Thymosin β4 and the Vasculature: Multiple Roles in Development, Repair and Protection against Disease.

ABSTRACT

Introduction: Formation of the vasculature is a complex process, defects in which can lead to embryonic lethality or disease in later life. Understanding mechanisms of vasculogenesis may facilitate the treatment of developmental defects and may be extrapolated to promote wound healing and tissue repair. Thymosin β4 (Tβ4) is an actin monomer binding protein with recognized roles in vascular development, neovascularization and protection against disease.

Areas covered: Vascular network assembly is complex, regulated by multiple signals and cell types; Tβ4 functions in many of the underlying processes, including vasculogenesis, angiogenesis, arteriogenesis, endothelial-mesenchymal transition and extracellular matrix remodeling. Loss of Tβ4 perturbs vessel growth and stability, whereas exogenous application enhances capillary formation and pericyte recruitment, during development and in injury models.

Expert opinion: Although vascular functions for Tβ4 have been well documented, the underlying molecular mechanisms remain obscure. While Tβ4-induced cytoskeletal remodeling likely mediates the directional migration of endothelial cells, paracrine roles have also been implicated in migration and differentiation of smooth muscle cells. Moreover, nuclear functions of Tβ4 have been described but remain to be explored in the vasculature. Delineating the molecular pathways impacted by Tβ4 to promote vascular growth and remodeling may reveal novel targets for prevention and treatment of vascular disease.

Keywords: Angiogenesis; Arteriogenesis; Endocardium; Endothelial cells; Endothelial-Mesenchymal Transition; Epicardium; Neovascularization; Smooth Muscle Differentiation; Thymosin β4; Vasculogenesis.

1. INTRODUCTION

The process of blood vessel growth is essential for development, disruption of which can lead to embryonic lethality, congenital defects or vascular disease in later life. Knowledge of the cellular and molecular mechanisms of vascular development may directly enable correction of developmental defects and, moreover, insight into these processes may be extrapolated to promote therapeutic neovascularization and tissue regeneration. Indeed, it has become apparent that roles in development are endogenously recapitulated in adulthood for repair, even if these intrinsic responses are frequently inadequate to elicit sufficient regeneration, for example in the heart post-myocardial infarction (MI). Novel therapeutic strategies are required as angiogenic growth factor therapy has been ineffective in clinical trials[1],[2] and progenitor cell transplantation approaches hindered by limited engraftment and trans-differentiation[3].

Thymosin β4 (Tβ4) has been implicated in vascular development[4-6] and in endogenous repair mechanisms[7]; moreover, its exogenous application has been shown to potently induce neovascularization and regeneration[8-13]. The multiple roles identified for Tβ4 will be reviewed, along with recently-acquired insights into the underlying mechanisms, which may reveal novel therapeutic targets for vascular repair.

1.1 Development and Maintenance of Blood Vessels

The arrangement of blood vessels in vertebrates is highly organized and optimally arranged such that flow can be achieved with minimal work (Murray's law) and physiological demands of all organs in the body can be met. This is achieved by tightly regulated, yet dynamic, morphological processes. For example, in the yolk sac vasculature, a simple honeycomb-like capillary plexus is transformed into an extensive hierarchical network of arteries and veins, structurally and functionally distinct; dysfunction during this phase greatly compromises embryonic viability. The process of vascular development (reviewed in[14]) is predicated upon successive phases: i) *vasculogenesis*, the *de novo* formation of mesoderm-derived angioblasts or endothelial cell (EC) precursors and assembly of a primitive capillary plexus; ii) *angiogenesis*, the dynamic remodeling of pre-existing vessels that drives expansion of the capillary network by sprouting and intussusception; iii) *arteriogenesis*, the increase in diameter of existing vessels, with accompanying recruitment of pericyte and smooth muscle coverage, resulting in formation of large conductance arteries. Once matured, blood vessels require turnover, with a reported contribution from circulating endothelial progenitor cells (EPCs) [15], and stability, by close association and reciprocal signaling with smooth muscle cells (SMCs), for maintenance throughout adulthood. It is now evident that failure of endothelial homeostasis underlies many of the clinical complications associated with peripheral vascular disease, sepsis, aneurysms and atherosclerosis[16].

While, in general, the systemic and organ-specific vasculatures form via the same fundamental mechanisms and fulfil similar roles, each organ requires unique and versatile properties to respond to changing systemic and local needs. A relevant example, the coronary

vasculature, is described here due to the prevalence of atherosclerosis and the multiple roles for Tβ4 in the heart[6, 8, 10, 17, 18].

