DEVELOPMENTAL DYNAMICS 239:2594–2602, 2010

RESEARCH ARTICLE

Adam10 Is Essential for Early Embryonic Cardiovascular Development

Chi Zhang,[1] Lei Tian,[1,2] Congwu Chi,[1,3] Xiaohui Wu,[1] Xiao Yang,[4] Min Han,[1,3] Tian Xu,[1,2] Yuan Zhuang,[1,5] and Kejing Deng[1]*

Notch pathway has been demonstrated to regulate cardiovascular development. One important step in Notch pathway is the cleavage of Notch receptor, during which an intracellular fragment of Notch protein is released to activate downstream genes. It is still uncertain whether *Adam10*, the mammalian homologue of Kuzbanian in *Drosophila*, is required to activate the Notch pathway during cardiovascular development. To further understand the physiological function of *Adam10* in vascular and cardiac development, we generated mice lacking the *Adam10* gene primarily in the endothelial compartment. We found that disruption of *Adam10* in endothelial cells resulted in embryonic death after embryonic day 10.5 due to multiple cardiac and vascular defects similar to *Notch1* mutants. We further showed that the expression of Notch target genes *Snail* and *Bmp2* are impaired in *Adam10*-deficient cardiac tissues. Finally, we provide experimental evidence to support that *Adam10* functions in a cell autonomous manner during mammalian cardiac development. *Developmental Dynamics 239:2594–2602, 2010.* © 2010 Wiley-Liss, Inc.

Key words: *Adam10*; cardiac development; Notch signaling; epithelial-to-mesenchymal transition; cell intrinsic

Accepted 12 July 2010

INTRODUCTION

The formations of the vascular and cardiac systems are two major processes essential to early embryonic development in mammals. During vascular system development, the endothelial precursors first differentiate into a primitive blood vessel network through the process of vasculogenesis. After a series of sprouting, branching, and splitting processes named angiogenesis, the primitive blood vessel network is then remodeled into a mature vascular system (Roca and Adams, 2007). The cardiac development begins with the appearance of cardiac progenitor cells and the formation of two primary heart fields (High and Epstein, 2008). These two fields join together to form a linear heart tube, which in turn are followed by a rightward folding and the addition of progenitor cells from the neural tube to form the secondary heart field. At this time the heart has been remodeled from a linear structure to an organ with four distinct chambers (Harvey, 2002). The atrioventricular canal inside the heart starts to take shape and the endocardium undergoes epithelial-to-mesenchymal transition

Additional Supporting Information may be found in the online version of this article.

[1]Institute of Developmental Biology and Molecular Medicine, School of Life Sciences, Fudan University, Shanghai, China
[2]Department of Genetics, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, Connecticut
[3]Department of Molecular, Cellular, and Developmental Biology, Howard Hughes Medical Institute, University of Colorado, Boulder, Colorado
[4]Genetic Laboratory of Development and Diseases Institute of Biotechnology, Beijing, China
[5]Department of Immunology, Duke University Medical Center, Durham, North Carolina

Grant sponsor: National Basic Research Program of China (973); Grant number: 2006CB806700; Grant sponsor: National Hi-tech 14 Research and Development Program of China (863); Grant number: 2007AA022101; Grant sponsor: National Natural Science Foundation of China; Grant number: 30228014; Grant sponsor: Shanghai Pujiang Program; Grant number: 09SG04; Grant sponsor: Shanghai Rising-Star Program; Grant number: 10QH1400100; Grant sponsor: The 211 and 985 projects of the Chinese Ministry of Education.

***Correspondence to: Kejing Deng, Institute of Developmental Biology and Molecular Medicine, School of Life Sciences, Fudan University, Shanghai 200433, China. E-mail: dengkj@fudan.edu.cn**

DOI 10.1002/dvdy.22391
Published online 27 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

(EMT) to form an endocardial cushion. The ventricular myocardium differentiates to form the ventricular tabulate. The embryonic heart development is completed with the formation of the outflow tract and remodeling of the aortic arch artery (Harvey, 2002; High and Epstein, 2008).

Notch pathway has been shown to play important roles in the different stages of vascular and cardiac development mentioned above (Roca and Adams, 2007; High and Epstein, 2008). For example, during the development of the cardiac cushion Notch activates the expression of the transcription factor *Snail* in the endocardium, which inhibits the transcription of *Vascular Endothelium Cadherin*. As a result the endocardium cells detach from each other and invade the cardiac jelly to form the cardiac cushion (Timmerman et al., 2004). Targeted disruption of Notch or Notch pathway genes, such as *Notch1*, *RBPJk*, and *Snail*, all lead to a block in the EMT process, resulting in impaired cardiac cushion development and embryonic death (Carver et al., 2001; Timmerman et al., 2004).

A critical step in the Notch activation is the proteolytic cleavages of the Notch receptor, which releases the Notch intracellular domain (NICD) into the cytoplasm and actives the expression of downstream genes. Three consecutive cleavage events of Notch have been defined. First, the Notch receptors undergo furin cleavage at the S1 site when they are transferred to the cell surface. ADAM proteases are involved in the second cleavage at the S2 site, resulting in removal of Notch extracellular domain. Finally an inner membrane cleavage mediated by γ-secretase at the S3 site releases the NICD (Bray, 2006). In the ADAM protease family, both ADAM10 and ADAM17 were predicated to be functional in Notch pathway. Kuzbanian (homolog of *Adam10*) in *Drosophila* is the functional shedding enzyme for the S2 cleavage of Notch (Pan and Rubin, 1997). Previous studies indicated that ADAM17 was able to cleave Notch at the S2 site in vitro (Brou et al., 2000). However, *Notch1*-deficient mice and *Adam17*-deficient mice showed completely different phenotypes (Zhao et al., 2001). More recent reports indicated that ADAM17 mediated S2 cleavage occurs without ligand engagement whereas ADAM10 mediated S2 cleave is strictly dependent on Notch ligands (Cagavi Bozkulak and Weinmaster, 2009; van Tetering et al., 2009). *Adam10*-deficient mice die during early embryogenesis with multiple defects (Hartmann et al., 2002) similar to *Notch1*-deficient mice (Krebs et al., 2000; Limbourg et al., 2005). Although these genetic studies support the idea that ADAM10 plays a major role in Notch signaling during embryogenesis, the exact role of *Adam10* in cardiovascular development and its relationship with the Notch pathway has not been clearly defined. Here, we used a conditional allele of *Adam10* (Tian et al., 2008) and a *Tie2-Cre* recombinase transgene (Li et al., 2005; Lan et al., 2007) to generate *Adam10* endothelial conditional knockout mice. With this model system, we explicitly examined the role of *Adam10* in vascular and cardiac development.

