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

Fu-Jung Lin,* Li-Ru You,* Cheng-Tai Yu, Wen-Hsin Hsu, Ming-Jer Tsai, Sophia Y. Tsai

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-TFI, NR2F2) in cardiac organogenesis.

Methods and Results—We analyzed embryos deficient in COUP-TFI 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-TFI is expressed in the endocardium and the epicardium but not in the myocardium of the ventricle. Using endothelial-specific COUP-TFI mutants and molecular approaches, we show that COUP-TFI 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-TFI 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-TFI impairs epicardial EMT and coronary plexus formation.

Conclusion—Our results reveal that COUP-TFI 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. (Arterioscler Thromb Vasc Biol. 2012;32:e135-e146.)

Key Words: atrioventricular septal defect ■ cardiac morphogenesis ■ chicken ovalbumin upstream promoter-transcription factor II ■ epicardium ■ epithelial-mesenchymal transformation

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. 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).\(^7\)

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

Here, we present data demonstrating novel roles of COPU-TFII in the formation of AVC and coronary vessels. Inactivation of \(\text{COPU-TFII}\) results in a variety spectrum of cardiac defects, including cardiac valve malformation, compact zone hypoplasia, and defective coronary vasculature. We discovered that COPU-TFII in the endocardium is important for appropriate EMT and is involved in \(\text{Snail1}\)-vascular endothelial (VE)-cadherin signaling to regulate endocardial cushion development. Furthermore, COPU-TFII in the epicardium is required for normal epicardial formation, epicardial EMT, and epicardium-derived mesenchymal cell migration. Collectively, our results indicate that endocardial COPU-TFII is important for AVC formation and that epicardial COPU-TFII is required for coronary vascular development.

**Methods**

**Animal Experiments**

\(\text{COUP-TFII}\)\(^{+/+}\) mice, \(\text{COUP-TFII}\)\(^{-/-}\) mice, \(\text{COUP-TFII}\)\(^{-/+}\)embryos, and Tie2Cre; \(\text{COUP-TFII}\)\(^{+/+}\) embryos were generated as previously reported.\(^{10-12}\) The \(\text{Gata5Cre}^{+/+}\) mouse strain was provided by Dr Pilar Ruiz-Lozano,\(^{13}\) and the \(\text{ROSA26Cre-ERT2}\)\(^{+/+}\) 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 deletion, 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-sarcromeric actin (Sigma, 1: 3000), phospho-Histone H3 (Upstate, 1:500), Wt1 (Santa Cruz, 1:500), COPU-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 5x with PBSMT, followed by biotinylated donkey anti-rat IgG secondary antibody (Jackson ImmunoResearch). 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://rsweb.nih.gov/ij/). Quantification of the percentage of the endothelium 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 3x 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.\(^{8}\)

**AVC Canal and Epicardial Explant Cultures**

AVC explants and epicardial explants cultures were established as previously 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 RNasy Mini Kit (QIAGEN) and TRIzol (Invitrogen), respectively. First-strand cDNA was synthesized by SuperScriptIII reverse
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 mice and anti-COX-2 antibody were used to analyze the expression patterns of COUP-TFII in the developing hearts. X-gal staining of COUP-TFII/LacZ+ 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 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 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+/− 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+/− by crossing COUP-TFII+/− mice with COUP-TFII+/− mice. The resulting COUP-TFII+/− 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+/− 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+/− 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-TFII+/− 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 line18 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−/−) 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
Lin et al  COUP-TFII Function in Heart Development  e139

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/+/embryos, supporting our previous finding that COUP-TFII negatively regulates Notch1 expression.

Snail, 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 Snail. In contrast to the robust expression of Snail1 in the endocardium of control embryos, Snail1 expression was reduced in the Tie2Cre; COUP-TFII/embryos. Snail1 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, Snail 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 Snail–VE-cadherin pathway to regulate endocardial cushion formation.