1.2 The Coronary Vasculature

Coronary vessel development proceeds via compartmentalized contribution of coronary ECs from the sinus venosus, a transient structure that returns venous blood to the embryonic heart[19], and the ventricular endocardium[20] (reviewed in[21]). In the early weeks after birth, the continued expansion of the capillary network also derives ECs from the endocardium, via a distinct mechanism; coincident with myocardial compaction, endocardial cells on the trabeculated surfaces are trapped within the muscle and coalesce to form new vessels[22]. A subcompartment of the proepicardium, a transient precursor of the epicardium, contributes a further 5-15% of coronary ECs[23, 24], as well as coronary SMCs and adventitial fibroblasts to support the forming vessels [25].

1.3 Thymosin β4

Originally isolated from calf thymus, Tβ4 is a highly conserved 4.9 kDa peptide comprising 44 amino acid residues[26]; upon synthesis, the initiator methionine residue is removed and the N-terminal serine becomes acetylated[27]. Tβ4 is expressed in all cell types, except erythrocytes[28], and abundant in serum, despite the absence of a signal peptide[29]. Tβ4 has been shown to be a glutaminyl substrate for transglutaminase, linking Tβ4 to both fibrin and collagen *in vitro*[30, 31], supporting the proposal that release of Tβ4 from dying cells and subsequent attachment to fibrin increases local Tβ4 concentrations to signal tissue damage[31]. Furthermore,

Tβ4 is markedly upregulated around the sites of inflammation[32] and has multifunctional actions in the context of wound healing (reviewed in [33]).

Historically, Tβ4 was considered to function solely as the major G-actin sequestering protein in the cytoplasm. Upon desequestration, monomeric actin subunits polymerize, to extend microfilaments of the cytoskeleton[34]; consistent with this, Tβ4 has been extensively implicated in cell motility[35, 36]. Although its role in actin-binding is well documented, a growing number of studies provide evidence of intercellular roles for Tβ4[6, 17]. Tβ4 has been shown to be an essential paracrine factor, secreted from EPCs[37], ECs [5] and cardiomyocytes[6], possibly utilizing an unconventional pathway for its secretion, along the lines of FGFs, which also lack signal peptides, or secreted via extracellular vesicles. While the mechanism of Tβ4 entry into cells is similarly unclear, exogenous Tβ4 has been shown to be rapidly internalized[38]. Moreover, despite the lack of a nuclear localization signal , Tβ4 has been reported to enter the nucleus[39, 40]. Consistent with a putative nuclear role, Tβ4-mediated regulation of a number of gene transcripts has been reported[41]. How Tβ4 modulates gene expression remains unclear, although recent studies implicating Tβ4 in chromatin remodelling[42] and putative roles in splicing[43] may point to mechanisms involving recruitment of actin to nuclear complexes.

There is now an extensive literature on the pleiotropic roles of Tβ4 in physiology, pathology and repair of various organ systems. This review will focus on identified roles for the peptide in the development, homeostasis and repair of the vasculature.

2. 1 The Endogenous Roles of Tβ4 in Vascular Development

2.1.1 The Coronary Vasculature

The first suggestion of a putative role for Tβ4 in cardiovascular development was the observation of high levels of *Tβ4* transcripts in developing blood vessels and the heart from mid-gestation murine embryos[44]. The functional role of Tβ4 in the heart was later confirmed in experiments in which Tβ4 expression was selectively knocked down in the embryonic myocardium[6]. Cardiac knockdown of Tβ4 was embryonic lethal and analysis of mutant embryos revealed impaired coronary vessel development. Mechanistically, malformed coronary vasculature was attributed to the observed failure in epicardial cell differentiation and migration into the myocardium since lack of Tβ4 disrupted myocardial compaction, with ECs and SMCs trapped in nodules on the epicardial surface and within the subepicardial space[6]. Notably, the effects of Tβ4 appeared non-cell autonomous since myocardial Tβ4 ablation resulted in an epicardial defect. It should be noted that, in contrast, a subsequent study found that Tβ4 was dispensable for cardiac development and adult heart function[45]. The disparity between these studies has been suggested[46] to result primarily due to a compensatory mechanism with gene deletion that is not induced with hypomorphic shRNA embryos[6]; genetic background differences may also contribute towards the exacerbated knockdown phenotype. In support of this, the gain-of-function roles of exogenously administered TB4, demonstrated in various contexts, are consistent with the roles inferred by loss-of-function. The demonstration that loss of Tβ4 can be compensated for does not preclude a role for Tβ4 to ordinarily function in such a context.

