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Thymosin Beta 4 is Dispensable for Murine Cardiac Development and Function

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Abstract

Rational—Thymosin beta 4 (Tβ4) is a 43 amino acid factor encoded by an X-linked gene. Recent studies have suggested that Tβ4 is a key factor in cardiac development, growth, disease, epicardial integrity and blood vessel formation. Cardiac specific shRNA knockdown of *tβ4* has been reported to result in embryonic lethality at E14.5-16.5, with severe cardiac and angiogenic defects. However, this shRNA *tβ4*-knockdown model did not completely abrogate Tβ4 expression. To completely ablate Tβ4 and to rule out the possibility of off-target effects associated with shRNA gene silencing, further studies of global or cardiac specific knockouts are critical.

Objective—Here, we examined the role of Tβ4 in developing and adult heart via global and cardiac specific *tβ4*-knockout mouse models.

Methods and Results—Global *tβ4*-knockout mice were born at Mendelian ratios and exhibited normal heart and blood vessel formation. Furthermore, in adult global *tβ4*-knockout mice, cardiac function, capillary density, expression of key cardiac fetal and angiogenic genes, epicardial marker expression, and extracellular matrix deposition were indistinguishable from that of controls. Tissue specific *tβ4*-deficient mice, generated by crossing *tβ4*-floxed mice to *Nkx2.5-Cre* and *αMHC*-Cre, were also found to have no phenotype.

Conclusion—Therefore, we conclude that Tβ4 is dispensable for embryonic viability, heart development, coronary vessel development and adult myocardial function.

Keywords

Cardiac Development; Cardiac Function; Epicardium; Thymosin Beta 4

Introduction

Insights into functions of molecular factors involved in cardiovascular development and healing can improve our understanding of cardiac homeostasis and disease.[1] Use of these

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Disclosures None

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factors can provide novel therapeutic approaches to treat myocardial dysfunction. Thymosin beta 4 (Tβ4) is a 43 amino acid long peptide that was originally shown to bind G-actin and play a role in F-actin formation.[2-6] Recent studies have suggested that Tβ4 is a key factor in cardiac development, growth, disease, epicardial integrity and all three phases of blood vessel formation (angiogenesis, vasculogenesis and arteriogenesis).[7-15] Indeed, murine and chick studies noted temporal and spatial regulation of Tβ4 expression during embryonic development, neonatal development and biomechanically induced pressure overload.[8-12] Moreover, murine and porcine myocardial infarction and ischemia/reperfusion studies have demonstrated that postoperative exogenous administration of Tβ4 improved cardiovascular function, abrogated scar formation, and improved myocyte survival.[8-10, 13] It has been reported that treatment with Tβ4 prior to myocardial infarction can promote *de novo* cardiomyocyte formation post-infarction; however, addition of Tβ4 post-infarct does not promote *de novo* cardiomyocyte formation.[14, 15] It has also been reported that administration of Tβ4 reactivates a proangiogenic cascade and improves cardiac capillary bed formation.[13] Together, these data indicate that stimulation with Tβ4 may have positive effects on vessel formation and may inhibit disease progression post cardiac injury.

Recently, it has been reported that shRNA knockdown of Tβ4 specifically in an *Nkx2.5-Cre* expression domain results in significant cardiac developmental defects.[9] These data also suggested that Tβ4 is pivotal in epicardial-derived progenitor cell mobilization and differentiation into endothelial and vascular smooth muscle cells. However, this shRNA *tβ4*-knockdown model did not completely abrogate Tβ4 expression. To completely ablate Tβ4 and rule out the possibility of off-target effects associated with shRNA gene silencing, further studies of global or cardiac specific knockouts of Tβ4 are critical.

To further investigate Tβ4-loss of function, we generated global and cardiac specific *tβ4*-knockout mice to examine cardiac function. Using these mice we examined myocardium and the coronary vasculature during embryonic development and in adult mouse. Results of our studies demonstrate that absence of *tβ4* does not perturb overall embryonic development, heart development, adult heart function, and does not alter capillary formation or density in murine heart. Thus, we conclude that Tβ4 is dispensable for normal cardiac development and function, including coronary vessel formation.

Methods

An expanded Methods section is available in the online-only Data Supplement

Animal care

All animal procedures were performed and approved by the UCSD Institutional Animal Care and Use Committee.

Generation of tβ4 floxed and knockout mice

tβ4 targeted mice were generated as previously described.[16]

Real-time PCR, Real Time PCR analyses

Transcript expression levels were determined using exon spanning primers (Supplementary Table I) and standard sybr green protocols.[17]

Histology and Immunofluorescence

Histology and Immunofluorescence was performed on embryonic and adult hearts and sections as previously described.[18]

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Cardiac perfusion of microspheres

Perfusion of hearts was performed as previously described.[19] Images were taken via confocal microscopy and processed using Velocity 3d software.

Transthoracic echocardiography

Analyses were performed using the VEVO 2100 Ultrasound (Visual Sonics) and a 45-Mhz transducer.

