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Endocardial Cushion Morphogenesis and Coronary Vessel Development Require Chicken Ovalbumin Upstream Promoter-Transcription Factor II

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Abstract

Objective—Septal defects and coronary vessel anomalies are common congenital heart defects, yet their ontogeny and the underlying genetic mechanisms are not well understood. Here, we investigated the role of chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII, NR2F2) in cardiac organogenesis.

Methods and Results—We analyzed embryos deficient in *COUP-TFII* and observed a spectrum of cardiac defects, including atrioventricular septal defect, thin-walled myocardium, and abnormal coronary morphogenesis. We show by expression analysis that COUP-TFII is expressed in the endocardium and the epicardium but not in the myocardium of the ventricle. Using endothelial-specific *COUP-TFII* mutants and molecular approaches, we show that *COUP-TFII* deficiency resulted in endocardial cushion hypoplasia. This was attributed to the reduced growth and survival of atrioventricular cushion mesenchymal cells and defective epithelial-mesenchymal transformation (EMT) in the underlying endocardium. In addition, the endocardial EMT defect was accompanied by downregulation of *Snai1*, one of the master regulators of EMT, and upregulation of vascular endothelial-cadherin. Furthermore, we show that although COUP-TFII does not play a major role in the formation of epicardial cell cysts, it is critically important for the formation of epicardium. Ablation of COUP-TFII impairs epicardial EMT and coronary plexus formation.

Conclusion—Our results reveal that COUP-TFII plays cell-autonomous roles in the endocardium and the epicardium for endocardial and epicardial EMT, which are required for proper valve and coronary vessel formation during heart development.

Keywords

atrioventricular septal defect; cardiac morphogenesis; chicken ovalbumin upstream promoter-transcription factor II; epicardium; epithelial-mesenchymal transformation

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Disclosures
None.

Congenital heart defects are the most common type of birth defects, occurring in 8 of 1000 newborns. It often results from abnormal development of the inner curvatures of the looping heart, including the inflow tract, atrioventricular (AV) cushion (AVC), and the outflow tract (OFT), as well as the malformation of coronary vessels.[1]

During cardiac morphogenesis, the endothelial cells lining the endocardial cushions respond to the signals emitted by the myocardium within AVC and OFT and undergo an endothelial-mesenchymal transformation (EMT). The transformed cells then migrate into the underlying extracellular matrix, eventually contributing to the leaflets of the mitral and tricuspid valves, as well as to the interatrial and interventricular septa.[2] The reciprocal signals between the endocardium and the myocardium were shown to induce EMT. Many genetic pathways/signals, transcription and growth factors, adhesion molecules, and proteases have been implicated in the formation of valves and septum.[2]

The epicardium is the primordial cell sheet in which coronary vessels derive from. It originates from the proepicardium (PE), which is a protrusion of the septum transversum near the venous pole of the developing heart.[3] At around mouse embryonic day (E) 9.5, proepicardial cells either migrate directly from the PE to the developing atrium or detach as cell cysts and float freely into the pericardial cavity to reach the myocardium. Soon after the attachment, the proepicardial cells flatten, spread, and eventually form a continuous epithelial sheet, the epicardium. In mice, the heart is entirely covered by the epicardium by E11. Similar to the process that occurs in the endocardium, between E11.5 and E12.5, a subpopulation of epicardial cells undergoes EMT soon after contacting the underlying myocardium. These epicardium-derived mesenchymal cells invade into the subepicardial space, which is rich in extracellular matrix proteins. A subset of these cells further migrates into the compact zone of the myocardium. The epicardium-derived mesenchymal cells are thought to be the sources of cardiac fibroblasts and coronary vascular smooth muscle cells.[4] Coronary vessels begin to form when PE-derived angioblasts or hemangioblasts differentiate into primitive vascular plexus in the subepicardial space and the myocardium. This process is called vasculogenesis. The vascular network then undergoes remodeling, an angiogenic process, to give rise to the mature coronary vascular tree. Several transcription factors regulating coronary vessel development have been characterized, including Wilms tumor 1 (Wt1), GATA4, and friend of GATA (FOG2).[5–7]

Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) is an orphan member of the nuclear receptor superfamily. Genetic analysis in the mouse uncovered essential roles of the transcription factor COUP-TFII in the development of numerous organ systems.[8] Previously, we have demonstrated that COUP-TFII plays an important role in angiogenesis and heart development.[9] Targeted disruption of *COUP-TFII* leads to embryonic lethality at midgestation, with severe hemorrhage and edema.[9] However, an intrinsic function of COUP-TFII during cardiac development is yet to be defined.

Here, we present data demonstrating novel roles of COUP-TFII in the formation of AVC and coronary vessels. Inactivation of *COUP-TFII* results in a variety spectrum of cardiac defects, including cardiac valve malformation, compact zone hypoplasia, and defective coronary vasculature. We discovered that COUP-TFII in the endocardium is important for appropriate EMT and is involved in *Snai1*–vascular endothelial (VE)-cadherin signaling to regulate endocardial cushion development. Furthermore, COUP-TFII in the epicardium is required for normal epicardial formation, epicardial EMT, and epicardium-derived mesenchymal cell migration. Collectively, our results indicate that endocardial COUP-TFII is important for AVC formation and that epicardial COUP-TFII is required for coronary vascular development.

Methods

Animal Experiments

COUP-TFII$^{F/F}$ mice, *COUP-TFII*$^{+/-}$ mice, *COUP-TFII*$^{-/-}$ embryos, and *Tie2Cre; COUP-TFII*$^{F/F}$ embryos were generated as previously reported.[10–12] The *Gata5Cre*^{Tg} mouse strain was provided by Dr Pilar Ruiz-Lozano,[13] and the *ROSA26CRE-ER*^{T2} knock-in mouse strain was provided by Dr Thomas Ludwig.[14] All mouse strains were maintained in a mixed genetic background (129/Sv×C57BL/6) and received standard rodent chow. A vaginal plug was set as E0.5. For inducible deletions during embryonic stages, tamoxifen (TAM) was dissolved in corn oil (Sigma; 10 mg/mL), and 3 mg of TAM was injected intraperitoneally into pregnant females starting at E9.5 to E15.5. For all studies, littermate controls were used. Experimental animals and studies were approved by the Institutional Animal Care and User Committee of Baylor College of Medicine.

Histological Analysis

Embryos were dissected and fixed in 4% paraformaldehyde in PBS overnight, dehydrated, embedded in paraffin, and then sectioned and stained with hematoxylin and eosin. For the frozen section, the dissected embryos were slightly fixed in 2% paraformaldehyde/PBS for 30 minutes at 4°C. After PBS wash, the embryos were cryoprotected in 30% sucrose/PBS solution overnight and embedded in optimal cutting temperature compound (Tissue Tek). Serial sections at 7 to 10 μm were made for X-gal staining, performed as described below, and counterstained with Nuclear Fast Red (Vector).

Immunohistochemistry

Immunohistochemistry analysis was performed, as described previously.[15] Antibodies to smooth muscle α-actin (Sigma, clone 1A4, 1: 3000), endoglin (R&D systems, 1:200), pan-cytokeratins (Sigma, 1:2000), α-sarcomeric actin (Sigma, 1: 3000), phospho-Histone H3 (Upstate, 1:500), Wt1 (Santa Cruz, 1:500), COUP-TFII (Perseus Proteomics, 1:1000), GATA4 (Santa Cruz, 1:200), cleaved Notch1 (Val1744; Notch1 intracellular domain [NICD], Cell Signaling, 1:20), platelet-endothelial cell adhesion molecule (PECAM; Abcam, 1:200), and platelet-derived growth factor receptor β (PDGFRβ; Abcam, 1:1000) were used.

Whole-Mount PECAM Immunohistochemistry

Hearts were isolated and fixed in 4:1 methanol:dimethyl sulfoxide overnight and incubated in 4:1:1 methanol:dimethyl sulfoxide:hydrogen peroxide for 5 hours, rehydrated, and blocked in 2% milk/PBS/0.5% Triton X-100 (PBSMT). Tissues were stained with rat anti-mouse PECAM antibody (BD Biosciences, 1:200), washed 5× with PBSMT, followed by biotinylated donkey anti-rat IgG secondary antibody (Jackson Immmunoresearch). Signals were amplified by Vectastain ABC-peroxidase reagent (Vector) and visualized by 3,3′-diaminobenzidine staining (Vector). After PECAM staining, hearts were photographed and analyzed using Image J software (http://rsbweb.nih.gov/ij/). Quantification of the percentage of the ventricle covered by blood vessels was examined by dividing the area covered by blood vessels by the total ventricular area. At least 3 hearts were analyzed. After photography, PECAM-stained hearts were paraffin embedded and sectioned. Paraffin sections (7 μm) were then dewaxed, rehydrated, counterstained with hematoxylin (Vector), and mounted.