2.1.2 The Systemic Vasculature

The requirement for Tβ4 in embryonic SMC differentiation is not limited to the developing coronary vasculature. Indeed, it has been shown to function more broadly throughout the systemic vasculature[5] and in the embryonic yolk sac vasculature[4]. Rossdeutsch *et al.* demonstrated that global knockout of Tβ4 ($T\beta4^{KO}$) resulted in embryonic lethality in a portion of mutant mice, coinciding with vascular hemorrhage and a reduction in SMC coverage surrounding the dorsal aorta[5]. EC-specific knockdown of Tβ4 was shown to phenocopy the global Tβ4 knockout, leading to the conclusion that, within the systemic vasculature, endothelial Tβ4 is required to induce surrounding mesoderm to differentiate into smooth muscle[5]. Exon array analyses revealed a disruption in the TGFβ pathway and relative expression of genes downstream of TGFβ signaling (*Pai-1*, *Id1* and *c-myc*) was reduced in Tβ4 null embryos. Furthermore, the extent of TGFβ pathway repression correlated with the severity of the phenotype[5].

2.1.3 The Yolk Sac Vasculature

The suggestion that Tβ4 may promote mural cell differentiation via the TGF-β pathway is consistent with a mechanism proposed for differentiation of the yolk sac vasculature. Tβ4 was identified by representational difference analysis[47], and confirmed by chromatin immunoprecipitation and transcriptional assays[4], to be a downstream target of the basic helix-loop-helix transcription factor, Hand1. *Hand1*-null embryoid bodies revealed vascular differentiation defects and *Hand1*-null embryos displayed defective yolk sac vasculogenesis[4]. Exogenous administration of TB4 rescued expression of EC and SMC markers in *Hand1*-null embryoid body

cultures and, moreover, rescued *Hand1*-null embryos to prolong survival. In contrast to control embryos, which developed within a yolk sac with an organized capillary plexus, null embryos lacked yolk sac vascular plexus formation and arrested in development by embryonic day (E)8.5. Injection of pregnant female mice with TB4 was sufficient to rescue *Hand1*-null embryos, which were recovered at E8.5 with an appropriately formed yolk sac capillary network and in which the embryos had developed beyond the arrested stage of the mutants. Mechanistically, genes of the TGFβ and Notch signaling pathways, which were found to be dysregulated in *Hand1* mutant yolk sacs, were rescued to control levels with TB4 treatment[4]. It is not known whether Hand1 also regulates *Tβ4* expression at later embryonic stages, perhaps at the time points when Tβ4 is required for systemic or coronary vasculogenesis, nor is it fully understood how Tβ4 impacts on the key vasculogenic signaling pathways, those that regulate EC proliferation, migration and differentiation, that include, but may not be limited to, TGFβ and Notch1.

2.2 The Endogenous Roles of Tβ4 in Neovascularization of the Ischemic Heart

Although it was previously reasoned that all new coronary vessels in the injured heart derive from pre-existing ECs[48], the precise sources of EC contribution had not been defined. Using *Pdgfb*CreERT2-based genetic lineage tracing, Dubé and colleagues labelled existing ECs before MI, and found that a significant proportion of vessels arise *de novo*, from reactivated developmental sources, alongside angiogenesis from existing capillaries[7]. The endocardium and coronary sinus were identified as primary sources of new vessel formation, recapitulating their developmental roles, with an injury-induced hypertrabeculation and subsequent compaction, leading to formation of subendocardial vessels. While the reactivated adult epicardium did not

directly contribute vascular cells to the ischemic heart it appeared to play a role in stimulating directional expansion of sprouting vessels towards the infarct[7].

The processes of epicardial activation, angiogenesis and endocardial neovessel formation were found to require Tβ4[7]. Tβ4 levels increased in the heart within 24h of MI; levels were highest in the expanded epicardium, in capillary ECs and in endocardial cells. Tβ4 was strongly expressed in cells that appeared to delaminate from the endocardium, many of which co-expressed the mesenchymal/SMC marker, αSMA. The putative role(s) of Tβ4 in neovascularization post-MI were explored in global Tβ4KO mice[7]. In Tβ4KO hearts, epicardial activation was diminished and fewer capillaries were present within the sub-epicardium; those present were poorly-formed and largely lacked supporting SMCs, essentially replicating the coronary defects in cardiac-specific Tβ4 knockdown embryos[6]. Post-MI, Tβ4KO cells failed to mobilize from the epicardium, lacking the extended actin cytoskeleton observed in wild type epicardial cells. Mutant cells remained spindle-shaped, failed to orientate for myocardial invasion and retained epicardial marker expression, suggesting an incomplete transition to a mesenchymal state. With regard to the endocardial requirement for Tβ4, KO mice displayed a comparable extent of induced trabeculation, however, they failed in compaction remodeling and coalescence of new vessels[7]. Multiple, large trabeculae and lumina persisted in Tβ4KO endocardium and this coincided with a significant reduction in the numbers of subendocardial vessels after MI. Examination of forming lumina showed fewer αSMA+ cells underlying the endocardium, suggesting a possible endothelial-mesenchymal defect. Within the infarct border zone, vessel density was significantly reduced in Tβ4KO hearts, and fewer new vessels acquired SMC support. Taken together, these data suggest a requirement for Tβ4 in angiogenesis, remodeling and SMC recruitment to vessels in the infarct border zone.

