Tbx5 is Required for Avian and Mammalian Epicardial Formation and Coronary Vasculogenesis

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ABSTRACT

Rationale: Holt-Oram syndrome (HOS) is an autosomal dominant heart-hand syndrome caused by mutations in the TBX5 gene. Overexpression of Tbx5 in the chick proepicardial organ (PEO) impaired coronary blood vessel formation. However, the potential activity of Tbx5 in the epicardium itself, and Tbx5’s role in mammalian coronary vasculogenesis, remains largely unknown.

Objective: To evaluate the consequences of altered Tbx5 gene dosage during PEO and epicardial development in the embryonic chick and mouse.

Methods and Results: Retroviral-mediated knockdown or upregulation of Tbx5 expression in the embryonic chick PEO as well as proepicardial-specific deletion of Tbx5 in the embryonic mouse (Tbx5<sup>epi−/−</sup>) impaired normal PEO cell development, inhibited epicardial and coronary blood vessel formation and altered developmental gene expression. The generation of epicardial-derived cells (EPDCs) and their migration into the myocardium was impaired between embryonic day (E) 13.5-15.5 in mutant hearts due to delayed epicardial attachment to the myocardium and subepicardial accumulation of EPDCs. This caused defective coronary vasculogenesis associated with impaired vascular smooth muscle cell recruitment, and reduced invasion of cardiac fibroblasts and endothelial cells into myocardium. In contrast to wildtype hearts that exhibited an elaborate ventricular vascular network, Tbx5<sup>epi−/−</sup> hearts displayed a marked decrease in vascular density that was associated with myocardial hypoxia as exemplified by HIF1α upregulation and increased binding of Hypoxyprobe-1. Tbx5<sup>epi−/−</sup> mice with such myocardial hypoxia exhibited reduced exercise capacity compared to wildtype mice.

Conclusion: Our findings support a conserved Tbx5 dose-dependent requirement for both proepicardial and epicardial progenitor cell development in chick and mouse coronary vascular formation.

Keywords: Tbx5, proepicardium, cell migration, epicardium, coronary vessels, cell adhesion molecule, myocardium, transcription factors
Nonstandard Abbreviations and Acronyms:

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<th>Abbreviation</th>
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<tr>
<td>αSMA</td>
<td>alpha-smooth muscle actin</td>
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<td>cTbx5</td>
<td>chick Tbx5</td>
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<td>cTbx5As–CXL</td>
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INTRODUCTION

Epicardial formation and coronary vasculogenesis are highly regulated processes that involve a series of spatiotemporally regulated molecular and cellular events.\(^1\,2\) These processes begin with formation of the proepicardial organ/proepicardium (PEO/PE) and involve epicardial-derived cell (EPDC) transition. The PEO is located at the venous pole of the heart and is part of the septum transversum (ST). This transitory structure is comprised of mesothelial cells morphologically visible in the mouse between embryonic day (E) 9 and 9.5.\(^1\) At E10.5, the epicardium is formed and fully matured by E11.5. A subset of epicardial cells undergoes epithelial-mesenchymal transition (EMT) to generate epicardial-derived cells (EPDCs) that delaminate and populate the subepicardial space and further migrate into the underlying myocardium.\(^1,3\) Here, they differentiate into the cellular elements of coronary blood vessels, i.e., endothelial cells, smooth muscle cells (SMC), and cardiac fibroblasts. Subepicardial endothelial cells contribute to both coronary arteries and veins.\(^4\) The sinus venosus and endocardium also contribute coronary endothelial cells to form the developing coronary arteries.\(^5\,6\) Subsequently, these cellular components coalesce and form the primitive vascular plexus that ultimately remolds to form the coronary blood vessels. Using various mouse models with Cre-recombinase expressed in epicardial and/or EPDCs, investigators have demonstrated multiple contributions of EPDCs to coronary smooth muscle, coronary endothelium, the interstitial fibroblast population and the atrioventricular junctional myocardium.\(^1,2,7-10\)

The epicardium plays a pivotal role in cardiac development because paracrine-mediated secretion of epicardial factors, such as Wnts, erythropoietin, fibroblast growth factors and retinoids, are important for embryonic compact myocardial growth\(^1,2\) and can be reactivated during cardiac injury and repair.\(^11\) PEO ablation/blockade that perturbs or delays epicardial formation and genetic ablation of various genes expressed in the PE or myocardium leads to abnormal coronary vasculogenesis, myocardial defects and potential embryonic lethality.\(^1,2\)

T-box transcription factor genes, including \(Tbx5\) and \(Tbx18\), are expressed in the PEO or septum transversum.\(^1,12-15\) We previously showed that manipulation of \(Tbx5\) gene dosage in the chick PEO affects proepicardial cell migration and can impair avian coronary vasculogenesis.\(^16\) However, these studies did not address the potential activity of \(Tbx5\) in the epicardium itself. The mechanisms by which \(Tbx5\) controls mammalian coronary vasculogenesis remain largely unknown, and the relevance of avian signaling to mammalian ontogeny was not tested. Holt-Oram syndrome (HOS) is a human inherited disorder caused by mutations in \(TBX5\) and manifests as congenital cardiac septation and conduction defects in the setting of limb deformities.\(^17,18\) Some Holt-Oram patients also exhibit left pericardium agenesis and anomalous coronary arteries along with ventricular septal defects (VSD)\(^19,20\) consistent with a role for \(TBX5\) in epicardial/pericardial and myocardial development. Early embryonic lethality resulting from germline inactivation of \(Tbx5\) in the mouse has precluded further investigation of \(Tbx5\)’s function in later stages of cardiac development, including epicardial formation and coronary vasculogenesis.\(^21\)

We have now developed strategies to investigate epicardial activity of \(Tbx5\) in the chick, and we have generated conditional mutant mice with \(Tbx5\) deletion from the PE and epicardial derivatives. We show that \(Tbx5\) expression in the PE/PEO and epicardium is required for normal development of PE/PEO cells as well as proper epicardial formation and maturation. \(Tbx5\) deficiency delays epicardial cell attachment to the myocardium, impairs production of EPDCs and their migration into the myocardium, and results in abnormal coronary vasculogenesis and murine ischemic cardiomyopathy. Our findings demonstrate that \(Tbx5\) is required both for proepicardial and epicardial development in avian and mammalian hearts and for establishment of the coronary vasculature.
METHODS

Tbx5lox/lox

and Wt1-Cre mice have been described. Generation of retrovirus and in ovo retroviral injection was performed as previously described with the exception of epicardial infection that was accomplished by retroviral injection into the pericardial space of the embryonic chick.

An expanded Methods section, including all experimental procedures, is available in the Online Data Supplement.

RESULTS

Tbx5 expression in chick and mouse Peo and derivatives.

Although Tbx5 myocardial and endocardial expression has been previously described during cardiogenesis, the detailed spatiotemporal patterns of chick and mouse Tbx5 expression in the PEO/PE and epicardium have not been well defined. In situ hybridization (ISH) in chick embryos revealed heterogeneous staining in the PEO at Hamburger-Hamilton (HH) stage 16 or E3 (Online Figure I A, E). By HH22 (E4), chick Tbx5 remained detectable in the PEO remnant and in migrating proepicardial cells contacting the myocardium. By HH26 (E5), Tbx5 was evident in the newly formed epicardium (Online Figure I B, F). A similar expression pattern for mouse Tbx5 (mTbx5) was observed by ISH at the corresponding developmental stages of E9.5 (Online Figure I C, G) and E10.5 in the mouse (Online Figure I D, H). Immunostaining for mTbx5 protein expression showed a spatiotemporal pattern similar to that observed with our ISH data. At E9.5, nuclear expression of mTbx5 protein was evident in the murine PE in a heterogeneous pattern (Online Figure I I, L). At E10.5, mTbx5 protein expression was evident in the nascent epicardium and pericardium (Online Figure I J). Whereas some epicardial cells were Tbx5-negative (Online Figure I M), all epicardial cells expressed Wt1 (Online Figure I N). Tbx5 expression was decreased in the epicardial cells by E11.5 (Online Figure I K, O), but persists into adulthood as previously shown. Thus, these analyses support spatial and temporal restriction of Tbx5 expression in the embryonic chick and murine PEO/PE and epicardium.

