Molecular Therapy
Original Article

Tβ4 Increases Neovascularization and Cardiac Function in Chronic Myocardial Ischemia of Normo- and Hypercholesterolemic Pigs

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Translations of new therapeutic options for cardiovascular disease from animal studies into a clinical setting have been hampered, in part by an improper reflection of a relevant patient population in animal models. In this study, we investigated the impact of thymosin β4 (Tβ4), which promotes collateralization and capillarization, during hypercholesterolemia, a known risk factor of coronary artery disease. Initial *in vitro* results highlighted an improved endothelial cell function upon Tβ4 treatment under control conditions and during hypercholesterolemic stress (scratch area [pixels]: oxidized low-density lipoprotein [oxLDL], 191,924 ± 7,717; and oxLDL + Tβ4, 105,621 ± 11,245). To mimic the common risk factor of hypercholesterolemia *in vivo*, pigs on regular (NC) or high-fat (HC) diet underwent chronic myocardial ischemia followed by recombinant adeno-associated virus (rAAV)-mediated transduction of Tβ4 or LacZ as a control. We show that Tβ4 overexpression improves capillarization and collateralization (collaterals: NC + rAAV.LacZ, 2.1 ± 0.5; NC + rAAV.Tβ4, 6.7 ± 0.5; HC + rAAV.LacZ, 3.0 ± 0.3; and HC + rAAV.Tβ4, 6.0 ± 0.4), ultimately leading to an improved myocardial function in both diet groups (ejection fraction [EF] at day 56 [%]: NC + rAAV.LacZ, 26 ± 1.1; NC + rAAV.Tβ4, 45 ± 1.5; HC + rAAV.LacZ, 26 ± 2.5; and HC + rAAV.Tβ4, 41 ± 2.6). These results demonstrate the potency of Tβ4 in a patient-relevant large animal model of chronic myocardial ischemia.

INTRODUCTION

Cardiovascular disease (CVD) has become the leading cause of morbidity and mortality worldwide, accounting for approximately one-third of all deaths.[1] The majority of CVD comprises 6 major conditions: ischemic heart disease, stroke, hypertensive heart disease, cardiomyopathy, atrial fibrillation, and rheumatic heart disease, all ultimately leading to heart failure.[1–3]

There is evidence that western-style diets (high fat and cholesterol, high protein, and high sugar)[4–6] can lead to an increased atherogenic lipid burden, which is characterized by high cholesterol, high low-density lipoprotein (LDL) levels, or high LDL/high-density lipoprotein (HDL) ratios in the circulation.[7,8] These parameters can be drawn upon as a reliable indicator of individual CVD risk.[9] Excess levels of circulating LDL can lead to cholesterol deposition onto arterial walls, resulting in leukocyte recruitment, prolonged vascular inflammation, endothelial dysfunction, and, ultimately, atherosclerosis leading to coronary artery disease (reviewed by Zarate et al.[10]). Areas of ischemic myocardium downstream of chronically occluded coronary vessels suffer from hypo-perfusion and, consequently, tissue hypoxia.[11] As a rescue strategy, such myocardium enters a state termed hibernating myocardium, characterized by a contractile dysfunction in still viable myocardium. It is postulated to represent an adaptive myocyte response to chronic stress in order to promote survival of the myocardium[12] and create a state where myocardial function can be restored by stimulating angiogenesis.

Strategies to recover the hibernating myocardium in CVD into functional tissue thus include surgical attempts at revascularization[13] as well as several molecular approaches[14] that target the aberrant vasculature characteristic for this condition. Among these molecules is thymosin β4 (Tβ4), a G-actin-sequestering peptide that has been shown to play a role in cell fate determination and angiogenesis in the heart. It has been successfully utilized in small as well as large animal models[15–17] to promote cardiomyocyte differentiation, angiogenic sprouting, improvement of vessel functionality, and enhancement of cardiac function.[17–20] Previous studies have demonstrated that recombinant adeno-associated viral vector-based regional application of Tβ4 may represent a viable method of vascular gene therapy.[15,21,22]

Even though measures that decrease serum lipid levels appear to lower the risk for acute and chronic ischemic cardiovascular disease