Whole-Mount X-gal Staining

Embryos or tissues were fixed in fresh 2% paraformaldehyde/PBS for 1 hour at 4°C, rinsed 3× with buffer A (100 mmol/L sodium phosphate at pH 8, 2 mmol/L $MgCl_2$, 0.01%

deoxycholate, and 0.02% NP-40) at room temperature, and stained with solution B (5 mmol/L potassium ferricyanide, 5 mmol/L potassium ferrocyanide, 1 mg/mL X-gal in rinse buffer) at 37°C. Postfixation was performed in fresh 4% paraformaldehyde/PBS at 4°C overnight.

Whole-Mount In Situ Hybridization

Whole-mount in situ hybridization was performed, as previously described.[9]

AVC Canal and Epicardial Explant Cultures

AVC explants and epicardial explants cultures were established as described.[13,16] Briefly, endocardial cushions in the AV canal of E9.5 embryos were explanted and placed on 1 mg/mL rat tail collagen surface (BD Biosciences). The AVC was then sliced open with tweezers along the junction line of the 2 adjacent cushions in the canal. The endocardial layer was placed in direct contact with the surface of the collagen gel. Opti-MEM medium with 1% FCS and antibiotics was added to the wells 1 hour after explantation. The explants were then incubated in 5% CO_2 at 37°C. Upon 24 to 48 hours of incubation, mesenchymal cells migrating from the endocardial cushions were counted using an inverted microscope that allows focusing on the mesenchymal cells present on different planes of the collagen gel.

Quantitative Reverse Transcription Polymerase Chain Reaction Assay

Total RNAs were extracted from ventricles and cultured cells using the RNeasy Mini Kit (QIAGEN) and TRIzol (Invitrogen), respectively. First-strand cDNA was synthesized by SuperScriptIII reverse transcriptase using random hexanucleotide primers according to the manufacturer's instructions (Invitrogen). Quantitative reverse transcription polymerase chain reaction (PCR) analyses were carried out with an ABI PRISM 7500 Fast Real-Time PCR System (Applied Biosystems) using the SYBR Green PCR master mix (Applied Biosystems). The expression data from heart tissues and cultured cells were normalized to β-actin and *18S* rRNA, respectively. The primer sequences are listed in Table I in the online-only Data Supplement.

Chromatin Immunoprecipitation and PCR

Chromatin immunoprecipitation (ChIP) assays were carried out with an EZ ChIP Kit (Millipore) following the manufacturer's protocol. The primer sequences are listed in Table I in the online-only Data Supplement.

Results

Expression Pattern of COUP-TFII in the Embryonic Mouse Heart

To dissect the role of COUP-TFII during heart development, *COUP-TFII/LacZ* knock-in mouse[17] and anti–COUP-TFII antibody were used to analyze the expression patterns of COUP-TFII in the developing hearts. X-gal staining of *COUP-TFIILacZ/+* mouse showed that COUP-TFII is expressed at the posterior cardiac crescent and the lateral plate mesoderm at E8 (Figure 1A and 1B). As development proceeds, high expression of COUP-TFII was detected in the head folds, the somites, the sinus horn region, and the lateral plate mesoderm at the 6-somite stage (Figure 1C). By E9.5, the expression domains of COUP-TFII were greatly expanded throughout the embryos (Figure 1D). High levels of COUP-TFII expression were detected in the brain vesicle and sinus venosus, and medium levels of expression were observed in the common atrium (Figure 1D). Transverse sections of embryonic heart at E9.5 showed that high levels of COUP-TFII were observed in the endocardium and the myocardium of the atrium (Figure 1E). COUP-TFII was also detected in the endocardium of the AVC next to the start of the atrium and in the endocardium of the ventricle at this age (Figure 1F). The expression level of COUP-TFII was lower in the

cushion mesenchymes compared with the endocardium at E10.5 (Figure 1G; Figure IA in the online-only Data Supplement). In addition, COUP-TFII was not detected in the endocardium of the OFT (Figure 1G; Figure IB in the online-only Data Supplement). Unlike the expression pattern in the atrium, COUP-TFII was only expressed in the endocardium but not detected in the myocardium of the ventricle (Figure 1H–1J). The colocalization of COUP-TFII with Tie2 in the endocardium (Figure 1H, arrow), but not with smooth muscle α-actin in the myocardium (Figure 1I), further demonstrated that COUP-TFII is specifically expressed in the endocardium but not in the myocardium of the ventricle. The expression of COUP-TFII was also detected in the epicardium (Figure 1H–1J, arrow in J), where COUP-TFII–positive cells were colocalized with the epicardium marker anti–pan-Cytokeratins (data not shown). Finally, X-gal staining of the embryonic heart at E15.5 further indicated that COUP-TFII is expressed in the endocardium and the epicardium of the ventricle (Figure 1J). The expression in both atrium and ventricles was maintained throughout development (Figure 1K). The expression patterns of COUP-TFII in the endocardium and the epicardium suggest that COUP-TFII may play important roles in the formation of the cardiac valves and coronary vasculature.

Cardiac Abnormalities of *COUP-TFII*$^{F/-}$ Embryos

To circumvent early embryonic lethality of *COUP-TFII* null mutants and to investigate the roles of COUP-TFII in the heart, we generated compound heterozygous mutants *COUP-TFII*$^{F/-}$ by crossing *COUP-TFII*$^{F/F}$ mice with *COUP-TFII*$^{+/-}$ mice. The resulting *COUP-TFII*$^{F/-}$ mutants died by E16 and exhibited severe peripheral hemorrhage and subcutaneous edema at E14.5, suggesting the impending heart failure (Figure 2A). The mutant hearts at E14.5 showed a small left atrium and round apexes (Figure 2A). The facts that *COUP-TFII*$^{+/-}$ mice can survive postnatally and that *COUP-TFII*$^{F/-}$ mutants die by E16 suggest that floxed *COUP-TFII* is a hypomorphic allele. Analysis of *COUP-TFII* transcripts in E12.5 ventricles revealed that *COUP-TFII* mRNA levels were reduced in the *COUP-TFII* hypomorphic mutant ventricles in comparison with the control ventricles (data not shown). Histological analysis revealed that *COUP-TFII*$^{F/-}$ embryos at E15.5 exhibited AV septal defect (Figure 2B). Furthermore, in the control embryos, the cardiomyocytes of the developing myocardium undergo extensive growth, differentiation, and maturation to form the muscularized compact zone, whereas the *COUP-TFI*$^{F/-}$ mutant hearts remain extensively trabeculated with a thin compact zone (Figure 2C). These results suggest that a decrease in *COUP-TFII* dosage in the heart contributes to congenital heart abnormalities.

Endocardial Cushion Hypoplasia in Endothelial-Specific *COUP-TFII* Mutants

During the formation of endocardial cushion, a subset of endocardial cells in the region of AV canal delaminates and transforms into mesenchymal cells.[2] Subsequently, the mesenchymal cells undergo extensive proliferation to generate the AVC. Through remodeling and maturation processes, the primitive cushion then develops into septum and valves. Thus, EMT is a critical step for valve leaflet formation during cardiac development. To study the roles of COUP-TFII on AVC morphogenesis, we inactivated *COUP-TFII* in the endocardium by crossing the endothelial-specific *Tie2Cre* transgenic mouse line[18] with the floxed *COUP-TFII* mice.[17] *Tie2Cre* efficiently inactivates target genes in the endothelium and its derivatives, and its activity is evident in endothelial cells by E8.0 (data not shown). The endothelial-specific *COUP-TFII* null mutants (*Tie2Cre; COUP-TFII*$^{F/F}$) die by E12 with a variety of cardiac defects, including pericardial effusion and widespread hemorrhage.[11] Histological analysis of E9.5 and E10.5 embryos showed that the number of mesenchymal cells in AVC was reduced by 50% in the endothelial-specific *COUP-TFII* null mutants (Figure 3A, arrows and data not shown). The AVC mesenchymal cells are identified based on the cell shape and location. In contrast, endocardial cushions in the OFT were not affected because of lack of COUP-TFII expression in the OFT (data not shown). The

hypoplastic endocardial cushions present in the *COUP-TFII* mutants could be attributable to a failure in EMT, a decrease in cell proliferation, and an increase in apoptosis within the mesenchymal cells of AVC. To directly address whether COUP-TFII is required for normal EMT, we conducted in vitro collagen gel analysis. Normally, the endocardial cells undergo EMT and invade into and migrate through the collagen gel. As shown in Figure 3B, AVC explants from E9.5 control embryos had well-formed mesenchymal cells. In contrast, fewer mesenchymal cells were evident in the mutant explants (Figure 3B, arrows), suggesting that endocardial COUP-TFII is necessary for normal EMT.