Western Blot Analysis

Western analysis was performed on adult heart, lung, spleen, kidney and brain from knockout and adult tissues as previously described.[20]

Statistics

The data obtained from all analyses were measured for significance using either a Student's t-test, or Chi-squared analyses with $P<0.05$ regarded as significant. All data is ± SEM.

Results

Generation of tβ4-global knockout mice

To explore the role of *tβ4* in mouse cardiovascular development and function, we generated conditional alleles for *tβ4* gene by homologous recombination in ES cells as described previously.[16] Briefly, we flanked the second exon for mouse *tβ4*, encompassing 75% of the coding sequence (Figure 1A). Targeted ES cells were confirmed using Southern blot analyses (Figure 1B). Chimeric males were then bred with Black Swiss females to establish $t\beta4^{f/+;neo+}$ mice. These mice were fertile, viable and phenotypically normal (data not shown). Global deletion of *tβ4* was achieved by crossing $t\beta4^{f/+;neo+}$ mice with *Mox2-Cre* deleter mice ($t\beta4^{f/y;cre+}$).[21] These mice were then crossed to Black Swiss mice to establish $t\beta4^{+/-}$ and $t\beta4^{-/y}$ mice. $t\beta4^{-/-}$ mice were established by crossing $t\beta4^{+/-}$ and $t\beta4^{-/y}$. Deletion of *tβ4* was confirmed using PCR from tails and Real-Time PCR from hearts (Figure 1C and 1D). Deletion was further confirmed by Western blot analyses of adult heart, thymus, lung, spleen and brain as well as immunostaining in E11.5 embryos (Figure 1E and 1F). Both $t\beta4^{-/-}$ and $t\beta4^{-/y}$ were born at predicted Mendelian ratios (Table 1). *tβ4*-deficient mice were then backcrossed to C57/B6 mice for eight generations. Again, we observed that both $t\beta4^{-/-}$ and $t\beta4^{-/y}$ mice were born at predicted Mendelian ratios (Table 1).

tβ4-knockout embryos are viable and have normal blood vessel development

Early murine studies detected Tβ4 in developing myocardium as early as E.8.0.[22] Later studies furthered these observations by characterizing temporal and spatial expression patterns of Tβ4 in developing heart from E9.5 to 12.5.[8] These data suggested that Tβ4 may be required for embryonic heart development. In keeping with this idea, embryonic lethality and significant cardiac defects were observed in mice with shRNA *tβ4*-knockdown in *Nkx2.5-Cre* lineages.[9] In contrast, our *tβ4*-knockout mice did not present with embryonic lethality, even in different genetic backgrounds. The possibility remained that global *tβ4*-knockout mice would display some cardiac abnormalities during embryonic development. To assess whether *tβ4*-knockout mice displayed any abnormalities during heart development, we examined global *tβ4*-knockout embryos at E14.5. No cardiac or other gross anatomical abnormalities were observed in global knockout embryos when compared to wild-type controls (Figure 2A). Histological examination of *tβ4*-knockout hearts did not exhibit abnormalities in development of compact myocardium, detachment of the epicardium, changes in the epicardial marker Wt1, or presence of nodules on the epicardial surface, (Figure 2B and 3A).

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Stimulation with Tβ4 recombinant protein activated VEGF and vessel formation in a chick chorioallantoic membrane angiogenesis model.[23] Previous studies suggested that Tβ4 activates epicardium-derived cells (EPDC)'s to migrate in and differentiate into endothelial (EC) or smooth muscle cells (SMC).[9] Therefore, we further characterized the embryonic heart by immunohistological examination of the microvasculature. Our analyses revealed that formation and distribution of CD31 positive cells throughout the myocardium was not changed in *tβ4*-knockout hearts relative to control littermate hearts (Figure 2C and 2D). These data suggested that *tβ4*-loss had no deleterious effects on coronary angiogenesis. Moreover, quantitative assessment demonstrated no difference in the number of CD31 positive EC's per field in *tβ4*-knockout E14.5 hearts relative to hearts from littermate controls (Figure 2E).

Given that EPDCs also contribute to SMCs, and that *tβ4*-loss may alter invasion of these cells into myocardium, we also examined the pattern and distribution of alpha-smooth muscle actin (αSMA) positive cells. These immunostaining data revealed that both control and knockout fetal hearts displayed normal distributions of αSMA+ cells (Figure 2C). Finally, immunostaining of large vessels at E14.5 revealed normal SMA+ SMC and CD31+ endothelial cell layers. Again, defects were not evident either at the histological or cellular levels (Figure 2B and 2D). Together, these data indicate that global loss of Tβ4 does not alter the development of coronary vessels.