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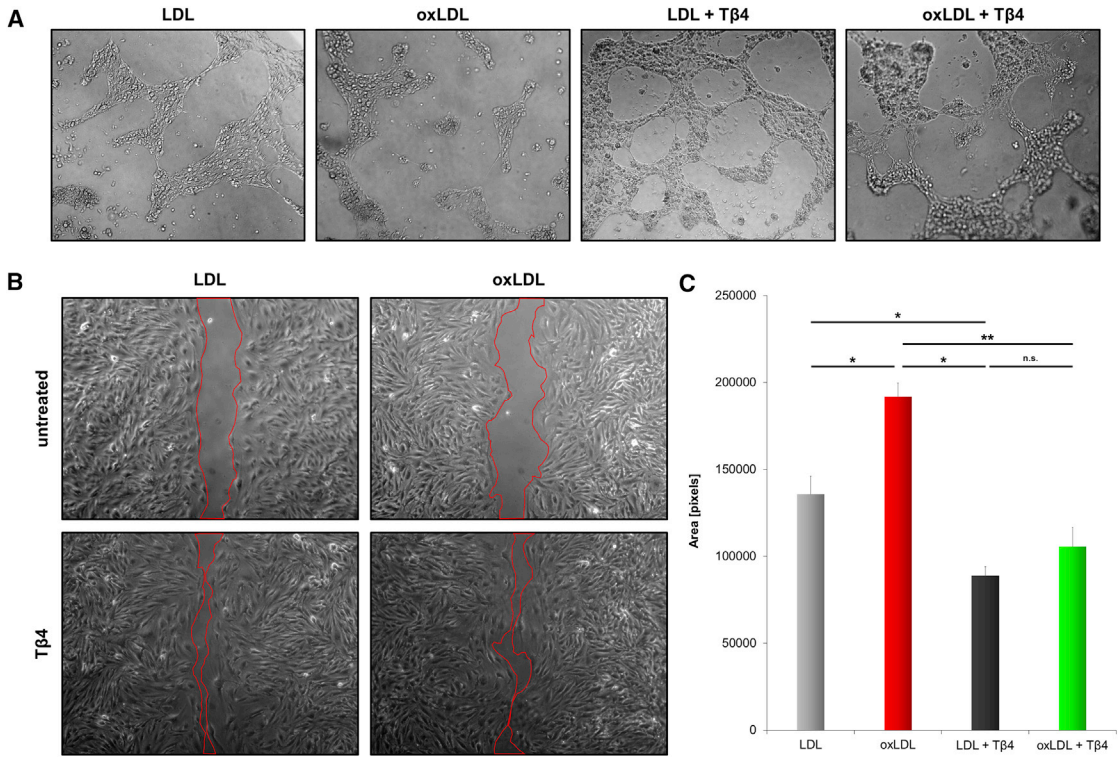

Figure 1. Thymosin β4 Improves Endothelial Function during oxLDL Stress

(A) Endothelial cells treated with oxidized LDL prompted a reduction in tube formation after seeding on matrigel, an effect ameliorated by the addition of Tβ4. (B and C) In a scratch assay (B, examples; C, quantification), endothelial cells displayed a significant reduction of migratory capacity upon treatment with oxLDL in comparison to LDL-treated cells, an effect sensitive to the treatment with thymosin β4 (mean ± SEM; *$p < 0.05$ and **$p < 0.001$; n.s., not significant).

(reviewed elsewhere[23–28]), patients with hypercholesterolemia still present with an enhanced incidence of cardiovascular events.

We thus studied chronic ischemic heart disease in a pre-clinical large animal model of hibernating myocardium[15] in pigs subjected to the single important individual risk factor for CVD: high-fat western-style diet. Our aim was to examine the capacity of Tβ4 treatment on vascular regeneration in chronic myocardial ischemia while more closely approximating the patient population suffering from cardiovascular disease.

RESULTS

Tβ4 Improves Endothelial Cell Function *In Vitro* during oxLDL Stress

To test the effect of Tβ4 treatment on endothelial cells both during resting conditions and under stress conditions that mimic *in vivo* hypercholesterolemia, we treated endothelial cells with oxidized LDL (oxLDL), a known contributor to endothelial dysfunction during atherosclerosis,[29] and non-oxidized LDL, as a control after Tβ4 application in an endothelial cell tube formation assay on matrigel. Here the treatment with oxLDL led to a reduction in tubes formed by endothelial cells compared to LDL-treated control cells. This effect was abolished after Tβ4 treatment, highlighting the efficacy of a Tβ4 treatment in inducing vessel formation even under pro-atherosclerotic conditions (Figure 1A). Further evidence of the positive effect on endothelial cell function during pro-atherosclerotic stimulation was attained in a scratch migration assay, where oxLDL led to a significant reduction in the ability of endothelial cells to traverse the induced gap. Both in oxLDL- as well as control-treated cells, Tβ4 significantly increased the migratory capacity of endothelial cells (Figures 1B and 1C).

