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Vascular Metabolomics —
gene regulation and role of VEGF-B in tissue fatty acid uptake

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To my parents and the Universe

Nearly 14 billion years ago, it all started with the Big Bang...

About the cover picture: NGC5897 is a globular cluster of stars, which symbolizes the “messy” metabolism regulatory networks. This deep-sky object, about 40,000 light-years away, is located in the constellation Libra, which symbolizes the metabolic balance elucidated in Paper II.

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科普综述

地球上的生命有着 36 亿年漫长的进化史。大约 10 亿年前，多个特化的系统逐渐衍生出来，这极大的推动了生物从单细胞到复杂多细胞结构的进化。其中之一就是存在于现代高等生物体内的血液循环系统。血液循环系统（依托血管和血液）向机体各部分输送氧气和营养，同时带走二氧化碳和代谢废物。血管中最靠近血液的一层细胞称为血管内皮，是血液循环系统中最重要的一部分。有一族蛋白分子，称为血管内皮生长因子。它们负责控制最初胚胎血管的形成和发育，以及维持成年动物体内血管的正常功能。1996 年，我们的实验室发现了血管内皮生长因子 B，这里简称为“B”。蛋白因子 B 就是本论文的研究对象。

本论文包含两篇文章，第一篇阐述了 B 在体内的生理功能，第二篇则阐述了 B 在体内是如何按需调节的。与同家族其它成员不同，B 不控制血管生长，而且完全缺乏 B 的小鼠没有任何明显的生理缺陷和异常。因此，在发现 B 后相当长的一段时间内，其生理作用和调控机制均未获得阐释。众所周知，细胞中的线粒体负责生产能量。我们在 2005 年意外发现 B 和线粒体存在高度的关联，这是其他任何生长因子都不具备的。有鉴于此，我们进而阐明了上述关于 B 生物学意义的最重要的两个问题。

线粒体主要通过“燃烧”两种物质来产生能量，分别是葡萄糖和脂肪酸。已知葡萄糖在体内主要通过胰岛素来进行调节，所以我们假设 B 应当负责脂肪酸的转运。经过一系列的实验，果然如此，问题一得到解答。问题二：既然 B 和线粒体关联度极高，那么 B 的调节应该和线粒体的调节类似。已知有一种调节线粒体功能的蛋白简称为“P”，经过一系列的实验，P 果然可以调节 B。两个基本问题都回答了，那么 B 在体内是如何发挥作用的呢？以肌肉为例：肌肉细胞经过锻炼后，需要更多能量，于是 P 被激活。它一方面促进线粒体数量的增加，一方面促进肌肉细胞分泌 B。B 虽然不能控制血管的生长，但可以指导血管把更多的脂肪酸从血液转运到肌肉细胞里。既增加了燃料（脂肪酸），又增强了引擎（线粒体），引擎得以加速运转，就产生了更多能量。这样，肌肉细胞有能力更快更多地产生能量，为下一次运动做好准备。

那我们的科研意义何在呢？目前，尤其是在中国等一些发展中国家，糖尿病已经发展成为一种常见病。营养过剩再加上运动匮乏，往往导致肥胖；长期过度肥胖极易罹患 II 型糖尿病。近十几年的研究表明，过多摄取碳水化合物（包括饮食中的糖，以及存在于面食和米饭中的淀粉等）并不是 II 型糖尿病的根本致病原因，这与人们通常的理解大相径庭。事实上，肥胖本身也不是根本病因。真正的原因是脂肪去错了地方：摄入脂肪等能量物质过多（多余的糖、淀粉等在体内也会转化成脂肪），会超过身体脂肪组织的存储能力，这就导致过多的脂肪进入肝、肌肉、心脏等组织，这就是“脂肪溢出假说”。这些组织中脂肪含量太多，会严重影响组织对葡萄糖的吸收，进而导致葡萄糖滞留在血液中，血糖也就升高了。

我们的研究对象 B，专门负责脂肪酸在肌肉和心脏等组织中的转运。如果能够阻断 B，上述组织就不会过量吸收脂肪酸，而会重新增加对葡萄糖的吸收，这样 II 型糖尿病的症状就会得到改善。实际上，本论文及本实验室最近发表于 Nature 的另一篇文章，已经通过多种方法证实了这一点。在大鼠或小鼠上的动物实验证明，即使过量进食高脂肪食物，缺乏运动，只要阻断 B 的作用，它们也会“胖并快乐着”，所有与 II 型糖尿病相关的症状都得到了显著的改善。总之，我们的研究成果为开发高效抗糖尿病药物提供了全新的思路。

ABSTRACT

Vascular endothelial growth factor B (VEGF-B) belongs to the VEGF family, which constitutes of five mammalian members. VEGFs exert pivotal roles in the formation, development and maintenance of the vascular and lymphatic vessels. Unlike VEGF-A, the first VEGF discovered and a close homologue, VEGF-B is poorly angiogenic in most tissues and not regulated by hypoxia. Gene regulation and physiological function of VEGF-B remained obscure for more than a decade after its discovery.

We identified an unexpected high correlation of expression of *Vegfb* with a large cluster of nuclear-encoded mitochondrial genes. This high correlation is not shared by any other VEGF gene. Based on this finding, we were able to answer two fundamental questions in VEGF-B biology in this thesis work: gene regulation and role of VEGF-B.

In Paper I, we identified an unexpected role of VEGF-B in tissue fatty acid (FA) uptake. VEGF-B induces endothelial FA uptake through upregulation of two fatty acid transporter proteins (FATPs), namely FATP3 and FATP4. This regulation is dependent on the two known receptors for VEGF-B, VEGF receptor 1 (VEGFR1) and neuropilin 1 (NRP1), and it is unique among the three VEGFR1 ligands. Genetically modified mouse models that are deficient in VEGF-B signaling showed reduced lipid accumulation in peripheral tissues. In *Vegfb* knockout mice, FA uptake capacity in heart, skeletal muscle and brown adipose tissue was reduced. The resulted excess FA was diverted to white adipose tissue for storage. As a consequence, the glucose uptake capacity in the heart was drastically increased in *Vegfb* knockout mice.

