAPT or an equal volume of DMSO for 48 h. The mRNA level of VE-cadherin, Slug, and SMA was examined by qRT-PCR and normalized with that of GAPDH. The relative mRNA levels were expressed as fold changes with that of vector/DMSO samples designated as 1 and shown as mean ± S.E. of three independent experiments. *, significantly different (p<0.05) from the respective vectors. C, HMEC transduced with RUNX3 or vector were treated with 10 μM DAPT or an equal volume of DMSO for 48 h. The protein expression of Tie2, VE-cadherin, Slug, SMA, and Calponin in the whole cell lysates was examined by immunoblotting. Tubulin was used as a loading control.

**RUNX3-expressing or vector-transduced HMEC with a γ-secretase inhibitor DAPT.** qRT-PCR and immunoblotting showed that blockade of Notch did not affect the down-regulation of endothelial markers and the up-regulation of Slug and mesenchymal markers by RUNX3 (Fig. 4, B and C). Therefore, our data suggest that RUNX3-induced EndMT is independent of Notch activation.

**RUNX3-induced Slug Expression Is Partially CSL-dependent—** Our finding that RUNX3 induces EndMT and regulates a subset of Notch target genes similar to Notch, but independent of Notch activation, suggested to us that RUNX3 might regulate the expression of some genes through interacting with CSL, the nuclear partner of Notch (6, 31). To examine the physical interaction between RUNX3 and CSL, we transiently transfected 293T cells with MSCVpac-RUNX3, pLNCx-FLAG-CSL, or both and conducted coimmunoprecipitation assays. As shown in Fig. 5A, IP-immunoblotting experiments showed that IP of RUNX3 pulled down FLAG-CSL, and a reciprocal IP using anti-FLAG antibody pulled down RUNX3, suggesting that RUNX3 and CSL indeed physically interact when overexpressed. Furthermore, RUNX3-induced Slug expression was at least partially CSL-dependent, because knockdown of CSL by two shRNA constructs targeting distinct CSL sequences (shCSL-A and shCSL-B versus shRandom) (15) inhibited RUNX3-induced Slug expression (Fig. 5B). We also examined whether RUNX3 interacts with NICD using the same approach described above in 293T cells but did not find an interaction between RUNX3 and NICD (data not shown). Interestingly, even though both Notch and RUNX3 regulate Slug expression through an interaction with CSL (15), their effects on downstream target genes appear to be gene-specific. In particular, RUNX3 showed a similar effect to Notch on genes in Fig. 5C, no effect on genes in Fig. 5D, and an opposite effect to Notch on genes in Fig. 5E. These data suggest that NICD and RUNX3 may recruit different co-activators or repressors to the target genes, thus exerting different effects on their expression. Alternatively, NICD and RUNX3 may regulate the expression of these genes through a CSL-independent mechanism (32).

**RUNX3 Has a Longer Half-life than Slug—** The finding that Notch induces the expression of RUNX3 which, similar to Notch, induces EndMT, prompted us to examine whether RUNX3 is required for the induction of EndMT by Notch. To this end, we knocked down the expression of RUNX3 in HMEC using two shRNA constructs. As shown in Fig. 6, A and B, both shRNA constructs were able to knock down the expression of RUNX3 protein to low levels, including in HMEC transduced with NICD or Jagged1. Knockdown of RUNX3 did not affect the induction of Slug or down-regulation of Tie2 and VE-cadherin by NICD or Jagged1 (Fig. 6, A and B), suggesting that RUNX3 may not be required for the induction of EndMT by Notch. One possible role for Notch–induced RUNX3 may be to sustain the expression of Slug and the mesenchymal phenotype induced by Notch. The half-life of NICD in the nucleus is short (33, 34). If RUNX3 is more stable than Slug, then the simultaneous induction of RUNX3 and Slug by Notch signaling is turned off. To determine their half-lives, we transduced HMEC with RUNX3, Slug, or empty vector and left the cells untreated or treated with 50 μg/ml cycloheximide for various times. Expression of RUNX3 and Slug was then examined by immunoblotting using anti-RUNX3 and anti-Slug antibodies, respectively. As shown in Fig. 6, C and D, the half-life of RUNX3 protein is >8 h, whereas that of Slug is only ~2 h,
indicating that RUNX3 has a much longer half-life than Slug. This finding suggests that RUNX3-mediated Slug expression probably accounts for persistence of the mesenchymal phenotype in Notch-transformed cells when Notch is no longer activated.