TABLE 1. Genotype Distribution of Embryos and Newborn Mice

	Tie2-Cre positive		*Tie2-Cre negative*	
Time point	$Adam10^{flox/flox}$	$Adam10^{flox/+}$	$Adam10^{flox/flox}$	$Adam10^{flox/+}$
E9.5	38(36.75)	38(36.75)	34(36.75)	37(36.75)
E10.5	31(26.25)	21(26.25)	31(26.25)	22(26.25)
E11.5	4(4.25)*	3(4.25)	6(4.25)	4(4.25)
E12.5	0(2)	3(2)	3(2)	2(2)
E14.5	0(9.75)	12(9.75)	13(9.75)	14(9.75)
At birth	0(6)	11(6)	7(6)	6(6)

Expected numbers showed in parentheses.
*Asystolic embryos.

RESULTS

Endothelial Specific Deletion of *Adam10* Leads to Embryonic Growth Retardation and Lethality

To examine the role of *Adam10* in cardiac and vascular development, we crossed the $Adam10^{flox/flox}$ mice (Tian et al., 2008) with *Tie2-Cre* mice (Li et al., 2005; Lan et al., 2007) to remove the *Adam10* gene in developing cardiovascular system, primarily in endothelial cells. The resulting $Adam10^{flox/+}$; *Tie2-Cre* male mice were crossed with $Adam10^{flox/flox}$ females to generate $Adam10^{flox/flox}$; *Tie2-Cre* animals, named AEKO as *Adam10* endothelial knockout. However, no AEKO mice alive were recovered from this breeding. Live AEKO embryos were identified with expected Mandelian distribution until embryonic day (E) 10.5 (Table 1). We further examined AEKO embryos from E9.5 to E11.5. At E9.5, the AEKO embryos appeared a little smaller than $Adam10^{flox/flox}$ littermates, and some of them showed larger precardiac sacs (Fig. 1A,B). At E10.5, the AEKO mice exhibited growth retardation and greatly enlarged precardiac sacs. The hearts of AEKO embryos also showed defects in looping compared with wild-types (Fig. 1C,D). At E11.5, only dead or severely retarded AEKO embryos could be found (data not shown). We further use in utero ultrasound microscopy to examine the AEKO mice. At E10.5, the hearts of AEKO embryos were poorly looped (Fig. 1E,F), and blood flow could hardly be found in AEKO embryos (Supp. Movie S1 and Movie S2, which are available online). These results indicated that the AEKO embryos had growth retardation and cardiovascular defects.

To verify the specificity of the *Tie2-Cre* line used in this study, we crossed the *Tie2-Cre* strain with the *ROSA26 LacZ Cre* reporter strain (from Jackson Lab) and confirmed that the activity of *Tie2-Cre* is indeed restricted to the cardiac and the vascular system (Supp. Fig. S1). Additionally, a polymerase chain reaction (PCR) -based assay (Tian et al., 2008) was performed on anti-PECAM (antibody to platelet endothelial cell adhesion molecule, a specific marker for vascular endothelial cells) antibody sorted endothelial cells

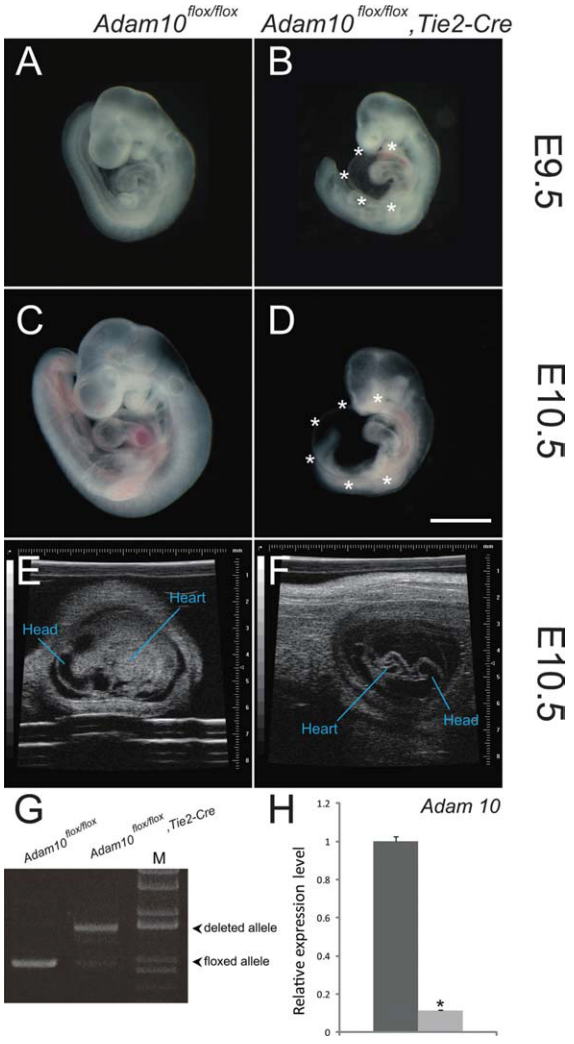

Fig. 1. Defective cardiovasculature in *Adam10* endothelial knockout embryos. **A–F:** Representative pictures of embryonic day (E) 9.5 (A,B), E10.5 embryos under stereomicroscope (C,D), and E10.5 embryos under ultrasound microscopy (E,F). Embryos are from the *Adam10*$^{flox/flox}$ control group (A,C,E) and *Adam10*$^{flox/flox}$; *Tie2-Cre* mutant group (B,D,F). B,D: The outlines of pericardial sacs in *Adam10* endothelial deficient embryos are highlighted (*). **G:** Deletion of *Adam10* in endothelial cells sorted from E10.5 mouse embryos. The floxed and deleted alleles were examined by polymerase chain reaction (PCR) with three primers (KOF, EKOB, and KOB), which produced 1,636 bp band for the *Adam10*^{flox} allele and a 954 bp band for the deleted allele. M, 1-kb DNA ladder. **H:** Real-time RT-PCR analysis of *Adam10* expression in the heart. RNAs were extracted from total hearts of E10.5 *Adam10*$^{flox/flox}$ (dark grey bar, n = 3) and *Adam10*$^{flox/flox}$; *Tie2-Cre* (light gray bar, n = 3) embryos. The average value and SEM are shown. $P = 0.0005$. Scale bar = 1 mm in A–D.