In Paper II, we demonstrated that *Vegfb* is regulated by peroxisome proliferator activated receptor coactivator 1 α (PGC-1 α) through coactivation of estrogen-related receptor α (ERR α). *Vegfb* was upregulated in parallel with *Pgc1 α* and mitochondrial genes upon nitric oxide simulation and serum deprivation in cells. ERR α , together with PGC-1 α , strongly activated the *Vegfb* promoter in luciferase assay. It is known that muscle creatine kinase PGC-1 α transgenic (MCK-PGC-1 α TG) mice become insulin resistant on a high-fat-diet (HFD). *Vegfb* deficiency in HFD-fed MCK-PGC-1 α TG mice greatly improved insulin sensitivity as well as other metabolic parameters. This improvement may be attributed to the reduction in muscular lipid accumulation.

PGC-1 α and ERR α are known major regulators of mitochondrial biogenesis. In this thesis, we have elucidated that they also regulate VEGF-B expression and hence endothelial FA uptake in parallel. The two pathways are tightly coordinated to maintain a balance of FA β -oxidation and lipid homeostasis in the body. These findings have opened up new horizons for finding therapeutic targets in treating metabolic disorders such as type 2 diabetes.

LIST OF PUBLICATIONS

- I. Hagberg CE, Falkevall A, **Wang X**, Larsson E, Huusko J, Nilsson I, van Meeteren LA, Samén E, Lu L, Vanwildemeersch M, Klar J, Genove G, Pietras K, Stone-Elander S, Claesson-Welsh L, Ylä-Herttuala S, Lindahl P, Eriksson U. Vascular endothelial growth factor B controls endothelial fatty acid uptake. *Nature*. 2010 Apr 8; 464(7290):917-21.

- II. **Xun Wang**, Annika Mehlem, Annelie Falkevall, Carolina Hagberg and Ulf Eriksson. VEGF-B mediates high-fat-diet-induced insulin resistance in PGC-1 α overexpressing muscle.
Manuscript.

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LIST OF ABBREVIATIONS

BAT	brown adipose tissue
¹⁴ C-OA	¹⁴ C-labeled oleic acid
cDNA	complementary deoxyribonucleic acid
<i>Cyts</i>	cytochrome c, somatic
ERR α	estrogen-related receptor α
FA	fatty acid
FATP	fatty acid transporter protein
[¹⁸ F]FDG	¹⁸ F-labeled deoxyglucose
HFD	high-fat diet
HIF-1 α	Hypoxia-inducible factor 1 α
HSPG	heparan sulfate proteoglycan
LCFA	long-chain fatty acid
MCK-PGC-1 α TG	muscle creatine kinase PGC-1 α transgene/transgenic
mRNA	messenger ribonucleic acid
ND	normal diet
NO	nitric oxide
NRF	nuclear respiratory factor
NRP	neuropilin
PC	parietal cell
PGC-1 α	PPAR γ coactivator 1 α
PIGF	placenta growth factor
PPAR	peroxisome proliferator-activated receptor
qPCR	quantitative polymerase chain reaction
siRNA	short interfering RNA
sVEGFR1	soluble VEGFR1
T2D	type 2 diabetes
<i>Vegfb</i> ^{-/-}	<i>Vegfb</i> knockout
VEGF(R)	vascular endothelial growth factor (receptor)
WAT	white adipose tissue
WT	wildtype

1 INTRODUCTION

During the 3.6 billion years history of life on Earth, several important specialized systems gradually evolved. These evolution processes started about 1 billion years ago, which helped the organisms to leap from single cellular to more complex multicellular structures. The circulatory system in modern higher animals is among these crucial systems. It supports transport of oxygen and nutrients, as well as carbon dioxide and waste metabolites. Vascular endothelial growth factors (VEGFs) and their receptors exert fundamental and crucial roles in the formation, development and maintenance of the circulatory system.

Individual introductions are blended into the chapters below. In Chapter 3 (Paper I), common fatty acid (FA) handling proteins and FA uptake hypotheses are introduced in Section 3.1, followed by an introduction to the endothelial barrier (Section 3.2). In Chapter 4 (Paper II), regulations of VEGFs are introduced in Section 4.1, and the major mitochondrial biogenesis regulator peroxisome proliferator-activated receptor γ coactivator 1 α (PGC-1 α) is introduced in Section 4.2.1. Randle's cycle and lipid-induced insulin resistance are introduced in Section 4.3.1. In Chapter 5, the roles of VEGFs in tumorigenesis are introduced in Section 5.1.4. Current treatments for insulin resistance and type 2 diabetes (T2D) are introduced in Section 5.2.3. In this chapter, the VEGF family and their receptors are introduced.

1.1 VEGFs and their receptors

There are currently five mammalian VEGFs in the family, namely VEGF-A, placenta growth factor (PlGF), -B, -C and -D (Figure 1). There are also two non-mammalian VEGFs, VEGF-E found in orf virus and VEGF-F discovered in snake venom. Three VEGF receptors, together with the co-receptors neuropilins (NRPs) as well as heparan sulfate proteoglycans (HSPGs) and integrins, master downstream VEGF signaling pathways^{1,2}.