Tbx5 contributes to in vitro and in vivo proepicardial cell development.

We previously demonstrated that Tbx5 overexpression disrupts chick proepicardial cell migration in vitro. To determine the requirement for Tbx5 in epicardial formation and coronary vasculogenesis, we used retroviral-mediated transgenesis with an antisense cTbx5As-CXL construct (Online Figure II A) to knock down cTbx5 expression in cultured PEO explants in a manner similar to our previous TBX5 overexpression studies. Retroviral-mediated cTbx5 knockdown produced a 45% reduction of in vitro migration of proepicardial cells to the periphery of PEO explants compared to control CXL PEOs (Online Figure III A-C). This demonstrated that cTbx5 expression is required for in vitro chick proepicardial cell migration.

To confirm that this requirement for cTbx5 was preserved in PEO cell migration and coronary vasculogenesis in vivo, we injected either control CXL or cTbx5As-CXL retrovirus into chick PEOs in ovo at HH17-18 (E3) (Online Figure II A). By 8 hours post-injection, we observed transgene expression in the PEO as shown by X-gal staining for β-galactosidase activity for both retroviruses (Figures A, E). Twenty-four hours after PEO injection with the control CXL virus, β-galactosidase-positive cells were visible on the surface of the heart (Figure 1B). However, embryos that received PEO injections of cTbx5As-CXL to knock down Tbx5 expression exhibited only scant β-galactosidase-positive cells on the surface of
the heart (Figure 1F). By 12 days post-injection, E15 CXL-infected embryos (15/15 embryos) displayed prominent β-galactosidase-positive staining in the coronary vasculature (Figure 1C, D). Hearts from E15 embryos infected with cTbx5As-CXL did not show any evidence of β-galactosidase staining in the coronary vessels (0/13 embryos; Figure 1G). All hearts exhibited minimal levels of myocardial staining at 24 hours to 12 days post-injection due to slight retroviral leakage into the myocardium during the initial pressure injection of the retrovirus as evidenced by β-galactosidase staining of myocardial cells in E15 hearts (Figure 1C, G). This staining confirmed the infectivity of all viruses. Thus, maintenance of normal Tbx5 expression in the chick PEO is required for proper development of the epicardium and coronary vasculature.

Tbx5 contributes to chick epicardial cell fate and coronary artery development.

To determine whether Tbx5 activity during coronary vasculogenesis is required in the epicardium independent of its activity in the PEO, we used retroviral-mediated transgenesis to directly augment or knock down Tbx5 in the nascent epicardium in vivo. We microinjected either the same retroviruses as described above or control CXIZ and TBX5-CXIZ retrovirus27 (Online Figure II A) into the pericardial space (Online Figure II B) at later stages of development (E4) when the epicardium had largely already formed. This produces transgenesis of the nascent epicardium (Figure 1I-P). Because epicardium formation was not fully completed at this stage, areas of naked myocardium were exposed to viruses with this strategy, and the presence of infected myocardium in each embryo provided a positive control for viral infectivity. β-galactosidase-positive cells demonstrated infectivity of the epicardium at 8 (Figure 1I) and 24 hours (Figure 1J) post-injection for all viruses. By 11 days post-injection, β-galactosidase-positive cells were visible in the coronary blood vessels of most E15 CXL- and CXIZ-injected control embryos [21/23 CXL embryos, Figure 1K, L; 22/23 CXIZ embryos (not shown)]. Altered embryonic epicardial Tbx5 expression [knockdown with cTbx5As-CXL (1/15 embryos; Figure 1M, N) or overexpression with TBX5-CXIZ (0/18 embryos; Figure 1O, P)] inhibited incorporation of infected cells into the coronary vasculature. These hearts displayed only sparse and patchy β-galactosidase-positive cells in the myocardium and epicardium (Figure 1N, P). Thus, maintenance of normal Tbx5 dosage in the epicardium is required for its proper maturation and the subsequent incorporation of EPDCs into coronary blood vessels.

To investigate cellular mechanisms underlying impaired epicardial cell development, we examined the effect of cTbx5 augmentation on in vitro PEO cell behavior. We previously demonstrated that in vitro TBX5 overexpression in PEO explants impaired migration of PE cells out of these TBX5-CXIZ-infected PEO explants, but did not affect PEO cell proliferation.16 In addition to our previous observations, we now detect some apoptotic migratory PE cells outside of the PEO explants (Online Figure III D-F). Apoptosis was not observed in control CXIZ-infected migratory PE cells. Thus, in vitro augmentation of proepicardial cTbx5 not only impaired migration of PE cells, but also caused apoptosis in some migratory PE cells.

Epicardial inactivation of Murine Tbx5 affects formation and maturation of the epicardium and migration of EPDCs.

In the mouse, ubiquitous deletion of mTbx5 caused embryonic lethality by E10.5 that previously precluded analysis of coronary vasculogenesis.21 We circumvented this barrier by conditional tissue-specific ablation of Tbx5 in the PE and its derivatives. We crossed C57Bl/6 female Tbx5 conditional knockout mice (Tbx5\textsuperscript{flox/flox}) with C57Bl/6 male Wt1-Cre driver mice that express Cre recombinase in PE and epicardial cells.8, 22 Male progeny (Tbx5\textsuperscript{epi+/-}) exhibiting excision of one Tbx5 floxed allele and carrying the Cre transgene were crossed against female Tbx5\textsuperscript{flox/flox} to generate mice with two floxed alleles excised in PE cells, and hereafter referred to as Tbx5\textsuperscript{epi-/-}. We confirmed Cre-mediated deletion of Tbx5 and diminished Tbx5 mRNA expression in PE cells of Tbx5\textsuperscript{epi-/-} versus wildtype control mice (Online Figure IV A-E). Tbx5\textsuperscript{epi-/-} mice were born at significantly reduced Mendelian ratios compared to control mice (p=0.03; Online Figure IV F).
We analyzed Tbx5epi-/− embryos at various developmental stages to gain insight into the cause of lethality. Examination of whole mount and sectioned hearts from E9.5 wildtype and mutant mice revealed that both wildtype (Figure 2A, B) and Tbx5epi-/− (Figure 2D, E) embryos form a PE and express the PE and epicardial cell marker, Wilms tumor 1 (Wt1), in the PE at E9.5 (Figure 2C, F). While PE specification appeared normal in Tbx5epi-/− embryos, mutant PEs (Figure 2D-F) often appeared smaller relative to wildtype PEs (Figure 2A-C). Analyses of in vivo cell behavior by Ki-67 immunostaining (Online Figure V A-D) and TUNEL staining (not shown) revealed unmodified rates of proliferation (Online Figure V E), and no evidence of increased apoptosis, respectively, in Tbx5epi-/− proepicardial cells. Furthermore, our examination of PE cell polarity in these embryos did not reveal altered expression or localization of apical (PAR3, ezrin), basolateral (α4-integrin) and cell-cell junctional (ZO-1) markers between wildtype and mutant PEs at E9.5. These data indicated that Tbx5 deficiency did not alter proepicardial cell proliferation, apoptosis or polarity.

