The Role of Thymosin β4 in Vascular Development

A thesis submitted for the degree of Doctor of Philosophy to
University College London

Alexander Rossdeutsch

Molecular Medicine Unit
UCL Institute of Child Health
University College London

December 2010

Declaration

I, Alexander Rossdeutsch confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Alexander Rossdeutsch, December 2010

Abstract

Thymosin β4 (Tβ4) is a 43 amino acid peptide encoded by the Tmsb4x gene located on the X-chromosome. It has previously been shown to act as a secreted factor from the myocardium to the overlying epicardium of the developing murine heart, to mediate transformation of epicardial derived progenitor cells (EPDCs) into the coronary vasculature. This PhD project seeks to build on these studies and characterises the function of Tβ4 in the developing systemic vasculature, using the mouse as a model system.. Expression analyses demonstrated specific localisation of Tβ4/Tβ4 in the endothelial cells of the embryonic vasculature. In order to ascertain the function of vascular Tβ4, global and endothelial cell specific in vivo Tβ4 loss of function models were examined. Both global and endothelial-specific Tβ4 mutant embryos displayed a reduced recruitment of vascular mural cells to developing blood vessels. Detailed phenotypic examination revealed that the mural cell deficit could be attributed to impaired differentiation of mature mural cells from undifferentiated mesoderm. This process was modelled in vitro, and it was discovered that treatment of the mural progenitor cell lines 10T1/2 and A404 with exogenous Tβ4 could promote their differentiation into mural cells. This process correlated with an increase in Smad phosphorylation and increased activity of the TGF-β pathway. Decreased levels of TGF-β target genes in vivo in Tβ4− null embryos indicated that TGF-β signalling was perturbed in the absence of Tβ4. These findings suggest a model whereby Tβ4 is secreted by the developing endothelium to stimulate the differentiation of uncommitted mesoderm into mature peri-vascular mural cells, via activation of the TGF-β pathway in the target cell population. As a consequence, Tb4 plays an essential role in vascular stability through mural cell support which has implications for vascular dysfunction in disease.

Table of Contents

DECLARATION 2

ABSTRACT 3

TABLE OF CONTENTS 4

LIST OF FIGURES AND TABLES 10

ACKNOWLEDGMENTS 16

LIST OF ABBREVIATIONS 17

1 INTRODUCTION 21

1.1 Historical Context 21

1.2 Thymosin Nomenclature 22

1.3 The Beta Thymosin Family 23

1.4 The Tβ4 Gene 24

1.5 The Structure of Tβ4 25

1.6 Tβ4 as an Actin Binding Molecule 25

1.7 The Dynamics of F-Actin Formation 25

1.8 The Function of Tβ4 as a G-Actin Buffer 26

1.9 Limitations of the Tβ4/G-Actin Model 28

1.10 Functional Consequences of the Tβ4/G-Actin Interaction 29

1.11 The Effects of the Tβ4/G-Actin Interaction on Tumour Metastasis 30

1.12 The Function of Extracellular Tβ4 31

1.13 The Effects of Tβ4 on Apoptosis 32

1.14 The Anti-Inflammatory Effects of Tβ4 32

1.15 The Anti-Inflammatory Effects of Tβ4 as a Treatment for Sepsis 33

1.16 Tβ4 as a Treatment for CNS Inflammation 34

1.17 The Action of Pharmacological Tβ4 on Wound Healing 34

1.18 Tβ4 in Neuroregeneration 35

1.19 Tβ4 in the Cardiovascular System 35

1.20 The First Embryonic Blood Vessels Are Formed By the Process of Vasculogenesis 36

1.21 The Role of Tβ4 in Vasculogenesis 37

1.22 Secondary Blood Vessel Formation Occurs Via Angiogenesis 39

1.23 The Molecular Events Underlying Sprouting Angiogenesis 39

1.24 A Role for Tβ4 in Sprouting Angiogenesis 40

1.25 A link between Tβ4 and MMP Secretion 41

1.26 The Function of Tβ4 in Endothelial Cell Migration 41

1.27 Mural Cell Recruitment is Essential for the Formation of Stable Blood Vessels 42

1.28 Mechanisms of Mural Cell Recruitment 43

1.29 PDGF-B Signalling in Mural Cell Development 43

1.30 The Mechanism of PDGF-B Signalling 44

1.31 The Biology of Notch Signalling 45

1.32 Arterio-Venous Specification by Notch Receptor Signalling 46

1.33 Notch is a Key Mediator of Arteriogenesis 47

1.34 An Essential Function for Notch Signalling in Sprouting Angiogenesis 47

1.35 Notch3 Signalling Stimulates Mural Cell Maturation 48

1.36 TGF-β is a Molecular Effector of Vascular Development 49

1.37 The Molecular Biology of TGF-β Signalling 50

1.38 The Termination of TGF-β Signalling 51

1.39 The TGF-β Pathway in Mural Cell Differentiation 52

1.40 Two Type I TGF-β Receptors Function in TGF-β Mediated Vascular Development 52

1.41 The Function of Alk-5 During Vascular Development 53

1.42 Two Models for the Function of TGF-β in Vascular Development 54

1.43 A Consensus Model for the Function of TGF-β in Vascular Development 55

1.44 Connexins Regulate the Bioavailability of TGF-β During Vascular Development 57

1.45 Evidence of a Role for Tβ4 in the Process of Mural Cell Development 57

1.46 Development of the Heart 58

1.47 Tβ4 Has an Essential Function During Coronary Vessel Development 59

1.48 Synthetic Tβ4 as a Treatment for Acute Myocardial Infarction 60

1.49 The Retinal Model of Vascular Angiogenesis 63

1.50 Formation of the Primary Retinal Vascular Plexus 63

1.51 Formation of the Secondary Retinal Vascular Plexus 65

1.52 Mural Cell Recruitment in the Retinal Vasculature 66

1.53 Aims and Objectives 66

2. MATERIALS AND METHODS 69

2.1 Immunofluorescence Staining 69

2.2 Whole Mount In Situ Hybridisation 69

2.3 In situ Hybridisation on Sections 70

2.4 Whole Mount PECAM Staining 71

2.5 Immunohistochemistry 71

2.6 Preparation of Tissue for Cryo-Sectioning: 72

2.7 Fluorescent Imaging 72

2.8 Quantification of NG2 Immunofluorescence 73

2.9 qRT-PCR 73

2.10 Quantification of Haemorrhage at E14.5 79

2.11 10T1/2 and A404 Cell culture 80

2.12 Western blotting 81

2.13 Cell Transfections 82

2.14 Retinal Immunostaining 82

2.15 Gene arrays 83

2.16 Antibodies 83

2.17 Recombinant Protein 84

2.18 Statistics 84

2.19 X-Gal Staining of Whole Embryos 84

2.20 MEF Isolation and Culture 85

2.21 ES Cell Culture 86

3 DEVELOPMENTAL EXPRESSION OF TB4 87

3.1 Introduction **87**

3.2 Results **88**
3.2.1 Vascular Expression of Tβ4 88
3.2.2 Cardiac Expression of Tβ4 91
3.2.3 Neural Expression of Tβ4 93
3.2.4 Gastrointestinal Expression of Tβ4 94
3.2.5 Lung Expression of Tβ4 95

3.3 Discussion **96**
3.3.1 Expression of Tβ4 in the Vasculature 96
3.3.2 Expression of Tβ4 in the Developing Heart 98
3.3.3 Expression of Tβ4 in the Neural Network 100
3.3.4 Expression of Tβ4 in the Gastrointestinal System 103
3.3.5 Expression of Tβ4 in the Respiratory System 104

4 GENETIC MODELS FOR TB4 LOSS OF FUNCTION 105

4.1 Introduction: **105**

4.2 Results **106**
4.2.1 Mouse Transgenesis Can Be Used to Achieve Germline Global Knockout of Tβ4 106
4.2.2 Knockout of the Tmsb4x Gene Abolishes Synthesis of the Tβ4 Protein In Vivo 107
4.2.3 Knockout of Tβ4 Results in Impaired Survival 108
4.2.4 E10.5 Tβ4 -/Y Embryos Display A Haemorrhagic Defect 109
4.2.5 Tβ4 -/Y Embryos Possess a Reduced Peri-Aortic Mural Cell Coverage 112
4.2.6 Tβ4 -/Y Embryos Display Dermal Haemorrhage at E16.5 114
4.2.7 Defects in Mural Cell Recruitment Coverage Underlie Dermal Haemorrhage in E14.5 Tβ4 -/Y Embryos 115
4.2.8 Tβ4 Can Be Knocked Down in a Tissue Restricted Fashion 116
4.2.9 E10.5 Tβ4-RIEK Embryos Rarely Reproduce the Same Haemorrhagic and Mural Cell Defects Observed in Tβ4 -/Y Mice 119
4.2.10 Targeting the Tβ4 shRNA Construct to an Open Genomic Locus Creates a More Reliable Tβ4 Tissue Specific Knockdown 124
4.2.11 Mutant Tβ4-HEK Mice Recapitulate the Mural Cell Defects of the Tβ4 -/Y Mouse 127
4.2.12 Mural Cell Defects in Tβ4 Loss of Function Models Are Due to Impaired Mural Cell Differentiation 128

4.3 Discussion **133**
4.3.1 Summary 133
4.3.2 Penetrance of the Haemorrhagic Phenotype in Tβ4 -/Y Embryos 134
4.3.3 Down Regulation of Mural Cell Markers in E10.5 Tβ4 -/Y Embryos 135
4.3.4 Impaired Mural Cell Development in Endothelial Cell Specific Tβ4 Knockdown Embryos 136
4.3.5 Aberrant Cellular Behaviour in Tβ4 -/Y Embryos 141

5 USE OF GENE EXPRESSION STUDIES TO UNDERSTAND THE MOLECULAR PHENOTYPE OF TB4 -/Y EMBRYOS 145

5.1 Introduction: **145**

5.2 Results **150**
5.2.1 Expression Analysis in E12.5 Limb Buds 150
5.2.2 Expression Analysis in Somite Matched E10.5 Embryos 162
5.2.3 Gene Array Analysis of Tβ4 -/Y Embryos and Adult Hearts 169
5.2.4 Symplekin is Expressed in Endothelial Cells 176

5.2.5 Bioinformatic Analysis of Array Dataset Reveals Dysregulation of the TGF-β Pathway in Tβ4 -/Y Mice 177

5.3 Discussion 179
5.3.1 Summary 179
5.3.2 Candidate Pathway Approaches 180
5.3.3 Gene Arrays 183

6 MODELLING THE FUNCTION OF TB4 *IN VITRO* 188

6.1 Introduction: 188

6.2 Results: 191
6.2.1 Tβ4 Treatment of ES Cells Has No Effect on the Expression of Mural Cell Markers 191
6.2.2 Treatment of 10T1/2 Cells with Tβ4 Can Induce the Expression of Mural Cell Markers 194
6.2.3 Treatment of A404 Cells with Tβ4 Can Induce the Expression of Mural Cell Markers 196
6.2.4 Tβ4 Treatment of Tβ4 +/Y and Tβ4 -/Y MEFs Fails to Induce the Expression of Mural Cell Markers 199
6.2.5 Tβ4 Can Stimulate TGF-β Signalling Pathway *In Vitro* 203
6.2.6 Tβ4 Stimulated TGF-β Pathway Activation Correlates with Increased Smad Phosphorylation 205
6.2.7 Tβ4 Can Stimulate the Activity of the TGF-β Pathway *In Vivo* 210

6.3 Discussion 211
6.3.1 Summary 211
6.3.2 Effects of Tβ4 on ES Cell Differentiation 213
6.3.3 Effect of Tβ4 on MEF Phenotype 215
6.3.4 Influence of Tβ4 on Alk-1/Alk-5 Signalling 216
6.3.5 Evidence for an *In Vivo* Defect in TGF-β Signalling 219
6.3.6 The Mechanism of Tβ4 Induced Smad Phosphorylation 220

7 THE ROLE OF TB4 IN PHYSIOLOGICAL ANGIOGENESIS 223

7.1 Introduction 223

7.2 Results 226
7.2.1 E10.5 Tβ4-RIEK Embryos Display No Defect in Angiogenesis 226
7.2.2 Tβ4 Is Expressed in the Developing Retinal Vasculature 228
7.2.3 Loss of Tβ4 Function Has No Effect on Retinal Vascular Angiogenesis 228
7.2.4 Tβ4-HEK Mice Display No Defect in Retinal Vascular Sprouting 233

7.3 Discussion 235
7.3.1 Summary 235
7.3.2 Tβ4 Loss of Function Does Not Lead to an Angiogenic Defect *In Vivo* 236
7.3.3 Sprouting of the Secondary Vascular Plexus 237

8 DISCUSSION AND FUTURE WORK 239

8.1 A Molecular Model for Tβ4 Action 239

A Receptor for Tβ4 242

A Potential Role for Tβ4 in the Nervous System 244

Tβ4 in Vascular Disease 246

Tβ4 in Aneurismal Disease 246

Tβ4 in Diabetic Retinopathy 247

Tβ4 in Tumour Angiogenesis 249

REFERENCES 251

APPENDIX 1: TOP 100 GENES DOWN REGULATED IN E12.5 TB4 -/Y EMBRYOS 270

APPENDIX 2: TOP 100 GENES UP REGULATED IN E12.5 TB4 -/Y EMBRYOS 276

APPENDIX 3: TOP 100 GENES DOWN REGULATED IN TB4 -/Y ADULT HEARTS 282

APPENDIX 4: TOP 100 GENES UP REGULATED IN TB4 -/Y ADULT HEARTS 288

List of Figures and Tables

Fig. 1.1	The sequence and structure of β-thymosin family proteins	**23**
Fig. 1.2	Tβ4 acts to maintain a buffered pool of G-actin	**28**
Fig. 1.3	The myriad functions of Tβ4	**31**
Fig. 1.4	Mechanisms of blood vessel development	**37**
Fig. 1.5	The role of PDGF-B in mural cell development	**45**
Fig. 1.6	Key molecular events in the Notch signalling cascade	**46**
Fig. 1.7	A consensus model of TGF-β mediated vascular development	**56**
Fig. 1.8	Tβ4 is secreted by the developing myocardium to stimulate EPDCs to form the coronary vasculature.	**60**
Fig. 1.9	Synthetic Tβ4 can improve cardiac function following MI	**62**
Fig. 1.10	Formation of the primary retinal vascular plexus can be used as a model to investigate many of the cellular processes of vascular development	**65**
Fig. 3.1	Tβ4 is expressed in the vasculature of midgestation mouse embryos	**89**
Fig. 3.2	Tβ4 protein is expressed in the walls of the dorsal aortae and cardinal veins	**90**
Fig. 3.3	Tβ4 is expressed in the endothelium but not the mural cell layer of developing blood vessels	**91**
Fig. 3.4	Tβ4 is expressed in the tissues of the developing mouse heart	**92**
Fig. 3.5	Tβ4 is expressed in both the central and peripheral nervous systems	**94**

Fig. 3.6	Tβ4 is expressed in the epithelial surface of the developing gastrointestinal tract	**95**
Fig. 3.7	Tβ4 is expressed on the in the alveolar surface epithelium of the developing lung	**96**
Fig. 4.1	A scheme to knockout Tβ4 through insertion of a neomycin resistance cassette in exon 2 of the Tmsb4x gene	**107**
Fig. 4.2	Knockout of the Tmsb4x gene results in ablation of Tβ4 protein expression *in vivo*	**108**
Table 4.1	Tβ4 -/Y mice die *in utero* between E10.5 and Birth	**109**
Fig. 4.3	Tβ4 -/Y embryos display overt pericardial Haemorrhage	**110**
Fig. 4.4	E10.5 Tβ4 -/Y embryos display pericardial and coelomic cavity haemorrhage	**111**
Fig. 4.5	E10.5 Tβ4 -/Y have a reduction in NG2+ mural cell staining around their dorsal aortas	**113**
Fig. 4.6	E10.5 Tβ4 -/Y display globally depressed levels of mural cell markers	**114**
Fig. 4.7	E14.5 Tβ4 -/Y embryos display cutaneous haemorrhage	**115**
Fig. 4.8	Skin vessels of E14.5 Tβ4 -/Y embryos possess reduced mural cell coverage	**116**
Fig. 4.9	Transcription of a Tβ4 shRNA initiated by Cre recombinase can induce knockdown of Tβ4 in vivo	**117**
Fig. 4.10	Tie2-Cre embryos express Cre recombinase in the developing endothelium	**119**
Fig. 4.11	E10.5 Tβ4-RIEK mutant embryos display pericardial haemorrhage	**120**
Fig. 4.12	Haemorrhagic E10.5 Tβ4-RIEK embryos have reduced mural cell investiture of their dorsal aortas	**121**

Fig. 4.13	Tβ4-RIEK embryos show no defect in mural cell coverage of their dorsal aortas at E14.5	**123**
Fig. 4.14	The Hprt targeted Tβ4 shRNA construct produces more effective Tβ4 knockdown in vivo than the randomly integrant Tβ4 shRNA construct	**126**
Fig. 4.15	E10.5 Tβ4-HEK mutant mice display reduced mural cell investiture of their dorsal aortas	**128**
Fig. 4.16	No apoptosis is observed in the mural cells of E10.5 Tβ4 -/Y embryos	**129**
Fig. 4.17	No apoptosis is observed in the mural cells of E10.5 Tβ4 -/Y embryos	**130**
Fig. 4.18	P6 Tβ4 -/Y mice display no mural cell deficit in their retinal vasculature	**131**
Fig. 4.19	Aortic mural cell differentiation does not take place until after E9.5	**132**
Fig. 5.1	Quantification of mRNA expression for Notch receptors in E12.5 Tβ4 -/Y limb buds	**151**
Fig. 5.2	Quantification of mRNA expression for Notch ligands in E12.5 Tβ4 -/Y limb buds	**152**
Fig. 5.3	Quantification of mRNA expression for Notch signalling intermediates in E12.5 Tβ4 -/Y limb buds	**153**
Fig. 5.4	Quantification of mRNA expression for Notch transcriptional targets in E12.5 Tβ4 -/Y limb buds	**155**
Fig. 5.5	Quantification of mRNA expression for PDGF-B, its receptor, and PDGF-B target genes in E12.5 Tβ4 -/Y limb buds	**157**
Fig. 5.6	Quantification of mRNA expression for TGF-β and its receptors in E12.5 Tβ4 -/Y limb buds	**159**
Fig. 5.7	Quantification of mRNA expression for Smad genes in E12.5 Tβ4 -/Y limb buds	**160**

Fig. 5.8	Quantification of mRNA expression for TGF-β target genes in E12.5 Tβ4 -/Y limb buds	**161**
Fig. 5.9	Quantification of mRNA expression for Notch receptors in E10.5 Tβ4 -/Y haemorrhagic embryos	**163**
Fig. 5.10	Quantification of mRNA expression for Notch ligands in E10.5 Tβ4 -/Y haemorrhagic embryos	**164**
Fig. 5.11	Quantification of mRNA expression for Notch target genes in E10.5 Tβ4 -/Y haemorrhagic embryos	**165**
Fig. 5.12	Quantification of mRNA expression for PDGF-B, its receptor and target genes in E10.5 Tβ4 -/Y haemorrhagic embryos	**166**
Fig. 5.13	Quantification of mRNA expression for TGF-β and its receptors in E10.5 Tβ4 -/Y haemorrhagic embryos	**167**
Fig. 5.14	Quantification of mRNA expression for Smad genes in E10.5 Tβ4 -/Y haemorrhagic embryos	**168**
Table 5.1	Dysregulated genes in Tβ4 -/Y mice selected for further investigation	**171**
Fig. 5.15(i)	Validation of gene array targets of interest - part 1	**173**
Fig. 5.15(ii)	Validation of gene array targets of interest - part 2	**174**
Fig. 5.15(iii)	Validation of gene array targets of interest - part 3	**175**
Fig. 5.16	Symplekin is expressed in the embryonic endothelium	**177**
Table 5.2	Bioinformatic analysis highlights dysregulated molecular pathways in Tβ4 -/Y mice	**178**
Table 6.1	Mural cell markers and their cell specific expression	**191**
Fig. 6.1	Wild type ES cells express mural cell marker genes under stimulation by TGF-β but not Tβ4	**193**

Fig. 6.2(i)	Tβ4 can induce up regulation of mural cell markers in 10T1/2 cells – part 1	**195**
Fig. 6.2(ii)	Tβ4 can induce up regulation of mural cell markers in 10T1/2 cells – part 2	**196**
Fig. 6.3(i)	Tβ4 can induce up regulation of mural cell markers in A404 cells – part 1	**198**
Fig. 6.3(ii)	Tβ4 can induce upregulation of mural cell markers in A404 cells – part 2	**199**
Fig. 6.4	Tβ4 has no effect on the expression of mural cell markers in primary MEFs	**201-202**
Fig. 6.5	Tβ4 can induce the expression of TGF-β responsive transcription factors in 10T1/2 and A404 cells	**204**
Fig 6.6	Tβ4 can stimulate the activity of a Smad responsive luciferase reporter	**205**
Fig. 6.7	Tβ4 cannot up regulate the expression of TGF-β receptors in 10T1/2 cells	**207**
Fig. 6.8	Tβ4 cannot up regulate the expression of Usp9x in 10T1/2 cells	**209**
Fig. 6.9	Tβ4 co-operates with TGF-β to stimulate Smad phosphorylation	**210**
Fig. 6.10	E10.5 Tβ4 -/Y embryos display a reduced level of TGF-β target genes *in vivo*	**211**
Fig. 7.1	E10.5 Tβ4 -/Y embryos display no angiogenic defect	**227**
Fig. 7.2	Tβ4 is expressed in the retinal primary vascular plexus	**228**
Fig. 7.3	The appearance of the retinal vasculature in Tβ4 -/Y mice is grossly normal	**229**
Fig. 7.4	There is no defect in the migration of the sprouting vascular front in Tβ4 -/Y retinas	**230**
Fig. 7.5	Tβ4 -/Y retinas display no abnormality in vascular branch point density	**231**

Fig. 7.6	Tβ4 -/Y retinal vascular tip cells possess a normal number of filopodia and lamellipodia	**232**
Fig. 7.7	Tβ4 -/Y retinas display a trend towards a reduction in secondary vascular plexus sprouting	**233**
Fig. 7.8	No angiogenic defect is observed in the Tβ4-HEK retinal vasculature	**234**
Fig. 8.1	A model for Tβ4 action in mural cell development.	**240**

Acknowledgments

I would like to thank my supervisor Professor Paul Riley for all the support he has shown to me over the course of my PhD studies. His intellectual and personal support has proved invaluable to me and allowed me to enjoy immensely the time spent in his research group. As my secondary supervisor, Professor Pete Scambler has always provided a fresh perspective on challenging data and has stimulated many new avenues of thought as recorded in this thesis.

I would also like to thank the other past and present members of the Riley lab and the Molecular Medicine Unit at the Institute of Child Health. In particular, I would like to single out Dr Nicola Smart and Dr Jonathan Tobin for particular gratitude. Dr Smart has painstakingly taught me the techniques of molecular biology whilst Dr Tobin has always given me enough perspective and enthusiasm to return to experiments which at first seemed impossibly challenging.

Several collaborators have assisted me with both technical and intellectual input into this project. Of these, I would like to highlight Dr Nick Henriquez at the UCL Institute of Neurology who performed much of the data analysis on gene array studies of the Tβ4 knockout mouse,. Dr Marcus Fruttiger and Dr Shalini Jadeja at the UCL Institute of Opthalmology taught me how to dissect neonatal mouse retinas and provided conceptual input into the analyses performed on these samples.

Finally, I'd like to thank my family, whose unwavering emotional and practical support has been key to pursuing my desire to become the best scientist I can.

List of Abbreviations

ADAM	A disintegrin and metalloproteinase domain containing protein
ADP	Adenosine diphosphate
Akt	Protein kinase B
Alk	Activin-like kinase
Ang1	Angiopoietin-1
Ap1	Activator protein 1
Ap3	Activator protein 3
Arp2/3	Actin related protein 2/3
ATP	Adenosine triphosphate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BMP	Bone morphogenetic protein
CADASIL	Cerebral autosomal dominant arteriopathy and leukencephalopathy with subcortical infarcts
CC3	Cleaved caspase 3
Ccbe1	Collagen and Calcium binding EGF domain containing 1
CD13	Alanyl aminopeptidase
CHAPS	3- ((3-Cholamidopropyl)dimethylammonio)-1- Propanesulfonic Acid)
c-jun	Activator protein 1
c-myc	Class E basic helix-loop-helix protein 39
c-ski	Ski oncoprotein
CNS	Central nervous system
Cre	Crenarchaea
CSL	Recombining binding protein suppressor of hairless homologue
DAB	Diaminobenzidine
Ddr2	Discoidin domain receptor tyrosine kinase 2
Dll	Delta-like ligand
DMEM	Dulbecco’s modified Eagle’s medium
DMSO	Di-methyl sulphoxide
DNA	Deoxyribonucleic acid
Dner	Delta/Notch-like EGF repeat containing
ECM	Extracellular matrix
EDD	End diastolic diameter
EDTA	Ethylenediaminetetraacetic Acid
EGF	Epidermal growth factor
EMT	Epithelial to mesenchymal transformation
Epas1	Endothelial PAS containing domain protein
EPDC	Epicardial derived progenitor cell
ER	Endoplasmic reticulum
ES cell	Embryonic stem cell
ESD	End systolic diameter
eYFP	Enhanced yellow fluorescent protein
FACS	Fluorescence associated cell sorting
F-actin	Filamentous actin
FCS	Foetal calf serum
FGF	Fibroblast growth factor
Foxo1	Forkhead box protein O1
G-actin	Globular actin

GDF	Growth differentiation factor
H&E	Haematoxylin and eosin
Hand1	Heart and neural crest derivatives expressed protein 1
HDL-1	High density lipoprotein 1
Hes	Hairy/enhancer of split
Hey	Hairy/enhancer of split related protein
HIF	Hypoxia inducible factor
HIF1an	Hypoxia inducible factor 1, alpha subunit inhibitor
His	Histidine
Hmgb1	High mobility group box 1
Hprt	Hypoxanthine phophoribosyltransferase
HRP	Horseradish peroxidase
HSPG	Heparan sulphate proteoglycan
HUVEC	Human umbilical endothelial cell
Id-1	Inhibitor of differentiation 1
IFT-20	Intraflagellar transport 1
IGFbp4	Insulin-like growth factor binding protein 4
IHC	Immunohistochemistry
IL-1α	Interleukin 1 alpha
IL-1β	Interleukin 1 beta
Ilb4	Isolectin B4
ILK	Integrin-linked kinase
ISH	In situ hybridisation
Jag	Jagged
KCl	Potassium Chloride
kDa	Kilo-Dalton
Ku80	X-ray repair complementing defective repair in Chinese hamster cells 5
LAP	Latency associated peptide
LIF	Leukocyte inhibitory factor
Lnfng	Lunatic fringe
LPS	Lipopolysaccheride
Ltbp4	Latent TGF-β binding protein 4
μg	Microgram
μm	Micrometer
Maml1	Mastermind like protein 1
MCP-1	Monocyte chemoattractant protein 1
MEF	Mouse embryonic fibroblast
MI	Myocardial infarction
MIP-1α	Monocyte inhibitory protein 1 alpha
MIP-1β	Monocyte inhibitory protein 1 beta
MIP-2	Monocyte inhibitory protein 2
MIS	Mullerian inhibiting substance
MMP	Matrix metalloprotease
Mnfng	Manic fringe
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NBT	Nitro-blue tetrazolium
NECD	Notch extracellular domain

NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
Ng	Nanogram
NG2	Chondroitin sulphate proteoglycan 4
NICD	Notch intracellular domain
Nkx2.5	Homeobox protein NK-2 homolog E
NO	Nitric oxide
Nrarp	Notch-regulated ankyrin repeat-containing protein
N-WASP	Neural Wiskott-Aldrich syndrome protein
OCT	Optimal cutting temperature
Oct-1	Octamer binding protein 1
PAI-1	Plasminogen activator inhibitor 1
PBS	Phosphate buffered saline
PDGF-B	Platelet derived growth factor B
PDGFRβ	Platelet derived growth factor receptor beta
PECAM	Platelet/endothelial cell adhesion molecule
PFA	Paraformaldehyde
PGF1α	Placental growth factor 1 alpha
PI3K	Phosphoinositide-3-kinase
Pim-1	Pim-1 oncogene
PINCH	Particularly interesting Cys-His protein
PLCγ	Phospholipase C gamma
PNS	Peripheral nervous system
PPH3	Phospho-histone H3
Pres1	Presenilin 1
qRT-PCR	Quantitative real time polymerase chain eaction
R26R	Rosa 26 reporter
R26R-eYFP	Rosa 26 reporter enhanced yellow fluorescent protein
RA	Retinoic acid
RBPj	Recombining binding protein suppressor of hairless
Rdfng	Radical fringe
RFC-1	Reduced folate carrier 1
RISC	Ribonucleic acid induced silencing complex
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
RP124	Ribosomal protein 124
Scar	Wiskott-Aldrich syndrome protein family member 1
SDS	Sodium deodocyl sulphate
shRNA	Short hairpin ribonucleic acid
siRNA	Short interfering ribonucleic acid
Skip	Ski-interacting protein
Slit3	Multiple EGF-like domains protein 5
SM22α	Transgellin
SMA	Smooth muscle actin
Smad	Mothers against decapentaplegic homolog
Smap2	Stromal membrane-associated protein 1-like
Smurf	Smad-ubiquitin regulatory factor
SRF	Serum response factor
TACE	Tumour necrosis factor alpha converting enzyme
Tβ4	Thymosin beta 4

Tβ4-HEK	Thymosin beta 4 Hprt targeted endothelial knockdown
Tβ4-RIEK	Thymosin beta 4 random integrant endothelial cell knockdown
Tβ4-SO	Thymosin beta 4 sulphoxide
Tβ10	Thymosin beta 10
TβR	Transforming growth factor beta receptor
TF5	Thymosin fraction 5
TGF-β	Transforming growth factor beta
Tie-2	Tunica interna endothelial cell kinase 2
TNF-α	Tumour necrosis factor alpha
tRNA	Transfer ribonucleic acid
Usp9x	Ubiquitin specific kinase 9, X-linked
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell
Wasf2	Wiskott-Aldrich protein family member 2
WASP	Wiskott-Aldrich syndrome protein
WH2	Wiskott-Aldrich syndrome protein homology domain 2
X-Gal	Bromo-chloro-indolyl-galactopyranoside

1 Introduction

Thymosin β4 (Tβ4) is a 43 amino acid peptide, the gene for which lies on the X chromosome in human and mouse. It was originally thought to be a thymic hormone, but is now known to be an important actin binding protein and modulator of the cellular cytoskeleton.

1.1 Historical Context

The studies, which eventually led to the discovery of Tβ4, were carried out in the lab of Abraham White. The researchers in his lab had postulated that the thymus, as well as being the organ source of mature T cells, also had a hormonal role in immune system development[1]. Evidence for this hypothesis came from the observation that neonatal thymectomized mice displayed a dramatic reduction in immune cell, particularly T cell, function. Treatment of these thymectomized mice with a preparation known as "thymosin", extracted from calf thymus, could restore immune cell function back to near normal levels[2,3].

The crude "thymosin" extract used in these studies, also known as thymosin fraction 5 (TF5), was initially thought to consist of just one polypeptide of around 15kDa in mass. However, subsequent analysis determined that this preparation actually consisted of around 40 polypeptides ranging, between 1-15kDa in mass.

1.2 Thymosin Nomenclature

The various proteins in the TF5 extract were then classified on the basis of their isoelectric points. α thymosins have an isoelectric point corresponding to a pH below 5.0, β thymosins a pH between 5.0 and 7.0 and γ thymosins a pH above 7.0. Thus, molecules may share the name "thymosin" despite being disparate in terms of their sequences, structures and biological properties. Subsequently the thymosins within each sub-family were numbered in order of their identification. Tβ4 shares sequence homology with thymosins β9, β10 and β15 (**Fig. 1.1**).

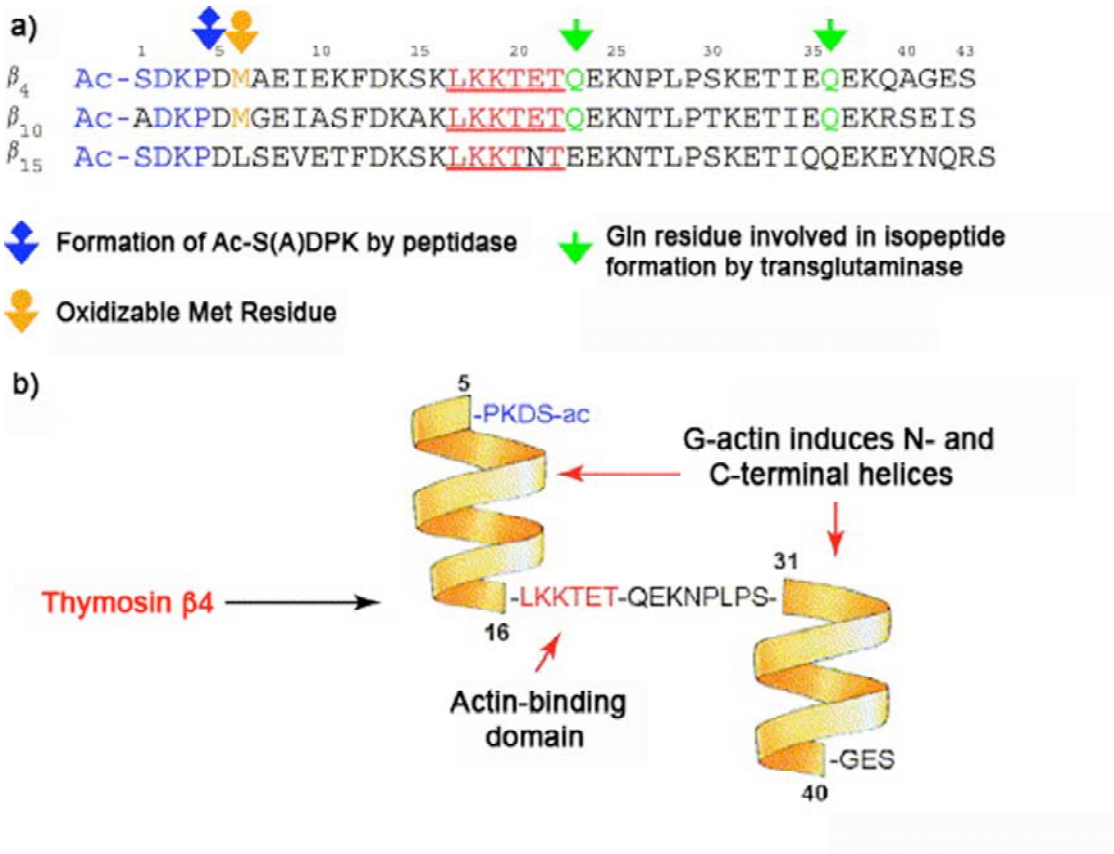


Fig. 1.1
The sequence and structure of β-thymosin family proteins

Diagram to demonstrate the amino acid sequence of the mammalian beta thymosin family of proteins (**a**), and the structure of the Tβ4 molecule (**b**). Blue sequence motifs denote the bioactive peptide Ac-SDKP which can be derived from Tβ4 via cleavage by prolyl-oligopeptidase, The red motifs represent the LKKTET actin binding domain of Tβ4. Adapted from [4].

1.3 The Beta Thymosin Family

β-thymosin molecules from the TF5 fraction are not all related to each other by sequence. For example, the original thymosin β1 molecule has been identified as a 72 amino acid fragment of the ubiquitin protein. As other beta thymosin molecules have subsequently been identified as belonging to other families, the term "beta thymosin family" has come to mean those molecules, which share homology to Tβ4. The beta thymosin family of proteins is highly conserved in eukaryotic species; present in

species ranging from mammalian human, mouse and rat through to echinoderms. Additionally, proteins have been identified in the model organisms *Drosophila Melanogaster* and *Caenorhabditis Elegans* bearing three beta thymosin like domain repeats. Beta thymosins have not been identified in yeast or prokaryotes.

In mammalian species, tissues tend to express two beta thymosin family members. This is usually Tβ4 plus one other family member. The principally expressed thymosins in human and mouse are Tβ4 and Tβ10.

1.4 The Tβ4 Gene

The gene for Tβ4 in humans is located on the X chromosome, specifically the locus Xq21.3-q22 [5]. In humans, a transcript for a Tβ4 variant encoded on the Y chromosome has been published, but a resultant protein product has never been isolated[6]. In the murine setting, the gene for Tβ4; *tmsb4x*, is again located on the distal arm of the X chromosome at Xq21.33-q22 and consists of three exons and two introns[7]. Alternative splicing is known to affect transcription of the *tmsb4x* gene, although this appears to be unique to the murine orthologue. In most tissues, a transcript including all three of the standard exons is produced. However, in lymphoid tissue, an alternative transcript, made by extending exon 1 with an alternative downstream splice site, is made[7]. The upstream 5' region to the *tmsb4x* gene possesses many of the properties of a promoter. Namely, it has an initiator site and several consensus sequences for transcription factors such as Oct-1, AP1, c-jun and AP3. Partial homologies to consensus sequences for retinoic acid and interferon response elements have also been identified[7].

1.5 The Structure of Tβ4

Tβ4 in its native form is 43 amino acids in length. The N-terminal serine always appears to be acetylated. In addition, the Tβ4 molecule can also exist in an oxidised form known as Tβ4 sulphoxide, wherein the methionine residue at position 6 of the protein is oxidised. In aqueous solutions, Tβ4 is unstructured and exists in a free conformation. However, in fluorinated alcohols, the N- and C- terminals of the molecule form α helices[8] (**Fig. 1.1**). It is thought that this reflects the structure of Tβ4 *in vivo* when bound to monomeric G-actin[9].

1.6 Tβ4 as an Actin Binding Molecule

Initial functional studies of Tβ4, identified it as being identical to the actin binding protein up until that point known as Fx[10]. Subsequent work has demonstrated that Tβ4 has a critical effect on the formation of F-actin and as a consequence influences the cytoskeletal architecture of cells. Actin is a 42kDa molecule ubiquitously present in eukaryotic cells. This monomeric or G-actin can polymerise into long, straight or branched filaments which are the building blocks for cellular structures such as microfilaments and thin filaments. The polymerised form is known as G-actin.

1.7 The Dynamics of F-Actin Formation

The formation of polymerised filamentous or F-actin is one of nucleation and elongation. Actin nucleating molecules such as Arp2/3 bind ATP-G-actin in association with WAVE proteins to create a new nucleus for actin polymeristaion.

Following the creation of actin nuclei, ATP-bound G-actin can associate with the growing filament, concomitant with the hydrolysis of ATP to ADP[9]. Thus, there exist two ends to a growing actin filament known as the + or "barbed" end and the - or "pointed" end. These two ends of the filament have different association and dissociation constants for the further addition/removal of G-actin. Net polymerisation of the filament occurs until the concentration of free G-actin drops to a level (known as the critical concentration) approaching that of the dissociation constant of the barbed end. At this point, an equilibrium is reached, whereby the rate of G-actin addition at the barbed end is equal to the rate of G-actin dissociation at the pointed end. In this state, a "treadmilling" occurs along the F-actin molecule as actin subunits migrate from the barbed to the pointed end of a filament of unchanged length[11]. However, rather than existing in this state *in vivo*, F-actin filaments are likely to be "capped" by capping proteins binding to the barbed end – decreasing the ability of G-actin to associate/dissociate from the barbed end. This reduces the critical concentration to a value close to the (much higher) dissociation constant of the pointed end[9].

1.8 The Function of Tβ4 as a G-Actin Buffer

One model for how Tβ4 might influence this process relies on Tβ4 binding G-actin in a 1:1 complex. Given that the dissociation constant for the Tβ4/G-actin interaction is lower than the dissociation constant for the F-actin/G-actin interaction at the pointed end, and that Tβ4 is usually present at high concentrations in cells, this means that most G-actin is bound in complex with Tβ4. This lowers the concentration of free G-actin to below that of the critical concentration, with the effect of stalling F-actin

polymerisation. As a result, a resting cell can maintain a constant cytoskeletal arrangement in the presence of a high G-actin concentration – although this G-actin exists in a Tβ4-sequestered state. Such a situation permits the cell, upon activation to rapidly reform a different cytoskeletal conformation without having to endure the lengthy process of *de novo* G-actin synthesis.

Upon cell stimulation, actin polymerisation can re-start through a variety of mechanisms, which rely on the production of new free barbed ends[12]:

- The removal of capping proteins.
- The cleavage of F-actin filaments to form daughter filaments each with a barbed end.
- Formation of new sites of actin nucleation.

Thus, as the number of free barbed ends increases, the critical concentration for the association between F-actin and G-actin decreases until it is below that of the dissociation constant for the G-actin/Tβ4 interaction. At this point, G-actin is free to dissociate from Tβ4 to become available for the formation of new actin filaments. It is thought that Tβ4 acts purely in a passive manner, to buffer the intracellular pool of G-actin and that post-translational modification of the Tβ4/G-actin interaction does not serve as a regulatory mechanism for F-actin polymerisation[9] (**Fig. 1.2**)

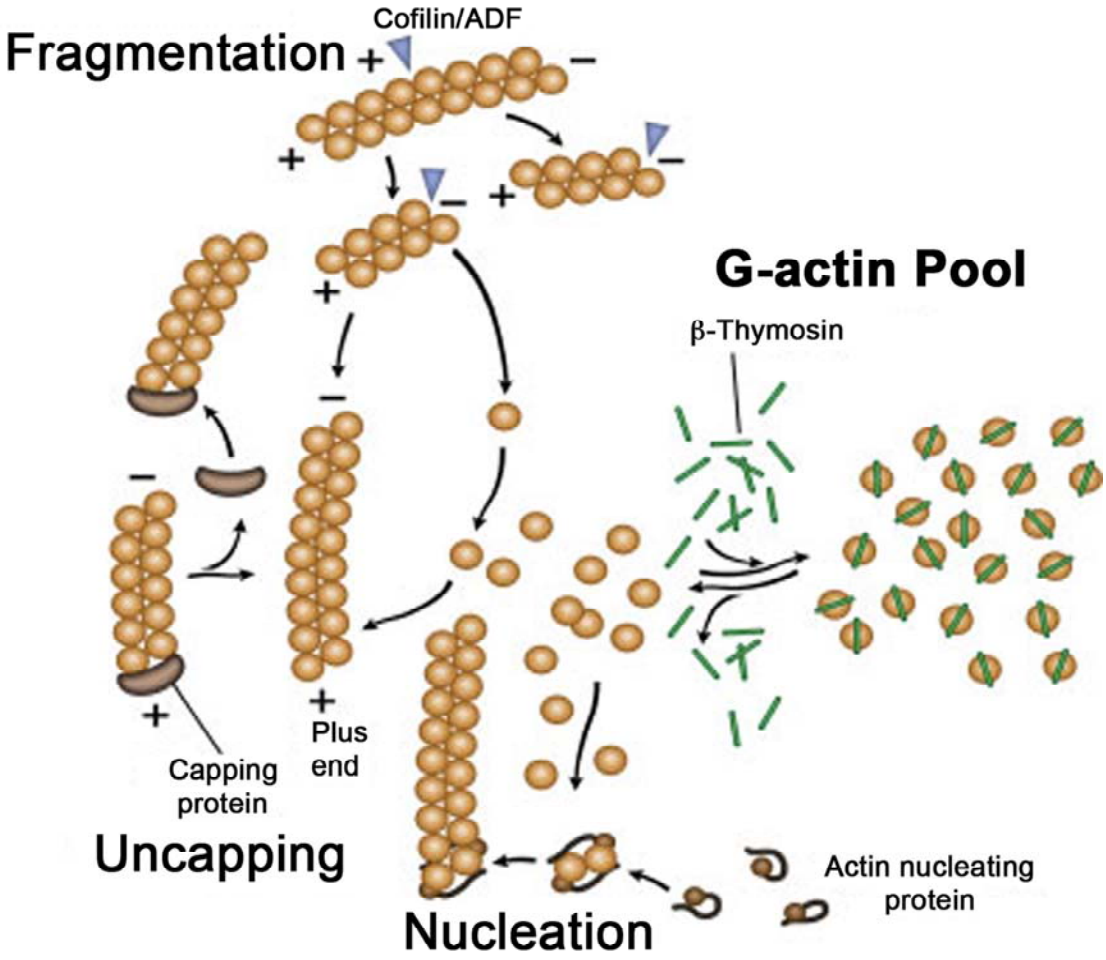


Fig. 1.2
Tβ4 acts to maintain a buffered pool of G-actin

Diagram to summarise the action of Tβ4 in control of actin polymerisation and depolymerisation. Adapted from [9].

1.9 Limitations of the Tβ4/G-Actin Model

Although there is considerable evidence for this mechanism of action in Tβ4 dependent cytoskeletal regulation[13], a number of observations do not fit this pattern. When Tβ4 is overexpressed in cells, it has been observed to bind to F-actin and promote the formation of stress fibres – large structures made up of polymerised F-actin. Indeed, Tβ4 can even be chemically crosslinked to F-actin[14,15].

An explanation for this activity perhaps lies in Tβ4's structure. Site directed mutagenesis studies have demonstrated that amino acids 17-22 of Tβ4 – the LKKTET domain, are responsible for Tβ4's ability to bind actin. This region, when supplemented with an N-terminal α-helix (as in Tβ4), is known as the beta thymosin domain, or alternatively as the WASP-homology (WH2) domain, as it is also found in proteins of the Wiskott-Aldrich syndrome protein (WASP) family. Many other proteins such as N-WASP, Scar/WAVE and the *drosophila melanogaster* protein Cibulot contain this WH2 domain and serve as actin nucleating factors[16,17]. It appears that, under certain circumstances, Tβ4 may also be able to function in this role.

1.10 Functional Consequences of the Tβ4/G-Actin Interaction

The ability of intracellular Tβ4 to affect cytoskeletal dynamics has been shown to have important consequences for the functional capabilities and phenotypes of cells. It has been demonstrated that photoactivation of caged Tβ4 in the lamellipodia of migrating cells can arrest their forward movement via a decrease in lamellipodial activity, presumably by an abrupt cessation of actin treadmilling[18]. Similar results have been obtained by transiently transfecting cells with Tβ4; leading to a rapid increase in intracellular Tβ4 concentration. This has the effect of abolishing the cells' directional movement[19]. Similarly, when HeLa cells, lining the edge of an *in vitro* induced wound, are injected with Oregon green labelled Tβ4, it is only uninjected cells, which are able to migrate and close the wound[9].

However, there is also evidence, which contradicts this particular interpretation of Tβ4 function. Knock down of Tβ4 through the use of siRNA in tumour cells,

inhibited the ability of these cells to migrate towards a scratch wound *in vivo*[20] – a function opposite to that expected if Tβ4 was acting to reduce lamelliopial formation. In addition, overexpression of Tβ4 by stable (as opposed to transient) transfection of cells, seems to lead to increased migratory behaviour[21]. The explanation for this paradoxical observation was that the increased levels of Tβ4 somehow stimulated increased expression of actin so that the free G-actin to F-actin ratio remained constant. Increased Tβ4 also stimulated the synthesis of other actin binding proteins leading to increased nucleation of F-actin filaments and consequent increased motility of cells[21].

1.11 The Effects of the Tβ4/G-Actin Interaction on Tumour Metastasis

Metastasis is the ability of tumour cells to form a cancerous lesion at a site distinct from its primary location. Amongst many factors, this process relies on cell motility and migration to take place – it has been noted that metastatic tumour cells are more motile than non-metastatic tumour cells[22]. Thus, one might expect that metastatic tumour cells might show decreased levels of expression and activity of Tβ4. This is exactly what has been observed by Yamamoto et al. in colorectal carcinomas[23]. However, these results were contradicted by Clark et al., who demonstrated that Tβ4 expression correlated with increased metastatic capability, in melanoma cells[24]. Thus, it is fair to state, that the effect of intracellular Tβ4 on actin cytoskeleton dynamics and cell migration is an, as yet, incompletely understood topic.

1.12 The Function of Extracellular Tβ4

In recent years much of the research focus on Tβ4 has shifted from understanding its role as an intracellular G-actin binding molecule to an extracellular pleiotropic factor. Not only does Tβ4 function as a G-actin buffer, but when it is applied pharmacologically to a variety of *in vitro*, *ex vivo* and *in vivo* models, it also displays a wide variety of effects on cell activity and phenotype (**Fig. 1.3**). Possession of such a range of functions in both the intra- and extracellular niche has led to Tβ4 being dubbed a moonlighting protein[4].

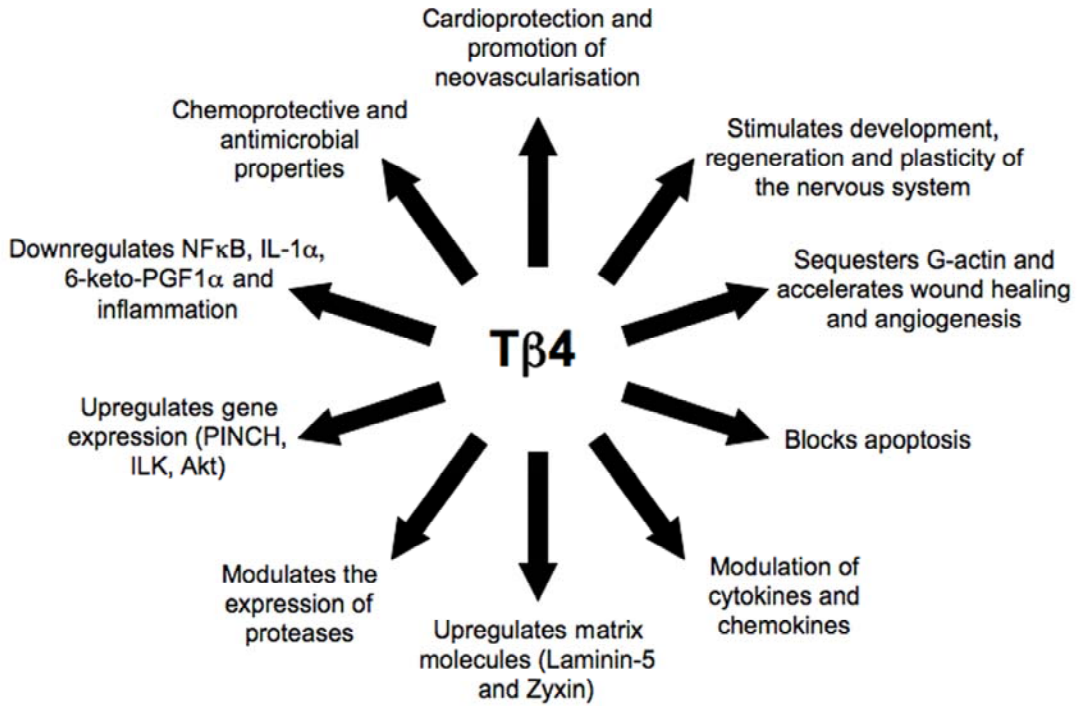


Fig. 1.3
The myriad functions of Tβ4

Schematic diagram summarising the biological actions of Tβ4. Based on [1].

1.13 The Effects of Tβ4 on Apoptosis

One of the primary effects that Tβ4 appears to have on cells is to prevent apoptosis. Sosne et al. found that both ethanol induced apoptosis of human corneal epithelial cells and chlorhexidine induced apoptosis of human dermal fibroblasts was reduced when these cells were treated with Tβ4 *in vitro*[25,26]. Furthermore, such an effect has been shown to have relevance *in vivo*. When myocardial infarction (MI) is experimentally induced in mice; systemic administration of Tβ4 through the intraperitoneal or intracardiac routes dramatically reduces apoptosis of cardiomyocytes in the infarct zone[27].

1.14 The Anti-Inflammatory Effects of Tβ4

It has also been demonstrated that Tβ4 exerts a number of anti-inflammatory effects on cells. One system in which the anti-inflammatory properties of Tβ4 have been studied extensively, is in the setting of corneal epithelial injury. When rat corneas are experimentally injured through exposure to alkali, twice daily topical treatment with Tβ4 leads to a reduction in levels of pro-inflammatory cytokines such as IL-1α, IL-1β, MIP-1α, MIP-1β, MIP-2 and MCP-1. Possibly as a consequence of this, The Tβ4 treated corneas show a reduction in the number of infiltrating polymorphonuclear neutrophils[28,29]. The oxidised form of Tβ4, Tβ4-SO, has also been shown to possess this same ability to inhibit neutrophil chemotaxis and as a result reduce carrageenin-induced oedema of the mouse paw[30].

The effects of Tβ4 treatment on inflammatory cytokine production may have several underlying causes. One possibility is that Tβ4 directly affects the expression and activity of the transcription factor NF-κB. NF-κB plays a central role in the inflammatory response and regulates immune functions as diverse as lymphoid organogenesis, development and maturation of innate and adaptive immune cells, and signalling through Toll-like receptors[31]. Corneal epithelial cells treated with Tβ4 demonstrate blunted responses in NF-κB activation and upregulation, in response to treatment with the pro-inflammatory mediator TNF-α[32]. This implies that Tβ4, under certain circumstances and in certain cell types, can directly regulate NF-κB.

1.15 The Anti-Inflammatory Effects of Tβ4 as a Treatment for Sepsis

As well as effects on rate of corneal wound healing, the anti-inflammatory properties of Tβ4 might have many other functional benefits in treating disease pathology. Septic shock is a syndrome of life threatening hypotension, caused by an inability to maintain an effective plasma volume due to leakage into the non-vascular extracellular space. This is the result of increased permeability of blood vessels stimulated by the toxic products of cell death and bacterial infection. Often, the clinical syndrome is complicated by the presence of high circulating levels of actin released into the bloodstream by dying cells[33]. It is thought that when released into the blood plasma, G-actin has a propensity to polymerise and form circulating F-actin filaments. These filaments can serve as nuclei for the formation of pulmonary clots and can lead to death[34].

It has been observed that in patients with septic shock, levels of naturally circulating Tβ4 are decreased, implying that Tβ4 has been used up in the circulation to buffer free G-actin and prevent deleterious F-actin formation[4]. Furthermore, induction of septic shock in mice through challenge with LPS, can be treated by the administration of Tβ4; thereby significantly improving their survival[35]. Given Tβ4's ability to also down regulate levels of the inflammatory mediator IL-1α in this setting, it becomes an interesting candidate for treatment of septic shock.

1.16 Tβ4 as a Treatment for CNS Inflammation

Tβ4's anti-inflammatory properties have also been tested to treat a mouse model of multiple sclerosis. Multiple sclerosis can be induced in wild type mice via co-injection of myelin basic protein and an adjuvant, to stimulate a host immune response against the myelin sheathing of oligodendrocytes. When animals such as these are pre-treated with Tβ4, there is a significant reduction in inflammatory infiltrate[36]. This indicates that Tβ4 may be a novel candidate for treatment of multiple sclerosis. As such, a company by the name of "RegeneRx Biopharmaceuticals Inc." are currently trialling recombinant Tβ4 in phase 2 clinical trials for multiple sclerosis (http://www.regenerx.com/wt/page/clinical_trials).

1.17 The Action of Pharmacological Tβ4 on Wound Healing

In addition to its properties as an anti-apoptotic and anti-inflammatory agent, much recent research has been conducted centring around the role of pharmacologically administered Tβ4 in regenerative medicine. Initial interest first appeared after it had

been demonstrated that topical or intraperitoneal administration of Tβ4 to full thickness dermal wounds in rats was able to accelerate re-epithelialisation of the lesions[37]. It is thought that Tβ4 acted as a migratory stimulus to keratinocytes in this model. Following this, it has also been observed that Tβ4 accelerates wound healing in several other model systems involving corneal damage[38], epidermolysis bullosa[39] and venous stasis ulcers[40].

1.18 Tβ4 in Neuroregeneration

Surface epithelium is not the only location in which Tβ4's regenerative properties have been observed – there is now much evidence, that suggests that Tβ4 may exert neuroregenerative effects. In the previously described animal model of multiple sclerosis, Tβ4 was not only able to dampen the inflammatory response but also appeared to stimulate the expansion of endogenous oligodendrocyte progenitor cells[36]. Tβ4 has also demonstrated regenerative activity in an animal model of stroke. After embolic occlusion of the middle cerebral artery in rats, Tβ4 treatment led to significantly improved functional recovery concordant with an increase in oligodendrocyte progenitor cells and remyelinating oligodendrocytes[41].

1.19 Tβ4 in the Cardiovascular System

Taken as a whole, these observations that pharmacological and extracellular Tβ4 can have profound roles on apoptosis, inflammation and wound healing imply that Tβ4 cannot simply be an intracellularly acting, G-actin buffering molecule but has potent and pleiotropic effects on cell signalling. Yet, there is currently a profound lack of

understanding about how these different functions of Tβ4 are regulated and segregated. This has been termed the "β thymosin enigma" in the literature[42]. The lack of a traditional secretory signal sequence in Tβ4's amino acid sequence have led some to postulate that Tβ4's apparent effects on extracellular signalling are artefactual and that Tβ4's function is solely intracellular[43]. Nowhere are these paradoxical views of Tβ4's function more apparent than studies of Tβ4's role in the developing cardiovascular system.

The formation of a competent and functional vasculature in the developing embryo is necessary to deliver oxygen and nutrients to the growing tissues once simple diffusion is no longer adequate for this purpose. Tβ4 is thought to play several critical roles in this process.

1.20 The First Embryonic Blood Vessels Are Formed By the Process of Vasculogenesis

The first blood vessels in the embryo proper arise as the concluding result of a process known as vasculogenesis. Vasculogenesis is the process by which *de novo* blood vessels arise out of previously undifferentiated tissue. It involves a number of linked events. First of all, progenitor angioblast cells must be specified and differentiated into endothelial cells from the surrounding tissue. These endothelial cells must then migrate towards one another in order to coalesce into cord-like structures. These cord-like entities must then transform themselves from solid cylinders to hollow, lumen bearing vessels (**Fig. 1.4**)[44,45].

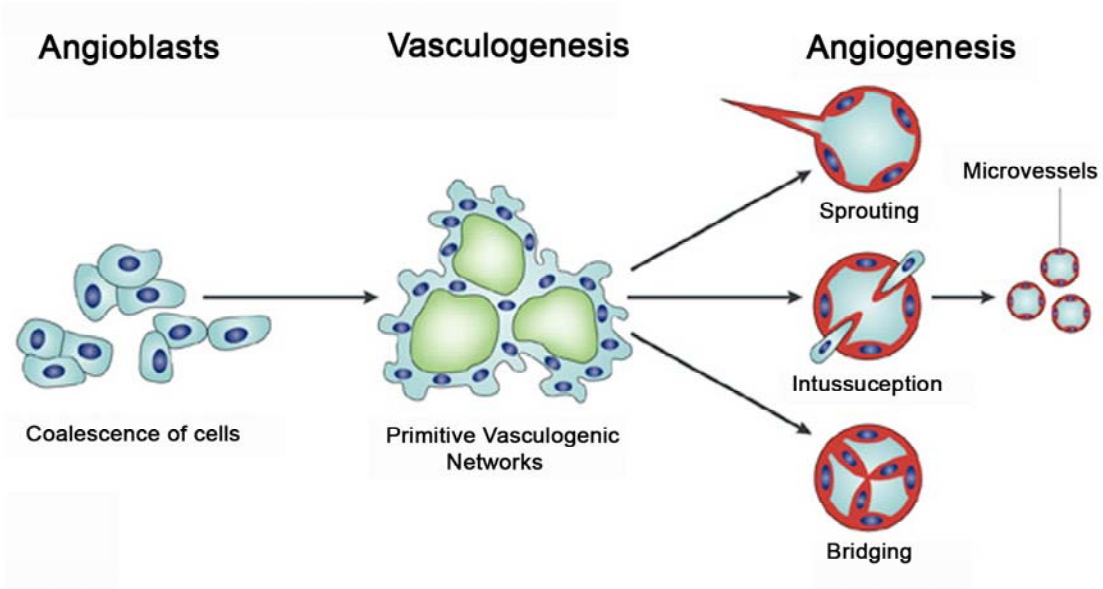


Fig. 1.4
Mechanisms of blood vessel development

Schematic diagram to demonstrate the principle mechanisms of vascular development in the embryo. Formation of the first blood vessels such as the dorsal aorta arise through coalescence of angioblasts in the process of vasculogenesis. Subsequent vessel formation occurs via angiogenesis in either its sprouting, intussuceptive or bridging forms. Adapted from[46].

1.21 The Role of Tβ4 in Vasculogenesis

Although definitive evidence that Tβ4 influences this process in the *in vivo* setting has not yet been obtained, there is a lot of evidence from *in vitro* and *ex vivo* experiments that Tβ4 might have a part to play in this process. When human umbilical vein endothelial cells (HUVECS) are cultured on an artificial extracellular membrane known as Matrigel, the endothelial cells form tube like structures. Thus, this system has been used as an *in vitro* model of vasculogenesis. It has been shown that when HUVECs are cultured on Matrigel, they upregulate their expression of Tβ4 concomitant with the attachment of the HUVECs to the artificial matrix and spreading of the endothelial cells[47]. These experiments also highlight the nature of the Tβ4 enigma. It is unclear whether the upregulated Tβ4 seen in these HUVECs is acting

intracellularly or as a paracrine factor. One might assume that given the migrating, attaching and spreading nature of the endothelial cells in this experiment, that Tβ4 must be acting in its role as a regulator of the intracellular cytoskeleton, yet this is disputed by other observations. Namely, when exogenous Tβ4 peptide is added to this system, the incorporation of HUVECs into tubes increases, implying that the upregulation of Tβ4 seen in these cells could be acting in its role as an extracellular signalling molecule[48].

It is also possible that Tβ4 may have a function in the initial endothelial differentiation stages of vasculogenesis. The N-terminus of Tβ4 can be cleaved to the short peptide Ac-SDKP by the enzyme prolyl oligopeptidase[49]. When this peptide is added exogenously to murine embryonic epicardial explants, it can stimulate the differentiation of epicardial derived progenitor cells (EPDCs) to an endothelial cell phenotype[50]. Similarly, when differentiating embryoid bodies are cultured in the presence of Tβ4, they upregulate a number of endothelial markers[51]. Such experimental data are consistent with a role for Tβ4 in the initial differentiation of endothelial cells in the embryo proper. It is only large, essential vessels such as the aorta, which form in the embryo via the process of vasculogenesis[44,45]. The aorta forms through the vasculogenic coalescence of endothelial cells arising from the aorta, gonad, mesonephros region of the splanchno-pleural mesoderm. Immediately prior to formation of the dorsal aorta, Tβ4 is expressed in this region of the mesoderm providing further evidence for a possible role of Tβ4 in this process[52].

1.22 Secondary Blood Vessel Formation Occurs Via Angiogenesis

Once the initial, major vessels of the embryos are formed, the other vessels are formed through the process of angiogenesis. Angiogenesis is the formation of two or more daughter vessels from an already formed vessel. This may be through sprouting angiogenesis whereby a new vessel buds out and branches off from an existing vessel, or through intussusceptive angiogenesis where a blood vessel is split into two or more separate conduits along its longitudinal axis through growth of an endothelial pillar down the centre of the lumen (**Fig. 1.4**)[44]. In practice, sprouting angiogenesis is thought to be the more common process and the best studied. It is also the angiogenic process in which Tβ4 has been most extensively studied.

1.23 The Molecular Events Underlying Sprouting Angiogenesis

Sprouting angiogenesis can be broken down into several molecular and cellular events. The first step in sprouting angiogenesis is thought to be mediated by a hypoxic stimulus, which acting through the hypoxia inducible factor (HIF) pathway, causes an increase in the production of nitric oxide (NO). This leads to an increase in vessel diameter. Concurrently, higher concentrations of the vascular endothelial growth factor (VEGF) family of molecules, act on VEGF receptors on the endothelium to mediate an increase in vascular permeability. This is combined with the release and activation of matrix metalloproteases and proteinases, which digest the extracellular matrix (ECM) surrounding the blood vessel. Endothelial cells on the existing vessel, which have become specified as endothelial tip cells, then invaginate

into the surrounding tissue and migrate along gradients of pro-angiogenic factors such as VEGF. In response to mitogenic molecules, endothelial stalk cells then proliferate along the path of the nascent vessel. It is usual for a plexus of new blood vessels to form in this situation before the process of remodelling decreases the amount of branches in the newly vascularised area. Finally, vessels mature through the recruitment of pericytes and smooth muscle cells[44].

1.24 A Role for Tβ4 in Sprouting Angiogenesis

It appears that Tβ4 can act on several steps in this process. The first point of intersection between the events of sprouting angiogenesis and Tβ4 function may be at the initial stage of HIF induction. Although not yet demonstrated in any endothelial system, Tβ4 has been shown to stabilise the formation of HIF1α in the HeLa human cervical tumour cell line[53]. If such a phenomenon occurs *in vivo* in endothelial cells, it is possible that paracrine Tβ4 could act as an initiating stimulus in sprouting angiogenesis. Compelling lines of evidence that Tβ4 can act as this initiating factor comes from several *ex vivo* experimental systems. First of all, when exogenous Tβ4 is added to coronary ring explants derived from the coronary arteries of pigs, it is able to stimulate the sprouting of capillary branches from the coronary ring[48,54]. Secondly, when Tβ4 peptide is added to chick chorioallantoic membranes, it can induce the appearance of vascular branches on the membrane surface[54].

1.25 A link between Tβ4 and MMP Secretion

Once sprouting angiogenesis has been initiated and endothelial tip cells specified, the new blood vessels form a sprouting front which migrates along a VEGF gradient into hypoxic tissue. This is typified by the complex of migrating endothelial tip cells from the optic neurovascular bundle to the periphery of the retina, in the model of postnatal retinal angiogenesis[55]. In order to accomplish this migration, endothelial cells must secrete matrix metalloproteases (MMPs) and proteinases to digest the tissue into which they are to migrate[44]. Treatment of cells with Tβ4 has been shown to stimulate such production. In particular it can induce the secretion of MMPs 1, 2 and 3 from endothelial cells[56]. Interestingly, the link between Tβ4 and MMP production has been shown to depend on both extracellular exogenous Tβ4 and intracellular Tβ4 as determined following RNAi mediated knockdown of Tβ4 in HUVECs[43].

1.26 The Function of Tβ4 in Endothelial Cell Migration

Tβ4 also seems to play a key role in the migratory mechanics of endothelial cells. Once more, this seems to be the case irrespective of whether Tβ4 is being exogenously added or endogenously deprived. In Boyden chamber assays, used to measure the *in vitro* migratory capacity of cells, exogenous Tβ4 was able to stimulate the migration of HUVECs and human coronary artery endothelial cells but not foreskin fibroblasts, aortic smooth muscle cells, monocytes or neutrophils indicating a specific stimulatory effect of Tβ4 on endothelial cells[57]. In other experiments, RNAi mediated knockdown of endogenous Tβ4 in endothelial cells, compromised the ability of these HUVECs to migrate efficiently. However, the effect of RNAi

knockdown of Tβ4 was not solely to inhibit the migration of these HUVECs – the precise nature of the migration abnormality was dependent on the degree of Tβ4 knockdown. In the case of partial knockdown due to transient transfection of HUVECs with the Tβ4 RNAi construct, HUVEC migration increased. This was thought to be due to a reduction in the ability of the cell to buffer monomeric G-actin, thus allowing the cell to form more F-actin and migrate faster. However, when Tβ4 was knocked down, almost completely, via stable transfection with the Tβ4 RNAi construct, the pro-migratory effects on G-actin were outweighed by the greatly reduced production of MMPs, leading to decreased migration of the cell[43]. Thus, it can be concluded that regulation of Tβ4 plays an essential role in controlling the migratory capacity of endothelial cells.

1.27 Mural Cell Recruitment is Essential for the Formation of Stable Blood Vessels

Once endothelial tubes have formed either through the processes of vasculogenesis or angiogenesis, it is necessary for them to recruit an outer layer of mural cells. Such mural cells exist on a phenotypic continuum between cells, with some cells possessing the character of vascular smooth muscle cells, whilst other cells display the characteristics of pericytes. Vascular smooth muscle cells provide structural support for endothelial tubes and have the capacity to contract with the purpose of either maintaining blood pressure or regulating blood flow into vascular beds. Pericytes meanwhile are thought to provide molecular interactions with the endothelial cell layer, necessary for maintenance of endothelial cell health[58,59].