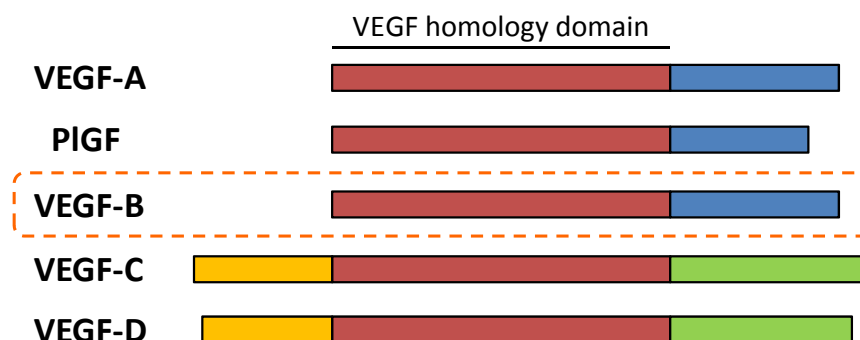


Figure 1. Schematic illustration of the domain structures of the five known mammalian VEGFs. The different domain structures include the VEGF homology domain (in red), the heparin-binding domains at the C-terminal in some splice isoforms of VEGF, PlGF and VEGF-B (in blue), the N-terminal propeptide domains (in yellow), and silk domain-containing C-terminal propeptides in VEGF-C and D (in green). The domains are not drawn in scale. Referenced and modified from Li et al., 2001³.

1.1.1 VEGF-A signaling through VEGFR2

VEGF-A, also known as VEGF, is the first identified member of the family⁴. Genetic deletion of a single *Vegfa* allele is enough to cause embryonic lethality⁵, indicating its fundamental and crucial functions during embryonic development. VEGF-A is regulated primarily by hypoxia inducible factor 1 α (HIF-1 α) in response to hypoxia^{6,7}. It binds to VEGFR2 and induces phosphorylation of the tyrosine kinase domain. VEGFR2 signaling, induced by the binding of VEGF-A, is responsible for tip cell formation, sprouting, migration, maturation and tube formation of endothelial cells during normal and pathological conditions^{1,2} (Figure 2).

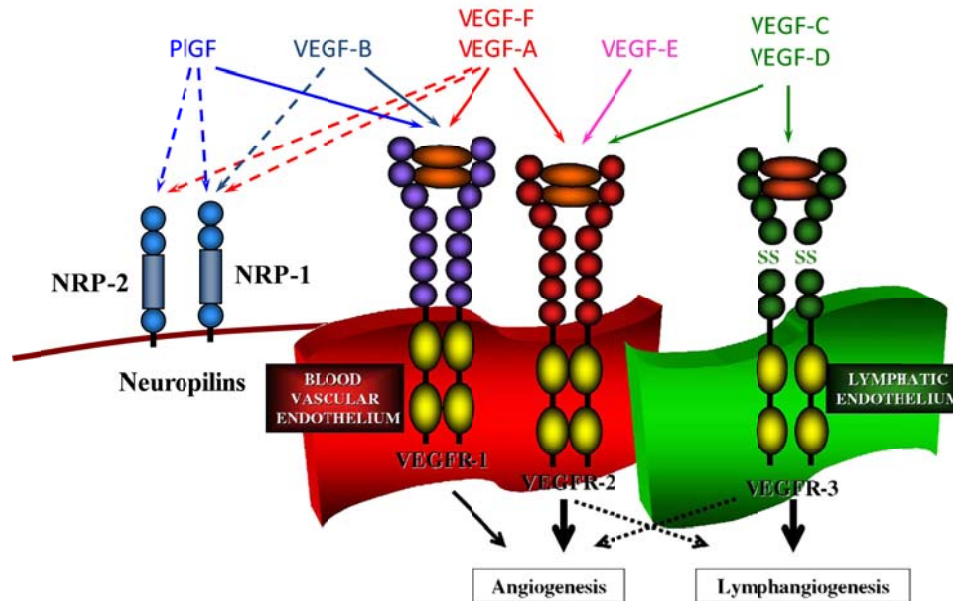


Figure 2. VEGF ligands and their receptors. Dotted arrows indicate that not all isoforms bind the NRPs. Referenced and modified from Tammela et al., 2005¹.

1.1.2 PIGF

PIGF, encoded by the *PGF* gene, was first isolated from a human placenta cDNA library⁸. PIGF expression is found predominantly in the placenta, heart and lungs⁹. *Pgf* knockout mice did not show any apparent pathological phenotype, indicating that PIGF being dispensable during embryonic development¹⁰. By studying *Pgf* knockout^{10,11} and skin-specific transgenic^{11,12} mouse models, PIGF was shown to exert important modifying roles in pathological angiogenesis during ischemia, inflammation, wound healing and cancer.

1.1.3 VEGF-B

VEGF-B was the third VEGF family member to be discovered. Being a close homologue, VEGF-B was expected to have similar and redundant functions to VEGF-A and PIGF during angiogenesis¹³. As VEGF-B is the focus of this thesis, it deserves a separate section below (Section 1.2).

1.1.4 VEGFR1 and its ligands

VEGFR1 binds VEGF-A, -B and PlGF with high affinity (Figure 2). Unlike VEGFR2, VEGFR1 shows very low tyrosine phosphorylation activity upon ligand binding¹⁴, which make its signaling cascade difficult to be captured and studied. When VEGFR1 dimerize with VEGFR2 however, the signaling properties are stronger than homodimers of either receptor¹. VEGFR1 expression is found primarily in endothelial cells, although it is also seen in other cell types¹. In Paper I, we found that *Vegfr1* expression was restricted to the cardiac endothelium in mouse heart. Among the three VEGFRs, only VEGFR1 was found to be induced by hypoxia via the HIF-1 α pathway¹⁵.

The three VEGFR1 ligands are found to exhibit distinct functions in various physiological and pathological processes^{1,2}. Binding of either PlGF or VEGF-A to VEGFR1 induces phosphorylation of distinct tyrosine residues and hence regulates different sets of genes¹⁶. In Paper I, we also showed a unique regulation of downstream target genes by VEGF-B among all three VEGFR1 ligands (Section 3.3.5). VEGF-A was recently shown to be regulated by the PGC-1 α /estrogen-related receptor α (ERR α) pathway besides the canonical HIF-1 α pathway¹⁷⁻²⁰. In Paper II, we elucidated that VEGF-B can also be regulated by the PGC-1 α /ERR α pathway. The two VEGFR1 ligands, VEGF-A and -B, have distinct expression patterns (Paper I), although both being regulated by PGC-1 α /ERR α . The hypothesis of the regulatory mechanisms behind this differential regulation is discussed in Section 4.2.3.