These promising *in vitro* results prompted us to conduct an *in vivo* large animal study in pigs suffering from chronic myocardial ischemia. To test the regenerative vascular potential of Tβ4 during chronic myocardial ischemia in a patient-relevant setting, animals on a high-fat western-style diet (HC) were compared to animals on a regular diet (NC). As expected, the high intake of fat induced a significant increase in cholesterol as well as triglycerides (Figures 2A and 2B), while glucose and insulin levels remained unchanged compared to animals on the control diet, indicating an isolated hypercholesterolemia without induction of metabolic syndrome (Figures 2C and 2D). Interestingly, already without the ischemic stimulus, hypercholesterolemic animals displayed a capillary rarefication compared to control pigs (Figure 2E). After the establishment of a porcine model accurately mimicking hypercholesterolemia, the animals underwent

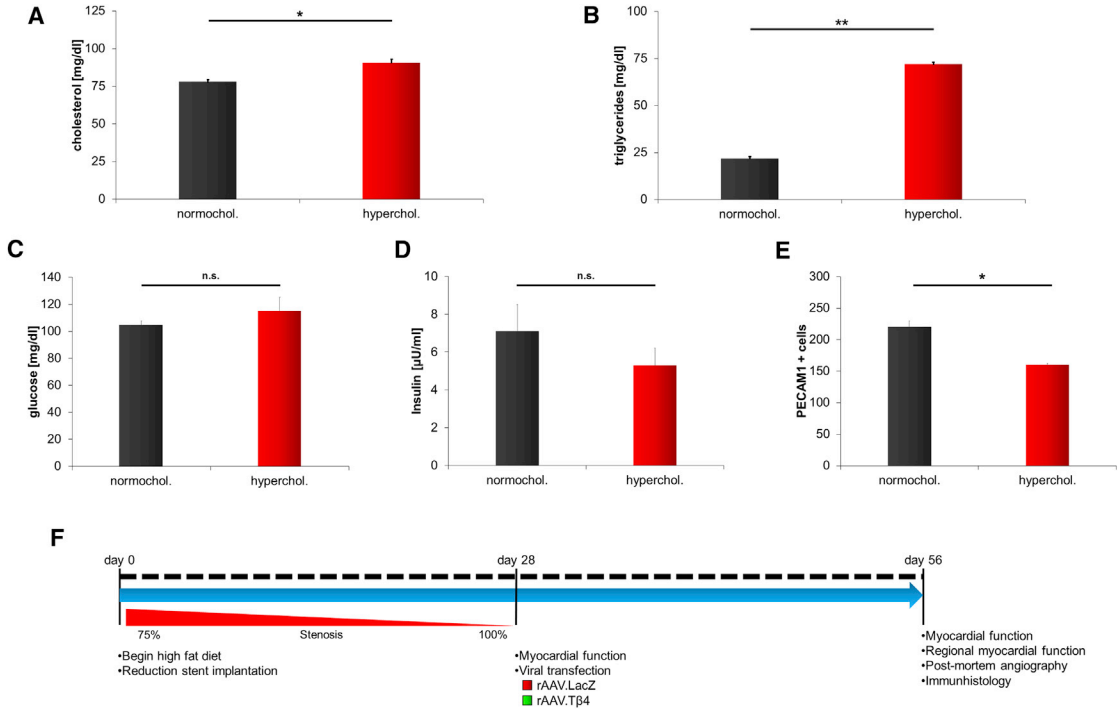

Figure 2. A Porcine Model of Hypercholesterolemia

(A and B) Pigs that were fed a high-fat diet developed increases in triglyceride levels (B) and cholesterol (A). (C and D) Pigs on a high-fat diet did not produce a metabolic syndrome with diabetic phenotype, as highlighted by unchanged glucose (C) and insulin (D) levels. (E) Hypercholesterolemic pigs developed capillary rarefication in the non-ischemic myocardium compared to pigs on a regular diet. (F) The experimental setup. On day 0, a reduction stent was implanted into the circumflex artery followed by the occlusion of the vessel over the next 28 days. Together with the stent implantation, the diet was changed to a high-fat diet in half of the animals. On day 28, pigs were transfected with either rAAV.LacZ or rAAV.Tβ4 together with a baseline measurement of global myocardial function. After 28 additional days (day 56), global myocardial function was assessed again together with regional myocardial function, and angiography was performed to determine collateralization (mean ± SEM; *$p < 0.05$, **$p < 0.001$ and n.s., non significant.

reduction stent implantation into the left circumflex coronary artery, leading to a complete vessel occlusion during the next 4 weeks, at which time recombinant adeno-associated virus (rAAV).Tβ4 or rAAV.LacZ (as the control) were retrogradely infused into the great cardiac vein. After 4 additional weeks, hemodynamic measurements were performed and organs were harvested for further analysis. During the whole observation period, animals were kept either on a regular diet or a high-fat diet (Figure 2F).