To ask whether decreased cell proliferation and increased cell apoptosis also contribute to the hypocellular endocardium cushions present in the *COUP-TFII* mutants, we measured proliferation and apoptotic rates by phosphorylated histone H3 immunostaining and terminal deoxynucleotidyl transferase dUTP nick end labeling assay, respectively. Our results showed that the proliferation of endocardial and cushion mesenchymal cells of the AVC region was significantly decreased in the mutants compared with the control embryos at E9.5 (Figure IIA in the online-only Data Supplement). Similarly, an enhanced apoptosis was observed in the mutant hearts at E10.5 (Figure IIB in the online-only Data Supplement). Therefore, loss of *COUP-TFII* in the endocardium affects not only EMT but also cell proliferation and cell apoptosis in the endocardium as well as in the underlying mesenchyme of AVC.

Notch signaling is involved in cell proliferation, survival, apoptosis, and differentiation, which affects the development of many organs.[19] It was also shown to be involved in cardiac EMT.[20,21] Our previous study demonstrated that COUP-TFII suppressed Notch signaling to specify venous identity during vascular development[11,22]; we then asked whether COUP-TFII also regulates Notch signaling during cardiac EMT. We used an antibody against NICD, the activated form of the Notch1 receptor. As shown in Figure 3C, NICD expression was increased in the AVC of *Tie2Cre; COUP-TFII*$^{F/F}$ embryos, supporting our previous finding that COUP-TFII negatively regulates Notch1 expression.

Snai1, a zinc-finger transcription factor, is expressed in the AV endocardium and has been implicated in the endocardial EMT and EMT in cancer metastasis.[21,23] We examined the expression level of *Snai1*. In contrast to the robust expression of *Snai1* in the endocardium of control embryos, *Snai1* expression was reduced in the *Tie2Cre; COUP-TFII*$^{F/F}$ mutant AV canal at E9.5 (23-somite stage; Figure 3D). *Snai1* is known as a negative regulator of epithelial-cadherin in epithelial cancer cells,[24,25] prompting us to examine the expression of VE-cadherin in the AV endocardium. As EMT proceeds, *Snai1* is normally upregulated and VE-cadherin is downregulated. As expected, the VE-cadherin expression is downregulated in controls by E9.75. However, in endothelial-specific *COUP-TFII* mutants, the expression of endocardial VE-cadherin persisted in AVC at 26-somite stage (Figure 3E), suggesting that COUP-TFII might be involved in *Snai1*–VE-cadherin pathway to regulate endocardial cushion formation.

To further test the above hypothesis, we examined the expression of *Snai1* and VE-cadherin in *COUP-TFII*–depleted endothelial cells by quantitative reverse transcription PCR. Because COUP-TFII is expressed in the venous but not arterial endothelium,[11] we used human umbilical venous endothelial cell as a cell model. Human umbilical venous endothelial cells were treated with specific small interfering RNA against *COUP-TFII* for 48 hours. Consistent with the in vivo result, when *COUP-TFII* was downregulated, the expression of *Snai1* was reduced (Figure 3F). To determine whether *Snai1* is a direct downstream target of COUP-TFII, we performed ChIP assays. We and others have previously demonstrated that when COUP-TFs act as a positive regulator to enhance its target gene expression, COUP-TFs interact with Sp1 on Sp1-binding sites to positively regulate their target genes.[8] To

assess whether COUP-TFII regulates *Snai1* expression, we first searched for potentially conserved Sp1-binding sites in the human, mouse, and rat sequences and found at least 3 conserved sites in the promoter and intron 2 of the *Snai1* gene locus (Figure 3G, red boxes). ChIP analysis showed that COUP-TFII was preferentially recruited to 3 regions containing Sp1-binding sites, site 1 in the promoter and sites 2 and 3 in the second intron, whereas COUP-TFII is not recruited to the region lacking Sp1-binding sites (Figure 3G). To further test whether COUP-TFII binding to the *Snai1* promoter leads to activation of transcription, we performed luciferase reporter assays using a 0.9-kb human *Snai1* promoter fragment that included the first conserved Sp1-binding sites (site 1). We transiently transfected a luciferase reporter containing the *Snai1* promoter (pGL3-hSnai1) in COUP-TFI–overexpressing and control human embryonic kidney 293 cells. Relative luciferase reporter activity driven by the *Snai1* promoter reporter was significantly increased in COUP-TFII–transduced cells compared with control cells (Figure III in the online-only Data Supplement). Collectively, these results strengthened our model that endogenous COUP-TFII is recruited to the *Snai1* promoter to modulate *Snai1* expression, implicating that COUP-TFII in the endocardium of the AVC region regulates proper cushion formation via *Snai1*–VE-cadherin pathway.

Myocardial Hypoplasia in *COUP-TFII* Mutant Embryos

In addition to AV septal defect phenotype, *COUP-TFII* hypomorphic mutants also display a thin compact zone (Figure 2D). It is well established that cardiac growth, contractile performance, and rhythmicity are determined by the cross talks between cardiac endothelial cells (endocardium) and cardiomyocytes and between epicardial cells and cardiomyocytes. Because COUP-TFII expression is not detected in the ventricular myocardium, the compact zone hypoplasia phenotype of *COUP-TFII* hypomorphic mutants is likely caused by indirect mechanisms. The epicardium is known to potentially direct the ventricular myocardium development and morphogenesis. Therefore, we deleted *COUP-TFII* in the epicardium using *Gata5Cre* mouse line. *Gata5Cre* activity is evident at E9 in PE and epicardium.[13] Although *Gata5Cre* fails to completely ablate *COUP-TFII* in the epicardium (Figure IV in the online-only Data Supplement), nevertheless, the *Gata5Cre; COUP-TFII*$^{F/F}$ mutants still developed a compact zone hypoplasia at E13.5 (Figure 4A), suggesting that COUP-TFII expression in the epicardium and its derivatives is required for normal development of the compact myocardium.

To investigate the time window in which inactivation of *COUP-TFII* caused a thin myocardium, TAM-inducible mouse model was used. As previously reported, a single dosage of 3 mg of TAM efficiently deletes *COUP-TFII* in embryos 2 days after administration of TAM to the pregnant female.[15] The untreated *ROSA26CRE-ER*^{T2}; *COUP-TFII*$^{F/F}$ mutant embryos were indistinguishable from *COUP-TFII*$^{F/F}$ control embryos, regardless of the developmental stage.[15] Induced *COUP-TFII* deletion at E12.5 resulted in a pronounced hypoplastic compact zone at E15.5 (Figure 4B), suggesting, during a time window between E12.5 and E15.5, that COUP-TFII is necessary for the normal development of compact myocardium. Compact zone hypoplasia and defective coronary development usually occur together in many experimental mouse models. Our results showed that COUP-TFII is highly expressed in the epicardium (Figure 1H–1J), which is necessary for the formation of coronary vessel. As will be described later, *COUP-TFII* mutants indeed have defective coronary vessel formation. It has been speculated that functional coronary vasculature may in fact be required for the myocardium growth and morphogenesis via the delivery of oxygen and nutrients. Therefore, the thin myocardium phenotype observed in the *Gata5Cre; COUP-TFII*$^{F/F}$ mutants might reflect a primary failure in coronary vascular development.