tβ4-global knockout mice do not have altered adult cardiac function or pathology

Given that *tβ4*-knockout animals were viable, without cardiac defects in mid-gestation embryos, and born at predicted Mendelian ratios, (Table 1) we examined adult *tβ4*-global knockout mice for the presence of cardiac abnormalities. *tβ4*-global knockout mice had normal morphology, and HW/BW, HW/TL ratios when compared to control mice (Figure 4A, 4B, 4F and 4G,). No changes were observed in expression of epicardial markers *wt1*, *tbx18* or the closely related Thymosin factor, *tβ10* via real-time PCR analyses (Figures 3B, 3C, and Supplementary Figure IA). Moreover, no significant changes were observed in cardiac fetal gene markers at 12 weeks of age (Supplemental Figure II A-D). Furthermore, no change in myocyte size was observed in global *tβ4*-knockout mice when compared to control mice (Figure 4E and 4H). Previous studies of Tβ4 stimulation during myocardial infarction and transverse aortic constriction in mice reported decreased fibrosis and *procollagen-1α1* and *−3α1* transcript activation.[8, 24] Histological and molecular examination of *tβ4*-global knockout hearts revealed no fibrosis or fibrotic gene activation (Figure 4C, 4D, Supplementary Figure IIE, and IIF). These data demonstrated that *tβ4*-loss does not result in morphological abnormalities in adult heart.

We also examined cardiac function via echocardiographic analyses of *tβ4*-global animals up to 12 months of age and did not observe any significant difference between knockout and wild-type control littermates (Supplementary Table II). In addition, no changes in survival were observed over the 12-month time course (data not shown).

tβ4-global knockout mice do not have altered coronary capillary bed density or angiogenic gene activation

Previous observations demonstrated that *tβ4* is significantly unregulated during neonatal development.[11] This expression correlates with the increase in coronary capillary density observed in the first three weeks of the murine neonatal period.[25] Given that Tβ4 simulation can activate proangiogenic markers in a murine myocardial adult infarct model, we postulated that *tβ4*-loss could cause a subtle decrease in the capillary bed volume in the adult mouse heart. To examine vascular density we employed coronary perfusion with FluroSpheres red (580/605 nm) and CD31 vessel staining as previously described.[19] To

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calculate volume, 30μm thick sections were imaged and three-dimensional reconstructions were examined. Representative reconstructions revealed no major changes in vessel arrangement. Moreover, quantitative analyses indicated that capillary volume or CD31 positive vessels were not reduced in the *tβ4*-deficient animals when compared to wild-type controls (Figure 5A and 5C). To further support these results, we examined key pro-angiogenic molecular markers found to either be upregulated by Tβ4 simulation or altered in vascular deficiency.[13] Real-time PCR analyses found no significant changes in factors previously found to be activated by Tβ4, including VEGF-A,-B or Flk-1. Furthermore, levels of CD31, Tie2 and Flt-1 were not altered in our *tβ4*-deficient mouse model (Figure 5B).

Cardiac specific deletion of tβ4 mice does not result in embryonic lethality or cause adult abnormal cardiac function

We generated cardiac specific *tβ4*-deficent mice using both the *Nkx2.5-Cre* and α*MHC-Cre* drivers. Initially, we crossed $t\beta4^{f/+;neo+}$ mice to FLPe recombinase mice[26] to delete the neo cassette, to avoid potential effects of the neo cassette on normal tβ4 expression. $t\beta4^{f/+}$ mice were then crossed to homozygosity and subsequently crossed to either *Nkx2.5-Cre*[27] or α*MHC-Cre*[28, 29] mice to generate cardiac specific *tβ4*-knockouts ($t\beta4^{f/y;nkx-cre+}$, $t\beta4^{f/y;amhc-cre+}$). Neither line displayed embryonic lethality and both were viable to adulthood. Given these observations, we examined $t\beta4^{f/y;nkx-cre+}$ mice in greater detail. $t\beta4^{f/y;nkx-cre+}$ knockout efficiency was confirmed in isolated adult cardiomyocytes and found to be ~80% (Supplemental Figure III). Adult $t\beta4^{f/y;nkx-cre+}$ mice did not have alterations in HW/BW or HW/TL compared to control mice ($t\beta4^{f/y)}$ (Figure 6A and 6B). Detailed histological observations as well as expression of fetal gene and fibrotic markers were also similar to control mice (Figure 6C-J). Echocardiographic analysis at 12 weeks of age demonstrated that $t\beta4^{f/y;nkx-cre+}$ mice have normal cardiac function, chamber size and wall thickness compared to their littermate controls (Supplementary Table III). Coronary perfusion with FluroSpheres red (580/605 nm), also demonstrated no changes in vessel volume in the $t\beta4^{f/y;nkx-cre+}$ mouse (Supplementary Figure IVA and IVB).

Discussion

Characterization of factors that drive heart formation, coronary angiogenesis and vasculogenesis is critical for understanding both development and disease progression. Indeed, pro-angiogenic factor expression can drive a physiological response to disease stimuli (hypertension/myocardial infarct).[19, 25, 30, 31] Recent studies have suggested that Tβ4 is essential for cardiac development and angiogenesis.[9] To understand the role of Tβ4 we generated global and cardiac specific knockout mice. We observed that global or cardiac specific loss of *tβ4* did not cause either a significant embryonic or adult cardiovascular phenotype, with no embryonic lethality, and no age related decline in cardiovascular function. Our data demonstrated that *tβ4* global knockout mice, regardless of genetic background (mixed 129svj and Black Swiss or pure C57/B6), were not embryonic lethal. Indeed, no cardiac or gross abnormalities were observed in global knockout embryos or hearts. Moreover *tβ4*-knockout hearts did not reveal any defects in compact myocardium, epicardial detachment or presence of nodules on the epicardial surface. We also performed quantitative assessment of CD31 positive endothelial cells in E14.5 hearts and found no significant differences between *tβ4*-knockout and control hearts. These data suggested that *tβ4*-loss had no deleterious effects on angiogenesis. These data are in contrast with data obtained with *Nkx2.5-Cre* specific shRNA *tβ4*-knockdown mice, which display significant lethality at E14.5-16.5, with severe cardiac developmental and angiogenesis defects.[9]