Improved Vascularization by Tβ4 during Hypercholesterolemia in the Hibernating Myocardium

To investigate the capillarization and collateralization in pigs undergoing reduction stent implantation, heart sections from the ischemic region were stained for PECAM-1, an endothelial cell marker, to assess the capillarization of the hibernating myocardium (left circumflex artery [LCx]-perfused area). These stainings supported the initial finding of decreased capillarization in hypercholesterolemic pigs. The ischemic region in hypercholesterolemic pigs already showed a significant reduction in capillary density in the control-treated group, a finding that was ameliorated by Tβ4 treatment, albeit at a significantly lower level in hypercholesterolemic pigs than in animals receiving a regular diet (Figures 3A and 3B). To test the inflammatory response of the microvasculature to the chronic ischemic stress, staining for CD14, an established monocyte marker, was performed, which displayed an increase in CD14-positive cells in control-transfected animals that was more pronounced in hypercholesterolemic pigs and reduced by the transduction of Tβ4. Furthermore, this staining revealed an enhanced CD14-positive cell accumulation in hypercholesterolemic pigs in comparison to pigs on a regular diet (Figure 3C), pointing to a chronic vascular activation upon enhanced cholesterol levels.

While these findings demonstrated an impairment in microcirculation and an increase in inflammatory cell recruitment during hypercholesterolemia, collateralization, at least by numbers, was not significantly different in hypercholesterolemic pigs compared to pigs on a control diet, both in rAAV.LacZ- as well as rAAV.Tβ4-transfected pigs, which was apparent in the number of collaterals (Figures 3D and 3E) as well as the Rentrop score (Figures 3D and 3F).

Tβ4 Ameliorates the Loss of Myocardial Function to a Lesser Extent in Hypercholesterolemic Pigs

Lastly, we investigated if this Tβ4-mediated microvascular regeneration translated into an improved myocardial function, which

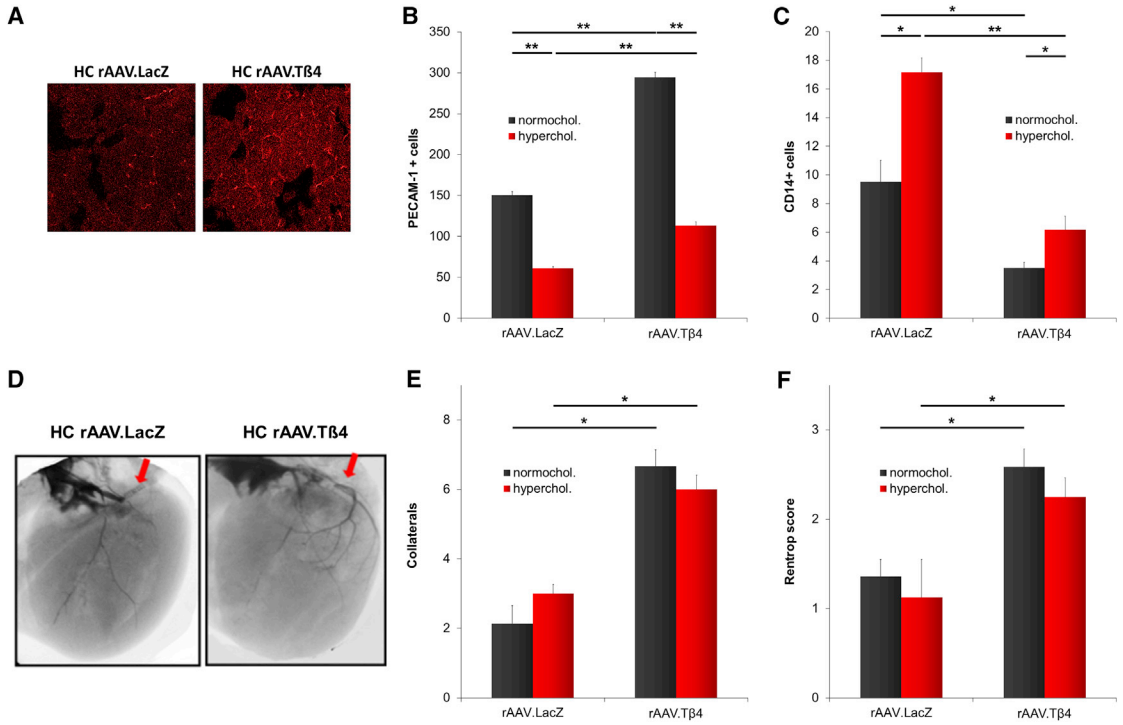

Figure 3. Thymosin β4 Enhances Collateralization and Capillarization during Chronic Myocardial Ischemia in Hypercholesterolemic Pigs

(A) Example pictures of staining for PECAM-1 from the ischemic region demonstrate an increase in capillarization after thymosin β4 treatment in hypercholesterolemic pigs. (B) The quantification of PECAM-1-positive cells demonstrated this increase in both normo- and hypercholesterolemic pigs, which, however, was less pronounced in hypercholesterolemic pigs. (C) Monocyte recruitment was reduced in thymosin β4 treatment in both diet groups compared to control-transduced animals. (D) Example pictures of post mortem angiographies (red arrow indicates the reduction stent), which highlight an increase in distal coronary artery perfusion upon Tβ4 treatment. (E and F) As quantified, this increase of collaterals (E) and perfusion (F) was independent of diet (mean ± SEM; *$p < 0.05$ and **$p < 0.001$).