VEGFR1 binds VEGF-A with higher affinity in comparison to VEGFR2²¹ (Figure 2). In angiogenic vasculature, VEGFR2 expression is more profound in the tip cell region whereas VEGFR1 expression is more retained in the stalk cell region²². PlGF deficiency impaired VEGF-A signaling¹⁰. Under certain pathological conditions, PlGF displaces VEGF-A from VEGFR1 and thereby allows higher VEGF-A/VEGFR2 signaling activity²³. VEGF-A mutants engineered to bind VEGFR1 specifically do not exhibit mitogenic signals in endothelial cells²⁴. Furthermore, unlike embryonic lethality of *Vegfr1* deletion due to vessel overgrowth^{25,26}, mice lacking the VEGFR1 intracellular kinase domain showed only minor defects in pathological angiogenesis, indicating that VEGFR-1 might function as an inert decoy^{10,27}. All these findings point to the “sink” theory that VEGFR1, without exhibiting significant downstream signals, competes the binding of VEGF-A with VEGFR2 and hence modifies its signaling^{1,2,28}.

VEGFR1 encodes a soluble variant through alternative splicing, namely soluble VEGFR1 (sVEGFR1), which contains only the extracellular domains of VEGFR1²⁹. Preeclampsia is a pregnancy-specific syndrome of hypertension and proteinuria. Placenta-derived sVEGFR1 has been shown to play an important role during the pathogenesis of preeclampsia³⁰. Although these advance, the precise biological role of sVEGFR1 remains to be elucidated.

1.1.5 NRPs and HSPGs

NRP1^{31,32} and NRP2^{31,33} were isolated from neurons, and were shown to mediate repulsive signals during neuronal axon guidance. Besides class 3 semaphorins³¹⁻³³,

NRP1 binds VEGF-A, -B and PlGF while NRP2 binds VEGF-A, -C and PlGF³⁴ (Figure 2). NRP1 acts as a co-receptor enhancing VEGFR2 signaling³⁵, although NRPs does not seem to show signal transduction properties upon VEGF-A binding³⁶. Later studies have shown that the PSD-95/Dlg/ZO-1 (PDZ) binding domain, which consists of three amino acids at the carboxyl-terminal of NRP1³⁷, is crucial for its signaling³⁸. Genetic deletion of *Nrp1* is embryonic lethal due to vessel malformation, indicating that NRP1 plays roles in embryonic vessel formation, as well as nerve fiber guidance³⁹.

HSPGs exist ubiquitously on the cell surface and extracellular matrix. HSPGs bind longer VEGF-A isoforms and facilitate spatial gradient forming, which is crucial for the angiogenesis processes like tip cell formation and sprouting⁴⁰. The shorter VEGF-B isoform, VEGF-B₁₆₇, also binds HSPGs through its hydrophilic carboxyl-terminal^{13,41}, but the biological significance of this binding is poorly understood.

1.1.6 VEGF-C, -D and VEGFR3

Both VEGF-C and -D bind to and signal through VEGFR2 as well as VEGFR3² (Figure 2). VEGF-C was shown to be required for sprouting of the first lymphatic vessels from embryonic veins⁴², indicating its pivotal role in lymphangiogenesis. VEGF-C was shown to be responsible for supporting lymphangiogenesis, tumor growth and metastases in various types of cancers^{1,2}. Similar to VEGF-C, both in molecular structure and function, VEGF-D can strongly induce angiogenesis and lymphangiogenesis, and also plays a vital role in lymphatic metastasis in a variety types of cancers². VEGFR3 was shown to be important in both angiogenesis and lymphangiogenesis, as genetic deletion of *Vegfr3* in mice was not phenocopied by the double-deletion of *Vegfc* and *Vegfd*⁴³.

1.1.7 VEGF-E, and -F

VEGF-E was found in a parapoxvirus, namely the orf virus. Although VEGF-E isoforms show low amino acid sequence identity, they are structurally highly similar to VEGF-A, and can strongly activate VEGFR2 phosphorylation with high binding affinities^{40,44} (Figure 2). VEGF-F is the most recently discovered VEGF member, found in snake venom. Like VEGF-E, it binds specifically to VEGFR2 without heparin-binding properties⁴⁵ (Figure 2). Since VEGF-E and -F are the only two VEGFs that bind to VEGFR2 specifically, potential usage in clinical pro-angiogenic therapies was proposed². The existence of the non-mammalian VEGFs is a good example of parallel evolution, which shows the power and beauty of natural selection.

1.1.8 Retrospective of the VEGFs

VEGF-A was identified already in 1983 as a VPF secreted by tumor cells⁴. In 1989, it was first named as VEGF and its cDNA was cloned⁴⁶. In 1992, VEGF-A was shown to function as a hypoxia-inducible angiogenic factor⁶. Later in 1995, a 28-bp element including HIF-1 consensus sequences in *Vegfa* promoter was identified, which was sufficient to regulate *Vegfa* expression in response to hypoxia⁷. Genetic deletion of *Vegfa* in mice was done in 1996. The study showed that deletion of a single *Vegfa* allele (*Vegfa*^{+/-}) was enough to cause embryonic lethality⁵. Coming to the end of the first decade of the 21st century, a few recent studies showed that VEGF-A can be

induced by PGC-1 α /ERR α in response to hypoxia and nutrient deprivation, independent of the canonical HIF-1 α pathway¹⁷⁻²⁰.

In 1991, PlGF was first isolated and cloned shortly after the identification of VEGF-A⁸. The knockout mice were generated in 2001 and showed no major abnormality under normal conditions¹⁰.