The epicardial cell layer, as indicated by Wt1-positive cells, began to form on the myocardial surface of E10.5 wildtype and Tbx5epi-/− hearts (not shown). Although a smooth-surfaced epicardium was observed in E11.5 wildtype hearts (Figure 2G, H), the epicardium in Tbx5epi-/− hearts appeared irregular and ruffled with focal regions that were unattached to the myocardium (Figures 2I, J). However, Wt1-positive epicardial cells were attached to the myocardium in both wildtype (Figure 3A) and Tbx5epi-/− (Figure 3D) hearts by E12.5. These data indicate that deletion of Tbx5 delayed adhesion of the epicardial cell layer to the myocardium and Tbx5 is important for proper structural formation of the epicardium.

Within the subepicardial space of E13.5 wildtype and mutant hearts, Wt1-positive epicardial cells began to delaminate and EPDCs began invading the compact myocardium (Figure 3B, 3E). Nonetheless, fewer Wt1-positive EPDCs invaded the compact myocardium of E13.5 mutant hearts (Figure 3E). We observed focal accumulation of Wt1-positive cells in the subepicardium of E14.5 mutant embryos (Figure 3F) compared to wildtype littermates (Figure 3C). Quantification of these cells in the compact myocardium of E14.5 mice revealed a 48.5% reduction in Wt1-positive EPDCs in Tbx5epi-/− hearts versus wildtypes (Figure 3G). Thus, Tbx5 is important for proper EPDC migration.

Migration of EPDCs into the myocardium occurs in a patterned manner that involves several mechanisms including epicardial cell proliferation, survival and EMT. Despite the absence of altered PE cell proliferation in E9.5 Tbx5epi-/− embryos, we observed a 42% reduction in Ki-67-positive proliferating epicardial cells in E12.5 Tbx5epi-/− embryos (Figure 4E, I) compared to wildtypes (Figure 4A, I). Examination of these hearts for potential EMT defects induced by proepicardial-specific deletion of Tbx5 did not reveal significant changes in expression of the EMT markers Slug, Snail and E-cadherin in E14.5 mutant versus wildtype embryos (not shown). These data demonstrate that proepicardial loss of Tbx5 produces developmental defects in the epicardium and EPDCs that may be precipitated by delayed epicardial cell adhesion to the myocardium and partially attributed to a reduction in epicardial cell proliferation.

Structural defects and impaired cardiac growth in Tbx5epi-/− mice.

We did not observe gross morphological differences in whole mount embryos or hearts from E12.5 wildtype and Tbx5epi-/− mice. Normal formation of the compact myocardium was observed in H&E stained wildtype E12.5 hearts (Figure 4B, C). However, we observed a thinned compact myocardium in E12.5 Tbx5epi-/− mice (Figure 4F, G) in comparison to wildtype littermates. A comparison of Ki-67-positive cardiomyocytes in E12.5 wildtype versus mutant mouse hearts revealed a 48% reduction in cardiomyocyte proliferation in Tbx5epi-/− hearts compared to wildtype hearts (not shown). TUNEL assays of sectioned hearts did not reveal myocardial apoptosis in the hearts of E12.5 wildtype or Tbx5epi-/− embryos. However, myocardial apoptosis was observed in E14.5 mutant hearts compared to wildtypes (not shown). In contrast to wildtype hearts (Figure 4D), muscular and membranous ventricular septal defects (VSDs) were seen in
15% of E15.5 Tbx5epi-/ embryos (Figure 4H). Collectively, these myocardial defects may result from both a decrease in myocardial cell proliferation and the onset of cardiomyocyte apoptosis secondary to impaired epicardial formation and EPDC development in Tbx5epi-/ embryos.

Epicardial inactivation of Tbx5 affects development of the EPDCs and coronary vasculogenesis.

Gross inspection of E15.5 wildtype and Tbx5epi-/ mice revealed areas of peripheral hemorrhage in mutant embryos (Online Figure VI E) that was not observed in wildtype littermates (Online Figure VI A). Overall cardiac patterning was similar in both wildtype and mutant mice, but ventricular chamber size appeared to be reduced in Tbx5epi-/ versus wildtype embryos. In contrast to E15.5 wildtype hearts (Online Figure VI B), Tbx5epi-/ hearts exhibited punctate areas of hemorrhaging (Online Figure VI F). Histology revealed abnormal vascular structures in mutant hearts. As opposed to E15.5 wildtype hearts (Online Figure VI C), Tbx5epi-/ hearts exhibited epicardial cyst-like structures lined by delaminated epicardium that were filled with erythrocytes (Online Figure VI G). PECAM-1 immunostaining revealed a subepicardial accumulation of vessels in mutant hearts (Online Figure VI H) compared to wildtypes (Online Figure VI D). Together, these data are indicative of a coronary vascular defect in Tbx5epi-/ hearts.

Given that EPDCs differentiate into smooth muscle cells (SMCs), cardiac fibroblasts and endothelial cells,1, 2, 7, 8 we determined the effects of proepicardial-specific deletion of Tbx5 on these cell populations. Histological analysis of E17.5 wildtype and Tbx5epi-/ hearts revealed nests of multiple contiguous subepicardial vessel-like structures that did not penetrate the myocardium of mutant hearts (Figure 5E) in contrast to wildtype hearts (Figure 5A). We determined that smooth muscle cell recruitment to nascent coronary vessels was affected in Tbx5epi-/ hearts via immunohistochemical analysis for smooth muscle cell-specific protein (SM22α). SMCs formed a continuous layer surrounding the coronary vessels of E17.5 wildtype hearts (Figure 5B), whereas the smooth muscle cell layer was either completely lost or discontinuous throughout the coronary vessels of Tbx5epi-/ hearts (Figure 5F). Immunostaining for periostin, a marker of the non-cardiomyocyte lineage that is enriched in cardiac fibroblasts, revealed a reduced fibroblast invasion of the cardiac interstitium in E17.5 Tbx5epi-/ hearts (Figure 5G) compared to wildtypes (Figure 5C). PECAM-1 (CD31) immunostaining revealed an elaborate ventricular capillary network in wildtype (Figure 5D) and Tbx5epi-/ hearts (Figure 5H). However, subsequent vessel quantification revealed a 45% reduction in PECAM-1-positive capillary density in Tbx5epi-/ hearts compared to wildtypes (Figure 5M). Together, these results suggest that a partial defect occurred during coronary vascular maturation in Tbx5epi-/ hearts that affected smooth muscle cell recruitment and caused reductions in formation of endothelial cells and other noncardiomyocytes that may include cardiac fibroblasts.

We sought to determine the physiologic manifestations of these vascular developmental anomalies. In fact, we observed hypoxia in Tbx5epi-/ mice. Compared to wildtype hearts (Figure 5I, J), E18.5 Tbx5epi-/ hearts displayed increased immunoreactivity for Hypoxia Inducible Factor-1α (HIF1α; Figure 5K) as well as increased binding of Hypoxpyrobe-1 (pimonidazole hydrochloride; Figure 5L), respectively. Hypoxpyrobe-1 forms protein adducts in cells with a pO2<10 mm Hg. To determine the physiological consequences of this myocardial relative hypoxic state, we evaluated the maximal exercise capacity of Tbx5epi-/ mice. 12-16 week old Tbx5epi-/ mice were subjected to graded treadmill exercise testing.33 In contrast to wildtype mice, Tbx5epi-/ mice exhibited a significantly reduced maximal exercise tolerance (Figure 5N).