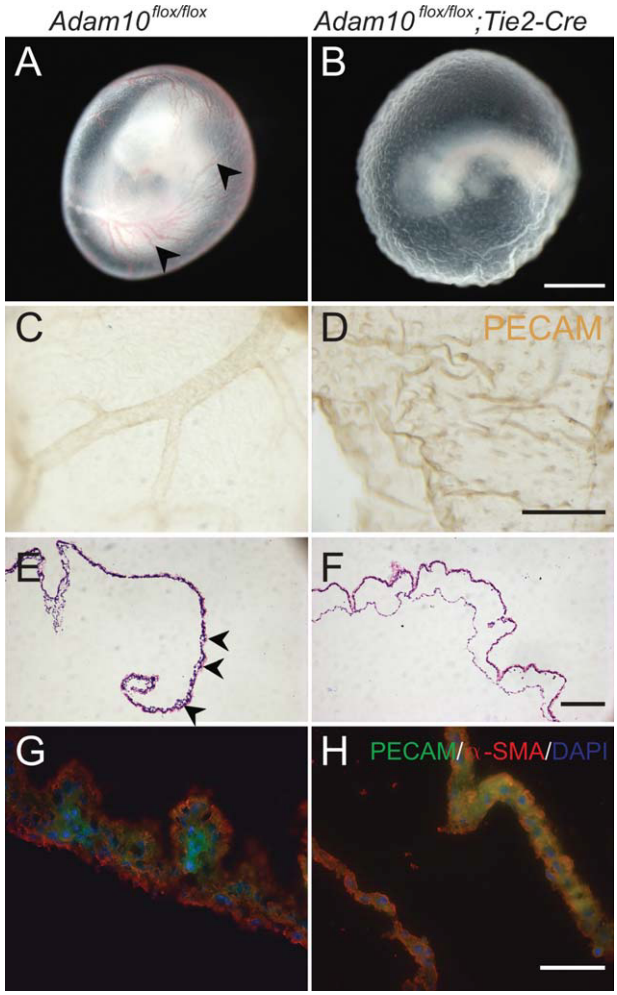

Fig. 2. Angiogenic defects in yolk sacs of *Adam10* endothelial mutant embryos. **A,B:** morphology of embryos in their yolk sacs at embryonic day (E) 10.5. Large vitelline blood vessels (arrowheads) are observed in the *Adam10*$^{flox/flox}$ (A) yolk sacs, but not in the *Adam10*$^{flox/flox}$; *Tie2-Cre* mutants (B). **C,D:** Platelet endothelial cell adhesion molecule (PECAM) - stained yolk sacs. The yolk sacs of the mutant embryos are at the primitive vascular plexus stage and have not undergone vascular remodeling to form the large and small blood vessels of the mature yolk sac. **E,F:** Hematoxylin and eosin (H&E) staining of histological sections of the yolk sacs. The mutant yolk sac (F) splits into two parts and cannot form vitelline collecting vessels. **G,H:** Anti-α-smooth muscle actin (α-SMA), PECAM antibody, and DAPI (4′,6-diamidine-2-phenylidole-dihydrochloride) staining shows the endothelial signal is diffused and cannot form vessels. Scale bar = 1 mm in A,B; 0.5 mm in C,D, 0.2 mm in E,F, 0.05 mm in G,H.

to evaluate the efficiency of *Adam10* deletion. The deleted allele became the major product in endothelial cells, indicating that most of the endothelial cells had lost the floxed allele (Fig. 1G). Real-time RT-PCR results of cardiac tissues further confirmed efficient deletion of *Adam10* in AEKO embryos (Fig. 1H). These findings indicate the growth defects were caused by deletion of *Adam10* in endothelial cells.

Defects in Angiogenic Vascular Remodeling in AEKO Embryos

Vascular defects in AEKO mice can first be found in the yolk sacs of the AEKO embryos. At E9.5 the structures of the mutants and controls were similar (Supp. Fig. S2), indicating the initial vasculogenesis was normal in AEKO yolk sac. We further examined the vascular development in the yolk sac at E10.5. At this time, we found that the large vitelline vessels had formed in yolk sacs of control embryos, but were absent in those of AEKO embryos (Fig. 2A,B). Immunohistochemistry analysis of the whole-mount embryos with the vessel specific anti-PECAM antibody identified the primary vascular plexus formation in control yolk sacs when

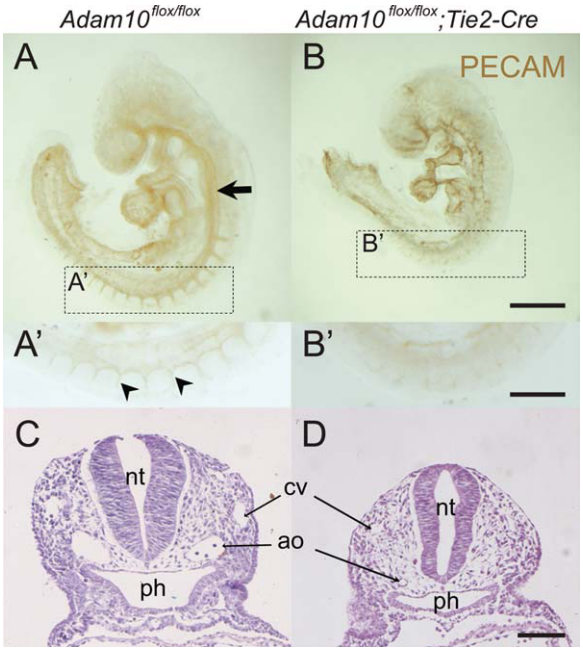

Fig. 3.