1.28 Mechanisms of Mural Cell Recruitment

In the developing embryo, mural cell recruitment tends to take place via one of two principal mechanisms. Blood vessels in mesodermal tissues tend to derive their mural cell coats through the *in situ* differentiation of mesoderm into mature mural cell tissue[60]. This process is thought to be largely dependent on endothelial secreted TGF-β, which has been shown to stimulate the differentiation of mesodermal progenitor cell lines into vascular smooth muscle tissue. In contrast, blood vessels of the central nervous system (CNS) recruit their mural cells via the migration of phenotypically mature mural cells from non-CNS vascular beds. This process is thought to be dependent on endothelial secreted PDGF-BB acting as a chemoattractant for mature mural cells[58,59]. Although there are other factors which affect and direct mural cell development, the best studied are components of the PDGF-B, Notch and TGF-β signalling axes.

1.29 PDGF-B Signalling in Mural Cell Development

The PDGF-B/PDGFRβ ligand/receptor pair is one of the most important molecular determinants of mural cell development. Most of the advances in the understanding of how PDGF-B/PDGFRβ affect vascular development have come from the observation of transgenic mice. Global knockout of either PDGF-B or PDGFRβ leads to perinatal lethality in mice due to endothelial hyperplasia, abnormal immature endothelial cell-cell junctions and excessive luminal invaginations – all secondary to a lack of mural cell ensheathment of blood vessels[61-66]. The model currently accepted to explain these data, is that sprouting endothelial tips secrete PDGF-B into the

extracellular space. It is then bound to heparan sulphate proteoglycans in the extracellular matrix and on the endothelial cell surface to create a chemoattractant gradient for migrating mural cells[58] (**Fig. 1.5**).

It is thought that PDGF-B facilitates the migration and proliferation of mural cells rather than their differentiation as, in the PDGF-B/PDGFRβ knockouts, initial induction of the mural cell population from the undifferentiated mesenchyme surrounding nascent blood vessels is not impaired[62]. These findings also seem to explain why the blood vessels of the CNS are most affected by the loss of PDGF-B/PDGFRβ signalling. The CNS lacks vasculogenesis competent mesenchyme and so must recruit all of the mural cells required for blood vessel investiture from outside sources. Thus the effect of loss of PDGF-B/PDGFRβ is most keenly felt in tissues such as the retina where no mural cell can be induced by PDGF-B independent means[58].

1.30 The Mechanism of PDGF-B Signalling

PDGF-B/PDGFRβ signalling is an example of receptor tyrosine kinase signalling. PDGF-B is generally secreted as a homodimer; PDGF-BB, which binds to a PDGFRββ homodimer[67]. Upon ligand binding, the receptor autophosphorylates at tyrosine residue 857 (in human)[68,69]. This allows the receptor to activate a number of downstream pathways including Src kinase, PI3K, PLCγ and Ras[67]. Such pathway activation will eventually lead to transcription of target genes, allowing the cell to proliferate and migrate in the direction of the growth factor gradient.

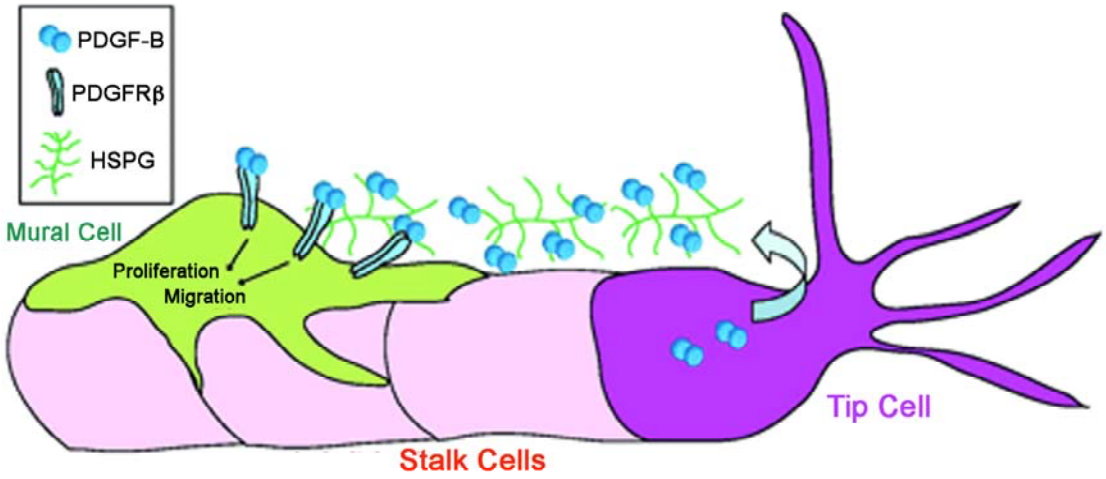


Fig. 1.5
The role of PDGF-B in mural cell development

PDGF-B is secreted by endothelial cells and acts as a proliferative and migratory stimulus for mural cells. PDGF-B may be retained in the peri-vascular extracellular space through associating with heparan sulphate proteoglycans. Adapted from [58].

1.31 The Biology of Notch Signalling

Members of the notch family of receptors play a crucial part in several aspects of vascular development. There are four Notch receptors; Notch1, 2, 3 and 4, each of which is synthesised as a single polypeptide chain before being cleaved into non-covalently linked intracellular (NICD) and extracellular (NECD) domains by the protease enzyme furin[70]. The NECD of each Notch receptor contains 29-36 EGF-like repeats which bind to one of five canonical Notch ligands; Delta-like ligand (DLL) 1, 3 or 4 and Jagged (Jag) 1 and 2. Each of these ligands also contain a variable number of EGF domain repeats (8 in Jag proteins, 15-16 in Dll) which bind to the NECD. Upon ligand binding, the NECD is cleaved by extracellular proteases of the ADAM/TACE family. This induces a conformational change in the remaining part of the protein, which allows the enzyme γ-secretase to perform a second proteolytic cleavage and release the NICD into the cytoplasm. The NICD then translocates to the

nucleus where it binds the DNA-binding protein RBP-J/CSL. The complex which forms is then able to bind DNA and stimulate transcription of stereotypical Notch target genes such as members of the Hairy/Enhancer of Split (Hes), Hes related protein (Hey) and Fringe proteins (**Fig. 1.6**)[70].

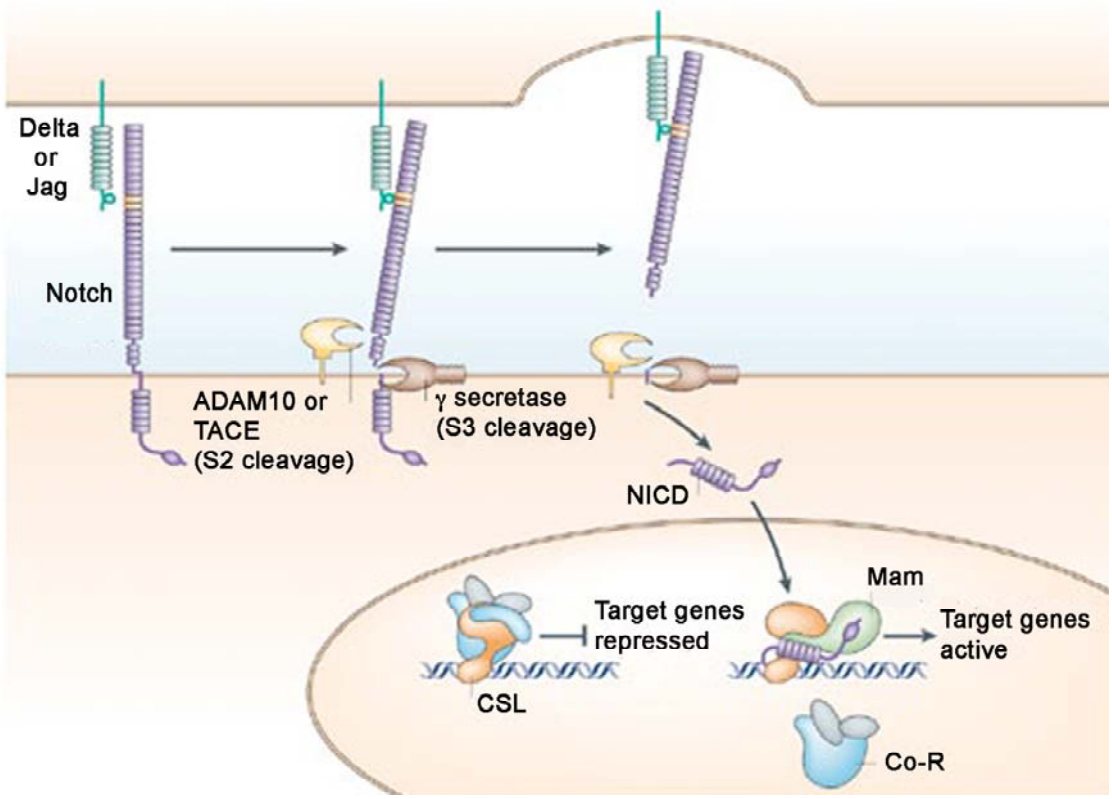


Fig. 1.6
Key molecular events in the Notch signalling cascade

Binding of a Notch ligand to a Notch receptor leads to sequential cleavage of the receptor by members of the ADAM/TACE and γ-secretase complexes. The free NICD is then able to translocate to the nucleus and stimulate Notch target gene transcription. Adapted from[71].

1.32 Arterio-Venous Specification by Notch Receptor Signalling

Different flavours of Notch signalling have different effects on the development of the vasculature. As previously mentioned, the first vessels of the embryo proper, the

dorsal aortae and the cardinal veins, are formed in a process known as vasculogenesis, where haemangioblast progenitors coalesce to form endothelial tubes[45]. Notch signalling is thought to play an essential role in whether early endothelial cells will assume a venous identity and become part of the cardinal veins or an arterial identity and help form the dorsal aortae. Various Notch ligands and receptors are expressed in arterial-fated endothelial cells and knockout of different Notch components, in several models, leads to vascular malformation and a deficiency in the expression of arterial identity genes in arterial endothelial cells, which take on a more venous molecular signature[70].

1.33 Notch is a Key Mediator of Arteriogenesis

The Notch ligand Dll1 is also a key regulator of arteriogenesis. This is the process by which small arteriolar vascular branches can transform into large calibre collateral arteries; usually in the setting of arterial blockade due to atheromatous vascular disease. Dll1 is thought to be essential to this process as Dll1 heterozygote mice fail to develop sufficient collaterals after experimentally induced hindlimb ischaemia[72]. This process is also compromised in Notch1 heterozygous mice[72].

1.34 An Essential Function for Notch Signalling in Sprouting Angiogenesis

Most recently, Notch signalling has been identified as one of the most important events in the initiation of sprouting angiogenesis. As mentioned earlier, when new blood vessels grow into an inadequately vascularised area of tissue by the process of

angiogenesis, it is endothelial cells, which have been selected as tip cells that lead the sprouting branches. Tip cells characteristically extend a number of dynamic filopodial processes into the surrounding tissue and migrate along VEGF gradients. Whilst doing this, they express the Notch ligand Dll4 at the cell surface boundary with endothelial stalk cells. The outcome of this Dll4 signalling event is to repress the tip cell phenotype in the endothelial stalk cells[63,73,74]. The effects of Dll4 in this setting, are thought to be mediated through interaction with Notch1, as in the endothelial specific inducible Notch1 knockout, which has mosaic expression of Notch1, Notch1 deficient cells are preferentially found at the tips rather than the stalks of endothelial sprouts. The Notch ligand Jag1 is thought to antagonise this process and thus appears to be a potent stimulator of angiogenesis and the tip cell phenotype.

1.35 Notch3 Signalling Stimulates Mural Cell Maturation

Signalling via the Notch3 receptor is thought to influence the process of mural cell development. The first evidence for this came from study of the human disease CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukencephalopathy). This disorder is characterised by progressive VSMC loss around arteries leading to micro-strokes and progressive dementia[75]. The causative mutations in CADASIL are always found in the human NOTCH3 gene[76]. Due to the effects of NOTCH3 receptor mutation on the vascular smooth muscle cell population in CADASIL patients, it was postulated that Notch3 might play a functional role in mural cell development in the embryo. This was confirmed by analysis of the Notch3 knockout mouse. Mural cells in the Notch3 knockout mouse appear to differentiate,

migrate and invest blood vessels as normal but appear to be defective in their maturation. They do not adhere to endothelial cells properly, display abnormal morphology and do not adopt a typical arterial mural cell gene signature[77]. It is thought that since Notch3 activation can regulate PDGFRβ gene transcription directly *in vitro*, that one reason for the defective mural cell maturation in Notch3 knockout mice may be a lack of PDGFRβ expression on mural cells. It has been confirmed that VSMCs in Notch3 knockout mice have reduced PDGFRβ expression and that primary VSMCs from a patient with CADASIL lacked PDGFRβ[78]. One as yet unsolved problem is that there is no known Notch ligand, whose expression correlates spatially and temporally with the onset of the VSMC maturation defects observed in the Notch3 knockout mouse[58]. Thus, one hypothesis which has been put forward is that Notch3 activation in this setting is dependent on the intraluminal blood pressure inside developing blood vessels[77].

1.36 TGF-β is a Molecular Effector of Vascular Development

Although it is thought that PDGF-B and Notch3 are the main molecules governing migration/proliferation and maturation of mural cells respectively, our understanding of the initial stages of mural cell differentiation from unspecified embryonic mesenchyme remains incomplete. However, it appears that TGF-β is a key molecular player in this process.

1.37 The Molecular Biology of TGF-β Signalling

The mammalian TGF-β signalling family consists of 42 different ligands divided into two families; the TGF-β/Nodal/Activin family and the BMP/GDF/MIS family. Mural cell differentiation has most closely been linked to function of the TGF-β/Nodal/Activin pathway[79]. These ligands have varying affinity for over 12 different TGF-β family receptors. These are divided into Type I receptors (7 members) and Type II receptors (5 members). Type III receptors such as cripto, endoglin and betaglycan also exist and whilst not participating directly in the process of ligand induced signal transduction, act to modulate the TGF-β signalling pathway[80].

Typically, TGF-β family ligands are active when present in their homodimeric form. In this situation, they are able to bind heterotetrameric complexes composed of two Type I and two Type II receptors with intrinsic serine/threonine kinase activity[79]. This binding stimulates the Type II receptor to phosphorylate the Type I receptor at multiple serine/threonine residues in the cytoplasmic domain of the receptor[79]. The phosphorylated Type I receptor is then able to phosphorylate a number of receptor-regulated (R-) Smad proteins. These Smad proteins include Smad 1, 2, 3, 5 and 8. Following phosphorylation, the R-Smad proteins go on to form homotrimers. These homotrimers then translocate to the nucleaus where they form heteromeric complexes with the co-Smad; Smad 4. The R-Smad/Smad 4 complex is able to bind DNA at locations where there is a minimal Smad binding element (SBE) consisting of the nucleotide sequence 5'-AGAC-3'[79]. Depending on the specific transcriptional co-activators or co-repressors present in the nucleoplasmic milieu, this binding of Smad

complexes to SBEs can either stimulate or suppress the transcription of target genes in a cell type specific manner.

1.38 The Termination of TGF-β Signalling

TGF-β signalling can be terminated in a number of ways. One of these is through the action of the I-Smads; Smad 6 and 7. Smad 6 achieves this by competing with Smad 1 for Smad 4 binding. The Smad 6/Smad 4 complex cannot modulate promotor activity and thus serves to downregulate TGF-β signalling. Smad 7 typically resides in the nucleus in the basal state, but moves to the plasma membrane upon TGF-β pathway activation. Here, it binds one of two ubiquitinating proteins Smurf 1 or 2. The Smad 7/Smurf complex can bind directly to the Type I TGF-β receptor and inhibit its ability to phosphorylate R-Smads. The Smurf proteins then tag the receptor for degradation in the proteasome. Often Smad 7 is transcribed as a result of TGF-β pathway activation to serve as a negative feedback loop to dampen pathway activity[79].

One other example of TGF-β pathway regulation of note, is that mediated by the deubiquitinating enzyme Usp9x. If Smad 4 is mono-ubiquitinated at lysine residue 519 by the nuclear factor ectodermin, it can no longer bind phospho-Smad 2 due to steric hinderance. The enzyme Usp9x reverses this ubiquitination; thus acting as a positive regulator of TGF-β pathway activity[81].

1.39 The TGF-β Pathway in Mural Cell Differentiation

Several members of the TGF-β pathway family have been implicated as having a functional role in the process of mural cell differentiation. TGF-β1 is thought to be the dominant ligand in TGF-β mediated vascular development as its mouse knockout has a lethality of 50% at the E10.5 stage of gestation. This is due to defective haematopoiesis and endothelial cell differentiation[82]. From these experiments it is clear that TGF-β has roles in endothelial cell as well as mural cell development. As normal endothelial cell development is a requirement for mural cell differentiation to take place, it is not clear from these studies alone whether TGF-β1 is the ligand responsible for TGF-β mediated mural cell induction, although when viewed in the context of other *in vivo* and *in vitro* studies, it is likely to be the responsible molecule.

Knockouts of the different TGF-β receptors expressed in the developing vasculature have different phenotypes. TGF-β RII is thought to be the Type II TGF-β receptor with the most important function in the events of vascular morphogenesis, as the vascular phenotype of the TGF-β RII null mouse is identical to that of the TGF-β1 mouse[83].

1.40 Two Type I TGF-β Receptors Function in TGF-β Mediated Vascular Development

At least two Type I TGF-β receptors appear to have importance in vascular developmental biology: Alk-1 and Alk-5. The Type I TGF-β receptor Alk1 is specifically expressed in the endothelium[84]. Knockout of Alk1 results in embryos,

which die during midgestation due to the formation of large arterio-venous shunts, as a consequence of impaired arterio-venous specification of endothelial cells[85]. Accompanying this phenotype are deficits in mural cell development. This is interesting as there is a defect in mural cell development as a consequence of absence of a gene expressed solely in the endothelium. Thus, it is thought that weakened cell autonomous TGF-β signalling in *cis* can lead to secondary defects for the mural cell population in *trans*.

1.41 The Function of Alk-5 During Vascular Development

There is controversy as to the role of the Type I TGF-β receptor Alk5. Early examination of the Alk5 knockout revealed an embryo that died between E10-E11 of gestation. Blood vessels in the yolk sacs of these embryos were large and dilated without any evidence mural cell investiture[86]. The authors of this study claimed to observe expression of Alk5 in the developing endothelium of wild types, and as such labelled the mural cell defects as being secondary to dysfunctional TGF-β signalling in the endothelial cells.

This interpretation has been disputed in a second study, in which the authors made use of transgenic Alk1-LacZ and Alk5-LacZ knock in mice[87]. These authors reported that the expression patterns of Alk1 and Alk5 did not overlap and that Alk1 expression was restricted to the endothelium, whilst vascular expression of Alk5 was confined exclusively to mural cells. The authors of this study also examined the vasculature of the embryo proper of the Alk5 knockout, rather than just the yolk sac vasculature. They observed that the endothelial morphology of blood vessels in the embryo itself

was proper and indistinguishable from wild type littermate controls with normal vessel lumen diameter and formation of a capillary plexus. However, mural cell investiture of blood vessels was dramatically reduced.

1.42 Two Models for the Function of TGF-β in Vascular Development

Thus, there are two competing models for the action of TGF-β on mural cell development. The first states that cell autonomous TGF-β signalling can take place in developing endothelial cells. This occurs through the interaction of endothelial derived TGF-β1 with endothelial cell membrane bound TGF-β RII complexed with either Alk1 or Alk5. Depending on the precise level of TGF-β stimulation, TGF-β would predominately activate one pathway preferentially over the other. If Alk1 becomes more strongly activated then it may be able to suppress Alk5 pathway activation and stimulate pro-migratory and pro-proliferative activity in the endothelial cells. Conversely, when Alk5 is more strongly activated at higher levels of TGF-β, the phenotype of the endothelial cell may switch to become matrix synthesising and allow the release of secondary factors, which stimulate mural cell development[88].

The second model for how TGF-β functions in vascular development, is that TGF-β has its primary effects on Alk1 signalling in endothelial cells. This mediates angiogenic effects such as increases in migratory and proliferative capacity. Endothelial cells will then secrete TGF-β1 which will act on undifferentiated mesodermal progenitor cells via an interaction with the Alk5 receptor expressed on these cells to stimulate their differentiation into mature mural cells[88].

Evidence for this second hypothesis comes from *in vitro* studies, which have used cell lines as surrogates for the process of mural cell differentiation from mesoderm. It has been observed that exposure of the mouse mesodermal 10T1/2 progenitor cell line to TGF-β *in vitro* upregulates expression of VSMC marker genes[89]. Moreover, if 10T1/2 cells are cocultured with endothelial cells in the absence of endothelial cell – 10T1/2 cell contact, then soluble factors produced by the endothelial cells can stimulate differentiation of the 10T1/2 cells into mural cells. This effect is abrogated by treating the cultures with anti-TGF-β antibody, thus implying that TGF-β is the soluble signal[90]. The effect is also apparent in other cell culture systems. Differentiation of embryoid body embryonic stem cells into VSMCs is impaired by interventions, which inhibit TGF-β signalling[91,92].

1.43 A Consensus Model for the Function of TGF-β in Vascular Development

Although it has been established that TGF-β does play a direct role in stimulating differentiation of a target progenitor cell population into mature mural cells, the most recent evidence suggests a consensus model with regards to the function of Alk5 in the process of vascular development. Carvalho et al. created mouse mutants in which Alk5 or TGF-β RII were conditionally deleted in either endothelial cells or VSMCs. In each of the four possible crosses, embryonic lethality due to abnormalities in mural cell development was observed[93]. This implies that intact Alk5 signalling is not only required in the mural cell progenitors or mature mural cells, but also in the endothelial cells in order for normal mural cell development to take place. Thus, the model, which is currently accepted, is one where cell autonomous TGF-β signalling in the endothelium, acting through either Alk1, Alk5 or both, stimulates secretion and

processing of TGF-β by the endothelial cell. This endothelial secreted TGF-β then acts on mural cell progenitors and immature mural cells to stimulate their differentiation into mature mural cells[88] (**Fig. 1.7**).

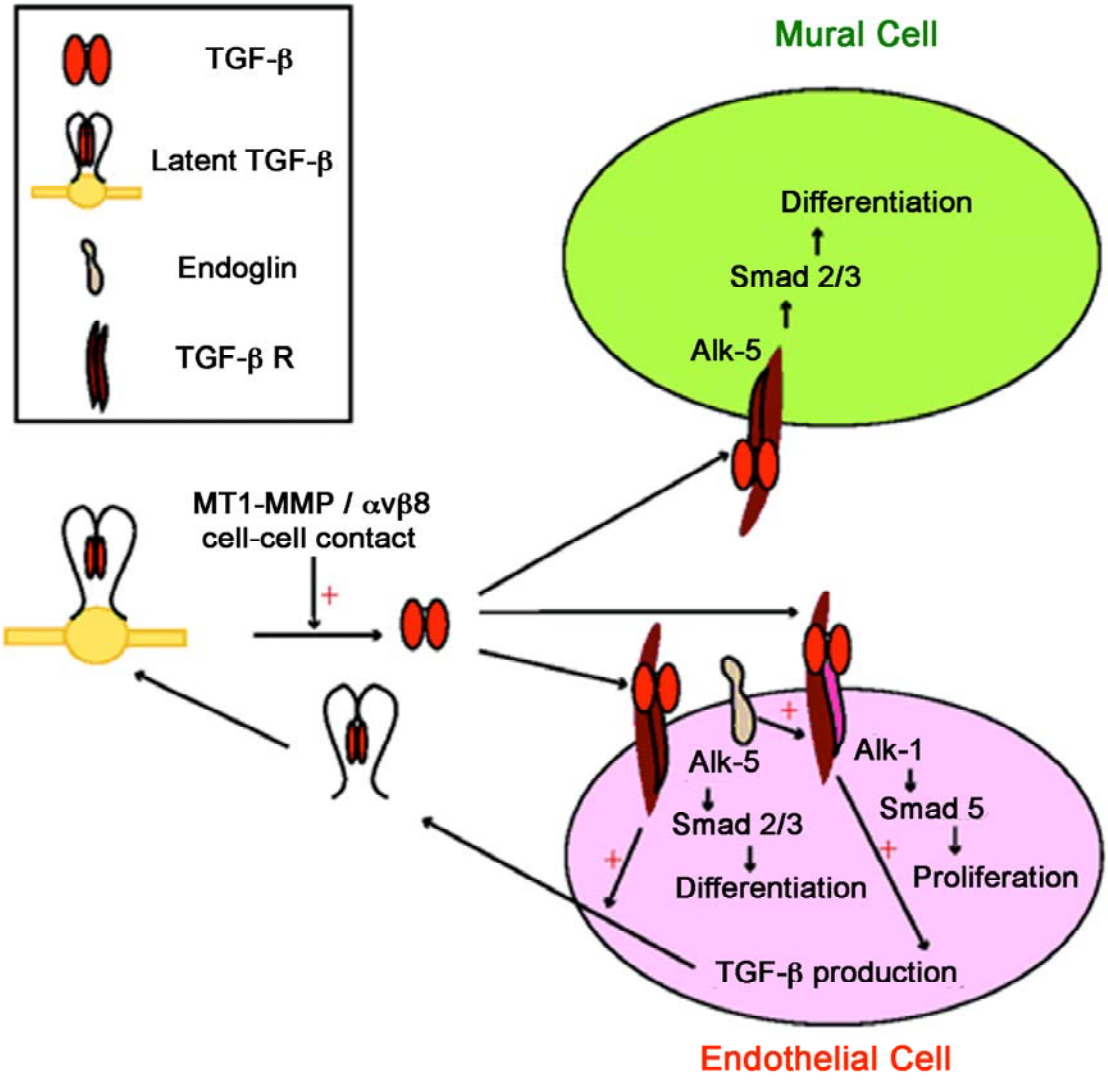


Fig. 1.7
A consensus model of TGF-β mediated vascular development

TGF-β has autocrine effects on endothelial cells and paracrine effects on developing mural cells. Endothelial autocrine signalling through Alk-5 promotes endothelial cell differentiation whilst signalling through Alk-1 is thought to stimulate proliferation. Endothelial secreted TGF-β can signal through Alk-5 on mural cell precursors to stimulate their differentiation into mature mural cells. Adapted from [58].

1.44 Connexins Regulate the Bioavailability of TGF-β During Vascular Development

Several other non-ligand, non-receptor molecules are also important for TGF-β mediated mural cell differentiation. Connexins 43 and 45 are components of cellular gap junctions with connexin 43 being expressed on endothelial cells and connexin 45 being expressed on mural cells. The phenotypes of both the connexin 43[94] and the connexin 45[95] knockout mice display lethality due to a lack of mural cell recruitment to developing blood vessels. It is thought, that the connexins either directly, or as a result of their signalling activity mediate cleavage of active TGF-β from its inactive secreted latency associated peptide (LAP) form[94].

1.45 Evidence of a Role for Tβ4 in the Process of Mural Cell Development

Evidence now exists that Tβ4 can, at least in certain stereotypical situations, influence this facet of vascular development. When embryonic epicardial explants are cultured in the presence of Tβ4, epicardial derived progenitor cells migrate out from the explant and are stimulated to differentiate into vascular smooth muscle like cells[96]. Moreover, when embryoid bodies deficient for the basic helix-loop-helix transcription factor *hand1* are cultured, it can be observed that they are also deficient in Tβ4 and do not express vascular smooth muscle cell markers. Culture of these embryoid bodies in the presence of exogenous Tβ4 restores the expression of vascular smooth muscle cell markers[51]. Taken as a whole, these data imply that Tβ4 has the potential to stimulate the differentiation of mural cells from progenitors under certain

circumstances. Whether this holds true *in vivo* as a wider function of Tβ4 in the vasculature is not yet known.

1.46 Development of the Heart

Development of the heart commences shortly after the process of gastrulation and formation of the three germ layers of the embryo. The first cells of the heart differentiate from a set of specialised progenitors present in an area of the anterior mesoderm known as the cardiac crescent or primary heart field[97]. Molecular cues involved in the initiation of this event are predominantly provided by members of the TGF-β and Wnt family of signalling cascades[98]. Following specification, the cardiogenic cells of the cardiac crescent coalesce in the ventral midline of the embryo to form the linear heart tube – the first structure which has contractile activity and is capable of circulating blood. Heart looping and remodelling then convert this simple cardiac tube into a four chamber entity resembling the fully mature adult heart. It is also likely that a second area of mesoderm known as the secondary heart field also contributes cells to the developing arterial and venous poles of the heart[99]. Once the cardiac mass has hypertrophied to the extent that simple diffusion of oxygen and nutrients is no longer able to sustain the metabolic requirements of the heart, EPDCs from the overlying epicardium invade into the myocardium to form the coronary vasculature[100].

1.47 Tβ4 Has an Essential Function During Coronary Vessel Development

The best evidence so far obtained that Tβ4 can affect the process of vascular development *in vivo* comes from studies carried out using a transgenic mouse in which Tβ4 can be knocked down in a tissue restricted fashion. This mouse relies on the expression of Tβ4 shRNA, which is transcribed in a Cre-dependent manner. Crossing this Tβ4 shRNA mouse with the Nkx2.5-Cre line causes a variable level of Tβ4 knockdown in the developing mouse heart. At the E14.5 stage of embryonic development Tβ4 shRNA Nkx2.5-Cre mouse hearts are hypoplastic and display aberrant blood filled nodules on their epicardial surfaces. These nodules are composed of endothelial and vascular smooth muscle cells. Normally, Tβ4 is secreted by the developing myocardium and signals to the overlying epicardial mesothelial layer. Epicardial derived progenitor cells (EPDCs) in this layer than migrate into the myocardium and differentiate into the endothelial and vascular smooth muscle cells of the coronary vasculature. In the absence of Tβ4, such a signalling event does not take place and the EPDCs differentiate *in situ* to form the non-patent nodules seen on the surface of the Tβ4 shRNA Nkx2.5-Cre hearts[50] (**Fig. 1.8**).

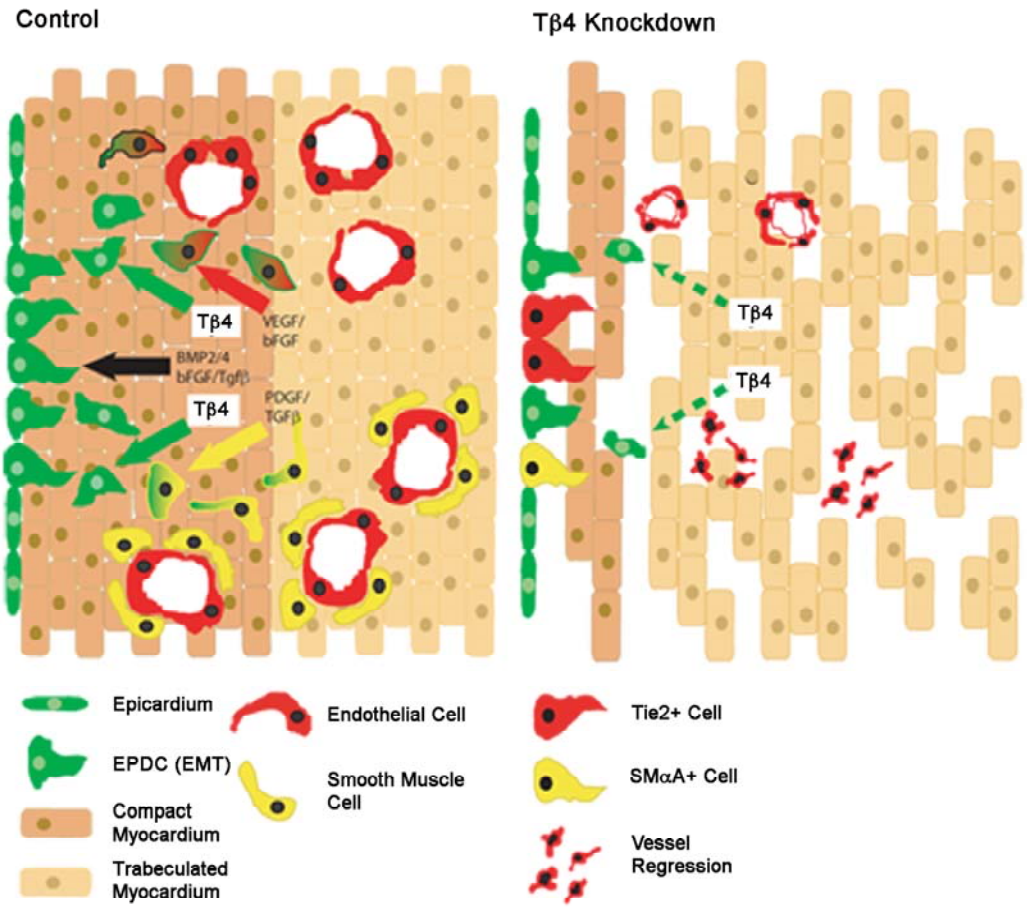


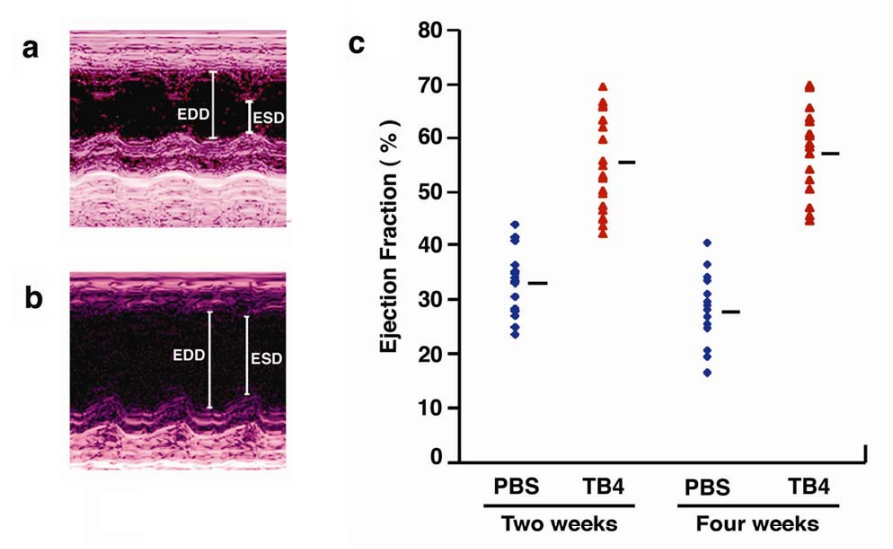
Fig. 1.8
Tβ4 is secreted by the developing myocardium to stimulate EPDCs to form the coronary vasculature.

In the normal situation, Tβ4 is secreted from the developing myocardium and signals to EPDCs in the epicardium. Tβ4 induces these EPDCs to migrate into the myocardium and differentiate into the cells of the coronary vasculature. In the absence of Tβ4, EPDCs differentiate *in situ* and form aberrant blood filled surface epicardial nodules. This failure of coronary vasculogenesis is accompanied by ventricular non-compaction. Adapted from [50].

1.48 Synthetic Tβ4 as a Treatment for Acute Myocardial Infarction

These findings, which demonstrate a role for Tβ4 in vascular development, have a great deal of relevance, not only for understanding the process of embryonic vascular development itself, but also for potential future therapeutics. It has been observed

that mice in which myocardial infarction (MI) has been experimentally induced through coronary artery ligation, can recover a high degree of their cardiac function after systemic administration of recombinant Tβ4 peptide[27] (**Fig. 1.9**). Several mechanisms have been postulated to account for this cardioprotective effect of Tβ4 including Tβ4 mediated activation of the Akt cell survival pathway[27]. However, one which is striking, and for which there is experimental evidence is the observed ability of Tβ4 to stimulate neovascularisation of the ischaemic region of the heart following MI[101]. Following systemic administration of Tβ4 after MI, endothelial cell and perfused capillary density was increased in the border zone of infarcted hearts compared to PBS treated controls. In other experiments, it has been demonstrated that embryonic endothelial progenitor cells can be cardioprotective when administered following MI. Their cardioprotective effects are abrogated when Tβ4 is knocked down via RNAi. Together, these pieces of evidence suggest that pharmacologically administered Tβ4 can mediate cardioprotective effects through the stimulation of new blood vessel growth in the ischemic heart[102].



d

			EDD	ESD	FS(%)	EF(%)	CUMULATIVE FS(%)	CUMULATIVE EF(%)	
CONTROL (n = 22)	Intraperitoneal	(n = 7)	.567 ± .055	.441 ± .051	22.2	33.3			
	Intraperitoneal & Intracardiac	(n = 7)	.530 ± .049	.412 ± .043	22.3	24.9	23.2 ± 1.2	28.2 ± 2.5	
	Intracardiac	(n = 8)	.470 ± .049	.354 ± .039	24.7	27.2			*
THYMOSIN B4 (n = 23)	Intraperitoneal	(n = 7)	.502 ± .069	.317 ± .045	36.9	54.6			
	Intraperitoneal & Intracardiac	(n = 8)	.451 ± .063	.296 ± .052	34.4	63.8	37.2 ± 1.8	57.7 ± 3.2	*
	Intracardiac	(n = 8)	.382 ± .043	.232 ± .038	39.3	54.0			

Fig. 1.9
Synthetic Tβ4 can improve cardiac function following MI

Representative echocardiographic M-mode images of left ventricles after coronary ligation with (**a**) or without (**b**) Tβ4 treatment. (**c**) Distribution of ejection fraction (EF) at 2 and 4 weeks after coronary ligation with (n = 23) or without (n = 22) Tβ4 treatment. Bars indicate means. **(d)** Echocardiographic measurements for intraperitoneal, intracardiac or intraperitoneal and intracardiac administration of Tβ4 or PBS (Control) at 4 weeks. Means and 95% confidence limits are shown. Asterisk, $P < 0.0001$. Adapted from [27].

1.49 The Retinal Model of Vascular Angiogenesis

When investigating the development of the vascular system it is important to make use of experimental models, which can provide a high yield of information with regards to the myriad of different processes involved. One commonly used model is the mouse model of neonatal retinal angiogenesis. This model provides a robust and commonly used tool to identify defects in the processes of angiogenesis, vascular cell migration and mural cell recruitment to name a few[55].

During mammalian retinal development, initial vascularisation of the inner eye is provided by the central hyaloid artery, which exits from the optic nerve and forms the arterial hyaloid vasculature. The hyaloid vasculature spreads through the vitreous before draining into an annular formation at the front of the eye. In mice and humans, at a point during development, the hyaloid vasculature regresses and is replaced by the retinal vasculature. In humans this takes place around mid-gestation whilst in mice it occurs shortly after birth[55].

1.50 Formation of the Primary Retinal Vascular Plexus

The retinal vasculature starts its course at the optic nerve, before spreading across the retina in a radial fashion with the optic nerve as its centre. The endothelial cells, which form the retinal vasculature migrate from the centre outwards to form a vascular plexus which covers the entire inner surface of the retina. During this initial phase of vascular sprouting, The morphology of the vascular tree can be distinguished into endothelial tip and endothelial stalk cells. The tip cells appear at the most

outward edge of the migrating vascular front where they project filopodial and lamellipodial processes towards hypoxic VEGF-secreting astrocytes (**Fig. 1.10**). The plexus is mostly composed of endothelial stalk cells, which actively proliferate to maintain a uniform tree behind the migrating tip cells[55].

This process tends to lay down a dense plexus of uniform endothelial tubes. As the plexus matures it remodels. Some vessels are strengthened and assume an arterial identity whilst others are selected to become veins. Some vessels are also pruned and regress, particularly in the areas immediately adjacent to large calibre vessels (**Fig. 1.10**).

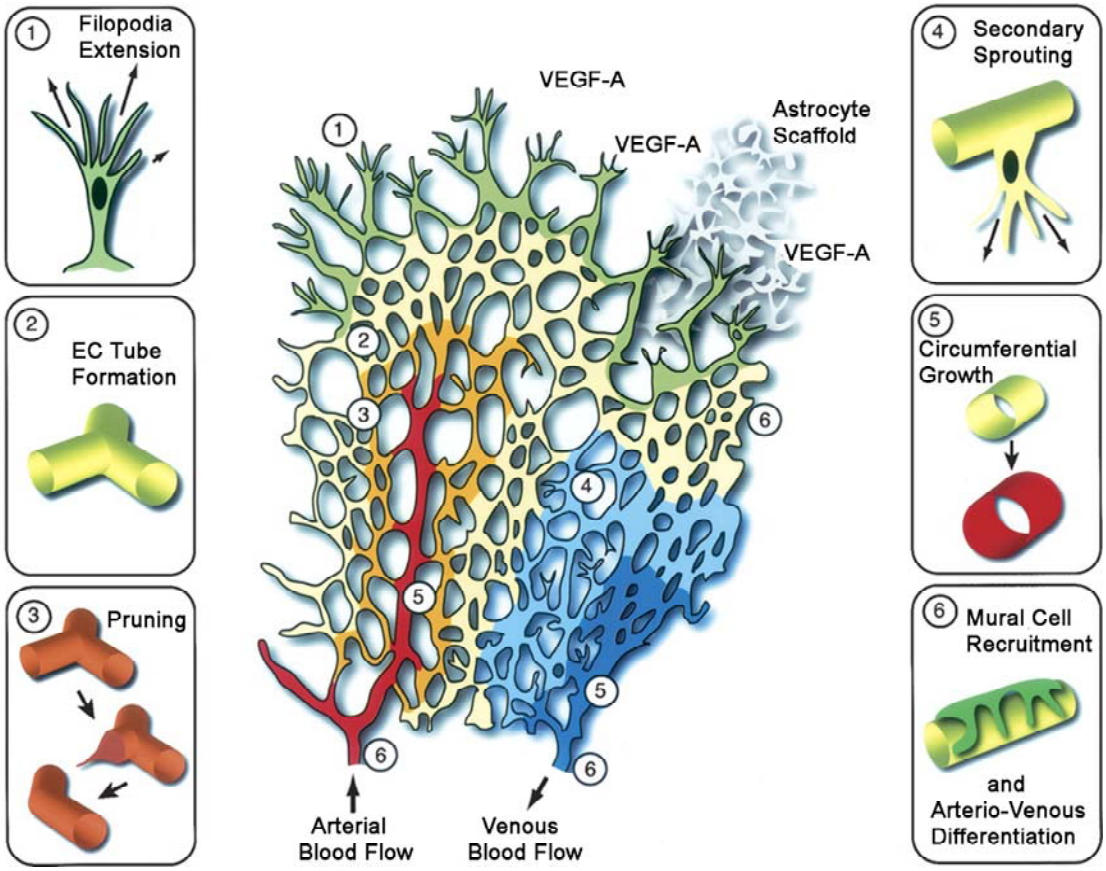


Fig. 1.10
Formation of the primary retinal vascular plexus can be used as a model to investigate many of the cellular processes of vascular development

As the primary plexus radiates out from the optic disc, many of the cellular processes underlying vascular development can be observed in a single vascular bed. These include filopodial extension, endothelial tube formation, vessel pruning, secondary sprouting, circumferential growth, mural cell recruitment and arterio-venous differentiation. Adapted from[70].

1.51 Formation of the Secondary Retinal Vascular Plexus

Once the primary plexus has reached the outer margins of the retina, a process which takes approximately one week in mice, a second deeper plexus sprouts downwards into the plexiform layers of the retina. This second deeper plexus is the result of selection of new endothelial tip cells on the veins and venules of the mature primary plexus and migration of the cells along the z-axis of primary plexus development.

Once these downward migrating cells encounter the inner nuclear layer of the retina they change direction again to form a migrating front in the usual centrifugal fashion[55]. Ultimately, two deeper plexi are formed from initial primary plexus sprouting.

1.52 Mural Cell Recruitment in the Retinal Vasculature

Mural cell recruitment to blood vessels of the retina is highly stereotyped. As the CNS does not contain a progenitor cell population capable of mural cell differentiation, all mural cell investiture in the developing retinal vasculature must take place via the central to peripheral migration and proliferation of mature mural cells[58,60]. The main molecule thought to govern this process is endothelial secreted PDGF-B. Indeed, intraocular administration of a blocking PDGFR-β antibody, inhibits mural cell recruitment and leads to vascular malformation[103].

1.53 Aims and Objectives

From the above literature review it can be seen that there is a strong likelihood that Tβ4 may play a functional role during physiological vascular development. Determining the function that Tβ4 might possess in this setting has implications beyond merely understanding how Tβ4 affects mammalian development. A greater knowledge of how blood vessels form normally is required to comprehend and therapeutically target the growth of tumour vasculature, as well as to understand how to grow new blood vessels in disease settings such as the infarcted heart. In order to

explore the role that Tβ4 plays in development of the systemic vasculature, the aims of this project are:

1. **To map the developmental expression pattern of Tβ4.** To date, the developmental expression pattern of Tβ4 has only been mapped out in a haphazard and piecemeal fashion. Comprehensively determining the pattern of Tβ4 developmental expression may provide clues as to what if any function Tβ4 has in vascular development and may highlight other potential functional roles in non-vascular systems.

2. **To use genetic loss of function mouse models to determine the role of Tβ4 in embryonic vascular development.** Genetic loss of function models in the mouse provide a powerful tool to assess the role of candidate genes during developmental processes. Examination of the phenotype of mutant embryos in which Tβ4 gene function has been disrupted may provide insight into the function of Tβ4 during normal vascular development.

3. **To determine molecular pathways on which Tβ4 may be acting to mediate its potential function in vascular development.** Presuming that a phenotype of aberrant vascular development is discovered in Tβ4 loss of function models, it will be necessary to assess how disrupted Tβ4 gene function impacts on the molecular pathways underlying blood vessel development. Insight into this may be gained by assaying gene expression changes in the Tβ4 mutants through the use of qRT-PCR and gene microarrays.

4. **To model the effects of Tβ4 on vascular development *in vitro*.** It is often the case that only limited information about gene function can be gleaned through passive observation of a mutant mouse phenotype. In order further understand the role of Tβ4 during vascular development, clues gained from analysis of the mouse phenotype will be used to attempt to model the vascular function of Tβ4 *in vitro*. Use of *in vitro* cell culture systems usually allows a more robust analysis of molecular and cellular behaviour due to the ability to manipulate the cells in ways, which are difficult to accomplish with murine *in vivo* models.

5. **To analyse the phenotype of the developing retinal vasculature in genetic Tβ4 loss of function models.** Developmental vascular processes such as sprouting angiogenesis are difficult to assess in the mouse embryo. Thus, neonatal retinas from Tβ4 genetic loss of function models will be analysed to more widely interrogate a role for Tβ4 in the various processes of vascular development.

2. Materials and Methods

2.1 Immunofluorescence Staining

E10.5 embryos were embedded in OCT and cryosectioned. Cryosections were washed for 10 minutes in PBS to remove OCT and then permeabilised in 0.5% Triton-X 100 in PBS for 10 minutes. After washing in PBS, sections were blocked at room temperature for 1 hour in blocking buffer (10% BSA, 10% sheep serum and 0.1% Triton-X 100 in PBS). Sections were then incubated in the primary antibody at dilution 1 in 100 overnight in blocking buffer at 4°C. Sections were then washed 5 times in 0.1% Triton-X 100 in PBS over the course of 1 hour. Incubation in the secondary antibody took place in blocking buffer for 1 hour at room temperature. Sections were again washed 5 times in 0.1% Triton-X 100 in PBS over the course of an hour. Sections were then mounted with coverslips with Vectashield plus DAPI (Vector labs).

2.2 Whole Mount In Situ *Hybridisation*

Embryos were dissected in calcium/magnesium free diethyl pyrocarbonate treated PBS (DepC PBS). Embryos were then fixed overnight in 4% PFA in DepC PBS and transferred to absolute methanol for storage at -20°C. Embryos were then rehydrated by incubating in a gradient of methanol diluted in PBT (DepC PBS + 0.1% Tween-20). Embryos were digested in proteinase K (10μg/ml in PBT) @ 18-22°C for 8-25 minutes depending on stage. Post-fixing was conducted for 20 minutes at room temperature in 4% PFA in PBT + 0.1% gluteraldehyde. After washing with PBT,

embryos were pre-hybridized in hybridization solution (50% formamide, 1.3x SSC, 5mM EDTA, 0.2% Tween-20, 0.5% CHAPS, 100μg/ml heparin in DepC water) overnight @ 68°C. Hybridisation was then carried out at 68°C overnight in probe diluted 1 in 100 in hybridisation mix. The following day, embryos were washed several times with hybridisation solution. After washing with TBS-T (0.8% NaCl, 0.02% KCl, 0.1M Tris-Cl pH7.5, 1.1% Tween-20 in DepC water) embryos were blocked overnight in 10% sheep serum + 1% BSA in TBS-T. Embryos were then incubated with anti-digoxigenin-AP Fab fragments (Roche) diluted 1 in 2,000 in block overnight at 4°C before washing and developing in NBT/BCIP solution until the desired colour change was achieved. Embryos were then dehydrated in a progressive methanol series before rehydrating and fixing in 4% PFA in PBS overnight. Embryos were then photographed.

2.3 In situ *Hybridisation on Sections*

Wax sections were cleared twice for 10 minutes in histoclear before rehydrating through a series of ethanol gradients. After washing with diethyl pyrocarbonate (DepC) treated PBS, sections were incubated for 8 minutes in proteinase K working solution (20μg/ml ProK, 50mM Tris pH8, 5mM EDTA) at 37°C before post-fixing in 4% PFA in DepC PBS with 0.2% gluteraldehyde. Pre-hybridisation took place in hybridisation solution (50% formamide, 5x SSC, 100% blocking reagent, 5mM EDTA, 0.1% Tween-20, 0.1% CHAPS, 0.1mg/ml heparin, 1mg/ml yeast tRNA in Depc water) for 2 hours at 70°C. Sections were then hybridised in hybridisation solution with the probe added at 1 in 80 overnight at 68°C. The next day, sections were washed once with 2x SSC pH7 before two washes at 65°C with 50% formamide

in 2x SSC pH7. Sections were then washed three times in PBS-T (PBS + 0.1% Tween-20). Sections were then blocked for 1 hour at room temperature in B-block (2% blocking reagent + 10% goat serum in PBS-T). After this sections were incubated overnight at 4°C anti-digoxygenin Fab fragments (500mU/ml) in B-block. Slides were then washed 3 times in PBS-T and twice in NTM-T (100mM Tris pH9.5, 100mM NaCl, 50mM Magnesium chloride, 0.05% Tween-20) before developing with NBT/BCIP. Sections were then counterstained with eosin before imaging.

2.4 Whole Mount PECAM Staining

Embryos were fixed overnight at 4°C in 1:4 DMSO:methanol. They were then incubated in 1:4:1 DMSO:methanol:hydrogen peroxide for 5 hours at room temperature. Embryos were then rehydrated in a gradient of decreasing methanol concentrations in water. Embryos were then permeabilised in PBS + 0.5% Tween-20 before incubating in block (PBS + 0.3% Tween-20 + 2% blocking reagent (Boehringer)) at room temperature for 1 hour. Embryos were then incubated in the primary biotinylated anti-mouse PECAM antibody (BD Pharminegn) diluted at 1 in 75 overnight at 4°C. After washing, embryos were incubated in streptavidin-HRP conjugate (Vector Labs) for 30 minutes before developing with DAB.

2.5 Immunohistochemistry

Frozen sections were heated at 37°C for 10 minutes on a slide dryer to allow the section to adhere to the slide before washing for 10 minutes in PBS. Sections were then treated with 3% hydrogen peroxide (Sigma) in PBS for 20 minutes to block

endogenous peroxidase activity before washing twice in PBS. Sections were then permeabilised in 0.5% Triton-X in PBS for 10 minutes before blocking with 10% sheep serum, 1% BSA in PBS for 1-2 hours. The sections were then incubated overnight at 4°C with the smooth muscle actin primary antibody diluted 1 in 700 in block. Slides were then washed three times for 5 minutes each in 0.1% Triton-X in PBS. Slides were then incubated with a biotinylated anti-mouse antibody diluted 1 in 100 in block for 30 minutes at room temperature, before incubation with a streptavdin-HRP conjugate for 30 minutes. 3 further washes in 0.1% Triton-X in PBS were performed before developing with DAB (Sigma). Slides were then counterstained with haematoxylin, mounted and imaged.

2.6 Preparation of Tissue for Cryo-Sectioning:

Embryos or organs were dissected out of their host before fixing in 4% paraformaldehyde (PFA) in PBS for 1-2 hours. Tissue samples were then left to equilibrate overnight in 30% sucrose in PBS at 4°C whereupon they were incubated in a 50:50 mixture of 30% sucrose in PBS and OCT embedding medium for 30 minutes. Samples were then transferred to OCT for a further 30 minutes before embedding and freezing at -80°C.

2.7 Fluorescent Imaging

Fluorescent images were captured on an upright Zeiss Z1 fluorescent microscope or an inverted Zeiss LSM 710 confocal microscope.

2.8 Quantification of NG2 Immunofluorescence

In order to quantify the mural cell density around aortas of E10.5 embryos, ten axial sections from each embryo providing sections throughout the length of the dorsal aorta were examined. Images were taken of the NG2 immunofluorescnce under constant exposure. Images were then thresholded to eliminate background fluorescence. Total channel fluorescence was then quantified with ImageJ software. Two perpendicular measurements of the diameter of each aortic section were averaged and used to calculate the vessel circumference. NG2 total fluorescence was then normalised to vessel circumference to produce a measure of mural cell density.

2.9 qRT-PCR

qRT-PCR was performed according to a standard ΔΔCT protocol using SYBR green (Applied biosystems).

Primers sequences used were as follows:

Smooth muscle actin:	F – GTCCCAGACATCAGGGAGTAA
	R – TCGGATACTTCAGCGTCAGGA
SM22α:	F – CAACAAGGGTCCATCCTACGG,
	R – ATCTGGGCGGCCTACATCA
NG2:	F – GGGCTGTGCTGTCTGTTGA
	R – TGATTCCCTTCAGGTAAGGCA
Endosialin:	F – CAACGGGCTGCTATGGATTG
	R – GCAGAGGTAGCCATCGACAG

CD13:	F – ATGGAAGGAGGCGTCAAGAAA
	R – CGGATAGGGCTTGGACTCTTT
Ang1:	F – CACATAGGGTGCAGCAACCA
	R – CGTCGTGTTCTGGAAGAATGA
Desmin:	F – GTGGATGCAGCCACTCTAGC
	R – TTAGCCGCGATGGTCTCATAC
PAI-1:	F – TTCAGCCCTTGCTTGCCTC
	R – ACACTTTTACTCCGAAGTCGGT
Id-1:	F – CCTAGCTGTTCGCTGAAG
	R – CTCCGACAGACCAAGTACCAC
c-myc:	F – ATGCCCCTCAACGTGAACTTC
	R – CGCAACATAGGATGGAGAGCA
Notch 1:	F – CCCTTGCTCTGCCTAACGC
	R – GGAGTCCTGGCATCGTTGG
Notch 2:	F – ATGTGGACGAGTGTCTGTTGC
	R – GGAAGCATAGGCACAGTCATC
Notch 3:	F – TGCCAGAGTTCAGTGGTGG
	R – CACAGGCAAATCGGCCATC
Notch 4:	F – CTCTTGCCACTCAATTTCCCT
	R – TTGCAGAGTTGGGTATCCCTG
Dll1:	F – CAGGACCTTCTTTCGCGTATG
	R – AAGGGGAATCGGATGGGGTT
Dll3:	F – CTGGTGTCTTCGAGCTACAAAT
	R – TGCTCCGTATAGACCGGGAC
Dll4:	F – TTCCAGGCAACCTTCTCCGA

	R – ACTGCCGCTATTCTTGTCCC
Jag1:	F – CCTCGGGTCAGTTTGAGCTG
	R – CCTTGAGGCACACTTTGAAGTA
Jag2:	F – CAATGACACCACTCCAGATGAG
	R – GGCCAAAGAAGTCGTTGCG
RBPj/CBF	F – ATGCCCTCCGGTTTTCCTC
	R – GGACAAGCCCTCCGAGTAGT
TACE	F – AGGACGTAATTGAGCGATTTTGG
	R – TGTTATCTGCCAGAAACTTCCC
Pres1	F – GGTGGCTGTTTTATGTCCCAA
	R – CAACCACACCATTGTTGAGGA
Pres2	F – GAAGACTCCTACGACAGTTTTGG
	R – CACCAGGACGCTGTAGAAGAT
Hes1	F – CCAGCCAGTGTCAACACGA
	R – AATGCCGGGAGCTATCTTTCT
Hey1	F – GCGCGGACGAGAATGGAAA
	R – TCAGGTGATCCACAGTCATCTG
Hey2	F – AAGCGCCCTTGTGAGGAAAC
	R – GGTAGTTGTCGGTGAATTGGAC
HeyL	F – CAGCCCTTCGCAGATGCAA
	R – CCAATCGTCGCAATTCAGAAAG
Lnfng	F – CGAGGTGCATAGCCTCTCC
	R – GCGAGGGGACAGAACTTCG
Mnfng	F – ATGCACTGCCGACTTTTTCG
	R – CCTGGGTTCCGTTGGTTCAG

Rdfng	F – CCACGGCAGACGTTCATTTTC
	R – GCAGAACCATTTTCGTCCAGA
Nrarp	F – AAGCTGTTGGTCAAGTTCGGA
	R – CGCACACCGAGGTAGTTGG
PDGF-B	F – AAGTGTGAGACAATAGTGACCCC
	R – CATGGGTGTGCTTAAACTTTCG
PDGFR-β	F – TTCCAGGAGTGATACCAGCTT
	R – AGGGGGCGTGATGACTAGG
Fibroglycan	F – TGTGTCCGCAGAGACGAGAA
	R – GGAATCAGTTGGGATGTTGTCA
FPP Synthetase	F – GGAGGTCCTAGAGTACAATGCC
	R – AAGCCTGGAGCAGTTCTACAC
Hmgb1	F – GGCGAGCATCCTGGCTTATC
	R – GGCTGCTTGTCATCTGCTG
Ste20	F – TCATTCGGCTACGGAACAAGA
	R – GACCTGCGACTCCAAAGTCTG
Tenascin C	F – ACGGCTACCACAGAAGCTG
	R – ATGGCTGTTGTTGCTATGGCA
TGF-β1	F – CTCCCGTGGCTTCTAGTGC
	R – GCCTTAGTTTGGACAGGATCTG
TGF-β RII	F – CCGCTGCATATCGTCCTGTG
	R – AGTGGATGGATGGTCCTATTACA
Alk-1	F – GGGCCTTTTGATGCTGTCG
	R – TGGCAGAATGGTCTCTTGCAG
Alk-5	F – TCCCAACTACAGGACCTTTTTCA

	R – GCAGTGGTAAACCTGATCCAGA
Smad1	F – GCTTCGTGAAGGGTTGGGG
	R – CGGATGAAATAGGATTGTGGGG
Smad2	F – ATGTCGTCCATCTTGCCATTC
	R – AACCGTCCTGTTTTCTTTAGCTT
Smad3	F – CACGCAGAACGTGAACACC
	R – GGCAGTAGATAACGTGAGGGA
Smad4	F – AGCCGTCCTTACCCACTGAA
	R – GGTGGTAGTGCTGTTATGATGGT
Smad5	F – TTGTTCAGAGTAGGAACTGCAAC
	R – GAAGCTGAGCAAACTCCTGAT
Smad6	F – GAGCACCCCCATCTTCGTCAA
	R – AACAGGGGCAGGAGGTGATG
Smad7	F – GGCCGGATCTCAGGCATTC
	R – TTGGGTATCTGGAGTAAGGAGG
Id-2	F – ATGAAAGCCTTCAGTCCGGTG
	R – AGCAGACTCATCGGGTCGT
Cadherin 3	F – CTGGAGCCGAGCCAAGTTC
	R – GGAGTGCATCGCATCCTTCC
Cadherin 5	F – CACTGCTTTGGGAGCCTTC
	R – GGGGCAGCGATTCATTTTTCT
Ccbe1	F – AAACAAGATCACCACGACCAAA
	R – CTCGCGGTCATATCGGTATCC
Ddr2	F – ATCACAGCCTCAAGTCAGTGG
	R – TTCAGGTCATCGGGTTGCAC

Dner	F – TGCCAGGACCAGTACATTGG
	R – GCAAGTGAAATTGCTCCCATCC
Epas1	F – CTGAGGAAGGAGAAATCCCGT
	R – TGTGTCCGAAGGAAGCTGATG
Fibullin5	F – GCTTGTCGTGGGGACATGAT
	R – TGGGGTAGTTGGAAGCTGGTA
Foxo1	F – CCCAGGCCGGAGTTTAACC
	R – GTTGCTCATAAAGTCGGTGCT
HIF1an	F – CAGTGTGATCGCGGCAAAC
	R – CTGAAGGGTCAACCGAGCAG
IGFbp4	F – AGAAGCCCCTGCGTACATTG
	R – TGTCCCCACGATCTTCATCTT
Integrin α1	F – CAAATGAGCCTGGAACCAAT
	R – CCATCCACGTTGAGGTCTTT
Integrin β1	F – CGTGGTTGCCGGAATTGTTC
	R – ACCAGCTTTACGTCCATAGTTTG
Ltbp4	F – CTGGGTGTCGCTATTGGTG
	R – GTTGTGACAGATCAAGGGACAT
Maml1	F – CGTAGCTCAGAGCAACCTCAT
	R – TTCATGTCTTCGTCGGGCAC
Paxillin	F – CAAACGGCCAGTGTTCTTGTC
	R – TGTGTGGTTTCCAGTTGGGTA
Pim1	F – CTGGAGTCGCAGTACCAGG
	R – CAGTTCTCCCCAATCGGAAATC
Plakoglobin	F – TGGCAACAGACATACACCTACG

	R – GGTGGTAGTCTTCTTGAGTGTG
Slit3	F – GCGCGATTTGGAGATCCTCA
	R – TGGAGTGTAGACGCAGAGTCC
Smap2	F – GGCCCTAGAGTGAGGCAAG
	R – GAAGGCTGACTGAAGCAGTGA
Sonic	F – AAAGCTGACCCCTTTAGCCTA
	R – TTCGGAGTTTCTTGTGATCTTCC
Symplekin	F – CGGAGTGTGGCATCACAGTTT
	R – CGCACTTCAATGGATTTGTCTG
Wave2	F – AGTAACCAGGAACATCGAGCC
	R – CTTGTATCGCTAGGCAACGTC

2.10 Quantification of Haemorrhage at E14.5

E14.5 embryos were harvested from pregnant female Tβ4 +/- mice after having been impregnated by Tβ4 -/Y males. Immediately after dissection, the amount of surface haemorrhage visible under a dissection stereomicroscope was quantified according to the following scheme. Score 0 – no visible haemorrhage, 1 – some small spots of dermal haemorrhage observed in a single location, 2 – some small spots of dermal haemorrhage observed in more than one location, 3 – a large area of haemorrhage observed in one location, 4 – a large area of dermal haemorrhage (usually flank or head) observed in one location with some small spots of dermal haemorrhage observed in at least one other location, 5 – more than one large area of dermal haemorrhage observed.

2.11 10T1/2 and A404 Cell culture

10T1/2 cells were maintained in Dulbecco's modified Eagles' medium plus Glutamax (DMEM-Gibco) supplemented with penicillin/streptomycin and 10% heat inactivated fetal calf serum. For stimulation experiments, cells were plated at 50,000 cells per well in a 6 well plate and serum starved in 0.5% fetal calf serum in DMEM supplemented with penicillin/streptomycin. For differentiation experiments, cells were then stimulated with either a control volume of PBS, Tβ4, TGF-β or a combination thereof. Cells were left to differentiate for 6 days. On each of these days, the medium was changed and the cells re-stimulated with the appropriate molecules. On the seventh day, medium was aspirated off, cells washed briefly with PBS and RNA extracted using Trizol reagent (Invitrogen).

A404 cells were maintained in α-modified Eagles' medium supplemented with 7.5% fetal bovine serum, 200mg/μl L-glutamine and penicillin/streptomycin. For stimulation experiments, cells were plated at 50,000 per well in a 6 well plate and maintained in complete medium. They were treated for 6 days with either a control volume of PBS or 1μg/mlTβ4. On each of these days, the medium was changed and the cells re-stimulated with the appropriate molecules. On the seventh day, medium was aspirated off, cells washed briefly with PBS and RNA extracted using Trizol reagent (Invitrogen).

2.12 Western blotting

10T1/2 cells were serum starved in 0.5% heat inactivated fetal calf serum in DMEM plus Glutamax (Gibco) overnight. The following day, cells were stimulated with 100ng/ml Tβ4, 2ng/ml TGF-β, 100ng/ml Tβ4 plus 2ng/ml TGF-β or a control volume of PBS. Cells were stimulated for 15 minutes. Following stimulation, medium was aspirated and cells briefly washed in PBS. Protein was extracted immediately by addition of hot (~90°C) laemmli buffer (250mM Tris-Cl pH 6.8, 4% SDS, 25% glycerol, 0.1% bromophenol blue, 5% β-mercaptoethanol). Samples were then run on a 10% acrylamide gel (Volume 10ml: 3.33ml 30% acrylamide, 50μl 20% SDS, 3.75ml 1M Tris-Cl pH8.8, 2.82ml double distilled water, 3.3μl Temed, 50μl 20% Amps). Proteins were then transferred to a nitrocellulose membrane. The membrane was then blocked for 2 hours in blocking buffer (5% milk in TBS (TBS volume 1l: 8g NaCL, 2g KCL, 3g Tris-Cl, pH 8)). Membranes were then incubated overnight at 4°C in primary antibody at 1 in 500 concentration in blocking buffer. The following day, membranes were washed 3 times over the course of 40 minutes in TBS plus 0.05% Tween-20. Membranes were then incubated in the secondary antibody at concentration 1 in 1,000 in blocking buffer at room temperature for 1 hour. Membranes were then washed 3 times over the course of 40 minutes in TBS plus 0.05% Tween-20. Protein bands were visualised by application of ECL western detection reagents (GE Healthcare). Bands on developed photographic film were then quantified using densitometry with ImageJ software.

2.13 Cell Transfections

10T1/2 cells were seeded in 24 well plates at 50,000 cells per well. The following day 1μg of the relevant plasmid was transfected into 10T1/2 cells with effectene transfection reagent (Qiagen) used according to the manufacturer's instructions. Smad activity luciferase reporter plasmids and appropriate positive and negative controls were purchased from SA Biosciences. Smad responsive constructs contained the Smad2/3/4 binding element AGCCAGACA. Following 16 hours of transfection, cells were serum starved by replacing the transfection medium with 0.5% FCS in DMEM + Glutamax. Cells were left overnight and the following day cells were stimulated for 6 hours in the presence of 100ng/ml Tβ4, 2ng/ml TGF-β, 100ng/ml Tβ4 plus 2ng/ml TGF-β or a control volume of PBS. Smad activity dependent firefly luciferase activity was then measured by means of a dual luciferase reporter assay (Promega) used according to the manufacturer's instructions. Renilla luciferase activity was also measured as a transfection efficiency control, and firefly luciferase activity expressed as a proportion on renilla luciferase activity.

2.14 Retinal Immunostaining

P6 mouse pups were culled by cervical dislocation and the globes enucleated. Retinas were dissected from globes in 2x PBS and subsequently stored in methanol at -20°C. For staining, methanol was aspirated off and the retinas were fixed for 2 minutes in 4% formaldehyde. Formaldehyde was then aspirated off and retinas left to block for 1 hour in retinal blocking buffer (2x PBS, 0.1% azide, 1% BSA, 3% Triton X -100, 0.5% Tween-20). Retinas were then incubated in primary antibodies at a

concentration of 1 in 200 in retinal blocking buffer overnight at 4°C. The following day, retinas were washed five times in retinal blocking buffer over the course of an hour. Retinas were then incubated in secondary antibody at a concentration of 1 in 200 in retina blocking buffer for 1 hour at room temperature. Retinas were then again washed 5 times in retinal blocking buffer over the course of an hour in the dark at room temperature. Retinas were then post-fixed for 2 minutes in 4% formaldehyde before mounting on a slide and coverslip.

