Supplementary Methods

Conditional knockdown of Tβ4 in the developing heart

The conditional RNAi approach was adopted following in vitro studies which demonstrated a putative role for Tβ4 in regulating cytokinesis and consequently cell survival (data not shown). Since Tβ4 maps to the X chromosome in the mouse, targeting of Tβ4 using either conventional or conditional approaches in ES cells could result in either a complete or partial loss of Tβ4 function respectively, and ultimately a failure in ES cell survival. Moreover, the use of conditional RNAi provided the possibility of generating a phenotypic range (dependent upon transgene copy number and insertion site), equivalent of a hypomorphic allelic series, for dissecting out Tβ4 function in the heart1.

Construction of Tβ4 shRNA transgene

The Tβ4 shRNA construct was prepared by modifying a RasGAP shRNA transgene1, kindly provided by G.Gish (S.L.R.I., Toronto). The RasGAP shRNA sequence was removed and replaced with sense and antisense Tβ4 Sequences of 21 base pairs in length, separated by a nine bp spacer, downstream of the H1 RNA pol III promoter, followed by a stretch of five thymidines which act to terminate transcription. A 5-thymidine stop termination sequence, flanked by 2 loxP recombination sequences, was inserted after the H1 RNA pol III promoter, upstream of the 21-mer Tβ4 hairpin sequences. Thus, in the absence of Cre recombinase, transcription will ordinarily be terminated prior to synthesis of the Tβ4 shRNA and Tβ4 expression unaffected. Transgenic mice were derived by genOway (France) using standard procedures.
Culture and Tβ4 treatment of C2C12 myoblast cells

C2C12 cells were cultured in DMEM containing 10% FBS. Tβ4 (100ng/ml, immundiagnostik AG) was added and cells harvested over a time course to assess the degree of phosphorylation (activation) of Akt.

Culture of epicardium derived cells (EPDCs) from embryonic and adult heart

Embryonic EPDCs were cultured and passaged as previously described. After first passage, cells were treated with or without Tβ4 (100ng/ml) and/or recombinant VEGF, FGF-7, FGF-8b and FGF-10 (all at 10ng/ml, R&D Systems) in DMEM containing 10% FBS for 48 hours, prior to harvesting of protein extracts. The protocol was extended to adult heart preparations: hearts were removed from 8 week old C57Bl/6 mice, cut into pieces (approximately 1mm³), rinsed in PBS to remove excess blood and plated onto gelatin-coated dishes in DMEM containing 15% FBS in the presence or absence of Tβ4 (100ng/ml) or AcSDKP (18µg/ml). Cultures were maintained with minimum disturbance to allow explants to adhere. After 72 hours, cells were gently washed and fresh medium added; after a further 24 hours, cells were fixed for 10 minutes in 4% paraformaldehyde (PFA) and cell types assessed by immunofluorescence.

RNA in situ hybridization

RNA in situ hybridization was performed on paraffin-embedded sectioned embryos, as previously described using a cDNA probe from the 3’UTR of Tβ4.

X-gal staining of R26R x Cre embryos
Embryos were equilibrated in 30% sucrose in PBS overnight at 4°C and embedded in OCT medium. 15μm cryostat sections were prepared, post-fixed in 4% PFA for 5 minutes and washed in PBS containing 2mM MgCl₂. Slides were incubated in X-gal stain solution (1mg/ml 4-chloro-5-bromo-3-indoly1-β-galactosidase, 4mM K₄Fe(CN)₆.3H₂O, 4mM K₃Fe(CN)₆, 2mM MgCl₂ in PBS) at 30°C for 24 hours, rinsed in PBS and counterstained with 0.1% nuclear fast red (Sigma).

**Immunohistochemistry and TUNEL staining**

E14.5 embryos were embedded in paraffin and sectioned at 10μm for immunohistochemistry using a polyclonal anti-Tβ4 antibody (abcam) and developed using a standard streptavidin-HRP method. DNA fragmentation was detected by TUNEL assay according to the manufacturer’s protocol (Promega).