2.3 The Effects of Exogenous Tβ4 on Endothelial Cells *in vitro*

Interest in the endothelial role of Tβ4 first arose with its identification from a screen of genes induced during endothelial capillary formation[49]. Tβ4 addition to human umbilical vein endothelial cells (HUVECs) increased the rate of attachment to extracellular matrix (ECM) components, accelerated the rate of tube formation on Matrigel, while Tβ4 knockdown abrogated sprouting[49]. A direct pro-angiogenic role for Tβ4 was demonstrated when addition of Tβ4 stimulated capillary sprouting in cultured coronary artery rings[50]. In addition to promoting endothelial migration, Tβ4 has been shown to protect endothelial progenitor cells from apoptosis[51], possibly via activation of ILK and AKT[52] since Tβ4 was found to co-immunoprecipitate with integrin-linked kinase (ILK)[17] and targeted ablation of ILK abolished the ameliorative effect of Tβ4 on apoptosis[52]. A further role for Tβ4 in angiogenesis was described when addition of Tβ4 to HUVECs was shown to increase expression of vascular endothelial growth factor (VEGF)-A[53], a highly vasculogenic and angiogenic protein. These findings have been corroborated *in vivo* and are discussed below.

In attempts to gain mechanistic insight into the ability of Tβ4 to promote capillary tube formation, many correlative studies were initially performed, essentially adding Tβ4 to cultured cells and selectively assaying processes relevant for angiogenesis, such as proliferation and migration, and the known regulatory pathways. For example, Tβ4-enhanced capillary formation has been associated with increased EC proliferation[10], activated AKT and ERK[54], downregulation of VE-Cadherin[55] and an increase in MMPs[56], HIF1α[57], Notch1[55], Notch4[55], Angiopoietin-1[58], insulin-like growth factor-1 (IGF-1)[57] and *Plasminogen activator inhibitor-1* (*Pai-1*)[59]. In some cases, pathway intermediates were directly implicated by loss-of-function studies. A requirement

for the Notch pathway was confirmed with the demonstration that inhibition of Notch1 or Notch4 with siRNA or the Notch receptor inhibitor DAPT significantly prevented Tβ4-induced VE-cadherin down-regulation, tube formation and lymphocyte transendothelial migration[55]. Tβ4-induced tube formation in HUVECs was also attenuated in the presence of IGF-1 siRNA[57], as was the Tβ4-induced protection of HUVECs against high glucose injury[57]. Further analysis *in vitro* linked IGF-1 receptor activation with the AKT-GSK3β signaling axis downstream[57] since HUVECs exposed to high glucose displayed reduced cell viability, IGF-1, IGF-1 receptor, phospho-AKT and phospho-GSK3β, all of which were reversed by exogenous Tβ4. The finding that IGF-1 induction by Tβ4 ameliorates endothelial damage cause by hyperglycemia may have implications for diabetic vascular disease.

The role of actin in Tβ4-enhanced endothelial migration and capillary formation is still unknown. While exogenous application of Tβ4 with a mutated actin binding domain was able to stimulate capillary formation on Matrigel[60], other researchers demonstrated that the adhesion and spreading effect of Tβ4 was abolished in the presence of exogenously added soluble actin, and that the LKKTETQ actin binding fragment of Tβ4 was sufficient and necessary to induce angiogenesis[61]. The Tβ4-derived tetrapeptide, Ac-SDKP, has been shown to stimulate EC migration and differentiation *in vitro*[62], supporting the notion that Tβ4-induced EC migration and subsequent angiogenesis is independent of actin binding. Work by Fan *et al.* may reconcile some of this disparity. These authors demonstrated that the interaction between actin and Tβ4 was polarized, with a diminished interaction at the leading edge of migrating ECs[63]. In lamellipodia, profilin-dependent release of Tβ4 from actin allowed Tβ4 to bind to ILK, thereby facilitating AKT activation and inducing MMP2 expression[63]. Corroborating the idea of an actin-independent mechanism, Tβ4 was identified to interact with F_1-F_o ATP synthase to increase EC surface ATP

levels[64]. The increase in ATP was proposed to signal via the ATP-responsive purinergic receptor (P2X4) to induce HUVEC migration[64]. However, this mechanism of Tβ4-induced HUVEC migration has been debated as P2X4 is a cell surface receptor that facilitates the entry of Ca^{2+} in response to ATP yet, in another study, Tβ4 was found to promote EC migration without any influx of Ca^{2+} [38].