2.15 Gene arrays

Gene arrays were performed on Affymetrix Mouse Exon 1.0ST arrays. Raw data was processed with Affymetrix expression console software before being analysed for gene expression changes in Partek. Statistics were performed in R. The top 200 up- and downregulated genes were fed into Metacore software from Genego and the reverse pathway analysis tool used to determine the most statistically perturbed signalling pathways in a combined data set from Tβ4 +/Y and Tβ4 -/Y E12.5 embryos and 10 week old hearts.

2.16 Antibodies

Antibodies were purchased as follows: rabbit anti-Tβ4 (Immundiagnostik), rat anti-endomucin (eBioscience), Cy3-conjugated mouse anti-smooth muscle actin (Sigma), rabbit anti-NG2 (Chemicon), rabbit anti phospho-Smad2 and rabbit anti phospho-Smad1/5 (Cell Signalling Technology), rat anti PECAM (BD Pharmingen), rabbit anti cleaved caspase 3 (Cell Signalling Technology), rabbit anti phopho histone H3 (Upstate), mouse anti Symplekin (BD Transduction) and mouse anti-GAPDH

(Chemicon). Secondary, alexafluor conjugated antibodies were purchased from Invitrogen.

2.17 Recombinant Protein

Recombinant Tβ4 was a kind gift from RegeneRX pharmaceuticals. Recombinant TGF-β 1 was purchased from R&D systems.

2.18 Statistics

Statistical analysis was performed with Graphpad Prism software. Contingency tables were analysed by the chi squared test. Two tailed, unpaired, non-parametric T tests were used for all other statistical tests.

2.19 X-Gal Staining of Whole Embryos

Embryos were dissected out into cold PBS. The embryos were fixed briefly for 5 minutes in 4% PFA. The embryos were washed twice for 5 minutes in PBS before staining with X-Gal solution at 30°C for 24 hours. Embryos were then washed in PBS before being photographed. They were subsequently embedded in wax and sectioned for analysis by bright field microscopy.

2.20 MEF Isolation and Culture

Individual embryos from mixed litters were dissected from the maternal uterus at E14.5. A small sample of each embryo was kept for genotypic analysis. Watchmaker's forceps were used to dissect away the placenta and membranes. Visceral organs were removed as far as possible from each embryo. Each embryo was then minced using curved iris scissors. 5ml of trypsin/EDTA was added and each embryo incubated at 37°C for 20 minutes. The embryo cell suspensions were then vigorously pippetted before incubating for a further 10 minutes. The EDTA was neutralised by adding DMEM supplemented with 10% FCS, non-essential amino acids and penicillin/streptomycin. Cells were then cultured overnight at 37°C. When the cells reached 80-90% confluent they were frozen in liquid nitrogen until results of the genotyping were available. After Tβ4 +/Y and Tβ4 -/Y embryos had been identified, MEFs were maintained in DMEM plus Glutamax supplemented with penicillin/streptomycin and 10% heat inactivated FCS. For stimulation experiments, cells were plated at 50,000 cells per well in a 6 well plate and serum starved in 0.5% fetal calf serum in DMEM supplemented with penicillin/streptomycin. For differentiation experiments, cells were then stimulated with either a control volume of PBS, Tβ4, TGF-β or a combination thereof. Cells were left to differentiate for 6 days. On each of these days, the medium was changed and the cells re-stimulated with the appropriate molecules. On the seventh day, medium was aspirated off, cells washed briefly with PBS and RNA extracted using Trizol reagent (Invitrogen).

2.21 ES Cell Culture

ES cells were maintained in the undifferentiated state by culture in medium consisting of Dulbecco's Modified Eagle's Medium supplemented with 20% ES qualified foetal bovine serum, 1% non-essential amino acids, 1% L-Glutamine, 1% 2-mercaptoethanol, 1% penicillin-streptomycin and 1,000units/ml ESGRO (Millipore). For differentiation assays, ES cells were plated on gelatin coated plates and cultured in medium with ES qualified serum replaced by non-ES qualified serum and with ESGRO removed. Cells were differentiated for 6 days.

3 Developmental Expression of Tβ4

3.1 Introduction

Mapping of the developmental expression pattern of Tβ4 has previously been performed, but has mostly been conducted in a piecemeal and haphazard manner across a wide variety of model organisms, particularly with reference to Tβ4 expression in the developing vascular system. For example, Dathe et al. performed Tβ4 expression studies on the chick and obtained data which may or may not be relevant to the mammalian system[52]. Gomez-Marquez et al. reported cardiovascular expression of Tβ4, but did not describe the expression pattern in detail and confined their studies to the early stages of mid-gestation[104].

In order to get a more complete picture about the expression pattern of vascular Tβ4, with regards to specific location and stage, a staged expression analysis for Tβ4 in the mouse embryo was performed. It was hoped that not only would such studies confirm previous reports of vascular Tβ4 expression, but also provide clues, gained from establishing the specific location of Tβ4 expression, as to the function of Tβ4 in the developing vasculature. For example, expression in early stage as opposed to fully developed vessels might indicate a role in the formation of a vascular system, whereas the converse might indicate a role for Tβ4 in physiological maintenance of a healthy vasculature. Similarly, if a predominantly arterial or venous expression pattern were to be observed, this might indicate a function in arteriovenous specification. Such a strategy might also allow the identification of other, non-vascular tissue in which Tβ4

is expressed. This might provide additional insight into the developmental function of Tβ4.

A strategy was thus devised to map the expression pattern of Tβ4. Initially, the techniques of whole mount and on section RNA *in situ* hybridisation (ISH) were used, due to the presence of functional, validated riboprobes and protocols in the lab. Immunohistochemistry was then used to confirm that the results observed at the mRNA level held true for protein expression. Finally, immunofluorescence co-staining would be used to identify individual cell lineages in which Tβ4 is expressed.

3.2 Results

3.2.1 Vascular Expression of Tβ4

Vascular expression of Tβ4 in the embryo proper was first observed at the embryonic E9.5 stage of development (**Figs. 3.1a and b**) by whole mount RNA ISH. Specifically, expression at this stage was localised to the dorsal aorta. Aortic expression was confirmed by sectioning the Tβ4 stained E9.5 embryos to reveal the internal pattern of Tβ4 expression (**Fig. 3.1c**). Expression was not confined solely to the dorsal aortae and was observed, at some stage in all blood vessels. Notably, expression was not restricted to arterial vessels but was also observed in veins, such as the umbilical vein (**Fig. 3.1d**) through the use of ISH performed on E11.5 wax embedded sections. Gradually, as development proceeded, expression of Tβ4 in the developing vasculature decreased, and by the E14.5 and E16.5 stages the expression of Tβ4 was negligible in large vessels such as the aorta (**Figs. 3.1e and 3.1f**).

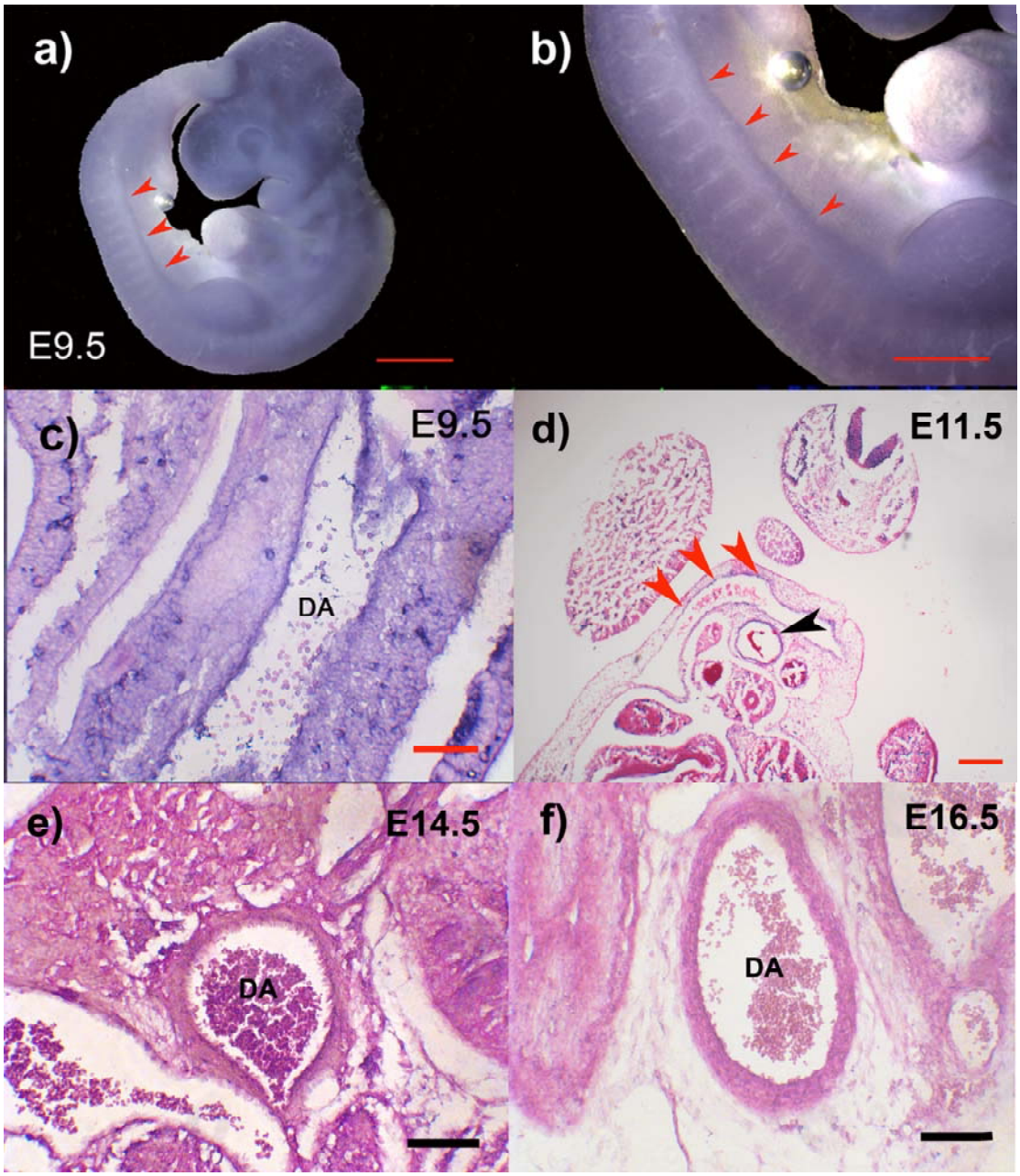


Fig. 3.1
Tβ4 is expressed in the vasculature of midgestation mouse embryos

Whole mount in situ hybridisation at E9.5 reveals Tβ4 staining in the dorsal aorta at at low (**a**) and high (**b**) magnification (red arrowheads). Sagittal sections through this embryo confirm the aortic expression of Tβ4 (**c**). Expression of Tβ4 is maintained in the developing aorta at E11.5 (black arrowhead) but can also be seen in venous tissue such as that of the umbilical vein (red arrowheads)(**d**). At later E14.5 (**e**) and E16.5 (**f**) stages of development the expression of Tβ4 becomes diminished in the dorsal aorta. DA (dorsal aorta). Scale bars: (a) 1mm, (b) 500μm, (c) 50μm, (d) 150 μm, (e) 100μm, (f) 200μm.

In order to confirm that the presence of Tβ4 mRNA, as revealed by RNA ISH, correlated with the production of properly synthesised Tβ4 protein, vascular expression of Tβ4 was validated by immunohistochemistry (IHC) conducted on wax sections from E11.5 embryos (**Figs. 3.2a and b**). Tβ4 protein was expressed in cells of the walls of both the dorsal aortae and the precursor vessels of the vena cava; the cardinal veins. Although expression was observed in both vessels, levels of Tβ4 appear to be higher in the arterial dorsal aortae than in the cardinal veins.

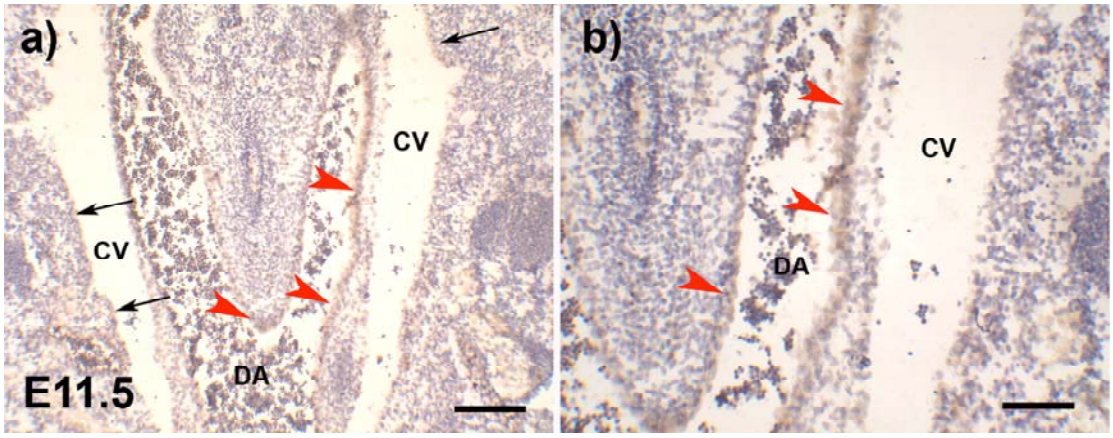


Fig. 3.2
Tβ4 protein is expressed in the walls of the dorsal aortae and cardinal veins

Immunohistochemistry for Tβ4 on E11.5 sagittal embryo sections demonstrate the expression of Tβ4 protein in the wall of the dorsal aorta (red arrowheads) and cardinal veins (black arrows) at low (**a**) and high (**b**) magnification. DA (Dorsal aorta), CV (Cardinal vein). Scale bars: (a) 200μm, (b) 100μm.

Although, the results above demonstrate unequivocal expression of Tβ4 in the developing vasculature, it is not clear from these data, in which specific cell lineage Tβ4 is expressed. Blood vessels are composed of numerous cell types. At this stage of development the two most prominent cell types are endothelial cells and mural cells (comprising cells which may have vascular smooth muscle cell or pericyte character). In order to determine, in which of these two cell types Tβ4 is primarily

expressed, immunofluorescence microscopy was performed on cryosections from E10.5 and E12.5 wild type mouse embryos for Tβ4 and endomucin to serve as a specific marker for endothelial cells[105] or smooth muscle actin to serve as a marker for mural cells. Tβ4 expression was observed only in the endothelial cell compartment of dorsal aorta as visualised by co-localisation of Tβ4 and endomucin, but was never observed in co-localising with smooth muscle actin in the mural cell component of the dorsal aorta vessel wall (**Figs. 3.3a and b**).

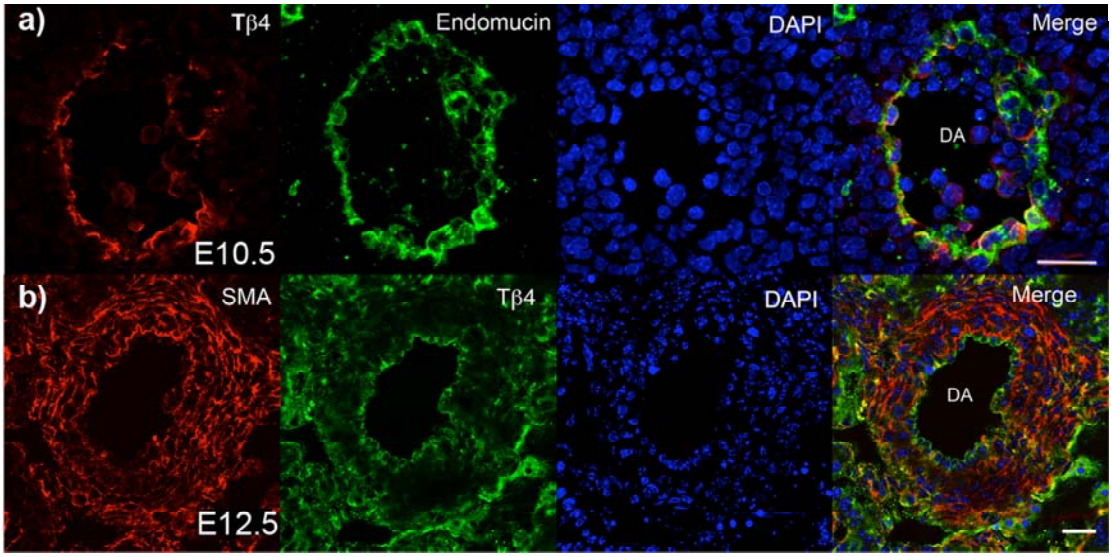


Fig. 3.3
Tβ4 is expressed in the endothelium but not the mural cell layer of developing blood vessels

Immunofluorescence studies reveal that expression of Tβ4 in the developing aorta co-localises with the endothelial marker endomucin (**a**) but not the mural cell marker smooth muscle actin (**b**) at E10.5 and E12.5 respectively. DA (dorsal aorta). Scale bars: (a) 20μm, (b) 100μm.

3.2.2 Cardiac Expression of Tβ4

Tβ4 expression was also observed by ISH, in non-vascular components of the cardiovascular system. This expression was seen in the tissues of the heart. Cardiac

expression of Tβ4 was first noted at E10.5 and was localised to the developing epicardial and endocardial surfaces of the heart (**Fig. 3.4a and b**). Tβ4 was also expressed at this stage in the endocardial cushions which will later go on to form the atrio-ventricular valves of the mature heart (**Fig. 3.4a**)[106]. At this early stage, Tβ4 does not appear to be expressed at high levels in the developing myocardium. However, by E11.5, as shown by Tβ4 IHC, Tβ4 protein is expressed at high levels throughout the myocardium (**Fig. 3.4c**).

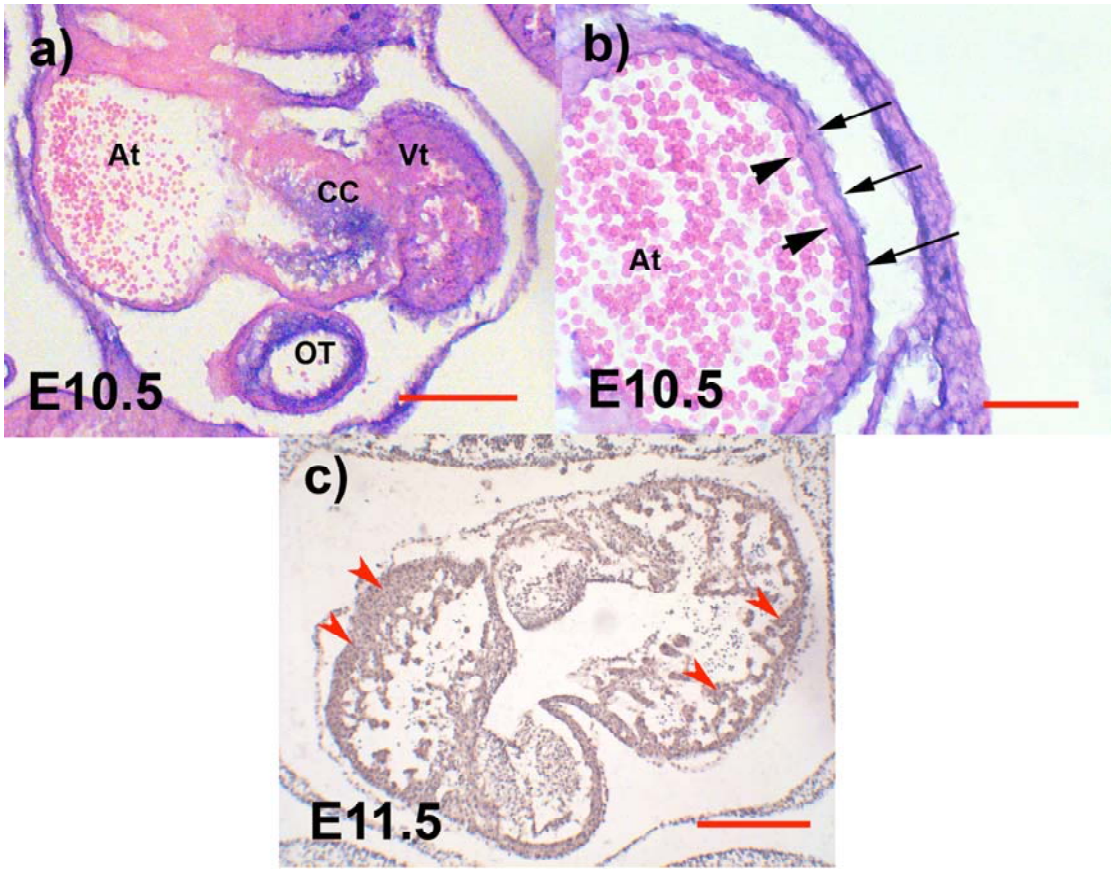


Fig. 3.4
Tβ4 is expressed in the tissues of the developing mouse heart

At the E10.5 stage of embryonic development, in situ hybridisation shows expression of Tβ4 mRNA in the cardiac cushions, outflow tract (**a**), endocardium (black arrowheads) and epicardium (black arrows) (**b**) of the heart. By E11.5, immunohistochemistry demonstrates expression of Tβ4 protein in the developing myocardium (red arrowheads) (**c**). At (Atrium), CC (Cardiac cushion, OT (Outflow tract), Vt (Ventricle). Scale bars: (a) 150 μm, (b) 50 μm, (c) 400μm.

3.2.3 Neural Expression of Tβ4

As well as being expressed in the developing cardiovascular system, in agreement with other sources[52,107,108], Tβ4 was expressed throughout the developing nervous system. The earliest expression of Tβ4 mRNA was detected in the developing neural tube at E9.5 where Tβ4 was expressed diffusely (**Fig. 3.5a**). Coincidently, expression of Tβ4 was also seen at this time point in the developing somites (**Fig. 3.5a**). Expression of Tβ4 mRNA was noted to be present in tissues of both the central nervous system (CNS) and the peripheral nervous system (PNS) at E10.5. CNS expression of Tβ4 is illustrated by ISH staining for Tβ4 mRNA in the region of the neural tube surrounding the fourth ventricle, which will go on to develop into the hindbrain (**Fig. 3.5b**). PNS expression of Tβ4 is visible as deep, intense staining of the presumptive trigeminal ganglia, which will house the cell bodies of sensory neurons innervating the face (**Fig. 3.5b**). Tβ4 expression in the CNS is not confined merely to the developing structures of the brain, but is also present in the spinal cord (**Figs. 3.5c and d**). It is notable, that at these stages of development Tβ4 is most strongly expressed in the inner sections of the spinal cord. This is the location of the spinal cord grey matter, which at this stage of development contains developing interneurons[109]. Tβ4 is expressed, qualitatively, to a lesser degree in the white matter tracts. There is also a matching Tβ4 expression pattern in the PNS, as the developing dorsal route ganglia, which contain the cell bodies of projecting sensory neurons are rich in Tβ4 mRNA (**Figs. 3.5c and d**).

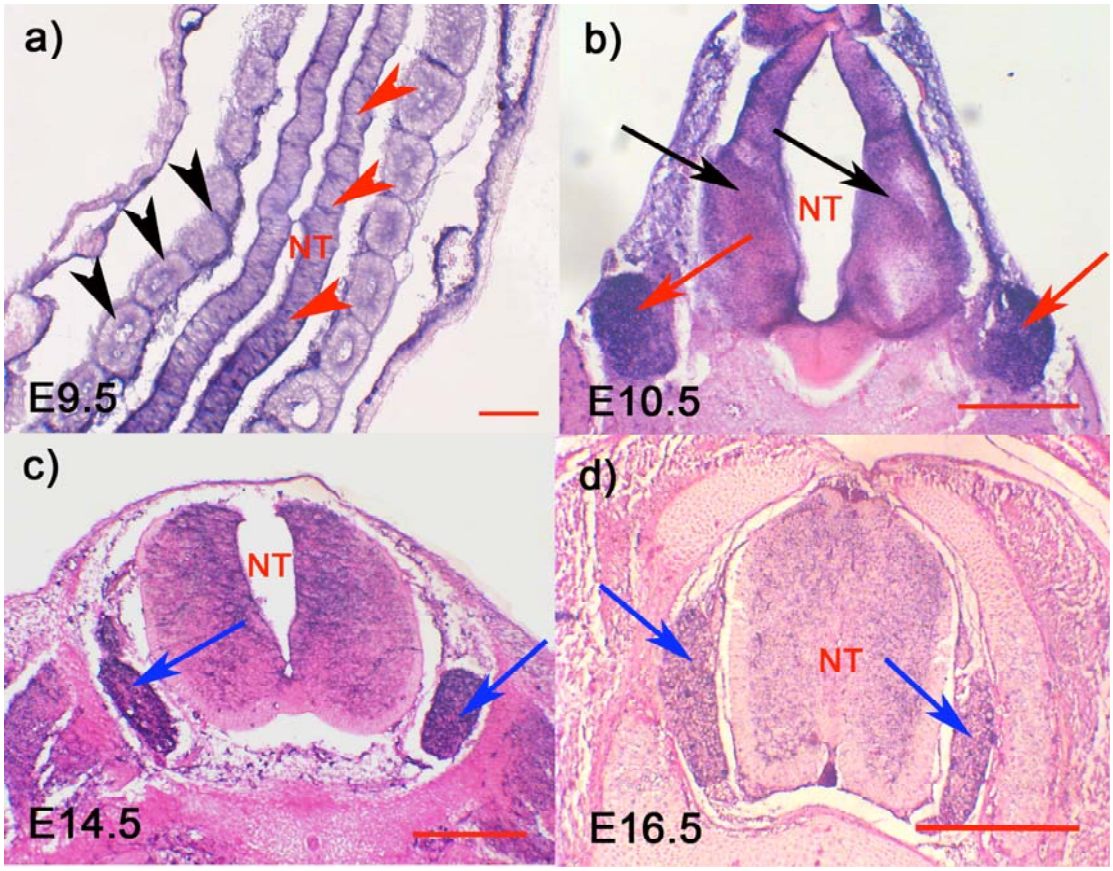


Fig. 3.5
Tβ4 is expressed in both the central and peripheral nervous systems

In situ hybridisation for Tβ4 mRNA, on E9.5 sagittal embryo sections shows expression in the developing neural tube (red arrowheads) and somites (black arrowheads) (**a**). Neural tube expression continues throughout development as demonstrated by expression in axial sections through the hindbrain at E10.5 (black arrows) (**b**). At this stage Tβ4 is also expressed in the developing cranial nerve motor ganglia such as the trigeminal ganglia (red arrowheads). In the spinal cord, Tβ4 expression becomes restricted to the developing grey matter at E14.5 (**c**) and E16.5 (**d**). Tβ4 is also expressed in the dorsal root ganglia at these stages (blue arrows). NT (Neural tube). Scale bars: (a) 50μm, (b) 200μm, (c) 200μm, (d) 200μm.

3.2.4 Gastrointestinal Expression of Tβ4

Another location, at which Tβ4 was markedly expressed, was the developing gastrointestinal tract. Expression of Tβ4 at this location first became apparent at E14.5 when Tβ4 mRNA was detected in the internal epithelial layer of the stomach

(**Fig. 3.6a**). This persisted until at least E16.5, where Tβ4 expression was again seen on this internal epithelial layer (**Fig. 3.6b**).

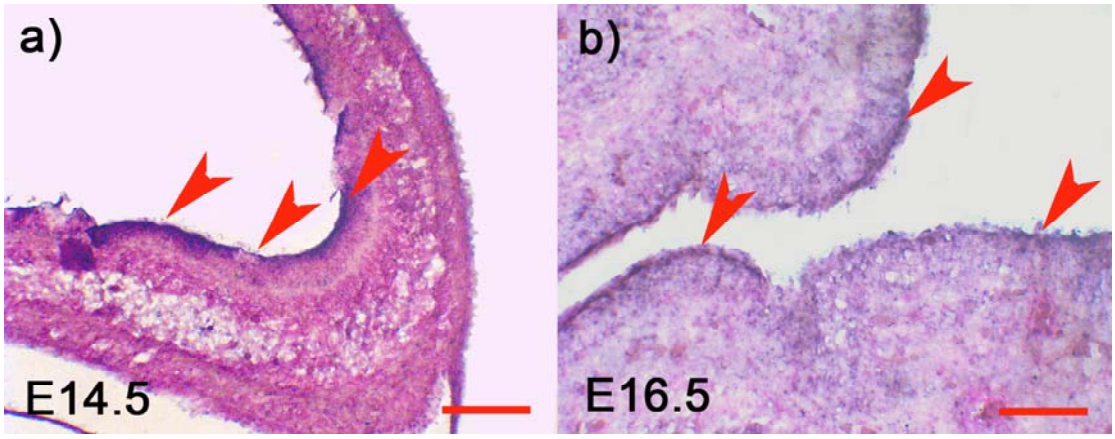


Fig. 3.6
Tβ4 is expressed in the epithelial surface of the developing gastrointestinal tract

Tβ4 in situ hybridisation on performed on axial sections, shows Tβ4 expression in the internal epithelial layer of the gastrointestinal tract at E14.5 (**a**) and E16.5 (**b**) (red arrowheads). Scale bars: (a) 100μm, (b) 100μm.

3.2.5 Lung Expression of Tβ4

At E16.5, tissues of the developing lung were also observed to express Tβ4 mRNA. Tβ4 expression was confined to the walls of small blood vessels in the lung and the epithelial surfaces of the developing airways (**Fig. 3.7a and 3.7b**).

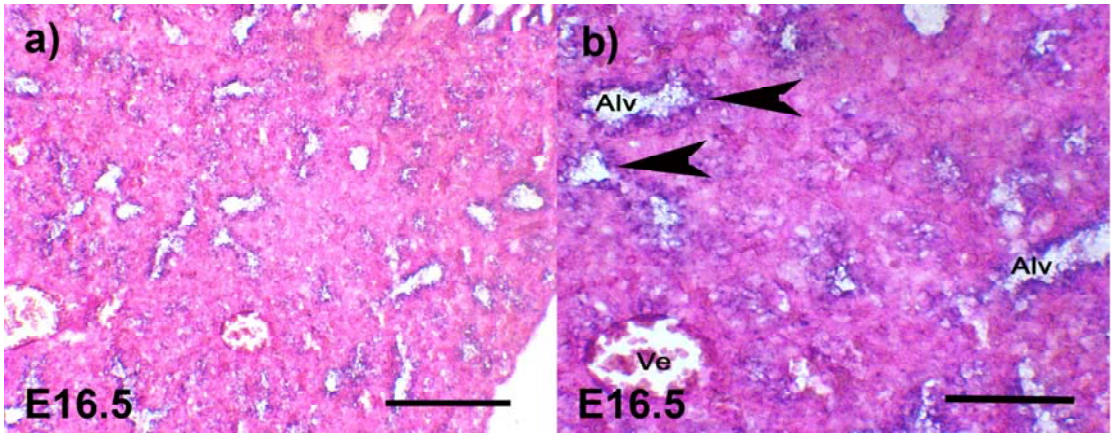


Fig. 3.7
Tβ4 is expressed on the in the alveolar surface epithelium of the developing lung

Low (**a**) and high (**b**) powered images of Tβ4 in situ hybridisation on E16.5 embryo sections demonstrated expression of Tβ4 in the developing alveolar surface epithelium of the lung (black arrowheads). Tβ4 is also expressed in the small vessels of the pulmonary vasculature. Alv (Alveolus), Ve (Blood vessel). Scale bars: (a) 400μm, (b) 150μm.

3.3 Discussion

3.3.1 Expression of Tβ4 in the Vasculature

The earliest expression of Tβ4 in the vasculature was detected at E9.5 in the developing dorsal aorta. In qualitative terms, high levels of Tβ4 expression in this large vessel continued until E14.5, when regions of the aorta began to reduce their Tβ4 mRNA levels. This pattern of expression agrees well with previous findings noted in chick and mouse[52,104]. Notably, Dathe et al. observed that, in the chick, at later stages of development, Tβ4 expression in established vessels diminished, in similar fashion to that observed here, in the dorsal aorta[52]. This is compelling evidence that suggests Tβ4 may play a role in blood vessel development. As

previously described, this can be separated into many different sub-processes, including but not limited to vasculogenesis, angiogenesis and vessel maturation.

In considering which of these processes Tβ4 may be involved with, further clues can be gathered from the expression pattern data. Tβ4 is expressed in both arteries and veins, largely ruling out the possibility that Tβ4 is involved in the specification between arteries or veins. This is because a difference in expression levels between arteries and veins appears to be a *de facto* prerequisite to have a specific role in this process. Examples of this principle include the arterial restricted expression of Ephrin-B2 and venous restricted expression of EphB4[110], as well as the preferential arterial expression of the arterial specifying transcription factor Gridlock[111]. It has also now been established that Tβ4 is expressed primarily in endothelial cells and not other vascular cell lineages including smooth muscle cells/pericytes.

It is thought that the very earliest blood vessels in the embryo form via vasculogenesis – the process of *in situ* differentiation of endothelial cells and their coalescence to form tubular structures[112]. As Tβ4 is expressed in vessels throughout all stages of development it becomes highly unlikely that Tβ4 is acting specifically and solely as a key molecule in vasculogenesis.

Because most blood vessels arise through one of the processes of angiogenesis, be it sprouting or intussucesptive[44], the near ubiquitous expression of Tβ4 in blood vessels could indicate an angiogenic function for Tβ4. Endothelial cell migration is an indispensable mechanism of this process. When this is combined with Tβ4's previously described roles in cell migration in general and endothelial cell migration

in particular, it is clear that the possibility that Tβ4 plays a part during *in vivo* angiogenesis is worthy of further investigation.

In order to form a stable blood vessel, endothelial cells need to become invested by mural cells. The pattern of aortic Tβ4 expression indicates a possible role in this process. Once endothelial cell tubes are formed through either angiogenesis or vasculogenesis, the process of mural cell recruitment begins straight away via the secretion of endothelial derived paracrine mediators such as TGF-β and PDGF-BB. Tβ4 being expressed in the early vasculature but not in later post-formation stages of the same vessel's lifespan is consistent with the molecule being required for the initial building of a mural cell wall but then becoming dispensable once this structure has formed. This type of vessel maturation relies on endothelial - mural cell interactions and cell autonomous functions in both cell types[58,88]. Given that Tβ4 is expressed in the endothelium and not significantly in mural cells, if Tβ4 plays a role at this level, it is likely due to a function of the endothelium rather than an autonomous role within mural cells.

3.3.2 Expression of Tβ4 in the Developing Heart

Cardiac expression of Tβ4 was observed in four locations during development:

- The myocardium
- The endocardial cushions
- The endocardium
- The epicardium

3.3.2.1 Myocardial Expression of Tβ4

The developing embryonic myocardium has been shown to recruit its coronary vasculature via the secretion of Tβ4. The paracrine secretion of Tβ4 acts on epicardial derived progenitor cells to stimulate their migration and differentiation into the cells of the coronary vasculature[50]. Consistent with this, Tβ4 expression was observed in the myocardium from E11.5.

3.3.2.2 Endocardial Cushion Formation

During cardiac development, the primitive atrio-ventricular chamber becomes separated by the endocardial cushions, which eventually go on to form the atrioventricular bicuspid and tricuspid valves[106]. This process starts following secretion of inductive molecular signals from the myocardium to the underlying endocardium. The endocardial cells then undergo an epithelial to mesenchymal (EMT) transformation and migrate inwards into the myocardium to form the cardiac cushion mesenchyme. Tβ4 is now shown to be expressed in these developing endocardial cushions. As the cardiac cushion cells consist of a population of migratory cells, clearly there is a possible role for Tβ4 in this system as a regulator of actin polymerisation and subsequent migratory capacity. However, one intriguing possibility is that Tβ4 itself may be involved in the regulation of EMT. Although there is, to date, no direct evidence to support this hypothesis, one evolving concept of cancer biology is that EMT facilitates neoplastic lesions to become metastatic[113]. Since an increased expression of Tβ4 has been shown amongst certain tumour types

to be correlated with an increase in metastatic potential[4]; Tβ4 mediated regulation of EMT provides an attractive common link between the two.

3.3.2.3 Epicardial expression of Tβ4

Interestingly, from the E10.5 stage onwards, qualitatively high levels of Tβ4 expression were observed in the epicardium of the heart. This layer consists of a population of mesothelial cells, which eventually undergo EMT and invade the myocardium to form the coronary vasculature[114]. However, this process is not thought to take place until approximately E14.5 in the mouse. One possible explanation for why the epicardium expresses Tβ4 at E10.5 may lie in the embryonic origins of the epicardium. The epicardium originates from a tissue known as the proepicardial organ. This is situated at the septum transversum in mammals[114]. At E10 in mice, the looping heart tube comes into contact with the proepicardial organ and cells migrate from the proepicardium to cover the surface of the myocardium and become the epicardium. It is possible that high levels of Tβ4 in the epicardium at E10.5 are present due to the migratory nature of the epicardial mesothelial cells and consequently the need for dynamic regulation of the actin cytoskeleton.

3.3.3 Expression of Tβ4 in the Neural Network

In situ hybridisation disclosed expression of Tβ4 in three locations throughout the developing nervous system. The first location identified was the embryonic hindbrain at E10.5. Tβ4 expression in the embryonic murine hindbrain has previously been described[108]. These studies carried out a more thorough analysis of Tβ4 expression in

the forebrain and hindbrain, with the finding that Tβ4 is expressed in both throughout development. However, the precise extent of Tβ4 expression was dependent on both developmental stage and spatial location. It appeared to these authors that Tβ4 expression was highest in regions experiencing ongoing neural corticogenesis. Although, in the limited expression analysis conducted in this manuscript Tβ4 was expressed in the hindbrain equally in mantle and ventricular zones of the neural tube, Carpenterio et al. showed a higher mantle than ventricular layer expression[108].

Tβ4 is also expressed in the spinal cord throughout development. As the neural tube separates into white and grey matter from E12.5 to E16.5[109], the area of expression becomes localised to the developing inner grey matter. This zone of the spinal cord at this stage of development is a site of ongoing neurogenesis, as this is the site of motor and inter neuron development[109]. These processes, particularly that of motor neuron development, are dependent on extensive cellular migration in order to form synapses/dendritic connections at the appropriate locations[115].

Another site of Tβ4 expression, and qualitatively, the site of highest Tβ4 expression throughout the embryo were the developing dorsal root ganglia. Once again, this pattern of expression agrees well with the published literature. In both chick and mouse embryo, dorsal root ganglia expression of Tβ4 has been observed[52,108]. The dorsal root ganglia are primarily composed of cells derived from migrating neural crest. These cells differentiate to form the afferent sensory neurons of the peripheral nervous system[109]. The cell bodies of these neurons are located in the dorsal root ganglia, and during embryonic development emit axonal projections, which follow

chemotactic signals to their target sites before terminating at neural sensory apparatus[115].

Although, to date, no functional studies have been conducted to elucidate the purpose of Tβ4 in the developing CNS and PNS, it is possible to speculate that Tβ4's canonical role in actin cytoskeleton formation may be at play in this system. The processes of axonal projection and migration, in the peripheral nervous system, are dependent on the formation of an axonal growth cone at the leading terminal end of the neuron. This growth cone comprises many actin dependent superstructures such as cellular filopodia, lammelipodia and actin bridges[115]. It is speculated, that due to its actin regulatory role, Tβ4 may perform a function in formation of these structures, critical for axonal outgrowth. The correlation between Tβ4 expression in the developing brain and the process of corticogenesis, identified by Carpintero et al. may also support a role for Tβ4 in neuronal migration[108]. During neurogenesis, cortical neuron subtypes differentiate in the ventricular zone before migrating, largely by the process of translocation, to the cortical zone[116]. The temporally overlapping events of Tβ4 expression and corticogenesis thus provide circumstantial evidence for Tβ4's function in the developing nervous system being a cell autonomous role in cell migration.

However, there is a case to be made against this. Carpintero et al. demonstrated that Tβ4 expression is higher, in general, in the mantle/cortical zone than the ventricular zone[108]. This layer consists largely of differentiated neurons, which have migrated into their positions. If Tβ4 is truly involved as an intracellular regulator of cell migration in the developing cortex, it might be expected that Tβ4 would be expressed

most highly in the actively migrating cells, and thus, presumably in the ventricular layer as opposed to the mantle layer. Some insight into possible alternative functions for Tβ4 comes from studies of Tβ4 in post-natal neural regeneration. Several groups have experimented with Tβ4 in the context of CNS injury. Pharmacologically administered Tβ4 protein has been shown to stimulate cellular mechanisms of regeneration in experimental autoimmune encephalitis[36] and traumatic brain injury[117]. Notably, Tβ4 was also able to stimulate the proliferation of oligodendrocyte precursor cells *in vitro*[36]. In contrast, during investigations into spinal cord regeneration, downregulation on Tβ4 via anti-sense viral transduction resulted in an increased ability of neural progenitor cells to differentiate into neurons and promote axonal regeneration[118]. Such observations support a hypothesis that Tβ4 may be acting in a paracrine fashion to control the fate of stem cell populations in the developing CNS.

3.3.4 Expression of Tβ4 in the Gastrointestinal System

Tβ4 is also shown here to be expressed in the developing gastrointestinal system, predominantly on the epithelial surface of gut. The gut epithelium is derived from the endodermal layer of the developing embryo. From E14.5 onwards, villous structures form due to projection of the underlying mesodermal layer into the gut lumen with the endodermal derived epithelial cells overlying these structures. The epithelial layer is then replenished in adult from a stem cell pool located in the villous crypts[119]. The observations made here agree well with immunohistochemical analysis of Tβ4 expression in the gut of foetal and adult humans[120]. Tβ4 in these systems was present mainly on the epithelial surfaces of each of the specialised regions of the human gut. At this stage, it is only possible to speculate on the functions of Tβ4 in these epithelial

linings. Although cell autonomous Tβ4 may be essential for cell migration, it is difficult to see how this is relevant to a collection of surface epithelial cells. It may be possible, that Tβ4 allows the cell to maintain effective cell structure and cellular adhesions, as these characteristics are dependent on an actin filament network. Alternatively, these cells could represent a secretory factory for Tβ4 to act in a paracrine fashion on other nearby cell populations – an effect that has been detected in the developing heart[50]. Consistent with this, Tβ4 has been identified as a component of the secreted gastric mucous[120].

3.3.5 Expression of Tβ4 in the Respiratory System

Tβ4 mRNA expression can be seen in epithelial cells of the developing airways of E16.5 mouse embryos. At this time point, the endodermally derived airway tissue is at the canalicular stage of development, at which the terminal lung buds narrow before entering the E18.5-P5 saccular stage characterised by the development of architecturally complete alveoli[121]. Interestingly, the E16.5 stage coincides with the period of development where airway smooth muscle cell progenitors expand, differentiate and migrate to the epithelial airway walls[122]. It is possible that Tβ4 may be playing a role in this process.

4 Genetic Models for Tβ4 Loss of Function

4.1 Introduction:

Results from the expression analysis of Tβ4 during embryonic development provide some intriguing clues as to the role that Tβ4 might play in the developing vasculature. Ubiquitous vascular expression suggests it may be involved in some universal process common to all blood vessels whether arteries or veins, big or small.

In order to further develop this insight into Tβ4 vascular function, further experiments need to be undertaken. One classical approach to dissecting the function of a gene and its protein product, is to generate *in vivo* loss of function models. The phenotype of the resulting organism can then be studied, and information about the gene's function can be gleaned. Mouse transgenesis is one of the most commonly used methods to create such loss of function models. The use of mouse as a model organism is often favoured due the wealth of knowledge, which has been accumulated regarding genetic modification of this organism. Its suitability as a model organism is further enhanced by its ease of maintenance, rapid breeding and high genetic homology to human[123].

For these reasons, it was decided to use mouse genetic loss of function models to further understand the role of Tβ4 in vascular development.

4.2 Results

4.2.1 Mouse Transgenesis Can Be Used to Achieve Germline Global Knockout of Tβ4

The first genetic loss of function model, which was examined, was a global knockout of Tβ4, received as a kind gift from Martin Turner at the Laboratory for Lymphocyte Signalling and Development, Babraham Institute, Cambridge, UK. Knockout of Tβ4 was achieved in this mouse by replacing exon 2 of the Tβ4 gene with a neomycin resistance cassette in order to create a non-functional allele (**Fig. 4.1**). This general strategy has been used on many occasions to great success with other genes[124]. As Tβ4 is located on the X chromosome, but not the Y chromosome of mice; male mice in possession of the knockout allele will be hemizygous and are denoted Tβ4 -/Y (Tβ4 +/Y being the wild type equivalent). Female mice, on the other hand possess two copies of the X chromosome and may be either homozygous knockouts (Tβ4 -/-), heterozygous for the knockout allele (Tβ4 +/-) or wild type (Tβ4 +/+).

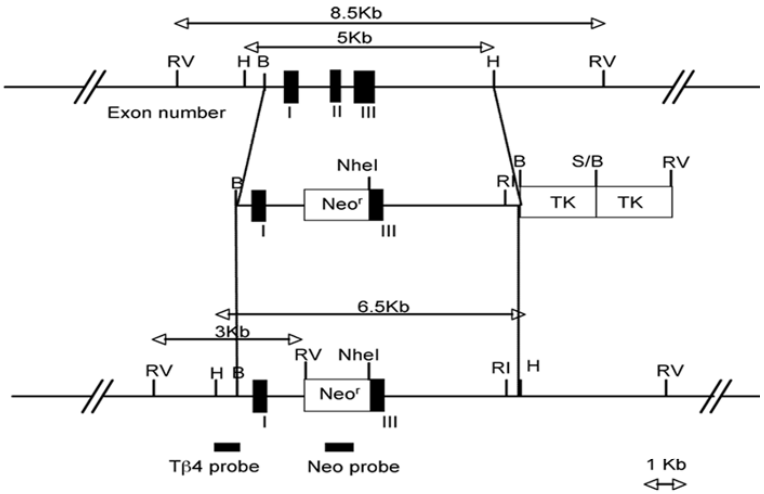


Fig. 4.1
A scheme to knockout Tβ4 through insertion of a neomycin resistance cassette in exon 2 of the Tmsb4x gene

Schematic diagram to demonstrate the strategy used to knockout Tβ4 in the mouse genome. Exon 2 of the murine Tβ4 gene was replaced by a neomycin resistance cassette, resulting in the creation of a non-functional allele. This figure was created by MartinTurner, Laboratory for Lymphocyte Signalling, Babraham Institute, Cambridge, UK.

4.2.2 Knockout of the Tmsb4x Gene Abolishes Synthesis of the Tβ4 Protein In Vivo

In order to ascertain that this strategy had been successful at producing the lack of a functional Tβ4 transcript, quantitative real time polymerase chain reaction (qRT-PCR) was performed on RNA extracted from E10.5 Tβ4 -/Y and Tβ4 +/Y embryos, for the presence of mature Tβ4 mRNA. Three Tβ4 +/Y embryos contained variable levels of the Tβ4 transcript, whilst in the Tβ4 -/Y embryos, this transcript could not be detected at all (**Fig. 4.2**).

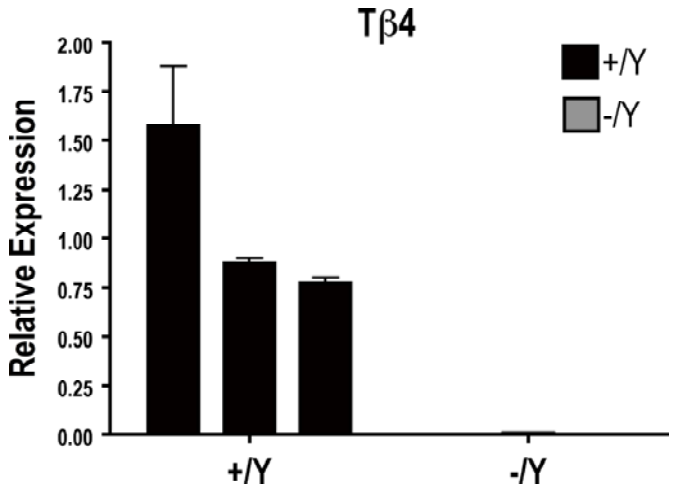


Fig. 4.2
Knockout of the Tmsb4x gene results in ablation of Tβ4 mRNA expression *in vivo*

E10.5 Tβ4 -/Y embryos expressed no detectable levels of Tβ4 mRNA as quantified by qRT-PCR in comparison to E10.5 Tβ4 +/Y controls. Error bars represent the standard error of four experimental replicates.

4.2.3 Knockout of Tβ4 Results in Impaired Survival

From the initial mouse colonies received, it became clear that Tβ4 knockout (in either the male or female) was compatible with viability and progression of the mice to adulthood. However, it was observed that numbers of knockout mice in litters appeared to be consistently reduced in comparison to their expected Mendelian ratios. In order to test whether Tβ4 knockout resulted in partially decreased viability of offspring, Tβ4 -/Y males were crossed with Tβ4 +/- females and the number of offspring of each expected genotype monitored. When this cross is set up, it is expected that equal ratios of Tβ4 +/Y, Tβ4 -/Y, Tβ4 +/- and Tβ4 -/- mice will be born, if there is no influence of the genotype on the viability of the offspring. In practice, it was observed that by the time of the early postnatal period, around half of the expected Tβ4 -/Y and Tβ4 -/- mice had failed to survive (**Table 4.1**).

	Expected	E10.5	Postnatal
Genotype			
+/Y	25%	23	40
-/Y	25%	19	19
+/-	25%	19	34
-/-	25%	28	24
Total		**89**	**117**
χ^2		2.46	9.26
p-value		0.482	**0.026**

Table 4.1
Tβ4 -/Y mice die *in utero* between E10.5 and birth

Offspring of crosses between Tβ4 -/Y male and Tβ4 +/- female mice are present in the correct Mendelian ratios at E10.5. However, by the early postnatal period, approximately half of the Tβ4 -/Y and Tβ4 -/- offspring have died.

Given the previously established strong expression of Tβ4 in the developing vasculature, it was hypothesised that lack of Tβ4 in the developing vasculature might be the cause of the embryonic lethality observed in the Tβ4 knockout mouse. Knockout of several genes involved in vascular development can lead to embryonic lethality[82,83,125,126]. Usually, when a failure of vascular development results in impaired survival, the defects manifest themselves at a mid-gestational stage. Thus, a decision was take to examine the phenotype of Tβ4 knockout embryos at the E10.5 stage of development.

4.2.4 E10.5 Tβ4 -/Y Embryos Display A Haemorrhagic Defect

In order to perform this analysis in a systematic way, it was necessary to generate litters with both null alleles and wild type littermate controls. As the Tβ4/Tβ4 null mutant allele is present only on the X chromosome, the only cross which can produce

sex matched wild type and null littermates is between Tβ4 -/Y and Tβ4 +/- parents. Thus, when analysing the Tβ4 null phenotype, Tβ4 -/Y males were always compared to Tβ4 +/Y littermates. Although, at the E10.5 stage of development no significant embryonic lethality is present (**Table 4.1**), it was observed that between 5-10% of Tβ4 -/Y embryos exhibited overt haemorrhage in their pericardial cavities (**Figs. 4.3a and b**).

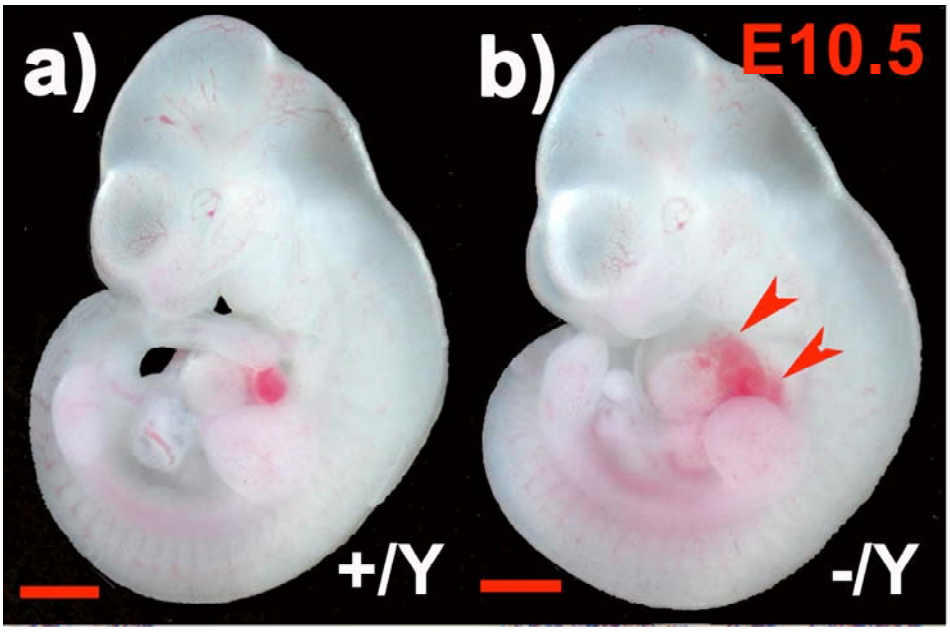


Fig. 4.3
Tβ4 -/Y embryos display overt pericardial haemorrhage

Wholemount pictures at E10.5 reveal that wild type Tβ4 +/Y embryos have developed normally (**a**), whilst a proportion of Tβ4 -/Y embryos display overt haemorrhage in the pericardial space (red arrowheads) (**b**). Scale bars: (a and b) 500μm.

In order to ascertain the extent of the vascular haemorrhage in these specimens, Tβ4 -/Y embryos, which displayed pericardial haemorrhage, were embedded and sectioned along with Tβ4 +/Y littermates. In haematoxylin and eosin (H&E) stained sections, the presence of blood can clearly be observed in the pericardial cavity of Tβ4 -/Y embryos, whilst the pericardial space in Tβ4 +/Y littermates is normal (**Figs. 4.4a and**

b). Noticeable also, is the presence of vascular haemorrhage into the coelomic cavity – the precursor of the peritoneum, in the affected Tβ4 -/Y embryos (**Fig. 4.4c**). This was never seen in Tβ4 +/Y wild type controls (**Fig. 4.4d**).

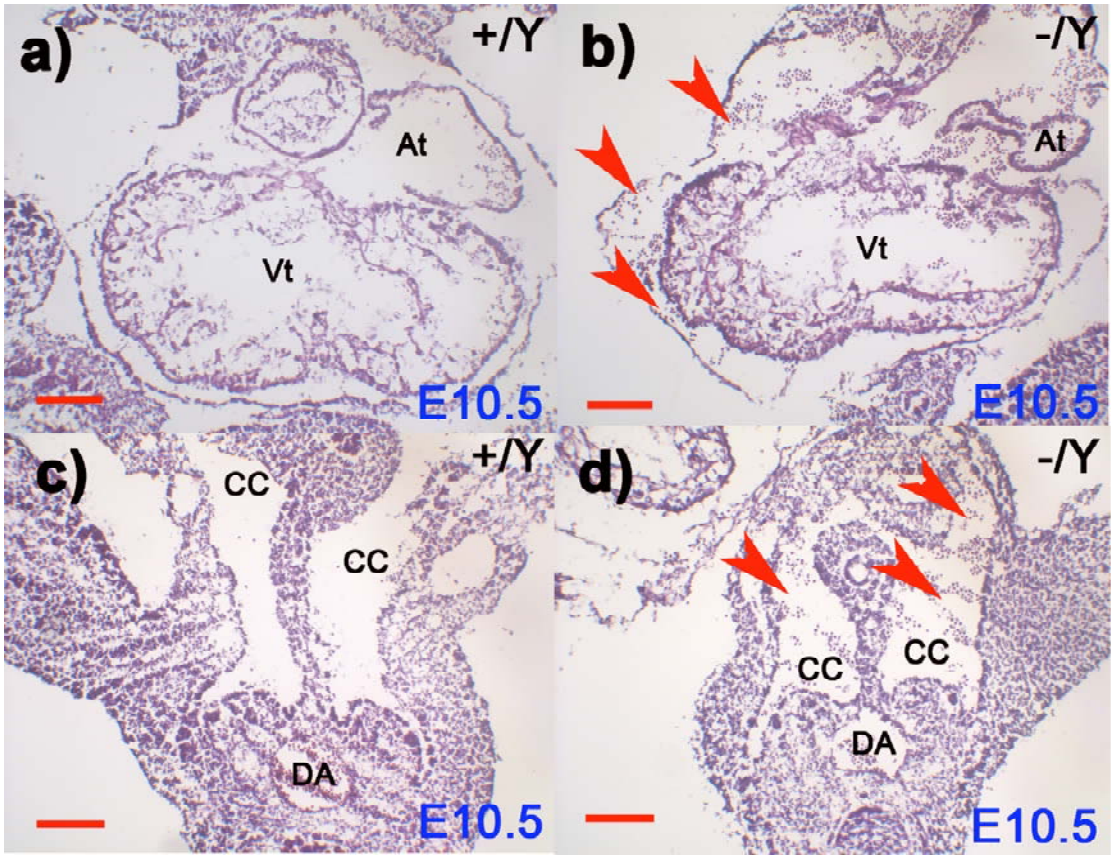


Fig. 4.4
E10.5 Tβ4 -/Y embryos display pericardial and coelomic cavity haemorrhage

H&E staining of axial sections demonstrates a pericardium free from haemorrhage in E10.5 Tβ4 +/Y embryos (**a**). However, the overt haemorrhage into the pericardial space observed on whole mount is confirmed by sectioning through haemorrhagic E10.5 Tβ4 -/Y embryos (red arrowheads) (**b**). Whilst these Tβ4 +/Y embryos possess a normal coelomic cavity (**c**), the coelomic cavities of the Tβ4 -/Y haemorrhagic mutants are also filled with blood (red arrowheads) (**d**). At (atrium), CC (Coelomic cavity), DA (Dorsal aorta), Vt (Ventricle). Scale bars: (a, b, c and d) 200μm.

4.2.5 Tβ4 -/Y Embryos Possess a Reduced Peri-Aortic Mural Cell Coverage

A haemorrhagic phenotype in embryos is very reminiscent of mouse mutants, published in the literature, which have inadequate investiture of their developing blood vessels with mural cell support[127,128]. In order to determine whether this was the cause of the haemorrhagic defects in the Tβ4 -/Y mouse, axial sections through the dorsal aortae of E10.5 Tβ4 -/Y and Tβ4 +/Y mice were stained with NG2 – a marker for mural cells, and examined by immunofluorescence microscopy for the presence of mural cells around the developing aorta (**Figs. 4.5a, b and c**). Quantification of the peri-aortic mural cells staining using ImageJ software disclosed that the aortae of E10.5 Tβ4 -/Y embryos had significantly fewer mural cells than Tβ4 +/Y littermate controls. It is thus likely that the presence of vascular haemorrhage in the E10.5 Tβ4 -/Y embryos is due to rupture of the aorta into the pericardial and coelomic cavities due to inadequate mural cell coverage of the vessel wall.

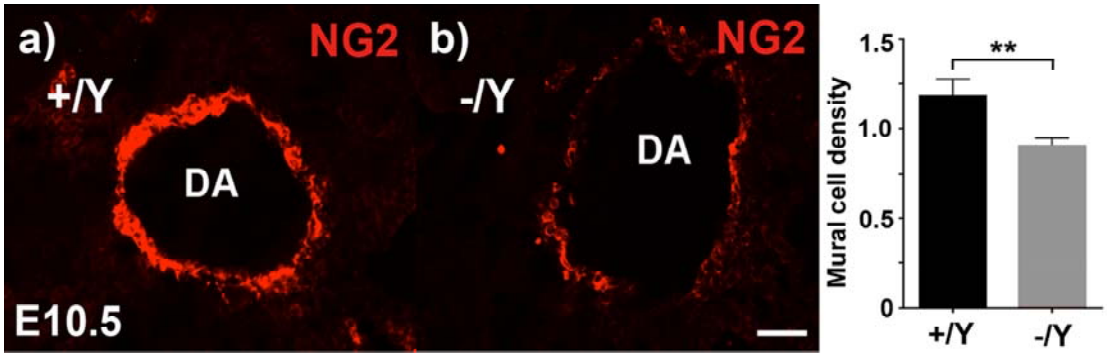


Fig. 4.5
E10.5 Tβ4 -/Y have a reduction in NG2+ mural cell staining around their dorsal aortas

NG2 immunostaining of axial sections revealed that E10.5 Tβ4 +/Y embryos had substantial coverage of their developing dorsal aortas with NG2 positive mural cells (**a**). However, Tβ4 -/Y dorsal aortas were incompletely invested by NG2 expressing mural cells (**b**). Quantification using ImageJ software of these mural cell defects across ten sections per embryo, with six embryos per group, demonstrated a significant and quantifiable reduction in mural cell coverage in Tβ4 -/Y embryos. DA (Dorsal aorta). Scale bars: (a and b) 20μm. ** $p<0.01$. Error bars represent standard error of the mean.

In order to provide further evidence for a global reduction in mature mural cells in the E10.5 Tβ4 -/Y embryo, RNA was extracted from whole somite matched Tβ4 -/Y and Tβ4 +/Y embryos. qRT-PCR was conducted on these samples for a large panel of mural cell marker genes. These marker genes included genes, which were representative of a VSMC type mural cell such as smooth muscle actin and SM22α as well as genes more indicative of pericytes such as NG2, endosialin, angiopoietin-1 and CD13. It was observed that Tβ4 -/Y embryos displayed significantly reduced expression of a number of these mural cell markers in comparison to their somite matched Tβ4 +/Y controls (**Fig. 4.6**). This indicates that mural cell marker genes, typical of both pericytes and VSMCs, are decreased globally in Tβ4 -/Y embryos.

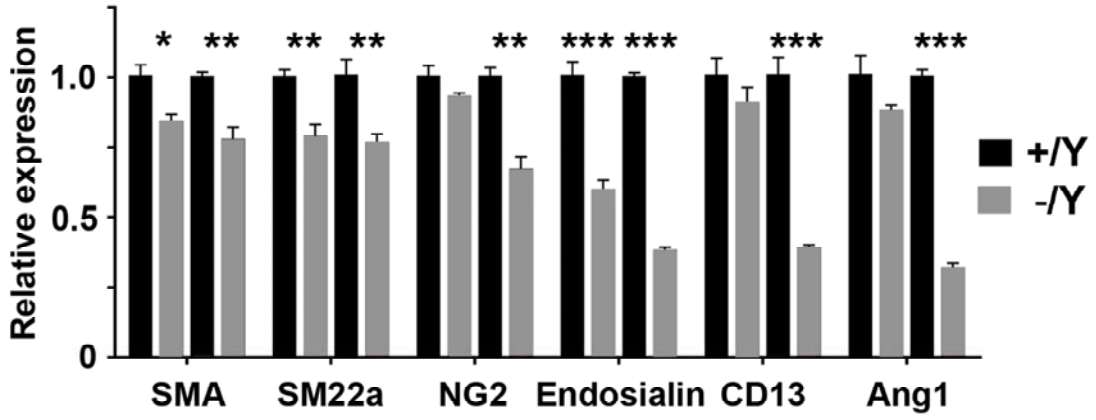


Fig. 4.6
E10.5 Tβ4 -/Y display globally depressed levels of mural cell markers

qRT-PCR analysis of somite matched pairs of E10.5 Tβ4 +/Y and Tβ4 -/Y embryos reveals reduced levels of mural cell marker genes in Tβ4 -/Y embryos. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Each pair of adjacent black and grey bars represent a pair of somite matched embryos. Error bars represent standard error of the mean.

4.2.6 Tβ4 -/Y Embryos Display Dermal Haemorrhage at E16.5

Haemorrhage in the embryos of genetic mouse mutants may manifest itself at any stage of mid or late gestational development from E10.5[129], through to E16.5[130]. Although it is likely that the 5-10% of Tβ4 -/Y mutants, which display overt haemorrhage at E10.5, will not survive to later stages of development, the incidence at this time point cannot account for all of the embryonic lethality observed in Tβ4 -/Y mutants. Thus, embryos from the E14.5 stage of development were assessed for the presence of a haemorrhagic phenotype. It was observed that Tβ4 -/Y mouse mutants at this E14.5 stage often exhibited a variable degree of dermal haemorrhage (**Fig. 4.7a and b**). However, as dermal haemorrhage was sometimes viewed in the Tβ4 +/Y littermate controls, a blinded scoring system was devised, whereby a numerical value would be assigned to an embryo based on the extent of dermal haemorrhage observed without the genotype being known. Analysis by this method

revealed that Tβ4 -/Y embryos incurred a significantly greater incidence of surface dermal haemorrhage than their wild type littermates (**Fig. 4.7c**).

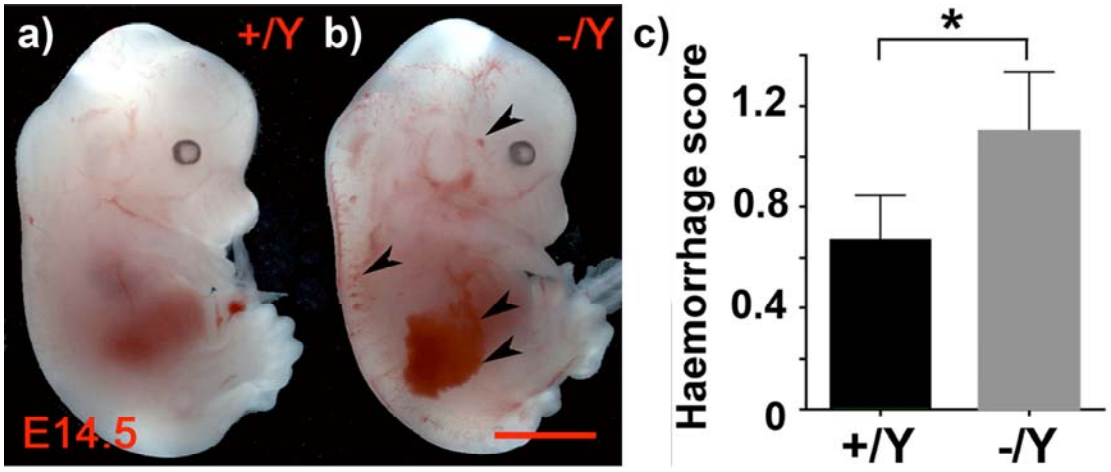


Fig. 4.7
E14.5 Tβ4 -/Y embryos display cutaneous haemorrhage
At E14.5, a proportion of Tβ4 -/Y embryos show dermal vascular haemorrhage (black arrowheads) (**a** and **b**). Whilst blinded to genotype, embryos were assigned a score out of 5 to quantify the extent of dermal haemorrhage observed. A significant difference was seen between the haemorrhage scores of Tβ4 +/Y and Tβ4 -/Y embryos (**c**)(n=21). Scale bars: (a and b) 2mm. Error bars represent standard error of the mean.

4.2.7 Defects in Mural Cell Recruitment Coverage Underlie Dermal Haemorrhage in E14.5 Tβ4 -/Y Embryos

It then became necessary to assess whether the haemorrhagic phenomena observed in the skin of Tβ4 -/Y mutants at E14.5 had the same aetiology as the haemorrhage seen at E10.5, namely one based in reduced recruitment of mural cells to the developing vessel wall. In order to investigate this, skin sections were dissected away from the E14.5 Tβ4 -/Y and Tβ4 +/Y embryos before being stained with the endothelial marker endomucin and the mural cell marker NG2. Confocal microscopy of these samples showed that whilst the dermal vessels of Tβ4 +/Y embryos had a near continuous coverage of mural cells, there were several gaps and interruptions in the mural cell

coverage of vessels from Tβ4 -/Y embryos (**Fig. 4.8a and b**). This implies that the origin of dermal haemorrhage in the E14.5 Tβ4 -/Y embryos is likely to be a lack of structural support in the blood vessels of the skin.