VEGF-B was discovered in 1996 as a partial mouse cDNA clone encoding a VEGF-related peptide¹³. Two independent *Vegfb* knockout (*Vegfb*^{-/-}) mouse lines were generated respectively in 2000⁴⁷ and 2001⁴⁸, which both showed a mild phenotype. The search for the genuine physiological role of VEGF-B lasted more than a decade. In 2005, we found a tight correlation of expression of *Vegfb* with a mitochondrial gene cluster, which is unique in the VEGF family. Subsequently in 2010, an unexpected role of VEGF-B controlling endothelial FA uptake was finally discovered (Paper I). Following this breakthrough finding, the role of VEGF-B in the pathogenesis of insulin resistance and T2D was unveiled this year⁴⁹. This role is further investigated in another high-fat diet (HFD)-induced insulin resistance mouse model in Paper II.

VEGF-C and -D were both discovered in 1996. VEGF-C was purified as a VEGFR3 ligand and had its cDNA cloned from human prostatic carcinoma cells⁵⁰. VEGF-D was isolated from fibroblasts, named as *c-fos*-induced growth factor (FIGF) and was linked to tumor malignancy at the very beginning of its discovery⁵¹. *Vegfc* knockout mice were generated in the same year and were shown to have severe edema and embryonic lethality⁴². *Vegfd* knockout mice were generated almost a decade later and showed only minor lymphatic phenotypes, indicating it being dispensable during development of the lymphatic system⁵².

VEGF-E and -F were discovered in 1994⁴⁴ and 2004⁴⁵ respectively. A VEGF-like gene was identified in the genome of orf virus in 1994⁴⁴. In 1998, Ogawa et al. first named the product of this gene as VEGF-E⁵³. Since these later VEGFs are not endogenous mammalian VEGFs, only a limited number of studies has been done in comparison to other VEGF members.

PlGF was shown to have modifying roles in pathological angiogenesis¹⁰⁻¹². Number of publications related to PlGF was quickly overtaken by that related to VEGF-C, which is known to exert important roles in lymphatic metastasis^{1,2}. VEGF-D has drawn much less attention in comparison to VEGF-C. This is probably due to the fact that VEGF-D is genetically and functionally similar to VEGF-C^{1,2}. Nonetheless, the number of publications on VEGF-D still outnumbers that on VEGF-B with a relatively high margin. This is a rough representation to the fact that the gene regulation and role of VEGF-B remained elusive for more than a decade after its discovery (Figure 3).

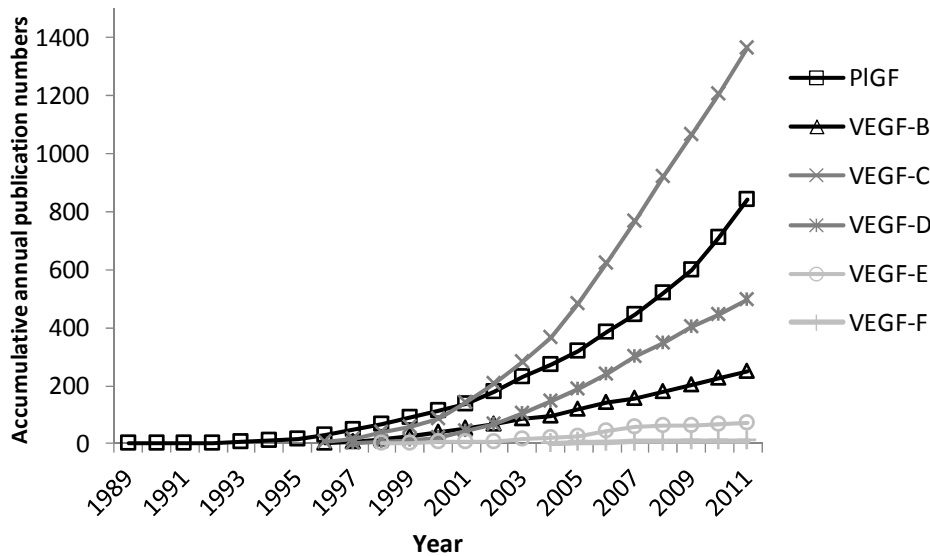


Figure 3. Accumulative annual publication numbers on PubMed in the field of VEGF research. Search criteria: numbers for each VEGF, of which respective abbreviated or full names appearing in the title or abstract in a publication, were counted. The number for VEGF-A is not shown here since it is difficult to distinguish with VEGF or VEGFR in general.

1.2 VEGF-B

VEGF-B binds to VEGFR1 and NRP1⁵⁴, and has two mRNA splicing variants⁴¹. The shorter form VEGF-B₁₆₇ binds to the extracellular matrix and is the predominant isoform under normal physiological conditions¹³. The longer form VEGF-B₁₈₆, on the other hand, is freely diffusible and was found to be upregulated in various forms of tumors⁵⁵. In Paper I, VEGF-B₁₈₆ was shown to induce higher fatty acid transporter protein (FATP) expression and FA uptake than VEGF-B₁₆₇ *in vitro*. Abundant VEGF-B expression was found in metabolic active tissues such as heart, skeletal muscle and brown fat⁴⁸. *Vegfb*^{-/-} mice were generated with the hope to reveal its function. In contrast to *Vegfa*, genetic deletion of *Vegfb* in mice resulted in a mild phenotype^{47,48}.

In the following years, the mystery of VEGF-B was unveiled piece by piece after another. Unlike VEGF-A, VEGF-B is poorly angiogenic and not regulated by hypoxia^{23,56,57}. In a rabbit hindlimb ischemia model however, VEGF-B gene transfer was shown to be beneficial⁵⁸. VEGF-B was also shown to stimulate neurogenesis⁵⁹ and have neuroprotective effects⁶⁰⁻⁶². A more recent study showed that VEGF-B inhibits apoptosis by suppression of BH3-only protein gene expression via VEGFR1 signaling⁶³. Heart-specific VEGF-B overexpression in mice was shown to alter cardiac ceramide accumulation and it induces myocardial hypertrophy⁶⁴. Although the advances, the whole picture of VEGF-B biology was still like a huge jigsaw puzzle with only a few pieces put in place. Even after the functions of the more recently identified VEGFs were well established, the role and gene regulation of VEGF-B remained enigmatic and controversial.