Loss of *COUP-TFII* Does Not Affect the Formation of PE

Coronary vascular system is derived from the epicardium, and the epicardium is derived from the PE. Migration of proepicardial cells to the naked heart tube begins with the formation of cell cysts with a central cavity. The cysts bud out from the pericardial surface of the septum transversum and either migrate directly or float freely to reach the myocardial surface of the hearts.[26] After attachment to the myocardial surface, the cells migrate laterally to form an epicardial sheet to envelope the entire heart. The high levels of COUP-TFII in the PE (Figure 5A) and epicardium (Figure 1H–1J) suggest that COUP-TFII may be involved in the normal function of epicardial cells. To test the above hypothesis, we performed the histological analysis on *COUP-TFII* null embryos at E9.5. *COUP-TFII* null embryos showed no obvious defects in the formation of heart chambers and OFT.[9] PE cell cysts were formed near the PE in E9.5 control and mutant embryos (Figure 5B and 5C, arrows). To investigate the epicardial development at the molecular level, we analyzed the expression of marker genes at E9.5. GATA4 is an essential factor for the formation of PE, and it is also expressed in the myocardium and the endocardium.[27] Wt1 is a marker of PE and epicardial cells. In both control and mutant embryos, the PE and PE-derived cells were positive for GATA4, suggesting that the epicardial cell fate is determined (Figure 5D). Wt1-positive epicardial cell derivatives budded out from the PE and attached to the myocardial surface in both controls and mutants, indicating cell cyst formation is unaffected (Figure 5E, arrows). Taken together, the initiation of epicardial development is likely unaffected in the absence of *COUP-TFII.*

COUP-TFII Deficiency Causes Abnormal Epicardial Morphogenesis

To ask whether inactivation of *COUP-TFII* affects epicardial development, the morphology of epicardium was compared between control and *COUP-TFII* hypomorphic mutants. By E12.5, when the epicardium completely formed in control embryos and as the heart increases in size, the subepicardial spaces become less apparent and the epicardium is more uniform over the myocardium surface, as evidenced by hematoxylin and eosin staining (Figure VA in the online-only Data Supplement). Although the epicardium largely covered the entire heart in E12.5 mutant embryos, there is an increase of subepicardial spaces in the mutant embryos, suggesting that the epicardium is malformed (Figure VA in the online-only Data Supplement, arrows). Similar results were observed in E11.5-inducible *COUP-TFII* knockout mutants when TAM was administrated to the pregnant dams at E9.5 and in *Gata5Cre; COUP-TFII*$^{F/F}$ mutants at E11.5 (Figure VB and VC in the online-only Data Supplement, arrows). Quantification of the number of Wt1-positive cells within a certain distance of epicardium was significantly reduced in E11.5-inducible *COUP-TFII* knockout mutants (Figure 5D in the online-only Data Supplement). In addition, scanning electron microscopy also showed that a bubble-like distension of the epicardium was evident in the *COUP-TFII* hypomorphic mutants at E12.5 (Figure VIA and VIB in the online-only Data Supplement). Histological analysis showed that large numbers of proepicardial cells were retained in the sinoatrial region of the hypomorphic mutant hearts at E15.5, implying that lack of COUP-TFII affects the proper migration of proepicardial cells from septum transversum mesenchyme to ensheathe the entire heart (Figure VIC in the online-only Data Supplement) Together, these results indicated the aberrant development of the epicardium in the mice lacking *COUP-TFII.*

Inactivation of *COUP-TFII* Leads to Abnormal Coronary Angiogenesis

To directly address whether COUP-TFII is required for coronary development, we performed whole-mount PECAM staining of E13.5 to E14.5 controls and the *COUP-TFII* hypomorphic mutant hearts to visualize the coronary vasculature. Analysis of E13.5 hearts demonstrated that although control hearts developed a vascular plexus that ensheathed the ventricle and was further remodeled to form a coronary vessel, the vascular plexus of

COUP-TFII$^{F/-}$ mutants failed to fully extend to the apices of the hearts (Figure 6A). Quantification of the percentage of coronary vessel coverage at E13.5 revealed that *COUP-TFII* mutants were ≈30% less in comparison with control littermates (Figure 6B). Analysis of coronary morphogenesis at E14.5 control hearts showed normal vascular plexus network in the ventricles (Figure 6C). A vessel with 4 major branches formed on the dorsal side of the control ventricles (Figure 6C, arrows). In contrast, *COUP-TFII* mutant ventricles exhibited abnormal coronary morphogenesis, with defective remodeling and maturation processes where the majority of vessels remain primitive plexus, suggesting an impairment in angiogenesis (Figure 6C). To further corroborate the above findings, E13.5 whole-mount stained hearts were sectioned, and multiple subepicardial (Figure 6D, asterisk) and intramyocardial coronary vessels (Figure 6D, arrows) were observed in the ventricles of the control embryos. Also, weak PECAM signals were detected in the endocardium (Figure 6D, arrowhead). In contrast, *COUP-TFII*$^{F/-}$ mutant ventricles contained little or no subepicardial coronary vessels, but with normal endocardium (Figure 6D, arrowhead), suggesting COUP-TFII is necessary for coronary angiogenesis. To study whether COUP-TFII is expressed in PECAM-positive vessels, COUP-TFII and PECAM antibodies were used. Immunohistochemistry showed that COUP-TFII is observed in PECAM-positive subepicardial vessels at E13.5 hearts (Figure 6E, asterisks). COUP-TFII is also expressed in the smooth muscle cells surrounding the intramyocardial vessel,[8] suggesting that COUP-TFII plays cell-autonomous functions on coronary angiogenesis. Collectively, these results indicate that loss of *COUP-TFII* results in defective coronary vasculature.

Epicardial EMT and Epicardial Migration Are Perturbed in Conditional *COUP-TFII* Mutant Hearts

Epicardial EMT has an important role in the formation of coronary vascular precursor cells. To test whether the coronary vascular defect of *COUP-TFII* hypomorphic mutants was a result of defective epicardial EMT, an ex vivo epicardial explant assay was performed. When E12.5 control epicardial explants were cultured on a collagen gel, a subset of cells delaminated, formed spindle-like cells that are 4′,6-diamidine-2′-phenylindole dihydrochloride positive, and migrated into the collagen gel (Figure 7A; Figure VII in the online-only Data Supplement). In contrast, epicardial explants from *COUP-TFII* mutant embryos formed less spindle-like mesenchymal cells, indicating that epicardial EMT was severely compromised in *COUP-TFII* mutant embryos. Next, we asked whether the migration of epicardial cell derivatives into the myocardium was also perturbed; Wt1 was used as a marker for epicardial cell derivatives. We observed a drastic reduction in the number of Wt1-positive cells inside the myocardium of E14.5-inducible *COUP-TFII* knockout mutant hearts and *COUP-TFII* hypomorphic mutant hearts (Figure 7B, arrows, and data not shown) compared with controls. Intriguingly, the expression of Wt1 was noticeably decreased in the epicardium of the mutant hearts compared with controls (Figure 7B, arrowheads). In addition to Wt1 signaling, PDGFRβ signaling has also been implicated in epicardial migration and coronary vessel maturation.[28] As expected, we observed a decrease in PDGFRβ-expressing cells in the myocardium of *COUP-TFII* hypomorphic mutant hearts compared with the control hearts at E13.5 (Figure 7C). To exclude systemic and other contribution, we examined the expression of Wt1 and PDGFRβ in E14.5 *Gata5Cre; COUP-TFII*$^{F/F}$ mutant hearts. Consistent with the above findings, there was a reduction of Wt1-expressing or PDGFRβ-expressing cells in the myocardium of *Gata5Cre; COUP-TFII*$^{F/F}$ mutant hearts. It is known that Notch signaling in the epicardium is important not only for coronary vascular morphogenesis but also for normal compact myocardium growth.[29] Interestingly, we also observed the ectopic expression of NICD in the myocardium of *Tie2Cre; COUP-TFII*$^{F/F}$ heart (Figure VIII in the online-only Data Supplement), suggesting that alteration of Notch signaling may contribute to the thin myocardium in COUP-TFII

mutants. These data also imply that the reduction of epicardial cell derivatives within the myocardium was attributable to the impaired epicardial EMT and epicardial migration.

Discussion

Cardiac development is regulated by an evolutionarily conserved transcriptional network throughout development. Aberrant transcriptional regulation contributes to the pathogenesis of congenital and acquired forms of heart diseases. Therefore, a better understanding of the function of members of this transcriptional network may provide insights into molecular pathways underlying cardiac diseases. Here, we report crucial roles for COUP-TFII in the endocardial cushion EMT and the subsequent AVC formation. COUP-TFII promotes EMT at least, in part, by directly regulating the expression of *Snai1*, an important EMT factor. In addition, we demonstrate that COUP-TFII in the epicardium is required for ventricular morphogenesis and development of coronary vessels. Exploring underlying mechanisms, we show that epicardial COUP-TFII promotes coronary angiogenesis by regulating epicardial EMT and epicardial cell migration.