Given that *tβ4*-knockout animals were viable, we examined adult *tβ4*-global and cardiac specific knockout mice for the presence of cardiac abnormalities. Our *tβ4*-knockout mice

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did not have any observable histological defects, and did not exhibit any abnormalities in fetal gene markers or cardiac function. Taken together these data demonstrated that global or cardiac specific deletion of *tβ4* did not result in a pathological phenotype, further illustrating that Tβ4 is dispensable for normal cardiac development and function.

Tβ4 has a suggested role in coronary angiogenesis.[9] We did not observe any defects in coronary vessel formation during embryonic development in our mutant mice. Given these observations, we studied adult global *tβ4*-knockout mice for the possibility of angiogenic defects. Upon examination of adult capillary bed density and volume we observed that *tβ4*-loss did not alter coronary vasculature density or volume. These data demonstrated that *tβ4*-loss does not affect blood vessel density in the adult animal

Published data shows that cardiac specific shRNA *tβ4*-knockdown mice utilizing an *Nkx2.5-Cre* driver display significant lethality at E14.5-15.5 with abnormal cardiac development.[9] However, our global *tβ4*-knockout mice have normal cardiac development and function without any embryonic lethality. To exclude the possibility that global deletion of *tβ4* results in a compensatory mechanism to replace *tβ4*-loss, thus accounting for the observed discrepancies between the two mouse models, we generated cardiac specific *tβ4*-deficent mice using either *Nkx2.5-Cre* or α*MHC-Cre* drivers. Our data demonstrated that cardiac specific deletion of *tβ4* did not result in embryonic lethality, defects in cardiac development and function, or coronary vasculature abnormalities; further illustrating that Tβ4 is dispensable for normal cardiac development and function.

Differences between results of our study and that of shRNA knockdown of Tβ4 likely result from the distinct experimental approaches used for inhibiting/blocking Tβ4. A strong possibility is that the shRNA approach had off-target effects. Our model is a total ablation of Tβ4 as demonstrated by Western blot and immunostaining analyses. Thus, we believe, given the model system and approach used in our study, that we have demonstrated that Tβ4 is dispensable for cardiac development, coronary vessel formation and cardiac function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations

ANP	Atrial Natriuretic Peptide
βMHC	beta myosin heavy chain
αMHC	alpha myosin heavy chain
Acta1	alpha skeletal actin 1
PECAM/CD31	Platelet endothelial cell adhesion molecule
VEGF	Vascular endothelial growth factor

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Flt-1	Vascular endothelial growth factor receptor 1
Flk-1	Vascular endothelial growth factor receptor 2
Tβ4	Thymosin Beta 4
Tβ10	Thymosin Beta 10
shRNA	Short Hairpin RNA
WGA	Wheat Germ Agglutinin
Wt1	Wilms tumor protein
Tbx18	T-box 18

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Novelty and Significance

What is known?

- Thymosin beta 4 (Tβ4) is an X-linked, small (44aa) ubiquitously expressed protein.
- Tβ4 has been shown to bind in a 1:1 ratio with G-actin.
- Cardiac specific shRNA knockdown of *tβ4* utilizing an Nkx2.5-cre driver in mice resulted in abnormal development of compact myocardium and coronary vasculature, and significant embryonic lethality between E14.5-E16.5

What New Information Does This Article Contribute

- Global and cardiac specific deletion of Tβ4 does not result in lethality.
- Global and cardiac specific *tβ4*-knockout mice do not display any cardiac pathology, coronary vasculature defects, or functional defects
- Tβ4 is dispensable for viability, heart development, coronary vessel development and adult myocardial function.

It has been previously shown that shRNA cardiac-specific knockdown of Tβ4 using the Nkx2.5-cre driver resulted in significant cardiac and coronary vessel defects. That study concluded that Tβ4 was critical for the formation of the coronary vasculature and the embryonic myocardium. Those data also suggested that Tβ4 is pivotal in epicardialderived progenitor cell mobilization and differentiation into endothelial and vascular smooth muscle cells. However, Tβ4 expression was not completely ablated and the shRNA might have had off-target effects. Thus to understand the role of Tβ4, we generated floxed alleles for *tβ4* and established global and cardiac specific deletion models of *tβ4*. Here we observed that both global and cardiac specific *tβ4*-knockout mice were viable. Detailed analyses of global *tβ4*-knockout embryos found no defects in coronary vasculature formation, cardiac development or epicardial development. Moreover, global *tβ4*-knockout mice did not exhibit adult heart pathology, cardiac functional defects, or alterations in the coronary vasculature. Cardiac specific *tβ4*-knockout mice had no overtly detectable pathologic phenotype, exhibiting normal heart and coronary vessel development. Thus we conclude that Tβ4 is dispensable for viability, heart development, coronary vessel development and adult myocardial function.