Transcriptome analysis of wildtype and Tbx5epi-/ mouse hearts.

To gain insight into Tbx5-dependent molecular pathways involved in epicardial and EPDC development during coronary vasculogenesis, we characterized the transcriptome of E11.5 wildtype and Tbx5epi-/ hearts. Expression of 57 genes was dysregulated in Tbx5epi-/ hearts (p<0.05). Among these dysregulated genes, 91.2% were downregulated and 8.8% were upregulated in Tbx5epi-/ versus wildtype hearts.
hearts (Figure 6A). Gene ontology analysis of significantly downregulated transcripts in Tbx5epi-/− mouse hearts revealed dysregulation of genes involved in vitamin A biosynthesis, cell adhesion, enhancer binding, RNA polymerase II transcriptional activity and developmental growth (Figure 6B). Genes encoding these biological functions, which correlate to retinoic acid signaling, gene transcription, development and cell-cell interactions, contribute to epicardial and EPDC development.1, 2, 29, 34-36 We previously observed focal regions of irregular, ruffled epicardium that were unattached to the myocardium in E11.5 Tbx5epi-/− hearts, but became attached by E12.5. Because changes in expression of cell adhesion and extracellular matrix (ECM) proteins could contribute to such abnormal epicardial integrity, we used quantitative rt-PCR to validate mRNA expression of the Ajap1, Frem3 and Reln genes encoding these cell adhesion and ECM proteins.37-39 Expression of these genes was significantly decreased in epicardial cells and EPDCs of E11.5 mutant versus wildtype hearts (Figure 6C) and this may contribute to the delayed epicardial cell attachment to the myocardium in these hearts.

DISCUSSION

In this study, we demonstrate a conserved requirement for Tbx5 in avian and mammalian proepicardial development as well as epicardial and coronary vascular formation and maturation. We show that Tbx5 displays a dynamic expression pattern in embryonic proepicardial cell lineages and regulates development of chick and mouse epicardial and coronary vascular progenitor cells from the PEO and epicardium, respectively. Moreover, genetically engineered loss of Tbx5 in the mouse PE results in defective epicardial formation due to delayed epicardial cell adhesion to the myocardium and altered developmental gene expression. We believe this consequently leads to impaired EPDC production and invasion into the myocardium, compromised establishment of the coronary vasculature, decreased myocardial growth, myocardial hypoxia and reduced maximal exercise tolerance in Tbx55mi-/- mice. We conclude that Tbx5 is indispensable for normal epicardial formation and coronary vasculogenesis during embryogenesis and this requirement may be reflected in the pericardial agenesis and anomalous coronary arteries seen in human Holt-Oram syndrome patients with defective TBX5.19, 20

Loss of murine Tbx5 in the PE did not prevent PE cell specification and formation in Tbx5epi-/− mouse embryos as exhibited in zebrafish with mutant Tbx5.30 The genetically heterogeneous nature of the PE was recently demonstrated to have molecularly distinct expression domains7 and our expression analyses similarly suggest that Tbx5 expression is also heterogeneous throughout the developing PE and epicardium. This may explain the normal specification and development of PE cells in Tbx55mi-/- embryos. We will further explore this heterogeneity of Tbx5 expression in the PE in future studies. Furthermore, we cannot exclude that other T-box transcription factors expressed in the PE and EPDCs, such as Tbx18,15, may partially compensate for the Tbx5 deficiency. Although PE development is molecularly conserved across species,40 we speculate that minor differences in Tbx5 expression and function occur during PE development across species. This was exemplified by the lack of a functionally conserved requirement for Tbx5 between our embryonic chicks and mice in regulating proepicardial cell apoptosis. Although we observed some apoptotic migratory PE cells outside of the TBX5-CXIZ-infected chick PEO explants, we did not observe apoptotic PE cells in Tbx55mi-/- mouse embryos. We speculate that apoptosis is an epiphenomenon of altered chick Tbx5 gene dose that reflects the untimely fate of PE cells with augmented cell migratory behavior. This may partially explain the reduced incorporation of EPDCs into the coronary vessels of hearts from E15 cTbx5As-CXL or TBX5-CXIZ-injected chick embryos.

The epicardial cell layer formed in E11.5 Tbx55mi-/- mice. However, its structural integrity appeared defective as exhibited by an irregular, ruffled epicardium with focal regions that delayed attachment to the myocardium until E12.5. Transcriptome analysis of our E11.5 mouse hearts demonstrated that PE-specific deletion of Tbx5 altered several biological functions, including cell-cell interactions. Ajap1, Frem3 and
Reln, which encode cell adhesion and ECM proteins, were among the downregulated genes identified in our Tbx5epi-/- mouse hearts. Epicardial cell adhesion to the myocardium is important for EPDC production and delamination into the subepicardial space, invasion into the myocardium and coronary vasculogenesis.1, 2, 29, 34-36 The contribution of these genes to cardiovascular development is unknown, but these structural adhesion molecules are known to mediate cell-cell interactions in extracardiac tissues37-39 and, thus, we have identified novel genes involved in a Tbx5-dependent pathway of epicardial development. We believe that downregulation of these cell adhesion and ECM genes may contribute to the delayed epicardial cell attachment to the myocardium in Tbx5epi-/- hearts. Proper establishment of epicardial structure provides the foundation for subsequent EPDC development, delamination into the subepicardial space and myocardial invasion to form coronary vessels. Thus, it is likely that the defective epicardial structure of our Tbx5epi-/- embryonic hearts precipitated the decrease in EPDC production, impaired myocardial migration of EPDCs and also perturbed coronary vasculogenesis. These findings are reminiscent of the structural and functional defects observed during epicardium and coronary vascular development of PE-specific Tbx18 deficient mice which similarly exhibited an irregular epicardial surface with frequent areas distended from the myocardium as well as subepicardial nodules containing erythroblasts.15 EPDC invasion of the myocardium involves several mechanisms including epicardial cell proliferation, survival29, 30 and EMT31, 32 Although expression of EMT and apoptotic markers were unaffected by PE-specific deletion of Tbx5 in our mice, epicardial cell proliferation was significantly reduced in E12.5 Tbx55mi-/- embryos. Tbx5 has been previously shown to regulate cell proliferation during morphogenesis of tissues, including the vertebrate heart.27, 41 The diminished epicardial cell proliferation observed in our mutant mice may underlie the impaired production of EPDCs given that epicardial cell proliferation is intricately linked to EPDC invasion of the myocardium.30, 42 Thus, we believe that the impaired EPDC development in our Tbx55mi-/- embryos is a consequence of a suboptimal environment previously established by delayed epicardial cell adhesion to the myocardium and this is compounded by reduced epicardial cell proliferation. The resultant inability of EPDCs to invade the myocardium is consistent with previous reports of altered deposition of adhesion molecules during mammalian cardiogenesis that hindered EPDC migration into the ventricular myocardium.29, 34, 43, 44 Altogether, these data indicate that Tbx5 expression is critical for establishing normal epicardial structure and subsequent EPDC production.