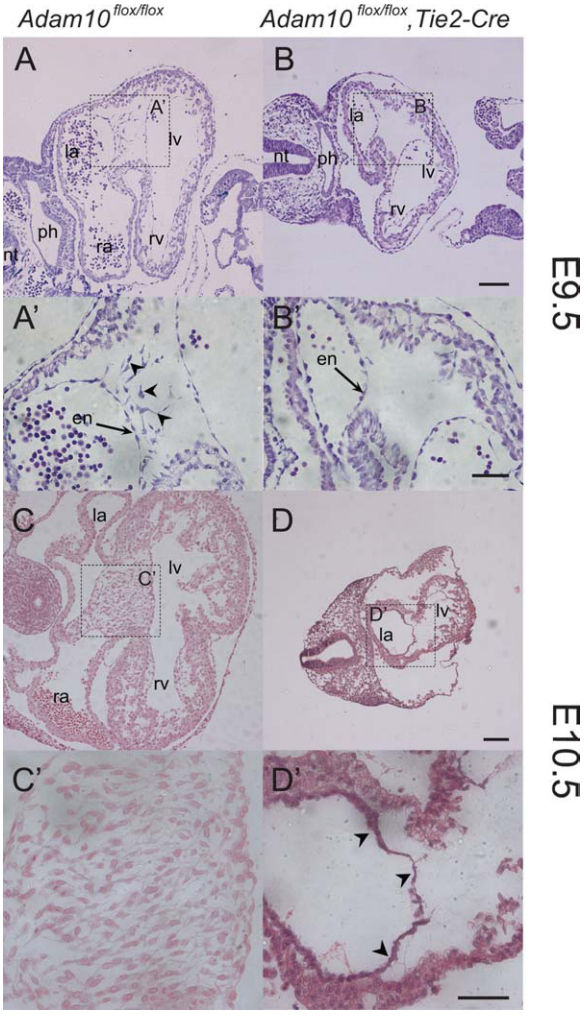

Fig. 4.

compared with the loose structures in AEKO ones (Fig. 2C,D). Histology sections revealed that the AEKO yolk sac was separated into two layers (Fig. 2F). The PECAM positive cells were typically located between the smooth muscle actin (α-SMA) -expressing cells (Fig. 2G). They are now exclusively associated with the outside layer in AEKO yolk sacs (Fig. 2H). Impaired vascular morphogenesis was also observed in AEKO embryos. At E9.5, the control embryos had clearly developed main trunks of the anterior cardinal vein (Fig. 3A) and intersomitic vessels (Fig. 3A′). However, these vessels were found either to be missing or poorly formed in AEKO littermates (Fig. 3B,B′). Transverse sections of AEKO mutants showed reduced size of the cardinal vein and aorta in comparison to the wild-type controls (Fig. 3C,D). The vascular defects became more pronounced in E10.5 AEKO embryos (Supp. Fig. S3). These results suggest that ADAM10 is involved in vascular development.

Impaired EMT and Ventricular Trabeculation in the Heart of AEKO Mice

To investigate the roles of ADAM10 in cardiac development, we analyzed

Fig. 3. Defects in vascular remodeling in *Adam10* conditional knockout mice. **A,B:** Representative results of platelet endothelial cell adhesion molecule (PECAM) -stained whole-mount embryos from either the *Adam10*$^{flox/flox}$ (A) or *Adam10*$^{flox/flox}$; *Tie2-Cre* (B) at embryonic day (E) 9.5 are shown. A: The arrow indicates the main trunks of anterior cardinal vein. The intersomitic vessels differentiate in *Adam10*$^{flox/flox}$ embryo (arrow heads in A′) but not in AEKO embryo (B′). **C,D:** Representative transverse sections of embryos at E9.5. The structure of aorta and cardinal vein are normal in *Adam10*$^{flox/flox}$ embryo, but collapsed in AEKO mutant. ao, aorta; cv, cardinal vein; nt, neural tube; ph, pharynx. Scale bar = 0.5 mm in A,B, 0.25 mm in A′,B′, 0.1 mm in C,D.

Fig. 4. Impaired epithelial-to-mesenchymal transition (EMT) after *Adam10* endothelial deletion. **A–D:** Representative transverse sections of hearts from either *Adam10*$^{flox/flox}$ (A,C) or *Adam10*$^{flox/flox}$; *Tie2-Cre* (B,D) embryos at E9.5 and E10.5. Arrows (A′,B′) indicate the endocardium. The mesenchymal cells start invading the cushion in *Adam10*$^{flox/flox}$ (arrowheads in A′) embryo but not in AEKO mutant at E9.5. D′: Arrowheads indicate collapsed endocardium at E10.5. lv, left ventricle; rv, right ventricle; la, left atrium; ra, right atrium; nt, neural tube; ph, pharynx. Scale bar = 0.1 mm in A–D, 0.05 mm in A′–D′.

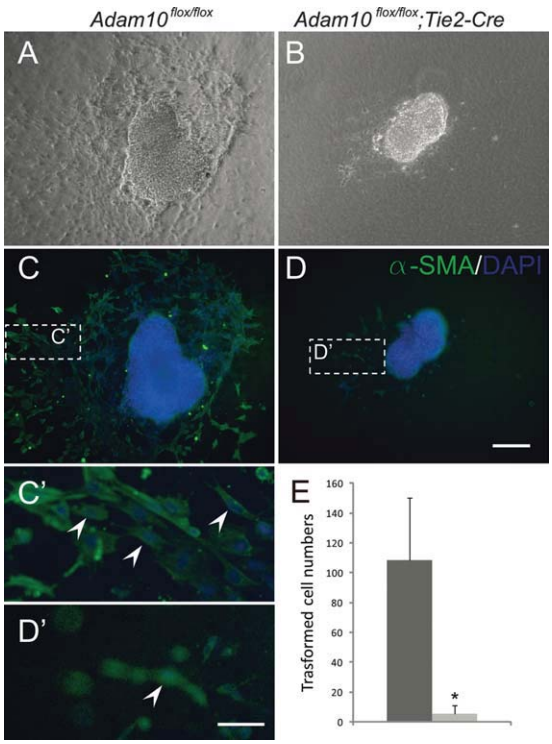

Fig. 5.