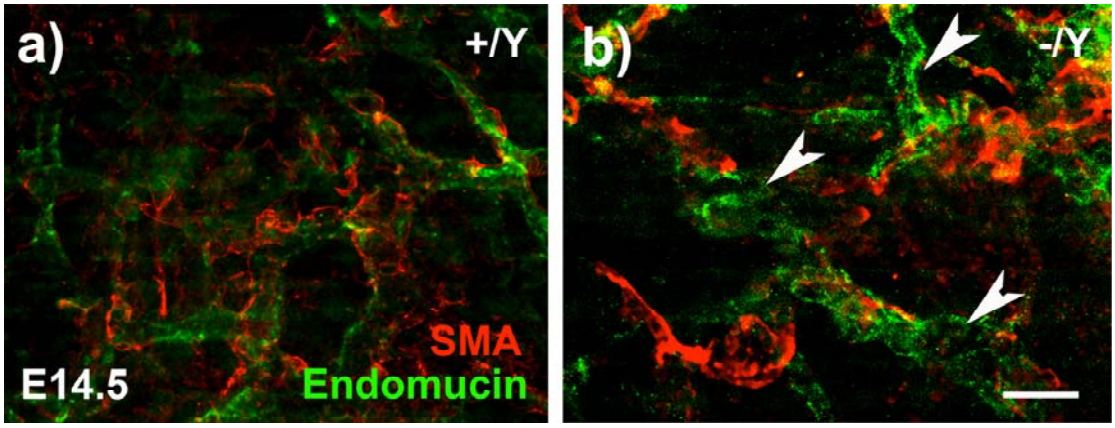


Fig. 4.8
Skin vessels of E14.5 Tβ4 -/Y embryos possess reduced mural cell coverage

Confocal microscopy of wholemount E14.5 dermal specimens, stained for endomucin and smooth muscle actin expression displayed a vascular plexus well invested by mural cells in Tβ4 +/Y samples (**a**), but a vascular plexus with several regions lacking mural cell coverage (white arrowheads) in Tβ4 -/Y samples (**b**). Scale bar applies to both a and b: 10μm.

4.2.8 Tβ4 Can Be Knocked Down in a Tissue Restricted Fashion

Given that vascular expression of Tβ4 appears to be confined exclusively to the endothelium and is not observed in the mural cells themselves, it is highly likely that the lack of endothelial Tβ4 is responsible for the vascular defects of Tβ4 -/Y embryos observed above. However, in order to provide further evidence for this conclusion, genetic loss of function models, which impaired Tβ4 expression specifically in the endothelium were analysed. The first strategy that was attempted, made use of a previously published mouse, which expressed a Tβ4 shRNA under the control of a tissue specific Cre recombinase[50].

When Cre recombinase is expressed in a cell where the conditional Tβ4 shRNA construct is also present, the Cre enzyme can mediate recombination between two LoxP sites on the transgene. This causes the excision of a transcription termination sequence, which brings transcription of the Tβ4 shRNA sequence under the control of the endogenously active H1 pol III promotor, leading to transcription of the short hairpin RNA (**Fig. 4.9**). This shRNA is then processed by the enzyme Dicer, to produce siRNAs against Tβ4. These siRNAs interact with the protein Argonaute to form an RNA induced silencing complex (RISC), which degrades Tβ4 mRNA[131].

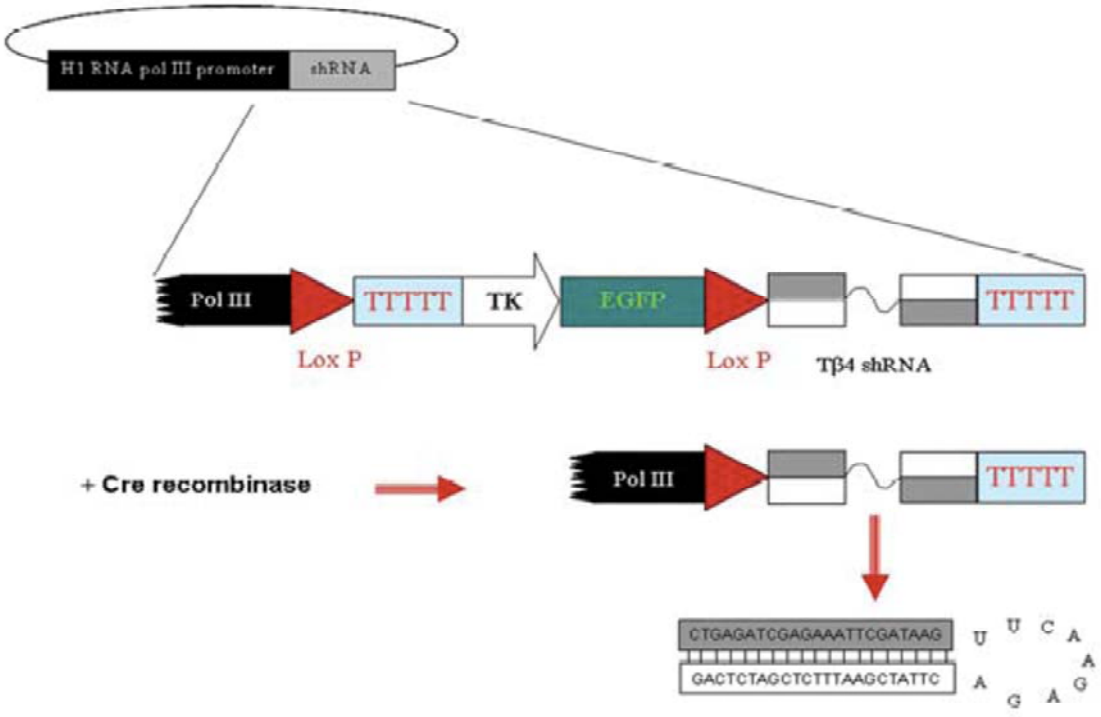


Fig. 4.9
Transcription of a Tβ4 shRNA initiated by Cre recombinase can induce knockdown of Tβ4 in vivo

Schematic to demonstrate the strategy used to create a tissue specific knockdown model of Tβ4. Cre recombinase mediated excision of the floxed region of the transgene will eliminate a transcriptional termination sequence. This allows the transgene for a Tβ4 shRNA to come under the control of the constitutively active Pol III promotor, allowing transcription of a Tβ4 shRNA. Taken from[50].

In order to knockdown Tβ4 via these means in the endothelium, the Tβ4 shRNA mouse was crossed with a Tie2-Cre mouse, which expresses Cre recombinase under the control of the Tie2-Cre promotor[132]. As Tie2 is predominantly expressed in the vascular endothelium, this should ensure that Cre recombinase will only be expressed in vascular endothelial cells. In order to assess whether the in-house Tie2-Cre mouse appropriately expressed Cre recombinase in the developing endothelium, it was crossed with the Rosa 26R (R26R)[133] and R26R-eYFP[134] reporter mouse strains. These mouse strains express either LacZ or eYFP in cells, which also express Cre recombinase, by virtue of the removal of a LoxP flanked stop codon upstream of the respective reporter encoding sequence. As LacZ can be visualised by X-Gal staining and eYFP can be visualised with fluorescent microscopy, this strategy allows cells, which express Cre recombinase to be identified and traced.

Tie2-Cre x R26R and Tie2-Cre x R26R-eYFP embryos were stained with X-Gal or examined by fluorescence microscopy respectively. E10.5 offspring of the Tie2-Cre x R26R cross, demonstrated expression of LacZ in agreement with previously published data[132]. Namely, LacZ was seen in the walls of small and large blood vessels systemically as well as the endocardium and endocardial cushions of the developing heart (Figs. **4.10a, b and c**). eYFP was observed to be co-expressed with the endothelial marker PECAM in sections through the E14.5 embryonic aorta of offspring resulting from the Tie2-Cre x R26R-eYFP cross, thus indicating that vascular expression of Cre recombinase is confined to the endothelium (**Fig. 4.10d**).

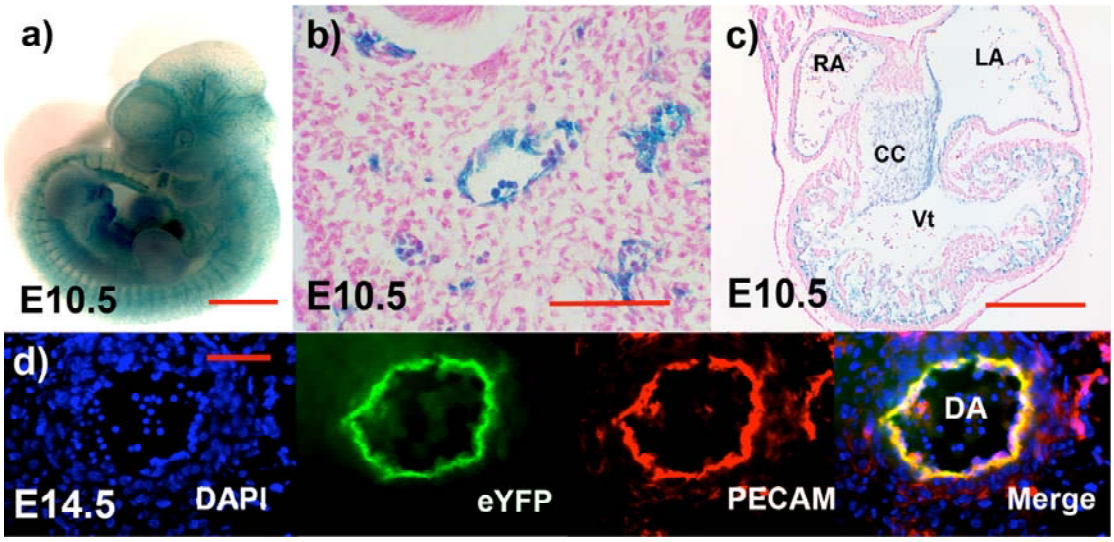


Fig. 4.10
Tie2-Cre embryos express Cre recombinase in the developing endothelium

Whole mount X-Gal staining of E10.5 Tie2-Cre R26R demonstrated Cre expression throughout the developing vasculature (**a**). Sectioning of this embryo followed by eosin counter staining revealed expression of cre in blood vessels (**b**) and the endocardium and cardiac cushions of the heart (**c**). Immunofluorescence staining of axial sections through thr terminal aorta of E14.5 Tie2-Cre R26R-eYFP mice revealed that Cre (eYFP) was confined to PECAM positive endothelial cells (**d**). CC (Cardiac cushion), DA (Dorsal aorta), LA (Left atrium), RA (Right atrium), Vt (Ventricle). Scale bars: (a) 1mm, (b) 100μm, (c) 500μm, (d) 50μm.

4.2.9 E10.5 Tβ4-RIEK Embryos Rarely Reproduce the Same Haemorrhagic and Mural Cell Defects Observed in Tβ4 -/Y Mice

Crossing the Tβ4 shRNA mouse with the Tie2-Cre mouse, leads to generation of Tβ4 shRNA random integrant endothelial knockdown mice (Tβ4-RIEK). When E10.5 Tβ4-RIEK embryos were bred, one of the mutant offspring displayed overt pericardial haemorrhage (**Figs. 4.11a and b**). This haemorrhage in the pericardial space was also visualised upon sectioning and H&E staining of the embryo (**Figs. 4.11c and d**).

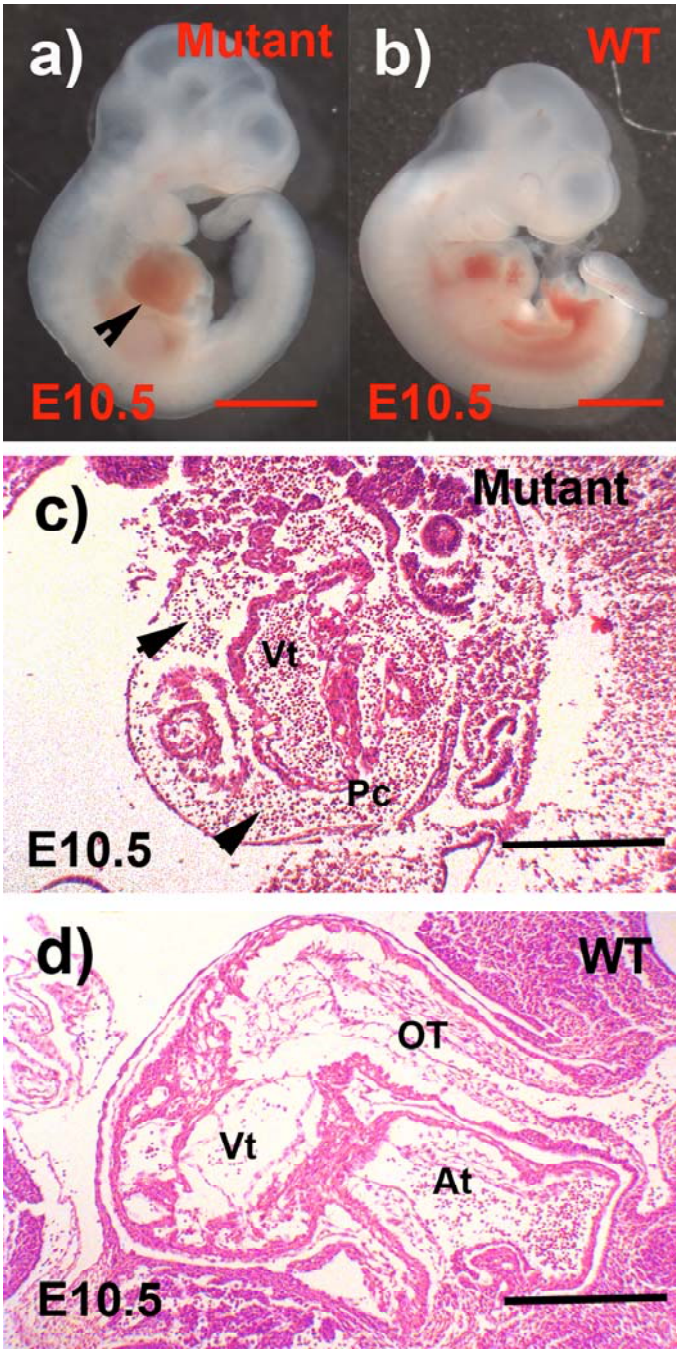


Fig. 4.11
E10.5 Tβ4-RIEK mutant embryos display pericardial haemorrhage

Whole mount images reveal one instance of an E10.5 Tβ4-RIEK embryo, which displays overt pericardial haemorrhage (**a**), in comparison to a wild type littermate control (**b**). Saggital H&E stained sections through these embryos confirm the presence of aberrant blood in the pericardial space of the Tβ4-RIEK mutant (black arrowheads) (**c**), but not in the wild type littermate control (**d**). At (Atrium), OT (Outflow tract), Pc (Pericardial cavity), Vt (Ventricle). Scale bars: (a and b) 1mm, (c and d) 500μm.

In order to assess whether this embryo displayed a coexisting mural cell defect, which could account for the pericardial haemorrhage that was observed, sagittal sections through the dorsal aorta of the affected embryo and a wild type littermate control were stained for the mural cell marker smooth muscle actin. The wild type embryo possessed a dorsal aorta which had a multi layered covering of smooth muscle actin positive mural cells whilst analysis of the mutant embryo showed a dorsal aorta where the investing smooth muscle cell layer was only ever one cell thick and had numerous gaps where no mural cell coverage was apparent (**Figs. 4.12a and b**).

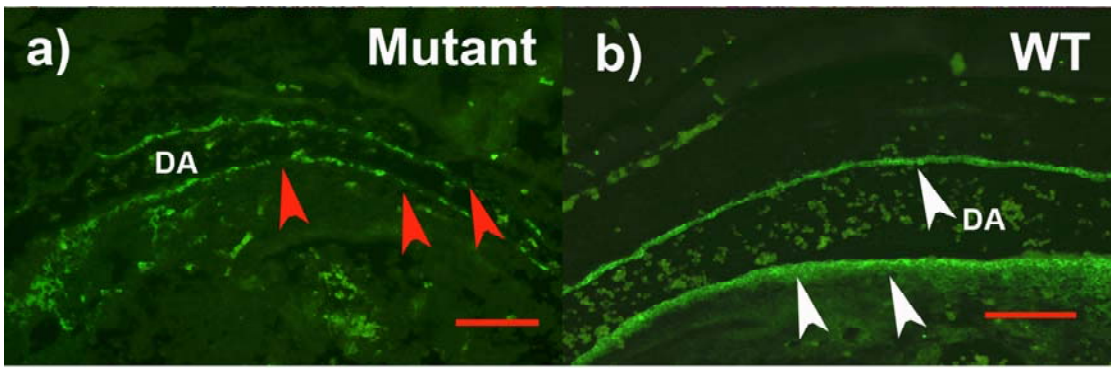


Fig. 4.12
Haemorrhagic E10.5 Tβ4-RIEK embryos have reduced mural cell investiture of their dorsal aortas

Sagittal sections through the dorsal aorta of the haemorrhagic Tβ4-RIEK mutant followed by Immunofluorescence staining for the mural cell marker smooth muscle actin show an aorta in possession of a mural cell layer only a single cell thick. Numerous gaps in the mural cell coverage are also observed (red arrowheads) (**a**). This is in comparison to the aorta of a wild type littermate control which has a uniform mural cell layer several cells thick (white arrowheads) (**b**). DA (Dorsal aorta). Scale bars: (a) 100μm, (b) 150μm.

In spite of analysing several more litters including approximately forty mutant E10.5 embryos, this overt haemorrhagic phenotype was never again observed in the Tβ4-RIEK embryos. As the Tβ4-shRNA construct can lead to variable levels of knockdown[50], it was hypothesised that endothelial cells continued to express Tβ4 at a

low level which might allow the embryos to maintain a normal phenotype until later stages of development. In order to test whether defects in the mural cell coverage of the developing dorsal aorta occurred at later stages in the Tβ4-RIEK mice, E14.5 mutant and wild type embryos were harvested, cryo embedded and sectioned axially through the dorsal aorta. These aortic sections were stained with smooth muscle actin by immunohistochemistry to visualise aortic mural cells. However, Tβ4-RIEK mutant vessels were identical to wild type littermates and no qualitative difference was observed between the mutants and wild types (**Figs. 4.13a, b, c and d**).

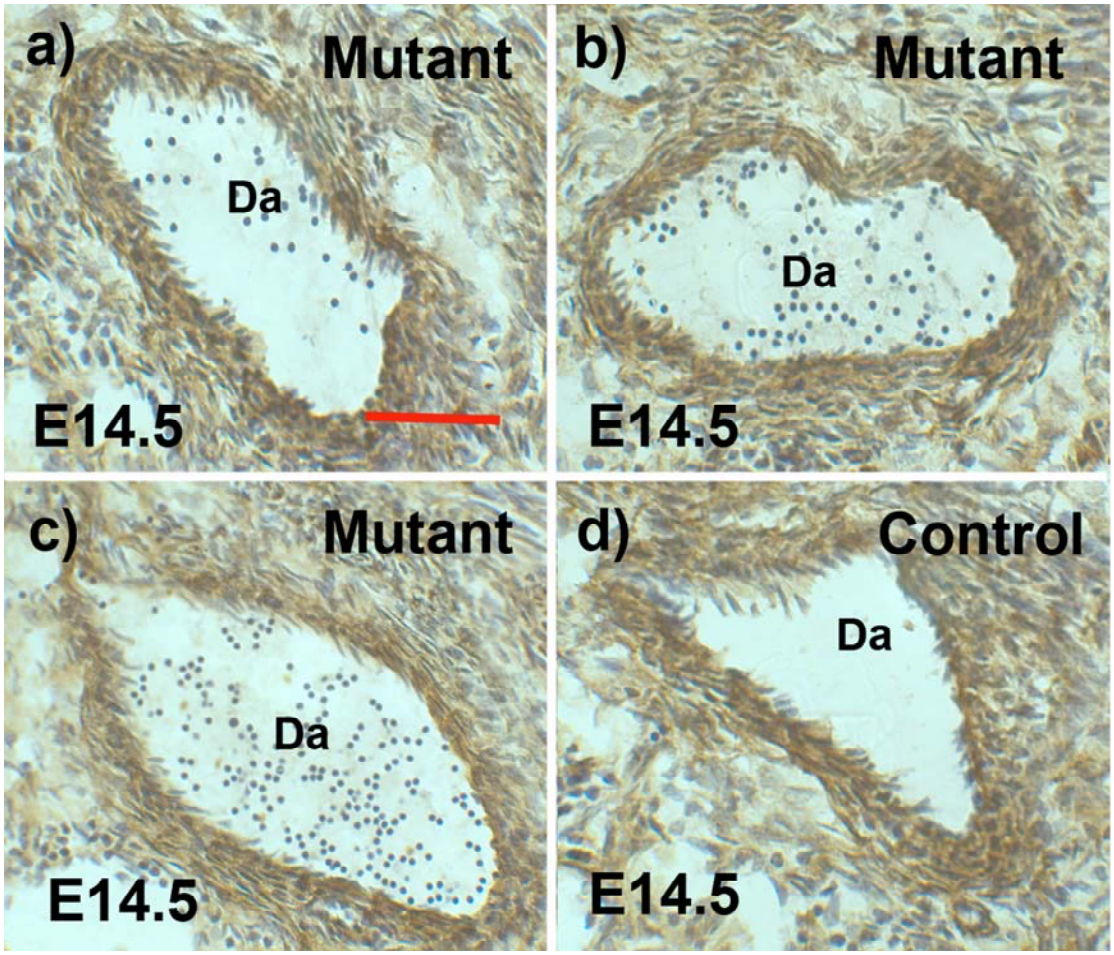


Fig. 4.13
Tβ4-RIEK embryos show no defect in mural cell coverage of their dorsal aortas at E14.5

Immunohistochemical staining for the mural cell marker smooth muscle actin on axial sections through the dorsal aortas of E14.5 Tβ4-RIEK mice revealed no qualitative difference in the mural cell investiture between mutants (**a, b, and c**) and littermate controls (**d**). DA (Dorsal aorta). Scale bars: bar in (a) applies to all figures, 150μm.

One explanation, which might underlie the poorly penetrant phenotypes of the Tβ4-RIEK mice is insufficient expression of the Tβ4 shRNA and inadequate Tβ4 knockdown in endothelial cells. This might be because the Tβ4 shRNA construct was not targeted to a specific locus in the mouse genome and integration took place at random locations when the mouse was generated[50]. It has been previously demonstrated that the expression of randomly inserted transgenes can be affected by generational dependent epigenetic gene silencing[135]. In order to attempt to overcome

this potential problem, use was made of commercially constructed transgenic mouse line, in which the Tβ4 shRNA transgene had been specifically targeted to locus for the gene Hprt, which codes for the protein hypoxanthine-guanine phosphoribosyltransferase. This locus was chosen as it is thought to be open, stable and not subject to epigenetic gene repression, due to its nature as a housekeeping gene[136].

4.2.10 Targeting the Tβ4 shRNA Construct to an Open Genomic Locus Creates a More Reliable Tβ4 Tissue Specific Knockdown

Crossing of the HPRT targeted Tβ4 shRNA mouse with the Tie2-Cre mouse led to the generation of the Tβ4 shRNA HPRT targeted endothelial cell specific knockdown mouse (Tβ4-HEK). In order to attempt to quantify the degree of Tβ4 knockdown achieved in the endothelial cells of both the Tβ4-RIEK and the Tβ4-HEK mice, both mouse strains were crossed with the R26R-eYFP mouse. E14.5 embryos from both Tβ4-RIEK R26R-eYFP and Tβ4-HEK R26R-eYFP were digested into single cell suspensions and flow sorted by FACS on the basis of their eYFP fluorescence. An attempt was then made to extract RNA from these cell-sorted samples and conduct qRT-PCR for the presence of Tβ4 mRNA to quantify the extent of Tβ4 knockdown achieved *in vivo* in these embryos. However, these attempts ultimately proved unsuccessful due to the inability to extract a large enough quantity of RNA from sorted cells to assess Tβ4 knockdown at the level of the individual embryo.

Thus, another approach was taken to attempt to quantify the activity of the random integrant Tβ4 shRNA and the HPRT targeted Tβ4 shRNA *in vivo*. Both the Tβ4 shRNA mouse and the HPRT targeted Tβ4 shRNA mouse were crossed with the

Nkx2.5-Cre mouse, which expresses Cre specifically in the myocardium[137]. This strategy has been previously used to knockdown Tβ4 in the developing embryonic myocardium[101]. Whole hearts were then dissected from E14.5 Nkx2.5-Cre Tβ4 shRNA and Nkx2.5-Cre HPRT Tβ4 shRNA embryos, protein extracted, and levels of Tβ4 in the myocardium detected by western blotting. The random integrant Tβ4 shRNA transgene under the control of the Nkx2.5-Cre exhibited a highly variable degree of Tβ4 knockdown. In this situation, paradoxically, the wild type tested actually displayed a lower level of Tβ4 than the mutants. However, although level of knockdown in the Nkx2.5-Cre HPRT Tβ4 shRNA embryos was variable, a high degree of Tβ4 knockdown was observed in one of the embryonic hearts examined (**Fig. 4.14**).

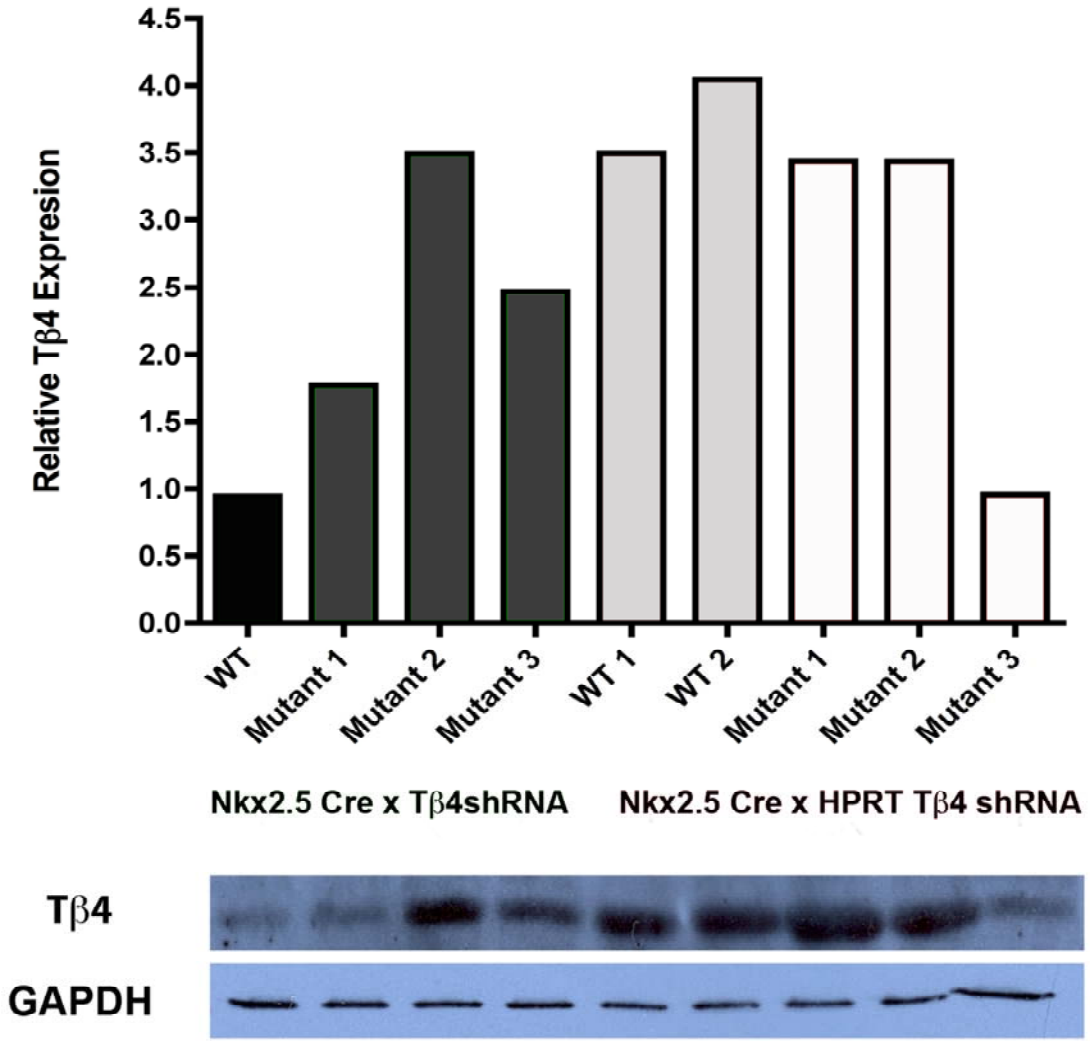


Fig. 4.14
The Hprt targeted Tβ4 shRNA construct produces more effective Tβ4 knockdown in vivo than the randomly integrant Tβ4 shRNA construct

Western blotting and scanning densitometry for the presence of Tβ4 in protein extracted from the E14.5 hearts of the offspring of Nkx2.5 Cre x Tβ4 shRNA and Nkx2.5 Cre x HPRT-Tβ4shRNA mice demonstrated a highly variable level of Tβ4 expression in hearts from the random integrant Tβ4 shRNA transgenic. In fact, levels of Tβ4 expression in the mutants tested were higher than that of the wild type littermate control. However, in one instance crossing the Nkx2.5 Cre with the HPRT targeted Tβ4 shRNA mouse resulted in a mutant with a dramatically reduced level of Tβ4 in its developing heart. This data is representative of two experiments performed.

4.2.11 Mutant Tβ4-HEK Mice Recapitulate the Mural Cell Defects of the Tβ4 -/Y Mouse

Thus, it was concluded that the Tβ4-HEK mouse might prove to be a better model for assessing the effects of endothelial cell specific Tβ4 knockdown, than the previously studied Tβ4-RIEK mouse. As such, a series of E10.5 Tβ4-HEK embryos were examined. Although none of these embryos displayed overt signs of haemorrhage above and beyond that observed in their wild type littermates, it was decided that a systematic analysis of the mural cell coverage of the aortas in E10.5 Tβ4-HEK mice should be made, in order to determine whether they possessed subtle vascular wall defects, which were insufficient to manifest themselves as explicit haemorrhage. In order to achieve this, ten non-consecutive axial sections from six mutants and six wild type E10.5 Tβ4-HEK embryos were stained with an antibody for the mural cell marker NG2, prior to examination of the mural cell layer by immunofluorescence microscopy. The mural cell coverage of the aortas of Tβ4-HEK embryos and littermate wild type controls was quantified using ImageJ software. It was determined, that the Tβ4-HEK mutants displayed a significantly reduced mural cell coverage compared to controls (**Figs. 4.15 a, b and c**).

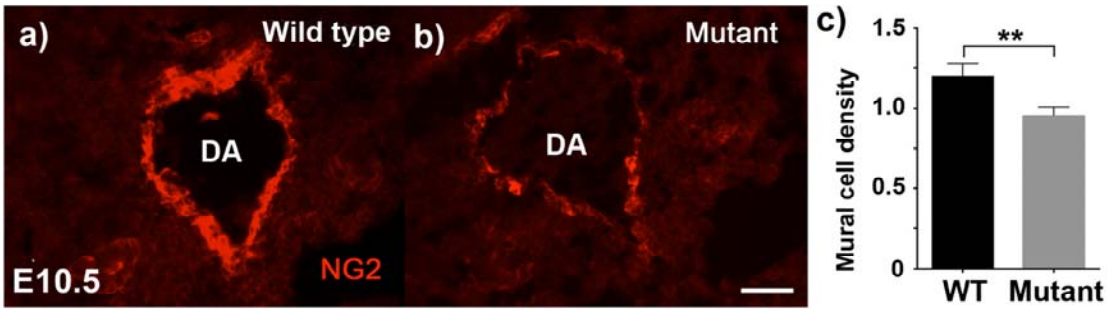


Fig. 4.15
E10.5 Tβ4-HEK mutant mice display reduced mural cell investiture of their dorsal aortas

Immunofluorescence examination of axial sections through the dorsal aorta revealed a robust coverage of NG2 positive mural cells in liitermate control wild type E10.5 control embryos (**a**), but a substantially reduced mural cell investment of the dorsal aorta in Tβ4-HEK mutants (**b**). Quantification of 10 sections per embryo, and 6 embryos per group, using ImageJ software demonstrated a significant reduction in the mural ell coverage of aortas from mutant Tβ4-HEK mice as compared to wild type littermate controls. DA (Dorsal aorta). Scale bars: (a and b) 50μm. ** $p<0.01$. Error bars represent standard error of the mean.

4.2.12 Mural Cell Defects in Tβ4 Loss of Function Models Are Due to Impaired Mural Cell Differentiation

Having established that haemorrhagic defects in Tβ4 -/Y embryos are likely due to a defect in mural cell investiture of developing blood vessels caused by a deficiency in endothelial Tβ4, it became necessary to further investigate the cellular behaviours underlying this phenomenon. Amongst other processes, the mural cell defects could arise because of aberrant apoptosis, impaired migration, under proliferation or compromised differentiation of mural cells. In order to test whether inappropriate apoptosis of mural cells was present in Tβ4 -/Y embryos, axial sections through E10.5 Tβ4 -/Y and littermate Tβ4 +/Y embryos were co-stained with anti-cleaved caspase 3 (CC3), a marker of apoptosis, and anti-smooth muscle actin before being examined with fluorescence microscopy (**Figs. 4.16a, b, c and d**). This staining revealed a complete absence of CC3 expression in peri-aortic mural cells in both Tβ4 +/Y and

Tβ4 -/Y embryos. This confirms that excess apoptosis of mural cells in Tβ4 -/Y embryos cannot underlie the vascular phenotype observed, due to the absence of apoptosis in either the Tβ4 -/Y or the Tβ4 +/Y embryo.

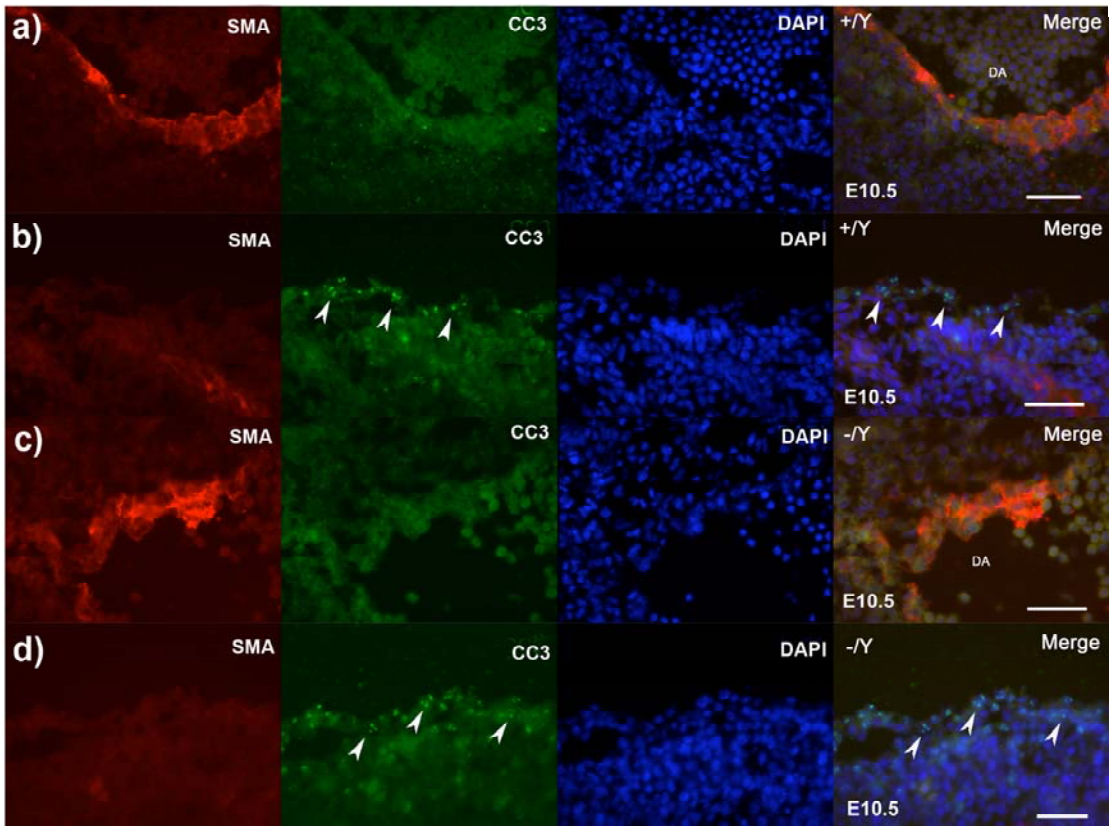


Fig. 4.16
No apoptosis is observed in the mural cells of E10.5 Tβ4 -/Y embryos

Co-immunofluorescence staining for the apoptotic marker cleaved caspase 3 and the mural cell marker smooth muscle actin revealed an absence of apoptosis in the mural cell layer of dorsal aortae in E10.5 Tβ4 -/Y embryos and Tβ4 +/Y controls (**a and c**). Punctate nuclear cleaved caspase 3 staining in the overlying surface epithelium of the embryo in Tβ4 +/Y and Tβ4 -/Y animals serve as a positive control for this staining (**b and d**). DA (Dorsal aorta). Scale bars: (a, b, c and d) 25μm.

Next, trans-aortic axial sections through E10.5 Tβ4 -/Y and Tβ4 +/Y embryos were stained with anti-phospho histone H3 (PPH3), a marker of proliferation and anti-smooth muscle actin before being examined by fluorescence microscopy (**Figs. 4.17a and b**). These investigations disclosed a complete absence of proliferation in the

peri-aortic mural cells of both Tβ4 -/Y and Tβ4 +/Y embryos. Similarly to the results from the assessment of apoptosis in the mural cells of Tβ4 -/Y embryos, a total lack of proliferation in this population of cells at this time point in both Tβ4 -/Y and Tβ4 +/Y embryos, effectively rules out proliferative defects as being causative in the mural cell defects observed in Tβ4 -/Y embryos.

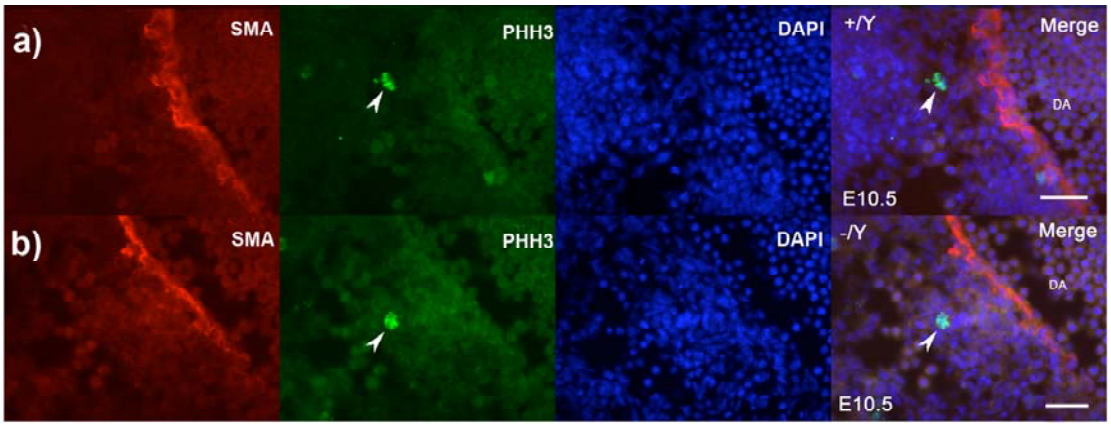


Fig. 4.17
No proliferation is observed in the mural cells of E10.5 Tβ4 -/Y embryos

Co-immunofluorescence for smooth muscle actin and the proliferative marker phospho-histone H3 demonstrates a lack of proliferation in the dorsal aorta mural cells of both Tβ4 +/Y (**a**) and Tβ4 -/Y (**b**) E10.5 embryos. Phospho-histone H3 staining in cells in the embryonic mesoderm serve as positive controls for this staining (white arrowheads). Scale bars: (a and b) 25μm.

In order to assess the extent to which a migratory defect might contribute to absence of mural cells in Tβ4 -/Y embryos, the postnatal retinal vasculature of P6 Tβ4 -/Y and Tβ4 +/Y animals was examined. The retina is part of the CNS, and like other CNS tissues, does not possess the vasculogenesis competent mesenchyme, from which mural cells can differentiate[58,60]. This implies that all mural cell investment of developing blood vessels, must derive from the migration of pre-existing mature mural cells from other non-CNS tissues. Thus, if knockout of Tβ4 causes a decreased mobility of mural cells, it might be expected that mural cell defects should manifest

themselves strongly in the developing retinal vasculature. Therefore, P6 retinas from Tβ4 -/Y and Tβ4 +/Y animals were stained with an anti-NG2 antibody to visualise mural cells and Isolectin B4 (Ilb4) to stain endothelial cells (**Figs. 4.18a, b and c**). This analysis demonstrated no difference in the coverage of the retinal vascular plexus with mural cells between Tβ4 -/Y and Tβ4 +/Y. This is evidence that absence of Tβ4 likely does not result in impaired mural cell migration.

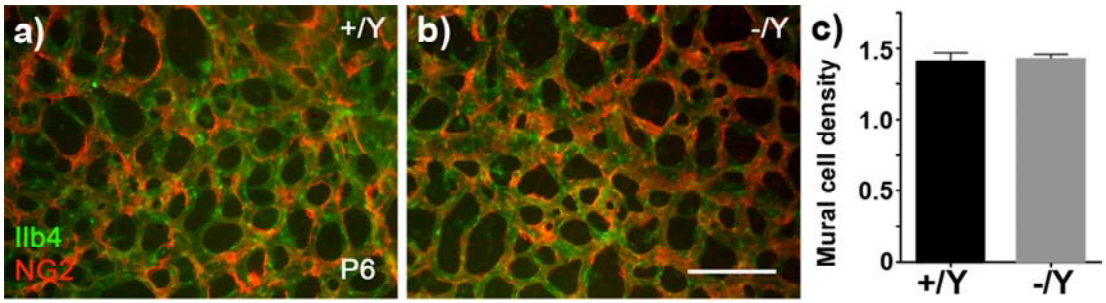


Fig. 4.18
P6 Tβ4 -/Y mice display no mural cell deficit in their retinal vasculature

NG2 immunofluorescence staining of P6 retinas revealed no difference in the mural cell coverage of the retinal vascular primary plexus between Tβ4 -/Y animals and Tβ4 +/Y controls, either by gross appearance (**a and b**) or by ImageJ quantification (**c**). Scale bars: (a and b) 50μm. Error bars represent standard error of the mean.

The final potential cellular pathway, which might be causative for mural cell defects in Tβ4 -/Y embryos, that was examined, was the process of mural cell differentiation. This process is difficult, if not impossible to visualise in *in vivo* samples, but tell tale signs of its disruption might be present. It has not yet been reported in the literature, at what stage initial development of the ensheathing mural cell layer of the aorta takes place. If E10.5 represents the earliest/one of the earliest stages at which mural cells are present around the aorta, then reduced numbers of mural cells at E10.5 might be the natural consequence of impaired mural cell differentiation. However, if mural cells are present at, for example, E9.5, and are present at their normal number in Tβ4

-/Y embryos, then their deficiency at E10.5 in Tβ4 -/Y mutants would likely not be due to impaired initial differentiation. In order to ascertain whether this might be the case, axial sections through E9.5 Tβ4 +/Y embryos were stained with anti-NG2 (**Fig. 4.19**). This staining showed that mural cells were not present in the wild type at the E9.5 stage. This result is consistent with a defect in mural cell differentiation first being able to manifest itself at the E10.5 stage in mutant Tβ4 -/Y embryos.

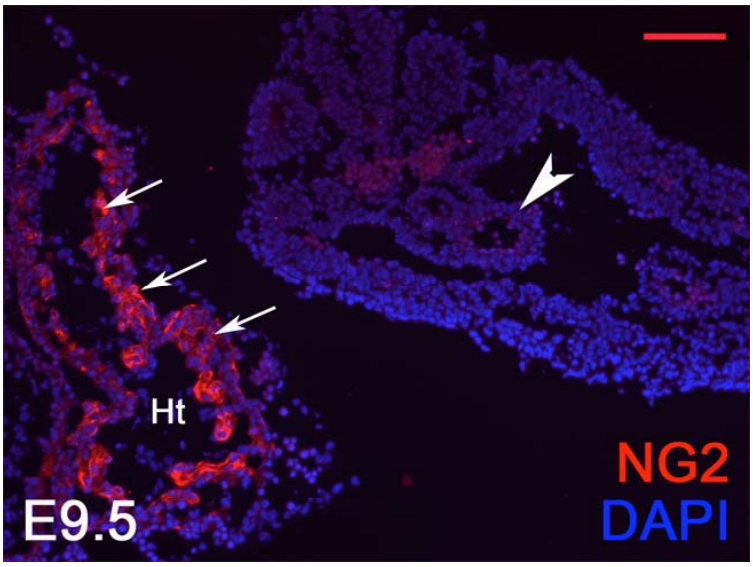


Fig. 4.19
Aortic mural cell differentiation does not take place until after E9.5

Immunofluorescence staining for NG2 on wild type E9.5 axial sections reveals a lack of any mural cell coverage around the dorsal aorta at this time point (white arrowhead). NG2 expression in the developing myocardium serves as a postive control for this staining (white arrows). Ht (Heart). Scale bar: 70μm.

4.3 Discussion

4.3.1 Summary

The above investigations focused on analysing the phenotypes of several mouse loss of function genetic models to gain insight into the function of Tβ4 during the process of vascular development. The first model used, was one in which the Tβ4 gene had been deleted globally in all tissues of the mouse. At E10.5, 5-10% of the Tβ4 -/Y embryos displayed overt pericardial and coelomic cavity haemorrhage. Assessment of the dorsal aortas of several Tβ4 -/Y embryos, both with and without the explicit haemorrhagic phenotype, revealed that these mutants display reduced mural cell coverage when compared to littermate Tβ4 +/Y controls. Embryos, which do not succumb to the likely lethal effects of haemorrhage at the E10.5 state, display dermal vascular haemorrhage at E14.5; a defect again related to a reduced mural cell investiture of dermal blood vessels. Although overt haemorrhage was not observed in embryos where Tβ4 has been knocked down specifically in endothelial cells, these embryos did still demonstrate a reduction in the mural cell coverage of their dorsal aortas. Aberrant proliferation and apoptosis of mural cells were ruled out as the causative cellular behaviour in production of the mural cell defect, on the basis of immunofluorescence analysis of E10.5 Tβ4 -/Y embryos. A migratory defect of mural cells in Tβ4 -/Y embryos is thought unlikely due to the normal mural cell coverage of P6 Tβ4 -/Y retinas. It is likely that the absence of Tβ4 in the endothelium of developing mouse embryos, leads to impaired differentiation of mural cells from the peri-aortic mesoderm.

4.3.2 Penetrance of the Haemorrhagic Phenotype in Tβ4 -/Y Embryos

A number of intriguing observations were made during analysis of the various Tβ4 genetic loss of function models studied. It was noted that only 5-10% of Tβ4 -/Y embryos displayed a haemorrhagic phenotype, consisting of pericardial and coelomic cavity haemorrhage. This implies that the phenotype observed is variably penetrant. Perhaps, an explanation for this variable penetrance can be ascertained by considering the abnormal cellular phenotype. It is thought that, since mural cells do not appear around the dorsal aorta until some point after the E9.5 stage of development, the mural cell deficiency apparent at E10.5 in Tβ4 -/Y embryos may be due to impaired differentiation of the peri-aortic mesoderm into mature mural cells. If this is the case, then the impenetrant haemorrhagic phenotype may have several aetiologies.

Differentiation of mesoderm into mural cell tissue is thought to be under the control, primarily, of endothelial secreted TGF-β[88]. One possibility, is that a compensatory upregulation of the TGF-β pathway, stimulates a degree of physiological, *in vivo* rescue of the phenotype in Tβ4 -/Y embryos. This compensatory increase might be enough to stimulate sufficient mural cell differentiation to prevent haemorrhage in a number of embryos. When Tβ4 -/Y embryos were analysed as a group for peri-aortic mural cell deficiency, with both haemorrhagic and non-haemorrhagic embryos included, it is observed that they have approximately 20% fewer mural cells. This implies that there may be a threshold of mural cell absence, below which haemorrhage will result, but above which will lead to a phenotypically normal appearing embryo.

Another possibility, is that lack of Tβ4 leads to a substantial decrease in the initial differentiation of mural cells. However, this could be compensated for by an increase in the proliferation of the mural cells, which have differentiated. However, the lack of any proliferation in the mural cell layer, as observed in wild type E10.5 embryos would argue against this. Nevertheless, it is possible that this compensatory increase in mural cell proliferation could occur prior to the E10.5 time point assessed in this study.

4.3.3 Down Regulation of Mural Cell Markers in E10.5 Tβ4 -/Y Embryos

Evidence of mural cell deficiency in E10.5 Tβ4 -/Y embryos, was first acquired by immunofluorescent examination of histological sections through E10.5 Tβ4 -/Y embryos. This was supported by data from qRT-PCR analysis of mural cell marker expression, performed on RNA extracted from whole E10.5 Tβ4 -/Y and Tβ4 +/Y embryos. This data showed a reduction in a number of mural cell markers in Tβ4 -/Y embryos compared to somite matched Tβ4 +/Y littermates. It is important to recognise the limitations of this type of analysis. As the experiment is conducted on RNA from whole embryo preparations, the experiment is not necessarily specific for vascular tissue alone. For example, smooth muscle actin is expressed in the developing heart at the E10.5 stage. Likewise, NG2 is also a marker of oligodendrocytes in the CNS[138]. It is possible that reduced expression of these mural cell markers in other tissues may confound a reduction in vascular tissue. This problem could be resolved by performing qRT-PCR on RNA derived from laser captured microdissection specimens which would allow isolation of the peri-aortic tissue from the rest of the embryo.

However, it can be argued that since the results of the qRT-PCR studies performed here are consistent with the histological evidence from immunofluorescence studies, that the presence of confounding factors is not likely. When the two pieces of data are taken together, they seem to support a strong conclusion that mural cell investiture of the dorsal aorta is compromised in E10.5 Tβ4 -/Y embryos.

4.3.4 Impaired Mural Cell Development in Endothelial Cell Specific Tβ4 Knockdown Embryos

In developing blood vessels, the previous chapter demonstrated that vascular Tβ4 expression is restricted to the endothelial cell lineage. Thus, it appears likely that mural cell defects present in Tβ4 -/Y embryos are due to an absence of Tβ4 in the endothelium. However, to provide additional evidence for this conclusion, two models of endothelial specific Tβ4 knockdown were examined for mural cell and haemorrhagic defects. In these models, the phenotypes observed were milder than that observed in the global Tβ4 knockout. Tβ4-RIEK mice were constructed through random genomic insertion of a transgene, which allowed expression of Tβ4 shRNA in cells in a Cre recombinase dependent manner. However, only one mutant Tβ4-RIEK mouse embryo studied displayed a haemorrhagic and mural cell phenotype that was similar to the phenotype observed in E10.5 Tβ4 -/Y embryos. The Tβ4-HEK mouse has a transgene inserted at the X-chromosomal locus of the Hprt gene, which stimulates production of a Cre dependent Tβ4 shRNA. Although this mouse never displayed any overt haemorrhage at E10.5, analysis of a group of mutants revealed that they did have a reduction in mural cell coverage of their developing aortas.

One reason that the endothelial specific Tβ4 knockdown models did not fully recapitulate the phenotype of the Tβ4 knockout mouse, might be due to insufficient reduction of Tβ4 levels in the endothelium. It proved difficult to assess the level of knockdown achieved in endothelial cells directly. Fluorescence sorting of Tβ4-RIEK and Tβ4-HEK endothelial cells did not yield enough mRNA to perform a qRT-PCR analysis of Tβ4 expression. In spite of this, it has previously been reported that the randomly inserted transgene does provide a variable level of knockdown in the developing heart[50]. In order to compare the ability of the randomly inserted and the Hprt targeted Tβ4 shRNA to mediate Tβ4 knockdown *in vivo*, each of these mouse strains was crossed with the cardiac specific Nkx2.5 Cre mouse. Hearts were isolated from E14.5 embryos and assayed for Tβ4 expression. The random integrant strain actually displayed higher levels of cardiac Tβ4 than wild type littermates, whilst the Hprt targeted strain displayed clear knockdown in only one out of three embryos. Thus, it is clear that the ability of the Tβ4 shRNA to knockdown Tβ4 *in vivo* is variable at best, and ineffective at worst.

One strategy that could be used is to directly assay levels of Tβ4 knockdown in endothelial cells would be to establish primary cultures of endothelial cells from Tβ4-RIEK and Tβ4-HEK embryos. The cells could then be grown in culture until enough cellular material was present to be able to conduct qRT-PCR or western blotting for Tβ4 levels. The cultures could be established by digesting Tβ4-RIEK and Tβ4-HEK embryos into single cell suspensions, and labelling endothelial cells with an anti-PECAM antibody conjugated to magnetic beads. The beads could then be sorted

magnetically, before eluting endothelial cells and culturing them in endothelial cell growth medium[139].

There are likely to be several factors, contributing to the inefficiency of Tβ4 knockdown in Tβ4-RIEK and Tβ4-HEK models. The first may be mosaicism of Cre expression in the Tie2-Cre mouse. It is common amongst Cre driver strains, that Cre is not expressed in all target cells. The reason for this is likely to be incomplete promotor activity of Cre transgenics. It has previously been reported that the Tie2-Cre mouse exhibits a degree of mosiacism[140], and as such it is plausible to assume that some endothelial cells in the Tβ4-RIEK and Tβ4-HEK strains will not express Cre and will therefore never be able to express the Tβ4 shRNA.

Another possible explanation is that levels of Cre recombinase in endothelial cells are insufficient to mediate recombination of the transgenic floxed transcriptional termination sequence upstream of the Tβ4 shRNA. Although, the levels of Cre expressed by the Tie2-Cre driver are high enough to excise the LoxP sites of the R26R and R26R-eYFP reporter transgenes, it is a well reported phenomenon that different floxed loci display variable sensitivity to Cre recombinase. For example, it appears that transgenic alleles of the transcription factors Mcl-1 and c-Myb are much more sensitive to Cre than the transgene at the R26R-eYFP reporter locus[141]. It is possible that the floxed Tβ4 shRNA transgene requires a higher level of cellular Cre to be expressed, than the Tie2-Cre mouse can produce, to excise the termination sequence.

As has been previously mentioned, epigenetic inactivation of randomly inserted transgenes is not an uncommon event[135]. This could underlie a lack of transgene expression in the Tβ4-RIEK mouse. However, in the Hprt Tβ4 shRNA model, stable targeting of the Tβ4 shRNA construct to the "open[136]" Hprt locus precludes epigenetic inactivation as an explanation for the incomplete knockdown of Tβ4. One factor, which could confound experiments with the Tβ4-HEK mice, is the X-chromosomal nature of the Hprt locus. Although the Hprt locus is not thought to be subject to epigenetic repression, it does not escape the process of X-inactivation[142]. X-inactivation is the process, by which the gene expression on one X-chromosome is largely silenced in cells with an XX karyotype. This means that in female mutants, even in cells, which express Cre, there is a 50% chance that the transgene will not be expressed, and consequently no Tβ4 knockdown will be induced.

These theories might explain why the Tβ4-HEK mouse does not possess an overtly haemorrhagic phenotype, but does show a defect in mural cell investiture of the dorsal aorta in grouped immunofluorescent analysis. Again, it could be postulated that deficiency of peri-aortic mural cells, below a certain threshold, is required for haemorrhage to be observed at the E10.5 stage. Due to the confounding effects of Cre mosaicism, Tβ4 shRNA inefficiency and X-inactivation, the mural cell count around the dorsal aorta in Tβ4-HEK mice never decreases to a low enough level to cause haemorrhage, but is sufficient to be detected by immunofluorescent analysis.

The experimental hypothesis underlying the use of the Tie2-Cre mouse to stimulate expression of a Tβ4 shRNA, was to knockdown Tβ4 in endothelial cells and prove that a deficiency in endothelial Tβ4 was responsible for the mural cell defects

observed in Tβ4 -/Y mice. However, one possible criticism of this approach, is that the Tie2-Cre mouse may not express Cre solely in endothelial tissues. Although, robust expression of Cre in the endothelium was demonstrated by crossing the Tie2-Cre with Rosa reporter strains, the Tie2-Cre mouse has also been used as a driver strain in haematopoietic cells[143,144]. Thus, it is possible that paracrine secretion of Tβ4 by haematopoietic cells in the developing embryo may drive mural cell development in the embryo. In order to test whether this could be the case, a robust expression analysis for Tβ4 in haematopoietic cells should be undertaken. An alternative approach would be to use a more tightly endothelial restricted Cre mouse strain to drive expression of the Tβ4 shRNA. This could be achieved using the tamoxifen inducible PDGF-B Cre mouse, which expresses Cre in a highly endothelial specific fashion[145].

However, in spite of these potential criticisms, it remains highly likely that depletion of Tβ4 in the endothelium, does underlie the vascular defects of Tβ4 -/Y embryos. This statement is justifiable as Tβ4 is highly expressed in the endothelium, whilst it has never been noted in the literature that Tβ4 is expressed in early embryonic haematopoietic cells. There is also little precedent for paracrine haematopoietic trophic factors playing a role in development of the systemic vasculature. There is tentative evidence that Neuropilin-1, secreted by liver haematopoietic progenitors at E12.5 may have an effect on vasculogenesis and angiogenesis[146]. Also, a lack of VEGF-164 in haematopoietic cells can lead to mild yolk sac vasculature defects at E10.5, but no defects in the systemic vasculature[147]. There are no examples found in the literature of a haematopoietic factor regulating systemic vascular development at

the E10.5 stage, and no examples found to date of haematopoietic cells being able to influence the process of mural cell development.

The Tie2-Cre mouse has been used by many groups, over many years, to demonstrate the effects of deleting genes in the endothelium[148-150]. Thus, the simplest explanation for the results observed, is that loss of Tβ4 expression in the developing endothelium leads to insufficient mural cell development around blood vessels.

4.3.5 Aberrant Cellular Behaviour in Tβ4 -/Y Embryos

Several analyses were conducted in order to determine the abnormal cellular behaviour that caused the deficiency of mural cells observed in Tβ4 -/Y embryos. By immunostaining the mural cell layer for CC3 and PPH3, abnormal apoptosis or proliferation of mural cells were ruled out as the causative processes in mural cell insufficiency of the Tβ4 -/Y mouse.

Several mouse genetic mutants display blood vessels, which lack mural cells due to impaired migration of mural cells around blood vessels[62,127,128]. In these genetic models however, the mural cell defects appear to manifest themselves at slightly later time points in development than E10.5. For example, vascular defects first appear at E16.5 in PDBF-B or PDGFR-β knockout mice[62], at E17.5 in the mural cell specific Integrin β1 knockout mouse[127], and at E18.5 in the mural cell specific Ephrin-B2 knockout[128]. This itself, makes it unlikely that impaired mural cell migration is responsible for the haemorrhagic and mural cell defects in the Tβ4 -/Y mouse at E10.5.

However, in order to further investigate this possibility, postnatal retinas from Tβ4 -/Y mice were studied. The adult retinal vasculature commences its development at the time of birth and consists of a radially sprouting plexus of endothelial tubes. These tubes, become concomitantly covered by mural cells, migrating from the centre of the arteriolar plexus[151]. Similarly to all other CNS tissues, the retina does not contain vasculogenesis competent mesenchyme, which can act as the cellular substrate for mural cell differentiation[58,60]. As such, the mural cells, surrounding retinal blood vessels, derive from cells, which have developed outside the organ bed and migrated into the retinal vasculature.

When retinas from P6 Tβ4 -/Y mice had the mural cell coverage of their retinal vasculature assessed, no difference was observed when compared to Tβ4 +/Y retinas. The absence of a mural cell defect in this vascular bed is further evidence against Tβ4 acting as a migratory stimulus for mural cells. In comparison, mice, in which the extracellular retention motif for PDGF-B has been deleted, display severely delayed and disrupted mural cell investiture of the retinal vasculature – a phenotype consistent with impaired mural cell migration[152].

Even though, it is unlikely that Tβ4 -/Y embryos have a defect in mural cell migration, there are two possible ways in which absence of Tβ4 might, theoretically, cause an impairment in this cellular function. The first, may be due to a lack of cell autonomous Tβ4 in the mural cells themselves. Given Tβ4's role as a G-actin sequestering molecule, this could lead to the disrupted formation of the cytoskeletal machinery necessary for migration. However, this is very unlikely to be a problem

for Tβ4 -/Y mice *in vivo*, as the experimental data presented here demonstrates Tβ4 to be expressed in the endothelium and not the mural cells themselves. Moreover, the phenotype of the Tβ4 -/Y mouse can be partially replicated by endothelial specific Tβ4 knockdown models. The more likely possibility is that Tβ4 is secreted by the endothelium and serves as an extracellular migratory stimulus for mural cells.

To provide further evidence that abrogation of mural cell migration, does not contribute to the phenotype of Tβ4 -/Y embryos, the Tβ4 knockout mouse could be crossed onto the "immorto" transgenic background. Primary mural cells could then be isolated from the aortas of adult mice, and an immortalised primary mural cell line established. Such a strategy has been used to great effect by other researchers[128]. These cells could then be used in Boyden chamber or scratch assays of cell migration, when stimulated in the presence or absence of recombinant Tβ4. Such an experimental approach is not likely to provide significant insight into the Tβ4 -/Y phenotype however, as the migration of human aortic smooth muscle cells is not affected by treatment with Tβ4[57].

In contrast to genetic mouse mutants, which have defects in the migration of mural cells; those mutants, which display defects in the differentiation of mural cells, present with a vascular phenotype at a much earlier stage of development. Examples include the Alk-5 null mouse, which displays a phenotype at E11.5[87] and the TGF-β RII null mouse, which shows vascular malformation at E10.5[93]. Thus, the defects in mural cell recruitment observed in the Tβ4 -/Y mouse are consistent with impaired differentiation of mural cells from the surrounding peri-aortic mesoderm. This argument is strengthened by the observation that mural cells are absent from the peri-

aortic region in the wild type setting at E9.5. This implies that the defect observed in Tβ4 -/Y embryos at E10.5 is likely to be due to a defect, which affects the initial process of mural cell development.

Thus, a model can be derived for how Tβ4 might be functioning in vascular development. It can be postulated that Tβ4 is secreted from the developing endothelium and acts on mural cell precursors in the peri-aortic mesenchyme to stimulate their differentiation into mature mural cells. There is supporting evidence for such a model in the literature. It has been noted that Tβ4 is secreted by the developing murine myocardium at E14.5, before acting on EPDCs to stimulate their differentiation into the cells of the coronary vasculature[50]. In addition, treatment with exogenous Tβ4 can rescue the decreased expression of VSMC markers present in differentiating Hand1 null embryoid bodies[51].

In order to test whether the mural cell defects present in Tβ4 -/Y mice are due to impaired mural cell differentiation, and thus validate the proposed model of impaired Tβ4 function, a robust *ex vivo* or *in vitro* culture system should be established. The ability of Tβ4 to differentiate progenitor cells into mural cells, in relevant model systems should be tested. This will be experimentally addressed in subsequent chapters.

5 Use of Gene Expression Studies to Understand the Molecular Phenotype of Tβ4 -/Y Embryos

5.1 Introduction:

It has been established that a global deletion of Tβ4 in the mouse genome, results in embryos which display haemorrhage due to a lack of mural cell investiture of their developing blood vessels. Endothelial specific knockdown of Tβ4 confirms a non-autonomous role for Tβ4 in mural cell development. The timing of phenotype manifestation in these Tβ4 loss of function models, suggests a specific role for Tβ4 in the regulation of mural cell differentiation. In order to gain greater insight into this process, and the underlying molecular biology of Tβ4 function, it is important to understand the molecular phenomena involved.

If the hypothesis that paracrine secretion of Tb4 by endothelial cells acts to promote mural cell differentiation is accurate, it is inevitable that Tβ4 will mediate gene expression in the target mural cell progenitor population, via an effect on one or more signalling pathways. Cell-cell signalling can occur through a multitude of mechanisms at a number of different pathway points. In order to attempt to gain a better understanding of the signalling pathways Tβ4 might be affecting in this situation, a two-pronged approach was employed.

The first strategy used was a biased approach to identify candidate pathways. The literature base was analysed to find molecular pathways, whose peturbation might be able to cause a phenotype similar to that observed in embryonic Tβ4 -/Y mice. Three

potential candidate pathways were identified. These were the Notch, PDGF-B and TGF-β signalling pathways.

As previously described, the Notch pathway has a plethora of functional roles in development of the vascular system including the regulation of arterial/venous vascular character and the specification of endothelial tip cell identity[70]. Most intriguingly however, knockout of the Notch 3 receptor in the developing mouse embryo leads to a phenotype involving incomplete differentiation and failed maturation of mural cells[77]. This makes Notch receptor signalling a potential candidate pathway upon which Tβ4 might be acting.

The phenotypes of genetic mouse mutants, for components of the PDGF-B signalling pathway, are more in keeping with an abnormality in migration/proliferation of mural cells, rather than an abnormality of mural cell differentiation [60,62,65,152]. However, there are some reports in the literature of PDBF-B being able to mediate mural cell differentiation of progenitor cell populations *in vitro*[89,153]. Thus, given that it is difficult to unequivocally rule out a migratory or proliferative defect in mural cells in the Tβ4 -/Y mouse based solely on the described *in vivo* phenotype, and that there is putative, albeit *in vitro*, evidence for PDGF-B as a mediator of mural cell differentiation, PDGF-B signalling is also a candidate for potential mediation of defects in the Tβ4 -/Y mouse.

As has been previously discussed, TGF-β signalling is the pathway most thoroughly linked with the process of mural cell differentiation[58,59,88]. Knockouts of several TGF-β receptors and ligands are thought to result in a failure of mural cell

differentiation[83,86,87,93]. Moreover, TGF-β has shown a direct ability to stimulate the *in vitro* differentiation of mural cells, from progenitor cell lines[90,92]. For these reasons, the TGF-β signalling pathway can be considered as potentially disrupted in Tβ4 -/Y embryos.

In order to test whether these candidate pathways might play a role in the formation of the Tβ4 -/Y phenotype, a qRT-PCR mini-screen of dysregulated receptors, ligands, and where appropriate, pathway intermediates and transcriptional targets was conducted. Such an approach has been used in several settings to determine the over or under activity of molecular pathways. Examples include the identification of p53 pathway disruption in E10.5 hearts of the ribosomal protein l24 (Rpl24) hympomorphic mouse[154], and quantification of Wnt pathway over activity in the intraflagellar transport 20 (IFT20) knockout mouse[155]. Thus, by quantifying gene expression of molecular members of the Notch, PDGF-B and TGF-β signalling pathways in embryonic material, it may be possible to determine their contribution to the Tβ4 -/Y phenotype.

The success of the candidate pathway approach in this instance, partly relies on Tβ4 affecting a molecular signalling pathway which has previously been identified as affecting mural cell development. However, it is plausible that Tβ4 exerts its effects on mural cells through a pathway, which has not previously been linked to this process. In order to determine whether this might be the case, a second approach was taken. This was to subject the Tβ4 -/Y mouse to gene array analysis. Gene arrays allow high throughput expression analysis of every known gene in the mouse genome[156,157]. This allows differential levels of specific mRNAs to be quantified

between samples. Comparing the global gene expression patterns of the Tβ4 +/Y and Tβ4 -/Y mice could facilitate identification of novel molecular pathways affected by Tβ4. This strategy has been used in several different contexts to identify original signalling pathways perturbed in knockout mice. As an example, global gene expression analysis of by microarray revealed alterations in sterol metabolism in high density lipoprotein (HDL-1) deficient mice[158]. In another case, microarray analysis identified altered expression of the cibulin-megalin multiligand endocytic receptor complex in the E9.5 embryos of the reduced folate carrier 1 (RFC-1) knockout mouse[159].

In order to test the gene expression levels of candidate molecular pathways in Tβ4 -/Y mice by qRT-PCR, a decision was made to analyse two sources of mRNA in parallel. Limb buds from E12.5 Tβ4 -/Y embryos and somite matched whole E10.5 Tβ4 -/Y embryos were used for this purpose. Each of these specimens has its potential advantages and disadvantages. E12.5 limb buds represent, in terms of cell variety, a much more homogeneous tissue than E10.5 embryos. In a heterogenous tissue such as the E10.5 whole embryo, gene expression changes in other tissues could potentially mask expression changes in the vasculature. The strategy of using E12.5 limb buds in such a manner was employed with success to understand the molecular pathways underlying the vascular phenotype of the endothelial specific SRF knockout mouse[160]. At E12.5, the limb bud also possesses the advantage of being a site of active vascular development[160]. This time point can also be thought of as representing an intermediate stage of development between the two time points, E10.5 and E14.5, at which mural cell defects were observed in the Tβ4 -/Y mouse. Thus any positive

findings observed are likely to be applicable to the times at which both these sets of defects were detected.