In this study, we found that endothelial-specific disruption of *COUP-TFII* showed markedly reduced cellularization from E9.5 in the AVC, a decrease in cell proliferation, and an increase in apoptosis in the endocardium as well as in the underlying mesenchyme of AVC. Concordant with the previous finding, we demonstrated that *Notch1*, an important regulator of heart development, is also a target of *COUP-TFII* during cardiac morphogenesis. In addition to the increased expression of NICD in the AVC of *Tie2Cre; COUP-TFII*$^{F/F}$ heart (Figure 3C), we also observed the ectopic expression of NICD in the myocardium of *Tie2Cre; COUP-TFII*$^{F/F}$ ventricles (Figure VIII in the online-only Data Supplement). Evidences from gene targeting studies in the mice highlighted the importance of the Notch pathway in the cardiovascular development and disease. Either disruption or constitutive activation of Notch signaling disrupted cardiac development, indicating that the level of Notch signaling influences different microenvironmental factors that may affect cell differentiation, induce cell cycle arrest, and inhibit cell proliferation, and result in the loss of survival signals and an increase in cell apoptosis, and eventually elicit abnormal cardiac phenotypes.[21,30–32] Interestingly, the AVC phenotypes of *COUP-TFII* mutants are similar to Notch inactivation.[21] Notch-promoting EMT is attributable to the fact that Notch is shown to enhance *Snai1* expression.[21] However, here, we showed that *COUP-TFII* directly regulated *Snai1*, and the expression of *Snai1* was downregulated in the absence of *COUP-TFII*. Therefore, we proposed that loss of COUP-TFII overrides the gain of Notch, resulting in reduced EMT, even when Notch is enhanced. Our present study suggests the possibility that activated Notch signaling contributes to cardiac abnormalities of *Tie2Cre; COUP-TFII*$^{F/F}$ mutants. Clearly, the COUP-TFII–*Notch1* transcription hierarchy in regulating cell differentiation, proliferation, and apoptosis during cardiac development will require additional investigation.

How COUP-TFII regulates epicardial EMT and coronary angiogenesis needs further investigation. Recent studies have established novel roles of Notch signaling in epicardial development and coronary vessel morphogenesis.[29,33] Ectopic expression of Notch1 in epicardium caused defective epicardial integrity, a thinning of ventricular myocardium, and premature coronary smooth muscle cell differentiation of epicardial cells.[29,33] Given that either epicardial-specific *COUP-TFII* mutants or ectopic *Notch1* activation mutants exhibited similar coronary phenotypes and that COUP-TFII suppresses Notch signaling to govern venous identity and to control progenitor Leydig cell differentiation,[11,34] it is reasonable to speculate that COUP-TFII may function upstream of the Notch signaling to regulate coronary angiogenesis. Indeed, we found the ectopic expression of NICD in endothelial-specific *COUP-TFII*–deleted ventricles. Intriguingly, knocking down *COUP-*

TFII in endothelial cells resulted in the increased expression of not only genes involved in Notch signaling but also smooth muscle lineage markers,[22] which correlated with the published results that Notch signaling promotes smooth muscle differentiation of epicardial cells. Additional experiments will be necessary to fully elucidate the regulation of Notch signaling by COUP-TFII.

The epicardium is a critical tissue that directs several aspects of heart development, including formation of coronary vasculature, as well as ventricular morphogenesis and maturation.[35] In this study, we identified not only the origin (the epicardium) but also the time window (E12.5–E15.5) of COUP-TFII, which is critical for ventricular myocardial morphogenesis. It is well established that the epicardium secretes mitogenic factors to promote compact zone cardiomyocyte proliferation. This raises an intriguing question on whether epicardial COUP-TFII regulates secreted mitogen(s) to induce myocardial growth. Members of fibroblast growth factor (FGF) family, 9, 16 and 20, have been implicated in promoting ventricular cardiomyocyte proliferation in vivo.[36] Although COUP-TFII was colocalized with FGF9, FGF16, and FGF20 in both endothelium and epicardium, we found no differences in the expression of FGF9, FGF16, and FGF20 between control and *COUP-TFII* hypomorphic ventricles at E12.5 as determined by quantitative reverse transcription PCR (data not shown). Similarly, the expression of erythropoietin was unchanged in *COUP-TFII* mutant ventricles (data not shown). These results suggest that COUP-TFII is unlikely a major regulator of the subgroup of FGFs and erythropoietin in the ventricle. Other secreted factors, for example, retinoic acid, Wnts, and PDGFs, remain to be further investigated. It has also been shown that valve defects or vascular deficiency could cause hemodynamic changes that result in the appearance of a thin-walled ventricular chamber. However, epicardial-specific knockout of *COUP-TFII* by *Gata5Cre* exhibits similar thin compact zone without apparent valve defects (Figure 4A), indicating that hemodynamic changes are unlikely to play significant role in this defect.

Despite the importance of epicardium itself in ventricular morphogenesis, the cross talks between both coronary endothelial cells and epicardium with the myocardium also affect the growth of cardiomyocytes. Functional coronary vasculature is required for the myocardium growth and morphogenesis via the delivery of oxygen and nutrients. Thus, failure in myocardial compaction in the *COUP-TFII* mutants is likely a manifestation of the disruption of these cross talks.

As we have shown, the decreased numbers of Wt1- and PDGFβ-positive cells within the compact myocardium in the COUP-TFII mutant hearts indicated an impairment of epicardial EMT and migration and, consequently, affected the maturation of intramyocardial vessels of COUP-TFII hypomorphic mutants and of epicardial deletion of *COUP-TFII* mutants. These results strongly suggest that COUP-TFII contributes to coronary angiogenesis. Like the expression domain in other vascular systems, COUP-TFII is expressed in the endothelial cells of the subepicardial vessels (cardiac veins) and the smooth muscle cells of the intramyocardial vessels (cardiac arteries).[8] Although the origin of coronary endothelial cells is controversial, recent lineage tracing experiments in avian and mouse models have demonstrated that the majority of coronary vascular smooth muscle cells arise from the epicardium.[37–40] Thus, based on the expression of COUP-TFII in specific cell types, we speculate that COUP-TFII affects coronary morphogenesis by at least 3 mechanisms: first, by recruiting COUP-TFII–positive vascular endothelial cells to promote the assembly of newly formed blood vessels; second, by promoting epithelial-mesenchymal transition to generate vascular smooth muscle cells and fibroblasts for the formation of vessel; third, by affecting the mobilization of the epicardial-derived cells from the epicardium into the myocardium. However, we could not distinguish the second and third views because the reduction of Wt1- and PDGFRβ-positive epicardial cell derivatives in the myocardium of

COUP-TFII mutant hearts could be simply a result of the reduction of Wt1-positive subepicardial cells rather than reflecting their migration defects.

A study has identified that a male patient with a 6.5-Mb deletion covering chromosome 15q26.2 displayed several malformations, including congenital heart defects (left atrial hypoplasia, ventral septal defect, mitral valve atresia, aorta hypoplasia) and congenital diaphragmatic hernia.[41] Intriguingly, *COUP-TFII* is one of the known genes residing within this critical region, and genetic studies showed that mouse lacking *COUP-TFII* in foregut mesenchyme exhibits Bochdalek-type congenital diaphragmatic hernia.[10] Strikingly, the clinical manifestations of the male patient are consistent with the phenotypes displayed by the *COUP-TFII* hypomorphic mouse mutants, such as a small left atria, AV septal defect, and valve defects. Supporting the above finding, the patients with deletion at a similar region but with intact *COUP-TFII* gene did not have cardiac abnormalities.[42] Therefore, it is likely that the malformation of heart and diaphragm in the human patients is attributable to hemizygosity/haploinsufficiency of the *COUP-TFII* gene.