EPDCs contribute to the formation of SMCs, endothelial cells and cardiac fibroblasts in avian and murine hearts during coronary vasculogenesis.1, 2 7, 45 In fetal Tbx5epi-/- mouse hearts, we observed impaired SMC recruitment and a reduction in endothelial cells and cardiac fibroblasts. Our data demonstrate that impaired development of these lineages subsequently leads to abnormal coronary vasculogenesis in Tbx55mi-/- hearts. Further analysis will need to be performed to determine whether Tbx5 acts directly or indirectly to influence differentiation of these lineages. However, we speculate that delayed epicardial cell attachment to the myocardium precipitated a cascade of events that lead to abnormal coronary vasculogenesis in Tbx5epi-/- hearts. Our data collectively support a model in which PE-specific Tbx5 deficiency inhibits expression of cell adhesion and ECM proteins and delays epicardial cell attachment to the myocardium to disrupt epicardium integrity. The epicardial defect is made worse by reduced epicardial cell proliferation. As a result, EPDC production, delamination into the subepicardial space and myocardial invasion are impaired. Together, these events contribute to abnormal coronary vasculogenesis in Tbx5epi-/- embryos. In addition, Tbx55mi-/- mice exhibit hypoplastic ventricular trabeculation and 15% of mice display VSDs consistent with paracrine effects of the epicardium on stimulating myocardial growth and development. To our knowledge, Tbx55mi-/- mice represent the first PE-specific deletion mouse model to exhibit cardiac septation defects. Atrial septal defects caused by apoptosis or reduced cell proliferation were previously observed upon Tbx5 deletion in the endocardium46 or posterior second heart field41, respectively. Therefore, it is plausible that EPDCs display similar non cell-autonomous growth-promoting activities on the IVS. In addition, our analysis of Wt1 expression in the developing mouse heart documented the presence of Wt1-positive EPDCs in the IVS, and reports have raised the possibility that EPDCs may differentiate into cardiomyocytes in the IVS.1, 8 Thus, septation defects observed in mice with ubiquitous Tbx5 deficiency and in HOS patients may result from reduced Tbx5 activity in the myocardium, as well as in the PE and epicardium.19, 21
Proepicardial-specific loss of Tbx5 in mice causes embryonic lethality that may result from cumulative effects on coronary vasculogenesis and epicardial cell growth and survival to, subsequently, impose myocardial growth defects that alter cardiac structure. Defective coronary vasculogenesis leads to ischemic cardiomyopathy in adult Tbx5<sup>epi-/-</sup> mice. These mice exhibit resting hypoxia and exercise-induced ischemia. It is not uncommon for human adults to present with poorly explained cardiomyopathy long after childhood repair of congenital heart malformations, and Holt-Oram patients have been reported to develop diastolic ventricular dysfunction even post repair of septation defects. Ischemia is a well-recognized cause of reduced ventricular compliance, and thus subclinical microvascular hypoplasia in HOS may contribute to chronic ischemia and the late onset cardiomyopathy that has been observed. Based upon our observations that augmentation of chick and murine Tbx5 affects proepicardial development, disrupts epicardial structural integrity, impairs EPDC development and decreases vascular investment of the myocardium, we conclude that Tbx5 is indispensable for normal epicardial and coronary vascular formation during embryogenesis. This requirement for Tbx5 in mouse and chick epicardial development may be reflected in the pericardial agenesis and anomalous coronary arteries seen in human HOS patients with defective TBX5. Future studies will determine the combined effects of epicardial and myocardial Tbx5 activity in order to dissect pathways in normal cardiogenesis and the phenotypes of HOS patients.

ACKNOWLEDGMENTS
This article is dedicated to the memory of our respected colleague and friend, Dr. Nata Diman, who passed away on March 20, 2014. We gratefully acknowledge his contribution to this study and his passion for science.

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DISCLOSURES
None.
REFERENCES


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FIGURE LEGENDS

Figure 1. Knockdown and overexpression of Tbx5 affects PEO cell development. A through P, Retroviral-mediated manipulation of Tbx5 gene dosage in ovo. β-galactosidase activity in whole mount hearts at 8 hours (A, E, I), 24 hours (B, F, J), and at E15 (C, D, G, H, K-P) following either in ovo PEO retroviral microinjection at HH16 with control CXL (A-D) and cTbx5As-CXL (E-H) retroviruses or in ovo epicardial infection at HH23 with control CXL (I-L), cTbx5As-CXL (M, N) and TBX5-CXIZ (O, P) retroviruses. Arrows indicate β-galactosidase-positive PEO cells (A, E), epicardial cells (H) and also coronary vessels with (D, L) or without (N, P) β-galactosidase activity. Scale bars=100μm (A-P).

Figure 2. Phenotypic characterization of E9.5 Tbx5epi-/- embryos. A through F, Representative images of whole mount and sectioned E9.5 embryos. Whole mount wildtype (A) and Tbx5epi-/- mutant (D) embryos. H&E stained sections of wildtype (B) and Tbx5epi-/- mutant (E) embryos. Immunofluorescent detection of Wt1 (pink) in sectioned wildtype (C) and Tbx5epi-/- (F) hearts. Nuclei counterstained with DAPI (blue). Scale bars=500μm (A, D) and 100μm (B, E). A, atrium; V, ventricle. Arrowheads (A, D) and asterisks (B, C, E, F) indicate PE outlined by dashes. G through J, H&E stained sections of E11.5 wildtype (G, H) and Tbx5epi-/- (I, J) hearts. Arrowheads indicate epicardium. Scale bar=200μm (G-J).

Figure 3. Altered migration of epicardial-derived cells (EPDCs) into the myocardium of Tbx5epi-/- hearts. A through F, Immunohistochemical detection of Wt1-positive epicardial cells (brown) in wildtype (A-C) and Tbx5epi-/- (D-F) sectioned embryonic hearts at E12.5 (A, D), E13.5 (B, F) and E14.5 (C, F). Nuclei counterstained with hematoxylin (blue). Scale bars=100μm. V, ventricle. Arrowheads denote Wt1-positive cells in the ventricle and epicardium. G, Quantification of Wt1-positive cells in the compact myocardium of E14.5 wildtype (black bar, n=4) and Tbx5epi-/- (white bar, n=4) hearts. Data represented as mean ± SEM. *p<0.05 versus wildtype.

Figure 4. Altered cell behavior, thinned myocardium and structural defects in Tbx5epi-/- hearts. A through H, Representative images of sectioned hearts. Immunofluorescent detection of Ki-67 (green) and cardiac actin (red) in E12.5 wildtype (A) and Tbx5epi-/- (E) hearts. Nuclei counterstained with DAPI. H&E stained sections in E12.5 (B, C, F, G) or E15.5 (D, H) wildtype (A-D) and Tbx5epi-/- (E-H) hearts. Myocardial wall thickness indicated by brackets. Arrowhead indicates epicardium (A, E) or VSD (H). I, Quantification of Ki-67-positive epicardial cells in wildtype (black bar, n=4) and Tbx5epi-/- (white bar, n=4) hearts. Scale bars=100μm (A, C, E, G) and 500μm (B, F, D, H). Data represented as mean ± SEM. *p<0.05 versus wildtype.

Figure 5. Defective EPDC-derived cell development and cardiac ischemia in Tbx5epi-/- mouse hearts. A through H, Representative images of H&E stained (A, E) and immunofluorescently stained sections of E17.5 wildtype (A-D) and Tbx5epi-/- (E-H) sectioned hearts. Immunofluorescent detection of the smooth muscle cell-specific protein (SM22α; B, F, green), periostin (C, G, green), cardiac actin (C, G, red) and PECAM-1/CD31 (D, H, red). Nuclei counterstained with DAPI (B, F, blue). I through L, HIF1α immunostaining (dark brown) of E18.5 wildtype (I) and Tbx5epi-/- (K) sectioned hearts. Nuclei counterstained with hematoxylin (blue). Hypoxyprobe-1 binding (green) on sectioned E18.5 wildtype (J) and Tbx5epi-/- mutant (L) hearts. Nuclei counterstained with DAPI (blue). M, Quantification of PECAM-1-positive vessels/high-powered field (HPF) in E17.5 wildtype (black bar, n=4) and Tbx5epi-/- (white bar, n=4) hearts. Data represent mean ± SEM. *p<0.05 versus wildtype. N, Absolute maximum exercise tolerance quantified in 12-16 week old adult wildtype (black bar; n=6) and Tbx5epi-/- (white bar; n=10) mice. Data represent mean ± SEM; *p<0.05 versus wildtype. Scale bars=100μm (A-L).