Whole E10.5 embryos were also assessed due to the variable penetrance of haemorrhagic and mural cell defects in the Tβ4 -/Y mouse. It is possible that those embryos, which present with the most severe phenotype also possess the greatest derangement in the molecular pathways responsible for such a deficit. Thus, as a phenotype has not been directly observed in E12.5 limb buds, it is possible that analysed samples will not disclose significant changes in gene expression due to being samples, in which the phenotype is left penetrant. This problem can be circumvented by using somite matched E10.5 embryos, which display overt pericardial haemorrhage. As the embryos with the most severe phenotype, they are likely to present with the greatest range of gene expression changes.

Although it is possible that the proposed approaches to quantify gene expression changes in the Tβ4 -/Y mouse may lead to identification of the molecular pathways upon which Tβ4 exerts an influence, there are limitations to this strategy. Tβ4 may stimulate pathway activation without directly affecting the transcriptional levels of pathways. For example, this approach will not necessarily detect a hypothetical ability of Tβ4 to stimulate PDGFR-β phosphorylation. Such an occurrence might only be detectable with a gene expression profiling approach, as a reduction in the mRNA levels of stereotypical PDGF-B responsive genes in the Tβ4 -/Y mouse. In spite of these limitations, the outlined strategies have the potential to assess the interaction between Tβ4 and molecular pathways in a high-throughput manner.

5.2 Results

5.2.1 Expression Analysis in E12.5 Limb Buds

The initial analysis of candidate pathway gene expression in E12.5 limb buds quantified mRNA expression in samples derived from the limb buds of two E12.5 Tβ4 -/Y embryos and samples from the limb buds of two E12.5 Tβ4 +/Y embryos. The limb buds of these embryos did not display an haemorrhagic phenotype. In order to be designated a "hit", and as such subject to further analysis, any interrogated gene had to display either significant up regulation of at least 20% in both Tβ4 -/Y samples compared to both Tβ4 +/Y samples, or significant down regulation of at least 20% in both Tβ4 -/Y samples compared to Tβ4 +/Y samples.

The first pathway to be examined was the Notch pathway. All four known canonical Notch receptors were tested but did not display gene expression changes significant enough to be labelled a "hit" (**Fig. 5.1**). The same result occurred for four out of the five canonical Notch ligands tested (Dll1, Dll4, Jag1 and Jag2) (**Fig. 5.2**). However, the gene for Dll3 appeared to be significantly upregulated by 25% in the both of the Tβ4 -/Y samples compared to the Tβ4 +/Y controls.

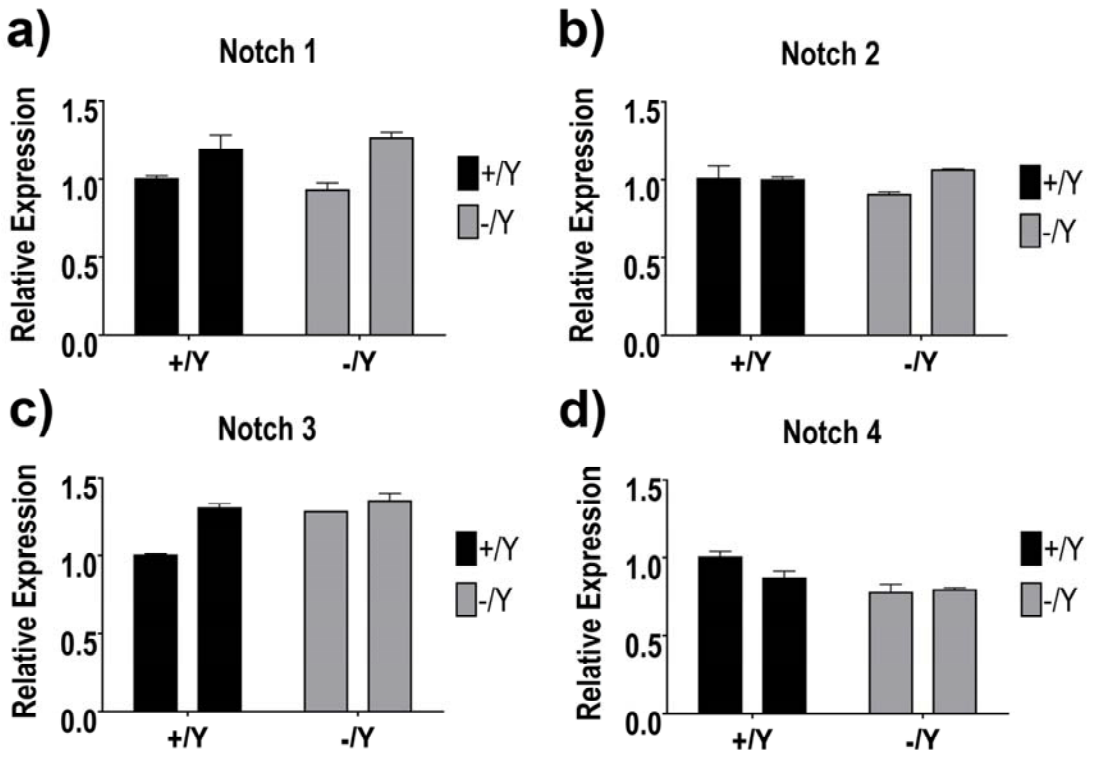


Fig. 5.1
Quantification of mRNA expression for Notch receptors in E12.5 Tβ4 -/Y limb buds

qRT-PCR quantification of mRNA expression levels for the Notch receptors Notch1 (**a**), Notch 2 (**b**), Notch 3 (**c**) and Notch 4 (**d**) in E12.5 Tβ4 -/Y and Tβ4 +/Y limb buds. Each bar represents mRNA expression from the limb buds of a single embryo. Error bars represent the standard error of four technical replicates. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in both Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

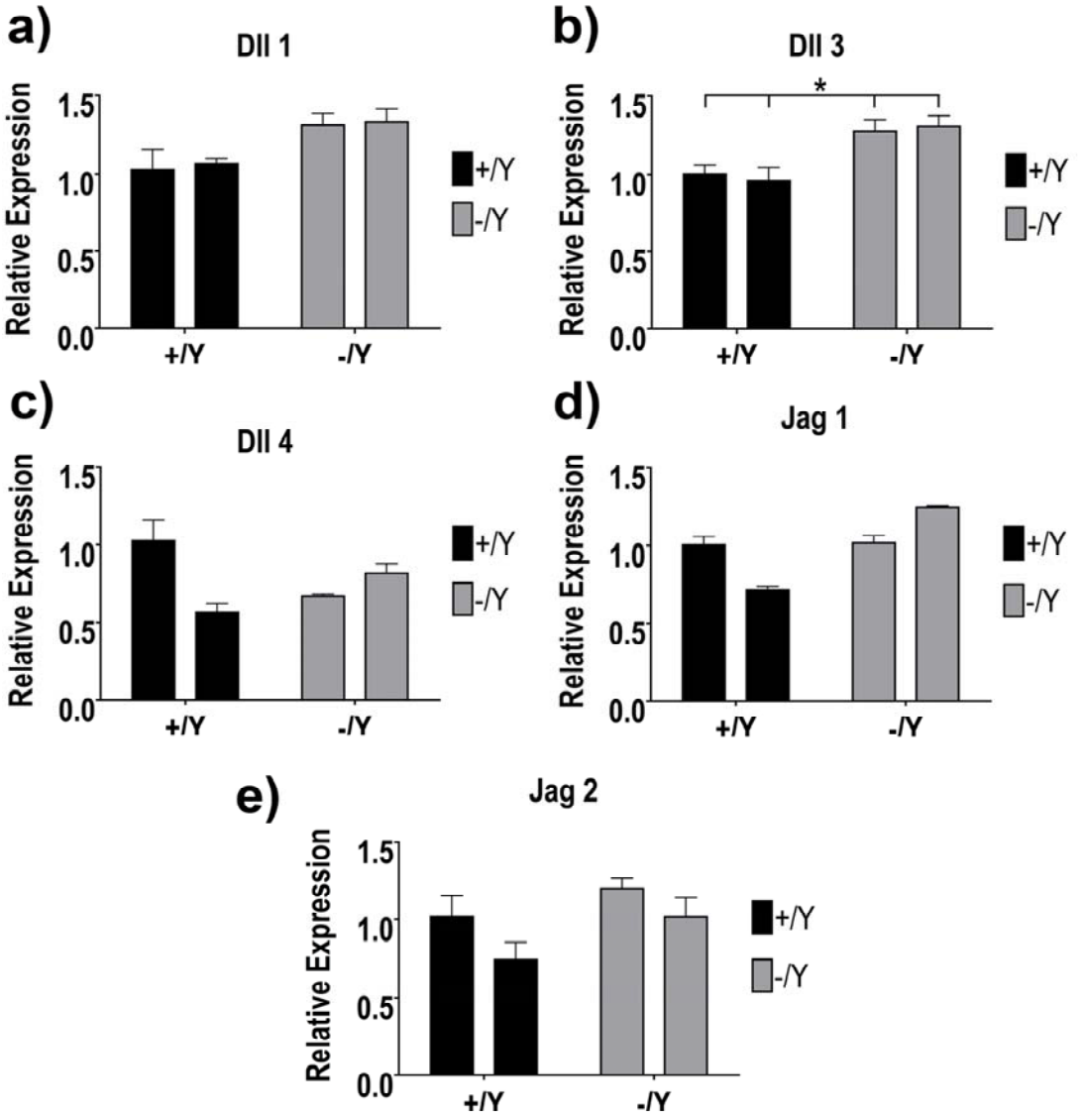


Fig. 5.2
Quantification of mRNA expression for Notch ligands in E12.5 Tβ4 -/Y limb buds

qRT-PCR quantification of mRNA expression levels for the Notch ligands Dll1 (**a**), Dll3 (**b**), Dll4 (**c**), Jag1 (**d**) and Jag2 (**e**) in E12.5 Tβ4 -/Y and Tβ4 +/Y limb buds. Each bar represents mRNA expression from the limb buds of a single embryo. Error bars represent the standard error of four technical replicates. * Only Dll3, but no other samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in both Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

Next, expression levels of Notch signalling intermediary and adaptor proteins were examined. The genes interrogated were TACE – the ADAM family protease which mediates the extracellular cleavage of Notch following ligand binding, Presenillin 1

and 2 – components of the γ secretase complex which mediates the cleavage of the Notch intracellular domain, and RBPj/CBF – an essential Notch ICD binding co-factor[70]. On testing, differences in expression levels of these genes between Tβ4 -/Y and Tβ4 +/Y samples did not meet the established "hit" criteria (**Fig. 5.3**).

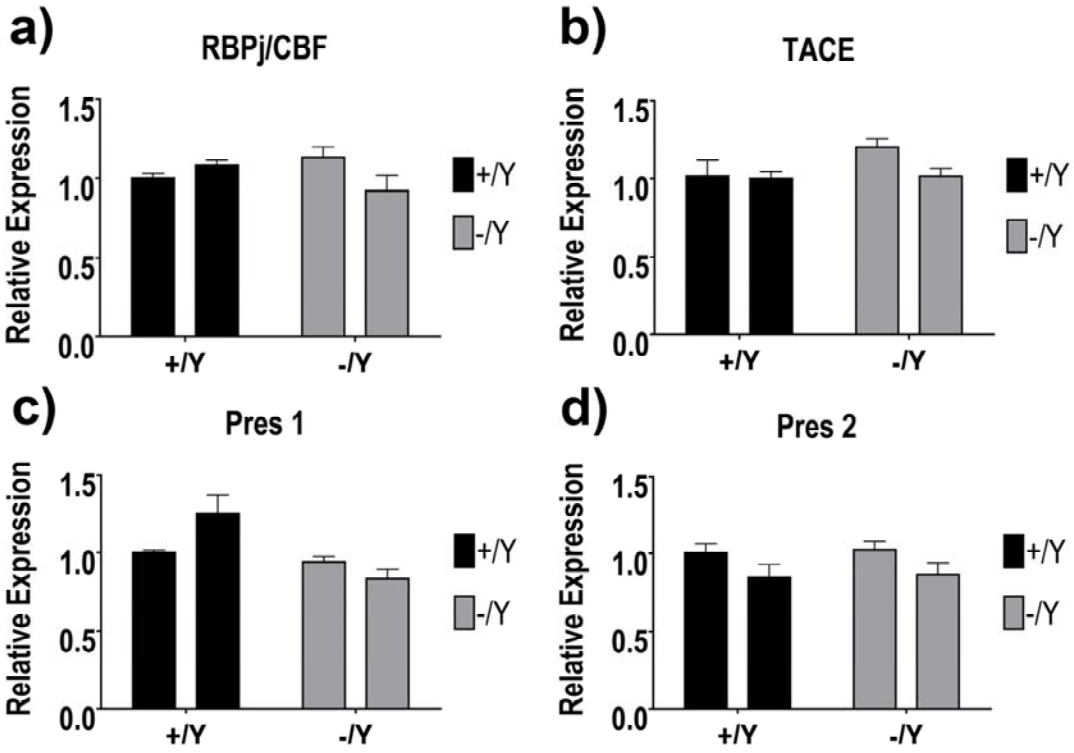


Fig. 5.3
Quantification of mRNA expression for Notch signalling intermediates in E12.5 Tβ4 -/Y limb buds

qRT-PCR quantification of mRNA expression levels for the Notch pathway intermediates RBPj/CBF (**a**), TACE (**b**), Presenillin 1 (**c**) and Presenillin 2 (**d**) in E12.5 Tβ4 -/Y and Tβ4 +/Y limb buds. Each bar represents mRNA expression from the limb buds of a single embryo. Error bars represent the standard error of four technical replicates. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in both Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

Finally, in order to provide a measure of global Notch signalling output, the expression levels of a number of Notch stereotypical transcriptional targets were quantified. The expression levels of these genes have been identified in the past as

being regulated predominantly by Notch signalling. They included members of the Hes/Hey family[70], members of the Fringe group of enzymes, and the ankyrin repeat domain protein Nrarp[161]. Once again, there was no consistent significant difference in the expression levels of these genes between Tβ4 -/Y and Tβ4 +/Y samples (**Fig. 5.4**). This suggests there was no difference in Notch pathway activity between the Tβ4 -/Y and Tβ4 +/Y samples tested, in spite of the apparent differences in Dll3 expression levels.

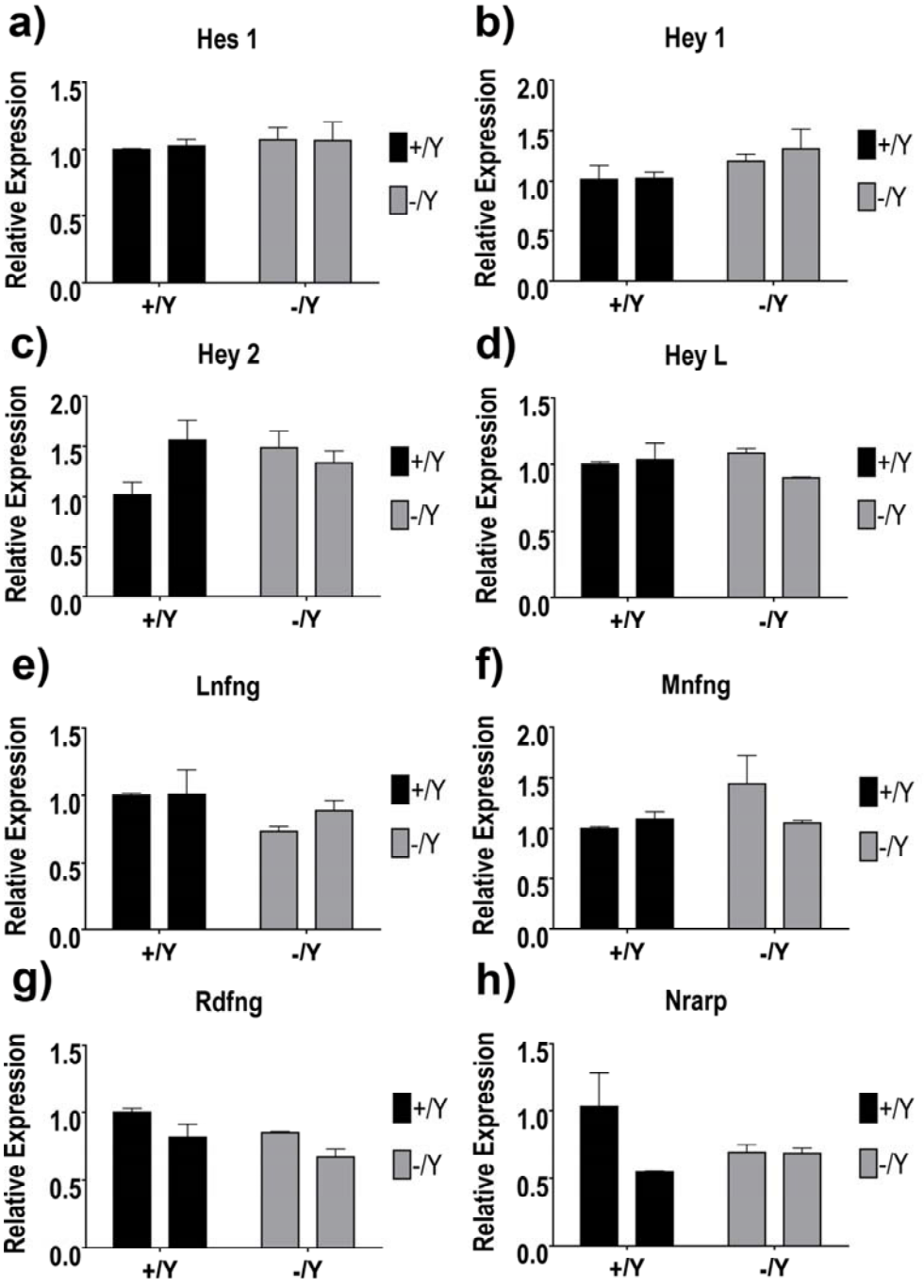


Fig. 5.4
Quantification of mRNA expression for Notch transcriptional targets in E12.5 Tβ4 -/Y limb buds

qRT-PCR quantification of mRNA expression levels for the Notch transcriptional target genes Hes1 (**a**), Hey1 (**b**), Hey2 (**c**), HeyL (**d**), Lunatic fringe (**e**), Manic fringe (**f**), Radical fringe (**g**) and Nrarp (**h**) in E12.5 Tβ4 -/Y and Tβ4 +/Y limb buds. Each bar represents mRNA expression from the limb buds of a single embryo. Error bars represent the standard error of four technical replicates. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in both Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

The next task undertaken was to quantify the expression levels of molecules involved in the PDGF-B pathway. As well as measuring expression levels of the PDGF-B ligand and the PDGFR-β receptor, expression levels of known PDGF-B transcriptional targets were also identified. These transcriptional target genes were Ste20, Hmgb1, Farnesyl PP synthetase, Fibroglycan and Tenascin C – all of which have been identified as being down regulated in the brains of PDGF-B deficient mice as compared to littermate wildtypes[162]. Once again, quantification disclosed no significant, consistent differences in expression levels between Tβ4 -/Y and Tβ4 +/Y samples which met the criteria for a positive "hit" (**Fig. 5.5**).

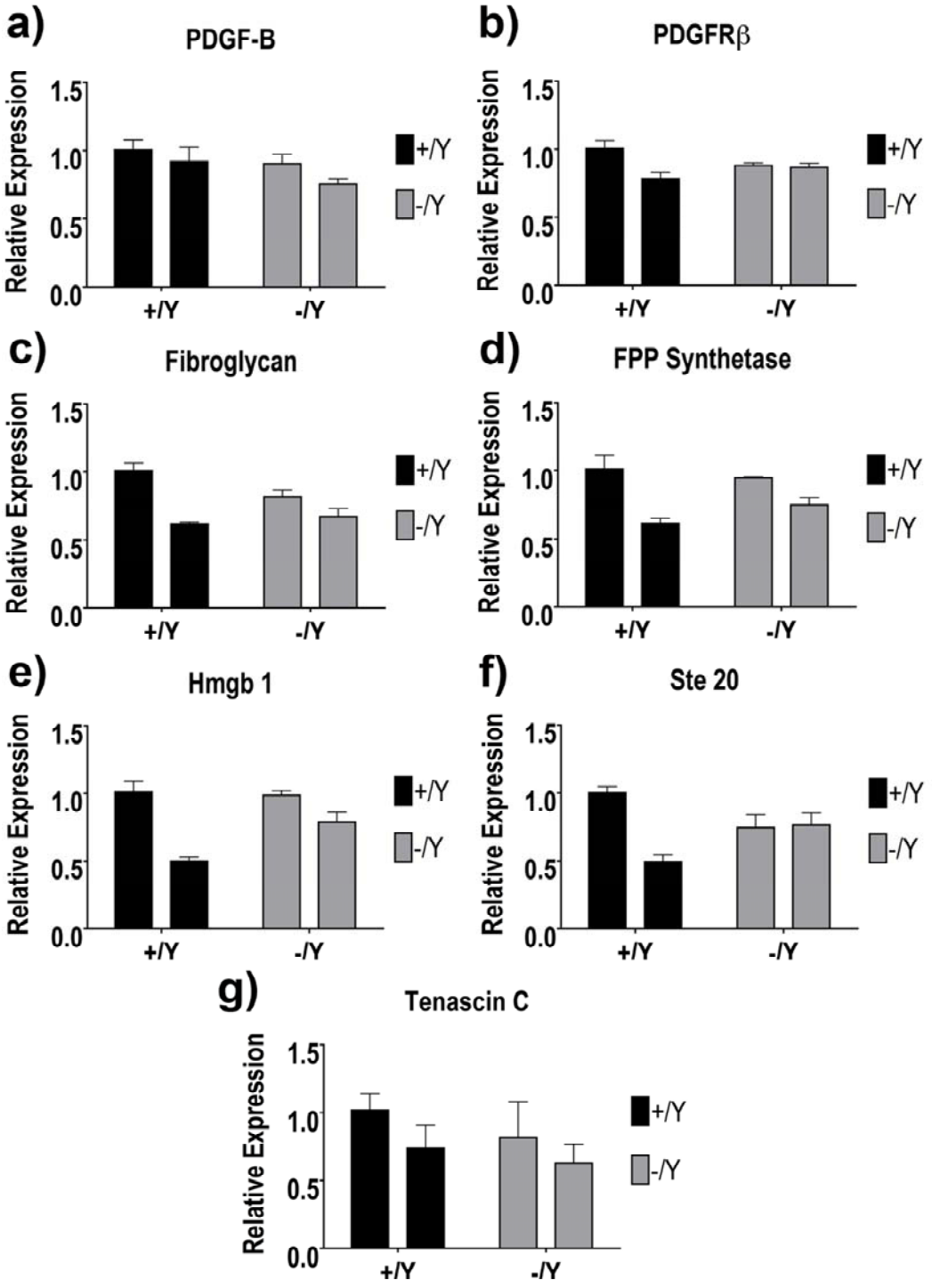


Fig. 5.5
Quantification of mRNA expression for PDGF-B, its receptor, and PDGF-B target genes in E12.5 Tβ4 -/Y limb buds

qRT-PCR quantification of mRNA expression levels for the PDGF-B ligand (**a**), the PDGFR-β receptor1 (**b**), and the PDGF-B transcriptional targets Fibroglycan (**c**), FPP Synthetase (**d**), Hmgb1 (**e**), Ste20 (**f**) and Tenascin C (**g**) in E12.5 Tβ4 -/Y and Tβ4 +/Y limb buds. Each bar represents mRNA expression from the limb buds of a single embryo. Error bars represent the standard error of four technical replicates. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or

down regulation in both Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

The TGF-β pathway was now probed for differences in expression levels of signal pathway members. For this pathway, the molecules tested were the TGF-β1 ligand and its receptors Alk-1, Alk-5 and TGF-β RII, all seven known Smad adaptor molecules and four TGF-β responsive transcriptional targets. The four transcriptional targets chosen were PAI-1 – known to be upregulated by Alk-5 activation[163], Id1 and Id2 – upregulated by Alk1 stimulation[163], and c-myc – often repressed[164], but sometimes induced by the action of TGF-β[165]. Upon quantification, no consistent difference was observed between Tβ4 -/Y samples and Tβ4 +/Y samples for expression of these genes (**Figs. 5.6, 5.7 and 5.8**).

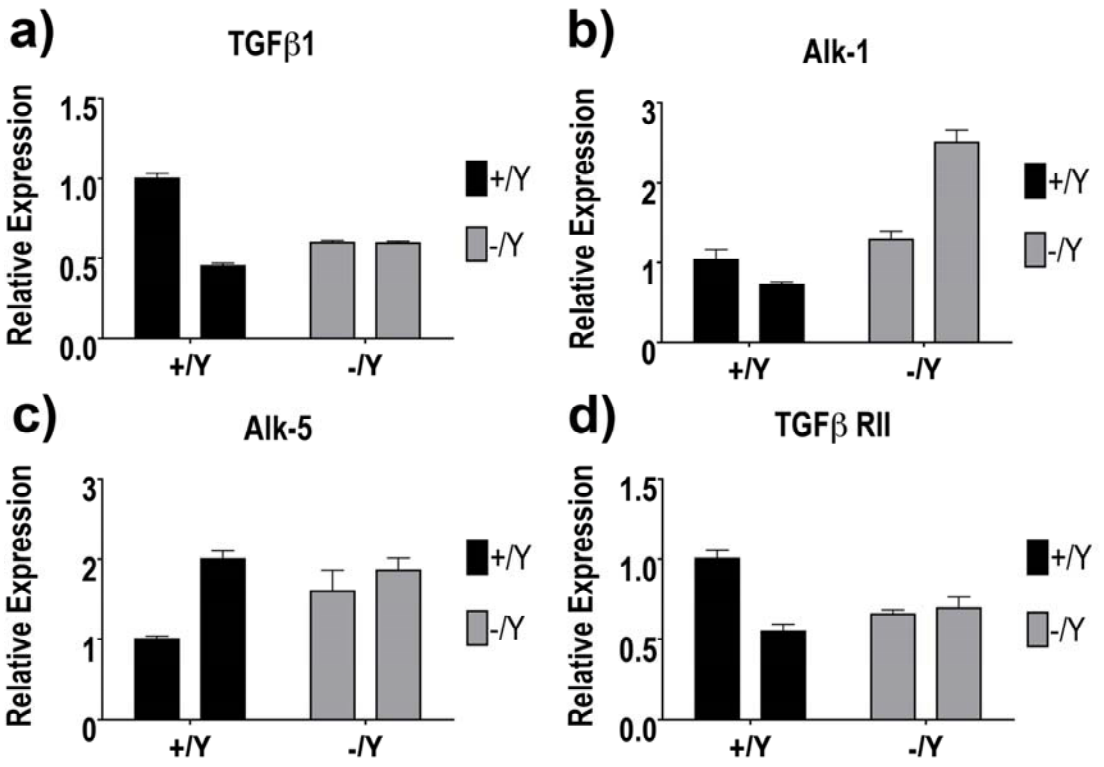


Fig. 5.6
Quantification of mRNA expression for TGF-β and its receptors in E12.5 Tβ4 -/Y limb buds

qRT-PCR quantification of mRNA expression levels for the TGF-β ligand TGF-β1 (**a**) and the TGF-β receptors Alk-1 (**b**), Alk-5 (**c**) and TGF-β RII (**d**) in E12.5 Tβ4 -/Y and Tβ4 +/Y limb buds. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in both Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

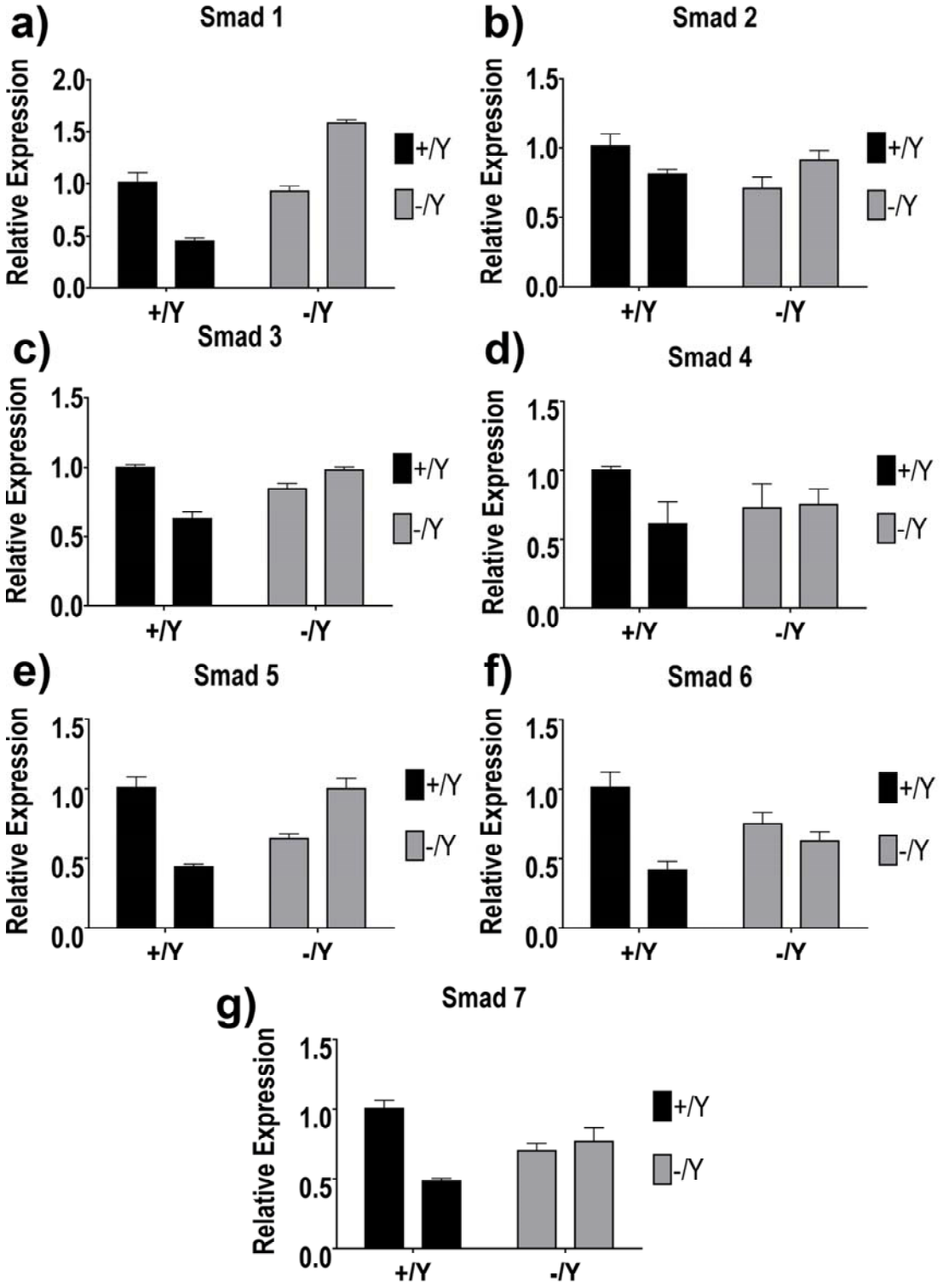


Fig. 5.7
Quantification of mRNA expression for Smad genes in E12.5 Tβ4 -/Y limb buds

qRT-PCR quantification of mRNA expression levels for the TGF-β pathway signalling adaptors Smad1 (**a**), Smad2 (**b**), Smad3 (**c**), Smad4 (**d**),Smad5 (**e**), Smad6 (**f**) and Smad7 (**g**) in E12.5 Tβ4 -/Y and Tβ4 +/Y limb buds. Each bar represents mRNA expression from the limb buds of a single embryo. Error bars represent the standard error of four technical replicates. No samples met the pre-specified "hit"

criteria of a consistent change of >20% up or down regulation in both Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

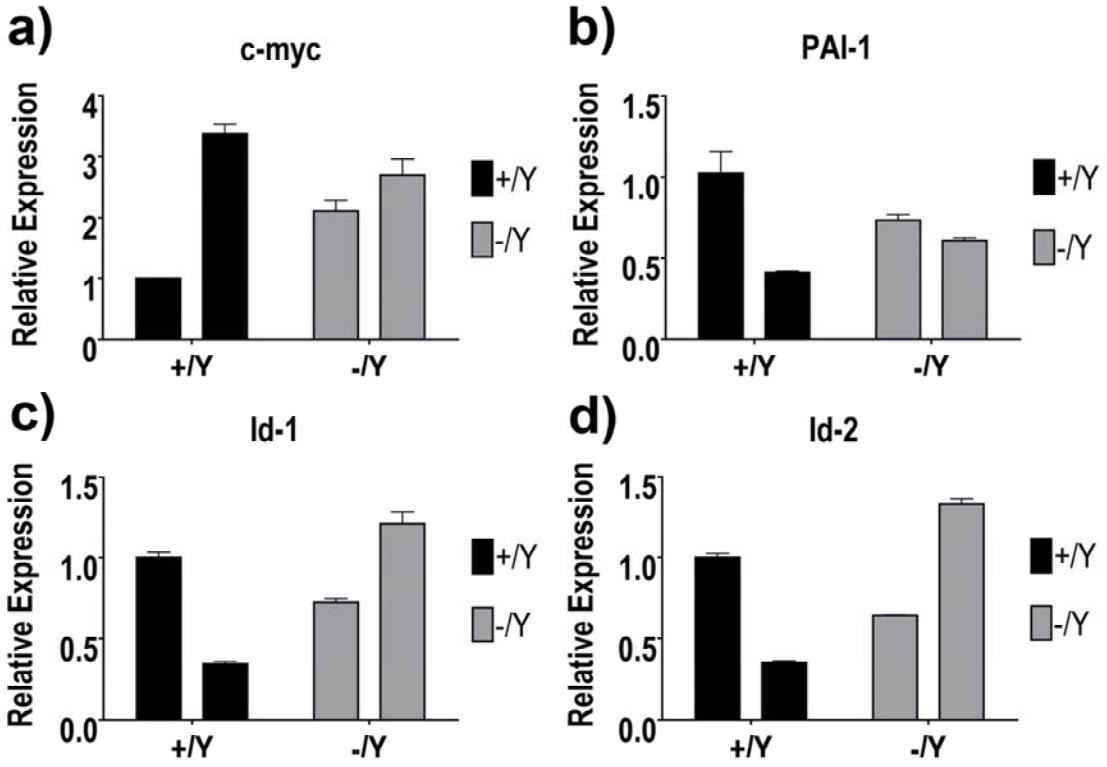


Fig. 5.8
Quantification of mRNA expression for TGF-β target genes in E12.5 Tβ4 -/Y limb buds

qRT-PCR quantification of mRNA expression levels for the TGF-β transcriptional target genes c-myc (**a**), PAI-1 (**b**), Id-1 (**c**) and Id-2 (**d**) in E12.5 Tβ4 -/Y and Tβ4 +/Y limb buds. Each bar represents mRNA expression from the limb buds of a single embryo. Error bars represent the standard error of four technical replicates. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in both Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

The only "hit" obtained in these expression assays was the Notch ligand Dll3. However, Dll3 is reported not to be expressed in the vasculature[70], and so differences in its expression seem unlikely to account for the mural cell phenotype in Tβ4 -/Y embryos.

5.2.2 Expression Analysis in Somite Matched E10.5 Embryos

As indicated previously, a similar candidate gene analysis would be performed on RNA derived from three sets of somite matched pairs of E10.5 Tβ4 -/Y and Tβ4 +/Y embryos, which displayed the overtly haemorrhagic phenotype, in parallel to the studies performed on E1.5 limb buds. As the Tβ4 -/Y embryo in each pair exhibited overt pericardial haemorrhage, they would represent embryos at the extreme end of the penetrance spectrum and likely display the most significant causative gene expression changes. Once again, for a gene expression change to be regarded as a "hit", the change would have to either be an upregulation of at least 20% in all three Tβ4 -/Y samples in comparison to their somite matched Tβ4 +/Y controls, or a similar 20% downregulation in all three Tβ4 -/Y embryos.

The previous qRT-PCR panel was repeated almost identically; with four Notch receptors, five Notch ligands, five Notch target genes, PDGF-B, PDGFR-β, TGF-β, three TGF-β receptors and five Smad adaptors all being quantitatively analysed. However, once again, there was no analysed molecule which manifested itself as an unequivocal "hit" as judged by the pre-specified criteria (**Figs. 5.9, 5.10, 5.11, 5.12, 5.13 and 5.14**).

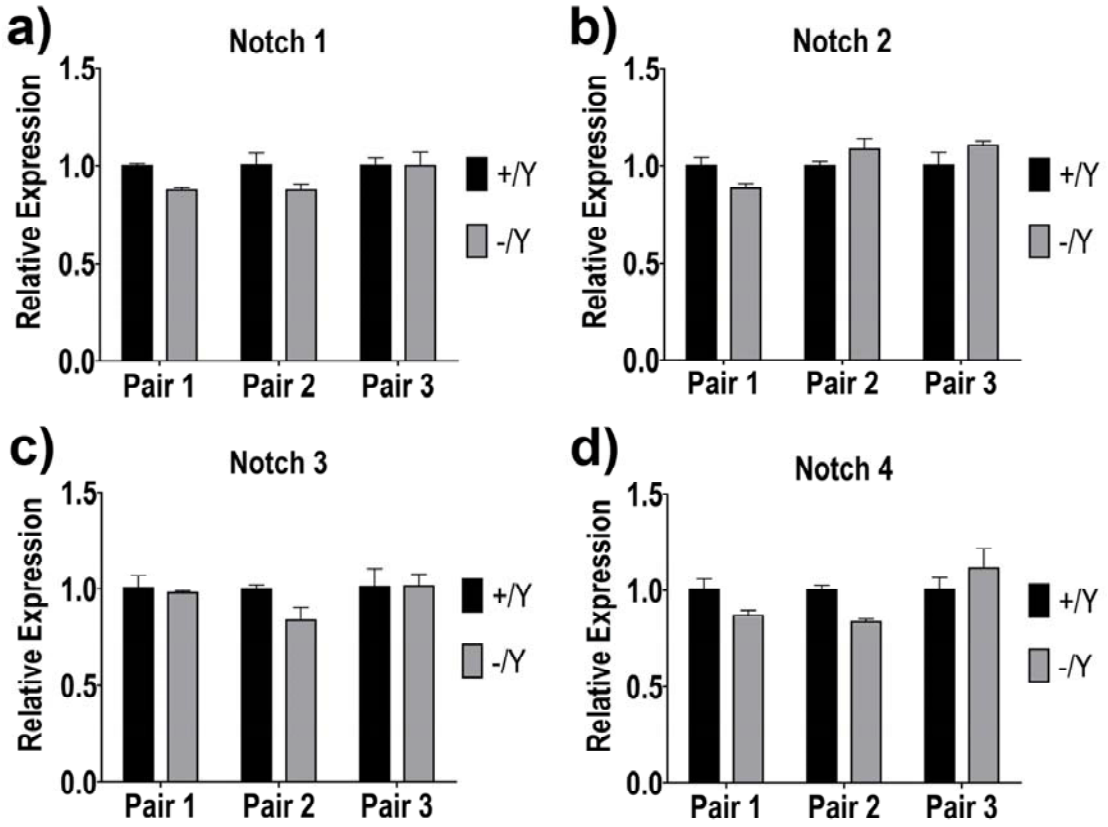


Fig. 5.9
Quantification of mRNA expression for Notch receptors in E10.5 Tβ4 -/Y haemorrhagic embryos

qRT-PCR quantification of mRNA expression levels for the Notch receptors Notch1 (**a**), Notch2 (**b**), Notch3 (**c**) and Notch4 (**d**) in three pairs of somite matched E10.5 Tβ4 -/Y and Tβ4 +/Y embryos. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in all three Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

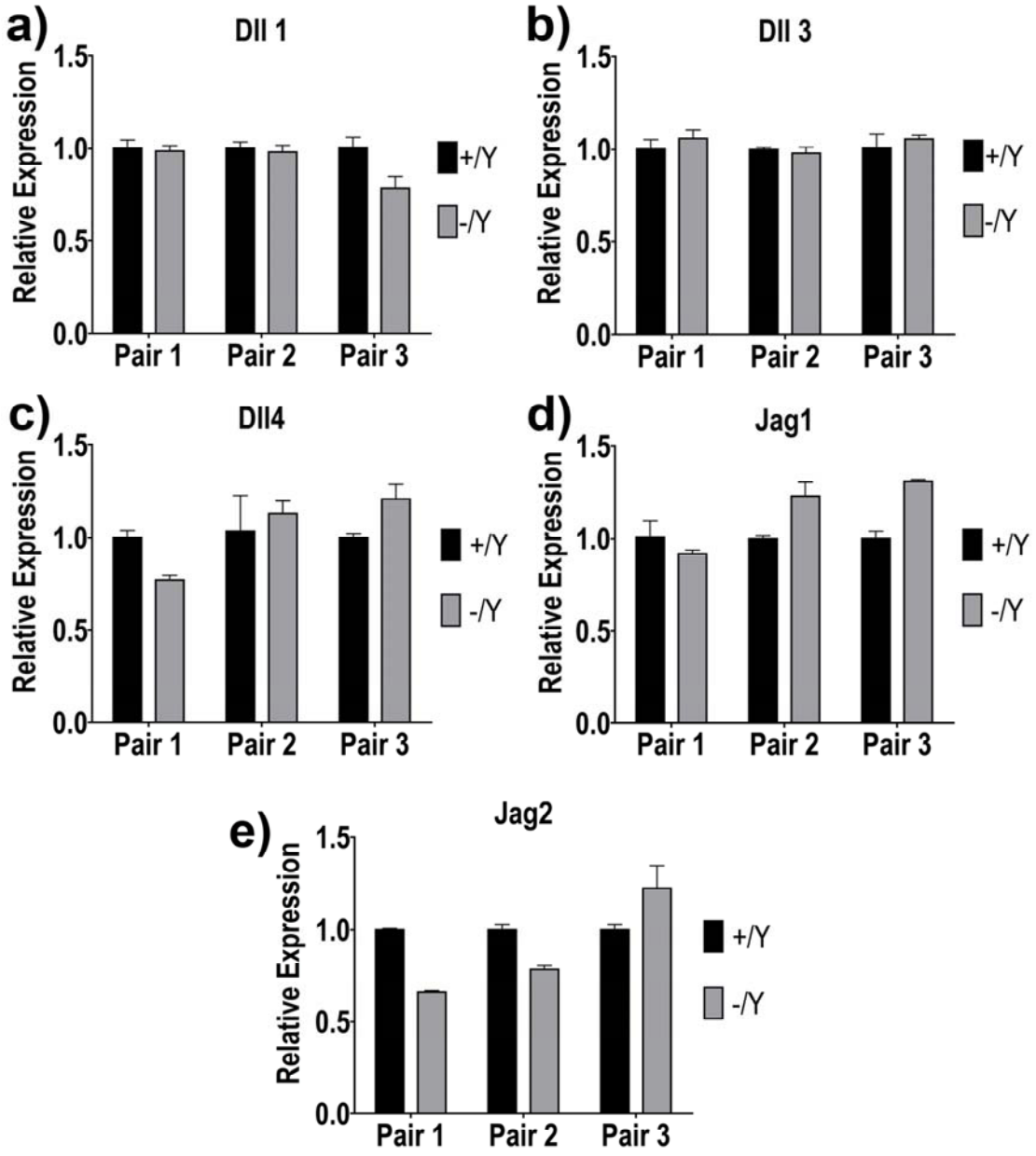


Fig. 5.10
Quantification of mRNA expression for Notch ligands in E10.5 Tβ4 -/Y haemorrhagic embryos

qRT-PCR quantification of mRNA expression levels for the Notch ligands Dll1 (**a**), Dll3 (**b**), Dll4 (**c**), Jag1 (**d**) and Jag2 (**e**) in three pairs of somite matched E10.5 Tβ4 -/Y and Tβ4 +/Y embryos. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in all three Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

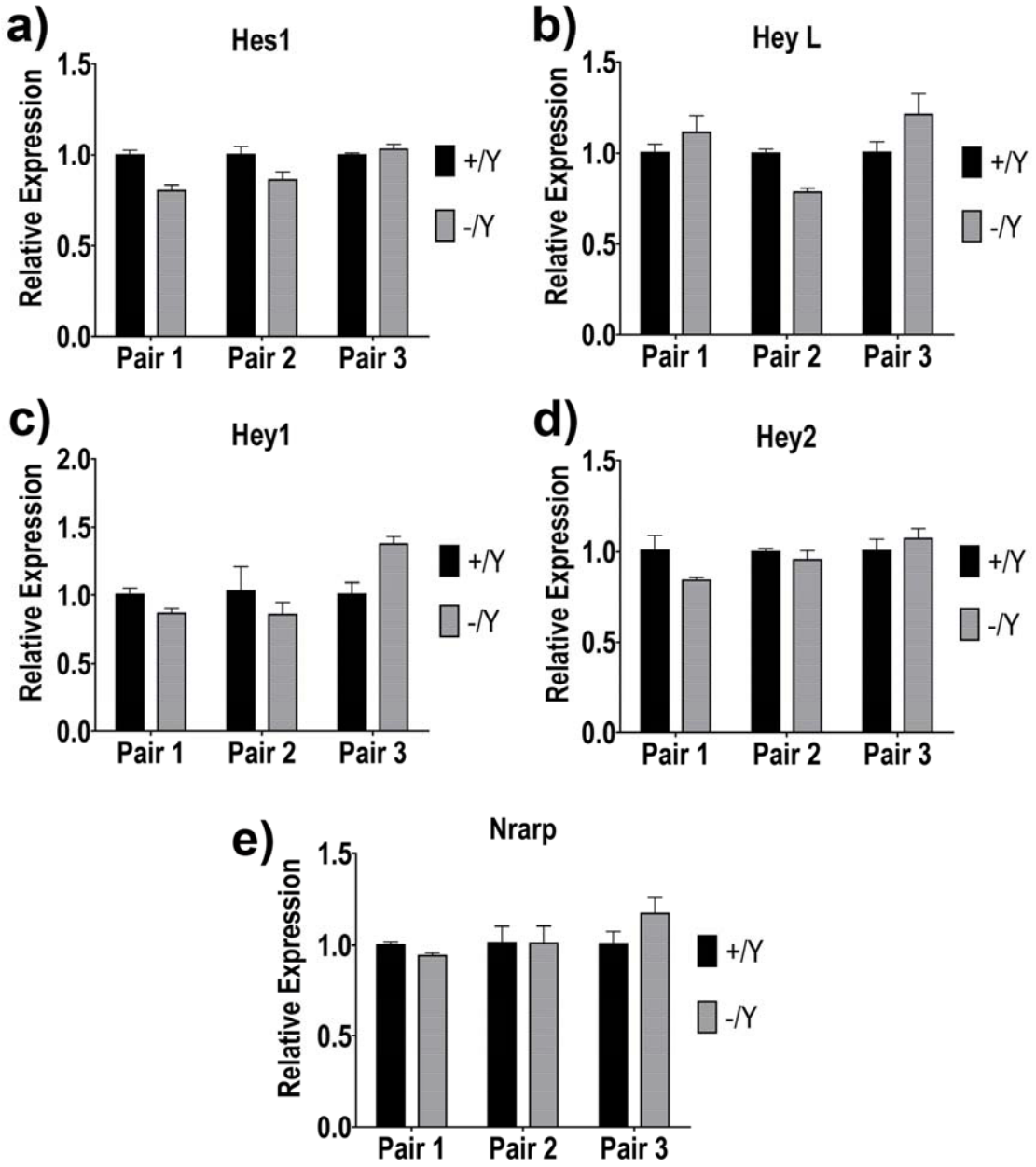


Fig. 5.11
Quantification of mRNA expression for Notch target genes in E10.5 Tβ4 -/Y haemorrhagic embryos

qRT-PCR quantification of mRNA expression levels for the Notch transcriptional targets Hes1 (**a**), HeyL (**b**), Hey1 (**c**), Hey2 (**d**) and Nrarp (**e**) in three pairs of somite matched E10.5 Tβ4 -/Y and Tβ4 +/Y embryos. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in all three Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

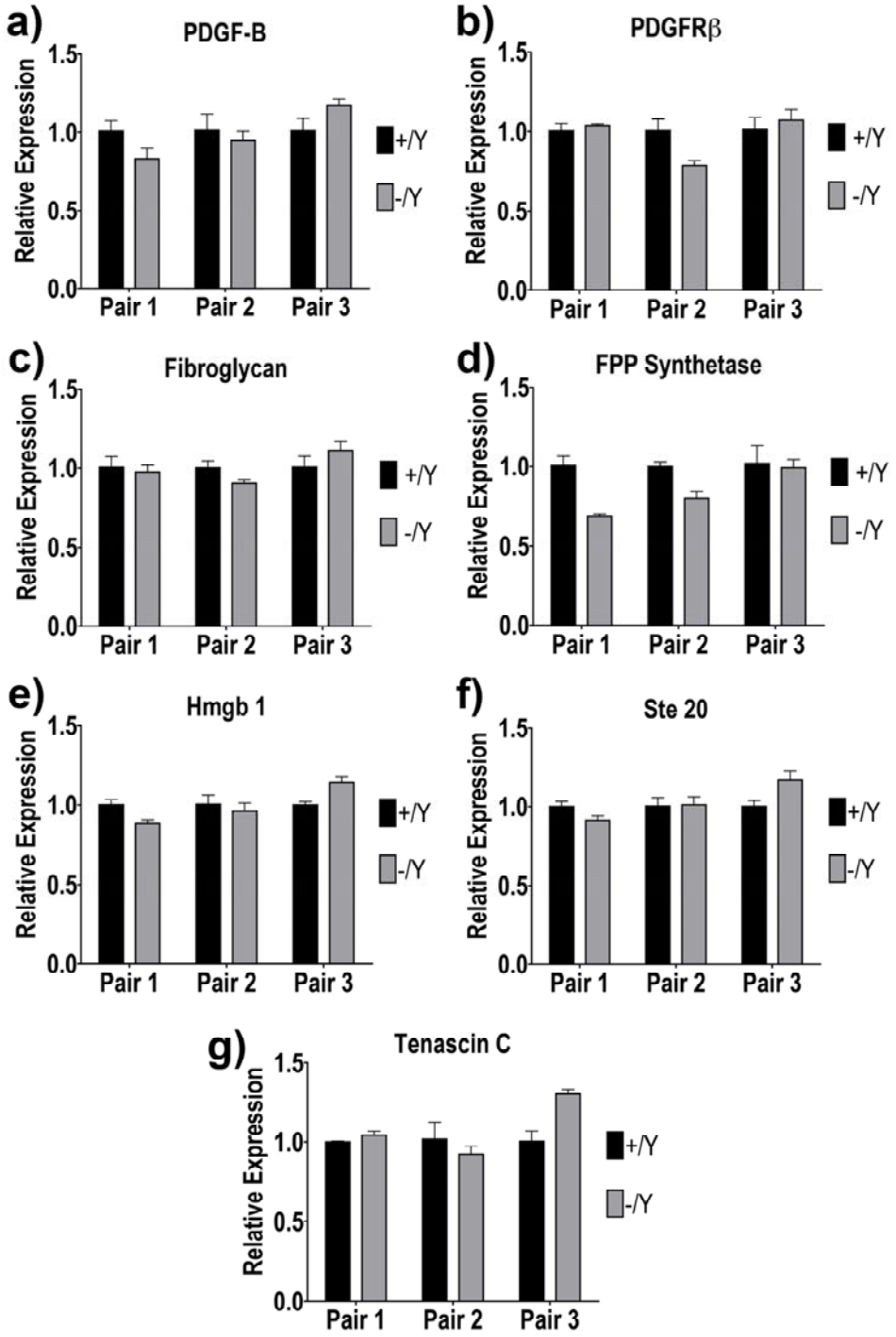


Fig. 5.12
Quantification of mRNA expression for PDGF-B, its receptor and target genes in E10.5 Tβ4 -/Y haemorrhagic embryos

qRT-PCR quantification of mRNA expression levels for the PDGF-B ligand (**a**), the PDGF receptor PDGFR-β (**b**) and the PDGF-B transcriptional targets Fibroglycan (**c**), FPP Synthetase (**d**), Hmgb1 (**e**), Ste20 (**f**) and Tenascin C (**g**) in three pairs of somite matched E10.5 Tβ4 -/Y and Tβ4 +/Y embryos. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in all three Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

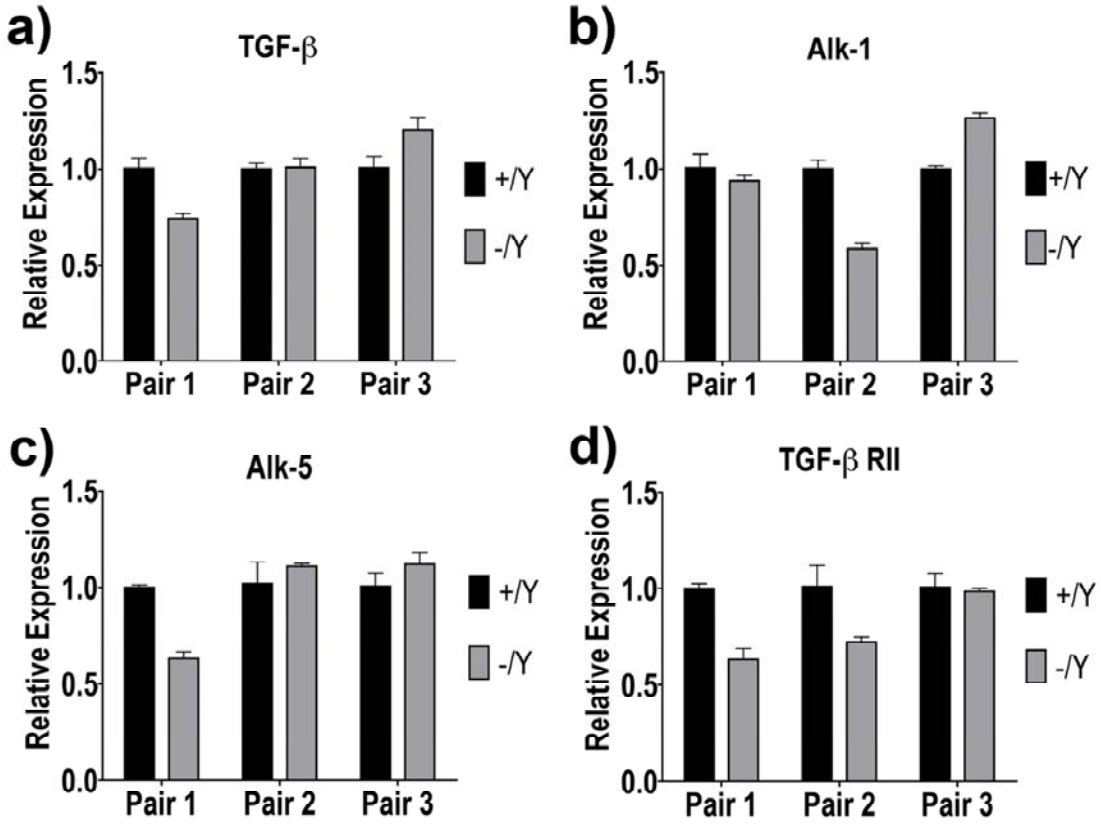


Fig. 5.13
Quantification of mRNA expression for TGF-β and its receptors in E10.5 Tβ4 -/Y haemorrhagic embryos

qRT-PCR quantification of mRNA expression levels for the TGF-β ligand TGF-β1 (**a**) and the TGF-β receptors Alk-1 (**b**), Alk-5 (**c**) and TGF-β RII (**d**) in three pairs of somite matched E10.5 Tβ4 -/Y and Tβ4 +/Y embryos. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in all three Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

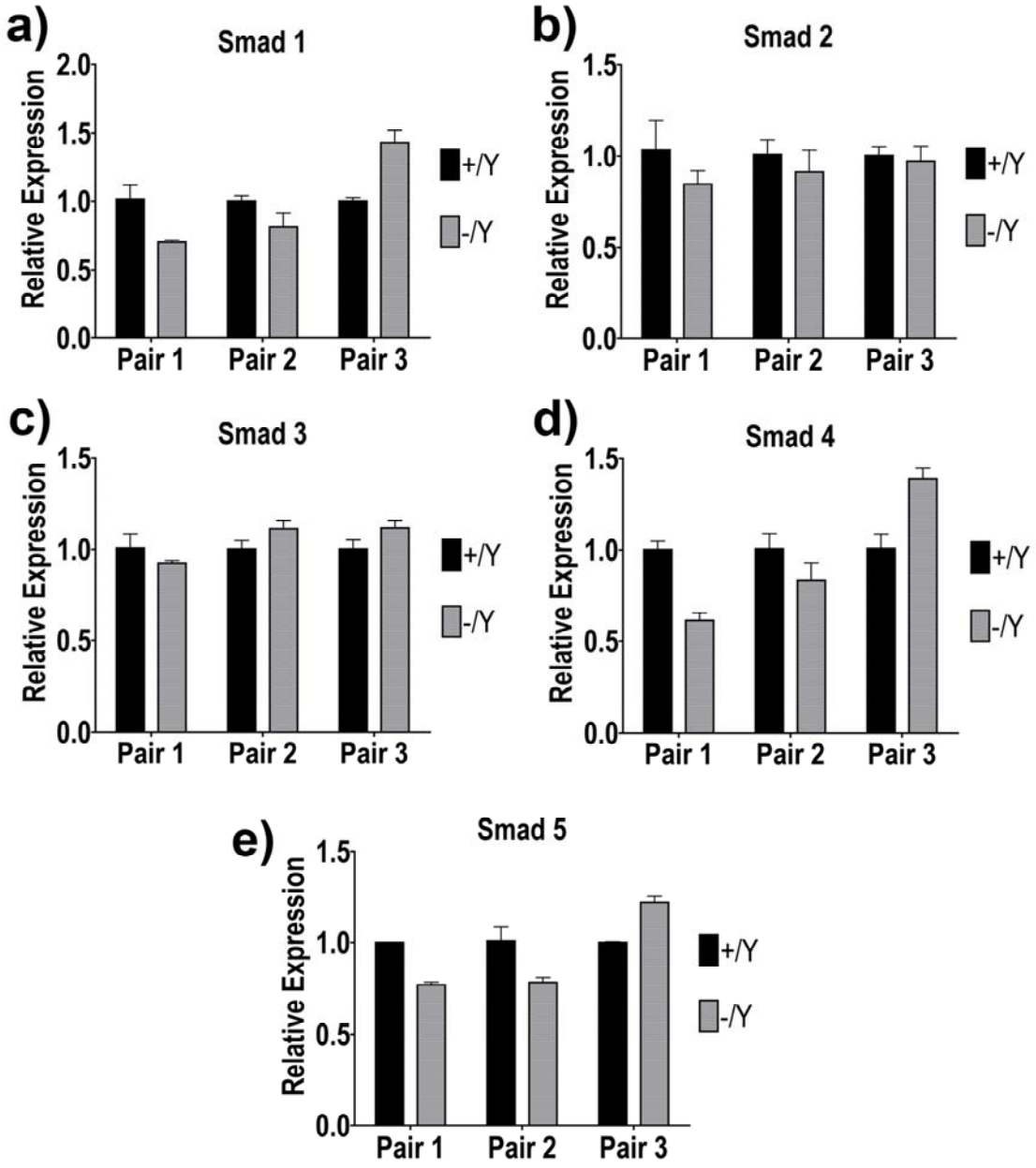


Fig. 5.14
Quantification of mRNA expression for Smad genes in E10.5 Tβ4 -/Y haemorrhagic embryos

qRT-PCR quantification of mRNA expression levels for the TGF-β pathway signalling adaptors Smad1 (**a**), Smad2 (**b**), Smad3 (**c**), Smad4 (**d**) and Smad5 (**e**) in three pairs of somite matched E10.5 Tβ4 -/Y and Tβ4 +/Y embryos. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in all three Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

Given the lack of success with the candidate pathway approach, attention was turned towards the unbiased gene array strategy. This line of enquiry had the advantage of

potentially being able to identify Tβ4 involvement in molecular pathways, which had not previously been linked to mural cell development. A two pronged analysis was performed with regards to these experiments. The first line of attack involved analysing the differential gene expression of E12.5 Tβ4 +/Y and Tβ4 -/Y embryos. This stage represented an intermediate between the E10.5 and E14.5 embryos where haemorragic and mural cell defects had been observed. It was hoped that using the E12.5 stage would provide insight into dysregulated genes, which might be applicable to both these previous observations. As however, performing array analysis on whole E12.5 embryos, involves assessing the gene expression of several distinct tissues, gene changes relevant to the vascular defect might be masked. Thus, the analysis was also performed on whole Tβ4 +/Y and Tβ4 -/Y eight week old postnatal hearts. Tβ4 has already been demonstrated to play a substantial role in cardiac development[50]. Moreover, Tβ4 -/Y mice maintain cardiac abnormalities into adulthood (Nicola Smart – personal communication). Therefore, if it is assumed that Tβ4 maintains similar cell signalling mechanisms across different tissues, then analysis of gene expression in an organ, which has a more uniform tissue distribution, might provide insight into the mechanism of embryonic vascular defects.

5.2.3 Gene Array Analysis of Tβ4 -/Y Embryos and Adult Hearts

RNA was extracted from three of each of the following; E12.5 Tβ4 -/Y embryos, E12.5 Tβ4 +/Y embryos, eight week old adult Tβ4 -/Y hearts and eight week old adult Tβ4 +/Y hearts. After RNA was tested for quality, it was hybridised by Priya Panchal at UCL Genomics, London, UK, onto Affymetrix mouse exon 1.0ST arrays. These arrays were used, as they possess high density coverage of the mouse genome and can

provide information about alternatively spliced transcripts, as well as global gene expression. Standardisation, extraction, normalisation and statistical analysis of the resulting data was performed by Nick Henriquez at the UCL Institute of Neurology, Queens Sq., London, UK.

A number of genes were identified as being differentially expressed between E12.5 Tβ4 -/Y and Tβ4 +/Y embryos, and Tβ4 -/Y and Tβ4 +/Y eight week old hearts respectively. As Bonferroni statistical testing was used to correct for multiple comparisons, and each experiment only used triplicate biological replicates, none of the adjusted p-values reached statistical significance. Therefore, an alternative approach was taken whereby genes were ranked in order of the magnitude of their differential expression, provided they displayed significance at the usual p=0.05 on standard t-testing. This represents a valid approach to identifying differentially regulated genes from microarrays, but does increase the false postitive discovery rate[156]. The top 100 up and down regulated genes in E12.5 Tβ4 -/Y embryos identified by these means, are listed in Appendices 1 and 2, and the top 100 up and down regulated genes in Tβ4 -/Y eight week old hearts are listed in Appendices 3 and 4.

From the lists of the top two hundred dysregulated genes in each category, twenty two genes of interest were identified for further validation and analysis. These genes were flagged up, on the basis of literature searching for a previous record of vascular expression, a role in vascular development or interaction with one of the three previously studied candidate pathways. The selected genes are listed in **Table 5.1**.

Name	Heart/Embryo	Up/Down	Function	Reference
Cadherin 3 (P-Cadherin)	Embryo	Up	Role in cell-cell adhesion. Reportedly expressed in bovine endothelial cells.	166
Cadherin 5 (VE-Cadherin)	Embryo	Up	Functions in endothelial cell adhesion and control of vascular permeability.	167
Ccbe1	Embryo	Down	Regulates lymphatic development and venous angiogenesis.	168
Ddr2	Embryo	Up	Controls VSMC migratory activity.	169
Dner	Embryo	Up	Delta/Notch like EGF related receptor.	170
Epas1 (HIF2α)	Embryo	Up	Essential role in vasculogenesis.	171
Fibulin 5	Embryo	Up	Forms part of the internal elastic lamina after being secreted by VSMCs.	172
Foxo1	Embryo	Up	Transcription factor which inhibits endothelial angiogenesis and VSMC proliferation	173,174
Hif1an	Embryo	Down	Hypoxia inducible factor alpha subunit inhibitor. Inhibits the activity of HIF1α.	175
IGFbp4	Embryo	Up	Inhibits VSMC proliferation and migration.	176
Integrin α1	Embryo	Down	Mediates adhesion to laminin and collagen I and IV on VSMCs.	177
Integrin β1	Embryo	Down	Role in mural cell adhesion to endothelial cells.	127
Ltbp4	Embryo	Up	Regulates bioavailability of TGF-β.	178
Maml1	Heart	Down	Transcriptional co-activator of the activated Notch receptor.	179
Paxillin	Embryo	Up	Signal adaptor protein in VSMCs required for focal adhesion formation.	180
Pim-1	Heart	Down	Transcription factor required for mural cell differentiation.	181
Plakoglobin	Embryo	Up	Structural component of endothelial cell junctions.	182
Slit 3	Embryo	Up	Axon guidance molecule with angiogenic activity.	183
Smap2	Heart	Down	Smooth muscle associated protein upregulated in neointima formation.	184
Sonic	Embryo	Down	Signalling molecule essential for vasculogenesis. Can also induce VSMC proliferation.	185,186
Symplekin	Heart	Down	Tight junction component.	187
Wasf2 (WAVE)	Embryo	Up	Wiskott-Aldrich syndrome protein necessary for endothelial cell migration.	188

Table 5.1
Dysregulated genes in Tb4 -/Y mice selected for further investigation

Twenty two genes were identified as being of interest in gene expression array studies on E12.5 Tβ4 -/Y embryos and 8 week old adult Tβ4 -/Y hearts. This table lists these target genes along with their purported relevant function.

Due to the possible existence of false positive results, an attempt was made to validate altered expression of these twenty two genes. In order to achieve this, gene expression was quantified in RNA from three E12.5 Tβ4 -/Y embryos and three E12.5 Tβ4 +/Y embryos, which were different to the embryos used for the arrays themselves, by qRT-PCR. In order to be regarded as "valid" each gene would have to be up regulated by at least 20% in all three Tβ4 -/Y embryos compared to controls, or down regulated by at least 20% in all three. The validation of potential genes of interest obtained from the analysis 8 week old hearts, was conducted on embryonic samples, as the use of the adult hearts in array studies were merely a tool to provide information about putative targets in the embryo. In order to establish that the gene expression changes observed in the adult heart are relevant to embryonic mural cell defects, it was necessary to demonstrate aberrant gene expression changes in the embryo. The results of this validation are displayed in **Fig. 5.15**.