represents the most important factor dictating patient prognosis and quality of life. Here a treatment with rAAV.Tβ4 led to an improved regional myocardial function at rest, as assessed by subendocardial segment shortening, only in normocholesterolemic pigs, whereas hypercholesterolemic pigs in this resting state did not show an improvement after transduction with Tβ4. During pacing at increased heart rates, to assess the myocardium's functional reserve, however, also hypercholesterolemic pigs transduced with Tβ4 displayed an increase in reserve capacity compared to rAAV.LacZ-transfected animals, whether on a regular or high-fat diet (Figure 4A). Consistently, left-ventricular end-diastolic pressure, a hallmark of diastolic dysfunction and a predictive parameter for the development of heart failure, was drastically reduced in both groups treated with rAAV.Tβ4 at day 56 compared to controls, with a trend toward an additional improvement in normocholesterolemic animals (Figure 4B). This cardioprotective effect of Tβ4 was also seen in the ejection fraction (EF) in an analogous manner, with an increase in EF in both Tβ4-treated groups and no significant difference between the hypercholesterolemic and normocholesterolemic pigs (Figure 4C).

DISCUSSION

In this study, we investigated the ability of myocardial Tβ4 overexpression to counteract myocardial dysfunction in a preclinical pig model of hibernating myocardium in combination with a single important individual risk factor for CVD, high-fat western-style diet. In our experimental *in vitro* setup, we wanted to determine the ability of Tβ4 to promote angiogenesis not only under resting conditions but also under stress conditions that mimic *in vivo* hypercholesterolemia. Oxidized LDL is known to contribute to endothelial dysfunction in atherosclerosis,[29] and it can thus provide a pro-atherosclerotic environment for endothelial cells in this assay. In contrast to other known molecules with pro-angiogenic potential, such as VEGF-A, which has been previously found to not yield sufficient micro- and macrovascular growth for distinct functional improvement in chronic ischemic pig hearts with or without the cardiovascular risk factor diabetes mellitus,[15,22] we demonstrate here that Tβ4 not only improves tube formation under resting conditions but also does so to a similar extent under pro-atherosclerotic stress *in vitro* and it may thus be a suitable compound for treating hibernating myocardium under pro-atherosclerotic conditions (Figure 1). By reduction stent implantation into the circumflex artery in pigs on a high-fat or regular diet, we were able to induce a hibernating myocardium phenotype in all animals, which collectively displayed a marked deterioration in vasculature as well as in regional and global myocardial function (Figures 3 and 4). These effects were more pronounced in pigs that presented with hypercholesterolemia, pointing to the

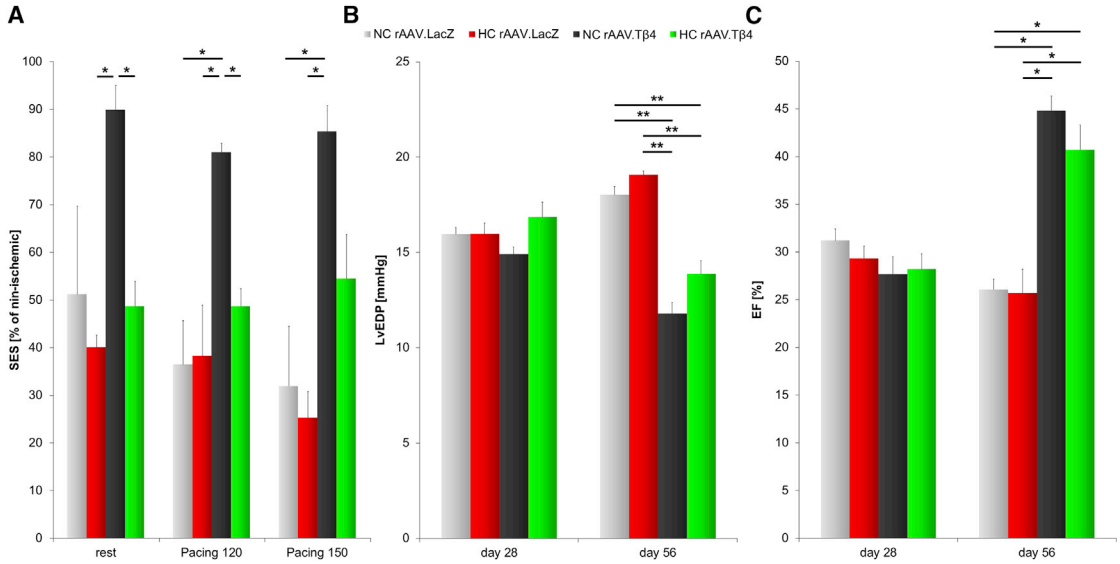

Figure 4. Improved Collateralization and Capillarization Ameliorate Ischemic Damage in Both Diet Groups