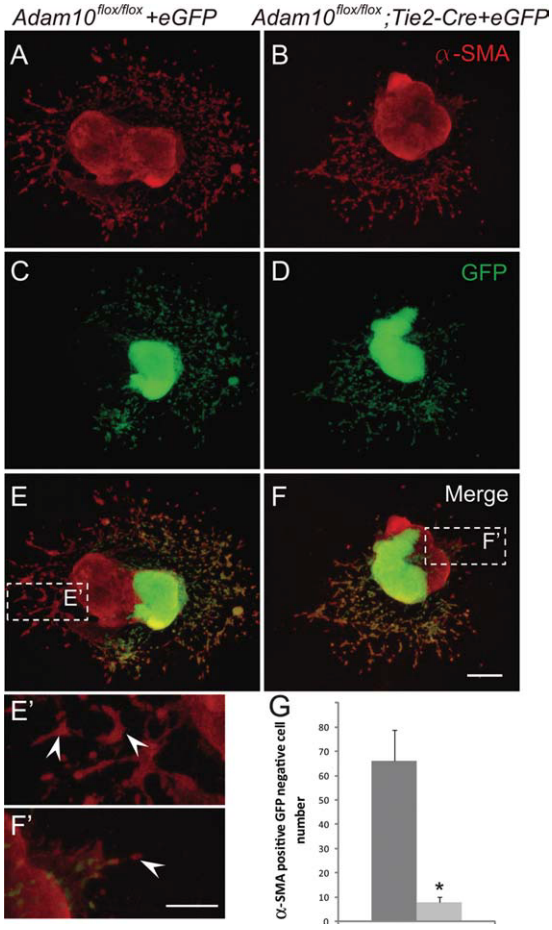

Fig. 6.

transverse sections of E9.5 and E10.5 embryos. At E9.5, the endocardial cells in the atrioventricular (AV) cushion had began the EMT process by invading the cardiac jelly in control embryos (Fig. 4A,A′) but not in AEKO mutants (Fig. 4B,B′). At E10.5, The cardiac regions of the AEKO mice were always smaller than that of the littermate controls. The four chambers of AEKO heart were not on the same plane, indicating that heart looping is incomplete (Fig. 4C,D). The endocardia of AEKO hearts were collapsed and lacked the mesenchymal cushion (Fig. 4D′). PECAM staining confirmed that the collapsed structure comprised of endocardium cells (Supp. Fig. S4). Another process in cardiac development at E10.5 is the ventricular trabeculation. In control embryos, both the left and right ventricles showed developing trabeculae. However, AEKO embryos exhibited poorly developed trabeculae and myocardium (Supp. Fig. S5). These

Fig. 5. Impaired epithelial-to-mesenchymal transition (EMT) in *Adam10* conditional knock out cardiac explants cultures. **A–D:** Light microscopic images and α-smooth muscle actin (α-SMA) staining, which labels the transformed mesenchymal cells, shows decreased number of transformed cells in embryonic day (E) 9.5 AEKO AV explants. **C′,D′:** Larger view of C, D (dash box) showing individual cells (arrowheads). **E:** Quantitative analysis of the transformed cell number. The number is significantly reduced in explants from the endothelial *Adam10* mutants (light gray bar) compared with wild-type controls (dark gray bar). The average value and SD are shown. n = 4, $P = 0.015$. Scale bar = 0.2 mm in A–D, 0.05 mm in C′,D′.

Fig. 6. *Adam10* plays a cell intrinsic role in epithelial-to-mesenchymal transition (EMT) process. A–F: Embryo cardiac explants from the enhanced green fluorescent protein (eGFP) knock in mouse were placed close to wild-type explants (A,C,E) or mutant explants (B,D,F). **A,B:** α-SMA signal labeled transformed mesenchymal cells. **C,D:** GFP signals. **E,F:** Merged results of A,C; B,D, respectively. **E′,F′:** Larger view of E, F (dash box) showing individual α-SMA positive GFP negative cells. Impaired EMT cannot be rescued by attachment of wild-type AV explants (arrowheads in E′ and F′). **G:** Quantitative analysis of the transformed α-SMA positive GFP negative cell number. The number is significantly reduced in explants from the endothelial *Adam10* mutants (light gray bar) compared with wild-type controls (dark gray bar). The average value and SD are shown. n = 5, $P = 0.0004$. Scale bar = 0.2 mm in A–F, 0.1 mm in E′, F′.

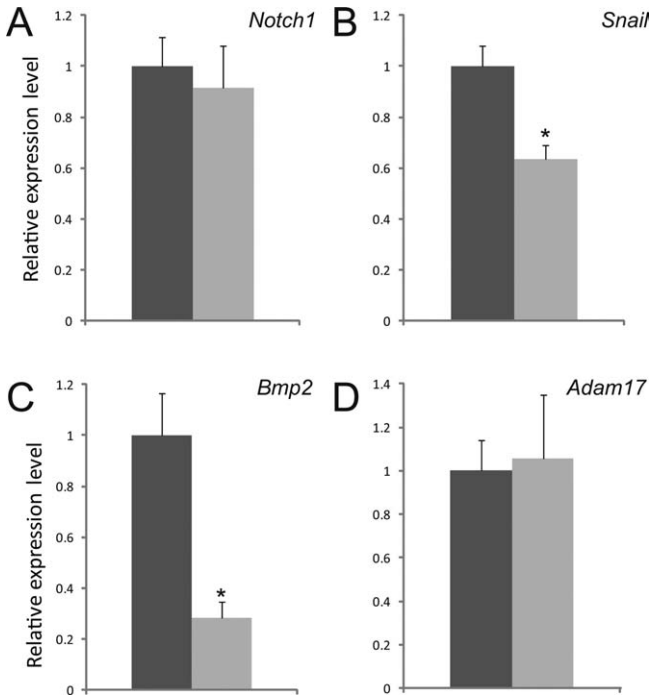

Fig. 7. Failure of Notch activation after deletion of *Adam10*. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of *Notch1*, *Snail*, *Bmp2*, and *Adam17* expression in the cardiac tissue. RNAs were extracted from total hearts of embryonic day 10.5 $Adam10^{flox/flox}$ (dark gray bar, n = 4) and $Adam10^{flox/flox}$; *Tie2-Cre* mice (light gray bar, n = 4). The expression level of *Notch1* ($P = 0.69$) and *Adam17* ($P = 0.88$) are unaffected while *Snail* ($P = 0.0002$) and *Bmp2* ($P = 0.016$) are significantly reduced. The average value and SEM are shown.

defects are very similar to the phenotypes of *Notch1* and *Notch* target *RBPJk* knockout mice (Timmerman et al., 2004; Limbourg et al., 2005; Grego-Bessa et al., 2007), supporting the idea that ADAM10 plays an essential role in the Notch pathway in regulating cardiac development.