2.4 The Effects of Exogenous Tβ4 on Endothelial Cells *in vivo*

In vivo, Tβ4 has demonstrated pro-angiogenic effects in a number of injury models. In particular, a number of studies document roles in models of epidermal wound healing, a process requiring controlled angiogenesis, and in models of tissue ischemia.

2.4.1 Tβ4 promotes angiogenesis in models of epidermal wound healing

In a number of animal models of cutaneous wound healing, including in the setting of diabetes mellitus (DM)-associated chronic, non-healing wounds, application of Tβ4 has been shown to promote angiogenesis[61], with increased vessel density coinciding with increased expression of angiogenic growth factors, for example VEGF-A[53] and PDGF-BB, as well as the receptor for advanced glycation end products (RAGE)[65], a marker normally elevated in wounds of diabetic mice and previously implicated in angiogenesis downstream of VEGF[66]. Consistency has been noted in at least three separate wound healing studies, whereby exogenous Tβ4-induced angiogenesis coincided with elevation of both VEGF and activated AKT[53, 57, 65], the latter in line with previous studies demonstrating ILK as a binding partner for Tβ4 to activate AKT[17]. It is noteworthy that the Tβ4-mediated increase in VEGF and activated AKT occurred irrespective of

the model of injury, whether inflicted by a burn[67], a burn in the setting of DM[65] or a biopsy punch in the setting of DM and hind limb ischemia[53]. Tβ4 was shown to induce heat shock protein (HSP)70 and knockdown of HSP70 in heat-injured HUVECs abrogated Tβ4's ability to increase VEGF and AKT, leading to a proposed HSP70-VEGF-AKT signaling axis[67]. Whether or not Tβ4-induced upregulation of VEGF is a direct mechanism remains unknown. In addition, the disruption of the F-actin:G-actin ratio, in favor of F-actin, was evident in HUVECs after heat treatment, however this was stabilized upon TB4 treatment, an observation corroborated *in vivo* as Tβ4 was shown to stabilize the decrease in F-actin:G-actin post-burn injury[67], signifying a role for Tβ4 in cytoskeletal homeostasis in the setting of burn induced dermal wounds. Aspects of dermal wound healing were previously reported to be dependent on the actin binding domain as synthetic LKKTETQ was sufficient to promote repair[68]. However, it remains entirely possible that synthetic LKKTETQ in this context serves to desequester Tβ4 from actin, allowing it to bind to its effector molecules.

2.4.2 Tβ4 promotes angiogenesis in models of ischemia

In a mouse model of MI, intraperitoneal injection of Tβ4 promoted neovascularization[8], yielding an increase in size and stability of the vascular plexus and enhanced collateral vessel growth, possibly via augmentation of suboptimal epicardial activation. In this context, Tβ4 was proposed to act, directly or indirectly, through protein kinase C (PKC) to increase capillary density via activation of myristoylated, Alanine-rich C Kinase Substrate-Phosphatidylinositol-4,5-diphosphate Signaling (MARCKS)[10]. Indeed, the increase in capillary density and the number of SMAα positive cells observed post-MI was attenuated by a PKC inhibitor[10]. The epicardium was proposed as a source of ECs, although this is not consistent with published lineage trace studies

post-MI[7]. An indirect role of the epicardium may be more likely, possibly promoting angiogenesis by paracrine action and deposition of conducive ECM components[69, 70]. As well as enhancing capillary density post-MI, Tβ4 improved cardiac function and reduced cardiac rupture post-MI in mice[9, 17, 71].

Heart transplantation is hampered by graft rejection and requires intensive immunosuppression[72]; survival of the allograft depends upon new blood vessel formation and allograft vasculopathy is the main reason for poor long-term survival of heart transplant patients[73]. Due to its recognized anti-inflammatory, pro-survival and neovascular properties, pre-transplant Tß4 gene therapy was investigated in a preclinical mini pig model[74]. Donor organs were transduced with a recombinant adeno-associated viral vector to drive Tß4 expression (rAAV2.9.Tß4) before transplantation; as well as attenuating inflammation, necrosis and acute vascular reaction, rAAV2.9.Tß4 pretreatment improved capillary density, graft function and survival, identifying Tß4 as a promising therapy to reduce graft rejection in allotransplantation of the heart, and possibly other organs.