Figure 6. Altered cardiac gene expression with proepicardial-specific deletion of mouse Tbx5. A. Representation of the percentage of genes upregulated (red) or downregulated (blue) in hearts of E11.5
Tbx5<sup>epi-/-</sup> versus wildtype mice. B. Gene ontology analysis of significantly enriched biological processes displayed as $-\log_{10}$ (p value). C. Quantitative rt-PCR analysis of Ajap-1, Frem3 and Reln gene expression in epicardial cells and EPDCs of E11.5 wildtype (black bar, n=4) and Tbx5<sup>epi-/-</sup> hearts (white bar, n=4). Data represent mean ± SEM, *p<0.05 versus wildtype.
Novelty and Significance

What Is Known?

- The proepicardial organ (PEO)/proepicardium (PE) is a grape-like structure located caudal to the heart that contributes precursor cells during cardiovascular development to form the epicardium, pericardium, coronary vessels and cardiac fibroblasts through a series of coordinated events involving regulation of gene expression and cell behavior.

- During cardiovascular development, Tbx5 mRNA is detected in the embryonic chick PEO and TBX5 protein is expressed in the epicardium and coronary vessels of the fetal human heart.

- TBX5 overexpression in the PEO disrupts proepicardial cell migration to the coronary vasculature in the embryonic chick heart whereas loss of Tbx5 in the developing zebrafish heart leads to the absence of PE formation.

What New Information Does This Article Contribute?

- We developed strategies to investigate Tbx5 activity in formation and maturation of the epicardium and coronary vessels through retroviral-mediated transgenesis in the embryonic chick and tissue-specific deletion of Tbx5 in the developing mouse PE.

- Our findings reveal that PE-specific loss of mouse Tbx5 causes delayed epicardial attachment to the myocardium and subepicardial accumulation of EPDCs that we believe precipitates a cascade of events to impair epicardial maturation and formation of the coronary vasculature, inhibit cardiac growth and elicit myocardial hypoxia in the mammalian heart.

- Tbx5 is indispensable for normal epicardial formation and coronary vasculogenesis during embryogenesis and this may be reflected by the pericardial agenesis and anomalous coronary arteries seen in some human Holt-Oram syndrome (HOS) patients with TBX5 mutations.

Previous studies identified Tbx5 roles in myocardial, endocardial and cardiac conduction system development. However, potential Tbx5 activity in the epicardium and mammalian coronary vasculogenesis remains unidentified. We show that Tbx5 expression in the PE and epicardium is heterogeneous. Our augmentation of Tbx5 expression in embryonic chick PEO and epicardium reveals its requirement in proepicardial cell migration for formation and maturation of the epicardium and incorporation of epicardial-derived cells (EPDCs) into coronary blood vessels. Mice genetically engineered for proepicardial-specific Tbx5 loss (Tbx5epi−/−) are born at sub-Mendelian ratios. Although the PE forms properly in these mice and PE cells begin migration toward the myocardium at embryonic day (E)10.5, Tbx5 deficiency delays epicardial cell attachment to the E11.5 myocardium. Cell adhesion and extracellular matrix gene expression are reduced in E11.5 Tbx5epi−/− hearts. Although epicardial cells attach to myocardium by E12.5, Tbx5epi−/− embryos exhibit other defects including impaired EPDC production and invasion into the myocardium, compromised establishment of coronary vessels, decreased myocardial growth and myocardial hypoxia. Adult Tbx5epi−/− mice are intolerant to exercise. We conclude that Tbx5 is required in normal epicardial formation and coronary vasculogenesis. This requirement provides insight into pericardial agenesis and anomalous coronary arteries observed in HOS patients with TBX5 mutations.
Figure 3

(A) E12.5 Wildtype

(B) E13.5 Wildtype

(C) E14.5 Wildtype

(D) Tbx5 epi-/- Wildtype

(E) Tbx5 epi-/- Wildtype

(F) Tbx5 epi-/- Wildtype

(G) Wt1-positive cells (%)

- Wildtype
- Tbx5 epi-/-

* Significance level
Tbx5 is Required for Avian and Mammalian Epicardial Formation and Coronary Vasculogenesis

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Supplemental Material

Methods

Generation of Proepicardial-Specific Tbx5-Deficient Mice
Mice with proepicardial-specific inactivation of Tbx5 were generated by crossing Tbx5<sup>lox/lox</sup> mice<sup>1</sup> with Wt1-Cre driver mice<sup>2-4</sup>. The Wt1-Cre mice were obtained from Dr. John B. Burch.

Apoptosis assay
Terminal Transferase dUTP Nick End Labeling (TUNEL) assays were performed using the In Situ Cell Death Detection Kit, Fluorescein per the manufacturer’s instructions (Roche Applied Science, Indianapolis, IN).

RNA Isolation, Library Preparation and RNA-Sequencing
Total RNA was isolated from E11.5 mouse wildtype and Tbx5epi-/- hearts using TRIzol (Invitrogen, Grand Island, NY) and purified using the PureLink RNA Mini kit (Invitrogen) according to manufacturer’s instructions. RNA quantity and quality were assessed using the Agilent Technologies 2100 Bioanalyzer (Santa Clara, CA). One microgram of total RNA with a RNA Integrity Number (RIN)>8 was used for subsequent library preparation. The Genomics Resources Core Facility at Weill Cornell Medical College performed all RNA-seq analyses. Briefly, mRNA was selected from the total RNA samples with Sera-mag magnetic oligo (dT) beads using the mRNA Sample Prep Kit in accordance with the RNA sequencing protocol provided by Illumina (San Diego, CA). An RNA fragmentation kit (Ambion, Grand Island, NY) was used to fragment the mRNA, followed by first- and second-strand cDNA synthesis using random hexamer primers. Klenow and T4 DNA polymerases were used to perform an “end repair” reaction to blunt the ends of all fragments and 3’- to 5’ exonuclease was used to create a 3’ adenine overhanging tail to facilitate ligation of the amplification adapters. Ligation products were purified on a 2% Tris-Acetate-EDTA-agarose gel to remove unligated adapters and size-select templates for downstream enrichment with a gel extraction kit. The purified ligation products were PCR amplified with primers complementary to the adapter ends and the resultant cDNA was purified with the QIAquick PCR purification kit (Qiagen, Valencia, CA). cDNA concentration was assessed on the Agilent 2100 Bioanalyzer. Samples were sequenced on an Illumina HiSeq 2000 per the manufacturer’s protocol for single-end, 51bp sequencing reactions. 60-80 M reads were obtained for each experiment and were aligned to the mouse (mm9) reference genome using TopHat with default parameters. All RNA-Seq experiments had more that 80% mappable reads. After alignment, transcript levels were then estimated and normalized using CuffLinks upper-quartile together with GC content normalization. Differential expression was analyzed by applying the Limma approach<sup>5</sup> to CuffLinks-derived FPKM values. All analyses were performed using the R statistical software. The data discussed in this publication will be deposited in NCBI’s Gene Expression Omnibus and made accessible upon publication.