AEKO Mice Show Impaired EMT In Vitro

We used an in vitro culture system (Runyan and Markwald, 1983) to further examine the role of *Adam10* in EMT. The left atriums, including the AV canals, were micro dissected out from E9.5 embryos and placed onto type I collagen gels. The cultured explants attached to the gel after 5 hr in culture and begun EMT subsequently. Successful cultures were confirmed by proper attachment and beating behavior of the explants. Anti-α-SMA antibody staining was performed 48 hr post adhesion to reveal the extent of EMT (Fig. 5C,D). A cell stained positive for both DAPI (4′,6-diamidine-2-phenylidole-dihydro chloride) and α-SMA signals (Camenisch et al., 2002) was counted as one transformed mesenchymal cell. Under our culture conditions, the average number of transformed cells is 108 (± 42) per explants for control samples and only 6 (± 5) for the AEKO embryos (Fig. 5E). This result indicates that ADAM10 is required for the EMT process during AV cushion development.

ADAM10 Functions in a Cell-Autonomous Manner

An important function of Notch is communication with neighboring cells through interaction with ligands expressed on adjacent cells (Bray, 2006). As a cell surface protease, ADAM10 has the potential to act on Notch in either a cell-autonomous or nonautonomous manner. To distinguish these two possibilities, we performed in vitro EMT essay by placing the mutant explant in physical contact with a wild-type tissue. The wild-type tissues were derived from an enhanced green fluorescent protein (eGFP) knock in embryos (Sun et al., 2008), which exhibited proper EMT and ubiquitous GFP expression during explant culture (Supp. Fig. S6). The eGFP positive controls were placed together with the AV cushions isolated from AEKO breeding, which produced either wild-type embryos or the AEKO embryos. GFP and α-SMA signals of the explants were examined after 48 hr in culture. Co-culture of GFP positive and GFP negative wild-type AV samples resulted in similar numbers of EMT cells from each sample (Fig. 6A,C,E). However, AEKO explants failed to show any significant EMT event even though the neighboring wild-type GFP tissues have undergone the normal EMT process (Fig. 6B,D,F,G). These results indicated that ADAM10 participates in the EMT process in a cell autonomous manner.

ADAM10 Is Required for Notch Activation in Developing Cardiac Tissues

The similar phenotypes between AEKO and Notch1 mutant embryos give the hint that defects of AEKO embryos are caused by perturbation of Notch1. To further explore whether the phenotypes of AEKO embryos are due to specific defect in ADAM10 mediated activation of Notch signal or simply a perturbation of Notch expression, we examined the expression of *Notch1* and *Notch* downstream genes. Real-time RT-PCR analysis showed that the transcription of *Notch1* was unaffected (Fig. 7A). It has been shown that Notch acts by means of *Snail* to promote and maintain the cardiac mesenchymal phenotype (Timmerman et al., 2004). The result showed that *Snail* in the heart of AEKO embryos was down-regulated to approximately 60% of the control (Fig. 7B). *Notch* target gene bone morphogenetic protein 2 (*Bmp2*) was also reported to be important for embryonic cardiac development, especially for the AV canal formation (Zhang and Bradley, 1996; Kokubo et al., 2007). AEKO cardiac tissues exhibited approximately 30% reduction of *Bmp2* expression in real-time RT-PCR analysis (Fig. 7C). ADAM17 could cleave Notch1 in vitro (Brou et al., 2000) while the *Adam17* mutant mice show no defect similar to Notch1 mutants (Zhao et al., 2001), indicating that ADAM17 may be functional

TABLE 2. Primer List

Name	Sequence(5'-3')	tm	Usage
KOF	ACCTCTTAGCGATACCACAAGCC	59	Adam10 targeting allele
EKOB	CCATGGAAGTGTCCCTCTTCATT CGTAGG		
KOB	CCAAGCGTCAAAGCGTTACAG		
Tek-F	GCCTGCATTACCGGTCGATGC	59	*Cre* transgene
Tek-R	CAGGGTGTTATAAGCAATCCC		
GAPDH-L1	TGTTCCTACCCCCAATGTGTCC	55	*Gapdh* QPCR
GAPDH-R1	GGAGTTGCTGTTGAAGTCGCAG		
Ae2F	ATTACACCAAAAACACCAGCG	55	*Adam10* QPCR
EKOB	CCATGGAAGTGTCCCTCTTCATT CGTAGG		
Notch1-F	TGCTGGAAGTATTTTAGCGACGG	58	*Notch1* QPCR
Notch1-R	CCACTCACATTCGGCACTGTTAC		
Snail-F	CCACACTGGTGAGAAGCCATTC	57	*Snail* QPCR
Snail-R	GCACTGGTATCTCTTCACATCCGAG		
BMP2-F	CCAGGTTAGTGACTCAGAACAC	54	*Bmp2* QPCR
BMP2-R	TCATCTTGGTGCAAAGACCTGC		
A17-L3	TCTTTGCTCTCAGACTACGACATCC	55	*Adam17* QPCR
A17-R3	CCACCACGACTCTCAAGTTTTGTG		

metalloprotease for Notch signaling in the absence of ADAM10. Our RNA expression analysis showed that the transcription of *Adam17* is unaffected in AEKO heart (Fig. 7D), suggesting that no compensating roles exist between *Adam10* and *Adam17*. These findings indicate that ADAM10 is directly involved in the activation of the Notch signaling pathway during cardiac development.

DISCUSSION

Previously the conventional knockout of *Adam10* in mice showed cardiac looping defects in cardiac development (Hartmann et al., 2002). However, the *Adam10* conventional knockout mice died at E9.5 with a severely retarded cardiac development equivalent to approximately the E8.0 stage. Many important cardiac developmental processes such as AV cushion EMT and ventricular trabeculation start at E9.5. Thus, the analysis of cardiovascular development in *Adam10* conventional knockout mice was hindered by the early embryonic death. Our investigation of *Adam10* endothelial knockout mouse model explicitly demonstrates that *Adam10* is essential for endocardial cushion formation, ventricular trabeculation, and vasculogenesis. The AEKO mice not only phenocopied the *Notch1* and *Notch* targets knockouts (Krebs et al., 2000; Fischer et al., 2004; Timmerman et al., 2004; Limbourg et al., 2005; Grego-Bessa et al., 2007), but also showed downregulated expression of Notch target gene *Snail* and *Bmp2* in the cardiac tissues. Because we delete *Adam10* primarily in the endothelial cells, which are only small fraction of the total cardiac, the expression changes of *Snail* and *Bmp2* could be more severe in *Adam10* deficient endocardium. These results indicated that the defect of AEKO mice is primarily due to the failure of Notch activation.