**Immunofluorescence**

10μm paraffin or cryostat sections were prepared for immunofluorescence using antibodies to SMαA (Sigma), Flk1 (BD Pharmingen), Fas, VEGF or Tie-2 (all Santa Cruz). Adult EPDCs were fixed in 4% PFA and incubated with antibodies against epicardin (TCF21, abcam), Flk1, SMαA or Procollagen type I (Santa Cruz). The following secondary antibodies were used: Cy3-conjugated anti-rabbit (Fas, Tie-2), TRITC-conjugated anti-mouse (VEGF, SMαA on embryo sections), FITC-conjugated anti-mouse (SMαA in EPDCs), Alexa 488-conjugated anti-goat (Procollagen type I) or Alexa 594-conjugated anti-rat (Flk1).

**Western blotting**
Western blotting was performed using standard methods (Tris-Tricine 4-20% gradient SDS-PAGE for blotting of Tβ4 or Tβ10 peptides and Tris-glycine SDS-PAGE for all other proteins) using antibodies against Tβ4 (abcam), Tβ10 (Biodesign International), Tie-2 (Santa Cruz), SMαA (Sigma), GAPDH (Chemicon), Caspase-8 (Santa Cruz), Cleaved caspase-3, total Akt and Phospho-Akt (both Cell Signalling Technology). HRP-conjugated secondary antibodies and ECL detection reagent were used to develop blots. Scanning densitometry was performed and quantified using Scion Image (Scion Corporation).

**AcSDKP enzyme immunoassay**

Protein extracts were prepared from 5 control, 5 Tβ4sh^Mlc^ and 4 Tβ4sh^Nk^ hearts from 6 different litters; 4 control (vehicle), 3 control (AcSDKP), 9 Tβ4sh^Nk^ (vehicle), 6 Tβ4sh^Nk^ (AcSDKP) from a total of 6 litters and adult hearts post MI, as previously described^4^. 8.2mg (E14.5 heart) or 20mg (adult heart) of total protein were extracted in methanol and the AcSDKP concentration in each sample was measured in triplicate using an enzyme immunoassay kit, according to the manufacturer’s protocol (SPI-Bio). Statistical analysis was performed using a t-test.

**AcSDKP rescue in Tβ4 knockdown embryos**

Timed matings were established between conditional Tβ4 shRNA transgenic and Nkx2-5 heterozygote mice. Four pregnant females were injected intraperitoneally with 36µg AcSDKP (SPI-Bio) in 0.1ml 0.85% saline and two with vehicle at E6.5, E8.5, E10.5 and E12.5. Embryos were harvested at E14.5 and hearts removed after assessment of severity of phenotype. Protein extracts were prepared for Western blotting and immunoassay to determine levels of Tβ4 and AcSDKP, respectively.
Myocardial Infarction

Adult heart samples post myocardial infarction (MI) were kindly provided by James Clark, Cardiovascular Division, King’s College London, St. Thomas’ Hospital. Briefly, MI was induced in anaesthetised C57Bl/6 male mice by ligation of the left anterior descending coronary artery for 30 minutes, followed by reperfusion. Animals were sacrificed one hour, one day or one week post MI and protein extracts prepared in Laemmli buffer for Western blotting and immunoassay to determine levels of Tβ4 and AcSDKP, respectively.

References


Supplementary Figure 1 | A conditional RNAi approach for knockdown of Tβ4.

Production of Tβ4 shRNA upon expression of Cre recombinase (a). The conditional Tβ4 shRNA construct was electroporated into 129Ola ES cells and, following neomycin selection, 99 clones were screened by Southern blotting. Three clones were injected into blastocysts and implanted into pseudo-pregnant females and three independent transgenic lines were derived. The severity of phenotype correlates with degree of Tβ4 knockdown, measured at the protein level by western blotting and scanning densitometry (b). Tβ10 levels are unaffected in severe Tβ4shMlc (1) and Tβ4shNk (2) hearts (c); these samples correspond to those showing the greatest degree of Tβ4 knockdown (1,2) in (b).

Supplementary Figure 2 | The thymus, representing the only other site of Tβ4 and Nkx2-5 co-expression, is severely disrupted in Tβ4shNk embryos. E14.5 embryos displayed thymic hypoplasia (a) and failure of the thymic lobes to migrate together at the midline (b), shown in frontal section. Nkx2-5CreKI driven Cre expression in the thymic lobes as well as the heart, shown by X-gal staining of embryos from a R26R cross (c).

