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# Overcoming disease-induced growth factor resistance in therapeutic angiogenesis using recombinant co-receptors delivered by a liposomal system

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ABSTRACT

Current treatment options for ischemia include percutaneous interventions, surgical bypass or pharmacological interventions aimed at slowing the progression of vascular disease. Unfortunately, while each of these treatment modalities provides some benefit for patients in the short-term, many patients have resistant or recurrent disease that is poorly managed by these therapies. A highly appealing strategy for treating ischemic disease is to stimulate the revascularization of the tissue to restore blood flow. While many techniques have been explored in this regard, clinically effective angiogenic therapies remain elusive. Here, we hypothesized that the presence of co-morbid disease states inherently alters the ability of the body to respond to angiogenic therapies. Using a mouse model of diabetes and obesity, we examined alterations in the major components for the signaling pathways for FGF-2, VEGF-A and PDGF under normal and high fat dietary conditions. In skeletal muscle, a high fat diet increased levels of growth factor receptors and co-receptors including syndecan-1, syndecan-4 and PDGFR-$\alpha$ in wild-type mice. These increases did not occur in Ob/Ob mice on a high fat diet and there was a significant decrease in protein levels for neuropilin-1 and heparanase in these mice. With the aim of increasing growth factor effectiveness in the context of disease, we examined whether local treatment with alginate gel-delivered FGF-2 and syndecan-4 proteoliposomes could overcome the growth factor resistance in these mice. This treatment enhanced the formation of new blood vessels in Ob/Ob mice by 6 fold in comparison to FGF-2 delivered alone. Our studies support that disease states cause a profound shift in growth factor signaling pathways and that co-receptor-based therapies have potential to overcome growth factor resistance in the context of disease.



## 1. Introduction

Peripheral arterial disease (PAD) affects about 30 million people worldwide and is estimated to affect 16% of the general population over 65 years of age [1]. The increase in prevalence of strong risk factors for PAD, including smoking, diabetes and obesity, indicate that the affected population will continue to grow [2]. A major sequela of PAD is the development of ischemia in the lower limb. Severe PAD has serious clinical consequences for patients including the formation of ulcers, pain from intermittent claudication and, ultimately, increased risk for limb amputation [3]. For many years the dominant clinical treatment for PAD was surgical revascularization through bypass grafting and endarterectomy. More recently, there has been a rapid growth in the number of endovascular treatments such as angioplasty, stenting and catheter-based atherectomy for PAD, although the overall benefit of these treatments versus surgery remains unclear [4]. A major limitation of these therapies is the limited clinical durability due to restenosis and continuation of the atherosclerotic disease process. Thus, an appealing alternative strategy for treating peripheral ischemia is the induction of angiogenesis through the exogenous delivery of growth factors, angiogenic genes or cells [5].

Angiogenesis is an intricate physiological process requiring the intricate coordination of endothelial cells, vascular smooth muscle cells, pericytes and macrophages under the control of environmental cues from the extracellular matrix and a host of growth factors/cytokines [6]. Among these, members of the FGF family bind

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to cell surface heparan sulfate proteoglycans, interactions that are essential to stabilize the formation of active FGF–FGF receptor complexes [7]. Consequently, cell surface heparan sulfate proteoglycans, such as the syndecans, can serve as essential co-receptors in this pathway. Vascular endothelial growth factor (VEGF) has also been recognized as a potent stimulator of endothelial proliferation/migration and plays an integral role in angiogenesis *in-vivo* through interactions with its two primary receptors Flt-1 (VEGFR-1) and KDR (VEGFR-2) [8]. Neuropilin-1 is a major co-receptor for VEGF acting to facilitate signaling with both Flt-1 and KDR [9]. In addition, syndecan-2 can bind VEGF and is essential for VEGF-mediated angiogenesis [10]. Platelet-derived growth factor-BB (PDGF-BB) is involved in pericyte recruitment around capillaries during angiogenesis and is consequently involved in blood vessel stabilization during angiogenesis and arteriogenesis [11]. The PDGF-β receptor has the high affinity for PDGF-BB, and this interaction has been linked to the control of cell migration and proliferation [12]. Both neuropilin-1 and the syndecans have been linked to regulation of PDGF activity [13–16]. In addition, PDGF-CC interacts with the PDGF-α and -β receptors, inducing angiogenesis [17] and revascularization of ischemic tissues [18].