To further test the above hypothesis, we examined the expression of Snail 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 Snail was reduced (Figure 3F). To determine whether Snail 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 Snail 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 Snail promoter leads to activation of transcription, we performed luciferase reporter assays using a 0.9-kb human Snail promoter fragment that included the first conserved Sp1-binding sites (site 1). We transiently transfected a luciferase reporter containing the Snail promoter (pGL3-hSnai1) in COUP-TFI–overexpressing and control human embryonic kidney 293 cells. Relative luciferase reporter activity driven by the Snail 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 Snail promoter to modulate Snail expression, implicating that COUP-TFII in the endocardium of the AVC region regulates proper cushion formation via Snail–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-TFIIfl/fl 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<sup>FL</sup>; COUP-TFIIfl/fl mutant embryos were indistinguishable from COUP-TFIIfl/fl control embryos, regardless of the developmental stage.15

![Figure 4](http://atvb.ahajournals.org/)

**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 Gata5Cre; COUP-TFIIfl/fl 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<sup>fl/fl</sup> and littermate CRE-ER<sup>FL</sup>; COUP-TFII<sup>fl/fl</sup> 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.
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<sup>−/−</sup> 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. 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.
Inactivation of COUP-TFIIF Leads to Abnormal Coronary Angiogenesis

To directly address whether COUP-TFIIF is required for coronary development, we performed whole-mount PECAM staining of E13.5 to E14.5 controls and the COUP-TFIIF 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-TFIIF−/− 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-TFIIF 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-TFIIF 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-TFIIF−/− mutant ventricles contained little or no subepicardial coronary vessels, but with normal endocardium (Figure 6D, arrowhead), suggesting COUP-TFIIF is necessary for coronary angiogenesis. To study whether COUP-TFIIF is expressed in PECAM-positive vessels, COUP-TFIIF and PECAM antibodies were used. Immunohistochemistry showed that COUP-TFIIF is observed in PECAM-positive subepicardial vessels at E13.5 hearts (Figure 6E, asterisks). COUP-TFIIF is also expressed in the smooth muscle cells surrounding the intramyocardial vessel, suggesting that COUP-TFIIF plays cell-autonomous

Figure 6. Inactivation of chicken ovalbumin upstream promoter-transcription factor II (COUP-TFIIF) 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-TFIIF 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-TFIIF−/− 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-TFIIF−/− 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-TFIIF is expressed in subepicardial vessels (asterisks), intramyocardial vessels (arrows), and endocardium (arrowhead). LV indicates left ventricle; RV, right ventricle; IVS, interventricular septum.
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

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/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 (PDGFRβ)-expressing epicardial-derived cells in the myocardium of COUP-TFII F/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.
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 formation 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 marked 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-TFIIf/f heart (Figure 3C), we also observed the ectopic expression of NICD in the myocardium of Tie2Cre; COUP-TFIIf/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-TFIIf/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 Notch signaling, 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 to be 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 PDGFRβ-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. As 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). 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. Thus, based on the expression of COUP-TFII, it is likely a manifestation of the disruption of these cross talks.

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. 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. Strikingly, the clinical manifestations of the male patient are consistent with the phenotypes displayed by 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. Therefore, it is likely that the malformation of heart and diaphragm in the human patients is attributable to hemizygosity/haplosufficiency 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.

Acknowledgments

We thank Dr Henry M. Sucov for the constructive comments on the manuscript, W. Chen, W. Qian, X.-F. Tong for their technical help, J. Hebert for proof reading of the article, and Dr Ching-Pin Chang for technical advice on the EMT assay. We also thank Dr Pilar Ruiz-Lozano for the Gata5CreERT2 mouse strain, Dr Thomas Ludwig for the ROSA26CreER-T2 knock-in mouse strain, and Dr Antonio Garcia de Herreros for the Snail promoter-driven reporter construct.

Sources of Funding

This work was supported by National Science Council of Taiwan grant NSC98-2320-B-010-010-MY3 and a grant from Ministry of Education, Aim for the Top University Plan (to L.-R. You), National Heart, Lung, and Blood Institute grant R01 HL076448 (to S.Y. Tsai), National Institute of Child Health and Human Development grant R01 HD17379, and National Institute of Diabetes and Digestive and Kidney Disease grant R01 DK54614 (to M.-J. Tsai), P01 DK059820 and U19 DK062434 (to S.Y. Tsai and M.J. Tsai), and core facilities of Diabetes and Endocrinology Research Center (DK079638).

Disclosures

None.