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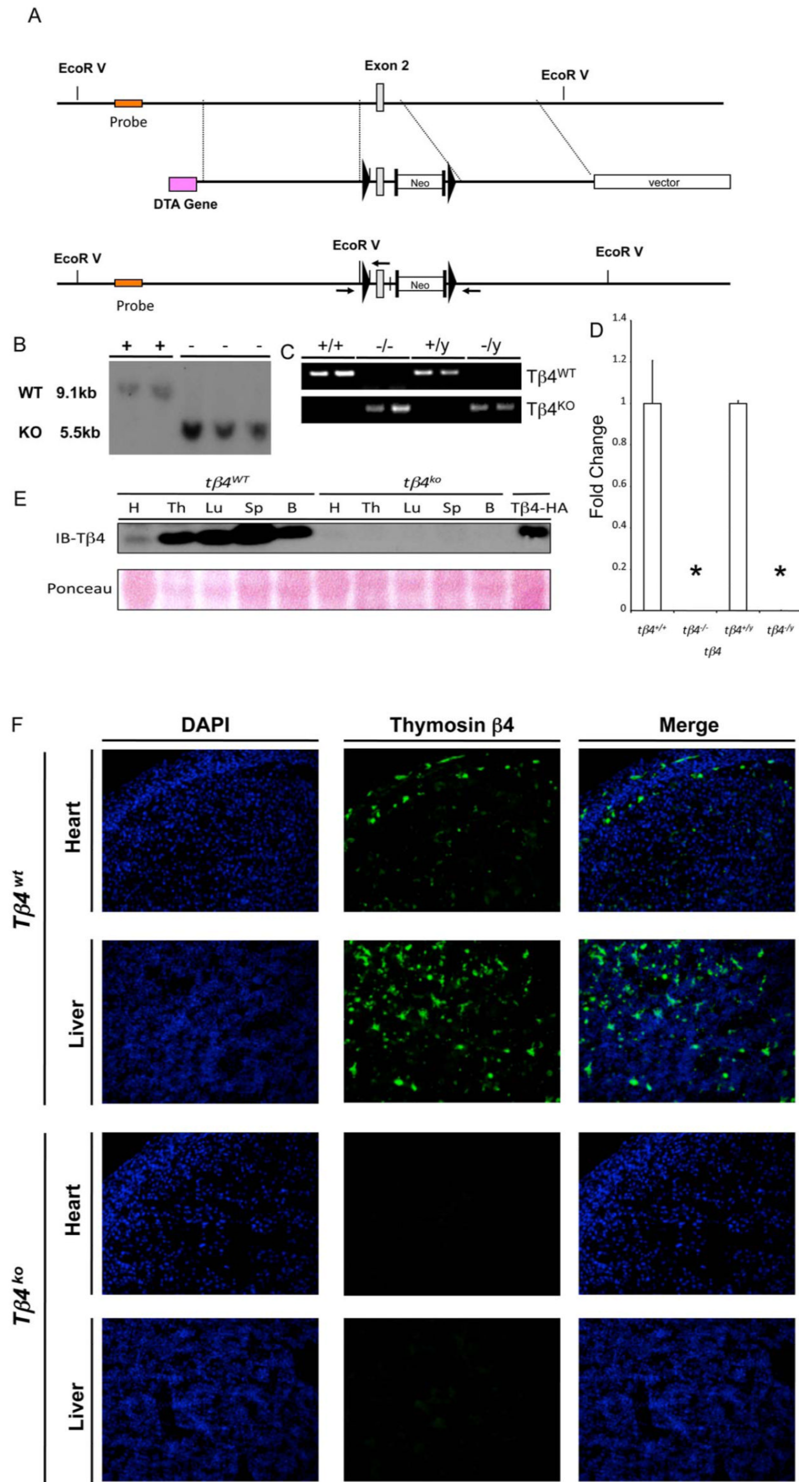

Figure 1. Generation of *tβ4*-knockout mice

(A) Targeting strategy, restriction map of genomic region of *tβ4*, in the middle is the targeted construct and at the bottom is the mutated loci after recombination. P1, P2 and P4 represent primer sites for genotyping. The grey rectangle denotes the targeted exon which is the 2nd exon encoding the majority of the coding region. Black triangles indicate LoxP sites,

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and black boxes indicate frt-NEO-frt cassettes. DTA, Diphtheria Toxic A chain gene; Neo, Neomycin resistance gene (B) Detection of wild-type and mutated alleles by Southern blot analysis. DNA from electroporated ES cells was digested with EcoR V and analyzed by Southern blot analysis with the probe diagrammatically represented in A. The 9.1 kb and 5.5 kb bands represent wild-type and mutated alleles, respectively. (C) PCR analysis of DNA isolated from Female ($t\beta4^{+/+}$, $t\beta4^{-/-}$) and Male ($t\beta4^{+/y}$ and $t\beta4^{-/y}$) mouse tails. (D) Real-time PCR confirmation of global gene deletion from Female ($t\beta4^{+/+}$, $t\beta4^{-/-}$) and Male ($t\beta4^{+/y}$ and $t\beta4^{-/y}$) mouse hearts. (E) Western analyses of adult Male ($t\beta4^{+/y}$ and $t\beta4^{-/y}$) mouse hearts (H), Thymus (Th), Lung (Lu), Spleen (Sp), Brain (B). HA tagged Tβ4 isolated from COS cells used as control. Observed at approximately 4kDa. (D) Immunostaining from Heart and liver from E11.5 $t\beta4^{Wt}$ and $t\beta4^{ko}$ mouse hearts and liver. Tβ4 (Green), DAPI (Blue).