**RUNX3 Expression in Cardiac Cushion of Mouse Embryonic Hearts Was Reduced by Notch Inhibition**—Our finding that CSL binds to the RUNX3 promoter in embryonic atrioventricular canals (Fig. 2E) suggests that Runx3 is a direct Notch target gene in embryonic hearts. To investigate whether RUNX3 expression is regulated by Notch in vivo, we examined the expression of RUNX3 in the cardiac cushion of mouse embryonic hearts from mice inducibly expressing the pan-Notch inhibitor, dnMAML1, from an endothelial promoter (22). Using this model, we have demonstrated that inhibition of Notch activity by the endothelial-specific expression of dnMAML1 impairs EndMT and cardiac cushion cellularization of mouse embryonic hearts (22). Endothelial-specific expression of dnMAML1 was induced at E9.5 and the expression of RUNX3 was examined at E10.5 by immunostaining using an anti-RUNX3 antibody (R3-5G4). As shown in Fig. 7A, anti-RUNX3 antibody, but not the IgG control, gave rise to strong nuclear staining of cardiac cushion cells. Quantitative analysis using the NIH ImageJ software showed that the expression of RUNX3 in the cardiac cushion was significantly reduced by dnMAML1 (Fig. 7B), suggesting that RUNX3 expression is indeed regulated by Notch in vivo. Taken together, our data suggest that Runx3 is a direct target gene of Notch in the mouse embryonic heart and may be necessary for sustaining the mesenchymal phenotype of cardiac cushion cells.

**DISCUSSION**

During heart development, a subset of endocardial cells undergoes EndMT, which is initiated at E9.5 in the atrioventricular canal and E10.5 in the outflow cardiac cushion and contributes to the formation of septa and valves of the adult heart.
heart (1). EndMT is regulated by multiple signaling pathways including Notch (5, 6). In humans mutations of Notch1 and Jagged1 causes several types of congenital heart defects (21, 35). The well characterized direct target genes of Notch, including Hey family transcriptional repressors and Snail family transcriptional repressor Slug (11, 15), have been shown to play a role in Notch-induced EndMT during heart development (12–15). However, deficiency of these genes does not completely recapitulate the phenotype of targeting Notch or CSL in the heart (31, 36), suggesting that these target genes mediate only a subset of functions of Notch.

RUNX1 is shown to be a critical downstream effector of Notch in hematopoiesis (20, 27), and RUNX3 is induced by Notch during human T-cell development (37), suggesting a potential role of RUNX proteins in Notch signaling. Here, we report that RUNX3, but not RUNX1, is the major RUNX protein to be induced by Notch in endothelial cells. Our data suggest that the functional ternary complex of NICD-CSL-MAML1 is required for the Notch-induced RUNX3 expression because it is blocked by either overexpression of a dominant-negative CSL (CSL179H) or a dnMAML. Furthermore, our ChIP data have shown that CSL directly binds to the CSL consensus sequences on the RUNX3 promoter in human endothelial cells and in the atrioventricular canals of mouse embryonic hearts. Therefore our data demonstrate that RUNX3 is a direct target gene of Notch in endothelial cells.

Several studies have shown that RUNX2 activity is repressed by Notch through physical interaction between NICD or Notch downstream target HES and Hey proteins with RUNX2 (21, 38, 39), and a recent study showed that inhibition of Notch by DAPT or Notch1 siRNA increases the expression of RUNX2 in sheep aortic valve interstitial cells (40). Increased expression and/or activity of RUNX2 due to the decreased Notch1 signaling contributes to aortic valve calcification in humans (21) and in mice (40). In this study, we show that the expression of RUNX2 is decreased by overexpression of NICD in endothelial cells (Fig. 1A), which is in accordance with the published study using sheep aortic valve interstitial cells (40). In contrast, RUNX2 expression is increased by Dll4-cocultures in endothelial cells suggesting that RUNX2 may not be a physiological Notch target in vivo.

The finding that RUNX3 is a direct target gene of Notch in endothelial cells prompted us to investigate whether RUNX3 plays a role in EndMT. Overexpression of RUNX3 significantly

**FIGURE 6.** RUNX3 protein has a longer half-life than Slug protein. A and B, expression of RUNX3 in HMEC was knocked down by two shRNA constructs, shRUNX3A and shRUNX3B. shRandom was the negative control. These cells were subsequently transduced with NICD (A) or Jagged1 (B). Transduced cells were purified by antibiotic selection. Expression of RUNX3, induction of Slug, and down-regulation of Tie2 and VE-cadherin by NICD or Jagged1 in the transduced cells were examined by immunoblotting. Tubulin was used as a loading control. C, HMEC transduced with RUNX3 or Slug were treated with 50 μg/ml of cycloheximide (CHX) for various hours. RUNX3 and Slug protein levels in whole cell lysates were examined by immunoblotting using anti-RUNX3 and anti-Slug antibodies, respectively. D, the density of the RUNX3 or Slug bands was measured by densitometry and normalized against that of the respective tubulin. The density of the RUNX3 or Slug protein at 0 h was designated as 100%. The relative density of RUNX3 or Slug protein at each time point was shown as mean ± S.E. of three independent experiments. *, significantly higher (p < 0.05) than Slug at the respective time points.
reduces the expression of endothelial markers and induces mesenchymal markers at both mRNA and protein levels, indicating that RUNX3 overexpression indeed induces EndMT. Interestingly, RUNX3 also induces the expression of *HEY1*, *HEY2*, *HEYL*, and Slug, which are primary Notch target genes (11, 15). Thus, we wanted to investigate whether RUNX3 induces EndMT by increasing Notch signaling in these endothelial cells. Our data suggest that this is not the case. First, RUNX3 overexpression decreases all the Notch components to be examined except for Notch3 (Fig. 4A). Dll4 is the major Notch ligand in vascular endothelial cells (41), and Notch4 is specifically expressed in vascular endothelial cells (42). The expression of both Dll4 and Notch4 is significantly decreased by RUNX3, which is more pronounced than the reduction of Notch1 and Notch2. The only receptor that is increased by RUNX3 is Notch3, which is predominantly expressed in vascular smooth muscle cells (30). Therefore, the increased expression of Notch3 appears to be a consequence, not the cause, of EndMT. More importantly, blockade of Notch by DAPT does not affect RUNX3-induced EndMT (Fig. 4, B and C). Therefore, we conclude that RUNX3 induces EndMT independent of Notch, suggesting that RUNX3 can induce EndMT even after Notch signaling becomes inactivated. Further, we find that RUNX3 is a novel CSL-interacting partner. We found that