Both *Adam10* and *Adam17* are implicated as the functional metalloprotease for the activation of Notch signaling. Before the study presented here, it was still uncertain which one was the functional protease for the activation of Notch during cardiac and vascular development. It has been clearly shown that ADAM17 can cleave Notch1 in vitro (Brou et al., 2000). Although the physiological relevance of this cleavage activity in vivo has been challenged by the fact that *Notch1* but not *Adam17* (Peschon et al., 1998) is essential for embryogenesis, these genetic studies did not rule out the possibility that *Adam17* and *Adam10* may functionally compensate each other in activating *Notch1*. Our study showed that *Adam17* is expressed in the embryonic heart tissues. However, disruption of *Adam10* is sufficient to block cardiac and vascular development without affecting *Adam17* expression. This result strongly indicates that ADAM10 is the primary protease involved in activation of the *Notch* signal during cardiac and vascular development. *Notch1* is one of four *Notch* genes found in mammals and is also the major Notch gene functioning in cardiac development (Hamada et al., 1999; Krebs et al., 2000; Domenga et al., 2004; Limbourg et al., 2005). The similar phenotypes observed between AEKO mice and *Notch1* knockout mice support the idea that ADAM10 is involved in activating Notch1 during embryonic cardiovascular development.

ADAM10 may function as a protease to cleave both Notch and its ligands based on the results from *kuz* in *Drosophila* (Pan and Rubin, 1997; Qi et al., 1999). Furthermore, ADAM10 itself may be cleaved by other ADAMs to become a soluble and catalytically active protease (Liu et al., 2009). It remains a possibility that ADAM10 expressed on neighboring cells or released by neighboring cells could be involved in Notch cleavage when Notch is engaged in ligand interaction. Our EMT co-culture assay partially addressed this question. Our study showed that the physical contact between wild-type tissues and the *Adam10* deficient tissues is not sufficient to provide any substantial rescue of EMT from the mutant tissues. This result supports the idea that ADAM10 primarily functions in a cell autonomous manner in regulating cardiac cushion development. It is possible that the physical contact provided in the explant culture was limited to few cells and thus the effect of rescue could not be easily detected under this assay condition. A more rigorous test would require generation of chimeric embryos in the future.

In summary, we provided in vivo evidence that *Adam10* is essential for Notch activation during cardiac and vascular development. Activation of Notch signals and Notch downstream targets require a functional ADAM10 in endothelium. These findings indicate that ADAM10 is a *bona fide* protease regulating Notch activity in cardiac and vascular development.

EXPERIMENTAL PROCEDURES

Mouse Strains and Embryo Genotyping

Adam10$^{flox/flox}$ mice (Tian et al., 2008), *Tie2-Cre* transgenic mice (Li et al.,

2005; Lan et al., 2007), and eGFP knock in mice (Sun et al., 2008) were described previously. *Adam10*$^{flox/flox}$ mice were on a C57/129SV6 mixed background, *Tie2-Cre* transgenic mice were on a C57/Kunming mixed background and the eGFP knock in mice were on a 129 SV6 pure background. All mice were bred in the animal facility of Institute of Developmental Biology and Molecular Medicine following the general guideline of AAALAC handbook and institutional regulations. Genotyping was performed by PCR analysis of the yolk sac DNA. The primer sequences for genotyping are listed in Table 2.

Ultrasound Backscatter Microscopy

The in utero ultrasound backscatter microscopy images and videos were acquired by Vevo 770 (Visualsonics) using standard procedures described before(Turnbull, 1999).

Cell Sorting

E10.5 embryos were dissected out and endothelial cells were isolated by PECAM (CD31 BD Pharmingen 550274), antibody to platelet endothelial cell adhesion molecule, a specific marker for vascular endothelial cells, and Dynabeads Sheep anti-Rat IgG (Invitrogen) according to manufacturer's instructions.

Histological Analysis and Immuno-fluorescence Staining

For paraffin sections, E9.5 or E10.5 embryos were dissected out, fixed in 4% formaldehyde over night at 4°C, dehydrated in ethanol series from 30% to 100%, cleared by xylene, and then embedded in paraffin. Paraffin sections (5–7 μm) were stained by hematoxylin and eosin staining. Frozen sections were prepared with OCT embedding of formaldehyde treated E10.5 embryos or yolk sacs in liquid-nitrogen. Frozen sections (5–7 μm) were then collected for immuno-fluorescence staining following standard protocols. The following commercial antibodies were used: PECAM (CD31 BD Pharmingen 558736); α-SMA (Sigma A5228), antibody to smooth muscle actin, a specific marker for mesenchymal cells.

Whole-mount Immunohistochemistry

Immnohistochemistry of whole embryos with PECAM (CD31 BD Pharmingen 550274) was performed following standard procedures (Urness et al., 2000; Hallaq et al., 2004).

Atrio-ventricular Canal Endocardial Cushion Explants Culture

The explant culture assay was performed as described (Runyan and Markwald, 1983; Hallaq et al., 2004; Timmerman et al., 2004). After 5 hr of adhering and another 48 hr of culturing, the explants were fixed in 4% formaldehyde, blocked in 0.05%Triton X-100 and 2% bovine serum albumin (Sigma) in phosphate buffered saline. Then the explant cultures were stained with DAPI and α-SMA (Sigma A5228). The transformed cell numbers were counted with Image-Pro plus 6.0 (Media Cybernetics).

Real-Time RT-PCR Analysis

Each RNA sample was prepared from four hearts of the same genotype. At least three independent samples were prepared for each genotype. RNA was extracted by RNeasy Mini Kit (Qiagen) and treated with RNase-free deoxyribonuclease I (TaKaRa) separately to avoid genomic DNA contamination. Reverse transcription (RT)-PCR was carried out with TaKaRa RNA PCR Kit (AMV) according to the manufacturer's instructions. Real-time PCR was performed with HotSybr PCR Reaction Mix (NuStar Laboratory). Expression of *Gapdh* was used as the baseline standard for real-time PCR. The primer sequences are listed in Table 2.

Statistics

P values were obtained with two-tailed unpaired Student's *t*-test on Microsoft Excel 2007 (Microsoft Corporation).

ACKNOWLEDGMENTS

We thank Raymond B. Runyan for the technical instructions of the in vitro EMT essay; Jing Yi for technical assistance of the histological sections; Ian Knisely for a critical reading of the manuscript; Huanhu Zhu, Chao Peng, Kai Lei, and all the other members in IDM for useful discussions.