(A) Regional myocardial function, displayed as subendocardial segment shortening in percentage of the non-ischemic LAD-perfused area, was increased in normocholesterolemic, rAAV.Tβ4-treated animals already at rest, an effect exacerbated during pacing. Hypercholesterolemic pigs with rAAV.Tβ4 treatment also showed an increase in regional myocardial function, albeit only during pacing, implying an increase in reserve capacity of those animals. (B and C) Left-ventricular end-diastolic pressure (B) was significantly decreased in both thymosin β4 groups, with no difference between hyper- and normocholesterolemic animals and with the ejection fraction showing a similar result (C) (mean ± SEM; *$p < 0.05$ and **$p < 0.001$).

significance of this risk factor for aggravating cardiac pathologies. Regional and long-term Tβ4 overexpression via recombinant adeno-associated viral vectors was able to improve microcirculation and collateralization and reduce the inflammatory activation of the endothelium (CD14-positive cells) in this hibernating myocardium (Figure 3). Importantly, loss of regional and global myocardial function could also be ameliorated by Tβ4 application. However, as for signs of neovascularization, this effect was attenuated in hypercholesterolemic animals compared to control-fed animals (Figure 4).

When promoting angiogenesis, one has to consider the distinct difference between pathological angiogenesis, as takes place, for example, in cancer and various ischemic and inflammatory diseases, and balanced angiogenesis with capillary growth (endothelial cells) and vessel maturation (mural cells), leading to an improved microcirculation (reviewed elsewhere[30,31]). The latter opens up the possibility of therapeutic neovascularization in ischemic tissues, such as hibernating myocardium. Achieving both angiogenesis and vessel maturation, however, requires different signaling pathways, and, therefore, it might require a combination of different pro-angiogenic factors. In recent years, besides studies of mono-therapies that revealed potential beneficial effects for angiogenesis, combinations of different pro-angiogenic factors for vessel growth and maturation were utilized to achieve therapeutic neovascularization.[32] Tao et al.[33] report that combined overexpression of VEGF and Ang1 via an adeno-associated viral vector in the ischemic myocardium improves the perfusion and function of porcine myocardial infarction (MI) heart through the induction of angiogenesis and cardiomyocyte proliferation. Furthermore, work from our group demonstrated the potential of a VEGF-A co-transfected with platelet-derived growth factor B (PDGF-B) for improving regional and global myocardial function in the hibernating myocardium.[34] These approaches led to an induction of capillarization as well as maturation of vessels, thus providing evidence for balanced angiogenesis taking place. However, these effects may not be sufficient under pro-atherosclerotic conditions. As pro-atherosclerotic conditions or atherosclerosis is present in the majority of patients suffering from coronary artery disease (CAD), effects of a potential therapeutic compound must be demonstrated under relevant conditions to improve pro-angiogenic therapy in clinical settings. Numerous animal models of atherosclerosis ranging across many species are available (reviewed in Kapourchali[35]), with pigs responding in a similar way to humans to an increase in dietary fat content. In accordance with other high-fat diet models of atherosclerosis in pigs[36–39] that describe early atherosclerotic lesions, such as inflammatory signals in the vessel walls after approximately 3 months of diet feeding, we were able to demonstrate increased serum cholesterol and triglyceride levels as early as 56 days after the switch to a high-fat diet (Figure 2).

The induction of chronic myocardial ischemia in high-fat and regular feeding groups led to impaired collateralization and capillarization in the myocardium, and it provoked an elevation in inflammatory activity in both groups. We were, however, able to demonstrate that the impairments in capillarization as well as inflammation are both exacerbated in hypercholesterolemic pigs while collateralization appears to be unaffected by the additional stress of hypercholesterolemia (Figure 3). While collateralization is not significantly different in

normocholesterolemic as well as hypercholesterolemic animals, there is a trend toward a less pronounced collateralization in hypercholesterolemic pigs upon Tβ4 treatment (as seen in Figures 3E and 3F). This effect is seen on the basis of a significantly reduced amelioration in capillary density in hypercholesterolemic pigs upon Tβ4 treatment. This discrepancy in the efficacy of Tβ4 treatment in improving endothelial function in different vascular beds might be due to the different biology of endothelial cells throughout the vascular tree. In a recent study by Vanlandenwijk,[40] it is demonstrated that endothelial biology differs substantially dependent on the localization of endothelial cells within the vascular tree, describing a seamless transition of expression profiles of endothelial cells through different zones in the vascular tree. The difference in endothelial biology in collateral-forming arteries in contrast to capillaries might explain the difference in the ability of Tβ4 to reverse endothelial dysfunction in hyper- or normocholesterolemic pigs, although the difference in the effect of Tβ4 treatment on subsets of endothelial cells is currently unknown. In addition, these findings may be explained by the short duration of high-fat diet application (8 weeks), which might be suitable to impair capillarization and monocyte recruitment but be too short a period to affect collateralization.