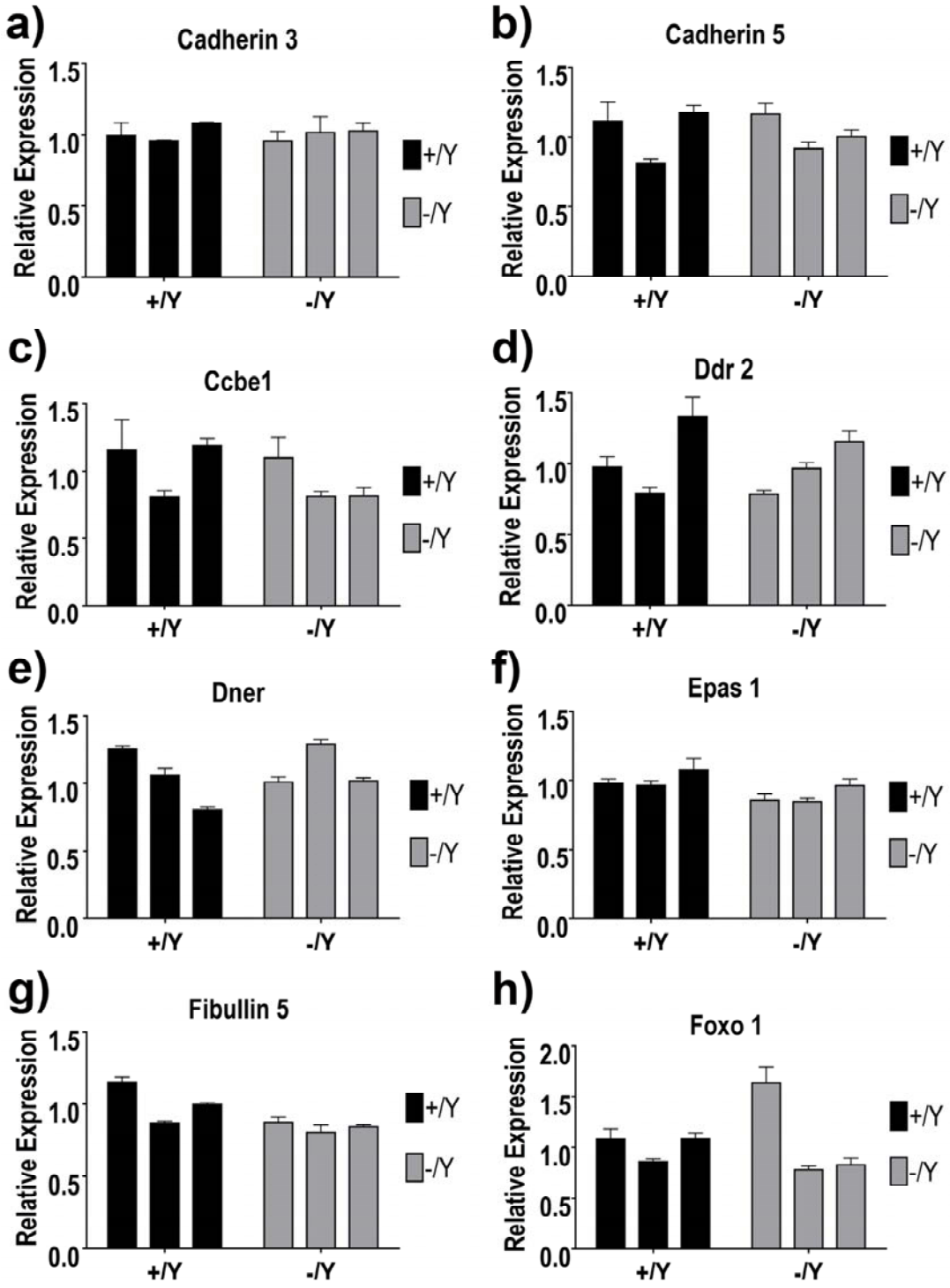


Fig. 5.15(i)
Validation of gene array targets of interest – part 1

Expression of the array identified genes Cadherin 3 (**a**), Cadherin 5 (**b**), Ccbe 1 (**c**), Ddr2 (**d**), Dner (**e**), Epas1 (**f**), Fibulin 5 (**g**) and Foxo1 (**h**) as measured by qRT-PCR performed on RNA from three E12.5 Tβ4 -/Y and three E12.5 Tβ4 +/Y embryos. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in all three Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

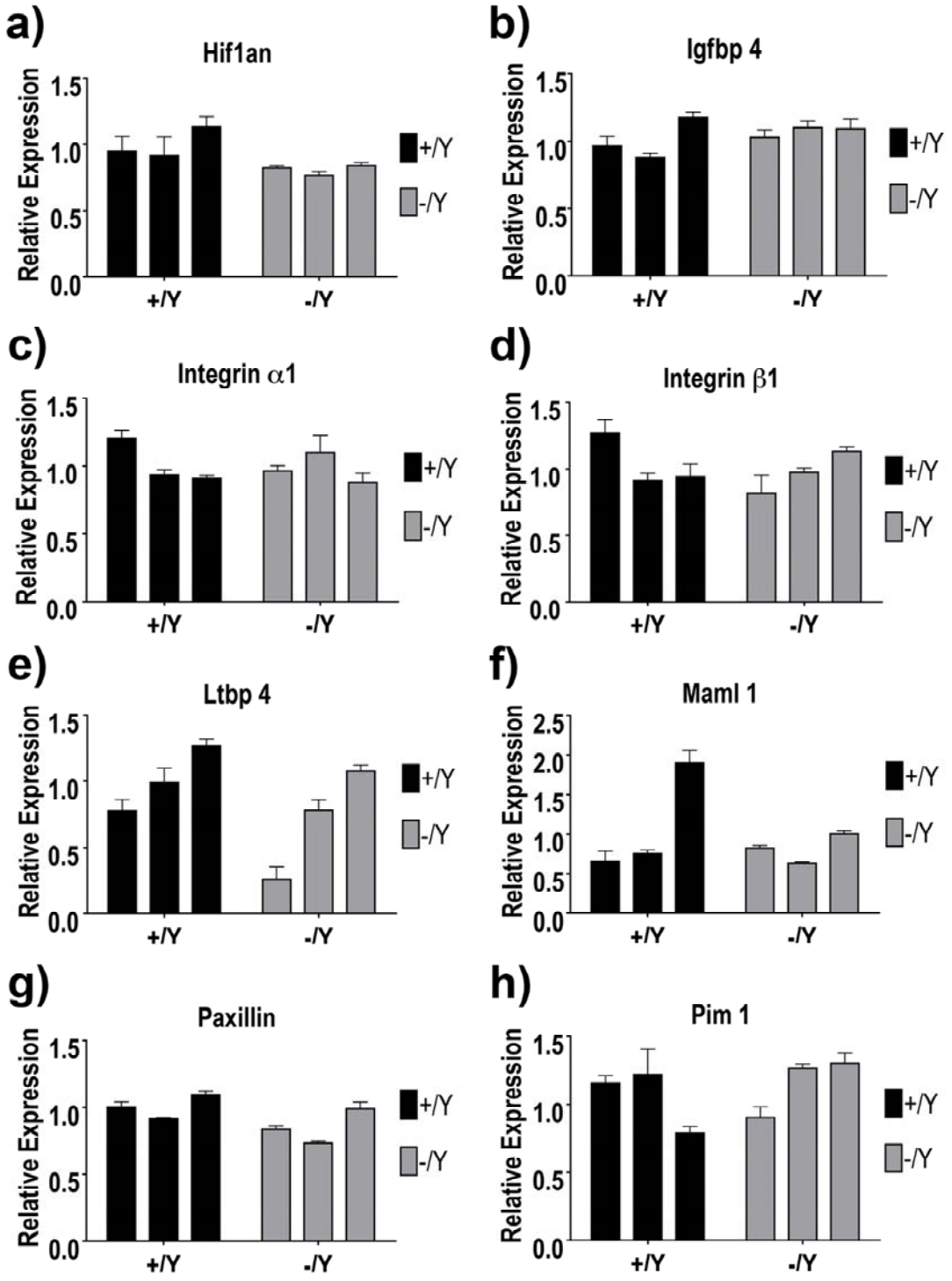


Fig. 5.15(ii)
Validation of gene array targets of interest – part 2

Expression of the array identified genes Hif1an (**a**), IGFbp 4 (**b**), Integrin α1 (**c**), Integrin β1 (**d**), Ltbp 4 (**e**), Maml 1 (**f**), Paxillin (**g**) and Pim1 (**h**) as measured by qRT-PCR performed on RNA from three E12.5 Tβ4 -/Y and three E12.5 Tβ4 +/Y embryos. No samples met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in all three Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

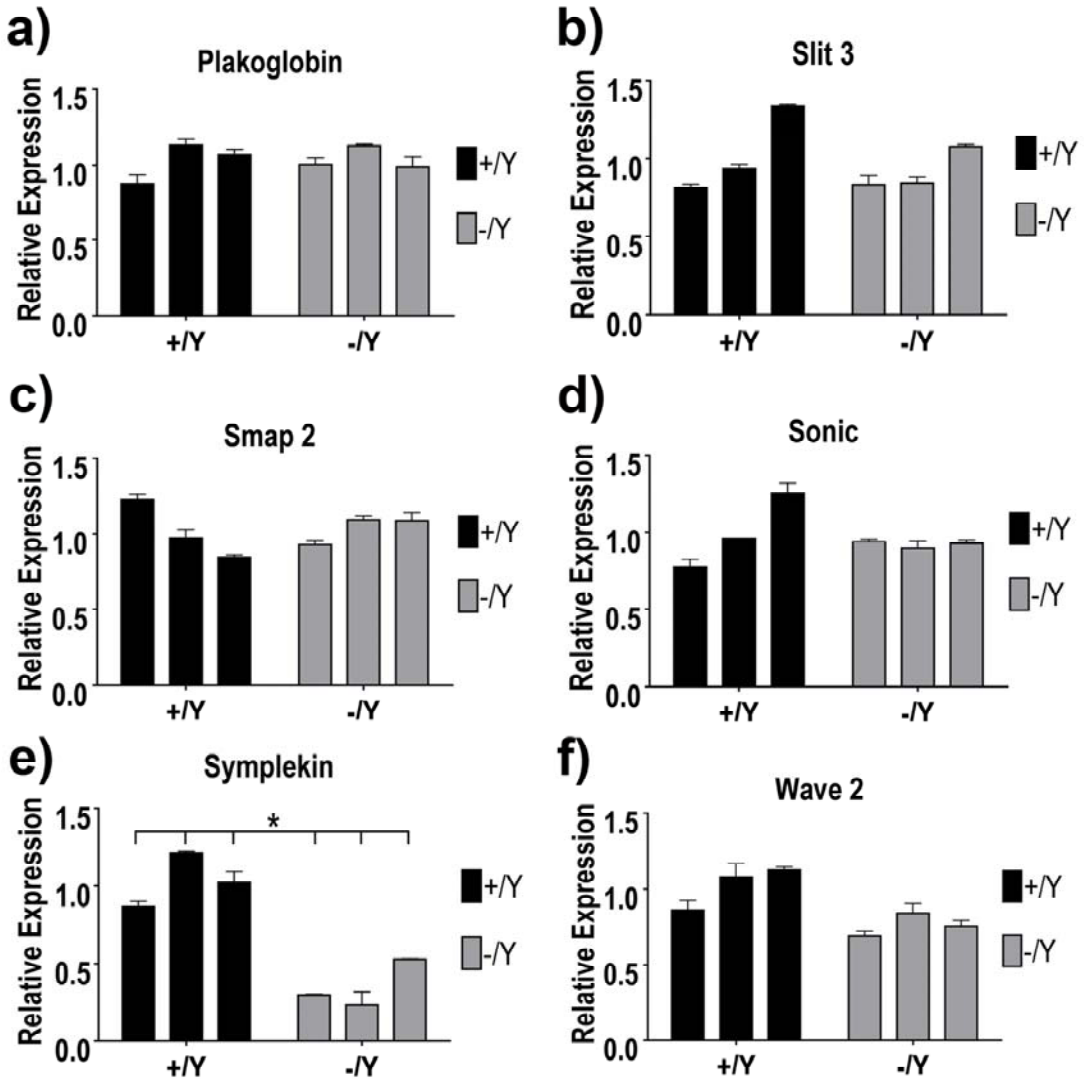


Fig. 5.15(iii)
Validation of gene array targets of interest – part 3

Expression of the array identified genes Plakoglobin (**a**), Slit 3 (**b**), Smap 2 (**c**), Sonic Hedgehog (**d**), Symplekin (**e**) and Wave 2 (**f**) as measured by qRT-PCR performed on RNA from three E12.5 Tβ4 -/Y and three E12.5 Tβ4 +/Y embryos. * Out of the targets examined, only the gene Symplekin met the pre-specified "hit" criteria of a consistent change of >20% up or down regulation in all three Tβ4 -/Y samples compared to Tβ4 +/Y samples, at a significance level of $p<0.05$.

5.2.4 Symplekin is Expressed in Endothelial Cells

The only gene assessed, which met the pre-specified validation criteria was that encoding the protein Symplekin. Symplekin is a protein, about which not much is known. It has been described as being involved in several biological processes including cytosolic polyadenylation of mRNA[189], inhibition of cellular differentiation[190], and promotion of tumourigenicity[191]. It is thought to often localise to cellular tight junctions[192], but analysis of its expression pattern initially appeared to suggest that it wasn't expressed in vascular tissue[187]. Therefore, wild type E10.5 embryo sections were stained with antibodies against Symplekin and the endothelial cell marker Endomucin. Immunofluorescence microscopy revealed expression of Symplekin in the endothelium of the E10.5 vasculature (**Fig. 5.16**).

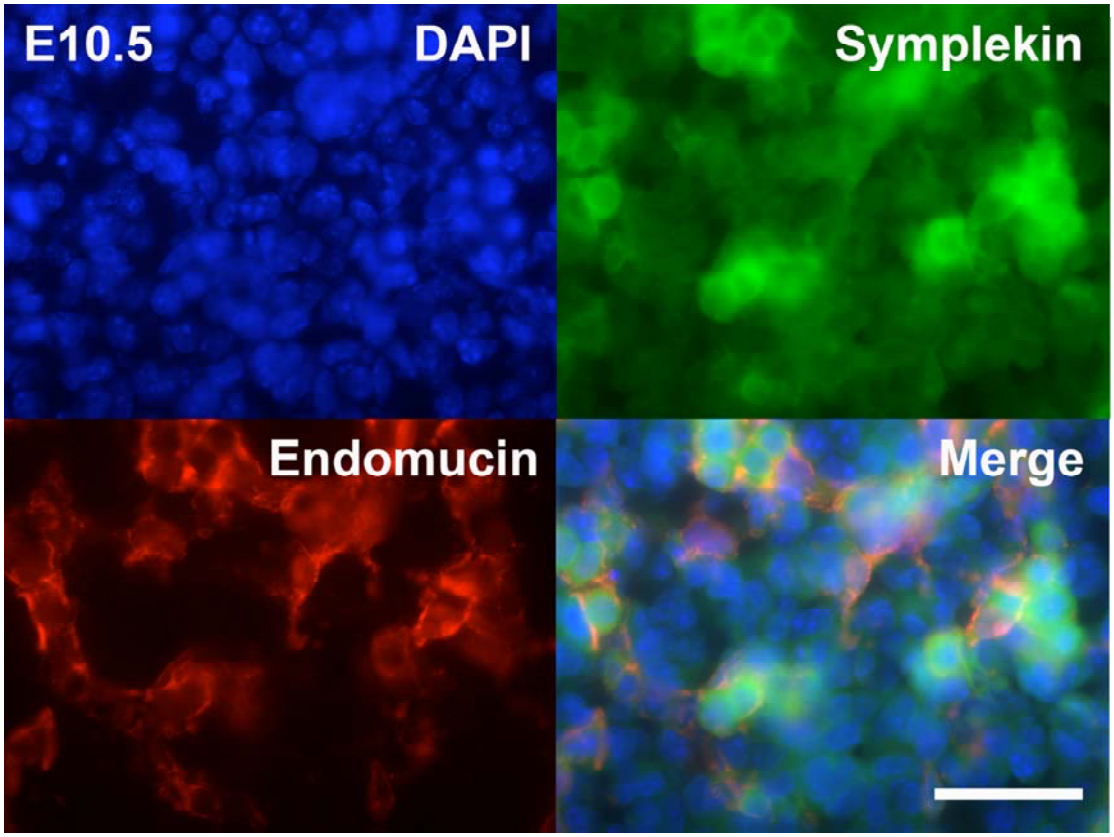


Fig. 5.16
Symplekin is expressed in the embryonic endothelium

Immunofluorecence microscopy reveals co-localisation of Symplekin with the endothelial cell marker endomucin, in the developing vasculature of the E10.5 wild type embryo. Scale bar: 20μm.

5.2.5 Bioinformatic Analysis of Array Dataset Reveals Dysregulation of the TGF-β Pathway in Tβ4 -/Y Mice

As the majority of genes identified from the gene array studies, as playing a potential role in the vascular phenotype of the Tβ4 -/Y mouse, could not be successfully validated in follow up qRT-PCR studies, an alternative bioinformatic approach was taken to analyse the array data. The top 200 up regulated and down regulated genes from both E12.5 Tβ4 -/Y embryos and eight week old Tβ4 -/Y hearts were fed into Metacore analysis software produced by Genego, St. Joseph, MI, USA. This software is able to conduct reverse pathway analysis by comparing the list of dysregulated

genes to a proprietary database. The software can then calculate the probability that peturbation of a known signalling pathway could cause those gene changes. Four out of the top five putative pathways identified by this software as being potential candidates for the gene changes observed in Tβ4 -/Y embryos and hearts involved TGF-β as a pathway node (**Table 5.2**).

Rank	Gene	Accession	Fold Change	Heart/ Embryo	Description
1	Actb	NM_007393	- 8.6	H	Actin B
	Pfn1	NM_011072	- 10.8	H	Profilin1
	Robo4	NM_028783	- 6.5	E	Roundabout homologue 4
	Tgfb1	NM_011577	- 8.8	H	Transforming growth factor beta 1
	Rac1	NM_009007	+ 6.8	E	Ras-related C3 botulinum toxin substrate 1
2	Gas8	NM_018855	+ 6.9	E	Growth arrest specific 8
	Tgfb1	NM_011577	- 8.8	H	Transforming growth factor beta 1
	Trp53	NM_011640	- 8.6	H	Transformation related protein 53
	Nme2	NM_008705	- 6.1	E	Non-metastatic cells 2, protein
3	Anxa1	NM_010730	+ 9.3	H	Annexin A1
	Stx4a	NM_009294	+ 7.0	E	Syntaxin 4a
	Rac1	NM_009007	+ 6.8	E	Ras-related C3 botulinum toxin substrate 1
4	Junb	NM_008416	+ 6.0	H	Jun-B oncogene
	Tgfb1	NM_011577	- 8.8	H	Transforming growth factor beta 1
	Adam12	NM_007400	- 7.0	E	A disintegrin and metallopeptidase 12
5	Nes	NM_016701	+ 8.6	E	Nestin
	Tgfb1	NM_011577	- 8.8	H	Transforming growth factor beta 1
	Rac1	NM_009007	+ 6.8	E	Ras-related C3 botulinum toxin substrate 1

Table 5.2
Bioinformatic analysis highlights dysregulated molecular pathways in Tb4 -/Y mice

The top 200 hundred up- and downregulated genes identified by Affymetrix exon array between Tβ4 +/Y and Tβ4 -/Y E12.5 embryos and Tβ4 +/Y and Tβ4 -/Y adult hearts were inputed into Genego Metacore software. Reverse pathway analysis based on altered transcription factor levels and comparison to a systems biology database identified potential disrupted signalling pathways in the Tβ4 -/Y embryos. Four out of the top five potentially disrupted pathways involved TGF-β as a major node in the signalling pathway. This table displays the nodes present in the top 5 ranked putative pathways and provides information about their transcript representation in the exon arrays.

5.3 Discussion

5.3.1 Summary

In order to understand the molecular basis for mural cell defects observed in the Tβ4 -/Y mouse, a number of strategies were undertaken. First, a candidate pathway approach was used to test whether ablation of Tβ4 expression had any effect on the expression levels of key genes known to be involved in the process of mural cell development. qRT-PCR analysis of limb buds from E12.5 Tβ4 -/Y and Tβ4 +/Y embryos revealed no notable differences in gene expression levels of several of the Notch, PDGF-B and TGF-β pathway components examined. The only exception to this was the identification of the Notch ligand Dll3 as being up regulated in the Tβ4 -/Y limb buds. Similar parallel analysis of somite matched pairs of E10.5 Tβ4 -/Y and Tβ4 +/Y embryos provided no clear, positive hits.

The second approach was to use gene arrays to assess expression level changes across the whole mouse genome. E12.5 embryos and eight week old hearts from Tβ4-/Y and Tβ4+/Y mice were compared by these means. Twenty two candidate genes were identified from this screen. However, only one gene could be validated in an independent set of E12.5 Tβ4 -/Y and Tβ4 +/Y embryos – symplekin. Symplekin was identified as being expressed in the embryonic endothelium during development. Further bioinformatic analysis of the gene changes observed in the arrays highlighted TGF-β signalling as being potentially disrupted in Tβ4 -/Y mice.

5.3.2 Candidate Pathway Approaches

The first strategy used to attempt to elucidate the molecular function of Tβ4 in vascular development was based on a qRT-PCR screen of candidate pathways, which had already had a pre-established function in mural cell development. Neither of the two approaches taken, analysing gene expression changes in E12.5 limb buds or E10.5 whole embryos, proved unequivocally successful. The only target that was identified as differentially regulated in the Tβ4 -/Y mouse embryo was Dll3. Dll3 is a canonical Notch ligand, but it is unlikely that its overexpression, as observed in E12.5 Tβ4 -/Y limb buds can account for the mural cell defects observed in Tβ4 -/Y mice. This is, in part, due to Dll3 expression never having been reported in the developing vasculature[70]. The change in expression levels of Dll3 is also unaccompanied by any consistent change in the expression levels of known Notch transcriptional targets such as members of the Hey, Hes or Fringe families of genes. Thus, it is unlikely that Dll3 plays any significant role in the genesis of mural cell defects in the Tβ4 -/Y mice.

In spite of none of the tested genes in these analyses meeting the pre-established criteria of up or down regulation of >20% in all tested Tβ4 -/Y samples, compared to Tβ4 +/Y samples, some trends were observed that are worth highlighting. Notably, when gene expression was assessed in E10.5 Tβ4 -/Y and Tβ4 +/Y embryos, several components of the TGF-β signalling pathway were highly and significantly down regulated in two out of three of the embryo pairs studied. These genes coded for the proteins TGF-β RII, Smad 1, Smad 4 and Smad 5 (**Figs. 5.13 and 5.14**). Given that these targets would have been designated "hits" if the third pair had shown an equivalent dysregulation, and that the TGF-β pathway was highlighted by subsequent

bioinformatics analysis of the datasets obtained from gene array studies on Tβ4 -/Y material, these data indicate a that a further, more thorough investigation of the TGF-β pathway in Tβ4 -/Y mice is warranted.

The approach used for testing candidate pathways was a valid one, and the generic strategy has been successful for other researchers[154,155]. If significant changes had occurred consistently in expression levels of ligands, receptors and target genes, across both experimental systems, then this would have been strong evidence for Tβ4's involvement in that particular pathway. However, no strong changes were observed. There may be two explanations for this; either the pathways tested are truly not involved in Tβ4 signalling, or that the experimental set up was not robust enough to detect alterations in the pathway.

Using RNA derived from either E12.5 limb buds or whole E10.5 embryos has its disadvantages. E12.5 limb buds were analysed for two reasons. First of all, it was a strategy previously used with success to characterise the molecular basis for the vascular defect in endothelial specific SRF knockout mice[160]. E12.5 was also a time point intermediate to the E10.5 and E14.5 stages at which mural cell defects had been observed in the Tβ4 -/Y mouse. Thus, it was hoped that by analysing the E12.5 stage, any molecular insight gleaned would be applicable to both the E10.5 and the E14.5 stage. However, from the previous chapter, it had been observed that Tβ4 -/Y embryos have a variable penetrance of the mural cell phenotype. Thus, it is plausible that the limb buds assayed, in this instance, may not have come from embryos, which were severely afflicted by the mural cell phenotype. If it is presumed that the embryos, which have the most severely affected phenotype, are likely to be the

embryos with the greatest degree of molecular derangement, then it is possible that the limb buds analysed would not have represented the best material on which to perform the experiment.

This problem was overcome by using somite matched E10.5 pairs of Tβ4 -/Y and Tβ4 +/Y embryos. The Tβ4 -/Y embryos chosen for this experiment all displayed overt pericardial haemorrhage and were as a consequence, likely to suffer from the mural cell defect. However, this analysis suffered from a different problem. The E10.5 embryo represents a heterogeneous population of tissues and cells. Therefore, it may have been difficult to isolate gene expression changes due to the vascular defect, from the noise produced by those genes being expressed in non-vascular tissues. The Notch ligand Dll4 can be used as a case in point. Dll4 is known to have a significant functional role in vascular development[70]. However, it is also expressed, during development, in cells of the nervous system, gut, kidney, eye, lung and thymus. Therefore, any subtle changes in levels of Dll4 gene expression occurring in the vasculature of Tβ4 -/Y mice, could be being drowned out by normal expression at other locations.

Another approach, which could be used to overcome this, would be to use laser capture microdissection techniques to isolate the aorta and peri-aortic region from Tβ4 -/Y embryonic sections before conducting candidate pathway gene expression analyses on amplified RNA from these samples.

One other limitation to the candidate gene approach that was employed, could be that gross changes in gene expression might not be the best way to determine the activity

of a given molecular pathway. For example, rather than assaying the levels of Notch target gene expression, an alternative approach could be to perform western blotting on appropriate biological samples for the cleaved Notch ICD. Similarly, western blotting for phospho-PDGFR-β or phospo-Smads could be used to measure pathway activity. In fact, an attempt was made to detect phospho-PDGFR-β and the Notch 3 ICD by western blotting on embryonic protein, but the technical aspects of this approach proved too challenging.

5.3.3 Gene Arrays

The second strategy used to obtain insight into the molecular phenotype of the Tβ4 -/Y embryo involved global expression mapping across the mouse genome with cDNA microarrays. E12.5 whole embryos were chosen as the source material, again with the intention that any positive data obtained would be applicable to both the E10.5 and E14.5 stages at which mural cell defects had been observed. However, as there were concerns about expression changes due to Tβ4 knockout being masked by the heterogeneous nature of the tissues in the E12.5 embryo, microarray analysis of eight week old postnatal Tβ4 -/Y and Tβ4 +/Y hearts was also performed. As the adult heart is more homogenous in terms of cell type relative to the embryo as a whole, it was hoped that any peturbation in gene expression in the mutant hearts via Tβ4 knockout would be less likely to be masked by normal expression in other tissues.

From these arrays, a number of differentially expressed genes were obtained. However, initial statistical testing of these expression changes did not highlight any

significant differences between the Tβ4 -/Y and Tβ4 +/Y samples. This is likely to be because the initial statistical testing was inappropriately stringent. Normally, when analysing cDNA microarray data, standard t-testing at the $p<0.05$ level has limited value. If $p<0.05$ is used as the statistical cut off point, then even when comparing two identical samples, 1 in 20 genes will display this level of significance purely through statistical error and random variation. When interrogating a whole genome of 30,000 targets, this will result in 1,500 false positives. Thus, a statistical correction for multiple comparisons has to be built into the analysis using Bonferroni statistical methods[156]. This, however, has the unfortunate effect of producing too many false negative errors, especially when the statistical power of the experiment is low, as in this case where samples were only compared in triplicate.

In order to overcome this, gene expression changes were ranked according to the magnitude of change, and included in analyses if they displayed a significance of $p<0.05$ on standard t-testing. This is held to be a valid method of interpreting gene expression data, provided that potential "hits" are validated by a more accurate method of quantifying gene expression changes, such as qRT-PCR[156].

Out of the top 200 upregulated and downregulated genes in Tβ4 -/Y hearts and embryos, 22 were selected for validation and further analysis, mostly on the basis of a previously described vascular expression pattern, functional activity in vascular development or involvement with one of the candidate pathways previously tested. All of the candidates identified through gene array studies were validated in material from E12.5 embryos, in order to establish the relevance of targets derived from

analysis of the adult Tβ4 -/Y heart to the embryonic situation. However, surprisingly only one of these identified genes, Symplekin, was successfully validated.

There are several possibilities for why the other twenty one genes could not be validated. One possibility is that their original appearances in the list of up regulated and down regulated genes was due to statistical or random error and they do not truly represent genes that had significant expression differences between Tβ4 -/Y and Tβ4 +/Y samples. As previously mentioned, this could be a possibility due to performing the experiment in triplicate. The power of the experiment would be boosted to detect true biological change, if the experiments had been conducted with six biological samples in each group. Alternatively, those genes acquired from assessment of the adult Tβ4 -/Y hearts may simply be dysregulated in the context of the adult heart, and may not be changed in the embryonic setting.

Another explanation could be that these genes truly were dysregulated in the samples subject to microarray testing. However, as the validation was performed on RNA derived from E12.5 Tβ4 -/Y and Tβ4 +/Y embryos separate from those which had been used for the microarray, it could be that the real differences observed in the array do not necessarily exist in the population of Tβ4 -/Y embryos as a whole. There is a possible mechanistic explanation of why this might occur.

Many of the gene expression changes revealed by the array studies could, rather than being directly regulated by Tβ4, be altered as a compensation mechanism for ablated Tβ4 expression. Hypothetically, it could be such compensatory mechanisms, which keep most E10.5 Tβ4 -/Y embryos from displaying overt haemorrhage. However,

such compensatory gene expression changes could vary from embryo to embryo resulting in a failure to validate the array data in independent samples. If this were the case, boosting the number of biological replicates used in the array could have the effect of reducing the significance of compensatory gene changes whilst highlighting gene expression directly influenced by the presence or absence of Tβ4.

The single gene, which was validated, codes for the protein Symplekin. The primary function of this protein has been described as involvement in polyadenylation, the process by which mRNA molecules acquire a poly-adenosine tail. It is thought to function mainly in cytoplasmic polyadenylation, allowing mRNAs to be retained and stored in the extranuclear cytosol[189]. As initial descriptions of gene expression stated that symplekin was a tight junction protein that was not expressed in the endothelium[187], it was deemed necessary to test whether this report was correct. Upon immunofluorescent examination, it became clear that symplekin was expressed in the endothelium of E10.5 wild type embryos. Several different functions have been ascribed to this protein including the promotion of tumourigenicity[191] and the repression of gastrointestinal stem cell differentiation[190], but it is difficult to understand how these roles could mediate the vascular defects observed in Tβ4 -/Y mice. Further work would either centre around the production of a murine Symplekin knockout, or if an *in vitro* model of Tβ4 mediated mural cell differentiation could be constructed, the effects of knocking down symplekin in such a system.

In order to analyse the gene expression changes observed in Tβ4 -/Y samples in a broader sense, than that achieved simply by focusing on changes at the individual gene level, Metacore software produced by the company Genego was used. One

feature of this software allows reverse pathway analysis. The gene expression changes observed in samples are compared to a proprietary database of gene expression changes catalogued from analysis of the literature. The software can then calculate which know pathways are most likely to have caused these gene expression changes. Four out of the five top ranked pathways produced by this software involve TGF-β as a signalling node.

This is highly interesting, as TGF-β signalling has been identified as the key molecular pathway in mural cell differentiation[88]. However, as with all bioinformatic analyses of gene expression data, this should not be taken as proof of Tβ4's involvement in TGF-β signalling at this stage. As mentioned above, the inability to adequately validate most of the observed gene expression changes of interest, suggests caution be exercised. However, a role for Tβ4 in TGF-β pathway signalling does provide an attractive mechanism to explain the mural cell defects observed in Tβ4 -/Y mice.

This result, once again highlights the need to create an adequate model of *in vitro* mural cell differentiation in which the effects of Tβ4 can be tested. Such a model would more readily allow measurement and manipulation of TGF-β signalling, through the use of western blotting for activated pathway components, qRT-PCR for target gene expression, pathway reporter constructs and specific pathway inhibitors.

6 Modelling the Function of Tβ4 *In Vitro*

6.1 Introduction:

Having observed that global or endothelial specific knockout of Tβ4 in the developing mouse embryo leads to a reduction in mural cell investiture of the developing vasculature; a search was conducted for the molecular and cellular basis of this phenotype. There are four main cellular processes in which, a defect could conceivably have led to such a phenotype, namely: increased apoptosis of mural cells, decreased proliferation of mural cells, impaired migration of mural cell progenitors or deficient differentiation of mesoderm into mature mural cells. The first two of these possibilities were eliminated via direct quantification of apoptosis and proliferation of mural cells as described earlier. Impaired migration of mural cell progenitors due to a deficiency in cell autonomous Tβ4 seems unlikely, due to the lack of a mural cell defect in the neonatal retina, the absent/low levels of Tβ4 expressed in wild type mural cells and the lack of any effect of Tβ4 on VSMC migration in the literature[57].

This leaves the intriguing possibility that mural cell defects in murine Tβ4 loss of function models, may be due to disrupted differentiation of mesoderm to a mature mural cell state. Such a hypothesis is difficult to confirm *in vivo* as no currently available technique can either image deeply placed murine embryonic cells intra vitally or readily ascertain cellular ancestry based on marker analysis. However, a number of *in vitro* models can be exploited to determine the effect of exogenous molecules on the differentiation of cells of a mesodermal and non-mesodermal nature.

In vitro models used to dissect out cellular mechanism also display the advantage of facilitating elucidation of molecular mechanisms. Candidate pathway analysis of E10.5 Tβ4 -/Y and Tβ4 +/Y embryos showed a trend to dysregulation of several components of the TGF-β signalling pathway. Subsequent bioinformatic pathway analysis of gene expression changes in E12.5 Tβ4 -/Y embryos determined by gene array, as previously described, revealed that changes in transcription factor expression within these embryos could most readily be explained by disturbance of TGF-β associated pathways. In order to test whether these results are relevant to the phenotype observed in the Tβ4 -/Y mouse, further experimental models must be used to obtain confirmatory data. Use of a cellular system of *in vitro* differentiation can enable the effects of Tβ4 on TGF-β pathway activity to be directly assayed by means of western blotting for phosphorylated signalling molecules, luciferase assays for Smad activation and qRT-PCR quantification of TGF-β transcriptional targets – all of which are very difficult to assess *in vivo*.

Thus, the following data will describe the effects of Tβ4 on four such systems of mural cell differentiation in order to resolve the question about whether paracrine endothelial secreted Tβ4 can stimulate the differentiation of primitive mesoderm to mature mural cells. The systems of cellular differentiation to be explored will involve differentiation of murine embryonic stem cells (ES cells), a P19 embryonal carcinoma cell line clonally selected for its propensity to differentiate to vascular smooth muscle known as A404, a mouse embryonic fibroblast-like cell line named 10T1/2 and primary isolated mouse embryonic fibroblasts (MEFs).

These cell lines were chosen as each of them has been shown to display the property of mural cell gene expression upon stimulation by exogenous trophic factors. Each cell type may also be viewed as a correlate of a different developmental stage or a representative of a different cell type within the embryo. *In vitro* differentiation of ES cells is a model for the initial differentiation of lineage uncommitted stem cells and may provide insight as to whether Tβ4 can affect the earliest stages of embryonic cell differentiation *in vivo*[92,193]. 10T1/2 cells are a cell line derived from the mesodermal tissue of E9.5 C3H mice. They may, thus, represent the truest *in vitro* model of differentiation of uncommitted mesodermal progenitor cells[89,90]. Embryonal carcinomas are germ cell tumours and as such can contain cells of any lineage normally present during development of an organism. The A404 cell line is a derivative of the P19 embryonal carcinoma cell line clonally selected for its ability to differentiate into vascular smooth muscle cells upon stimulation by retinoic acid[194]. Such cells represent, similarly to the 10T1/2 line, an intermediate stage of differentiation between the undifferentiated ES cell and the fully differentiated functional mural cell. Primary mouse embryonic fibroblasts (MEF) have also been used to represent mural cell precursors in differentiation assays, notably by injecting them into the tail veins of adult mice in a cocktail with mouse fibrosarcoma cells. It has been shown that these fibrosarcoma cells can recruit the co-injected MEFs to newly formed blood vessels at a location similar to that occupied by mural cells[195]. It is not yet clear to what extent this phenomenon applies only to the development of tumour vasculature.

It is for these reasons that ES cells, 10T1/2 cells, A404 cells and MEFs were initially used to examine the induction of mural cell markers when cultured *in vitro* with Tβ4.

Mural cell phenotype of the Tβ4 stimulated cells was assayed by the quantification of mRNAs of mural cell associated marker genes by qRT-PCR. As mural cells exist on a phenotypic continuum between vascular smooth muscle cells and pericytes, markers were chosen to represent a wide range of differentiated mural cell phenotypes. These markers are summarised in **Table 6.1**.

Marker name	Phenotype	Reference
Smooth muscle actin (SMA)	VSMC	[196]
SM22 alpha (SM22α)	VSMC	[153]
NG2	VSMC/Pericyte	[197]
Endosialin	Pericyte	[198]
Angiopoietin 1 (Ang1)	Pericyte	[199]
CD13	Pericyte	[196]
Desmin	Pericyte	[200]

Table 6.1
Mural cell markers and their cell specific expression

Summary of mural cell markers used during Tβ4 stimulated in vitro differentiation studies.

6.2 Results:

6.2.1 Tβ4 Treatment of ES Cells Has No Effect on the Expression of Mural Cell Markers

The first cell type on which Tβ4 mediated mural cell induction was tested was the wild type ES cell. Two ES cell lines, R1 and E14, were maintained under non-differentiating culture conditions. In order to test the ability of Tβ4 to induce the expression of mural cell markers in these ES cells, cells were transferred into

differentiating medium which lacked the LIF containing ESGro culture additive and was supplemented with 100ng/ml Tβ4, TGF-β or a combination of both. Cells were cultured under these conditions for six days before RNA was extracted and analysed for mural cell marker upregulation. Consistent with previously published data[92], TGF-β was able to upregulate expression of the VSMC marker genes SMA and SM22α in both ES cell lines tested (**Fig. 6.1**). However, Tβ4 was not able to induce upregulation of any of the mural cell markers probed. Culture with Tβ4 plus TGF-β was not able to significantly increase mural cell marker expression in these cells above and beyond that achieved through TGF-β alone.

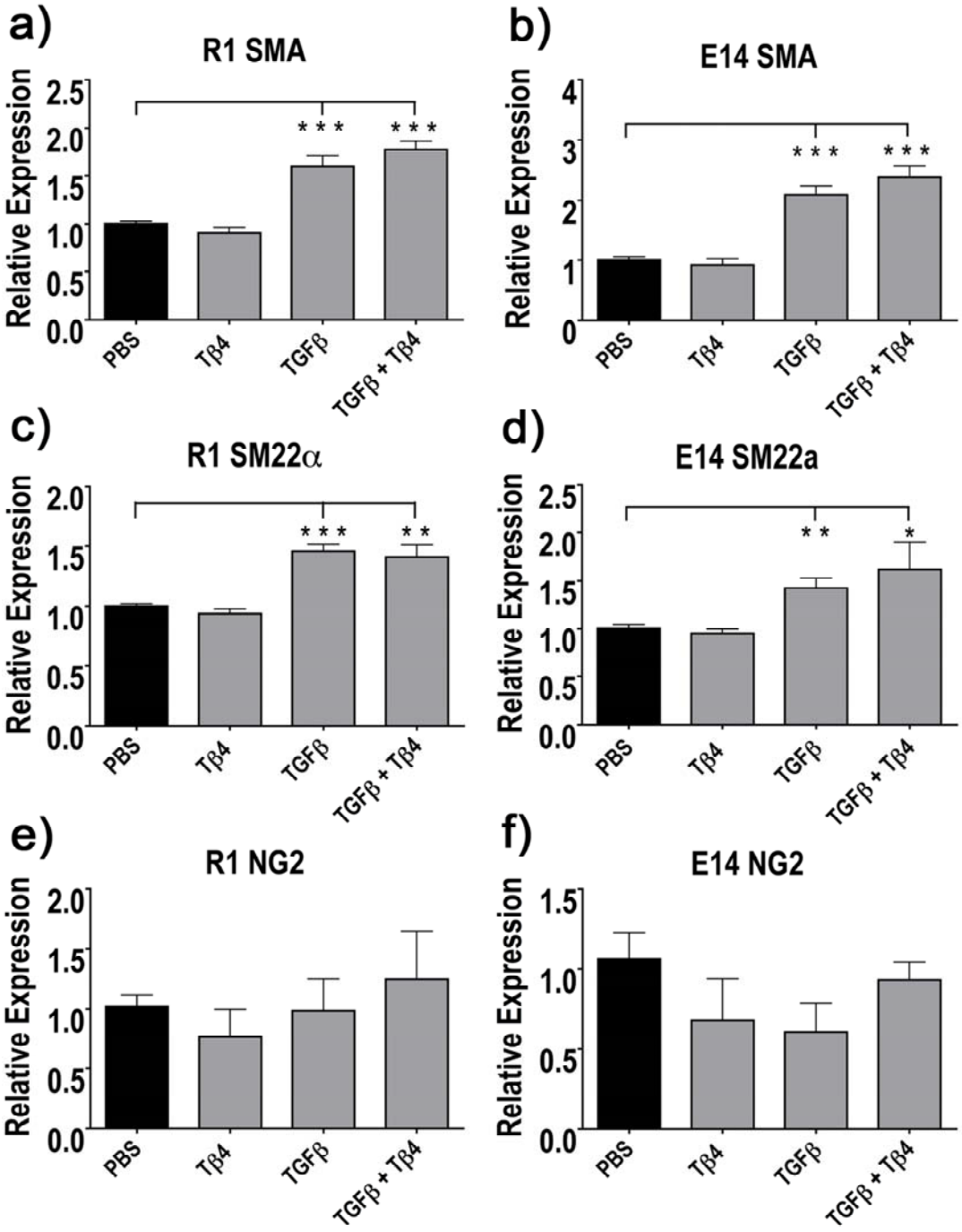


Fig. 6.1
Wild type ES cells express mural cell marker genes under stimulation by TGF-β but not Tβ4

Six day culture of wild type R1 (**a, c and e**) and E14 (**b, d and f**) ES cells in the presence of PBS, 100ng/ml Tβ4, 2ng/ml TGF-β or 2ng/ml TGF-β plus 100ng/ml Tβ4 revealed that TGF-β could induce significant expression of the mural cell markers SMA and SM22α, but not NG2 in ES cells. Tβ4 had no effect on this process. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error bars represent standard error.

6.2.2 Treatment of 10T1/2 Cells with Tβ4 Can Induce the Expression of Mural Cell Markers

Next, the ability of Tβ4 to induce mural cell marker upregulation in 10T1/2 cells was tested. These cells are thought to represent an *in vitro* population of mesodermal progenitor cells. Cells were maintained in their progenitor state under standard culture conditions supplemented with 10% heat inactivated FCS. In order to induce mural cell differentiation, culture medium was replaced with medium that contained only 0.5% FCS supplemented with either 0.1μg/ml Tβ4, 1μg/ml Tβ4, 2ng/ml TGF-β or a combination thereof. TGF-β was included in this assay in order to test whether TGF-β and Tβ4 had additive/synergistic effects on mural cell marker expression. Cells were grown in this differentiating medium for three days, with the medium being replaced each day, before being cultivated for a further three days in maintenance medium. At this point, RNA was extracted. Mural cell marker upregulation was prominent in several of the growth factor combinations tested, but was most consistently upregulated in 10T1/2 cells treated with 0.1μg/ml of recombinant Tβ4. Markers significantly upregulated in this population of cells included the VSMC markers SMA and SM22α, the pan-mural cell marker NG2, and pericyte markers Endosilain, CD13 and Angiopoietin-1 (**Fig. 6.2**).

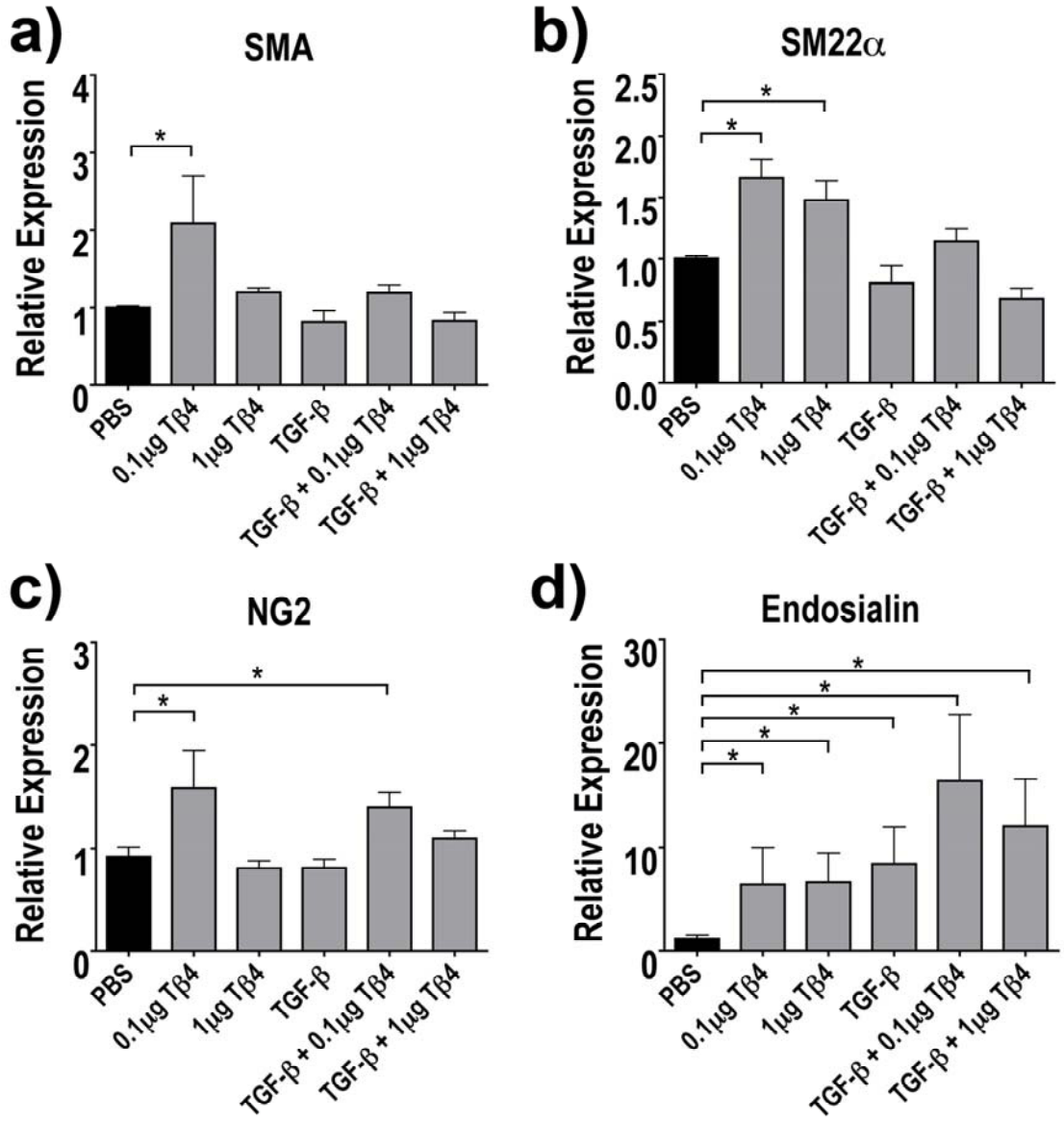


Fig. 6.2(i)
Tβ4 can induce up regulation of mural cell markers in 10T1/2 cells – part 1

10T1/2 cells were cultured in the presence of PBS, 0.1μg/ml Tβ4, 1μg/ml Tβ4, 2ng/ml TGF-β, 2ng/ml TGF-β plus 0.1μg/ml Tβ4 or 2ng/ml TGF-β plus 1μg/ml Tβ4 for six days. qRT-PCR revealed that culture in 0.1μg/ml Tβ4 alone could upregulate expression of the mural cell marker genes SMA (**a**), SM22α (**b**), NG2 (**c**) and Endosialin (**d**). * $p<0.05$. Error bars represent standard error.

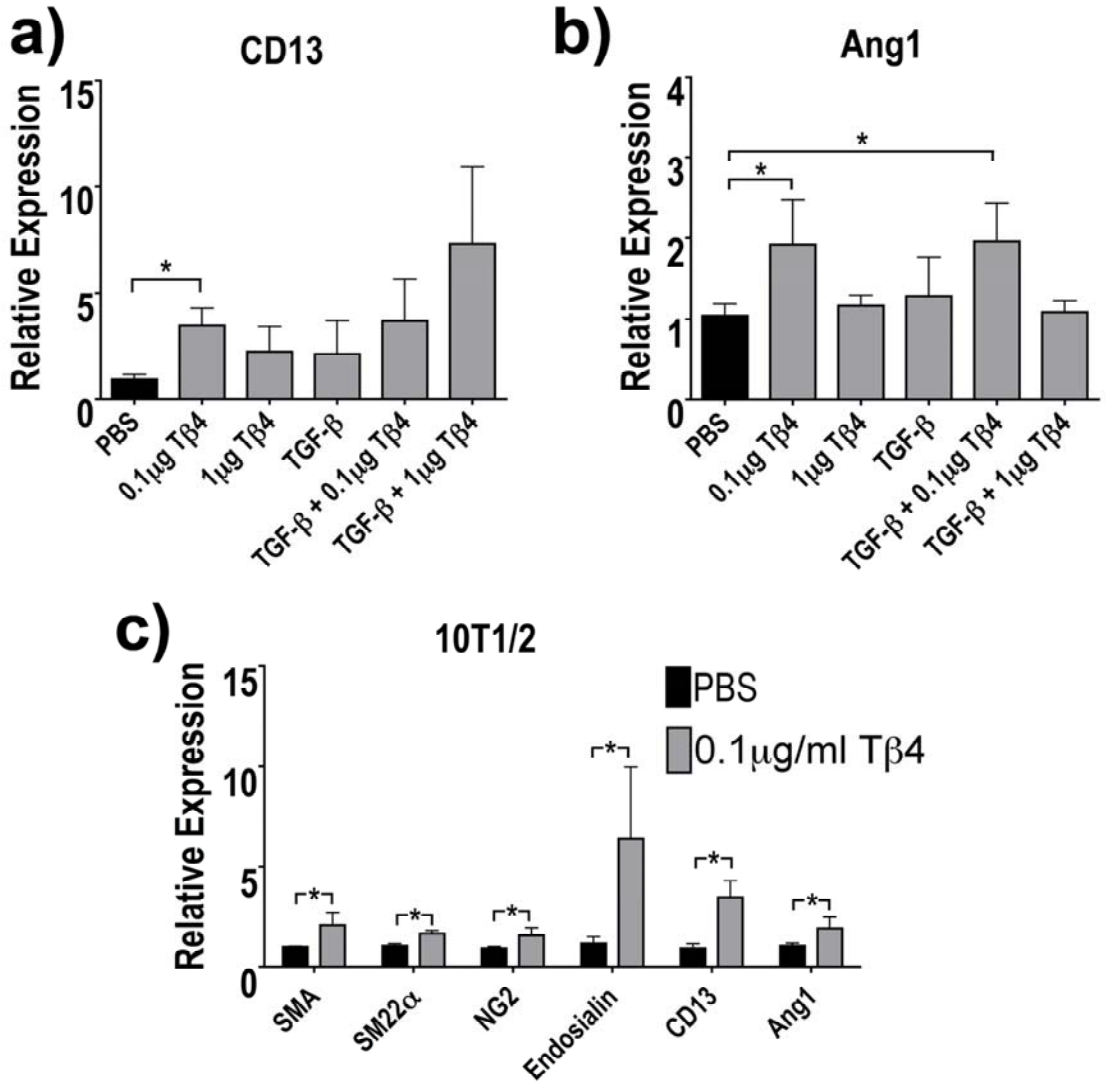


Fig. 6.2(ii)
Tβ4 can induce up regulation of mural cell markers in 10T1/2 cells – part 2

10T1/2 cells were cultured in the presence of PBS, 0.1μg/ml Tβ4, 1μg/ml Tβ4, 2ng/ml TGF-β, 2ng/ml TGF-β plus 0.1μg/ml Tβ4 or 2ng/ml TGF-β plus 1μg/ml Tβ4 for six days. qRT-PCR revealed that culture in 0.1μg/ml Tβ4 alone could upregulate expression of the mural cell marker genes CD13 (**a**) and Ang1 (**b**). (**c**) Summarises the results obtained for PBS treatment and treatment with 100ng/ml Tβ4. * $p<0.05$. Error bars represent standard error.

6.2.3 Treatment of A404 Cells with Tβ4 Can Induce the Expression of Mural Cell Markers

A404 cells were also used as a substrate for Tβ4. Cells were maintained in an undifferentiated state under standard culture conditions. In order to induce mural cell

differentiation, A404 cells were treated with 0.1μg/ml Tβ4, 1μg/ml Tβ4, 1nmol/ml all trans retinoic acid, or a combination thereof or control PBS. Cells were treated for six days, with the culture medium and differentiation stimulus replaced each day. Consistent with previous reports[194], retinoic acid induced the expression of mural cell markers SMA, SM22α and Endosialin by several hundred fold, as compared to PBS treated controls (**Fig. 6.3**). Treating cells with Tβ4 in addition to retinoic acid had no statistically significant effect on the level of mural cell gene expression, over and above that achieved with retinoic acid alone. Retinoic acid did not induce upregulation of all the mural markers tested, with no increase in Angiopoietin-1, NG2 or Desmin being observed. However, treatment with 1μg/ml of Tβ4 alone, even without the addition of RA was able to induce highly significant degrees of mural cell marker expression. This was true for all of the markers tested, with the exception of Desmin.

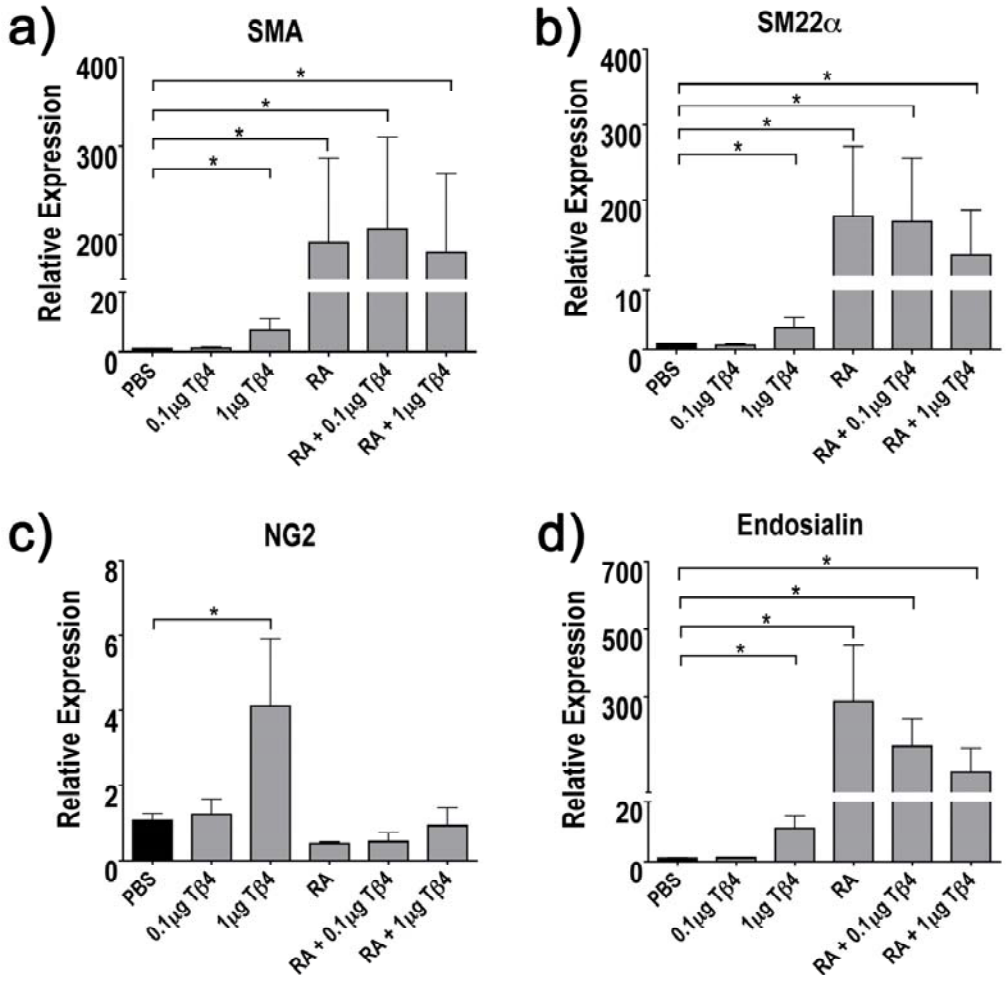


Fig. 6.3(i)
Tβ4 can induce upregulation of mural cell markers in A404 cells – part 1

Culture of A404 cells for six days in the presence of PBS, 0.1μg/ml Tβ4, 1μg/ml Tβ4, 1nmol/ml RA, 1nmol/ml RA plus 0.1μg/ml Tβ4 or 1nmol/ml RA plus 1μg/ml Tβ4 revealed that 1μg/ml Tβ4 could stimulate significant upregulated expression of the mural cell marker genes SMA (**a**), SM22α (**b**), NG2 (**c**) and Endosialin (**d**). * $p<0.05$, ** $p<0.01$. Error bars represent standard error.

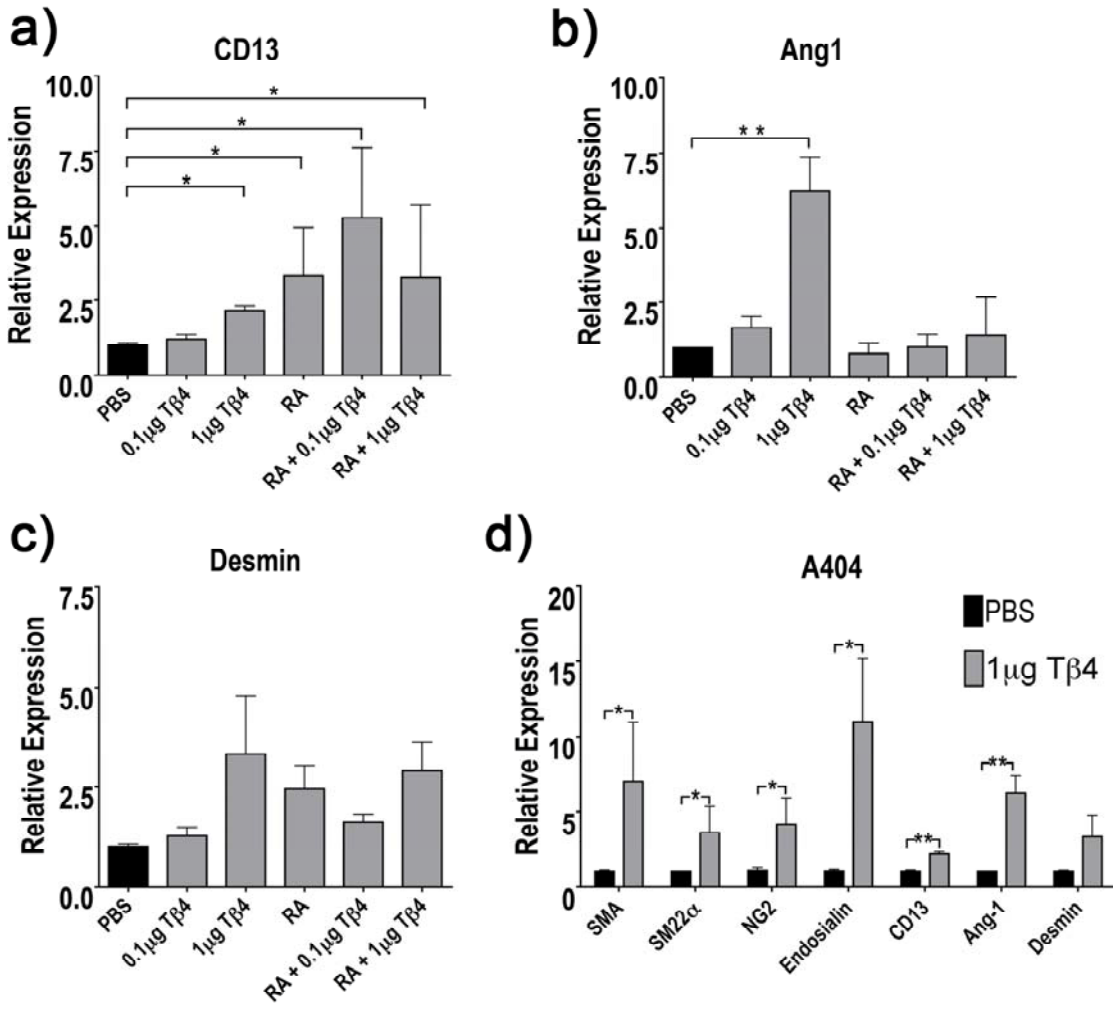


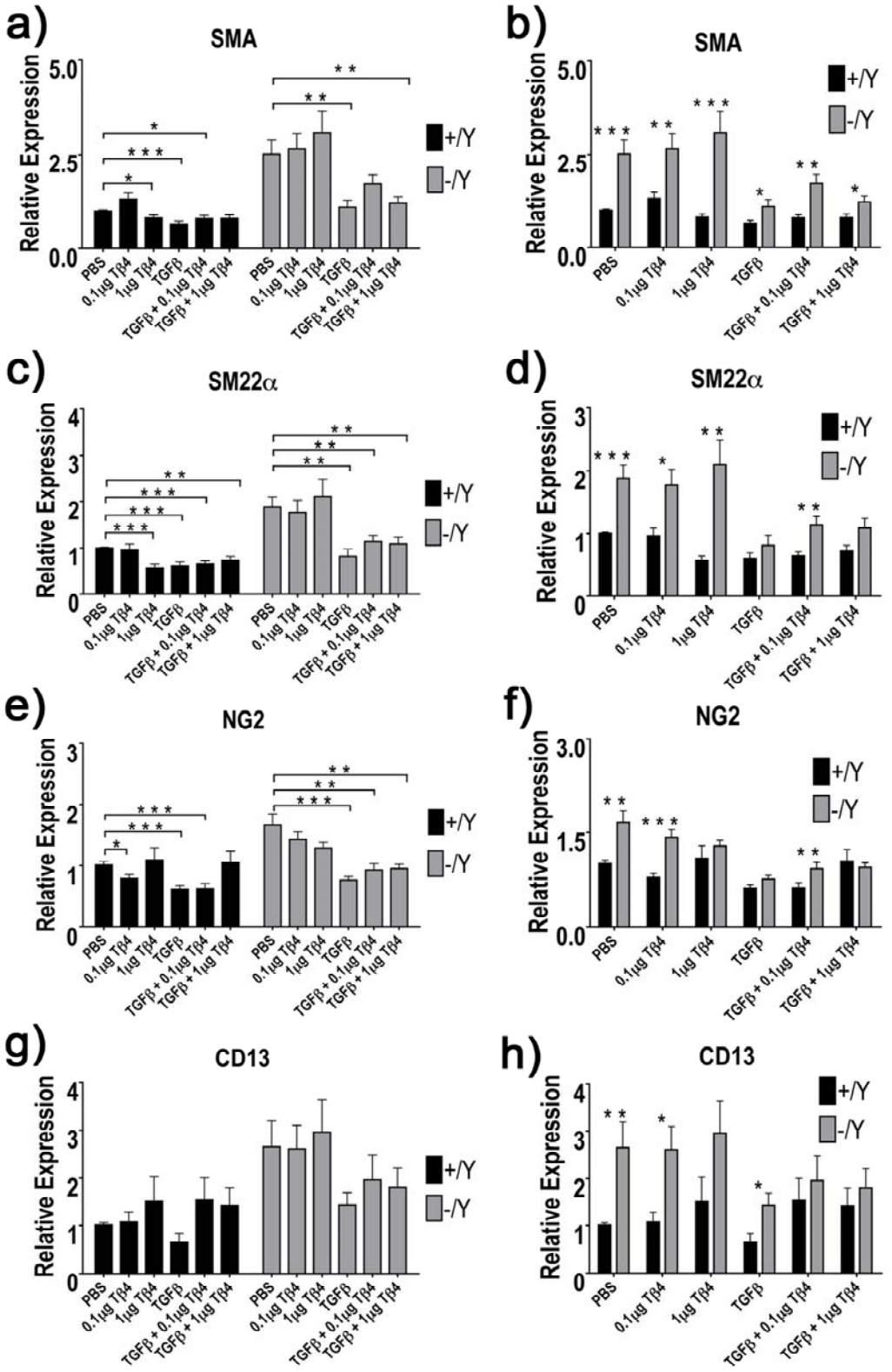
Fig. 6.3(ii)
Tβ4 can induce upregulation of mural cell markers in A404 cells – part 2

Culture of A404 cells for six days in the presence of PBS, 0.1μg/ml Tβ4, 1μg/ml Tβ4, 1nmol/ml RA, 1nmol/ml RA plus 0.1μg/ml Tβ4 or 1nmol/ml RA plus 1μg/ml Tβ4 revealed that 1μg/ml Tβ4 could stimulate significant upregulated expression of the mural cell marker genes CD13 (**a**) and Ang1 (**b**), but not desmin (**c**). (**d**) Summarises the results obtained for treatment of A404 cells with 1,000ng/ml Tβ4 or PBS. * $p<0.05$, ** $p<0.01$. Error bars represent standard error.

6.2.4 Tβ4 Treatment of Tβ4 +/Y and Tβ4 -/Y MEFs Fails to Induce the Expression of Mural Cell Markers

Next, the ability of Tβ4 to induce mural cell marker expression in primary mouse cells rather than a cell line was tested. It was decided to test mouse embryonic fibroblasts (MEFs) as they have been shown to localise to a mural cell typical location

both *in vitro*[193] and *in vivo*[195]. MEFs were isolated from both Tβ4 -/Y and Tβ4 +/Y mice to test if endogenous expression of Tβ4 made any difference to this process. MEF cells were serum starved and treated for six days with 0.1μg/ml Tβ4, 1μg/ml Tβ4, 2ng/ml TGF-β or a combination thereof. Once again, TGF-β was tested to observe whether co-treatment with Tβ4 and TGF-β led to any additive or synergistic upregulations in mural cell markers. Surprisingly, not only was TGF-β generally unable to upregulate any mural cell markers other than Angiopoietin-1, but it actually repressed the expression of mural cell markers such as SMA, SM22a, NG2 and Endosialin, both in Tβ4 +/Y cells and Tβ4 -/Y cells (**Fig. 6.4**). In general, culture with Tβ4, had little, if any effect on MEF mural cell marker expression. Interestingly, and again surprisingly, it appeared that Tβ4 -/Y MEFs expressed mural cell markers at a consistently, significantly higher level than Tβ4 +/Y MEFs.


a)
SMA
Relative Expression
5.0
2.5
0.0
+/Y
-/Y
PBS
0.1μg Tβ4
1μg Tβ4
TGFβ
TGFβ + 0.1μg Tβ4
TGFβ + 1μg Tβ4
b)
SMA
c)
SM22α
d)
SM22α
e)
NG2
f)
NG2
g)
CD13
h)
CD13

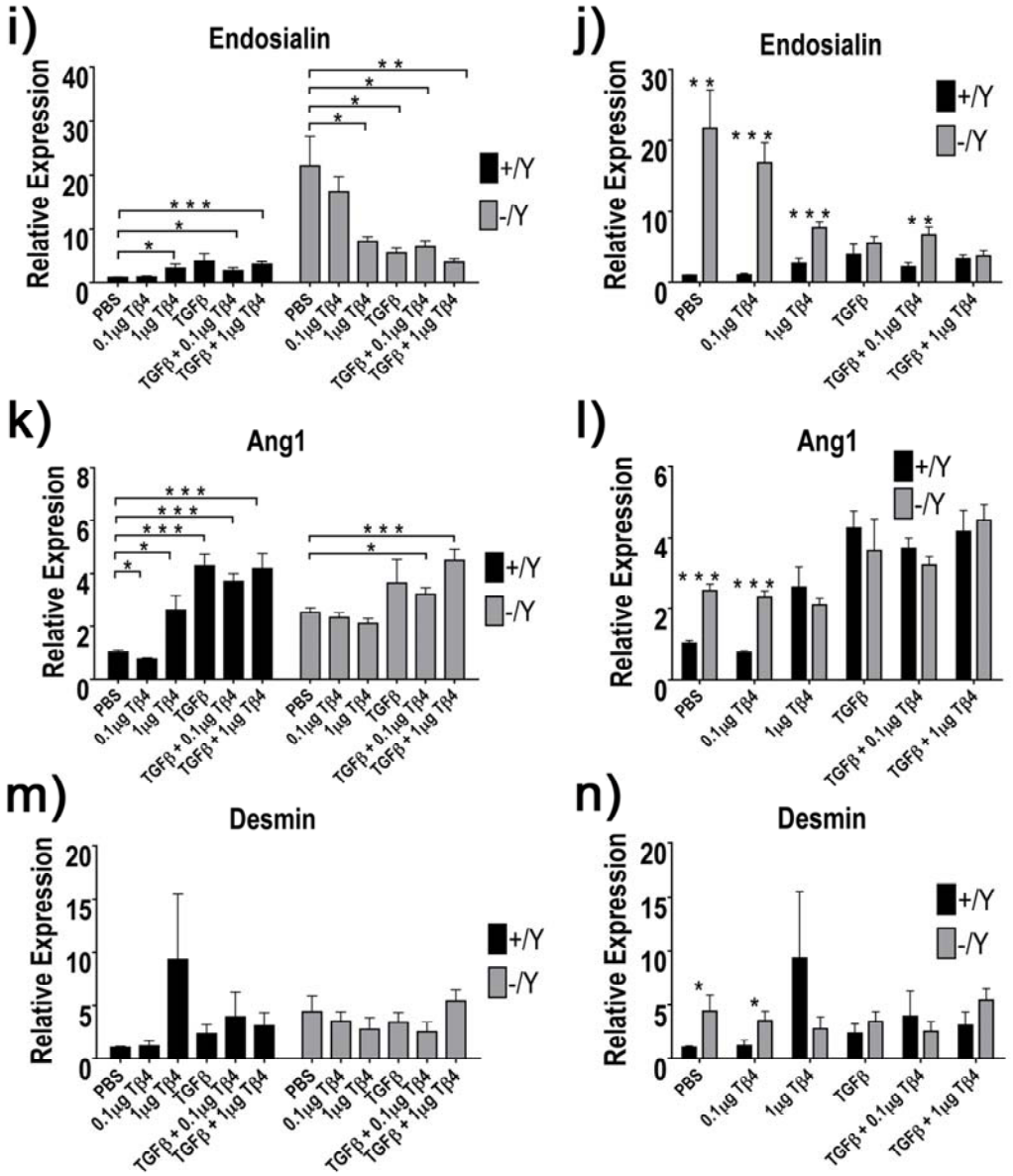


Fig. 6.4
Tβ4 has no effect on the expression of mural cell markers in primary MEFs

qRT-PCR on RNA extracted from Tβ4 +/Y or Tβ4 -/Y MEFs revealed that Tβ4 was not able to induce significant upregulation of the mural cell markers genes SMA (**a and b**), SM22α (**c and d**), NG2 (**e and f**), CD13 (**g and h**), Endosialin (**i and j**), Ang1 (**k and l**) or Desmin (**m and n**) in either cell type. Left hand columns allow comparisons to be made within a cell type. Right hand columns present the same data, but with it arranged to aid appreciation that in nearly all circumstances Tβ4 -/Y MEFs expressed higher levels of mural cell markers than Tβ4 +/Y MEFs. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error bars represent standard error.