Isolation and Culture of Embryonic Epicardial Cells and EPDCs
Hearts from E11.5 wildtype and Tbx5epi-/- mice were dissected and ventricular chambers were placed epicardial side down on 0.1% fibronectin-coated cell culture dishes and cultured as described by van Tuyn et al.<sup>6</sup> After 24-48 hours in culture, monolayer epicardial cells and EPDCs were collected from 4 hearts for subsequent RNA isolation and analyses by quantitative real time PCR as described below.

Quantitative real time PCR
Gene expression was further validated using quantitative reverse transcriptase (rt)-PCR. Total RNA was isolated from epicardial cells and EPDCs isolated in tissue culture from E11.5 mouse hearts as described above. 1 µg of total RNA isolated from E11.5 wildtype and Tbx5epi-/- mouse epicardial cells and EPDCs were reverse transcribed into cDNA (iScript cDNA synthesis kit, BioRad, Hercules, CA) that served as a template for real-time PCR analysis of mRNA expression (LightCycler 480 SYBR Green I
Master kit, Roche). PCR reactions were performed on a Roche LightCycler under the following cycle conditions: 95°C for 5 mins; followed by 95°C for 10 secs, 55°C for 20 secs, and 72°C for 30 secs for 45 cycles. Primer sequences used to amplify mouse genes Ajap1, Frem3, Reln, GAPDH and β-actin are listed in the Table. Changes in gene expression were determined by averaging threshold values (C_T) for duplicate PCR reactions from 4 wildtype control or 4 Tbx5epi-/- mouse samples and calculated as % change compared with control samples and expressed in values normalized to GAPDH or β-actin mRNA expression as previously described. Prior to quantitative PCR analyses, primer pairs were tested by standard reverse transcriptase (rt) PCR (OneStep RT-PCR kit, Qiagen) and shown to amplify a single product by agarose gel electrophoresis under the following cycle conditions: 95°C for 15 mins; followed by 95°C for 30 secs, 55°C for 30 secs, and 72°C for 30 secs for 45 cycles.

**Biological Pathway Analysis**

Gene ontology analysis was performed using iPAGE. Gene ontology gene sets used in our analysis were curated so as to remove all electronic annotations (IEA codes). Randomizations were used to estimate the false-discovery rate (FDR<10%). After identification of significant pathways and gene sets, we further quantified the significance of the association between the data sets and the biological pathways using hypergeometric p-values. P Values were represented as −log(p value).

**Histochemistry**

Mouse embryos were isolated at various stages of development and fixed in 4% paraformaldehyde (PFA) at 4°C overnight, washed in PBS, dehydrated through a graded ethanol series before paraffin embedding. Whole embryos or hearts were sectioned at 5-7μm, and stained with hematoxylin and eosin (H&E) according to standard protocols. Chick and mouse Tbx5 in situ hybridizations (ISH) were performed as previously described. After ISH, samples were post-fixed with 4% PFA at 4°C overnight (O/N), dehydrated, paraffin-embedded and sectioned at 7μm.

**Immunohistochemistry**

For immunohistochemistry and immunofluorescence, cardiac sections were deparaffinized and rehydrated. Following antigen retrieval in 10 mM citric acid (pH 6.0), sections were blocked with either 5% donkey serum, 3% bovine serum albumin, or 5% rabbit serum in PBS for 30 minutes to 1 hour at room temperature and then incubated with various primary antibodies.

For immunohistochemical detection of α4-integrin, cleaved Caspase-3, HIF-1α and Wt1 expression, the following antibodies were diluted in a solution of 0.1% Triton X-100 with 5% rabbit serum/PBS, and used at 4°C overnight (O/N) at the dilutions shown: anti-α4-integrin (rabbit, 1:100, Abbiotec, San Diego, CA), cleaved Caspase-3 (rabbit, 1:100, Cell Signaling, Danvers, MA), anti-HIF-1α (rabbit, 1:100, Novus Biologicals, Littleton, CO), anti-Tbx5 (rabbit 1:200, Abcam, Cambridge, MA) and Wt1 (mouse, 1:100, DAKO, Carpenteria, CA). The primary antibodies were detected with biotinylated goat anti-rabbit or mouse IgG secondary antibody (1:200, Vector Laboratories, Burlingame, CA) followed by avidin-biotin binding (Vectastain ABC kit, Vector Laboratories) and application of 3,3'-diaminobenzidene (Vector Laboratories).

For immunofluorescence, the following antibodies were used at the dilutions shown: anti-α4 integrin (rabbit, 1:100, Abbiotec), anti-cardiac actin (mouse, 1: 600, Sigma, St. Louis, MO), anti-E-cadherin (rabbit, 1:100, Cell Signaling), anti-ezrin (mouse, 1:100, Molecular Probes, Grand Island, NY ) anti-Ki-67 (rat, 1:100, DAKO), anti-PAR3 (rabbit, 1: 100, Millipore), anti-periostin (rabbit, 1:100, Novus Biologicals), anti-PECAM-1 (rabbit, 1:100, Santa Cruz, Santa Cruz, CA), anti-Slug (rabbit, 1:100, Cell Signaling), anti-Snail (Rabbit, 1: 100, Novus Biologicals) anti-SM22α (goat, 1:100, Abcam) and anti-ZO-1 (rabbit, 1:200, Molecular Probes). The following fluorescently labeled secondary antibodies (Molecular Probes) were used at a 1:300 dilution to detect primary antibodies: Alexa 488- and 555-conjugated donkey anti-mouse, Alexa 488-conjugated donkey anti-rat and, Alexa 488- and 555-conjugated donkey anti-rabbit (Molecular Probes). Sections were washed in PBS, mounted in Prolong...
with DAPI (Molecular Probes) and imaged on a DM 5000B Leica fluorescent microscope for analysis. For immunofluorescent detection of Tbx5, 6µm sections were deparaffinized and rehydrated. Following antigen retrieval with Antigen Unmasking Solution (Vector Laboratories) in a pressure cooker for 30 minutes per the manufacturer’s instructions, sections were either further processed as described above for immunohistochemical detection or sections were blocked in 5% goat serum for 30 minutes and incubated with anti-rabbit polyclonal Tbx5 antibody at a 1:1000 dilution O/N at 4°C. Next, the sections were processed using a combination of the Vectastain Elite ABC (Vector Laboratories) and Tyramide Signal Amplification Biotin System (Perkin Elmer Life Science, Waltham, MA). The primary antibody was detected with fluorescently labeled streptavidin (Alexa 488-conjugated streptavidin, Invitrogen) bound to a biotinylated secondary antibody. Sections were mounted and imaged as described above.

For fetal tissue hypoxia, pregnant female mice received intraperitoneal injections of pimonidazole (200 µg/g, Hypoxyprobe-1 kit; HPI, Inc., Burlington, MA) dissolved in a 0.9 % saline solution at 17.5-18.5 days post-coitum. They were sacrificed 3 hours after the injection. Fetuses were removed and fixed in 4% PFA at 4°C O/N and processed as described above. Pimonidazole binding was detected by hybridization of sections with a mouse monoclonal antibody at a 1:50 dilution O/N. Fluorescently labeled secondary antibody (1:300, Alexa 488-conjugated donkey anti-mouse, Molecular Probes) was used to detect the primary antibody.

Proliferation and Apoptosis Assay

Proliferation assays were performed on wildtype and Tbx5epi/- cardiac tissue sections. For epicardial proliferation, the ratio of Ki-67-positive cells to total Wt1-positive epicardial progenitor cells represented the %Ki-67-positive epicardial cells. For myocardial cell proliferation, we examined tissue sections previously immunostained for both Ki-67 and cardiac actin and counterstained with DAPI as described above. The ratio of Ki-67-positive cardiomyocytes to total cardiac-actin-positive cardiomyocytes represented the %Ki-67-positive myocardial cells. Three 250µm² high powered fields (HPFs) were counted per E12.5 sectioned heart (n=3-4 per genotype).