Studies of transcription factors and signaling molecules in heart development will enable us to understand the complexity and underlying mechanisms of the pathogenesis of congenital heart defect in humans. Further information regarding *COUP-TFII* mutations in humans will also facilitate the genetic counseling on affected individuals. The goal of future study will be to integrate *COUP-TFII*, together with preexisting molecules, into a comprehensive model of the regulatory hierarchy of cardiac development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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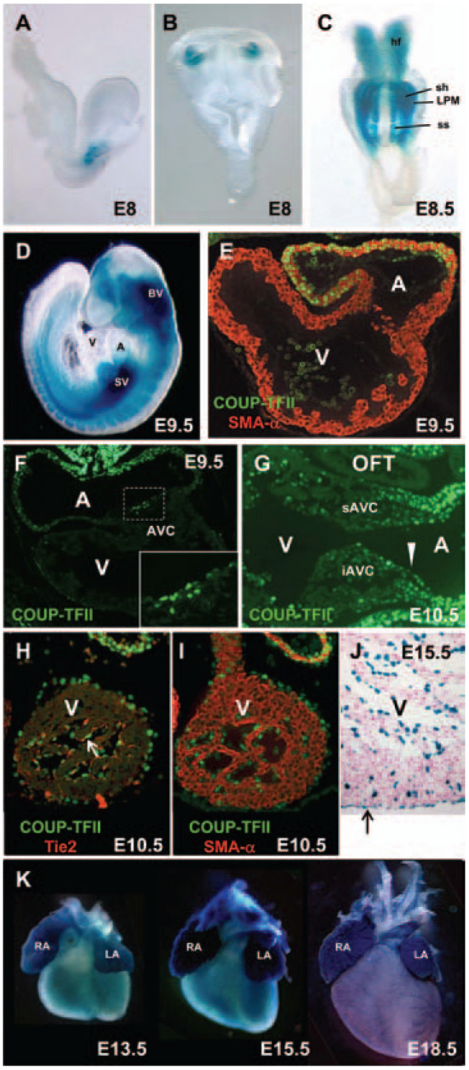

Figure 1.

The expression domains of chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) during cardiac development. **A** and **B**, Using *COUP-TFII/lacZ* knock-in mice, COUP-TFII (blue) is first noted at the posterior cardiac crescent of E8 embryo shown in lateral (**A**) and ventral (**B**) views. **C**, The expression of COUP-TFII is largely localized in the sinus horn region at the 6-somite stage. Its expression is also shown in lateral plate mesoderm and somites. **D**, At E9.5, COUP-TFII is highly expressed in the sinus venosus and the atrium. **E**, High expression level of COUP-TFII (green) is detected in the atrium and is colocalized with the myocardium marker smooth muscle actin-α (SMA-α; red) at E9.5 (21 somites). **F**, COUP-TFII (green) begins to express in the endocardial atrioventricular cushion (AVC) toward the atrium later at E9.5. Area surrounding AVC (dotted rectangle) is magnified in the bottom panel. **G**, Sagittal section from E10.5 embryo shows high expression level of COUP-TFII (green) in the endocardium (arrowhead), atrial myocardium, and epicardium, whereas weak signal is detected in the myocardium underlying AVC. **H**, COUP-TFII (green) is detectable in the endocardium of the ventricle (arrow) as it is colocalized with the endothelial marker Tie2 (red). **I**, COUP-TFII (green) is not colocalized with the myocardial marker SMA-α in the ventricle (red). **J**, COUP-TFII is expressed in the endocardium and epicardium (**arrow**) of the ventricle at E15.5. **K**, From E13.5 to E18.5,

high levels of COUP-TFII expression are observed in the atrium, whereas weak COUP-TFII expression is detected in the ventricle. A indicates atrium; Hf, head fold; iAVC, inferior atrioventricular cushion; LA, left atrium; LPM, lateral plate mesoderm; OFT, out-flow tract; RA, right atrium; sAVC, superior atrioventricular cushion; sh, sinus horn; ss, somites; SV, sinus venosus; V, ventricle.

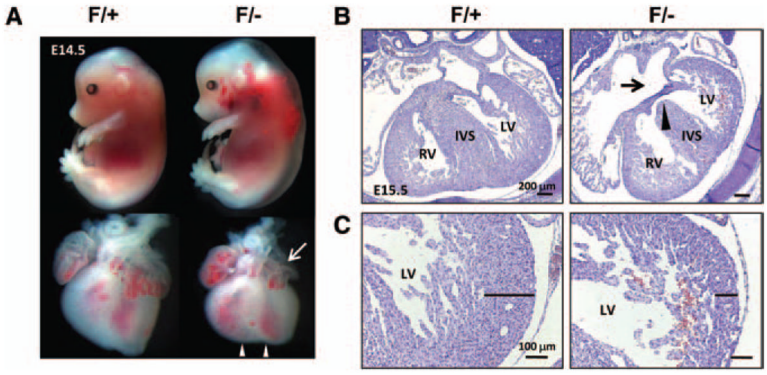

Figure 2.

Cardiac abnormalities in chicken ovalbumin upstream promoter-transcription factor II (*COUP-TFII*) hypomorphic mutant embryos. **A**, Gross appearance and whole hearts of *COUP-TFII*$^{F/+}$ control and *COUP-TFII*$^{F/-}$ mutants at E14.5. *COUP-TFII*$^{F/-}$ mutants exhibit extensive peripheral hemorrhage and edema. Mutant heart has round apexes of the ventricle (**arrowheads**) and reduced left atrium (**arrow**). **B**, Hematoxylin and eosin (H&E) stained transverse sections from control and *COUP-TFII*$^{F/-}$ mutant hearts at E14.5. Fusion of atrial septum and atrioventricular cushion is completed in *COUP-TFII*$^{F/+}$ littermate control. *COUP-TFII*$^{F/-}$ mutants display an atrioventricular septal defect with openings between left and right atria (**arrow**) and left and right ventricles (**arrowhead**). The atrial septum is absent, and the central mesenchymal mass is suspended in the mutant embryo. **C**, H&E-stained transverse sections of control and *COUP-TFII*$^{F/-}$ mutant hearts at E15.5. Black line indicates the thickness of the compact zone. Control embryos show normal compact zone of myocardium, whereas compact zone hypoplasia is observed in *COUP-TFII*$^{F/-}$ mutants. IVS indicates interventricular septum; LV, left ventricle, RV, right ventricle.

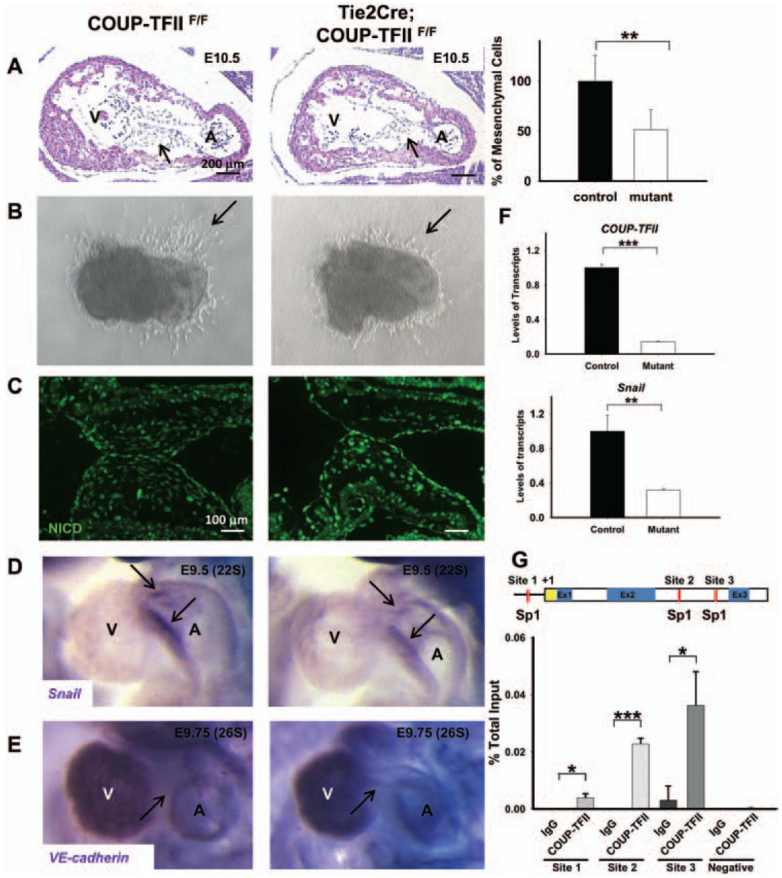

Figure 3.