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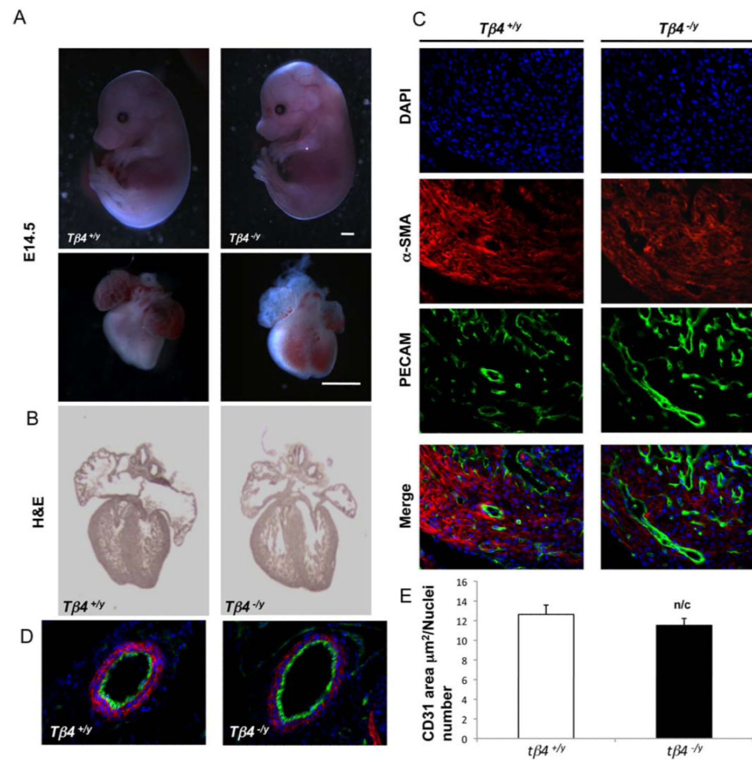

Figure 2. Global *tβ4*-loss does not cause embryonic lethality

(A) E14.5 Gross images of embryos and hearts, top and bottom respectively. No pathology was observed between conditions. Scale bar 1mm (B) Frozen H&E sections from $t\beta4^{+/y}$ and $t\beta4^{-/y}$ E14.5 hearts, images at 1x. (C&D) Representative immunofluorescence staining of (C) Left ventricular free wall and (D) Aorta of $t\beta4^{+/y}$ and $t\beta4^{-/y}$ mice E14.5. 40x CD31 (Green) α–SMA (Red) and DAPI (blue). (E) Quantification of CD31 positive area normalized to nuclei numbers. n=3-5. N/C= No significant change.

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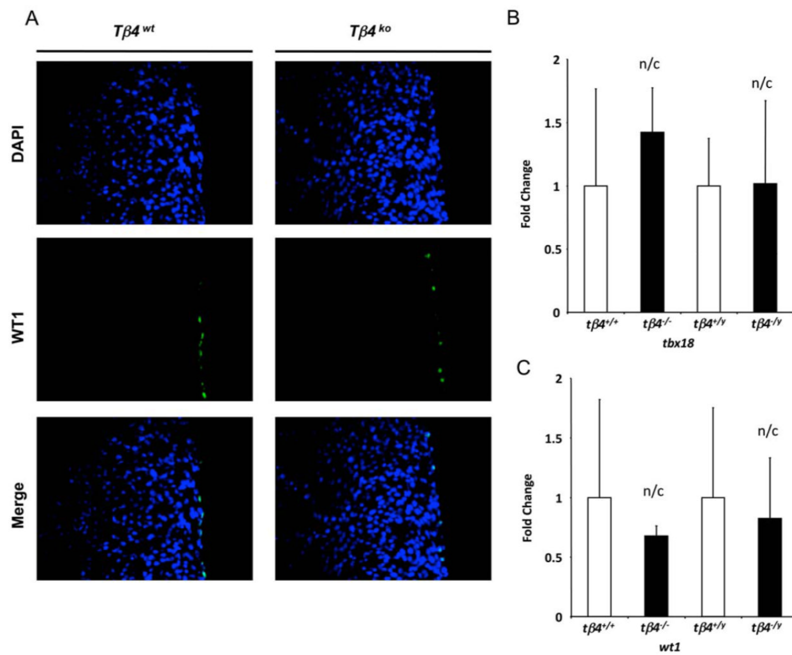

Figure 3. Analyses of Epicardial markers in the adult and embryonic heart

(A) Immunostaining of E15.5 hearts from both Male ($t\beta 4^{Wt}$ and $t\beta 4^{ko}$) mouse hearts. DAPI (Blue), Wt1 (Green). (B&C) Real-time PCR analyses of tbx18 and wt1 in the adult Female ($t\beta 4^{+/+}$, $t\beta 4^{-/-}$) ,and Male ($t\beta 4^{+/y}$ and $t\beta 4^{-/y}$) mouse hearts. N/C= No significant change.