Current therapies for peripheral ischemia are composed either of pharmacological interventions aimed at treating the progress of vascular disease/co-morbidities or interventional treatments such as angioplasty, stenting, endarterectomy or surgical bypass. However, for a significant portion of the clinical population these methods are insufficient to restore blood flow over the long-term course of their disease [19]. An appealing and potentially revolutionary strategy for treating ischemia is the stimulation of angiogenesis within the ischemic tissue, harnessing the body's own regenerative capacity to restore blood flow [20]. Previous studies have explored this strategy using exogenous applied growth factors [21–23], viral vectors to express growth factor/angiogenic transcription factor genes [23–32], or the implantation or mobilization of progenitors cells [25]. Unfortunately, while many of these strategies have shown promise in animal studies or small-scale clinical trials, none have found efficacy with significant clinically improvement in large randomized clinical trials [5].

Given the intense study of the process of angiogenesis and the evidence for the potent induction of angiogenesis by growth factors in experimental models, we hypothesized that the reason for this therapeutic failure may lie in disease mediated alterations in target tissue signaling. In animal models, ischemia is typically induced in a healthy animal by surgically ligating an artery either in the peripheral muscle or coronary arteries. Consequently, ischemia develops acutely in an animal that is often otherwise healthy. In human clinical use, the patient has developed ischemia most often through a long-term disease process. Thus, by the time patients have developed clinically recognizable symptoms they have had the disease for an extended period of time and the compensatory mechanisms of the human body may have been overwhelmed considerably. These mechanisms include, in all likelihood, the induction of the very angiogenic factors that we are attempting to use as therapeutic inducers of blood vessel growth. Accordingly, the presence of long-term ischemic disease in humans likely implies the presence of mechanisms to defeat growth factor therapy without modification. Here, we explored this hypothesis by examining how the expression of signaling components of FGF-2, VEGF-A and PDGF pathways change with diseased state caused by a high fat diet in the Ob/Ob mouse model. Our group has also recently shown that delivery of liposomally embedded co-receptors are effective in enhancing growth factor-induced signaling and trafficking in cell as well as revascularization in healthy rats [33]. To examine if this strategy could overcome disease-induced growth factor resistance, we developed an alginate-based hydrogel system for the local delivery of syndecan-4 (sdc-4) proteoliposomes in combination with FGF-2.

## 2. Materials and methods

### 2.1. Animal studies

All animal studies were performed with the approval of the University of Texas at Austin Institutional Animal Care and Use Committee (IACUC) and in accordance with NIH guidelines "Guide for Care and Use of Laboratory Animals" for animal care. Wild-type mice - C57BL/6J (Jackson Labs) and Ob/Ob mice - B6.Cg-Lep$^{ob}$/J (Jackson Labs) were used in these studies. The animals were fed normal chow diet (Lab Diet – Prolab RMH 1800) and high fat diet (Research Diets – D12331). The animals were fed a particular diet for 10 weeks and then sacrificed so that heart and quadriceps muscle could be harvested for downstream processing, as described below. We fed the animals for 15 weeks prior to the subcutaneous implantation surgery. For the subcutaneous implantation model, the dorsal surface of the mouse was shaved and prepared with three swabs of Betadine and 70% ethanol. A skin incision was made on the back with a scalpel and blunt dissection was used to create a subcutaneous pocket. An alginate bead containing growth factors or a control solution was implanted in the subcutaneous space. The wound was closed using resorbable sutures (Ethicon 5-0 polydioxanone). After seven days, the animals were sacrificed. The alginate gels were imaged and tissue samples were flash frozen in liquid $N_2$ cooled isopentane for subsequent analysis.