FIGURE 7. Endothelial-specific inhibition of Notch activity reduces RUNX3 expression in mouse embryonic hearts. A, Notch activity in endothelial cells was inhibited by inducible expression of dnMAML1 at E9.5 and RUNX3 expression in cardiac cushion cells was examined at E10.5 by immunofluorescence staining using a RUNX3 antibody. IgG was used as a negative control for RUNX3 staining. Red, RUNX3; blue, DAPI. A, atrium; V, ventricle; AVC, atrioventricular cushion. Bars, 50 μm. B, RUNX3 expression in cardiac cushion cells from 17 sections of four control hearts and 10 sections of four dnMAML hearts was examined by immunofluorescence staining. The intensity of RUNX3 staining was analyzed using ImageJ software, normalized against the cell numbers in the same area, and expressed as intensity per cell (arbitrary units, mean ± S.E.). *, significantly lower (p < 0.05) than control.
RUNX3 physically interacts with CSL, but not NICD, and RUNX3-induced Slug expression is partially inhibited by CSL knockdown. The partial inhibition suggests that the interaction between CSL and RUNX3 enhances but is not required for the RUNX3-induced gene expression. A recent study showed that RUNX3 directly interacts with NICD1, but not CSL, and suppresses Notch signaling in hepatocellular carcinoma cells (43). Therefore, the interaction between RUNX3 and CSL and NICD and the role for RUNX3 in Notch signaling appears to be cell type-specific.

To investigate the role of RUNX3 in Notch-induced EndMT, we knocked down the expression of RUNX3 in HMEC and activated Notch in these cells by NICD or Jagged1. Our data show that RUNX3 knockdown did not affect Notch-induced Slug expression and down-regulation of endothelial markers (Fig. 6, A and B), suggesting that RUNX3 may not be required for Notch-induced initiation of EndMT. However, because the shRNA knockdown of RUNX3 was not complete, whether the remaining RUNX3 is adequate to mediate Notch-induced EndMT needs to be further investigated. Nevertheless, our finding that RUNX3 has a longer half-life than Slug suggests that the simultaneously induced RUNX3 by Notch sustains Slug expression and the mesenchymal phenotype of Notch-transformed cells after Notch is activated. In most cells, Notch activation is under tight control through proteasomal degradation to ensure that the half-life of NICD is short (33, 34) because the sustained accumulation of NICD in the nucleus is deleterious in vivo (44–46). Appropriate spatial and temporal activation of Notch has been shown to be critical for proper heart development (7). Our finding that Notch and RUNX3 have both similar and distinct effects on target genes expression (Fig. 3, B and C and Fig. 5, C–E) (5) suggests that, after the Notch signal is inactivated, RUNX3 may act to sustain the expression of a subset of Notch target genes, such as those associated with EndMT, reducing the expression of Notch target genes whose sustained expression will have deleterious effects. Thus, the sequential activation of Notch and RUNX3 could be a delicate mechanism to initiate and complete EndMT and maintain the mesenchymal phenotype of cardiac cushion cells. In support of a role for RUNX3 in EndMT and heart development, we have found that CSL directly binds the Runx3 promoter in mouse embryonic hearts and that endothelial-specific inhibition of Notch reduces RUNX3 expression in the cardiac cushion of mouse embryonic hearts, thus providing in vivo evidence for the regulation of RUNX3 by Notch.

In summary, we have demonstrated that RUNX3 is a direct downstream target of Notch in endothelial cells and may play a role in maintaining the mesenchymal phenotype of Notch-transformed cells during heart development. Our study has revealed a novel role for RUNX3 in EndMT in addition to the well documented role in neurogenesis and tumorigenesis (18). Because EndMT is not only a critical process for embryonic development but also plays a role in cardiac fibrosis and cancer progression, a better understanding of the role of RUNX3 in EndMT may provide insight into these processes.

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