Multiple lines of evidence support the hypothesis that Tβ4-induced angiogenesis requires transcriptional activation of the myocardin-related transcription factor (MRTF)-serum response factor (SRF) pathway, via Tβ4-dependent sequestering of actin leading to nuclear accumulation of MRTF-A[13]. In cultured human microvascular cells, both Tβ4 and MRTF-A induced migration and tube formation and, in HL-1 cells, transfection of Tβ4 induced nuclear translocation of MRTF-A and activation of a MRTF-SRF-dependent luciferase reporter; both effects were abolished when a Tβ4 construct lacking the G-actin binding site was co-transfected. In addition, enhanced tube formation and pericyte recruitment in co-culture, upon MRTF or Tβ4 transfection, was abolished with knockdown of SRF target genes CCN1 and CCN2, respectively. In mice and rabbits,

transduction of Tβ4 or MRTF-A stimulated capillary formation and improved perfusion in models of hind limb ischemia, effects that were attenuated with co-application of rAAV.Tβ4 with MRTF-shRNA in mice or co-application of angiopoietin-2 in rabbits[13]. Consistent with these findings, rAAV.Tβ4 was unable to induce angiogenesis in either MRTF- or CCN1- deficient mice. Furthermore, in a pig model of myocardial ischemia, rAAV.Tβ4-induced neovascularization, smooth muscle recruitment and enhanced myocardial function, were attenuated by shRNA knockdown of MRTF[13]. Of note, mutating the actin binding domain of rAAV.Tβ4 abolished the *in vivo* neovascular benefits. In a further study from the same authors, the pro-angiogenic capacity of Tβ4 was shown to be Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/AKT-dependent, as Tβ4-induced neovascularization in a hind limb ischemia model was blunted by a dominant negative rAAV.AKT, or by inhibitors of Rho-associated protein kinase (ROCK) or PI3K[75].

Molecules that can enhance both vasculogenesis and smooth muscle support are of particular interest for treating diseases of microvascular destabilization. For example, myocardial tissue from diabetic patients demonstrated capillary rarefaction and pericyte detachment[12]. While both VEGF-A and Tβ4 could treat high glucose-induced endothelial dysfunction *in vitro*, only Tβ4 was able to promote pericyte recruitment to endothelial rings. *In vivo*, while rAAV.Tβ4 and rAAV.VEGF-A treatment of diabetic pigs with chronic myocardial ischemia increased coronary EC number, only Tβ4 increased pericyte number and collateral vessels, and ultimately improved myocardial function. Tβ4 was also shown to improve the peripheral vascular complications that occur in sepsis. Pretreatment with AAV.Tβ4, prior to the induction of sepsis in mice, reduced the associated pericyte loss. As a result, Tβ4-treated mice displayed reduced perivascular leakage, improved systemic blood pressure and ultimately increased survival rate[76].

2.5 Tβ4 Mediates the Vascular Benefits of Stem Cell Therapy

The regenerative properties of stem cell therapy are now considered to derive primarily from the rich cocktail of paracrine factors that they secrete (reviewed in[77]). Tβ4 has been shown to be expressed and secreted at high levels from a number of stem cell populations, including mesenchymal stem cells[78], amniotic fluid stem cells[79] and embryonic endothelial progenitor cells (eEPCs)[80]. Infusion of eEPCs into the ischemic pig heart decreased infarct size, enhanced neovascularization and improved cardiac function; however, these effects were abolished after shRNA-mediated knockdown of Tβ4[37], confirming that eEPCs induced neovascularization and cardiac protection in a Tβ4-dependent manner. As an alternative form of cell-based therapy, surgically transplanted omental pedicles are used to repair injured tissues[81]. In a murine carotid artery injury study, transplanted omental grafts, pre-treated with Tβ4, were found to directly contribute regenerative smooth cells to the injured vessels, restoring function to the level of uninjured controls[81].

2.6 Potential for Tβ4 to Protect Against Aortic Disease?

The requirement for Tβ4 for formation of stable vessels in the embryo and capacity to induce vascular repair may suggest a hitherto unidentified role in vascular protection. Although not yet directly implicated in either pathogenesis or repair, expression of Tβ4 in animal models and patients is consistent with one or more roles in disease. *Tmsb4x*, the gene encoding Tβ4, was identified as the most abundant transcript in human aorta from patients with abdominal aortic aneurysm (AAA)[82]. In a rat model, induction of hypertension resulted in increased secretion of Tβ4 from the aorta[83], indicating its potential for use as a clinical biomarker. By mass spectrometry

analysis, Tβ4 was identified as the most significantly up-regulated protein in atherosclerotic aortic tissue, from both a rabbit model and in human patient samples[84]. These descriptive studies strongly support a role for Tβ4 in aortic disease and highlight the need for further investigation, given its role in promoting SMC differentiation, as well as the highly relevant anti-inflammatory, anti-apoptotic and pro-survival effects of the peptide.