Supplementary Figure 3 | Conditional knockdown of Tβ4 in ventricular cardiomyocytes. R26R crosses with Nkx2-5CreKI (a-c) and MLC2vCreKI (d-f) mice and X-gal staining to reveal Cre recombinase expression in cardiomyocytes of the ventricular myocardium (my), and exclusion from the epicardium (epi; c). Cre expression/X-gal staining is mosaic following crosses with either Cre knock-in strain
Following a Nkx2-5CreKI cross expression is relatively widespread whereas expression is restricted to a proportion of ventricular cardiomyoctes following a MLC2vCreKI cross. The cohort of positive cells arising from a MLC2vCreKI cross express Cre at a higher level (d) relative to those from a Nkx2-5CreKI cross (a, b). Tβ4 is expressed throughout most cells of the ventricular myocardium (my; g) but is excluded from Flk1-positive endothelial cells (h, j).

Supplementary Figure 4| Phenotype of Tβ4 knockdown embryos over the time course of cardiac morphogenesis. The earliest manifestation of cardiac defects in Tβ4shNk, demonstrated by pericardial oedema at E10.5 (a-c). Epicardial defects are apparent by E12.5, indicated by surface nodules and haemorrhaging in Tβ4shNk embryos (d, e). Leftward displacement of the Tβ4shMlc heart (f). Cardiac morphology is not impaired prior to failure of coronary vessel development, shown in frontal section of E12.5 knockdown hearts (g-i). Epicardial nodules, misshapen hearts and reduced ventricular compaction are prominent features of mutant hearts at E15.5 (j-n) while the most severely affected are dying (o).

Supplementary Figure 5| Tβ4 promotes the differentiation of vascular smooth muscle and endothelial cells. Smooth muscle cell differentiation is delayed in Tβ4 knockdown hearts, indicated by the near absence of SMαA-positive cells in Tβ4shNk myocardium (my) and a considerable reduction in Tβ4shMlc myocardium, compared with control (a). Cultured EPDCs from E10.5 heart explants display maximum potential for differentiation into SMαA- and Tie2-positive cells (b); the potential for differentiation of these cell types diminishes by E12.5 (b). Tβ4 promotes
differentiation of SMαA- and Tie2-positive cells, the latter additionally requiring VEGF and FGFs (b). VEGF may mediate aspects of Tβ4-induced coronary vessel formation. Expression of VEGF was reduced in the myocardium (my) of Tβ4shNk hearts, compared with the same region of control myocardium; white boxes indicate magnified region (c).

**Supplementary Figure 6** Tβ4 is expressed in the myocardium and great vessels of the developing heart. RNA *in situ* hybridization (a-c) and immunohistochemical (d-f) detection of Tβ4 in wild-type E14.5 embryo. Tβ4 is expressed throughout the ventricular myocardium (a), robustly in the interventricular septum (ivs; b) and the myocardial compact layer (a). Further domains of Tβ4 expression in the vascular smooth muscle lining of the aorta (ao) and pulmonary artery (pa; c-f).

**Supplementary Figure 7** Disruption of the actin cytoskeleton, a direct consequence of Tβ4 knockdown, results in apoptosis of ventricular cardiomyocytes. Tβ4 knockdown led to severe disruption of the F-actin cytoskeleton, as determined by phalloidin staining (a-c). Fas activation (clustering) is greatly elevated in the myocardium (my) of Tβ4shMlc (e,f) and Tβ4shNk embryos; white arrowheads (h,i), compared with control (d,g). Initiator caspase-8 (j) and effector caspase-3 (k) are activated, resulting in increased apoptotic cell death, determined by TUNEL staining (l-n). TUNEL-positive cells are abundant in the epicardium (ep) and myocardium (my) and relatively scarce in the compact zone (cz).

**Supplementary Figure 8** Tβ4 does not appear to directly regulate the survival kinase Akt. (a). Tβ4 activates Akt (P-Ser 473) within 10 min of treatment of C2C12
myoblasts (b). However, increased Akt activation was observed in Tβ4sh^{Mlc} and Tβ4sh^{Nk} hearts, arguing against a direct requirement for Akt activation in coronary vessel development.

**Supplementary Figure 9** | Tβ4 and AcSDKP are up-regulated following myocardial infarction. Myocardial infarction induces an increase in endogenous Tβ4 (a) and AcSDKP (b) expression levels in the adult heart, determined by western blot and enzyme immunoassay, respectively.