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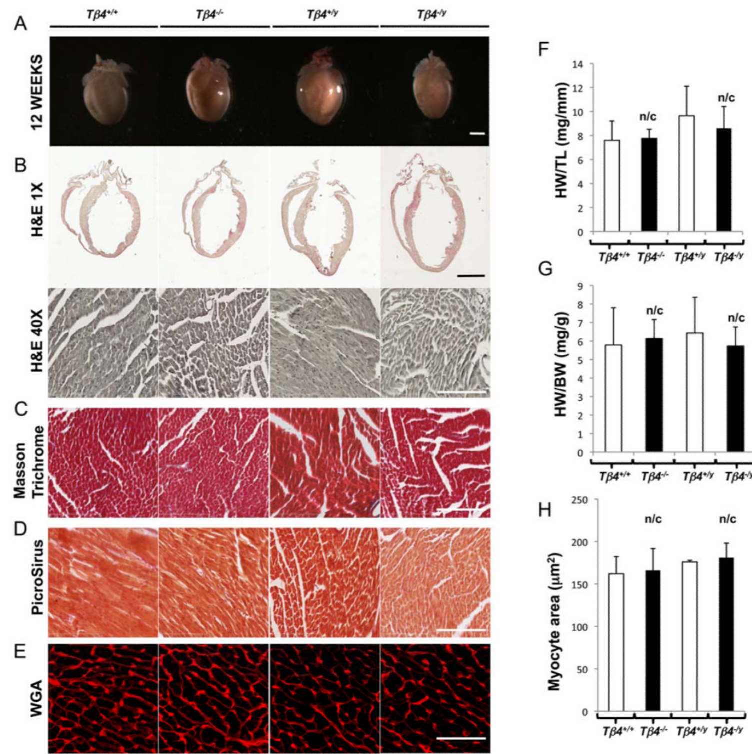

Figure 4. Global *tβ4*-loss does not cause adult pathology, fibrosis, alter myocyte size or morphology

(A) 12 week old hearts from *tβ4*$^{+/+}$, *tβ4*$^{-/-}$, *tβ4*$^{+/y}$ and *tβ4*$^{-/y}$ hearts. (B) 1 × and 40× Parrafin H&E sections from *tβ4*$^{+/+}$, *tβ4*$^{-/-}$, *tβ4*$^{+/y}$ and *tβ4*$^{-/y}$ hearts Scale bars 2mm and 200μm. (C&D) Masson's trichrome (C), and Picrosirius red stained 40X Images. Scale bars 200μm (E) Wheat Germ Agglutinin staining from left ventricular frozen sections, original magnification 40X. Scale bar 50μm. (F&G) Heart Weight (mg)/Tibia length (mm) (F) and Heart Weight (mg)/Body Weight (g) (G) from 12 week old mice. (H) Myocyte size from WGA stained sections, 100 random cells per field, quantification from 10 random fields per heart, n=3-10 hearts per experiment. N/C= No significant change.

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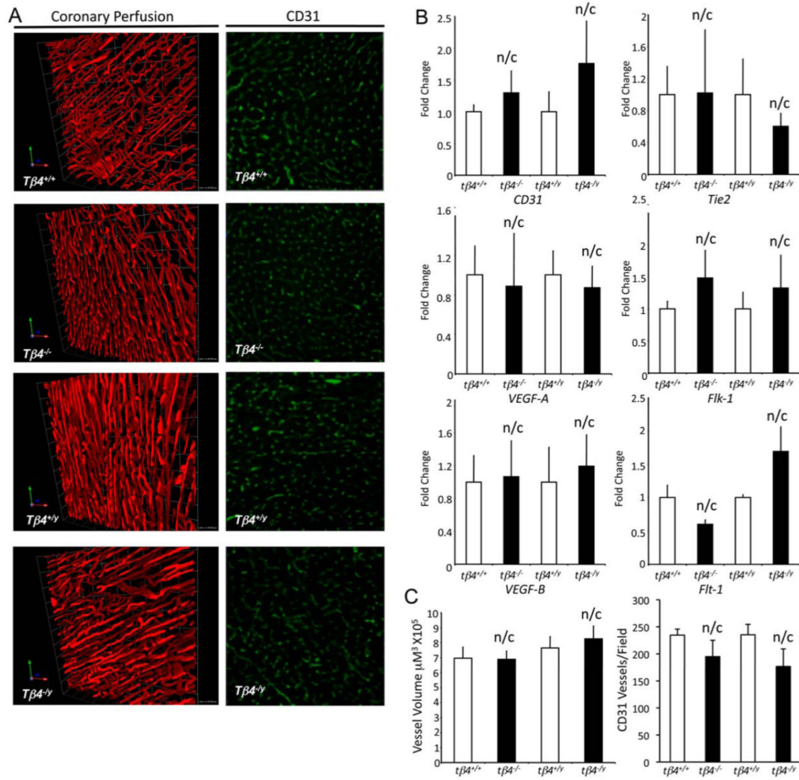