3. CONCLUSION

The assembly of vascular networks is a tissue-specific, complex process, orchestrated by tightly regulated signaling pathways and multiple cell types, whereby a rudimentary capillary plexus is formed via vasculogenesis, remodeled via angiogenesis and arteriogenesis and maintained, with turnover of constituent cells, throughout adulthood. Tβ4 has been implicated in all stages of vessel development and, when added exogenously, these roles can be recapitulated to promote aspects of vascular repair, including angiogenic sprouting, pericyte recruitment and SMC differentiation (**Figure 1**). Efficacy in preclinical models supports clinical application of Tβ4 and its use to enhance cell- and tissue engineering-based therapies. Abundant expression in the aorta and up-regulation in hypertension, AAA and atherosclerosis suggests its involvement in vascular homeostasis and potential to either protect against disease or, even, to contribute towards its progression. Given that known Tβ4 functions uphold reparative processes, notably suppression of inflammation and SMC differentiation, Tβ4 would appear more likely to favor repair of intimal ECs and maintenance of medial SMCs in a stable, contractile state. Understanding mechanisms of Tβ4 function in the vasculature will inform optimal therapeutic strategies for vascular stabilization and regeneration.

4. EXPERT OPINION

Since the earliest demonstration of its ability to induce angiogenesis in cultured ECs, many laboratories have focused on the vascular roles of Tβ4. The peptide is required for development of the earliest vessels of the embryonic yolk sac, the coronary circulation and systemic vasculature. In the adult, Tβ4 is similarly required for endogenous vascular remodeling to enhance revascularization of the ischemic heart. Intrinsic roles have been extrapolated for therapeutic application of Tβ4 to enhance vessel growth in epidermal wounds and in myocardial and peripheral ischemia. Interest in Tβ4 and other small molecule regulators of vascular growth and stability is set to intensify as cardiovascular diseases remain the primary cause of mortality worldwide, with atherosclerosis triggering most cases of MI and stroke. Moreover, it is increasingly apparent that vasculopathies underlie many of the complications associated with diabetes, sepsis, local ischemia and chronic wounds. Conversely, under disease conditions of excessive vascular growth, such as tumor angiogenesis or age-related macular degeneration, Tβ4 may emerge as target for attenuating new vessel development.

Despite the wide-ranging evidence that Tβ4 promotes vessel protection, growth and stabilization, the literature on the subject has, for almost 20 years, remained largely descriptive, with little insight into the underlying mechanisms. The molecular functions of Tβ4 are pleiotropic and incompletely understood. The established role in binding monomeric actin, to direct the necessary cytoskeletal remodeling to enable EC migration in angiogenesis, would be expected to contribute and studies interrogating the actin binding domain support this supposition, albeit the downstream consequences of actin binding remain equivocal. At the same time, other functions appear to be independent of actin binding and, in some cases, known to involve paracrine

mechanisms, yet few targets of secreted Tß4 have been identified. Tβ4 translocates to the nucleus and may function to sequester nuclear actin, a key component of the transcriptional machinery[85], as well as splicing[86] and chromatin remodelling[87] complexes. Regulation of nuclear actin dynamics by Tβ4 is consistent with its recently demonstrated role in chromatin remodelling[42], association with alternative splicing events[43] and reports of altered gene expression downstream of Tβ4 treatment. The putative nuclear and paracrine functions remain to be substantiated on a molecular level, however, these roles are difficult to replicate *in vitro* and distinguishing primary from secondary effects *in vivo* remains a considerable challenge. Interaction analyses to identify direct Tβ4 binding partners might enable the delineation of the relevant pathways, along with *in vitro* co-culture techniques to probe heterotypic cell signaling interactions and transcriptomic analysis and chromatin immunoprecipitation to validate target genes. Given the almost ubiquitous expression of Tβ4 and intrinsic, yet inadequate, reparative responses involving Tβ4, understanding the endogenous mechanisms through which Tβ4 acts will allow the selective targeting and augmentation of the relevant processes to achieve maximal therapeutic benefit.

Article Highlights

- Tβ4, an actin monomer binding peptide, has been shown to play multiple key roles in the vasculature, including vasculogenesis, angiogenesis and smooth muscle cell differentiation.
- Tβ4 plays an essential role in development of the yolk sac, coronary and systemic vasculature.