In this study, Tβ4 treatment ameliorated loss of regional and global myocardial function in high-fat and normal feeding groups, even though the positive impact of a Tβ4 treatment was reduced in hypercholesterolemic pigs. The reversal of the severe heart failure in our pig model, from a profound reduction of cardiac function to a moderate impairment, is demonstrated in a variety of pig models. This might be due to the relative young age of the pigs (3 months), at which time these animals might have a higher regenerative potential than adult pigs We do not provide data on electrophysiological parameters and the potential induction of arrhythmias in this paper, but, in the admittedly short observation period, we did not detect differences in episodes of severe arrhythmias in the four studied groups.

As to the safety profile of rAAV-mediated Tβ4 overexpression, possible safety concerns can either originate in the overexpression of Tβ4 or the use of the rAAV as a delivery system. To this end, Tβ4-overexpressing transgenic pigs and mice are not reported to be impaired.[15] Furthermore, rAAVs as gene therapy delivery systems are generally deemed safe, since they show a limited immunogenic potential, have a low degree of genomic integration (about 0.1%–0.01%), and, in our case, are delivered locally to the ischemic region exclusively. Thus, while we do not provide data on electrophysiological stability in our report, we deem the rAAV-mediated overexpression of Tβ4 safe based on prior publications and our own experience. These findings mirror results from our recent study, in which Tβ4 was upregulated via rAAVs in diabetic pigs undergoing chronic myocardial ischemia. In this model, similar to the data presented here, Tβ4 increased collateralization, capillarization, and myocardial function, albeit to a lesser extent in diabetic animals.

While there are differences in the endothelial dysfunction induced by hypercholesterolemic or hyperglycemic stress, there appear to be common downstream events in both situations. To this end, both diabetes and hypercholesterolemia induce a change in the ratio of Angiopoietin-1 to Angiopoietin-2, which leads to vascular destabilization and capillary rarefication,[41,42] as well as reducing nitric oxide synthase (NOS) activity in dysfunctional endothelial cells. Both NO production and the restoration of the Ang1/Ang2 balance have been shown to be facilitated by Tβ4.[22,43] These considerations imply that Tβ4 improves endothelial function during hypercholesterolemia as well as diabetes by ameliorating endothelial dysfunction, independently of the initially damaging pathology. Tβ4 may thus have cardioprotectant properties in patients suffering from ischemic heart disease and concomitant cardiovascular risk factors, such as diabetes mellitus or hypercholesterolemia, highlighting recombinant adeno-associated viral transduction of Tβ4 as an attractive therapeutic target in the treatment of ischemic heart disease.

MATERIALS AND METHODS

Adeno-Associated Viral Vector Generation

Recombinant adeno-associated viral vectors were produced as described earlier.[21] In short, U293 cells were transfected with three plasmids, one carrying the gene of interest (Tβ4 or LacZ) controlled by a cytomegalovirus (CMV) promoter flanked by *cis*-acting internal terminal repeats (ITRs) based on AAV2 together with one plasmid encoding the AAV2 rep and AAV9 cap sequences in *trans*.[44] Adenoviral helper function was provided by the third plasmid, delta F6. After transfection of the three plasmids, cells were cultured for 48 hr, at which point they were harvested and purified via a cesium chloride gradient centrifugation.[45] The viral titers of the purified rAAVs were quantified by real-time qPCR using primers for the polyA tail of the vector bGH (forward, 5′-TCTAGTTGCCAGCCATCTG TTGT-3′; and reverse, 5′-TGGGAGTGGCACCTTCCA-3). The *trans* and helper plasmids were provided by James M. Wilson, University of Pennsylvania.

Cell Culture

Murine brain endothelial cells (bEnd3) were cultured in DMEM. For the ring formation assay, 10,000 cells were seeded on matrigel-coated μ-slides (μ-slides angiogenesis, Ibidi, Munich, Germany) in endothelial growth medium supplemented with Tβ4 (1 μg/mL) together with either LDL or oxLDL (50 μg/mL, Life Technologies, Waltham, MA, USA). Images of ring formation were taken 18 hr after cell seeding. For migration assays, 70,000 bEnd3 cells were seeded on both sides of a 35-mm culture dish with a 2-well culture insert, which was removed upon confluency of the cells (24 hr), at which time cells were treated, again with Tβ4 (1 μg/mL) together with LDL/oxLDL (50 μg/mL) for 22 hr in starving medium. Pictures were taken and the area between the cell sheets was quantified using ImageJ.

Chronic Myocardial Ischemia in Pigs

Animal care and all experimental procedures were performed in strict accordance with the German and NIH animal legislation guidelines and were approved by the Bavarian Animal Care and Use Committee. All pig experiments were conducted at the Walter-Brendel Centre for Experimental Medicine at the LMU Munich.