References

Endocardial Cushion Morphogenesis and Coronary Vessel Development Require Chicken Ovalbumin Upstream Promoter-Transcription Factor II
Fu-Jung Lin, Li-Ru You, Cheng-Tai Yu, Wen-Hsin Hsu, Ming-Jer Tsai and Sophia Y. Tsai

Arterioscler Thromb Vasc Biol. 2012;32:e135-e146; originally published online September 6, 2012;
doi: 10.1161/ATVBAHA.112.300255
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/32/11/e135

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2012/09/06/ATVBAHA.112.300255.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Endocardial Cushion Morphogenesis and Coronary Vessel Development Require COUP-TFII

Fu-Jung Lin, Li-Ru You, Cheng-Tai Yu, Wen-Hsin Hsu, Ming-Jer Tsai and Sophia Y. Tsai

SUPPLEMENTAL METHOD

Luciferase Assay

The pGL3 plasmid containing a 0.9 kb human Snai1 promoter fragment that included the first conserved Sp1 binding sites was used (pGL3-hSnai1) (1) (A gift from Dr. Antonio Garcia de Herreros). HEK 293 cells were transfected with pGL3-Snai1-Luc using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Supplemental Table I. The primer sequences for Q RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences</th>
<th>Size (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCOUP-TFII</td>
<td>F: GCCATAGTCCTGGTCCACCTC</td>
<td>79</td>
<td>(2)</td>
</tr>
<tr>
<td></td>
<td>R: CTGAGACTTTTTCTGCAAGC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hSnai1</td>
<td>F: GCTGAGAATCTGAGTCCAGCC</td>
<td>130</td>
<td>(3)</td>
</tr>
<tr>
<td></td>
<td>R: GACAGATCCAGAGCCAGATGCAATTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>h18sRNA</td>
<td>F: TCCGATAACGAACGAGACTCC</td>
<td>81</td>
<td>(4)</td>
</tr>
<tr>
<td></td>
<td>R: CAG GGAATTTAATCAACGCAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChIP-hSnai1-Sp1-1</td>
<td>F: GGAGACGAGCGCTCCGATT</td>
<td>167</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AGCAGTGGCCGCAAGAAGACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChIP-hSnai1-Sp1-2</td>
<td>F: GCATGGCTGAGACACGAA</td>
<td>199</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CGATATCCCCTCGGATTAAGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChIP-hSnai1-Sp1-3</td>
<td>F: AAGGATGGCCTAACCAGGTT</td>
<td>163</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: CCAAGGCTCGAGGTTAACACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ChIP-Neg</td>
<td>F: AACGCGCAGAAGGAGAAGACCA</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td></td>
<td>R: AAGATGACCCGAGGGTGG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

REFERENCES


SUPPLEMENTAL FIGURES

Supplemental Figure I. COUP-TFII expression at E10.5.

(A) Tie2Cre; COUP-TFII^{F/+} whole mount β-galactosidase stained sagittal sections label endocardial COUP-TFII. COUP-TFII is observed in the endocardium of the atrioventricular cushion toward the atrium. (B) Immunofluorescence for COUP-TFII sagittal sections of E10.5 wild-type embryo. Nuclei were counterstained with DAPI. Scale bar, 100 μm. A, atrium; iAVC, inferior atrioventricular cushion; OFT, outflow tract; sAVC, superior atrioventricular cushion; V, ventricle.

Supplemental Figure II. A decrease in cell proliferation and an increase in apoptosis in Tie2Cre; COUP-TFII^{FF} mutants.

(A) Immunohistochemistry of phospho-histone H3 and quantification of phospho-histone H3 positive cells, showing a decrease on cell proliferation in AV cushions of Tie2Cre; COUP-TFII^{FF} embryos at E9.5 compared to the controls. N=3. (B) TUNEL assay and quantification of TUNEL positive cells were performed at E10.5 AV cushions. Control AV cushions are negative for TUNEL assay, while mutant AV cushions are positively stained with TUNEL assay (arrows). N=3. A, atrium and V, ventricle. Error bars indicate standard deviation; *P<0.05; ***P<0.001.

Supplemental Figure III. COUP-TFII binds to Snai1 promoter and activates its transcription.