### 2.2. Gene expression analyses

Slices of the tissue were sectioned (10–20 μm) with a Leica CM 1850 cryotome equipped with a steel knife. The sections were dissolved in the RLT buffer (Qiagen) using a Qiagen Tissuelyzer with a stainless steel bead. RNA was isolated using the Qiagen RNeasy Midi kit and purity checked on a UV–Vis spectrophotometer (Thermo Scientific Nanodrop 2000c). Pure RNA was reverse transcribed into cDNA using the TaqMan Reverse Transcription reagents (Applied Biosystems). The cDNA was then used with SYBR Green PCR master mix (Life Technologies) for real time qPCR quantification using the Applied Biosystems ViiA™ 7 system. GAPDH was used as a reference gene. The cycling conditions used were 95°C for 10 min for initialization followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Primers for the PCR reactions are listed in Supplemental Table S3.

### 2.3. Western blotting analyses

The tissues were cryosectioned and the slices lysed in a buffer containing the following: 20 mM Tris at pH 8, 150 mM NaCl, 1% triton, 0.1% SDS, 2 mM sodium orthovanadate, 2 mM PMSF, 50 mM NaF, and protease inhibitors (Roche). Qiagen Tissuelyzer was used with stainless steel beads to facilitate tissue lysis. The lysates were normalized to the amount of protein loaded into the wells by performing Micro-BCA assay (Thermo-Scientific). The samples were then separated by gel electrophoresis (NuPAGE® Novex 10% Bis–Tris Midi Gel) and transferred to a nitrocellulose membrane using the iBlot system (Life Technologies). The membranes were then blocked for 1 h in 5% nonfat milk in PBS with 0.01% Tween-20 and exposed to primary antibodies (see Supplementary Table S4 for details) overnight at 4 °C. The membranes were washed and incubated at room temperature for 2 h at room temperature with secondary antibody and were detected using a chemiluminescence substrate (Thermo Fisher Scientific). Imaging was performed using the FluorChem HD2 system (Protein Simple). Quantification of the blots was done by densitometric analysis using Metamorph.

### 2.4. Histological analysis and immunostaining

Eight-micron thick sections were obtained from frozen tissues using the Leica CM 1850 Cryotome equipped with steel knife. For H&E staining, the sections were fixed in 10% formalin for 10 min, washed in 1× PBS for 5 min and then air dried at 60 °C for 1 h. The standard H&E protocol was then followed and imaged with an upright compound microscope. For immunohistochemical staining the sections were fixed in 4% paraformaldehyde for 5–10 min, blocked with 25% FBS for 45 min and then exposed to a 1:50 dilution of primary antibody (see Supplementary Table S4 for details) for overnight at 4 °C. The samples were washed three times with PBS and treated with a 1:500 dilution of secondary antibody conjugated to a fluorescent marker (see Supplementary Table S4 for details) for 2 h at room temperature. The slides were then rinsed with PBS and cover slipped with using DAPI containing anti-fade mounting medium (Vector Labs). Imaging was performed with the Zeiss Axiovert or Leica SP2 AOBS, and images were analyzed using Photoshop and Metamorph.

### 2.5. Recombinant protein production

A constitutive expression vector containing the full-length syndecan-4 gene (Genecopoeia) was transduced into HEK-293Ta cells (Genecopoeia) using the Lenti-Pac transduction kit (Genecopoeia). Two days post-transduction, cell lysis was performed with a buffer containing the following: 20 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 2 mM sodium orthovanadate, 2 mM PMSF, 50 mM NaF, and protease inhibitors (Roche). The lysates were clarified by centrifugation for 15 min at