6.2.5 Tβ4 Can Stimulate TGF-β Signalling Pathway *In Vitro*

Tβ4 dependent mural cell marker induction had been most robustly observed in 10T1/2 and A404 cells. One explanation for why mural cell marker up regulation was more prominently demonstrated in 10T1/2 and A404 cells, as opposed to ES cells and primary MEFs, could be that they represent an intermediate stage of differentiation more closely resembling the state of undifferentiated mesoderm than either of the other two cell types. As such, these *in vitro* models became the focus of investigation. In the previous chapter, the TGF-β pathway, using Metacore software, was identified as being potentially disrupted in Tβ4 -/Y embryos. It therefore became necessary to assess whether treatment with Tβ4 could activate the TGF-β pathway in 10T1/2 and A404 cell models of Tβ4 induced mural cell differentiation. In order to accomplish this, qRT-PCR was used to quantify the expression levels of three TGF-β responsive transcription factors; PAI-1, Id-1 and c-myc. Treatment of 10T1/2 cells for six days with 0.1μg/ml Tβ4 led to significant induction of Id-1 and c-myc, (**Fig. 6.5a**) whilst treatment of A404 cells with 1μg/ml Tβ4 for six days led to statistically significant upregulation of all three genes studied (**Fig. 6.5b**). These results provided evidence that Tβ4 can stimulate TGF-β pathway activation in *in vitro* models of mural cell progenitor differentiation.

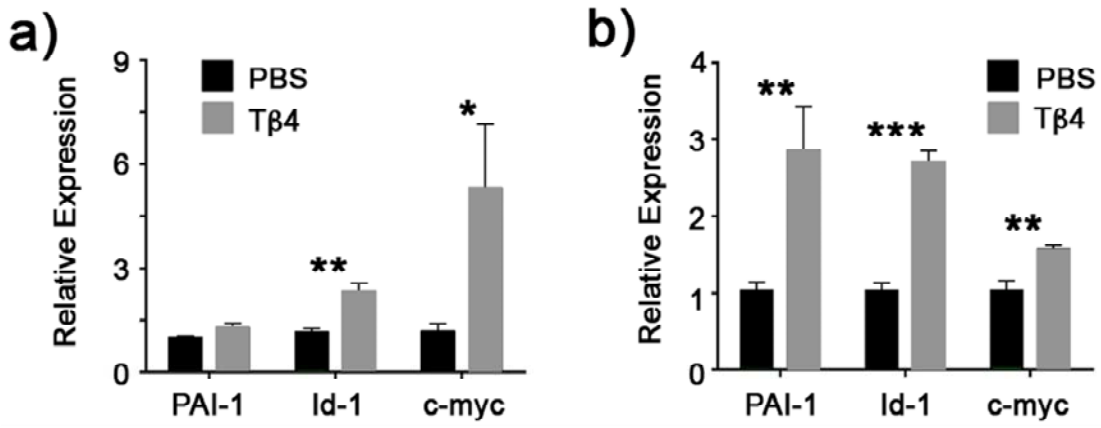


Fig. 6.5
Tβ4 can induce the expression of TGF-β responsive transcription factors in 10T1/2 and A404 cells

qRT-PCR analysis reveals that following six days in culture with 0.1μg/ml Tβ4 for 10T1/2 cells (**a**) and 1μg/ml Tβ4 for A404 cells (**b**), significant upregulation of the TGF-β responsive genes PAI-1, Id-1 and c-myc was observed. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error bars represent standard error.

In order to provide further evidence for the ability of Tβ4 to activate the TGF-β signalling pathway in mural cell progenitors, 10T1/2 cells were transfected with a Smad reporter luciferase construct. This construct permits transcription of firefly luciferase under the control of a Smad binding element. Thus, when the TGF-β pathway is activated, this will lead to transcription of firefly luciferase, the extent of which can be measured via luciferin stimulated luminescence. Treatment for 18 hours with 100ng/ml or 2ng/ml TGF-β induced a statistically significant four fold and six fold increase in luciferase activity over and above that achieved with control PBS (**Fig. 6.6**). Interestingly, treatment with a combination of 0.1μg/ml Tβ4 plus 2ng/ml TGF-β significantly increased luciferase activity to a higher level than that achieved with Tβ4 of TGF-β alone, demonstrating an additive effect. These data conclusively show that treatment of 10T1/2 cells with Tβ4 stimulates an up regulation of TGF-β pathway activity.

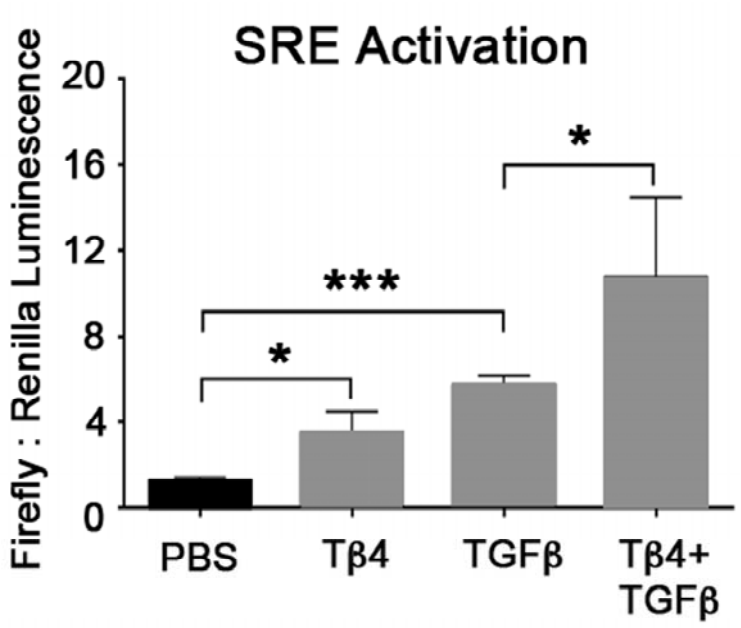


Fig 6.6
Tβ4 can stimulate the activity of a Smad responsive luciferase reporter

Treatment of 10T1/2 cells for 18 hours with 0.1μg/ml Tβ4 or 2ng/ml TGF-β could significantly upregulate activity of a Smad responsive luciferase reporter to a level above that achieved with PBS alone. Treatment of the cells with both 0.1μg/ml Tβ4 plus 2ng/ml TGF-β could increase the levels of Smad reporter activity significantly higher than that achieved with 2ng/ml TGF-β alone. * $p<0.05$, *** $p<0.001$. Error bars represent standard error.

6.2.6 Tβ4 Stimulated TGF-β Pathway Activation Correlates with Increased Smad Phosphorylation

Having established that Tβ4 could induce TGF-β pathway activity at the level of transcriptional outputs, further mechanistic insight into how Tβ4 was accomplishing this was sought. One possibility, which was considered, was that Tβ4 was somehow stimulating the expression of TGF-β signalling pathway components, such as TGF-β receptors. This, in theory, could lead to pathway activation by allowing TGF-β secreted cell autonomously by the 10T1/2 cells themselves to have a greater effect. This could also explain the additive effects of Tβ4 on TGF-β induced luciferase expression in the previous experiment. Thus, cells treated with 0.1μg/ml Tβ4, 2ng/ml

TGF-β, or a combination thereof were analysed by qRT-PCR for expression of TGF-β receptors. Levels of neither Alk-1 (**Fig. 6.7a**) nor Alk-5 (**Fig. 6.7b**), increased with any of the treatments tested. Although treatment of 10T1/2 cells with TGF-β, induced upregulation of the TGF-β RII, treatment with Tβ4 alone had no discernible effect on expression levels (**Fig. 6.7c**). Furthermore, addition of Tβ4 to TGF-β had no greater effect on TGF-β RII upregulation than TGF-β alone. This indicates that Tβ4 mediated activation of the Tβ4 signalling pathway is not likely to arise due to increased TGF-β receptor expression.

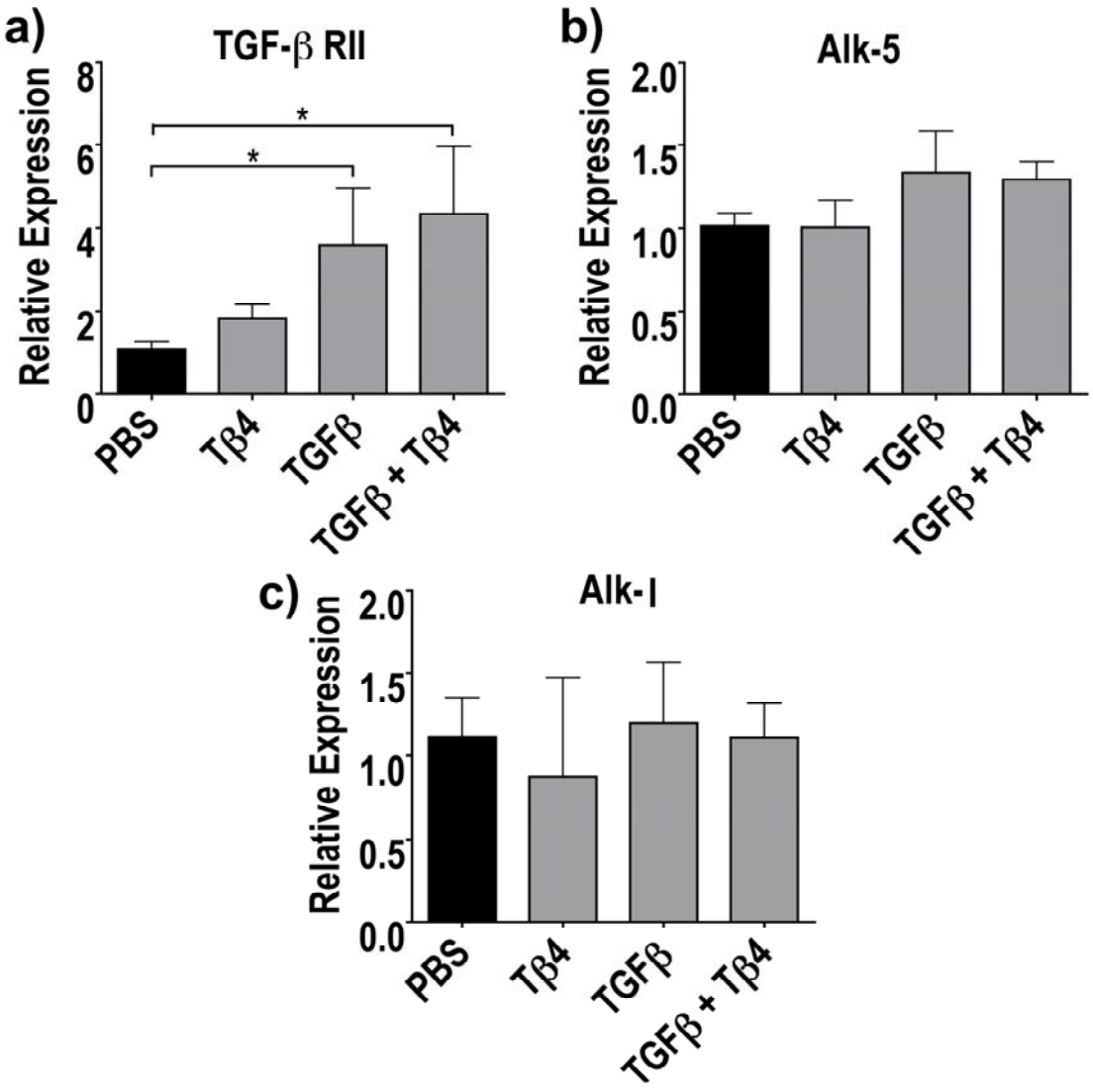


Fig. 6.7
Tβ4 cannot up regulate the expression of TGF-β receptors in 10T1/2 cells

Treatment of 10T1/2 cells for 6 days with PBS, 0.1μg/ml Tβ4, 2ng/ml TGF-β or 2ng/ml TGF-β plus 100ng/ml revealed that treatment with Tβ4 alone could not significantly upregulate mRNA expression levels of the TGF-β receptors TGF-β RII (**a**), Alk-5 (**b**) or Alk-1 (**c**) compared to treatment with PBS as measured by qRT-PCR. * $p<0.05$. Error bars represent standard error.

One of the genes identified by gene array screening as being upregulated in E12.5 Tβ4 -/Y embryos was Usp9x. This gene codes for a deubiquitinase enzyme. Often, *in vivo*, Smad 4 is monoubiquitinated at lysine residue 519. This inhibits TGF-β signalling by preventing physical interaction between Smad 4 and phospho-Smad 2. Usp9x acts as a positive modulator of TGF-β signalling by maintaining Smad 4 in its

deubiquitinated form[81]. Thus it was hypothesised, that Tβ4 might exert its effects on the TGF-β pathway by stimulating increased expression of Usp9x in 10T1/2 cells, thereby making cells more responsive to cell autonomous or exogenous TGF-β signalling. 10T1/2 cells were stimulated for six days with 0.1μg/ml Tβ4, 2ng/ml TGF-β, or a combination thereof and levels of Usp9x measured by qRT-PCR (**Fig. 6.8**). Studies disclosed that treatment with TGF-β could stimulate increased Usp9x, presumably as part of a positive feedback signalling loop, but that Tβ4 could not do the same. Combination treatment with TGF-β plus Tβ4 did not stimulate Usp9x expression beyond that of TGF-β alone. Thus, it is unlikely that Tβ4 exerts its effects on the TGF-β pathway by stimulating increased expression of Usp9x.

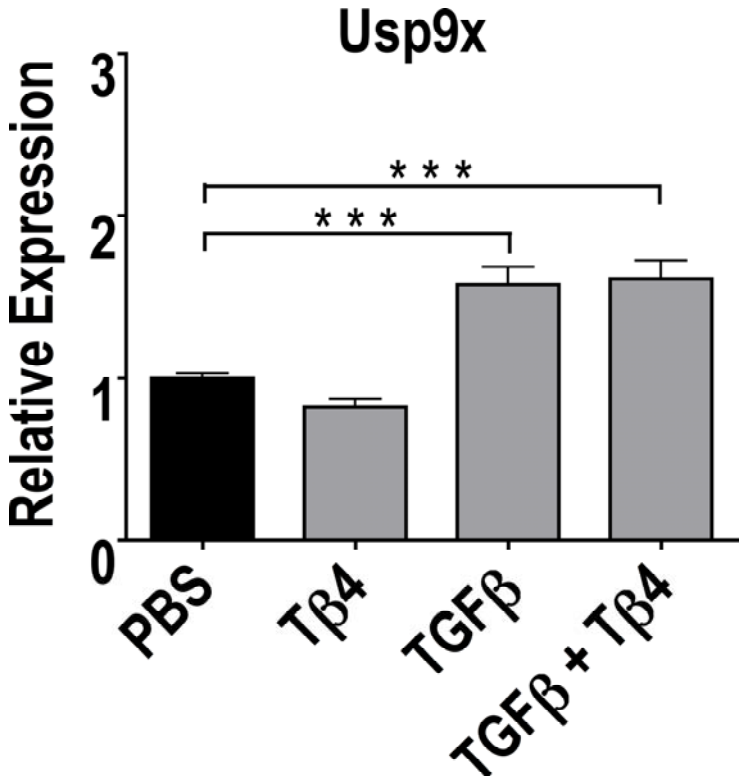


Fig. 6.8
Tβ4 cannot up regulate the expression of Usp9x in 10T1/2 cells

qRT-PCR revealed that treatment of 10T1/2 cells for six days with 2ng/ml TGF-β or 2ng/ml TGF-β plus 0.1μg/ml Tβ4 could upregulate levels of Usp9x mRNA higher than that achieved by PBS treatment. However, treatment with 0.1μg/ml Tβ4 could not achieve this. ** $p<0.01$, *** $p<0.001$. Error bars represent standard error.

An alternative mechanistic explanation for Tβ4 induced TGF-β pathway activation is that Tβ4 acts directly to activate TGF-β receptors, leading to increased Smad phosphorylation and pathway stimulation. In order to test this, 10T1/2 cells were serum starved overnight and treated for 30 minutes with control PBS, 0.1μg/ml Tβ4, 2ng/ml TGF-β, or 0.1μg/ml Tβ4 plus 2ng/ml TGF-β. Protein was rapidly extracted from these cells and western blotting conducted for activated phospho-Smad complexes (**Fig. 6.9**). It is typically thought that the type1 TGF-β receptor Alk-5 signals via phosphorylation of Smads 2 and 3, whilst the type 1 TGF-β receptor Alk-1 signals via phosphorylation of Smads 1, 5 and 9[163]. It was observed that whilst treatment with Tβ4 alone could not stimulate Smad2 or Smad1/5 phosphorylation,

treatment of cells with TGF-β plus Tβ4 could stimulate both Smad2 and Smad1/5 phosphorylation to a level greater than that achieved by TGF-β alone. This implies, that Tβ4 acts cooperatively with TGF-β to induce Smad phosphorylation and TGF-β pathway activation.

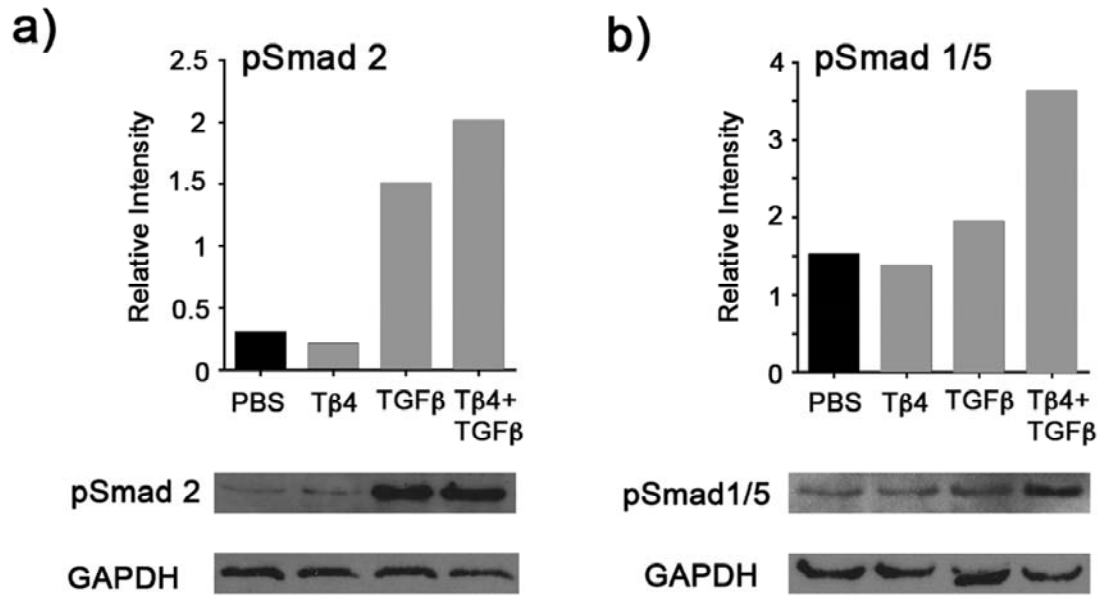


Fig. 6.9
Tβ4 co-operates with TGF-β to stimulate Smad phosphorylation

Western blotting followed by quantification with scanning densitometry demonstrated that although treatment of 10T1/2 cells for 30 minutes with 0.1μg/ml Tβ4 alone could not upregulate levels of phospho-Smad2 (**a**) or phospho-Smad1/5 (**b**) greater than that achieved with PBS treatment, treatment with a combination of 0.1μg/ml Tβ4 plus 2ng/ml TGF-β could induce phospho-Smad2 and phospho-Smad1/5 at levels higher than that achieved by treatment with 2ng/ml TGF-β alone.

6.2.7 Tβ4 Can Stimulate the Activity of the TGF-β Pathway *In Vivo*

In order to attempt to assess whether the effects of Tβ4 on TGF-β pathway activation *in vitro* held any relevance *in vivo*, RNA derived from somite matched pairs of E10.5 Tβ4-/Y and Tβ4 +/Y embryos were assayed by qRT-PCR for levels of the TGF-β responsive transcriptional targets PAI-1, Id-1 and c-myc (**Fig. 6.10**). In all cases

tested, Tβ4 -/Y embryos expressed significantly lower levels of these three TGF-β responsive transcriptional targets than somite matched Tβ4 +/Y controls. This is good evidence that TGF-β signalling is diminished in E10.5 Tβ4 -/Y embryos *in vivo*.

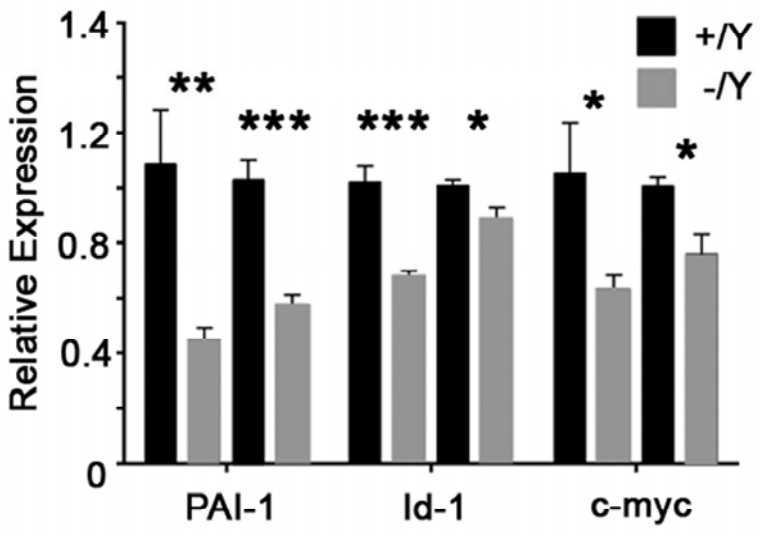


Fig. 6.10
E10.5 Tβ4 -/Y embryos display a reduced level of TGF-β target genes *in vivo*

qRT-PCR on RNA extracted from somite matched pairs of E10.5 Tβ4 +/Y and Tβ4 -/Y embryos demonstrated that Tβ4 -/Y embryos express significantly lower levels of the Smad responsive genes PAI-1, Id-1 and c-myc. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Error bars represent standard error.

6.3 Discussion

6.3.1 Summary

In order to investigate whether Tβ4 could stimulate the differentiation of progenitors into mature mural cells, a number of *in vitro* models of mural cell differentiation were explored. A number of cell types were treated with Tβ4 and the induction of mural cell markers assessed by qRT-PCR. Wild type ES cells were stimulated to differentiate through the withdrawal of LIF and grown to confluence. Tβ4 had no

effect on the expression of mural cell markers under this protocol but treatment with TGF-β resulted in up regulation of SMA and SM22α. 10T1/2 cells represent a mesodermal progenitor cell line, whilst A404 cells are P19 embryonal carcinoma cells, clonally selected for their ability to differentiate into VSMCs. Upon serum starvation and subsequent treatment with Tβ4, both of these cell types expressed a wide range of mural cell markers at significantly higher levels than control PBS treated cells. This indicates that Tβ4 can stimulate differentiation of progenitor cells into mature mural cells. Primary MEFs were also isolated from E14.5-16.5 Tβ4 -/Y and Tβ4 +/Y embryos. These cells did not modulate their expression of mural cell marker genes in response to Tβ4, and surprisingly down regulated the expression of mural cell markers when exposed to TGF-β. Also, somewhat surprisingly, Tβ4 -/Y MEFs expressed higher levels of mural cell marker genes than Tβ4 +/Y MEFs.

In order to further understand the molecular mechanism behind Tβ4 stimulated induction of mural cell markers in 10T1/2 and A404 cells, and to assess whether bioinformatic evidence of TGF-β pathway abnormalities in the Tβ4 -/Y mouse had any relevance to this process, the levels of TGF-β pathway transcriptional targets were quantified in Tβ4 stimulated 10T1/2 and A404 cells. It was observed that treatment with Tβ4, did indeed stimulate expression of TGF-β target genes in these cells. Transfection of A404 cells with a luciferase Smad activity reporter construct revealed that treatment of 10T1/2 cells with exogenous Tβ4 could directly stimulate Smad reporter activity – an effect that was additive with TGF-β. Altered expression levels of TGF-β receptors or the Smad4 deubiquitinating enzyme Usp9x could not account for the TGF-β pathway stimulatory effects of Tβ4. Co-treatment of 10T1/2 cells with Tβ4 and TGF-β was shown to induce higher levels of Smad2 and Smad 1/5

phosphorylation than treatment with Tβ4 alone. Finally, it was observed that the expression levels of TGF-β pathway transcriptional targets were reduced in E10.5 Tβ4 -/Y embryos compared to somite matched Tβ4 +/Y controls.

6.3.2 Effects of Tβ4 on ES Cell Differentiation

In keeping with previously published studies[92], TGF-β supplementation of ES cell differentiating medium was able to induce expression of the mural cell markers SMA and SM22α in wild type differentiating ES cells. However, treatment of these ES cells with Tβ4, either alone or in combination with TGF-β did not have any inductive effect on mural cell markers. One explanation for why Tβ4 had no effect on ES cells, but did act as a stimulus for mural cell differentiation in other cell types such as 10T1/2 and A404 cells, could be that the differentiating ES cell represents a cell population that exists in too undifferentiated a state, to provide an accurate model for the process of *in vivo* mural cell induction. It is possible that Tβ4 acts *in vivo* to induce differentiation from mesodermal progenitors and that some cellular differentiation/specification already needs to be in place, for Tβ4 induced mural cell differentiation to occur. TGF-β may be able to exert its effects on a less differentiated substrate both *in vitro* and *in vivo*.

These experimental data can be placed in the context of previously published observations. Smart et al. noted that embryoid bodies derived from ES cells deficient for the basic helix-loop-helix transcription factor Hand1, had diminished expression of SMA. However, this deficiency could be rescued by the addition of Tβ4 to the culture medium[51]. This would imply that under certain circumstances Tβ4 can induce

mural cell marker expression in differentiating ES cells – although this has not, as yet, been shown in wild type ES cells.

Differences in these two studies might be explained through analysis of the differentiation protocols that were used. Smart et al. cultured ES cells as embryoid bodies before dispersing them and re-culturing them in differentiating medium for 16 days. This might have two possible consequences. First of all, the effect of embryoid body aggregation followed by prolonged re-culture might serve as a significantly stronger signal to differentiation than the protocol of monolayer culture for 6 days used here. This could mean that Tβ4 only effects ES cell differentiation at a time point later than the 6th day or that the Tβ4 differentiation signal needs to be placed in the context of other, stronger signals to differentiation, in order to exert its effect.

The second possibility is that ES cells which have been cultured as embryoid aggregates represent a partially differentiated population of cells, some of which are already lineage committed to the mesoderm. Thus, they may represent a more viable substrate for Tβ4 induced mural cell differentiation than truly totipotent ES cells. In order to resolve these disparities, further work could be conducted in which ES cells were cultured as a monolayer for a prolonged period – a strategy that has yielded mural cell differentiation for workers researching other aspects of this process[201]. Alternatively, wild type ES cells could be cultured as embryoid bodies before being cultured in Tβ4 supplemented differentiation medium. In order to resolve the question of whether Tβ4 can only influence this process in cells, which are mesoderm lineage committed, clonal analyses could be preformed on cells with or without the expression of mesodermal markers such as Brachyury (T).

6.3.3 Effect of Tβ4 on MEF Phenotype

Although, Tβ4 was able to upregulate the expression of mural cell markers in 10T1/2 and A404 cells, surprising results were obtained when mural differentiation of MEFs was examined. Apart from Tβ4 being unable to consistently, significantly induce mural cell marker genes in this population, the first strange observation is that TGF-β has a repressive effect on mural cell marker expression in these cells. The experimental basis for using these cells as a substrate for mural cell differentiation, is work, which showed that they could localise to the wall of ES cell derived vascular sprouts *in vitro*[193]. Furthermore, these cells could localise to the blood vessel walls of tumours when co-injected with T241 tumour cells into the tail veins of mice[195]. However, unlike ES, 10T1/2 and A404 cells, MEFs have never been shown before to induce mural cell marker upregulation upon TGF-β treatment. In fact, TGF-β treatement of MEFs causes them to adopt a synthetic and proteolytic phenotype, much more consistent with the typical phenotypic profile of activated fibroblasts rather than vascular mural cells[202,203]. Thus it is unclear, whether MEF cell culture represents a robust, physiologically relevant model of mural cell development. An alternative explanation is that mural cells can be recruited from different cell pools during development, and that TGF-β responsive mural cell progenitors represent a different set of cells than that which the MEF model represents.

The second surprising observation encountered in the MEF model of mural cell differentiation, was that Tβ4 -/Y MEFs express higher levels of mural cell marker genes, in general than Tβ4 +/Y MEFs. This observation is puzzling due to the fact

that, established in chapter 3, Tβ4 -/Y embryos as a whole express lower levels of mural cell markers. One explanation for this may lie in the fact that markers of mural cells and of myofibroblasts may overlap[204]. Experimental data have shown that one route of origin of myofibroblasts is via the differentiation of MEFs[205]. It has also been demonstrated that incisional wounds in rats attract fewer myofibroblasts, when treated with Tβ4[206]. Thus, it is possible that Tβ4 may repress the differentiation of myofibroblasts, thus enabling excess myofibroblast differentiation from MEFs in Tβ4 -/Y mice and consequent higher expression overlapping mural cell/myofibroblast markers. One possible way of determing if this is the case, would be to analyse the Tβ4 -/Y and Tβ4 +/Y MEFs for the expression of markers, which are thought to be exclusively expressed by myofibroblasts, such as vimentin and fibronectin[204].

Thus, there are great difficulties in interpreting the data from MEF culture experiments, given that the phenotype of these cells in unclear due to their lack of TGF-β responsiveness. As such, at this time, it seems prudent to discard any notion that these cells are a suitable model, for the process of mural cell development from uncommitted mesodermal progenitor cells,.

6.3.4 Influence of Tβ4 on Alk-1/Alk-5 Signalling

Typically, expression of the molecule PAI-1 is thought to be the result of TGF-β binding to the Alk-5 receptor, causing subsequent downstream activation of the Smad2/3 signalling cascade. Meanwhile, stimulation of the Alk-1 receptor results in phosphorylation of Smad1/5/9 signalling components and subsequent transcription of the gene Id-1[207]. TGF-β signalling also has the ability to repress or induce expression

of the molecule c-myc dependent on the cell type being studied[164,165]. Induction of all three of these TGF-β pathway marker genes was observed in Tβ4 treated 10T1/2 and A404 cells. Furthermore, all three of these genes were observed to be downregulated in E10.5 Tβ4 -/Y mice. This indicates that Tβ4 may be exerting an influence on both the Alk-1 and the Alk-5 TGF-β signalling pathways. Further evidence for such an effect came from the observations that combination treatment with TGF-β and Tβ4 could lead to both Smad2 and Smad1/5 phosphorylation to a level, greater than that achieved by treatment with TGF-β alone. Though, such findings should be qualified by recognition that differences in Smad2 and Smad1/5 phosphorylation between TGF-β treated and Tβ4 plus TGF-β treated cells are small and come from only a single experiment.

Such findings have implications for understanding how the ability of Tβ4 to stimulate mural cell differentiation, when added exogenously to cells *in vitro*, applies to the situation *in vivo*. Mouse knockouts of both Alk-1[85,208] and Alk-5[87,93] embryonically lethal at midgestation due to vascular defects. Here, 10T1/2 cells are used as an *in vitro* surrogate for mesodermal mural progenitor cells. Reports concerning the expression patterns of Alk-1 and Alk-5 *in vivo* are contradictory. Some researchers maintain that Alk-1 is expressed in a multitude of tissues, including cells of the vasculogenesis competent mesenchyme[209], whilst others report that it is expressed very specifically in developing vascular endothelial cells[87]. Thus, it is difficult to fully appreciate the consequence of Alk-1 pathway upregulation in 10T1/2 cells for mural cell differentiation *in vivo.*

Causing further confusion, are contradictory reports regarding the expression of Alk-5. Some studies indicate that that Alk-5 expression is not present in the endothelium and is predominantly expressed in mural cells and the surrounding mesenchyme[87], whilst others demonstrate its endothelial expression and the vascular defects which result as a consequence of its endothelial specific knockout[93].

It is therefore unclear whether the ability of Tβ4 to stimulate Alk-5 pathway activation in 10T1/2 cells is more applicable to an *in vivo* phenomenon of endothelial to mural cell progenitor signalling, or a defect in endothelial cell autonomous signalling. The key facts to be appreciated, when reaching a conclusion in this matter, are that 10T1/2 cells are more indicative of mesodermal progenitors than mature endothelial cells[210], and that Tβ4's effects on mural cell differentiation occur when Tβ4 is added exogenously. Thus, it can be argued, that the simplest interpretation of these experiments is that they offer evidence that Tβ4 secreted by endothelial cells can act as a paracrine factor to stimulate the differentiation of mesodermal progenitors into mature mural cells via activation of the Alk-5 (and possibly Alk-1) pathway.

However, defective endothelial cell autonomous Tβ4 signalling (via Alk-1 or Alk-5) has not yet been unequivocally ruled out by these experiments. In order to do this, a number of further experiments could be undertaken.

One avenue of inquiry could take the form of endothelial cell and 10T1/2 cell co-culture experiments. Using this established technique[89], endothelial cells could be isolated from Tβ4 +/Y and Tβ4 -/Y embryos via magnetic bead separation, before being co-cultured with 10T1/2 cells. The endothelial cells can be kept separate from

the 10T1/2 cells through the use of transwell plates which allow diffusible factors to pass from one cell population to the other but inhibit direct cell to cell contact. If Tβ4 +/Y endothelial cells were able to induce mural cell differentiation of 10T1/2 cells under these conditions, but Tβ4 -/Y endothelial cells were not, then this would provide even more compelling evidence that loss of Tβ4 in endothelial cells causes reduced secretion of a trophic factor, resulting in impaired mural cell differentiation. Blunting of mural cell differentiation of 10T1/2 cells when co-culured with wild type endothelial cells, in response to administration of an α-Tβ4 antibody would provide further evidence that this trophic factor is Tβ4.

Further insight into whether signalling, through either Alk-1, Alk-5 or both receptors, is critical for mural cell marker gene induction in 10T1/2 cells, could also be gleaned through loss of function experiments. In the standard *in vitro* model of Tβ4 induced 10T1/2 cell differentiation, components of the Alk-1 and Alk-5 signalling pathways could be knocked down via transfection with siRNA, and any impairments in mural cell differentiation noted. Another approach would be to use specific chemical inhibitors of each TGF-β pathway such as the Alk-1 specific inhibitor RAP-041[211] and the Alk-5 specific inhibitor SB-431542[212].

6.3.5 Evidence for an *In Vivo* Defect in TGF-β Signalling

One possible criticism of the experiments reported in this chapter, is that qRT-PCR analysis of TGF-β target genes in somite matched pairs of E10.5 Tβ4 +/Y and Tβ4 -/Y embryos, is insufficient evidence to claim the existence of an unequivocal defect in vascular TGF-β signalling *in vivo*. Further evidence for this proposition comes

from work recently performed by Nicola Smart in Paul Riley's lab. She has demonstrated, through immunofluorescent analysis of sections through E10.5 Tβ4 -/Y aortas that mural cells in the Tβ4 -/Y embryo reside in two distinct populations. In E10.5 Tβ4 -/Y embryos, which display a reduced degree of mural cell coverage of the developing aorta, levels of phospho-Smad2 are significantly down regulated in peri-aortic mural cells compared to Tβ4 +/Y controls. However, in Tβ4 -/Y embryos, which display a normal number of mural cells around their dorsal aortas, Smad2 phosphorylation is slightly, but significantly up regulated in comparison to Tβ4 +/Y controls. These data, not only provide compelling evidence of a role for Tβ4 in regulation of mural cell TGF-β signalling *in vivo*, but also help to explain the incompletely penetrant mural cell phenotype in Tβ4 -/Y embryos, as they indicate that compensatory mechanisms exist to up regulate TGF-β signalling in the absence of Tβ4.

An alternative method, which would provide additional evidence for this conclusion, would be to perform laser capture microdissection on sections through E10.5 Tβ4 -/Y and Tβ4 +/Y embryos. qRT-PCR could then be conducted on amplified RNA collected from these samples, to determine whether the decrease in TGF-β target gene expression observed in material from whole Tβ4 -/Y embryos was due to a decrease in vascular TGF-β pathway activity.

6.3.6 The Mechanism of Tβ4 Induced Smad Phosphorylation

The precise mechanism underlying the ability of Tβ4 to induce higher levels of phosphorylated Smad2 and Smad1/5 in 10T1/2 cells, which were cultured with

TGF-β and Tβ4 as compared to TGF-β alone, is not as yet clear. One possibility is that Tβ4 interacts directly with TGF-β or with one of the TGF-β receptors and forms a complex that increases the affinity of the receptor for the ligand. Such a hypothesis could be explored by performing co-immunoprecipitation assays, to test whether a physical interaction can occur between Tβ4 and either the TGF-β1, Alk-1, Alk-5 or TGF-β RII molecules. Further work could involve performing surface plasmon resonance assays[213] on the relevant molecules, to test whether binding of Tβ4 to either the TGF-β ligand or the TGF-β receptors could alter the affinity of the ligand-receptor interaction.

In these experiments, the somewhat paradoxical observation was made that treatment with Tβ4, in the absence of TGF-β, could not stimulate phosphorylation of Smad2 or Smad1/5, above the level caused by PBS treatment, and yet Tβ4 alone could stimulate TGF-β pathway activation as measured by a Smad activity luciferase reporter. This observation suggests that Tβ4 may exert other effects on the TGF-β signalling cascade than merely receptor or ligand interaction. In relation to this, one striking observation has come from a co-worker in Paul Riley's group, who conducted a yeast two hybrid screen for molecular interactors of Tβ4 (Karina Dube). From this work, it appears likely that Tβ4 posesses an *in vivo* physical interaction with a molecule known as Ski-interacting protein (Skip). Smad2 and Smad3 can both interact with the c-Ski oncoprotein. When a Smad-Ski complex is formed, it attracts a nuclear co-repressor complex and transcription of Smad target genes is repressed[214]. However, Skip can compete with Ski for the Ski binding site on Smad proteins, and by preventing Ski mediated repression can serve as a co-activator for TGF-β signalling.

Thus, one plausible hypothesis is that Tβ4 can bind to Skip, enhancing its ability to act as a Smad activator and increasing the gain of the signalling system.

This could be tested in several ways. One approach would be to knockdown Skip in 10T1/2 cells by siRNA transfection, with a view to testing whether this could abolish Tβ4 stimulation of mural cell differentiation. Subsequently, cells could be transfected with a dominant negative version of the Skip protein, which would in theory be able to bind Tβ4 but could no longer act as a Smad co-activator. This experiment would demonstrate that a functional Tβ4-Skip-Smad interaction was necessary for Tβ4 dependent mural cell induction.

7 The Role of Tβ4 in Physiological Angiogenesis

7.1 Introduction

Up until this point, the focus of investigation in this study, with regards to the role of Tβ4 in the developing vasculature has been guided by the haemorrhagic phenotype and mural cell defects observed in Tβ4 -/Y embryos. However, as of yet, no attempt has been made to evaluate a potential role for Tβ4 in the process of angiogenesis, from *in vivo* genetic loss of function models.

Such an approach seems appropriate, given the volume of research, which has been published on the role of Tβ4, both as a critical intracellular mediator[43] and as an extracellular stimulus of angiogenesis[215].

The process of angiogenesis comprises a series of highly ordered cellular events, which result in the formation of new blood vessels. During physiological angiogenesis, blood vessel sprouting into an, as yet, unvascularised tissue, is led by endothelial tip cells. These tip cells have a specialised cytoskeletal structure. They possess many veil-like membranous protrusions known as lamellipodia. Out of these, arise thin filamentous processes known as filopodia, which project into the extracellular space. It is thought that it is through these structures, that tip cells are able to sense such chemotactic signals as VEGF gradients[216].

Lamellipodia and filopodia are both manifestations of their underlying cytoskeletal scaffold. Lamellipodia possess a highly branched actin network, whilst filopodia

consist of tight bundles of filamentous actin[216]. The formation of both of these structures, is highly dependent on the activity of molecules which modulate intracellular actin dynamics. The initial stimulatory signal for their formation arises from members of the Rho family of GTPasaes. These molecules can stimulate the formation of lamellipodia and filopodia through their interaction with actin binding molecules such as Arp2/3 and Wasp.

In theory, one might expect intracellular Tβ4 to be able to influence the process of lamellipodial and filopodial formation, and consequently the processes of endothelial cell migration and angiogenesis, through its G-actin sequestering properties. It could be hypothesised that an overexpression of Tβ4 might lead to decreased cell migration, as it could lead to a greater proportion of the intracellular G-actin pool being sequestered, thereby slowing or halting the production of actin filaments in lamellipodia and filopodia. Conversely, a decrease in the expression of intracellular Tβ4 could exert a migratory drive due to the increased availability of unsequestered G-actin for filament formation. Such an effect has already been demonstrated by transfecting HUVECs *in vitro* with Tβ4 siRNA[43].

However, the situation does not appear quite this simple, potentially because of the diverse functions of Tβ4. Whilst transiently transfecting endothelial cells with Tβ4 siRNA does indeed stimulate increased migratory behaviour, stable transfection of these HUVECs with Tβ4 siRNA has the opposite effect and decreases migratory capacity. This effect is thought to be due to a lack of Tβ4 stimulated MMP secretion, resulting in endothelial cells which are unable to turnover the ECM[43]. To date, all of

these findings have been elucidated from *in vitro* studies, and so testing which of these effects Tβ4 is able to mediate *in vivo* is a highly relevant question.

One unresolved issue in endothelial Tβ4 biology is the extent to which extracellular or paracrine Tβ4 plays a role during *in vivo* angiogenesis. The addition of exogenous Tβ4 to aortic ring explants[217] and chick chorioallantoic membranes[54], results in the formation of new blood vessels, in these *ex vivo* models. As such, it is plausible that Tβ4 may act as an *in vivo* trophic stimulus for angiogenesis similar to VEGF.

In order to investigate the possible roles of Tβ4 during developmental angiogenesis, use was made of a number of *in vivo* models. Each of these assays relies on measuring parameters associated with active angiogenesis. For example, in the neonatal retinal model of angiogenesis, endothelial tip cells migrate from the centre of the optic disc to the periphery of the retinal cup in a radial fashion[55]. Thus, one parameter that can be measured is the distance from the central optic disc to the migratory front of the vascular plexus at any given time point. This will provide an *in vivo* measure of the migratory activity of endothelial cells and thus a quantification of the rate of angiogenesis[161].

An alternative metric of angiogenesis is to count blood vessel intersections, also known as branch points. When the filopodial processes of two tip cells meet, they can either separate or fuse together[218]. If they fuse together, a new vessel is formed linking the two existing pillars of endothelial stalk cells that each tip cell was leading. For this reason, the density of vascular intersections can also be used as a surrogate measure of angiogenesis[161].

7.2 Results

7.2.1 E10.5 Tβ4-RIEK Embryos Display No Defect in Angiogenesis

It was noted on whole mount examination of E10.5 embryos that the region surrounding and overlying the somites was rich in branching blood vessels. Quantification of the branch point density in this region, has been used by other groups as a surrogate measure for angiogenesis at this time point[219,220]. Therefore, E10.5 Tβ4 RIEK and wild type embryos had their blood vessels highlighted through the use of whole mount immunohistochemistry for the endothelial cell marker PECAM, and had their vessel branch points counted in the somatic region of the embryo (**Fig. 7.1**). However, no difference was observed in the branch point densities of Tβ4-RIEK mutant and wild type embryos.

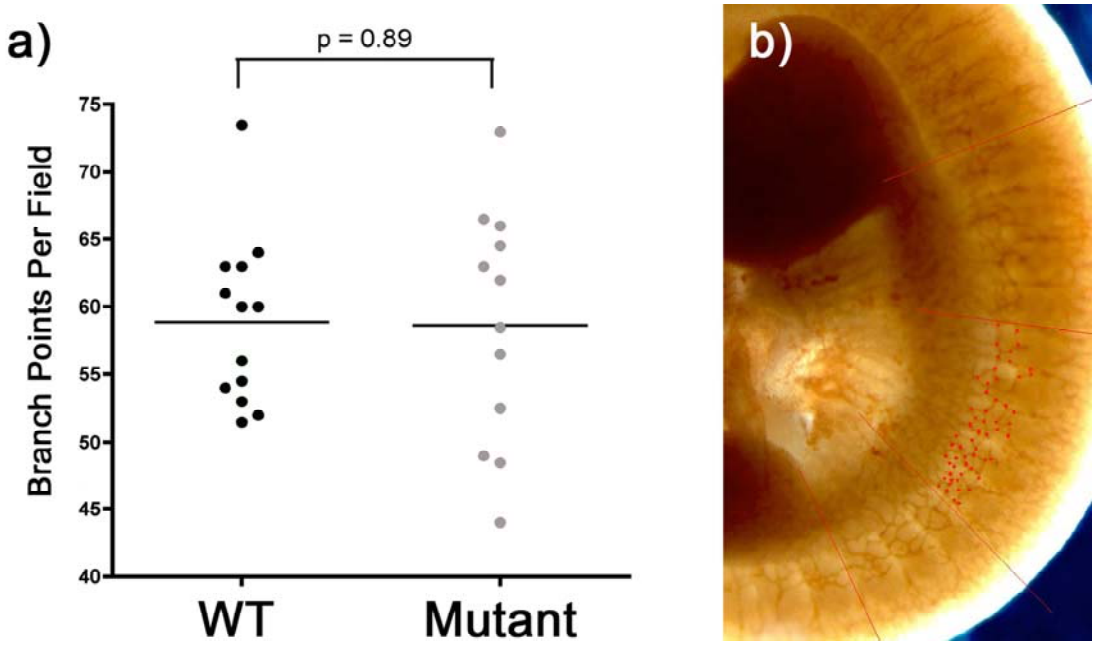


Fig. 7.1
E10.5 Tβ4 -/Y embryos display no angiogenic defect

Whole mount PECAM immunohistochemistry of E10.5 Tβ4-RIEK mutant and control embryos revealed that angiogenesis was not impaired in the mutants (**a**). This was performed by counting the number of intersecting branch points over a defined section of the somatic region of the embryo, as shown in (**b**).

Due to the previously described concerns about the efficiency and magnitude of Tβ4 knockdown in the Tβ4-RIEK mouse a decision was made to abandon this model and move to a different one. As the Tβ4 global loss of function mouse had presented with a mural cell phenotype, this was thought to be the best genetic mutant to pursue. The decision was also taken to make use of the neonatal retinal angiogenesis model. This is because several more parameters can be measured in the retinal model than the embryo/somite model. For example, as well as branch point density; migratory distance, tip cell filopodial number and secondary vascular sprouting can all be quantified.

7.2.2 Tβ4 Is Expressed in the Developing Retinal Vasculature

In order to gain further insight into what role Tβ4 might play in retinal angiogenesis, and to confirm its expression in the retinal vasculature, whole mount in situ hybridisation for Tβ4 mRNA was conducted on P6 wild type retinas. Wild type P6 retinas displayed staining for Tβ4 throughout the retinal vascular plexus, but could also be observed expressed in a speckled pattern in cells of the unvascularised retinal periphery (**Fig. 7.2**). Subjectively, it was thought that Tβ4 might be slightly higher in those vascular cells closest to the sprouting front.

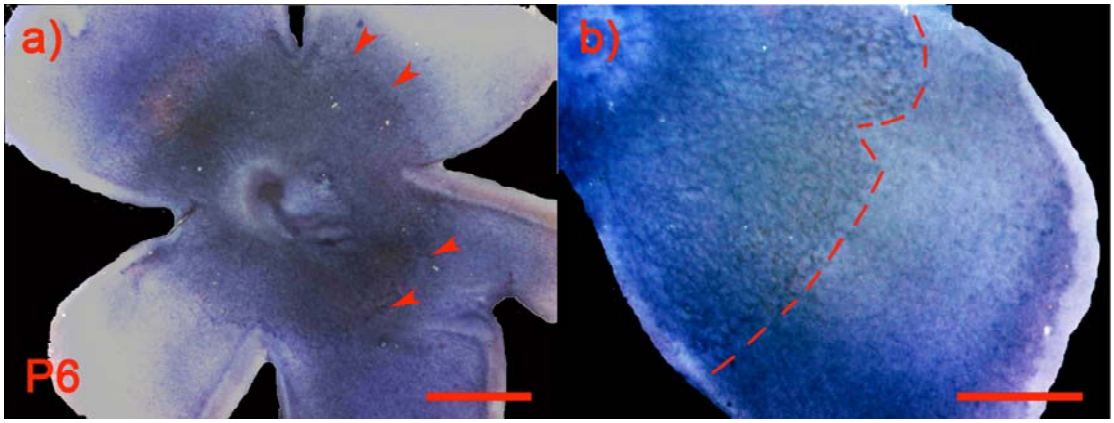


Fig. 7.2
Tβ4 is expressed in the retinal primary vascular plexus

Whole mount in situ hybridisation for Tβ4 mRNA reveals expression of Tβ4 in the developing vascular plexus of wild type P6 retinas at low magnification (red arrowheads) (**a**), and at high magnification (**b**). The dashed red line in (**b**) represents the age of the sprouting vascular front. More peripheral to this front, weaker Tβ4 expression can be viewed in non-vascular cells. Scale bars: (a) 100μm, (b) 50μm.

7.2.3 Loss of Tβ4 Function Has No Effect on Retinal Vascular Angiogenesis

In order to determine whether deletion of the Tβ4 gene, could affect physiological angiogenesis *in vivo*, whole mount retinas from P6 Tβ4 -/Y and Tβ4 +/Y mice were

stained with the endothelial cell marker Isolectin B4 and imaged using fluorescent microscopy. On gross examination, no differences could be observed between the retinas from Tβ4 -/Y and Tβ4 +/Y mice (**Fig. 7.3**).

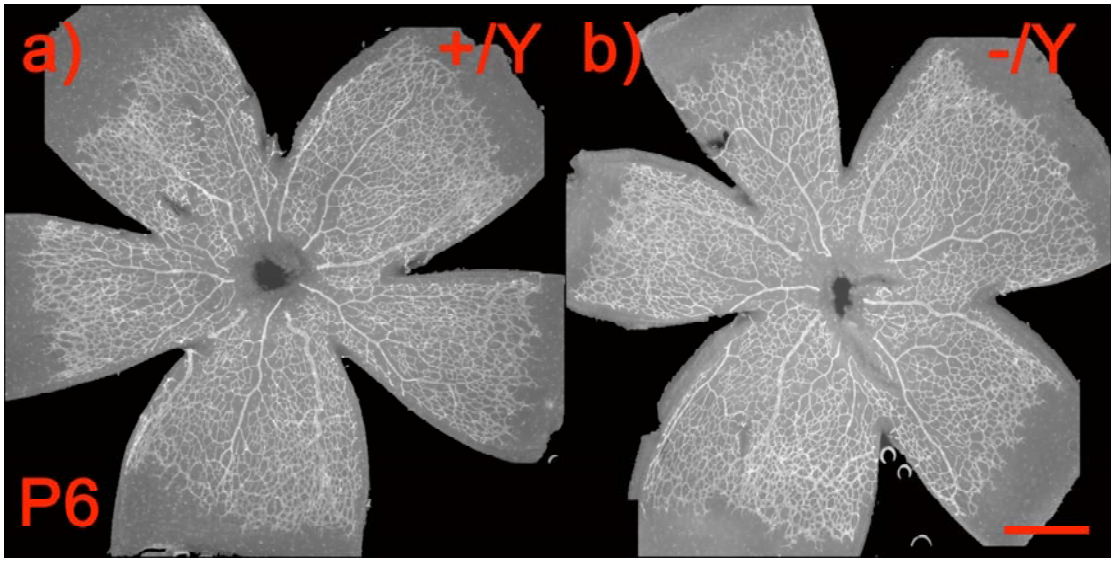


Fig. 7.3
The appearance of the retinal vasculature in Tβ4 -/Y mice is grossly normal

Whole mount staining of P6 Tβ4 +/Y (**a**) and Tβ4 -/Y (**b**) retinas with the endothelial specific lectin Isolectin B4 reveals no gross differences between Tβ4 +/Y and Tβ4 -/Y specimens. Scale bar: 100μm.

In order to provide a more systematic and quantitative analysis of angiogenesis in Tβ4 -/Y and Tβ4 +/Y retinas, P2, P4 and P6 Tβ4 +/Y and Tβ4 -/Y retinas were stained with Isolectin B4. From the pictures, which resulted, the first parameter that was measured was the radial distance that the vascular sprouting front had migrated. There was no significant difference observed in the distances achieved between the two genotypes examined at any of the time points that were assessed (**Fig. 7.4**).

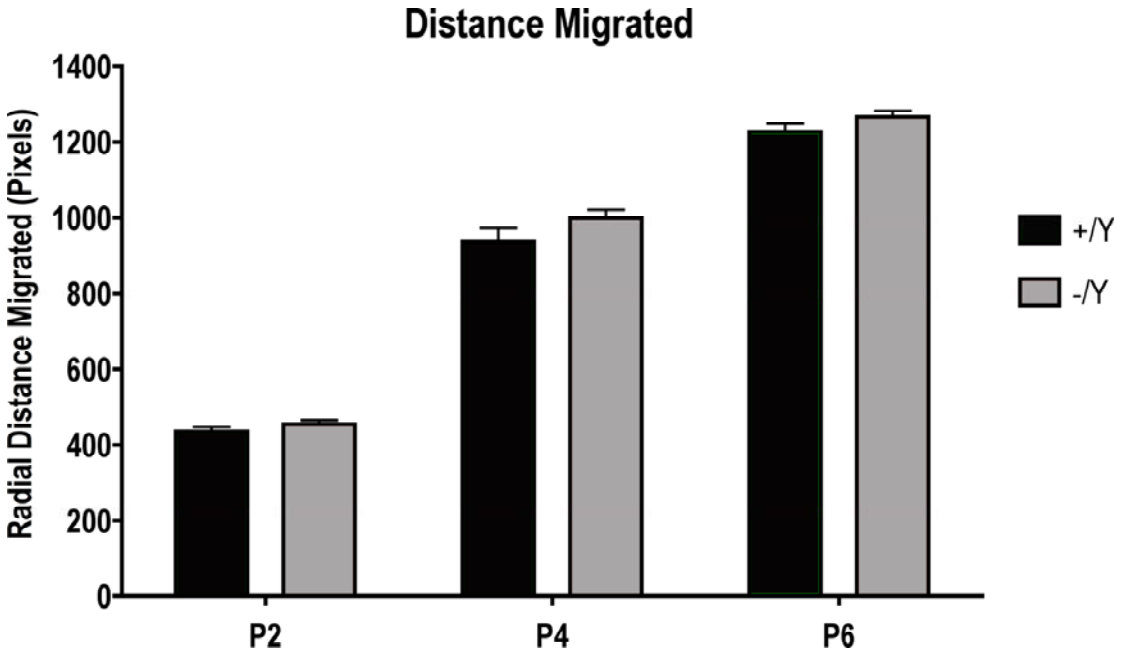


Fig. 7.4
There is no defect in the migration of the sprouting vascular front in Tβ4 -/Y retinas

Quantification of the radial distance migrated by the endothelial sprouting front in Tβ4 +/Y and Tβ4 -/Y mice at P2, P4 and P6 demonstrates no difference between the two genotypes.

The next step was to assess whether there were any differences in the branch point density between the Tβ4 +/Y and Tβ4 -/Y retinas. Branch point density was measured in the vascular plexus just proximal to the sprouting front in P2, P4, and P6 Tβ4 +/Y and Tβ4 -/Y retinas. Once again, no difference could be observed between the wild type and Tβ4 null specimens (**Fig. 7.5**).

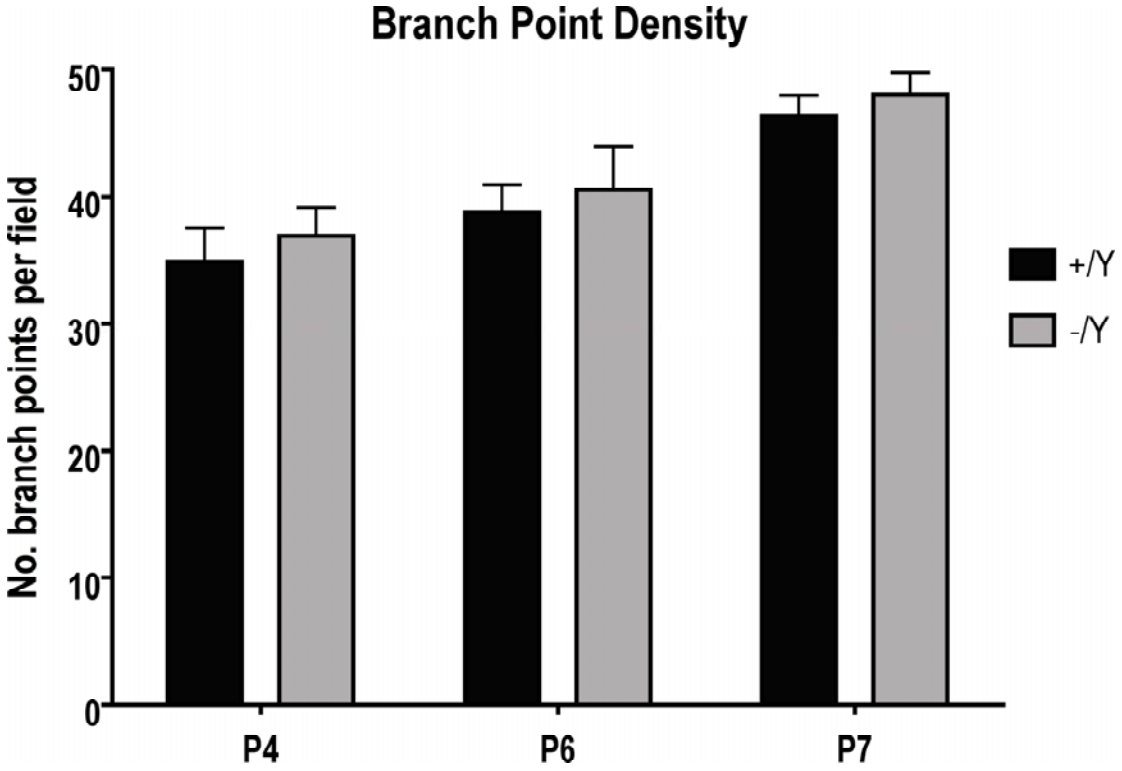


Fig. 7.5
Tβ4 -/Y retinas display no abnormality in vascular branch point density

Quantification of branch points in the sub tip cell layer of Tβ4 +/Y and Tβ4 -/Y mice at P4, P6 and P7 again reveals no significant differences between the genotypes.

Given the necessity of a functional actin cytoskeleton for the formation of lamellipodia and filopodia as previously described, it was hypothesised that Tβ4 -/Y tip cells might display a greater number of lamellipodia or filopodia due to a theoretical increase in intracellular free G-actin to allow formation of actin filaments. In order to assess this, high powered pictures of the Isolectin B4 labelled sprouting front were taken and the lamellipodial and filopodial density along the vascular front calculated. Somewhat surprisingly, there was no difference observed in the number of lamellipodia per field or filopodia per tip cell between the Tβ4 +/Y and Tβ4 -/Y mice (**Fig. 7.6**).

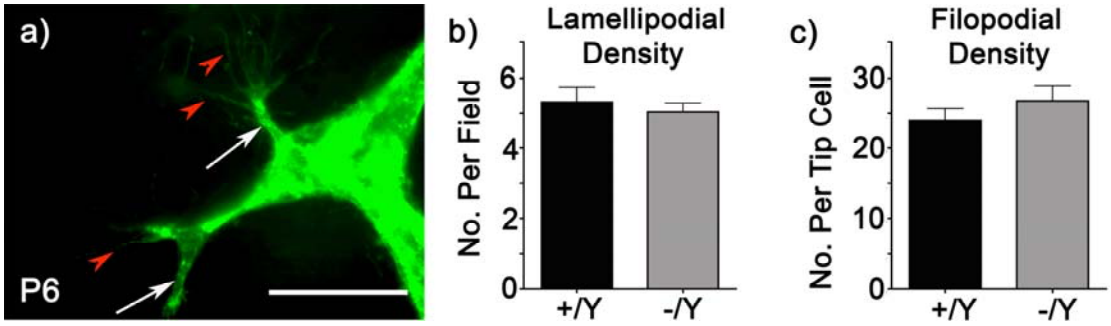


Fig. 7.6
Tβ4 -/Y retinal vascular tip cells possess a normal number of filopodia and lamellipodia

Endothelial tip cells possess characteristic lamellipodia (white arrows) and filopodia (red arrowheads) (a). Microscopic examination of Isolectin B4 stained retinas reveals no significant difference in the density of lamelipodia along the sprouting front (b) and no significant difference in the number of filopodia per tip cell (c) between P6 Tβ4 +/Y and P6 Tβ4 -/Y retinas. Scale bar: 10μm.

Following development of the primary vascular plexus, a deeper vascular plexus starts to form at around P7 in the neonatal mouse retina[55]. If the flat mounted retina is viewed as in **Fig. 7.3**, then initial sprouting takes place downwards along the z axis into the page. This makes formation of the secondary vascular plexus a difficult phenomenon to observe with fluorescence microscopy. However, there are parameters which can be measured which act as a sufficient markers for deeper vascular plexus development. The initial downward sprouts from the primary plexus can be viewed as small circular foci of fluorescence hyperdensity. The density of these across the retinal field where sprouting is taking place can act as a surrogate for secondary plexus formation. Likewise, the percentage of the primary plexus, which is producing downward sprouts can also be assessed. These concepts are illustrated in **Fig. 7.7a**.

When P9 Tβ4 +/Y retinas were compared to P9 Tβ4 -/Y retinas, it was observed that the Tβ4 -/Y retinas appeared to have both a decreased density of downward sprouts

and a reduced area of downward sprouting than Tβ4 +/Y mice (**Fig. 7.7b and c**). However, it is difficult to draw any firm conclusions from these observations as the differences did not reach statistical significance. This is likely due to the low N numbers used in this analysis (3 Tβ4 +/Y retinas versus 3 Tβ4 -/Y retinas). As there is a lot of variation from animal to animal, this sample was not of sufficient size to be sure of the observation.

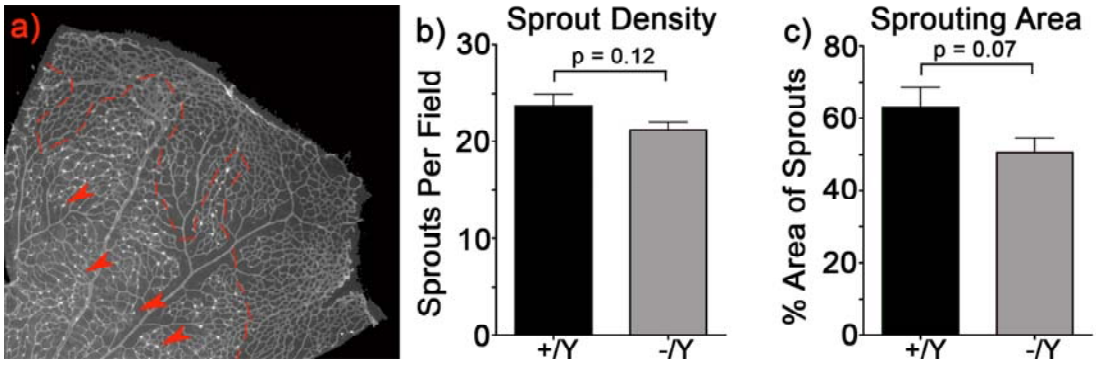


Fig. 7.7
Tβ4 -/Y retinas display a trend towards a reduction in secondary vascular plexus sprouting

The boundary of the secondary retinal vascular plexus (red dashed line) can be defined as the tightest surface area which encloses a region of small round fluorescent hyperdensities (red arrowheads) on whole mount retinal Isolectin B4 staining (**a**). Quantification of the density of downward sprouts (**b**) and the area of downward sprouting (**c**) show a non-significant reduction in downward sprouting in P9 Tβ4 -/Y as compared to Tβ4 +/Y retinas.

7.2.4 Tβ4-HEK Mice Display No Defect in Retinal Vascular Sprouting

It has been reported that the migratory phenotype of endothelial cells is extremely sensitive to the gene dose of Tβ4[43]. *In vitro* studies show that transient transfection of endothelial cells with a Tβ4 shRNA expressing construct leads to an increase in migratory capacity, whilst stable transfection with the same Tβ4 shRNA construct

leads to dramatically decreased migration of the cells. This variability in endothelial cell migration was ascribed to a difference in Tβ4 knockdown of about 30% between the two cell lines. Thus, a decision was made to investigate the phenotype of P6 retinas in Tβ4 HEK-mice. The rationale behind this strategy, was that, due to the Tβ4-HEK mouse being a model of Tβ4 knockdown, rather than knockout, the vasculature in the Tβ4-HEK mouse would display a different level of Tβ4 reduction to the Tβ4 -/Y mouse. This alteration in gene dose might manifest itself an endothelial migratory phenotype different to that observed in the Tβ4 -/Y mouse.

The mutant Tβ4-HEK mutant specimens, again displayed no significant difference in the magnitude of sprouting front migration compared to controls. Nor, did they show any change in branch point density in both the peripheral and central areas of the plexus compared to wild type littermates (**Fig. 7.8**).

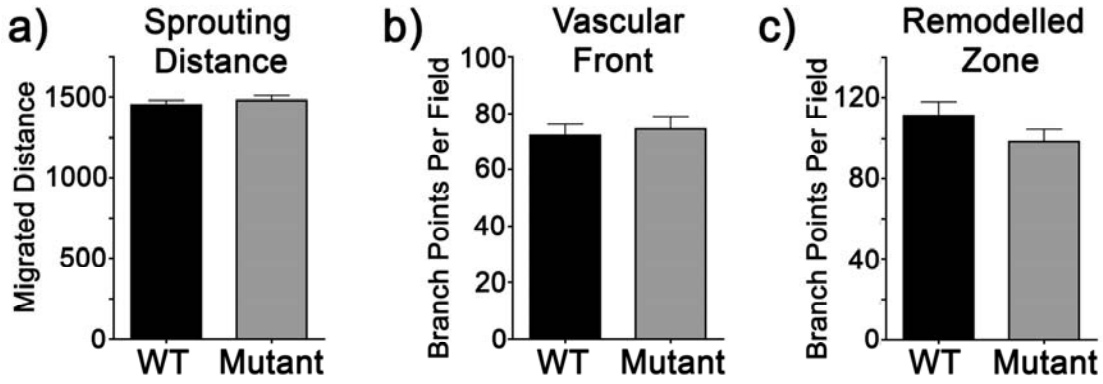


Fig. 7.8
No angiogenic defect is observed in the Tβ4-HEK retinal vasculature

Examination of Isolectin B4 stained whole mount P6 Tβ4 HEK mutant and control retinas disclose no differences in migratory distance of the sprouting front (**a**), branch point density at the vascular front (**b**) or branch point density at the central region (**c**) between mutants and controls.