HEK293 cells were transiently transfected with the Snail promoter-luciferase reporter (pGL3-hSnail) with a control vector (Ctrl) or a COUP-TFII-expressing vector (COUP-TFII). The results are expressed as relative luciferase activity after correction for protein concentration. COUP-TFII enhances Sani1 transcriptional activity. Error bars represent standard deviation. ***P<0.001.

Supplemental Figure IV. COUP-TFII expression is slightly reduced in the epicardium of E11.5 Gata5Cre; COUP-TFII^{FF} mutants.

(A) Immunofluorescence for COUP-TFII in transverse sections of E11.5 control and Gata5Cre; COUP-TFII^{FF} embryos. COUP-TFII is expressed in the endocardium and the epicardium (arrows) of controls, whereas COUP-TFII expression is slightly reduced in the epicardium of E11.5 Gata5Cre; COUP-TFII^{FF} embryos, indicating the incomplete deletion of COUP-TFII in the epicardium. (B) Nuclei were counterstained with DAPI. Scale bar, 100 μm.
Supplemental Figure V. Malformation of epicardium in the COUP-TFIIF deficiency hearts.
(A-C) H&E stained images of transverse sections of control COUP-TFIIF/F and COUP-TFIIF/F- (A), and of control COUP-TFIIF/F and littermate CRE-ER<sup>T2</sup>; COUP-TFIIF/F mutant embryos at E11.5 (with Tam administration at E9.5) (B), and of control COUP-TFIIF/F and Gata5Cre; COUP-TFIIF/F mutant hearts at E11.5 (C). In control hearts, the epicardium is uniform over the myocardium surface and the subepicardial spaces become less apparent. The detachments of the epicardium (arrow) from the underlying myocardium are observed in mutant hearts. (D) Immunofluorescence for Wt1 (green) and for pan-cytokeratin (red) in transverse sections of control COUP-TFIIF/F and littermate CRE-ER<sup>T2</sup>; COUP-TFIIF/F mutant embryos E11.5 (with Tam administration at E9.5). The number of Wt1 positive epicardial cells is reduced in the mutant hearts as compared to controls. Scale bar, 100 μm; ***P<0.001.

Supplemental Figure VI. COUP-TFIIF deficiency causes abnormal epicardium morphogenesis.
(A, B) Scanning electron microscopy of control and mutant hearts at E12.5. (B) Higher magnification pictures of (A). COUP-TFIIF/F- mutant hearts display abnormal epicardial features at E12.5. (C) The retention of a large sum of cells in sinoatrial region of the heart (arrows) was observed in COUP-TFIIF/F- mutant, but not in the control littermate, suggesting that cell migration in the epicardium is altered in the mutant heart. LV, left ventricle; RV, right ventricle.

Supplemental Figure VII. Epicardial mesenchymal cells derived from in vitro explants are DAPI positive.
(A) E12.5 control epicardial explants were cultured on a collagen gel: a subset of cells delaminated, formed spindle-like shape cells (arrows) and migrated into the collagen gel. (B) DAPI staining revealed epicardial delaminated cells (arrows) invading the collagen gel. Nuclei were counterstained with DAPI.

Supplemental Figure VIII. NICD expression is increased in the ventricles of E11.5 Tie2Cre; COUP-TFIIF/F mutants.
Immunofluorescence for NICD in transverse sections of E11.5 littermate control and Tie2Cre; COUP-TFIIF/F mutant embryos. Scale bar, 100 μm.
Lin et al., Supplemental Figure I
Lin et al., Supplemental Figure III

The diagram shows a luciferase assay with hSna1 elements. The x-axis represents the region from -869 to +59 of the hSna1 promoter, with luciferase activity indicated by a bar graph comparing pGL3-hSna1 vectors with or without COUP-TFI treatment. The y-axis represents relative fold changes.
Lin et al., Supplemental Figure IV

**A**

**COUP-TFII**<sup>F/F</sup> vs **Gata5Cre; COUP-TFII**<sup>F/F</sup>

**B**

**COUP-TFII**<sup>red</sup> vs **DAPI**<sup>blue</sup>

Scale bar: 100 μm
Lin et al., Supplemental Figure VII