In Paper I, a number of published microarray data sets were pooled and analysed. The original aim of the analysis was to identify sets of co-expressed genes, including new mitochondrial genes. However, VEGF-B, a VEGF member was never thought to be

correlated with mitochondrial functional, and it surfaced with an astonishing high correlation coefficient ($r = 0.90$). In comparison, other VEGF family members showed much lower or even no correlation at all (VEGF-A, $r = 0.30$; PlGF, $r = -0.18$; and VEGF-C, $r = -0.10$). Early VEGF-B studies have already showed this once overlooked this correlation as high expression of VEGF-B was found in tissues with high mitochondrial content, such as heart, skeletal muscle, brown fat and kidney. This was the starting point for the discovery of the function of VEGF-B. Following these initial findings, the thesis work has answered two fundamental questions that remained unclear in VEGF-B research: the physiological role (Paper I) and gene regulation (Paper II) of VEGF-B.

2 AIMS

The correlation of *Vegfb* expression with mitochondrial gene expression was unexpected. Based on this finding, we have established the following aims:

To characterize the role of VEGF-B in tissue FA uptake and its signaling pathways (**Paper I**);

To phenotype *Vegfb*^{-/-} mice in identifying the physiological consequences of genetic deletion of *Vegfb* (**Paper I**);

To identify the molecular regulatory mechanism of *Vegfb* (**Paper II**).

After identifying PGC-1 α as a major regulator of *Vegfb*, we set yet another aim:
To characterize the physiological consequences of genetic deletion of *Vegfb* in a PGC-1 α transgenic mouse model (**Paper II**).

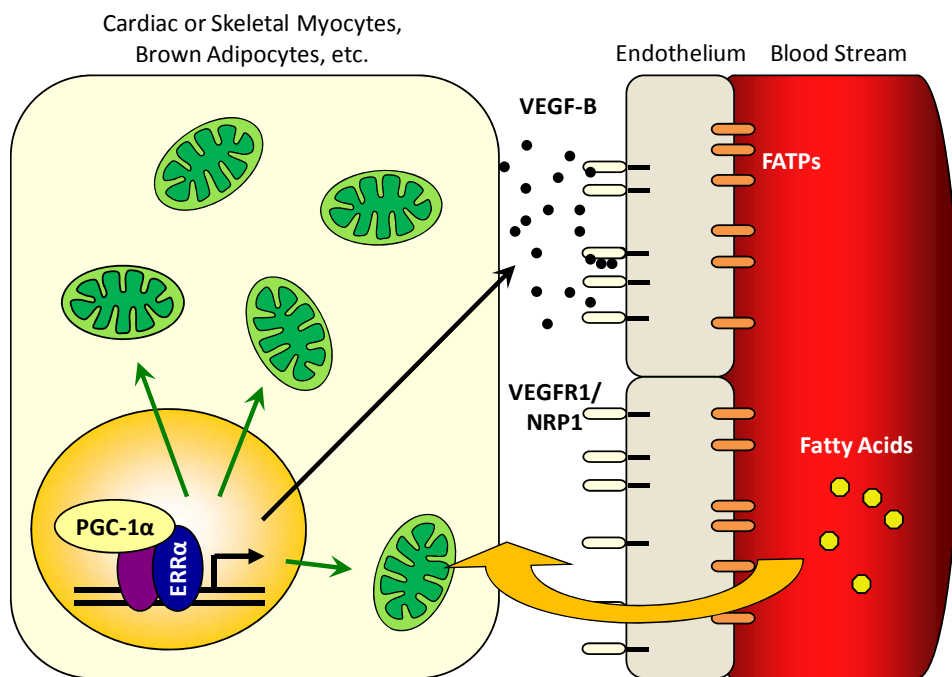


Figure 4. Schematic illustration of the working hypothesis on gene regulation and role of VEGF-B. VEGF-B is secreted in parallel with mitochondrial biogenesis upon activation of ERR α coactivated by PGC-1 α . VEGF-B then instructs the endothelium to upregulate FATPs for an increase of FA influx from the blood stream via VEGFR1 and NRP1 signaling. As a consequence, the increase in FA uptake matches elevated β -oxidation capacity in the mitochondria to fulfill the higher energy demand in the tissue cell.

3 PAPER I: VEGF-B CONTROLS ENDOTHELIAL FA UPTAKE

3.1 Current view and hypotheses of FA transport

Due to the bipolar nature of FA molecules, it was believed that FA transport across mammalian cell membranes is a combination of passive flip-flop and protein-mediated diffusion^{65,66}. Several membrane proteins associated with long-chain fatty acid (LCFA) uptake have been identified including FATPs and CD36⁶⁵.

Due to the intrinsic very long-chain acyl-CoA synthetase (VLACS) activity, it was debated whether FATPs are also solute carriers⁶⁷. Among the six FATPs identified, FATP1 and 6 express in the heart, while FATP1, 3 and 4 express in the skeletal muscle⁶⁵. In Paper I, we have shown that *Fatp3* expression is restricted in the cardiac and muscular endothelium.

The transmembrane glycoprotein CD36 has been identified as a putative transporter of LCFAs⁶⁸. Subsequent *in vitro* and *in vivo* studies have provided strong support for a role of CD36 in FA transport⁶⁹. Studies on various transgenic and genetic deletion mouse models of *Cd36* have confirmed the hypothesis that it facilitates a major fraction of FA uptake in heart, skeletal muscle, and adipose tissues, where it is highly expressed⁶⁹.

Tight endothelial cell layer exists between the blood and the tissue cells in most of the organs. Despite of this fact, the dominant consensus in the field of FA transport is that the rate-limiting step of FA uptake occurs at the plasma membrane of the tissue cells, with large ignorance of the endothelium.