Endothelial-specific chicken ovalbumin upstream promoter-transcription factor II (*COUP-TFII*) null mutants exhibited endocardial cushion hypoplasia. **A**, Hematoxylin and eosin–stained sagittal sections of control and *Tie2Cre; COUP-TFII*$^{F/F}$ embryos at E10.5 and the quantification of mesenchymal cells in atrioventricular cushions (AVCs). n=3 for control and n=4 for mutants. The data indicated that the mesenchymal cells in the AVCs are hypoplastic (**arrows**). **B**, AVC explants were harvested at E9.5 and cultured on 3-dimensional collagen gels for 24 hours. The number of transformed cells invading the collagen gel (**arrows**) is significantly reduced in *Tie2Cre; COUP-TFII*$^{F/F}$ mutants compared with littermate controls. **C**, Increased expression of Notch1 intracellular domain (NICD) in AVCs of *Tie2Cre; COUP-TFII*$^{F/F}$ mutant hearts at E10.5 compared with littermate control *COUP-TFII*$^{F/F}$ hearts. **D** and **E**, Whole-mount in situ hybridization analysis with *Snai1* (**D**) and vascular endothelial (VE)-cadherin (**E**) riboprobes at E9.5 and E9.75, respectively. *Snai1* expression is down-regulated, whereas VE-cadherin expression persists in AVC endocardium. **Arrows** donate hybridization signal in AVC. **F**, Quantitative reverse transcription polymerase chain reaction analysis of *COUP-TFII* and *Snai1* transcripts in RNA isolated from *COUP-TFII* or scrambled small interfering RNA–transfected human umbilical venous endothelial cells (HUVECs; normalization to 18S rRNA). **G**, Chromatin immunoprecipitation analysis of COUP-TFII binding to the putative Sp1-binding sites of human *Snai1* promoter in HUVECs. A indicates atrium; Ex, exon; V, ventricle. Error bars indicate SD; **P*<0.05; ***P*<0.01; ****P*<0.001.

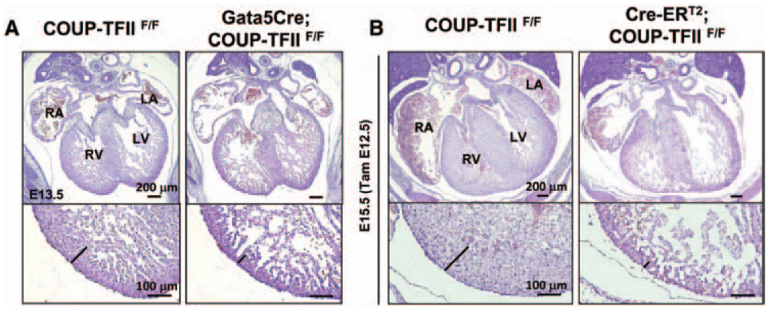

Figure 4.

A myocardial hypoplasia in epicardial-specific chicken ovalbumin upstream promoter-transcription factor II (*COUP-TFII*) mutants and inducible *COUP-TFII* mutant embryos. **A**, Hematoxylin and eosin (H&E) stained transverse sections from control and *Gata-5Cre; COUP-TFII$^{F/F}$* mutant hearts at E13.5. Mutant hearts exhibit a thinned compact zone of myocardium. **B**, H&E staining of transverse sections of control *COUP-TFII$^{F/F}$* and littermate *CRE-ER^{T2}; COUP-TFII$^{F/F}$* mutant embryos at E15.5 (with tamoxifen administration at E12.5). Black line indicates the thickness of the compact zone. Induced *COUP-TFII* deletion at E12.5 causes a compromised compact zone phenotype. LA indicates left atrium; LV, left ventricle; RA, right atrium, RV, right ventricle.

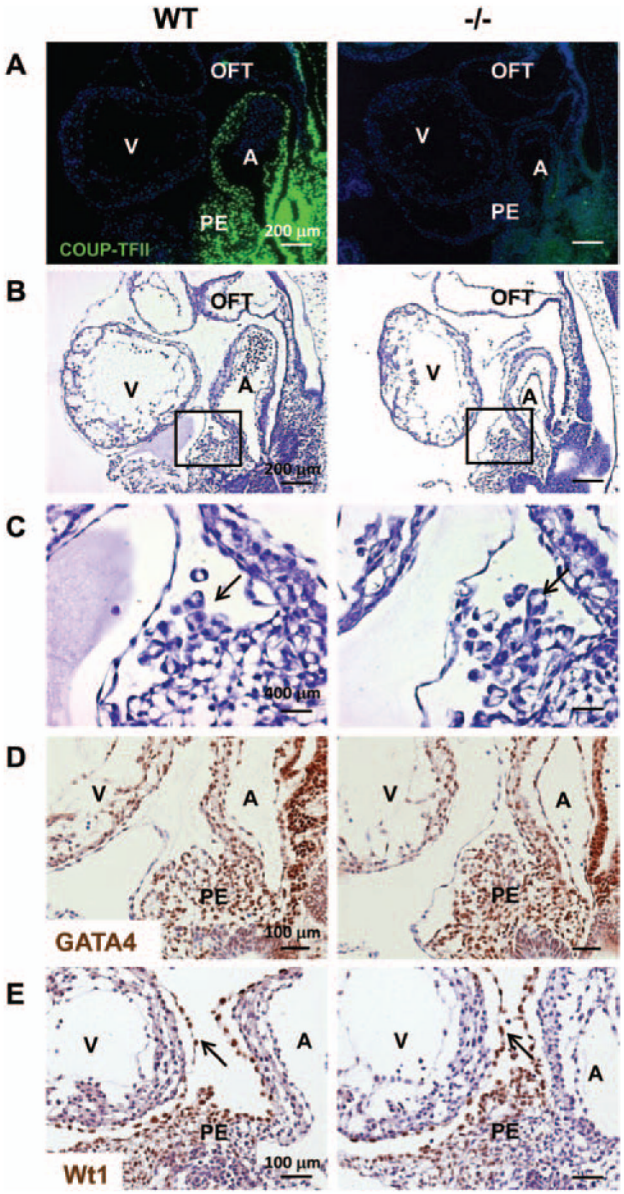

Figure 5.

Loss of chicken ovalbumin upstream promoter-transcription factor II (*COUP-TFII*) does not affect the formation of proepicardium. **A**, Immunofluorescent staining for COUP-TFII (green) in wild-type and *COUP-TFII*$^{-/-}$ littermates at E9.5. Nuclei were counterstained with 4′,6-diamidine-2′-phenylindole dihydrochloride. **B** and **C**, Hematoxylin and eosin staining of sagittal sections obtained from wild-type and COUP-TFII$^{-/-}$ mutants at E9.5. **C**, Higher magnification of the regions shown in **B**. In both wild-type and *COUP-TFII*$^{-/-}$ mutants, a substantial number of epicardial progenitors cell cysts are formed and bud out from the proepicardium (PE). **D**, Immunohistochemistry for GATA4 in sagittal sections of E9.5 wild-type and *COUP-TFII*$^{-/-}$ embryos. All tissues are labeled with GATA4. **E**, Immunohistochemistry reveals Wilms tumor 1 expression in the PE and epicardial cells in wild-type and *COUP-TFII*$^{-/-}$ littermates at E9.5. A indicates atrium; OFT, outflow tract; V, ventricle.