- Exogenously applied Tβ4 promotes neovascularization in ischemic tissues and accounts, at least in part, for the pro-angiogenic effects of stem cell therapy.
- Expression of Tβ4 in the aorta and up-regulation in disease settings suggests a possible role in homeostasis and vascular protection and warrants further investigation.
- Further insight into the role of Tβ4, such that it may be targeted therapeutically, requires a comprehensive understanding of its molecular mechanism of action.

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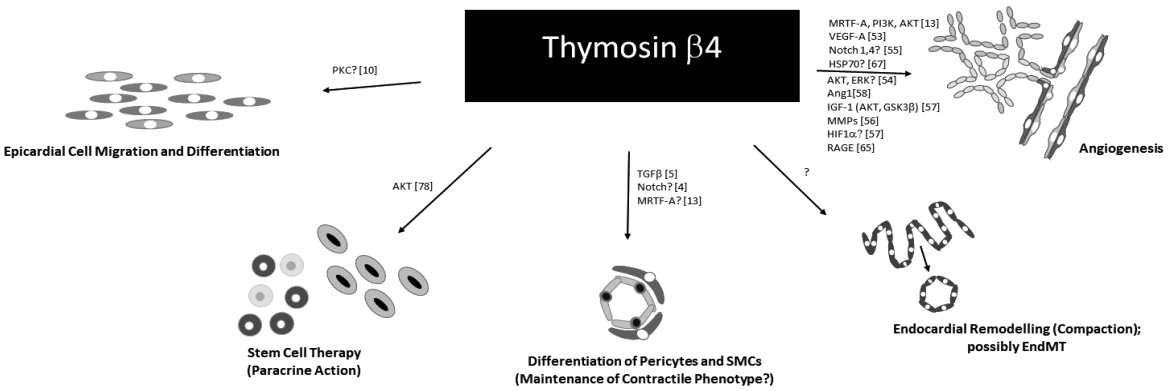
FIGURE LEGEND

Figure 1. Proposed Mechanisms of Tβ4-induced Vascular Development, Repair and Regeneration.

Tβ4 stimulates resident epicardial progenitor cells to contribute SMCs to the coronary vasculature during development; following myocardial infarction, Tβ4-re-activated epicardial cells enhance repair, via cardiomyocyte contribution (not shown) and paracrine signaling, to sustain myocardial vascularization and enhance functional recovery. Angiogenesis, the first vascular function ascribed to Tβ4, is promoted by the peptide in a range of *in vitro* and *in vivo* settings. Tβ4 has been shown to be, in part, responsible for the pro-angiogenic effects of stem/progenitor cell therapy. Tβ4 is required to induce differentiation of SMCs in the embryo and can perform the same role during regeneration of damaged tissues in adulthood. In the ischemic heart, remodeling of the ventricular endocardium to contribute new vessels requires Tβ4 for compaction and possibly for EndMT. The molecular mechanisms underlying the effects of Tβ4 in the vasculature remain to be fully elucidated. The putative regulatory signaling pathways, associated or implicated from experimental studies, are indicated, with the relevant references cited. Abbreviations: AKT: Protein Kinase B; Ang1: Angiopoietin 1; EndMT: Endothelial to Mesenchymal Transition; ERK: Extracellular Signal–Regulated Kinase; GSK3β: Glycogen synthase kinase 3β; HIF-1α: Hypoxia-Inducible Factor 1α; HSP70: Heat Shock Protein 70; IGF-1: Insulin-like Growth factor 1; ILK: Integrin-linked Kinase; MMPs: Matrix Metalloproteinases; MRTF-A: Myocardin-related Transcription Factor A; PI3K: Phosphatidylinositol 3-kinase; PKC: Protein Kinase C; RAGE: Receptor for Advanced Glycation End Products: SMCs: smooth muscle cells; Tβ4: Thymosin β4; TGFβ: Transforming Growth Factor β; VEGF-A: Vascular Endothelial Growth Factor A


Thymosin β4
PKC? [10]
Epicardial Cell Migration and Differentiation
MRTF-A, PI3K, AKT [13]
VEGF-A [53]
Notch 1,4? [55]
HSP70? [67]
AKT, ERK? [54]
Ang1[58]
IGF-1 (AKT, GSK3β) [57]
MMPs [56]
HIF1α? [57]
RAGE [65]
Angiogenesis
AKT [78]
Stem Cell Therapy
(Paracrine Action)
TGFβ [5]
Notch? [4]
MRTF-A? [13]
Differentiation of Pericytes and SMCs
(Maintenance of Contractile Phenotype?)
?
Endocardial Remodelling (Compaction);
possibly EndMT