3.2 Post-angiogenesis: the endothelial barrier

The process of vessel sprouting was documented as early as in the 17th century⁷⁰. In the late 1960s, a diffusible angiogenic factor derived from tumors was identified and the term “tumor angiogenesis” was first coined⁷¹. Blood vessels, which are composed of endothelial cells and mural cells, support normal and tumor tissue with oxygen and nutrient. This simple fact has led the field of vascular biology research focused on studying the pure extension of the endothelium without considering phenotypical changes at early years. In the early 1970s, Folkman proposed targeting angiogenesis as a treatment for malignant tumors⁷². Later studies on anti-angiogenesis therapies have brought more attentions to vessel maturation after the initial growth, including changes in endothelial cell junctions, pericyte coverage as well as functional changes such as blood perfusion^{70,73}.

Except for the smallest molecules like oxygen, carbon dioxide and nitric oxide (NO), the transport of most of molecules across the cell membranes are tightly controlled processes. Even water molecules were found to be transported in a controlled manner in certain tissues⁷⁴. With the notion of the endothelial barrier in mind, we hypothesized that the transport of FA across the endothelial cell layer in most tissues is also a tightly controlled mechanism.

3.3 Physiological role of VEGF-B

3.3.1 The starting point and the working hypothesis

Two independent bioinformatic analyses of published data had pointed to the same conclusion: VEGF-B, but not any other VEGFs, is highly correlated with mitochondrial genes. Two additional qPCR analyses have also shown similar gene expression patterns of *Vegfb* with two mitochondrial markers, *Ndufa5* (encodes NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 5) and *Cytc* (encodes cytochrome c, somatic), across a variety of mouse tissues and nutritional states. Genetic deletion of *Vegfb* has no influence on mitochondria copy number in mouse heart. These findings indicate that VEGF-B is closely related to mitochondrial function and there is no direct feedback loop between *Vegfb* and mitochondrial gene expression.

We then hypothesized that in the most energy demanding tissue cells, while mitochondrial biogenesis is underway, VEGF-B is secreted in parallel to instruct the endothelium for more FA transport from the blood stream. In this way, increased FA uptake matches up with higher mitochondrial content in the tissue cells for elevated energy production.

3.3.2 VEGF-B controls endothelial FA uptake *in vitro*

We first tested if VEGF-B regulates FA handling genes in endothelial cells *in vitro*. Both VEGF-B isoforms increased mRNA and protein levels of several FATPs across a number of endothelial cell lines. Pre-incubation of respective neutralizing antibodies with the endothelial cells prior to addition of VEGF-B unveiled that this effect is dependent on VEGFR1 and NRP1, but not VEGFR2. In contrast, neither VEGF-A nor PlGF, the other VEGFR1 ligands, can upregulate these FATPs. Furthermore, applying VEGF-B to a fibroblast cell line (NIH/3T3) did not induce FATP expression, indicating endothelial-cell-specific signaling pathway and/or transcription machinery being involved. A kinase inhibitor screening unveiled that the induction of FATP expression by VEGF-B is dependent on phosphatidylinositol-3-OH kinase (PI3K) pathways.

Since the exact mechanism of cellular FA uptake by FATPs is debated^{65,67}, we examined whether VEGF-B can induce LCFA accumulation through FATPs in cells, with a fluorophore-labeled LCFA analogue as the marker. By overexpressing (transient transfection) or silencing (using siRNA) *Fatp3/4* in endothelial cells, with or without addition of VEGF-B, we have shown that this LCFA uptake is VEGF-B-dependent via the FATPs. Interestingly, similar experiments done on HL-1 cells, a cardiomyocyte cell line, did not alter LCFA uptake. This gives more evidence that the induction of FA uptake by VEGF-B via FATPs is endothelial-cell-specific.

To closer mimic *in vivo* FA transport across the endothelium, we utilized cell culture inserts. Cultured endothelial cells can form a tight monolayer which creates two isolated liquid compartments. VEGF-B treatment increased ¹⁴C-OA transport across the endothelial cell layer in a NRP1-dependent manner, indicating VEGF-B controls trans-endothelial LCFA transport.

3.3.3 VEGF-B controls endothelial FA uptake *in vivo*

To determine whether VEGF-B signaling is endocrine or paracrine manner, we isolated endothelial cells from mouse heart. In *Vegfb*^{-/-} heart, only endothelial expression of *Fatp3* and *Fatp4* was decreased, indicating that VEGF-B signals in a paracrine fashion. Adenoviral administration of VEGF-B in mouse heart increases *Fatp* expression whereas genetic deletion of *Vegfb* in mice decreased it. Intracellular lipid accumulation was reduced in *Vegfb*^{-/-} heart, soleus and brown adipose tissue (BAT) compared to the wildtype (WT) counterparts. A similar decrease of *Fatp* expression and lipid accumulation was also found in tamoxifen-treated EC-SCL-Cre-ER^T-positive *Nrp1*^{fl/fl} (endothelial-cell-specific *Nrp1* knockout, *Nrp1-EC*^{-/-}) mice. Overexpressing VEGF-B by systemic adenoviral infection in *Vegfb*^{-/-} mice rescued *Fatp* expression and lipid accumulation, in but not *Nrp1-EC*^{-/-} mice. These results indicate that VEGF-B regulates endothelial FA uptake *in vivo* in a NRP1-dependent manner.

3.3.4 Phenotyping of the *Vegfb*^{-/-} mice

Vegfb^{-/-} mice showed less lipid accumulation in energy demanding tissues and were 15% heavier compared to WT littermates at 16 to 18 weeks of age. We then tested kinetics of tissue FA uptake with oral gavage of radio-labeled LCFA. Two hours after the gavage, less ¹⁴C-OA accumulation was seen in *Vegfb*^{-/-} heart, soleus and BAT comparing to the WT counterparts while the accumulation in white adipose tissue (WAT) remained the same. However, after 24 hours, ¹⁴C-OA accumulation in *Vegfb*^{-/-} WAT increased drastically and was significantly higher than in the WT. Magnetic resonance imaging (MRI) analysis also showed a higher body fat percentage in *Vegfb*^{-/-} mice compared to WT. These findings indicate that excess fat in *Vegfb*^{-/-} mice is shunted to WAT for storage.