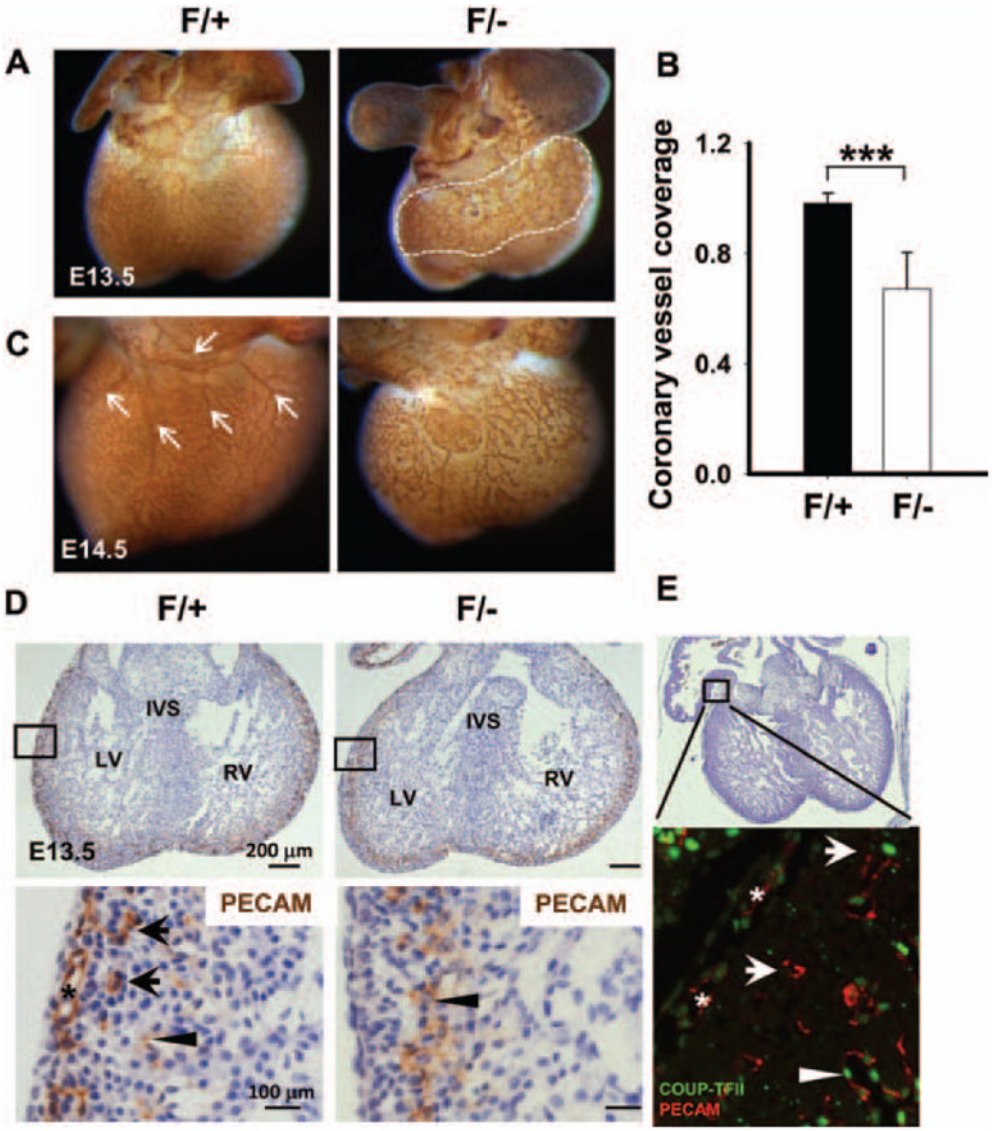

Figure 6.

Inactivation of chicken ovalbumin upstream promoter-transcription factor II (*COUP-TFII*) leads to abnormal coronary angiogenesis. **A** and **C**, Whole-mount immunostaining of E13.5 and E14.5 mouse hearts with platelet-endothelial cell adhesion molecule (PECAM) illustrates vascular endothelium. **A**, A representative littermate control heart shows the normal vascular plexus in the ventricles. The vascular plexus of *COUP-TFII* mutant ventricle fails to extend to the apex of the heart. **B**, The coronary plexus coverage (ratio of region within white dotted line to total projected ventricular area) at E13.5 was significantly decreased in mutants. Error bars indicate SD; ***P*<0.01. **C**, A vessel with 4 major branches (arrows) forms on the dorsal side of the control ventricle, whereas *COUP-TFII*$^{F/-}$ mutant ventricles at E14.5 present abnormal coronary morphogenesis, with less extensive vascular network and defective branching. **D**, Histological sections of PECAM-stained control and mutant hearts at E13.5 indicating that *COUP-TFII*$^{F/-}$ mutants lack subepicardial vessels (asterisk) and intramyocardial vessels (**arrows**). **Arrowheads** denote endocardial PECAM staining. **E**, Hematoxylin and eosin and immunofluorescence stainings of wild-type mouse hearts at E13.5 reveal that COUP-TFII is expressed in subepicardial vessels (asterisks),

intramyocardial vessels (**arrows**), and endocardium (**arrowhead**). LV indicates left ventricle; RV, right ventricle; IVS, interventricular septum.

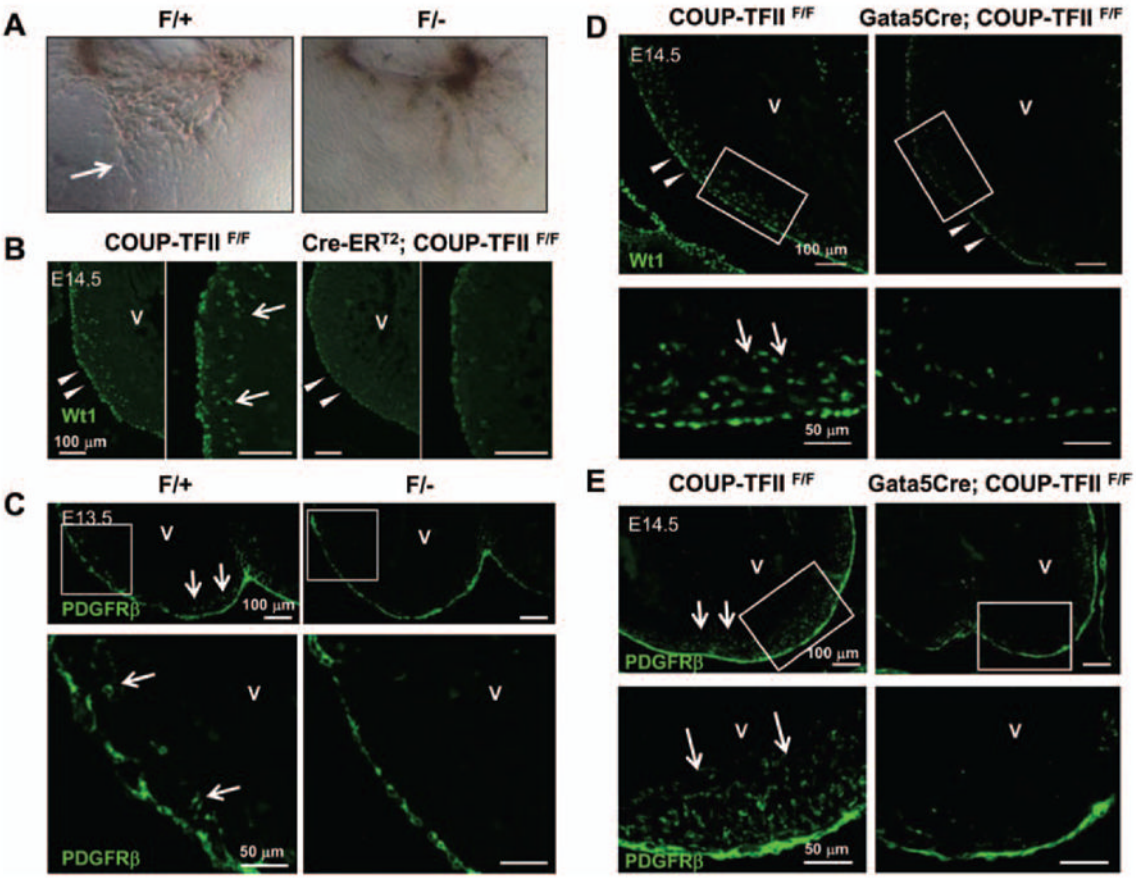

Figure 7.

Epicardial-mesenchymal transformation (EMT) and epicardial migration are perturbed in chicken ovalbumin upstream promoter-transcription factor II (*COUP-TFII*) mutant hearts. **A**, Impaired EMT in *COUP-TFII*$^{F/-}$ mutant epicardium. Representative figures of ex vivo heart explants on collagen gel. Cells migrate out of the control heart explants and transform to spindle-shaped mesenchymal cells (**arrow**), whereas little or no mesenchymal cells are observed in mutant heart explants. **B** and **D**, Decreased expression of Wilms tumor 1 (Wt1) in the epicardium of *Cre-ER*^{T2}*; COUP-TFII*$^{F/F}$ mutant hearts at E14.5 (with tamoxifen administration at E11.5) (**B**) or *Gata5Cre; COUP-TFII*$^{F/F}$ mutant hearts at E14.5 (**D**) compared with littermate control *COUP-TFII*$^{F/F}$ hearts. Numerous Wt1-positive epicardial derivatives were detected in the myocardium of controls (**arrows**), whereas decreased number of Wt1-positive epicardial derivatives was found in mutant hearts (**white arrows**). **C** and **E**, A decreased number of platelet-derived growth factor receptor β (PDGFRβ)–expressing epicardial-derived cells in the myocardium of *COUP-TFII*$^{F/-}$ mutant hearts at E13.5 (**C**) or *Gata5Cre; COUP-TFII*$^{F/F}$ mutant hearts at E14.5 (**E**) compared with littermate control hearts. Numerous PDGFRβ-positive epicardial derivatives were detected in the myocardium of controls (**arrows**), whereas a decreased number of PDGFRβ-positive epicardial derivatives were found in mutant hearts. V indicates ventricle.