7.3 Discussion

7.3.1 Summary

In order to determine whether Tβ4 played an important role in physiological angiogenesis *in vivo*, Tβ4 genetic loss of function models were interrogated. Analysis of the peri-somitic vasculature of E10.5 Tβ4 RIEK embryos, through whole mount PECAM staining revealed no defect in angiogenesis.

Attention was then switched to the Tβ4 -/Y mouse, and the retinal vasculature used as a model system. Whole mount in situ hybridisation showed prominent expression of Tβ4 in the developing retinal vascular plexus but careful phenotyping demonstrated no defect in migration of the endothelial sprouting front, nor an abnormality in branch point density in Tβ4 -/Y specimens. Somewhat surprisingly the Tβ4 -/Y mice possessed the same density of filopodia and lamellipodia on their endothelial tip cells as did Tβ4 +/Y mice. In fact, the only possible abnormality observed in the Tβ4 -/Y mice, might be a reduction in secondary vascular plexus sprouting. However, unfortunately the sample sizes tested were not sufficient to be able to exclude random variance about the mean as a cause for this difference.

The final model system employed was the Tβ4 HEK mouse. Once again, no difference could be observed in either the migratory distance of endothelial cells, nor the density of branch points in Tβ4 HEK mutant mice.

7.3.2 Tβ4 Loss of Function Does Not Lead to an Angiogenic Defect *In Vivo*

Somewhat surprisingly, no statistically significant and major angiogenic defect exhibited itself in Tβ4 loss of function models. This was unexpected, as a role for Tβ4 as both a stimulator of *ex vivo* vasculogenesis[215] and a critical intracellular mediator of endothelial cell migration[43] has already been established in the literature.

One explanation for the failure to observe a phenotype in the Tβ4 shRNA models tested could be due to incomplete and inefficient knockdown of Tβ4, for reasons previously discussed in Chapter 4. However, this does not explain the lack of phenotype in Tβ4 -/Y specimens. One plausible explanation could lie in the array data first presented in Chapter 5. Gene array analysis of Tβ4 -/Y hearts revealed that they possessed reduced amounts of mRNA for the genes Arp2/3 and profilin (**Appendix 3**). Arp2/3 is an actin nucleating factor, which promotes the addition of branching chains to F-actin, whilst profilin has the almost exact opposite effect to canonical Tβ4 action, as it desequesters G-actin and promotes its polymerisation to F-actin.

The result of these gene expression changes could be that they effectively maintain the ratio of actin depolymerising molecule to actin polymerising molecules constant, thus keeping the dynamic F-actin/G-actin equilibrium in balance. There is some precedent for such an effect. Through an unknown transcriptional regulatory mechanism, overexpression of Tβ4 in NIH 3T3 cells was able to stimulate an increase in the expression of β-actin, vinculin and Talin, with the result that G-actin to F-actin ratio was kept constant[21]. This hints at the existence of some as yet unexplained

transcriptional feedback loop, which keeps the cytoskeletal machinery in perfect balance. In order to determine whether this could be the explanation for normal endothelial cell migration in Tβ4 -/Y mice, qRT-PCR could be conducted on RNA extracted from Tβ4 +/Y and Tβ4 -/Y retinas. If levels of molecules such as Arp2/3, β-actin, and profilin were down regulated, this would provide good evidence for compensatory changes in actin dynamics following loss of Tβ4.

7.3.3 Sprouting of the Secondary Vascular Plexus

The only possible defect observed in the retina of the Tβ4 -/Y mouse was potentially a reduction or delay in sprouting of the secondary vascular plexus. The molecular mechanisms, which govern sprouting of the secondary deeper vascular plexus are not well understood. Although, it is speculated that there might be a molecular trigger which reactivates quiescent endothelium in the primary plexus to initiate downward sprouting (Fruttiger M. – personal communication).

The next experimental step would be to conduct a full characterisation of the secondary vascular plexus in Tβ4 -/Y pups. The same experiments as performed here could be conducted on a greater number of specimens to improve the statistical power of the experiment. In addition, confocal microscopy could be used to visualise the secondary plexus directly and note any branching abnormalities which were confined to this vascular bed.

One possible hypothesis, as to how Tβ4 could be functioning to induce secondary plexus sprouting defects, is that Tβ4 is secreted by non-endothelial cells, and acts in a

paracrine extracellular role to stimulate angiogenesis from the quiescent cells of the primary plexus. Such a niche would correspond well to experiments performed by other researchers, which have marked Tβ4 as a potent extracellular driver of angiogenesis[54,215]. Circumstantial evidence that this is how Tβ4 might function comes from the observation, made here, that Tβ4 does not appear to be expressed solely in the endothelial cells of the retina. **Fig. 7.2b**, certainly shows expression of Tβ4 in cells which are not part of the vascular plexus. Key to further exploration of this theory, would be identification of the non-endothelial cells which express Tβ4. This could be achieved by performing immunostaining for astrocyte, neuronal or macrophage markers on whole mount retinas, which have already undergone in situ hybridisation for Tβ4.

8 Discussion and Future Work

8.1 A Molecular Model for Tβ4 Action

The favoured model for the action of Tβ4 proposed in this thesis is that, Tβ4 is secreted by the endothelial cells of the developing vasculature. This paracrine Tβ4 signal then acts on mesodermal progenitors, to stimulate their differentiation into mature mural cells by activating the TGF-β signalling pathway (**Fig. 8.1**).

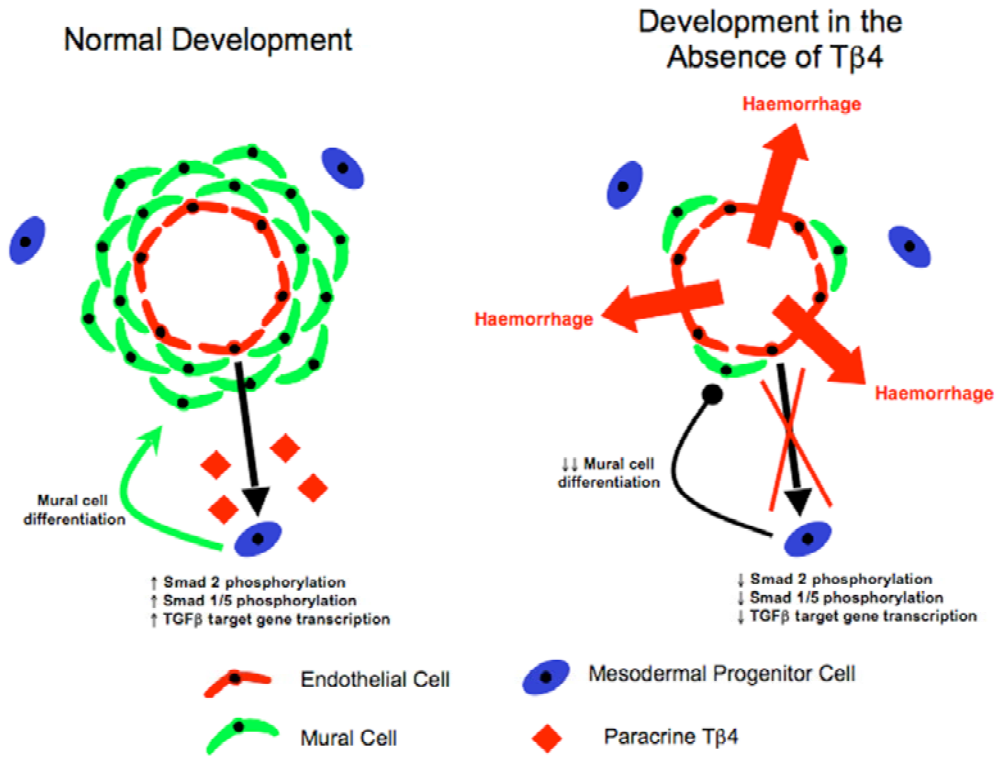


Fig. 8.1
A model for Tβ4 action in mural cell development

A schematic to explain the proposed mechanism of Tβ4 action in vascular development. Tβ4 is secreted by the developing endothelium. This Tβ4 acts on mesodermal progenitor cells to stimulate TGF-β target gene transcription through a co-operational effect on Smad phosphorylation with TGF-β ligand. This results in increased transcription of typical mural cell genes such as smooth muscle actin, NG2, endosialin and others. This leads to mural cell differentiation and investiture of the endothelium with a fully formed mural cell coat. In the absence of Tβ4, the effect of endothelial secreted TGF-β is reduced leading to decreased Smad phosphorylation in mesodermal progenitors resulting in lower TGF-β target gene activation. This causes less mural cell differentiation and reduced mural cell recruitment to the developing vessel wall. If a critical threshold of mural cell coverage is not achieved, the vessel loses structural integrity and haemorrhage results.

Such a model is plausible, but is also illustrative of a number of unresolved issues with regards to Tβ4 biology. The first of these regards the secretion of Tβ4. It is clear, for several reasons, that Tβ4 is secreted during physiological processes. First of all, levels of Tβ4 can be measured in the serum[221,222], salivary fluid and lacrimal secretions[223]. Secondly, cells in culture have been shown to secrete Tβ4 into the

culture medium[224]. Finally, the effects on target cells of exogenous Tβ4 are too numerous and too well described to not, in many instances, be representative of true physiological phenomena[1].

In eukaryotes, the majority of secreted proteins possess a secretion signal peptide, which allows them to be packaged in vesicles and exported via the Golgi/endoplasmic reticulum (ER)[225]. Tβ4 does not possess such a secretory peptide, and thus, how Tβ4 can be secreted remains unknown. However, Tβ4 is not the only molecule, which is secreted from cells without possessing a classical secretory sequences. In recent times a number of proteins such as the fibroblast growth factor (FGF) and the inflammatory cytokine IL-1β have been identified as being secreted by non-classical secretory pathways. Such non-classical secretory pathways are typically thought to rely on secretory lysosomes, membrane blebbing or transport by specific transmembrane channel proteins[226]. Some indication that Tβ4 can be secreted by one of these non-classical means comes from a screen, which identified the closely related molecule Tβ10, as being secreted by keratinocytes in a manner dependent on the activity of the enzyme Caspase-1[227]. It is plausible to suggest that Tβ4 might be secreted via a similar mechanism.

In order to fully validate the model for vascular Tβ4 action presented here, it would be necessary to provide evidence of endothelial Tβ4 secretion above and beyond that implied by the effects of exogenous Tβ4 on 10T1/2 and A404 cells. The ideal, would be to elucidate the non-classical mechanism of Tβ4 secretion, and then to perturb it. For example, if Tβ4 was found to be secreted in a Caspase-1 dependent fashion, then mutant constructs coding for forms of either Caspase-1 or Tβ4 which did not interact

with each other could be created. These could be transfected into endothelial cells and used in 10T1/2 cell co-culture assays to determine if they abrogated the effect of Tβ4 on mural cell differentiation.

An alternative approach could be to inject recombinant, synthetic Tβ4 into the pregnant mothers of E9.5 Tβ4 -/Y embryos. Maternal injection of Tβ4 has previously been shown to be able to cross the placental barrier and rescue the yolk sac vascular defects of Hand1 null embryos[51]. The rationale behind such an experiment, is that Tβ4 would be able to enter the foetal circulation, cross the endothelial and rescue the mural cell defect apparent in E10.5 Tβ4 -/Y mice. This would provide good evidence that the mural cell defect in E10.5 -/Y embryos is due to a paracrine rather than a endothelial cell autonomous loss of Tβ4.

A Receptor for Tβ4

To date, no cell surface receptor has been definitively identified for Tβ4. Although, treatment of A404 and 10T1/2 cells with exogenous Tβ4 activates the TGF-β signalling pathway, it is difficult to confirm the validity of the model of action proposed beyond all reasonable doubt, without insight into how Tβ4 mediates this effect.

A number of molecules, have been put forward as candidates for a Tβ4 receptor. The first of these is known as Integrin linked kinase (ILK). It has been demonstrated that in adult hearts, Tβ4 can bind to ILK and form a complex which can phosphorylate and activate the survival kinase Akt[27]. This observation is interesting, as considerable

evidence exists for crosstalk between Akt and the TGF-β signalling pathway[228]. However, it is not clear whether the effect of Akt activation is activatory[229] or inhibitory on the TGF-β pathway[230]. Another potential receptor is the Ku80 subunit of the DNA Helicase II complex[56,231]. Tβ4 has been shown to bind to this molecule *in vitro* and knockdown of Ku80 by siRNA has been shown to inhibit Tβ4 stimulated PAI-1 expression in colorectal carcinoma cells.

One common theme, which emerges from these observations, is that interactors of exogenous Tβ4 are generally intracellular. Ku80 is predominantly a nuclear protein, although it can also be expressed on the cell surface[232]. In addition, functional interaction of Tβ4 with ILK and subsequent activation of Akt2 in endothelial cells was thought to be localised, to the intracellular side of the cell membrane in HUVECs[43]. This raises the intriguing possibility that Tβ4 exerts its paracrine effects by binding to an internal rather than an external molecular target.

Further evidence for this hypothesis comes from observations that Tβ4 mediated protection of human corneal epithelial cells from ethanol induced apoptosis, is correlated with the accumulation of Histidine tagged Tβ4 in the cell cytoplasm. Furthermore, Tβ4 apoptotic protection was abolished by treatment of the corneal epithelial cells by cytochalasin D, which as an inhibitor of endocytosis, prevented intracellular accumulation of Tβ4[233].

Thus, it is possible that Tβ4 exerts its effects on mural cell differentiation and TGF-β pathway activation in 10T1/2 and A404 cells via intracellular uptake by endocytosis. This presents an attractive model, as it would allow for a functional interaction

between Tβ4 and the protein Skip/SNW1 as speculated in the discussion of chapter 6. This could be tested by treating A404 and/or 10T1/2 cells with Histidine or FLAG tagged Tβ4, and observing whether labelled Tβ4 was taken up by target cells. Various inhibitors of endocytosis such as cytochalasin D could be administered to these cells to observe whether they prevented Tβ4 uptake and suppressed Tβ4 induced mural cell differentiation.

In theory, one could test the *in vivo* relevance of a potential internalisation dependent Tβ4 signalling mechanism by creating a transgenic Tβ4-GFP or Tβ4-6His mouse. If Tβ4 internalisation does take place *in vivo* in mural cells, then the presence of GFP or Histidine tagged Tβ4 inside mural cells, without the expression of Tβ4 mRNA in these cells, would provide good evidence that this phenomenon has *in vivo* relevance.

A Potential Role for Tβ4 in the Nervous System

The studies reported in this thesis also report expression of Tβ4 in the nervous system consistent with other entries in the literature[52,108]. It appears to be expressed predominantly in grey matter at sites of active neurogenesis. These observations are striking, as many parallels can be drawn between Tβ4 expression and function in the CNS and Tβ4 expression and function in the cardiovascular system.

In the heart, Tβ4 is secreted by the developing myocardium, in order that it can signal to the overlying epicardium to stimulate the migration of EPDCs into the myocardium and their differentiation into the cells of the coronary vasculature[50]. In the systemic

vasculature, as shown in this thesis, Tβ4 is secreted by endothelial cells to signal to mesodermal progenitors, to induce their differentiation into mature mural cells.

However, Tβ4 also plays a role in the setting of adult cardiovascular disease. Upon surgically induced myocardial infarction in mice, administration of Tβ4 can restore cardiac function partly through stimulation of revascularisation[101], and partly through the promotion of myocardial regeneration from adult EPDCs (Paul Riley – personal communication). Thus, it is thought that Tβ4 exerts its cardioprotective effects by re-induction of the embryological mechanisms of cardiac development.

Tβ4 has also been shown to possess therapeutic function in treatment of neurological disorders. In an embolic model of stroke conducted in the rat, administration of synthetic Tβ4 was shown to promote functional outcome. This was correlated with an increase in myelinated axons in the ischaemic boundary[41]. In another model of neurological disease, in this case experimental autoimmune myeloencephalitis – a mouse model for multiple sclerosis, Tβ4 was able to induce functional recovery. Once again, this was correlated with an ability to induce proliferation of an N20.1 oligodendrocyte progenitor line[36].

Thus Tβ4 appears to possess similar functions in the adult central nervous and cardiovascular systems. It can stimulate the proliferation and differentiation of adult progenitor cells to promote recovery in a damaged target tissue. Given that Tβ4 seems to have the same function in the adult CNS and heart, it is very possible that they may have a similar function during development. This makes a study of the functional role of Tβ4 in the developing neural system an attractive proposition. In

order to determine whether Tβ4 has a function during embryonic neurogenesis the random integrant or Hprt targeted Tβ4 shRNA mice could be crossed with a Cre driver strain, which expresses Cre recombinase in the neural lineage of interest. As an alternative, the brains of Tβ4 -/Y embryos could be examined for subtle defects in neurogenesis.

Tβ4 in Vascular Disease

The findings outlined in this thesis may have relevance for understanding the pathogenesis and treatment vascular disease. Preliminary results from Paul Riley's lab, indicate that adult Tβ4 -/Y mice, which survive the process of embryogenesis have abnormally dilated aortas, somewhat reminiscent of aortic aneurysm. Co-incident with this, the mural cells of these aortas appear to express lower levels of differentiation markers such as smooth muscle actin. Thus, if these preliminary results hold true, it appears that Tβ4 -/Y mice may display an adult vascular disease with an aetiology of abnormal development due to a lack of Tβ4.

Tβ4 in Aneurismal Disease

Such a finding may have applicability to understanding the formation of aneurysmal disease of the aorta in humans. Several syndromic forms of aortic aneurysm, including those of Loeys-Dietz syndrome are caused by mutations in components of the TGF-β signalling pathway[234]. Given that, a role for Tβ4 in TGF-β signalling has been demonstrated in this thesis, it is plausible that Tβ4 modulation of TGF-β signalling has activity in human aortic aneurysm. Such a hypothesis is given more

credence by the observation that Tβ4 is one of the most highly expressed transcripts in human abdominal aortic aneurysms[235].

This theory becomes all the more important, when placed in the context that endovascular administration of VSMCs to aneurismal xenografts in rats was able to prevent aneurysm progression[236]. Administration of TGF-β was also able to achieve a similar effect[237]. Although, it has not been concluded that TGF-β exerts its protective effect on aortic aneurysm through the *de novo* induction of stabilising mural cells, this is a possibility. As such, if Tβ4 -/Y animals do possess significant aneurysm, it would be intriguing to determine whether systemic administration of recombinant Tβ4 could halt or reverse the disease through stimulation of appropriate ad full mural cell differentiation. Moreover, it would be interesting to test whether Tβ4 could exert therapeutic effects on other models of aortic aneurysm.

Tβ4 in Diabetic Retinopathy

The endothelial-pericyte signalling axis has become a critical focus for understanding the pathogenesis of several diseases. One of these is proliferative diabetic retinopathy. Vascular insufficiency in the retina is thought to stimulate a hypoxic drive for the proliferation of new blood vessels across the retinal surface. However, fibrous traction caused by these new blood vessels can lead to retinal detachment whilst growth of blood vessels into the vitreous can cause blindness. The first pathological step in this process is thought to be death of vascular pericytes in the retinal vascular plexus[238]. Evidence that pericyte loss is causative for the pathogenesis of diabetic retinopathy comes from studies involving mice heterozygous

for a PDGF-B null allele. These mice have reduced retinal pericyte coverage and are more susceptible to proliferative change in hypoxia induced retinopathy[239].

Thus, one approach to treat proliferative diabetic retinopathy might be to prevent pericyte loss or to replace pericytes that have died. Although, in this thesis, no defect was observed in the mural cell coverage of Tβ4 -/Y mice, it is not implausible that Tβ4 may be able to act as a stimulus for mural cell differentiation in the adult retina. The explanation for why loss of Tβ4 during development does not affect mural cell coverage of neonatal retinas is that the retina lacks vasculogenesis competent mesenchyme, which can act as a substrate for mural cell development[151]. However, in the adult, there might potentially be other sources of progenitor cells, which can undergo mural cell differentiation. For example, there are reports that bone marrow cells can act as pericyte precursors[240].

Therefore, it is possible that Tβ4 might play a role in the pathogenesis or treatment of diabetic retinopathy. In order to test whether loss of Tβ4 makes animals more susceptible to diabetic retinopathy, the retinas of old Tβ4 -/Y adult mice could be examined for the presence of vascular proliferation, aneurysm and haemorrhage. Tβ4 -/Y mice could also be exposed to a hyperoxic environment followed by a hypoxic environment to promote proliferative change. The degree of proliferative change and pericyte dropout could be assessed and compared to that present in Tβ4 +/Y mice.

In order to test whether Tβ4 might be protective against diabetic retinopathy, PDGF-B heterozygous null mice, which have a 30% reduction in vascular pericyte coverage, could be injected with Tβ4 to observe if exogenous Tβ4 could stimulate re-coverage

of the vasculature with newly differentiated mural cells, perhaps arising from bone marrow precursors.

Tβ4 in Tumour Angiogenesis

Another disease system where the endothelial-pericyte signalling axis is important is the tumour vasculature. Tumours require a blood supply in order to grow. Most tumours display abnormal vascularisation, being in possession of highly tortuous, leaky and incomplete blood vessels[241]. Recently, anti-VEGF immunotherapy has proved a very effective adjunct to typical surgical and radiotherapy treatments for cancer, by targeting the blood supply of growing tumours[242]. However, some tumours become resistant to anti-VEGF therapy. It is thought that this may be due, in part, to the endothelial-pericyte interaction, as this relationship stabilises the endothelial vasculature and makes the cells less dependent on VEGF[243]. Thus, combination immunotherapy targeting both the endothelial and the pericyte components of the tumour vasculature has increasingly become a focus of interest.

Treatment of mice on the tumourigenic RIP1Tag2 genetic background with a combination of anti-VEGF and anti-PDGFR-β was more effective at treating tumours than therapy with either one of these agents alone[244]. However, recent data suggest that this approach may not be as beneficial as originally perceived as reductions in primary tumour size are offset by an increased propensity for tumours to metastasise haematogenously, due to weakened blood vessel walls[243].

Due to the key nature of the endothelial-pericyte relationship in tumour biology, it will be important to explore a potential role for Tβ4 in this interaction. In order to explore a possible role for Tβ4 in recruitment of mural cells to tumour blood vessels, Tβ4 -/Y and Tβ4 +/Y mice could be inoculated with a tumour cell line. As, in this animal model of cancer, the vasculature is derived from the host animal, a reduction in mural cell coverage of the tumour vasculature in Tβ4 -/Y mice would indicate a role for host Tβ4 in establishment of a competent neoplastic blood supply. An alternative approach would be to treat tumour, which have been induced in wild type mice with an anti-Tβ4 antibody to observe whether this had any effect on the structure of the tumour vasculature. Such experiments would be particularly appropriate given that Tβ4 is over expressed in many types of primary tumour[23,245].

References

1. Goldstein AL. History of the discovery of the thymosins. Ann N Y Acad Sci. 2007;1112:1-13.

2. Asanuma Y, Goldstein AL, White A. Reduction in the incidence of wasting disease in neonatally thymectomized CBA-W mice by the injection of thymosin. Endocrinology. 1970;86:600-610.

3. Goldstein AL, Asanuma Y, Battisto JR, Hardy MA, Quint J, White A. Influence of thymosin on cell-mediated and humoral immune responses in normal and in immunologically deficient mice. J Immunol. 1970;104:359-366.

4. Goldstein AL, Hannappel E, Kleinman HK. Thymosin beta4: actin-sequestering protein moonlights to repair injured tissues. Trends Mol Med. 2005;11:421-429.

5. Huff T, Müller CS, Otto AM, Netzker R, Hannappel E. beta-Thymosins, small acidic peptides with multiple functions. Int J Biochem Cell Biol. 2001;33:205-220.

6. Lahn BT, Page DC. Functional coherence of the human Y chromosome. Science. 1997;278:675-680.

7. Li X, Zimmerman A, Copeland NG, Gilbert DJ, Jenkins NA, Yin HL. The mouse thymosin beta 4 gene: structure, promoter identification, and chromosome localization. Genomics. 1996;32:388-394.

8. Zarbock J, Oschkinat H, Hannappel E, Kalbacher H, Voelter W, Holak TA. Solution conformation of thymosin beta 4: a nuclear magnetic resonance and simulated annealing study. Biochemistry. 1990;29:7814-7821.

9. Mannherz HG, Hannappel E. The beta-thymosins: intracellular and extracellular activities of a versatile actin binding protein family. Cell Motil Cytoskeleton. 2009;66:839-851.

10. Safer D, Elzinga M, Nachmias VT. Thymosin beta 4 and Fx, an actin-sequestering peptide, are indistinguishable. J Biol Chem. 1991;266:4029-4032.

11. Wegner A. Head to tail polymerization of actin. J Mol Biol. 1976;108:139-150.

12. Condeelis J. How is actin polymerization nucleated in vivo? Trends Cell Biol. 2001;11:288-293.

13. Weber A, Nachmias VT, Pennise CR, Pring M, Safer D. Interaction of thymosin beta 4 with muscle and platelet actin: implications for actin sequestration in resting platelets. Biochemistry. 1992;31:6179-6185.

14. Carlier MF, Didry D, Erk I, et al. Tbeta 4 is not a simple G-actin sequestering protein and interacts with F-actin at high concentration. J Biol Chem. 1996;271:9231-9239.

15. Sun HQ, Kwiatkowska K, Yin HL. beta-Thymosins are not simple actin monomer buffering proteins. Insights from overexpression studies. J Biol Chem. 1996;271:9223-9230.

16. Boquet I, Boujemaa R, Carlier MF, Préat T. Ciboulot regulates actin assembly during Drosophila brain metamorphosis. Cell. 2000;102:797-808.

17. Paunola E, Mattila PK, Lappalainen P. WH2 domain: a small, versatile adapter for actin monomers. FEBS Lett. 2002;513:92-97.

18. Roy P, Rajfur Z, Jones D, Marriott G, Loew L, Jacobson K. Local photorelease of caged thymosin beta4 in locomoting keratocytes causes cell turning. J Cell Biol. 2001;153:1035-1048.

19. Sanger JM, Golla R, Safer D, et al. Increasing intracellular concentrations of thymosin beta 4 in PtK2 cells: effects on stress fibers, cytokinesis, and cell spreading. Cell Motil Cytoskeleton. 1995;31:307-322.

20. Ryu Y-K, Im Y-S, Moon E-Y. Cooperation of actin-sequestering protein, thymosin β-4 and hypoxia inducible factor-1α in tumor cell migration. Oncol Rep. 2010;24:1389-1394.

21. Golla R, Philp N, Safer D, et al. Co-ordinate regulation of the cytoskeleton in 3T3 cells overexpressing thymosin-beta4. Cell Motil Cytoskeleton. 1997;38:187-200.

22. Klein O, Savion N, Staroselsky A, et al. Metastasis-related cell functions in primary and metastatic tumor cells of AKR lymphoma. Cancer Lett. 1996;101:219-225.

23. Yamamoto T, Gotoh M, Kitajima M, Hirohashi S. Thymosin beta-4 expression is correlated with metastatic capacity of colorectal carcinomas. Biochem Biophys Res Commun. 1993;193:706-710.

24. Clark EA, Golub TR, Lander ES, Hynes RO. Genomic analysis of metastasis reveals an essential role for RhoC. Nature. 2000;406:532-535.

25. Sosne G, Qiu P, Goldstein AL, Wheater M. Biological activities of thymosin beta4 defined by active sites in short peptide sequences. FASEB J. 2010;24:2144-2151.

26. Sosne G, Siddiqi A, Kurpakus-Wheater M. Thymosin-beta4 inhibits corneal epithelial cell apoptosis after ethanol exposure in vitro. Invest Ophthalmol Vis Sci. 2004;45:1095-1100.

27. Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. Nature. 2004;432:466-472.

28. Sosne G, Christopherson PL, Barrett RP, Fridman R. Thymosin-beta4 modulates corneal matrix metalloproteinase levels and polymorphonuclear cell infiltration after alkali injury. Invest Ophthalmol Vis Sci. 2005;46:2388-2395.

29. Sosne G, Szliter EA, Barrett R, Kernacki KA, Kleinman H, Hazlett LD. Thymosin beta 4 promotes corneal wound healing and decreases inflammation in vivo following alkali injury. Exp Eye Res. 2002;74:293-299.

30. Young JD, Lawrence AJ, MacLean AG, et al. Thymosin beta 4 sulfoxide is an anti-inflammatory agent generated by monocytes in the presence of glucocorticoids. Nat Med. 1999;5:1424-1427.

31. Hayden MS, West AP, Ghosh S. NF-kappaB and the immune response. Oncogene. 2006;25:6758-6780.

32. Sosne G, Qiu P, Christopherson PL, Wheater MK. Thymosin beta 4 suppression of corneal NFkappaB: a potential anti-inflammatory pathway. Exp Eye Res. 2007;84:663-669.

33. Lee P-S, Patel SR, Christiani DC, Bajwa E, Stossel TP, Waxman AB. Plasma gelsolin depletion and circulating actin in sepsis: a pilot study. PLoS ONE. 2008;3:e3712.

34. Lee WM, Galbraith RM. The extracellular actin-scavenger system and actin toxicity. N Engl J Med. 1992;326:1335-1341.

35. Badamchian M, Fagarasan MO, Danner RL, Suffredini AF, Damavandy H, Goldstein AL. Thymosin beta(4) reduces lethality and down-regulates inflammatory mediators in endotoxin-induced septic shock. Int Immunopharmacol. 2003;3:1225-1233.

36. Zhang J, Zhang ZG, Morris D, et al. Neurological functional recovery after thymosin beta4 treatment in mice with experimental auto encephalomyelitis. Neuroscience. 2009;164:1887-1893.

37. Malinda KM, Sidhu GS, Mani H, et al. Thymosin beta4 accelerates wound healing. J Invest Dermatol. 1999;113:364-368.

38. Sosne G, Chan CC, Thai K, et al. Thymosin beta 4 promotes corneal wound healing and modulates inflammatory mediators in vivo. Exp Eye Res. 2001;72:605-608.

39. Fine J-D. Epidermolysis bullosa: a genetic disease of altered cell adhesion and wound healing, and the possible clinical utility of topically applied thymosin beta4. Ann N Y Acad Sci. 2007;1112:396-406.

40. Guarnera G, DeRosa A, Camerini R, sites E. The effect of thymosin treatment of venous ulcers. Ann N Y Acad Sci. 2010;1194:207-212.

41. Morris DC, Chopp M, Zhang L, Lu M, Zhang ZG. Thymosin beta4 improves functional neurological outcome in a rat model of embolic stroke. Neuroscience. 2010;169:674-682.

42. Sun HQ, Yin HL. The beta-thymosin enigma. Ann N Y Acad Sci. 2007;1112:45-55.

43. Fan Y, Gong Y, Ghosh PK, Graham LM, Fox PL. Spatial coordination of actin polymerization and ILK-Akt2 activity during endothelial cell migration. Dev Cell. 2009;16:661-674.

44. Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003;9:653-660.

45. Risau W, Flamme I. Vasculogenesis. Annu Rev Cell Dev Biol. 1995;11:73-91.

46. Hendrix MJC, Seftor EA, Hess AR, Seftor REB. Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. Nat Rev Cancer. 2003;3:411-421.

47. Grant DS, Kinsella JL, Kibbey MC, et al. Matrigel induces thymosin beta 4 gene in differentiating endothelial cells. J Cell Sci. 1995;108 (Pt 12):3685-3694.

48. Grant DS, Rose W, Yaen C, Goldstein A, Martinez J, Kleinman H. Thymosin beta4 enhances endothelial cell differentiation and angiogenesis. Angiogenesis. 1999;3:125-135.

49. Cavasin MA. Therapeutic potential of thymosin-beta4 and its derivative N-acetyl-seryl-aspartyl-lysyl-proline (Ac-SDKP) in cardiac healing after infarction. Am J Cardiovasc Drugs. 2006;6:305-311.

50. Smart N, Risebro CA, Melville AAD, et al. Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. Nature. 2007;445:177-182.

51. Smart N, Dubé KN, Riley PR. Identification of Thymosin β4 as an effector of Hand1-mediated vascular development. Nat Commun. 2010;1:1-10.

52. Dathe V, Brand-Saberi B. Expression of thymosin beta4 during chick development. Anat Embryol. 2004;208:27-32.

53. Oh J-M, Moon E-Y. Actin-sequestering protein, thymosin beta-4, induces paclitaxel resistance through ROS/HIF-1alpha stabilization in HeLa human cervical tumor cells. Life Sci. 2010;87:286-293.

54. Koutrafouri V, Leondiadis L, Avgoustakis K, et al. Effect of thymosin peptides on the chick chorioallantoic membrane angiogenesis model. Biochim Biophys Acta. 2001;1568:60-66.

55. Fruttiger M. Development of the retinal vasculature. Angiogenesis. 2007;10:77-88.

56. Cierniewski CS, Malinowski M, Bednarek R, Cierniewska-Cieslak A. Adhesive and proteolytic phenotype of migrating endothelial cells induced by thymosin beta-4. Ann N Y Acad Sci. 2007;1112:123-139.

57. Malinda KM, Goldstein AL, Kleinman HK. Thymosin beta 4 stimulates directional migration of human umbilical vein endothelial cells. FASEB J. 1997;11:474-481.

58. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. Circ Res. 2005;97:512-523.

59. von Tell D, Armulik A, Betsholtz C. Pericytes and vascular stability. Exp Cell Res. 2006;312:623-629.

60. Stenzel D, Nye E, Nisancioglu M, Adams RH, Yamaguchi Y, Gerhardt H. Peripheral mural cell recruitment requires cell-autonomous heparan sulfate. Blood. 2009;114:915-924.

61. Crosby JR, Seifert RA, Soriano P, Bowen-Pope DF. Chimaeric analysis reveals role of Pdgf receptors in all muscle lineages. Nat Genet. 1998;18:385-388.

62. Hellström M, Kalén M, Lindahl P, Abramsson A, Betsholtz C. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. Development. 1999;126:3047-3055.

63. Hellström M, Phng L-K, Hofmann JJ, et al. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. Nature. 2007;445:776-780.

64. Levéen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev. 1994;8:1875-1887.

65. Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. Science. 1997;277:242-245.

66. Soriano P. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. Genes Dev. 1994;8:1888-1896.

67. Tallquist M, Kazlauskas A. PDGF signaling in cells and mice. Cytokine Growth Factor Rev. 2004;15:205-213.

68. Fantl WJ, Escobedo JA, Williams LT. Mutations of the platelet-derived growth factor receptor that cause a loss of ligand-induced conformational change, subtle changes in kinase activity, and impaired ability to stimulate DNA synthesis. Mol Cell Biol. 1989;9:4473-4478.

69. Kazlauskas A, Durden DL, Cooper JA. Functions of the major tyrosine phosphorylation site of the PDGF receptor beta subunit. Cell Regul. 1991;2:413-425.

70. Roca C, Adams RH. Regulation of vascular morphogenesis by Notch signaling. Genes Dev. 2007;21:2511-2524.

71. Bray SJ. Notch signalling: a simple pathway becomes complex. Nat Rev Mol Cell Biol. 2006;7:678-689.

72. Limbourg A, Ploom M, Elligsen D, et al. Notch ligand Delta-like 1 is essential for postnatal arteriogenesis. Circ Res. 2007;100:363-371.

73. Lobov IB, Renard RA, Papadopoulos N, et al. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proc Natl Acad Sci USA. 2007;104:3219-3224.

74. Suchting S, Freitas C, le Noble F, et al. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. Proc Natl Acad Sci USA. 2007;104:3225-3230.

75. Kalaria RN, Viitanen M, Kalimo H, Dichgans M, Tabira T, Vas-Cog CGo. The pathogenesis of CADASIL: an update. J Neurol Sci. 2004;226:35-39.

76. Joutel A, Monet M, Domenga V, Riant F, Tournier-Lasserve E. Pathogenic mutations associated with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy differently affect Jagged1 binding and Notch3 activity via the RBP/JK signaling Pathway. Am J Hum Genet. 2004;74:338-347.

77. Domenga V, Fardoux P, Lacombe P, et al. Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. Genes Dev. 2004;18:2730-2735.

78. Jin S, Hansson EM, Tikka S, et al. Notch signaling regulates platelet-derived growth factor receptor-beta expression in vascular smooth muscle cells. Circ Res. 2008;102:1483-1491.

79. Shi Y, Massagué J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell. 2003;113:685-700.

80. Moustakas A, Heldin C-H. The regulation of TGFbeta signal transduction. Development. 2009;136:3699-3714.

81. Dupont S, Mamidi A, Cordenonsi M, et al. FAM/USP9x, a deubiquitinating enzyme essential for TGFbeta signaling, controls Smad4 monoubiquitination. Cell. 2009;136:123-135.

82. Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development. 1995;121:1845-1854.

83. Oshima M, Oshima H, Taketo MM. TGF-beta receptor type II deficiency results in defects of yolk sac hematopoiesis and vasculogenesis. Dev Biol. 1996;179:297-302.

84. Seki T, Yun J, Oh SP. Arterial endothelium-specific activin receptor-like kinase 1 expression suggests its role in arterialization and vascular remodeling. Circ Res. 2003;93:682-689.

85. Urness LD, Sorensen LK, Li DY. Arteriovenous malformations in mice lacking activin receptor-like kinase-1. Nat Genet. 2000;26:328-331.

86. Larsson J, Goumans MJ, Sjöstrand LJ, et al. Abnormal angiogenesis but intact hematopoietic potential in TGF-beta type I receptor-deficient mice. EMBO J. 2001;20:1663-1673.

87. Seki T, Hong K-H, Oh SP. Nonoverlapping expression patterns of ALK1 and ALK5 reveal distinct roles of each receptor in vascular development. Lab Invest. 2006;86:116-129.

88. Gaengel K, Genové G, Armulik A, Betsholtz C. Endothelial-mural cell signaling in vascular development and angiogenesis. Arterioscler Thromb Vasc Biol. 2009;29:630-638.

89. Hirschi KK, Rohovsky SA, D'Amore PA. PDGF, TGF-beta, and heterotypic cell-cell interactions mediate endothelial cell-induced recruitment of 10T1/2 cells and their differentiation to a smooth muscle fate. J Cell Biol. 1998;141:805-814.

90. Darland DC, D'Amore PA. TGF beta is required for the formation of capillary-like structures in three-dimensional cocultures of 10T1/2 and endothelial cells. Angiogenesis. 2001;4:11-20.

91. Han Y, Li N, Tian X, Kang J, Yan C, Qi Y. Endogenous transforming growth factor (TGF) beta1 promotes differentiation of smooth muscle cells from embryonic stem cells: stable plasmid-based siRNA silencing of TGF beta1 gene expression. J Physiol Sci. 2010;60:35-41.

92. Sinha S, Hoofnagle MH, Kingston PA, McCanna ME, Owens GK. Transforming growth factor-beta1 signaling contributes to development of smooth muscle cells from embryonic stem cells. Am J Physiol, Cell Physiol. 2004;287:C1560-1568.

93. Carvalho RLC, Itoh F, Goumans M-J, et al. Compensatory signalling induced in the yolk sac vasculature by deletion of TGFbeta receptors in mice. J Cell Sci. 2007;120:4269-4277.

94. Hirschi KK, Burt JM, Hirschi KD, Dai C. Gap junction communication mediates transforming growth factor-beta activation and endothelial-induced mural cell differentiation. Circ Res. 2003;93:429-437.

95. Krüger O, Plum A, Kim JS, et al. Defective vascular development in connexin 45-deficient mice. Development. 2000;127:4179-4193.

96. Smart N, Risebro CA, Melville AA, et al. Thymosin beta4 induces adult epicardial progenitor mobilization and neovascularization. Nature. 2007;445:177-182.

97. Olson EN, Schneider MD. Sizing up the heart: development redux in disease. Genes Dev. 2003;17:1937-1956.

98. Olson EN. Development. The path to the heart and the road not taken. Science. 2001;291:2327-2328.

99. Dyer LA, Kirby ML. The role of secondary heart field in cardiac development. Dev Biol. 2009;336:137-144.

100. Ratajska A, Czarnowska E, Ciszek B. Embryonic development of the proepicardium and coronary vessels. Int J Dev Biol. 2008;52:229-236.

101. Smart N, Risebro CA, Clark JE, et al. Thymosin beta4 facilitates epicardial neovascularization of the injured adult heart. Ann N Y Acad Sci. 2010;1194:97-104.

102. Hinkel R, El-Aouni C, Olson T, et al. Thymosin beta4 is an essential paracrine factor of embryonic endothelial progenitor cell-mediated cardioprotection. Circulation. 2008;117:2232-2240.

103. Uemura A, Ogawa M, Hirashima M, et al. Recombinant angiopoietin-1 restores higher-order architecture of growing blood vessels in mice in the absence of mural cells. J Clin Invest. 2002;110:1619-1628.

104. Gómez-Márquez J, Franco del Amo F, Carpintero P, Anadón R. High levels of mouse thymosin beta4 mRNA in differentiating P19 embryonic cells and during development of cardiovascular tissues. Biochim Biophys Acta. 1996;1306:187-193.

105. Morgan SM, Samulowitz U, Darley L, Simmons DL, Vestweber D. Biochemical characterization and molecular cloning of a novel endothelial-specific sialomucin. Blood. 1999;93:165-175.

106. Eisenberg LM, Markwald RR. Molecular regulation of atrioventricular valvuloseptal morphogenesis. Circ Res. 1995;77:1-6.

107. Anadón R, Rodríguez Moldes I, Carpintero P, et al. Differential expression of thymosins beta(4) and beta(10) during rat cerebellum postnatal development. Brain Res. 2001;894:255-265.

108. Carpinterio P, Anadón R, del Amo FF, Gómez-Márquez J. The thymosin beta 4 gene is strongly activated in neural tissues during early postimplantation mouse development. Neurosci Lett. 1995;184:63-66.

109. Jessell TM. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. Nat Rev Genet. 2000;1:20-29.

110. Gerety SS, Wang HU, Chen ZF, Anderson DJ. Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. Mol Cell. 1999;4:403-414.

111. Zhong TP, Childs S, Leu JP, Fishman MC. Gridlock signalling pathway fashions the first embryonic artery. Nature. 2001;414:216-220.

112. Jain RK. Molecular regulation of vessel maturation. Nat Med. 2003;9:685-693.

113. Iwatsuki M, Mimori K, Yokobori T, et al. Epithelial-mesenchymal transition in cancer development and its clinical significance. Cancer Sci. 2010;101:293-299.

114. Winter EM, Gittenberger-de Groot AC. Epicardium-derived cells in cardiogenesis and cardiac regeneration. Cell Mol Life Sci. 2007;64:692-703.

115. Pak CW, Flynn KC, Bamburg JR. Actin-binding proteins take the reins in growth cones. Nat Rev Neurosci. 2008;9:136-147.

116. Gupta A, Tsai L-H, Wynshaw-Boris A. Life is a journey: a genetic look at neocortical development. Nat Rev Genet. 2002;3:342-355.

117. Xiong Y, Mahmood A, Meng Y, et al. Treatment of traumatic brain injury with thymosin beta(4) in rats. Journal of neurosurgery. 2010.

118. Mollinari C, Ricci-Vitiani L, Pieri M, et al. Downregulation of thymosin beta4 in neural progenitor grafts promotes spinal cord regeneration. J Cell Sci. 2009;122:4195-4207.

119. de Santa Barbara P, van den Brink GR, Roberts DJ. Development and differentiation of the intestinal epithelium. Cell Mol Life Sci. 2003;60:1322-1332.

120. Nemolato S, Cabras T, Cau F, et al. Different thymosin Beta 4 immunoreactivity in foetal and adult gastrointestinal tract. PLoS ONE. 2010;5:e9111.

121. Morrisey EE, Hogan BLM. Preparing for the first breath: genetic and cellular mechanisms in lung development. Dev Cell. 2010;18:8-23.

122. Lindahl P, Karlsson L, Hellström M, et al. Alveogenesis failure in PDGF-A-deficient mice is coupled to lack of distal spreading of alveolar smooth muscle cell progenitors during lung development. Development. 1997;124:3943-3953.

123. Qureshi ST, Skamene E, Malo D. Comparative genomics and host resistance against infectious diseases. Emerging Infect Dis. 1999;5:36-47.

124. J. Tymms M, Kola I. Gene knockout protocols‚- Page ii. 2001:431.

125. Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature. 1996;380:435-439.

126. Ferrara N, Carver-Moore K, Chen H, et al. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature. 1996;380:439-442.

127. Abraham S, Kogata N, Fässler R, Adams RH. Integrin beta1 subunit controls mural cell adhesion, spreading, and blood vessel wall stability. Circ Res. 2008;102:562-570.

128. Foo SS, Turner CJ, Adams S, et al. Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly. Cell. 2006;124:161-173.

129. Albinsson S, Suarez Y, Skoura A, Offermanns S, Miano JM, Sessa WC. MicroRNAs are necessary for vascular smooth muscle growth, differentiation, and function. Arterioscler Thromb Vasc Biol. 2010;30:1118-1126.

130. Kono M, Mi Y, Liu Y, et al. The sphingosine-1-phosphate receptors S1P1, S1P2, and S1P3 function coordinately during embryonic angiogenesis. J Biol Chem. 2004;279:29367-29373.

131. Hannon GJ. RNA interference. Nature. 2002;418:244-251.

132. Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. Dev Biol. 2001;230:230-242.

133. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999;21:70-71.

134. Srinivas S, Watanabe T, Lin CS, et al. Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. BMC Dev Biol. 2001;1:4.

135. Calero-Nieto FJ, Bert AG, Cockerill PN. Transcription-dependent silencing of inducible convergent transgenes in transgenic mice. Epigenetics Chromatin. 2010;3:3.

136. Heaney JD, Rettew AN, Bronson SK. Tissue-specific expression of a BAC transgene targeted to the Hprt locus in mouse embryonic stem cells. Genomics. 2004;83:1072-1082.

137. Moses KA, DeMayo F, Braun RM, Reecy JL, Schwartz RJ. Embryonic expression of an Nkx2-5/Cre gene using ROSA26 reporter mice. Genesis. 2001;31:176-180.

138. Chang A, Nishiyama A, Peterson J, Prineas J, Trapp BD. NG2-positive oligodendrocyte progenitor cells in adult human brain and multiple sclerosis lesions. J Neurosci. 2000;20:6404-6412.

139. Matsubara TA, Murata TA, Wu GS, Barron EA, Rao NA. Isolation and culture of rat retinal microvessel endothelial cells using magnetic beads coated with antibodies to PECAM-1. Curr Eye Res. 2000;20:1-7.

140. Holtz ML, Misra RP. Endothelial-specific ablation of serum response factor causes hemorrhaging, yolk sac vascular failure, and embryonic lethality. BMC Dev Biol. 2008;8:65.

141. Schmidt-Supprian M, Rajewsky K. Vagaries of conditional gene targeting. Nat Immunol. 2007;8:665-668.

142. Ciavatta D, Kalantry S, Magnuson T, Smithies O. A DNA insulator prevents repression of a targeted X-linked transgene but not its random or imprinted X inactivation. Proc Natl Acad Sci USA. 2006;103:9958-9963.

143. Priestley GV, Ulyanova T, Papayannopoulou T. Sustained alterations in biodistribution of stem/progenitor cells in Tie2Cre+ alpha4(f/f) mice are hematopoietic cell autonomous. Blood. 2007;109:109-111.

144. Schlaeger TM, Mikkola HKA, Gekas C, Helgadottir HB, Orkin SH. Tie2Cre-mediated gene ablation defines the stem-cell leukemia gene (SCL/tal1)-dependent window during hematopoietic stem-cell development. Blood. 2005;105:3871-3874.

145. Claxton S, Kostourou V, Jadeja S, Chambon P, Hodivala-Dilke K, Fruttiger M. Efficient, inducible Cre-recombinase activation in vascular endothelium. Genesis. 2008;46:74-80.

146. Yamada Y, Oike Y, Ogawa H, et al. Neuropilin-1 on hematopoietic cells as a source of vascular development. Blood. 2003;101:1801-1809.

147. Drogat B, Kalucka J, Gutiérrez L, et al. Vegf regulates embryonic erythroid development through Gata1 modulation. Blood. 2010;116:2141-2151.

148. Anggrahini DW, Emoto N, Nakayama K, et al. Vascular endothelial cell-derived endothelin-1 mediates vascular inflammation and neointima formation following blood flow cessation. Cardiovasc Res. 2009;82:143-151.

149. Katz SG, Williams A, Yang J, et al. Endothelial lineage-mediated loss of the GATA cofactor Friend of GATA 1 impairs cardiac development. Proc Natl Acad Sci USA. 2003;100:14030-14035.

150. Tanjore H, Zeisberg EM, Gerami-Naini B, Kalluri R. Beta1 integrin expression on endothelial cells is required for angiogenesis but not for vasculogenesis. Dev Dyn. 2008;237:75-82.

151. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development. 1998;125:1591-1598.

152. Lindblom P, Gerhardt H, Liebner S, et al. Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. Genes Dev. 2003;17:1835-1840.

153. Miyata T, Iizasa H, Sai Y, Fujii J, Terasaki T, Nakashima E. Platelet-derived growth factor-BB (PDGF-BB) induces differentiation of bone marrow endothelial progenitor cell-derived cell line TR-BME2 into mural cells, and changes the phenotype. J Cell Physiol. 2005;204:948-955.

154. Barkić M, Crnomarković S, Grabusić K, et al. The p53 tumor suppressor causes congenital malformations in Rpl24-deficient mice and promotes their survival. Mol Cell Biol. 2009;29:2489-2504.

155. Jonassen JA, San Agustin J, Follit JA, Pazour GJ. Deletion of IFT20 in the mouse kidney causes misorientation of the mitotic spindle and cystic kidney disease. J Cell Biol. 2008;183:377-384.

156. Leung YF, Cavalieri D. Fundamentals of cDNA microarray data analysis. Trends Genet. 2003;19:649-659.

157. Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science. 1995;270:467-470.

158. Callow MJ, Dudoit S, Gong EL, Speed TP, Rubin EM. Microarray expression profiling identifies genes with altered expression in HDL-deficient mice. Genome Res. 2000;10:2022-2029.

159. Gelineau-van Waes J, Maddox JR, Smith LM, et al. Microarray analysis of E9.5 reduced folate carrier (RFC1; Slc19a1) knockout embryos reveals altered expression of genes in the cubilin-megalin multiligand endocytic receptor complex. BMC Genomics. 2008;9:156.

160. Franco CA, Mericskay M, Parlakian A, et al. Serum response factor is required for sprouting angiogenesis and vascular integrity. Dev Cell. 2008;15:448-461.

161. Phng L-K, Potente M, Leslie JD, et al. Nrarp coordinates endothelial Notch and Wnt signaling to control vessel density in angiogenesis. Dev Cell. 2009;16:70-82.

162. Renner O, Romero L, Carnero A, Betsholtz C, Euler M. Identification of transcripts specific for physiological gene activation by platelet-derived growth factor (PDGF)-B in intact brain tissue. J Cell Biochem. 2005;95:859-867.

163. Goumans M-J, Valdimarsdottir G, Itoh S, Rosendahl A, Sideras P, Ten Dijke P. Balancing the activation state of the endothelium via two distinct TGF-beta type I receptors. EMBO J. 2002;21:1743-1753.

164. Pietenpol JA, Stein RW, Moran E, et al. TGF-beta 1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming proteins with pRB binding domains. Cell. 1990;61:777-785.

165. Matsuyama S, Iwadate M, Kondo M, et al. SB-431542 and Gleevec inhibit transforming growth factor-beta-induced proliferation of human osteosarcoma cells. Cancer Res. 2003;63:7791-7798.

166. Liaw CW, Cannon C, Power MD, Kiboneka PK, Rubin LL. Identification and cloning of two species of cadherins in bovine endothelial cells. EMBO J. 1990;9:2701-2708.

167. Dejana E, Tournier-Lasserve E, Weinstein BM. The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications. Dev Cell. 2009;16:209-221.

168. Hogan BM, Bos FL, Bussmann J, et al. Ccbe1 is required for embryonic lymphangiogenesis and venous sprouting. Nat Genet. 2009;41:396-398.

169. Chen S-C, Wang B-W, Wang DL, Shyu K-G. Hypoxia induces discoidin domain receptor-2 expression via the p38 pathway in vascular smooth muscle cells to increase their migration. Biochem Biophys Res Commun. 2008;374:662-667.

170. Eiraku M, Hirata Y, Takeshima H, Hirano T, Kengaku M. Delta/notch-like epidermal growth factor (EGF)-related receptor, a novel EGF-like repeat-containing protein targeted to dendrites of developing and adult central nervous system neurons. J Biol Chem. 2002;277:25400-25407.

171. Duan L-J, Zhang-Benoit Y, Fong G-H. Endothelium-intrinsic requirement for Hif-2alpha during vascular development. Circulation. 2005;111:2227-2232.

172. Yanagisawa H, Schluterman MK, Brekken RA. Fibulin-5, an integrin-binding matricellular protein: its function in development and disease. J Cell Commun Signal. 2009;3:337-347.

173. Abid MR, Yano K, Guo S, et al. Forkhead transcription factors inhibit vascular smooth muscle cell proliferation and neointimal hyperplasia. J Biol Chem. 2005;280:29864-29873.

174. Potente M, Urbich C, Sasaki K-i, et al. Involvement of Foxo transcription factors in angiogenesis and postnatal neovascularization. J Clin Invest. 2005;115:2382-2392.

175. Shin DH, Chun Y-S, Lee DS, Huang LE, Park J-W. Bortezomib inhibits tumor adaptation to hypoxia by stimulating the FIH-mediated repression of hypoxia-inducible factor-1. Blood. 2008;111:3131-3136.

176. Hsieh T, Gordon RE, Clemmons DR, Busby WH, Duan C. Regulation of vascular smooth muscle cell responses to insulin-like growth factor (IGF)-I by local IGF-binding proteins. J Biol Chem. 2003;278:42886-42892.

177. Clyman RI, Turner DC, Kramer RH. An alpha 1/beta 1-like integrin receptor on rat aortic smooth muscle cells mediates adhesion to laminin and collagen types I and IV. Arteriosclerosis. 1990;10:402-409.

178. Heydemann A, Ceco E, Lim JE, et al. Latent TGF-beta-binding protein 4 modifies muscular dystrophy in mice. J Clin Invest. 2009;119:3703-3712.

179. Wu L, Aster JC, Blacklow SC, Lake R, Artavanis-Tsakonas S, Griffin JD. MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. Nat Genet. 2000;26:484-489.

180. Turner CE, Pietras KM, Taylor DS, Molloy CJ. Angiotensin II stimulation of rapid paxillin tyrosine phosphorylation correlates with the formation of focal adhesions in rat aortic smooth muscle cells. J Cell Sci. 1995;108 (Pt 1):333-342.

181. Zippo A, De Robertis A, Bardelli M, Galvagni F, Oliviero S. Identification of Flk-1 target genes in vasculogenesis: Pim-1 is required for endothelial and mural cell differentiation in vitro. Blood. 2004;103:4536-4544.

182. Lampugnani MG, Corada M, Caveda L, et al. The molecular organization of endothelial cell to cell junctions: differential association of plakoglobin, beta-catenin, and alpha-catenin with vascular endothelial cadherin (VE-cadherin). J Cell Biol. 1995;129:203-217.

183. Zhang B, Dietrich UM, Geng J-G, Bicknell R, Esko JD, Wang L. Repulsive axon guidance molecule Slit3 is a novel angiogenic factor. Blood. 2009;114:4300-4309.

184. Nishimoto S, Hamajima Y, Toda Y, Toyoda H, Kitamura K, Komurasaki T. Identification of a novel smooth muscle associated protein, smap2, upregulated during neointima formation in a rat carotid endarterectomy model. Biochim Biophys Acta. 2002;1576:225-230.

185. Li F, Duman-Scheel M, Yang D, et al. Sonic hedgehog signaling induces vascular smooth muscle cell proliferation via induction of the G1 cyclin-retinoblastoma axis. Arterioscler Thromb Vasc Biol. 2010;30:1787-1794.

186. Vokes SA, Yatskievych TA, Heimark RL, et al. Hedgehog signaling is essential for endothelial tube formation during vasculogenesis. Development. 2004;131:4371-4380.

187. Keon BH, Schäfer S, Kuhn C, Grund C, Franke WW. Symplekin, a novel type of tight junction plaque protein. J Cell Biol. 1996;134:1003-1018.

188. Yamazaki D, Suetsugu S, Miki H, et al. WAVE2 is required for directed cell migration and cardiovascular development. Nature. 2003;424:452-456.

189. Barnard DC, Ryan K, Manley JL, Richter JD. Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. Cell. 2004;119:641-651.

190. Buchert M, Darido C, Lagerqvist E, et al. The symplekin/ZONAB complex inhibits intestinal cell differentiation by the repression of AML1/Runx1. Gastroenterology. 2009;137:156-164, 164.e151-153.

191. Buchert M, Papin M, Bonnans C, et al. Symplekin promotes tumorigenicity by up-regulating claudin-2 expression. Proc Natl Acad Sci USA. 2010;107:2628-2633.

192. Mitic LL, Anderson JM. Molecular architecture of tight junctions. Annu Rev Physiol. 1998;60:121-142.

193. Lindskog H, Athley E, Larsson E, Lundin S, Hellström M, Lindahl P. New insights to vascular smooth muscle cell and pericyte differentiation of mouse embryonic stem cells in vitro. Arterioscler Thromb Vasc Biol. 2006;26:1457-1464.

194. Manabe I, Owens GK. Recruitment of serum response factor and hyperacetylation of histones at smooth muscle-specific regulatory regions during differentiation of a novel P19-derived in vitro smooth muscle differentiation system. Circ Res. 2001;88:1127-1134.

195. Abramsson A, Lindblom P, Betsholtz C. Endothelial and nonendothelial sources of PDGF-B regulate pericyte recruitment and influence vascular pattern formation in tumors. J Clin Invest. 2003;112:1142-1151.

196. Paradis H, Liu C-Y, Saika S, et al. Tubedown-1 in remodeling of the developing vitreal vasculature in vivo and regulation of capillary outgrowth in vitro. Dev Biol. 2002;249:140-155.

197. Ozerdem U, Grako KA, Dahlin-Huppe K, Monosov E, Stallcup WB. NG2 proteoglycan is expressed exclusively by mural cells during vascular morphogenesis. Dev Dyn. 2001;222:218-227.

198. MacFadyen J, Savage K, Wienke D, Isacke CM. Endosialin is expressed on stromal fibroblasts and CNS pericytes in mouse embryos and is downregulated during development. Gene Expr Patterns. 2007;7:363-369.

199. Wakui S, Yokoo K, Muto T, et al. Localization of Ang-1, -2, Tie-2, and VEGF expression at endothelial-pericyte interdigitation in rat angiogenesis. Lab Invest. 2006;86:1172-1184.

200. Nehls V, Denzer K, Drenckhahn D. Pericyte involvement in capillary sprouting during angiogenesis in situ. Cell Tissue Res. 1992;270:469-474.

201. Xiao Q, Luo Z, Pepe AE, Margariti A, Zeng L, Xu Q. Embryonic stem cell differentiation into smooth muscle cells is mediated by Nox4-produced H2O2. Am J Physiol, Cell Physiol. 2009;296:C711-723.

202. Gomez-Duran A, Mulero-Navarro S, Chang X, Fernandez-Salguero PM. LTBP-1 blockade in dioxin receptor-null mouse embryo fibroblasts decreases TGF-beta activity: Role of extracellular proteases plasmin and elastase. J Cell Biochem. 2006;97:380-392.

203. Karlsson G, Liu Y, Larsson J, et al. Gene expression profiling demonstrates that TGF-beta1 signals exclusively through receptor complexes involving Alk5 and identifies targets of TGF-beta signaling. Physiol Genomics. 2005;21:396-403.

204. Eyden B. The myofibroblast: phenotypic characterization as a prerequisite to understanding its functions in translational medicine. J Cell Mol Med. 2008;12:22-37.

205. Greenberg RS, Bernstein AM, Benezra M, Gelman IH, Taliana L, Masur SK. FAK-dependent regulation of myofibroblast differentiation. FASEB J. 2006;20:1006-1008.

206. Ehrlich HP, Hazard SW. Thymosin beta4 enhances repair by organizing connective tissue and preventing the appearance of myofibroblasts. Ann N Y Acad Sci. 2010;1194:118-124.

207. Velasco S, Alvarez-Muñoz P, Pericacho M, et al. L- and S-endoglin differentially modulate TGFbeta1 signaling mediated by ALK1 and ALK5 in L6E9 myoblasts. J Cell Sci. 2008;121:913-919.

208. Oh SP, Seki T, Goss KA, et al. Activin receptor-like kinase 1 modulates transforming growth factor-beta 1 signaling in the regulation of angiogenesis. Proc Natl Acad Sci USA. 2000;97:2626-2631.

209. Roelen BA, van Rooijen MA, Mummery CL. Expression of ALK-1, a type 1 serine/threonine kinase receptor, coincides with sites of vasculogenesis and angiogenesis in early mouse development. Dev Dyn. 1997;209:418-430.

210. Konieczny SF, Emerson CP. 5-Azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. Cell. 1984;38:791-800.

211. Cunha SI, Pardali E, Thorikay M, et al. Genetic and pharmacological targeting of activin receptor-like kinase 1 impairs tumor growth and angiogenesis. J Exp Med. 2010;207:85-100.

212. Inman GJ, Nicolás FJ, Callahan JF, et al. SB-431542 is a potent and specific inhibitor of transforming growth factor-beta superfamily type I activin receptor-like kinase (ALK) receptors ALK4, ALK5, and ALK7. Mol Pharmacol. 2002;62:65-74.

213. Maynard JA, Lindquist NC, Sutherland JN, et al. Surface plasmon resonance for high-throughput ligand screening of membrane-bound proteins. Biotechnol J. 2009;4:1542-1558.

214. Leong GM, Subramaniam N, Figueroa J, et al. Ski-interacting protein interacts with Smad proteins to augment transforming growth factor-beta-dependent transcription. J Biol Chem. 2001;276:18243-18248.

215. Smart N, Rossdeutsch A, Riley PR. Thymosin beta4 and angiogenesis: modes of action and therapeutic potential. Angiogenesis. 2007;10:229-241.

216. De Smet F, Segura I, De Bock K, Hohensinner PJ, Carmeliet P. Mechanisms of vessel branching: filopodia on endothelial tip cells lead the way. Arterioscler Thromb Vasc Biol. 2009;29:639-649.

217. Philp D, Nguyen M, Scheremeta B, et al. Thymosin beta4 increases hair growth by activation of hair follicle stem cells. FASEB J. 2004;18:385-387.

218. Jakobsson L, Franco CA, Bentley K, et al. Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. Nat Cell Biol. 2010;12:943-953.

219. Lu X, le Noble F, Yuan L, et al. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. Nature. 2004;432:179-186.

220. Ruhrberg C, Gerhardt H, Golding M, et al. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes Dev. 2002;16:2684-2698.

221. Hannappel E, van Kampen M. Determination of thymosin beta 4 in human blood cells and serum. J Chromatogr. 1987;397:279-285.

222. Weller FE, Shah U, Cummings GD, Chretien PB, Mutchnick MG. Serum levels of immunoreactive thymosin alpha 1 and thymosin beta 4 in large cohorts of healthy adults. Thymus. 1992;19:45-52.

223. Badamchian M, Damavandy AA, Damavandy H, Wadhwa SD, Katz B, Goldstein AL. Identification and quantification of thymosin beta4 in human saliva and tears. Ann N Y Acad Sci. 2007;1112:458-465.

224. Huang WQ, Wang QR. Bone marrow endothelial cells secrete thymosin beta4 and AcSDKP. Exp Hematol. 2001;29:12-18.

225. Schatz G, Dobberstein B. Common principles of protein translocation across membranes. Science. 1996;271:1519-1526.

226. Nickel W. Unconventional secretory routes: direct protein export across the plasma membrane of mammalian cells. Traffic. 2005;6:607-614.

227. Keller M, Rüegg A, Werner S, Beer H-D. Active caspase-1 is a regulator of unconventional protein secretion. Cell. 2008;132:818-831.

228. Guo X, Wang X-F. Signaling cross-talk between TGF-beta/BMP and other pathways. Cell Res. 2009;19:71-88.

229. Asano Y, Ihn H, Yamane K, Jinnin M, Mimura Y, Tamaki K. Phosphatidylinositol 3-kinase is involved in alpha2(I) collagen gene expression in normal and scleroderma fibroblasts. J Immunol. 2004;172:7123-7135.

230. Song K, Wang H, Krebs TL, Danielpour D. Novel roles of Akt and mTOR in suppressing TGF-beta/ALK5-mediated Smad3 activation. EMBO J. 2006;25:58-69.

231. Cierniewski CS, Papiewska-Pajak I, Malinowski M, et al. Thymosin beta4 regulates migration of colon cancer cells by a pathway involving interaction with Ku80. Ann N Y Acad Sci. 2010;1194:60-71.

232. Muller C, Paupert J, Monferran S, Salles B. The double life of the Ku protein: facing the DNA breaks and the extracellular environment. Cell Cycle. 2005;4:438-441.

233. Ho JH-C, Chuang C-H, Ho C-Y, Shih Y-RV, Lee OK-S, Su Y. Internalization is essential for the antiapoptotic effects of exogenous thymosin beta-4 on human corneal epithelial cells. Invest Ophthalmol Vis Sci. 2007;48:27-33.

234. Milewicz DM, Guo D-C, Tran-Fadulu V, et al. Genetic basis of thoracic aortic aneurysms and dissections: focus on smooth muscle cell contractile dysfunction. Annu Rev Genomics Hum Genet. 2008;9:283-302.

235. Tung WS, Lee JK, Thompson RW. Simultaneous analysis of 1176 gene products in normal human aorta and abdominal aortic aneurysms using a membrane-based complementary DNA expression array. J Vasc Surg. 2001;34:143-150.

236. Allaire E, Muscatelli-Groux B, Guinault A-M, et al. Vascular smooth muscle cell endovascular therapy stabilizes already developed aneurysms in a model of aortic injury elicited by inflammation and proteolysis. Ann Surg. 2004;239:417-427.

237. Dai J, Losy F, Guinault A-M, et al. Overexpression of transforming growth factor-beta1 stabilizes already-formed aortic aneurysms: a first approach to induction of functional healing by endovascular gene therapy. Circulation. 2005;112:1008-1015.

238. Motiejūnaite R, Kazlauskas A. Pericytes and ocular diseases. Exp Eye Res. 2008;86:171-177.

239. Hammes H-P, Lin J, Renner O, et al. Pericytes and the pathogenesis of diabetic retinopathy. Diabetes. 2002;51:3107-3112.

240. Rajantie I, Ilmonen M, Alminaite A, Ozerdem U, Alitalo K, Salven P. Adult bone marrow-derived cells recruited during angiogenesis comprise precursors for periendothelial vascular mural cells. Blood. 2004;104:2084-2086.

241. Hashizume H, Baluk P, Morikawa S, et al. Openings between defective endothelial cells explain tumor vessel leakiness. Am J Pathol. 2000;156:1363-1380.

242. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nature clinical practice Oncology. 2006;3:24-40.

243. Gerhardt H, Semb H. Pericytes: gatekeepers in tumour cell metastasis? J Mol Med. 2008;86:135-144.

244. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. J Clin Invest. 2003;111:1287-1295.

245. Zhang Y, Feurino LW, Zhai Q, et al. Thymosin Beta 4 is overexpressed in human pancreatic cancer cells and stimulates proinflammatory cytokine secretion and JNK activation. Cancer Biol Ther. 2008;7:419-423.

Appendix 1: Top 100 Genes Down Regulated in E12.5 Tβ4 -/Y Embryos

Appendix 2: Top 100 Genes Up Regulated in E12.5 Tβ4 -/Y Embryos

Appendix 3: Top 100 Genes Down Regulated in Tβ4 -/Y Adult Hearts

Appendix 4: Top 100 Genes Up Regulated in Tβ4 -/Y Adult Hearts