REFERENCES

Bray SJ. 2006. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol 7:678–689.

Brou C, Logeat F, Gupta N, Bessia C, LeBail O, Doedens JR, Cumano A, Roux P, Black RA, Israel A. 2000. A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. Mol Cell 5: 207–216.

Cagavi Bozkulak E, Weinmaster G. 2009. Selective use of Adam10 and Adam17 in activation of Notch1 signaling. Mol Cell Biol 29:5679–5695.

Camenisch TD, Schroeder JA, Bradley J, Klewer SE, McDonald JA. 2002. Heart-valve mesenchyme formation is dependent on hyaluronan-augmented activation of ErbB2-ErbB3 receptors. Nat Med 8:850–855.

Carver EA, Jiang R, Lan Y, Oram KF, Gridley T. 2001. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. Mol Cell Biol 21:8184–8188.

Domenga V, Fardoux P, Lacombe P, Monet M, Maciazek J, Krebs LT, Klonjkowski B, Berrou E, Mericskay M, Li Z, Tournier-Lasserve E, Gridley T, Joutel A. 2004. Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. Genes Dev 18: 2730–2735.

Fischer A, Schumacher N, Maier M, Sendtner M, Gessler M. 2004. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. Genes Dev 18:901–911.

Grego-Bessa J, Luna-Zurita L, del Monte G, Bolos V, Melgar P, Arandilla A, Garratt AN, Zang H, Mukouyama YS, Chen H, Shou W, Ballestar E, Esteller M, Rojas A, Perez-Pomares JM, de la Pompa JL. 2007. Notch signaling is essential for ventricular chamber development. Dev Cell 12:415–429.

Hallaq H, Pinter E, Enciso J, McGrath J, Zeiss C, Brueckner M, Madri J, Jacobs HC, Wilson CM, Vasavada H, Jiang X, Bogue CW. 2004. A null mutation of Hhex results in abnormal cardiac development, defective vasculogenesis and elevated Vegfa levels. Development 131: 5197–5209.

Hamada Y, Kadokawa Y, Okabe M, Ikawa M, Coleman JR, Tsujimoto Y. 1999. Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. Development 126: 3415–3424.

Hartmann D, de Strooper B, Serneels L, Craessaerts K, Herreman A, Annaert W, Umans L, Lubke T, Lena Illert A, von

Figura K, Saftig P. 2002. The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. Hum Mol Genet 11:2615–2624.

Harvey RP. 2002. Patterning the vertebrate heart. Nat Rev Genet 3:544–556.

High FA, Epstein JA. 2008. The multifaceted role of Notch in cardiac development and disease. Nat Rev Genet 9:49–61.

Kokubo H, Tomita-Miyagawa S, Hamada Y, Saga Y. 2007. Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2. Development 134:747–755.

Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP, Gallahan D, Closson V, Kitajewski J, Callahan R, Smith GH, Stark KL, Gridley T. 2000. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev 14: 1343–1352.

Lan Y, Liu B, Yao H, Li F, Weng T, Yang G, Li W, Cheng X, Mao N, Yang X. 2007. Essential role of endothelial Smad4 in vascular remodeling and integrity. Mol Cell Biol 27:7683–7692.

Li WL, Cheng X, Tan XH, Zhang JS, Sun YS, Chen L, Yang X. 2005. Endothelial cell-specific expression of Cre recombinase in transgenic mice. Yi Chuan Xue Bao 32:909–915.

Limbourg FP, Takeshita K, Radtke F, Bronson RT, Chin MT, Liao JK. 2005. Essential role of endothelial Notch1 in angiogenesis. Circulation 111:1826–1832.

Liu Q, Zhang J, Tran H, Verbeek MM, Reiss K, Estus S, Bu G. 2009. LRP1 shedding in human brain: roles of ADAM10 and ADAM17. Mol Neurodegener 4:17.

Pan D, Rubin GM. 1997. Kuzbanian controls proteolytic processing of Notch and mediates lateral inhibition during Drosophila and vertebrate neurogenesis. Cell 90:271–280.

Peschon JJ, Slack JL, Reddy P, Stocking KL, Sunnarborg SW, Lee DC, Russell WE, Castner BJ, Johnson RS, Fitzner JN, Boyce RW, Nelson N, Kozlosky CJ, Wolfson MF, Rauch CT, Cerretti DP, Paxton RJ, March CJ, Black RA. 1998. An essential role for ectodomain shedding in mammalian development. Science 282:1281–1284.

Qi H, Rand MD, Wu X, Sestan N, Wang W, Rakic P, Xu T, Artavanis-Tsakonas S. 1999. Processing of the notch ligand delta by the metalloprotease Kuzbanian. Science 283:91–94.

Roca C, Adams RH. 2007. Regulation of vascular morphogenesis by Notch signaling. Genes Dev 21:2511–2524.

Runyan RB, Markwald RR. 1983. Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue. Dev Biol 95:108–114.

Sun L, Wu X, Han M, Xu T, Zhuang Y. 2008. A mitotic recombination system for mouse chromosome 17. Proc Natl Acad Sci USA 105:4237–4241.

Tian L, Wu X, Chi C, Han M, Xu T, Zhuang Y. 2008. ADAM10 is essential for proteolytic activation of Notch during thymocyte development. Int Immunol 20:1181–1187.

Timmerman LA, Grego-Bessa J, Raya A, Bertran E, Perez-Pomares JM, Diez J, Aranda S, Palomo S, McCormick F, Izpisua-Belmonte JC, de la Pompa JL. 2004. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev 18: 99–115.

Turnbull DH. 1999. In utero ultrasound backscatter microscopy of early stage mouse embryos. Comput Med Imaging Graph 23:25–31.

Urness LD, Sorensen LK, Li DY. 2000. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. Nat Genet 26:328–331.

van Tetering G, van Diest P, Verlaan I, van der Wall E, Kopan R, Vooijs M. 2009. The metalloprotease ADAM10 is required for notch1 S2 cleavage. J Biol Chem 284:31018–31027.

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

Zhao J, Chen H, Peschon JJ, Shi W, Zhang Y, Frank SJ, Warburton D. 2001. Pulmonary hypoplasia in mice lacking tumor necrosis factor-alpha converting enzyme indicates an indispensable role for cell surface protein shedding during embryonic lung branching morphogenesis. Dev Biol 232:204–218.