The induction of chronic myocardial ischemia was performed as described.[15,22] Landrace pigs aged 3 months were subjected to chronic myocardial ischemia via the implantation of a polytetrafluorethylene (PTFE) membrane-covered stent into the proximal circumflex coronary artery (Ramus circumflexus [RCx]). Pigs were anesthetized followed by the instrumentation of the right common carotid artery (A. carotis communis). Proper placement of the stent was surveyed via coronary artery angiography. The implantation of the reduction stent led to an immediate reduction of the vessel area by 75%, followed by a total occlusion of the vessel after 28 days.[46,47] The periprocedural medication consisted of a loading with Clopidogrel 75 mg daily for 3 days prior to reduction stent implantation. Furthermore, during the procedure, pigs received 10,000 IU heparin and 500 mg acetylsalicylic acid (ASS) intravenously (i.v.) directly before stent implantation and, additionally, 10,000 IU heparin at the end of the procedure. After the procedure, pigs received ASS 100 mg and clopidogrel 75 mg for 7 days.

Upon reduction stent implantation, pigs were split into two groups, one receiving regular diet and the other a high-fat diet. 28 days after the implantation of the reduction stent, occlusion of the LCx was verified via angiography; thereafter, baseline measurements of EF and left-ventricular end-diastolic pressure (LvEDP) were performed. After these initial measurements, rAAV vectors (5×10^{12} viral particles per pig) were injected into the great cardiac vein via pressure-regulated retroinfusion (either rAAV.Tβ4 or rAAV.LacZ). Pigs were kept on their assigned diet for the next 28 days, upon which, on day 56, measurements of EF and left-ventricular end-diastolic pressure were repeated, followed by the assessment of regional myocardial function by measuring subendocardial segment shortening in the ischemic and non-ischemic areas of the heart.

In general, during this experimental setup, some pigs died within the first 10-day period of the experiment. The rate of death was less than 20% with no difference between the groups. At day 28 all stents were occluded. Furthermore, at day 56, the extent of infarct size (percentage of left ventricle) was obtained, and animals displaying more than 5% infarct size were excluded from the study to assure that the obtained results reflected the reversal of hibernating myocardium and not the remodeling after an acute myocardial infarction. Finally, post mortem angiographies were performed to measure collateralization in the ischemic area and to evaluate the Rentrop score, a reading that estimates the filling of collaterals (0, no filling; 1, side branch filling; 2, partial main vessel filling; and 3, complete main vessel filling). Calculation of the collateral growth was performed in the ischemic area and border zone. More specifically, vessels showing the well-known screwdriver-like shape and vessels that directly connected the left anterior descending (LAD) or the proximal LCx with the vessel after the occlusion were counted as collaterals. Therefore, the post mortem angiography was in all pigs performed with the same orientation of the heart. After these measurements, organs were harvested for further histological analysis. Group sizes were as follows: NC + rAAV.LacZ, 7; NC + rAAV.Tβ4, 6; HC + rAAV.LacZ, 4; and HC + rAAV.Tβ4, 5.

Histological Analysis

After explantation of the heart, the left ventricle was cut in 5 slices from apex to bases in a 90-degree angle to the LAD. Thereafter, tissue samples from slices 1–3 of the LCx area (ischemic) as well as the LAD area (non-ischemic) were harvested. Tissue samples of the non-ischemic (LAD-perfused) and ischemic (LCx-perfused) areas of the heart in normal and high-fat diet pigs were analyzed for capillary density and inflammation. Calculation of capillarization was performed in the ischemic area. Capillaries were stained with a CD31 (PECAM) antibody (red fluorescent, SC1506, Santa Cruz Biotechnology, CA, USA). Pictures were taken with high-power field magnification (40-fold), and 5 independent pictures per region and animal were quantified. Pro-inflammatory cells (macrophages) were stained with a CD14 antibody (AbD Serotec, MCA1218F, Puchheim, Germany) in the RCx-perfused ischemic tissue of normal and high-fat diet with rAAV.Tβ4 or rAAV.LacZ transduction. Pictures were taken with low-power field magnification (10-fold) and 5 independent pictures per region and animal were quantified.

Statistical Analysis

Data are given as mean ± SEM. Differences among several groups were tested using ANOVA and Student Newman Keul's post hoc analysis. A p value of < 0.05 was considered statistically significant. All data were assessed using the SPSS software package (version 20.0; https://www.ibm.com/analytics/data-science/predictive-analytics/spss-statistical-software). Sample sizes are provided in the figure legends.

AUTHOR CONTRIBUTIONS

R.H. and C.K. conceived the project, designed and performed experiments, coordinated collaborations, and wrote the manuscript. T.Z. and A.B. performed the experiments, analyzed data, and wrote the manuscript. W.H., A.H., and K.K. analyzed pig experiments and performed histology analysis. C.W. and K.L.L. gave technical support and conceptual advice, interpreted results, and critically reviewed the manuscript.

CONFLICTS OF INTEREST

There are no conflicts of interest.

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