PE Cell and Vessel Quantification

Quantification of PECAM-1-, and Wt-1-positive cells was performed. Following Wt1 immunostaining, tissue sections were counterstained with hematoxylin in order to visualize all the cells in the section. We determined the %Wt1- positive cells by counting the number of Wt1-positive cells as well as the total number of cells throughout 3 250µm² HPFs of E14.5 sectioned hearts (n=3-5 per genotype). Vessel density was calculated by quantification of PECAM-1-positive cells per 250µm² HPF in E17.5 sectioned hearts. Three representative fields were analyzed (n=3-4 per genotype).

Retroviral Tbx5 Constructs and Infection of Chick Embryos In Ovo

We PCR amplified the first 200 bp of chick Tbx5 cDNA (NCBI accession # 204173) and cloned it in the antisense direction into the CXL vector containing the lacZ reporter gene to create the antisense (As) cTbx5 retrovirus, cTbx5As-CXL. High titer retrovirus was produced as previously described. CXIZ and TBX5-CXIZ vectors and retroviruses have been previously described.

Mouse Treadmill Exercise Testing

Wildtype (n=6) and Tbx5epi/- (n=10) mice were exercised on a Columbus Instruments Exer 6/3 rodent treadmill as previously described in detail by Hernandez et al. In brief, animals were trained to run on the treadmill for 2 days prior to experimental trials twice a day for 20 minutes at a fixed speed of 16m/min for a total of 450m/day. Each training session was separated by at least 3 hours. The slope of the treadmill was kept constant at a 15° inclination. Mice began running at an initial speed of 16m/min with incremental increases in treadmill belt speed by 2m/min every 2 minutes until the mouse exhibited signs of exhaustion. The total running distance was recorded and used as an indicator of maximum exercise tolerance.
**Statistical Analysis**

All data are expressed as mean ± standard error (SEM). Statistical analysis was performed using Student’s t-test to compare data between two groups. p < 0.05 was considered statistically significant.
Supplemental References


Table. Primers used for evaluating gene expression in E11.5 wildtype and \( Tbx5^{\text{epi-}} \) mouse hearts.

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Online Figure I. **Tbx5 mRNA and protein expression in the developing heart. A through H.** In situ hybridization analysis of *Tbx5* mRNA expression (purple) in embryonic chick heart at HH16 (A, E), HH26 (B, F) and mouse heart at E9.5 (C, G) and E10.5 (D, H). *Tbx5* mRNA expression was detected in the atrial and ventricular myocardium and endocardium of developing chicks at HH16 (E) and HH26 (F) and mice at E9.5 (G) and E10.5 (H) as well as in the PEO (A, C, E, G, arrowhead), forming epicardium (F, H, arrowheads) and pericardium (H, arrow) of embryonic chick and mouse hearts, respectively. **I through O.** Immunofluorescent detection of Tbx5 protein (green), cardiac actin protein (red) and Wt1 protein (pink) in the embryonic mouse heart. Nuclei counterstained with DAPI (I-O). Tbx5 was detected in the myocardium and endocardium of the atrium, left ventricle and PE at E9.5 (I, L). Tbx5 was also detected at E10.5 in the epicardium (J, M, arrowhead) and pericardium (J, M, arrow) with heterogeneous Tbx5 expression noted in the PE (L) and epicardium (M). Tbx5 expression was diminished in the epicardium at E11.5 (K, O, arrowhead). Wt1 was uniformly expressed in the epicardium at E10.5 (N). A, atrium; AVC, atrioventricular canal; E, epicardium; En, endocardium; m, myocardium; PE, proepicardium; OFT, outflow tract; V, ventricle. Scale bars=200µm (A-D), 100µm (E, H-O), 50µm (F, G).
Online Figure II. Schematic representation of retroviral expression of wildtype and antisense-Tbx5 in the developing chick proepicardial organ or epicardium. Antisense-chick Tbx5 (cTbx5As) or wildtype human TBX5 was cloned into either the pCXL or pCXIZ replication-defective retroviral vector, respectively, that also expresses a lacZ reporter gene (A). Retroviruses were microinjected either into the proepicardial organ (B) or into the pericardial space to target nascent epicardium (C).
Online Figure III. Knockdown and overexpression of Tbx5 affects in vitro PEO cell behavior. 

A through C, PEO cell migration and quantification of migrating β-galactosidase-positive cells (blue) in E3 chick PEO explants infected with either control CXL (A, C, black bar, n=12) or cTbx5As-CXL (B, C, white bar, n=22) retrovirus. Data shown are mean ± SEM. *p< 0.001 versus CXL.  

D through F, E3 chick PEO explants infected with either CXIZ (D) or CXIZ-TBX5 (E) retrovirus and immunostained for an apoptotic marker, cleaved caspase-3 (brown, arrowheads). Quantification of caspase-3-positive cells in E3 chick PEO explants infected with either control CXIZ (F, black bar, n=4) or CXIZ-TBX5 (F, white bar, n=4) retrovirus. Data shown are mean ± SEM. *p<0.05 versus CXIZ.
Online Figure IV. Wt1-Cre mediated deletion of *Tbx5* diminishes mouse PE *Tbx5* mRNA expression. A. Genotypes of mice that are wildtype (lane 1) or homozygous null (lane 2) for *Tbx5*. PCR amplification of the *Tbx5* lox and Cre-mediated deletion alleles result in 194bp (lox) and 480bp (del) products, respectively. B through E. In situ hybridization analysis of *Tbx5* mRNA expression (purple) in hearts of whole mount and sectioned wildtype (B, D) and *Tbx5*epi-/- sectioned (C, E) PEs (arrowhead) demonstrating a marked diminution of *Tbx5* mRNA expression in PE cells of sectioned *Tbx5*epi-/- (E, arrowhead) versus wildtype hearts (D, arrowhead). F. Chart representation of Mendelian ratios of mice obtained from matings that are wildtype (*Tbx5*lox/+, *Tbx5*lox/lox), heterozygous (*Tbx5*epi+/+) or homozygous (*Tbx5*epi/-) for the Wt1-Cre-mediated deleted *Tbx5* allele. A, atrium; V, ventricle. Scale bars=200µm. (B, C) and 50µm (D, E).
Online Figure V. PE cells in E9.5 Tbx5epi/- mice exhibit normal cell proliferation. A through D, Immunofluorescent detection of Wt1 (red) and Ki-67 (green) in PE cells (arrowheads) of sectioned E9.5 wildtype (A, B) and Tbx5epi/- (C, D) embryos. E, Quantification of Ki-67-positive PE cells in wildtype (black bar, n=5) and Tbx5epi/- embryos (white bar, n=5). NS=p value not significant. V, Ventricle. Scale bar=100µm (A-D).
Online Figure VI. Tbx5 mutant mice exhibit defects in coronary vasculogenesis. A through H, Representative images of embryos (A, E), whole mount hearts (B, F) and sectioned hearts from E15.5 wildtype (A-D) and Tbx5^{epi-/-} (E-H) mice. H&E staining (C, G) and immunofluorescent detection of PECAM-1(red, D, H) sections of wildtype (C, D) and Tbx5^{epi-/-} (G, H) hearts revealed subepicardial accumulation of vessels in mutant hearts compared to wildtypes. Arrow (E) indicates peripheral hemorrhaging. Arrowhead (F) indicates peripheral hemorrhaging as well as myocardial (C, D) and subepicardial vessels (G, H). Scale bars=500µm (A, B, E, F) and 50µm (C, D, G, H).