Figure 5. Global $t\beta4$-loss does not result in loss of capillary bed density

(A) Representative 3D reconstruction of the capillary bed and CD31 staining of the murine left ventricular free wall at 12 weeks. (B) Real-time PCR analyses of angiogenic cascade from 12 week–old. (C) Quantification of capillary bed analyses/vessel volume (μm^3) and CD31 positive areas per field. n-3-4 hearts per experiment. N/C= No significant change.

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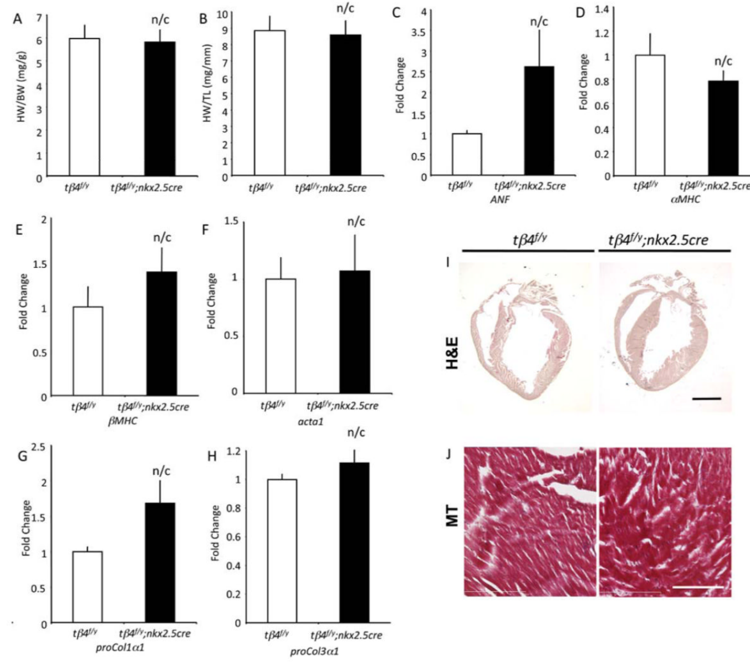

Figure 6. Cardiac-specific *tβ4*-loss does not cause adult pathology, fibrosis, alter myocyte size or morphology

(A&B) 12 week old hearts from Male (*tβ4*$^{f/y}$ and *tβ4*$^{f/y;nkx2.5cre}$) hearts morphological analyses HW/BW, HW/TL (C-F) Real-time PCR analyses of fetal gene expression in 12 week old hearts from Male (*tβ4*$^{f/y}$ and *tβ4*$^{f/y;nkx2.5cre}$) hearts. (G&H) Real-time PCR analyses of fibrotic genes from 12 week old Male (*tβ4*$^{f/y}$ and *tβ4*$^{f/y;nkx2.5cre}$) hearts. (I) H&E stained 12 week old hearts from *tβ4*$^{f/y}$ and *tβ4*$^{f/y;nkx2.5cre}$ hearts, 1X. Scale bar, 2mm (J) Masson Trichrome stained sections from 12 week old hearts from *tβ4*$^{f/y}$ and *tβ4*$^{f/y;nkx2.5cre}$ hearts, 40×. Scale bar, 200μm. n-3-4 hearts per experiment. N/C= No significant change.

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Table 1

Mendelian Ratios of Global-Tβ4 Knockout Mice.

Background: Mixed 129svj and Black Swiss		
	Cross $t\beta 4^{+/-} \times t\beta 4^{+/y}$	
Genotype	% Observed	Expected
+/+	24.04% (44/183)	25%
−/+	25.14% (46/183)	25%
+/*Y*	23.50% (43/183)	25%
−/*Y*	27.32% (50/183)	25%
	Background: C57/b6	
	Cross $t\beta 4^{+/-} \times t\beta 4^{+/y}$	
Genotype	% Observed	Expected
+/+	21.31% (13/61)	25%
−/+	26.23% (16/61)	25%
+/*Y*	29.51% (18/61)	25%
−/*Y*	22.95% (14/61)	25%
Background: Mixed 129svj and Black Swiss		
	Cross $t\beta 4^{+/-} \times t\beta 4^{-/y}$	
Genotype	% Observed	Expected
+/−	18.75% (12/64)	25%
−/−	26.56% (17/64)	25%
+/*Y*	29.69% (19/64)	25%
−/*Y*	25.00% (16/64)	25%
	Background: C57/b6	
	Cross $t\beta 4^{+/-} \times t\beta 4^{-/y}$	
Genotype	% Observed	Expected
+/−	29.51% (18/61)	25%
−/−	26.23% (16/61)	25%
+/*Y*	19.67% (12/61)	25%
−/*Y*	24.59% (15/61)	25%