Back in 1963, Randle et al. have proposed a mechanism that glucose and FA competes for substrates in metabolic processes, named the Randle's cycle^{75,76}. Randle's cycle is one of the most important metabolic processes during the development of insulin resistance and T2D⁷⁵⁻⁷⁸. The involvement of Randle's cycle in lipotoxicity-induced muscular insulin resistance will be discussed in more details below (Section 4.3.1).

In a micro positron emission tomography (micro-PET) scan analysis, *Vegfb*^{-/-} mice accumulated significantly more [¹⁸F]FDG, a glucose homologue, within 60 min in heart compared to WT mice. This finding indicate that genetic deletion of *Vegfb* results in the reduction of FA uptake in tissues, and in turn induces a metabolic shift towards more glucose usage. Except for the VEGF-B-mediated pathway, there are more regulatory pathways controlling FA uptake in various tissues, which will be discussed in Paper II. Despite of these facts, inactivation of a single gene *Vegfb* alone, is enough to alter whole-body fat distribution and tissue preferences for energy molecules. These findings hinted that VEGF-B also plays a role in insulin resistance induced by muscular lipid accumulation and pathogenesis of T2D. We have further tested this hypothesis in one of our recent studies⁴⁹ as well as in Paper II.

3.3.5 Unique downstream effects of VEGF-B via VEGFR1

Among all the VEGFR1 ligands tested, only VEGF-B upregulated *Fatps* in endothelial cells *in vitro* and *in vivo*. Ten times molar excess of PlGF did not even attenuate this effect *in vitro*. Furthermore, adenoviral administration of PlGF into mouse heart did influence *Fatp* expression. These results indicate differential receptor binding sites and/or structural variants. Recruitment of unidentified unique co-receptors is another intriguing hypothesis. These findings are in line with the notion that the three VEGFR1 ligands activate distinct signaling pathways and induce different downstream effects.

3.4 Conclusion

In conclusion, we elucidated a VEGFR1- and NRP1-dependent FA uptake in endothelial cells controlled by VEGF-B via FATPs. This regulation of FA uptake is unique to VEGF-B in contrast to VEGF-A and PlGF. *Vegfb*^{-/-} mice had less FA uptake in the most energy-demanding tissues but shunted the excess FA to WAT. *Vegfb*^{-/-} mice had a metabolic shift towards more glucose usage in the heart compared to WT mice. Our study here has established a direct link for two research fields, angiogenesis and metabolism.

4 PAPER II: *Vegfb* IS REGULATED BY PGC-1 α

4.1 Gene regulation of VEGFs

VEGF-A is known to be regulated by the traditional “hypoxia - HIF-1 α ” pathway^{6,7} as well as the recently found “hypoxia/nutrient deprivation - PGC-1 α /ERR α ” pathway¹⁷⁻²⁰. VEGF-A can also be induced by a number of cytokines including interleukins (ILs), insulin-like growth factor 1, basic fibroblast growth factor, epidermal growth factor and transforming growth factors (TGFs)⁷⁹. PlGF is readily upregulated in pathological conditions by stimuli such as hypoxia, NO, inflammatory cytokines (IL-1 and tumor necrosis factor α , TNF α), oncogenes (*HRAS*) and growth factors (VEGF-A and TGF β)⁸⁰. Pro-inflammatory cytokines were shown to regulate *Vegfc* expression⁸¹. Putative nuclear factor-kappa B (NF- κ B) binding sites were further identified in *Vegfc* promoter⁸². This finding indicates that the induction of *Vegfc* by TNF α and IL-1 may be NF- κ B-mediated². VEGF-D expression has been shown to correlate with lymphatic metastasis across a variety of tumors^{1,2}, but its gene regulatory mechanism is still poorly understood.

Although quite a few studies have explored *Vegfb* expression in different contexts, its molecular regulatory mechanism remained enigmatic. Not long after its discovery, it has already been shown that, unlike VEGF-A, VEGF-B is not regulated by hypoxia⁵⁷. Early tissue expression studies have hinted a correlation of VEGF-B with metabolism⁴⁸. Several studies have implied this correlation: a microarray study in the aim of identifying potential peroxisome proliferator-activated receptor γ (PPAR γ) target genes has shown an upregulation of *Vegfb* by rosiglitazone, a PPAR γ agonist in mouse aorta⁸³; another microarray study showed that *Vegfb* was downregulated by experimental type 1 diabetes and attenuated by long-term endurance training⁸⁴; a more recent study showed that muscle-specific loss of nuclear receptor corepressor 1 in mice could induce mitochondrial function in parallel with *Vegfb* expression⁸⁵; two independent muscle-specific PGC-1 α transgenic mouse lines have been shown to have elevated *Vegfb* expression levels in the muscle^{86,87}. Furthermore, in Paper I, we identified a tight correlation of *Vegfb* with a large cluster of mitochondrial genes. All these findings point to a regulation of VEGF-B in parallel with mitochondrial biogenesis.

4.2 PGC-1 α regulates *Vegfb* and mitochondrial biogenesis

4.2.1 PGC-1 α and mitochondrial biogenesis

PGC-1 α , a major regulator in mitochondrial biogenesis, coactivates a number of transcription factors including PPARs, ERR α and nuclear respiratory factors (NRFs). ERR α is a ligand-independent orphan nuclear receptor which exerts vital roles in various physiological conditions such as exercise and cold adaptation. The PGC-1 α /ERR α transcription complex is a known regulator of mitochondrial biogenesis⁸⁸⁻⁹⁸ and in Paper I, *Vegfb* expression was found to be tightly correlated with mitochondrial genes. We then tested the hypothesis that *Vegfb* is co-regulated with mitochondrial genes by ERR α when coactivated by PGC-1 α (Figure 5).

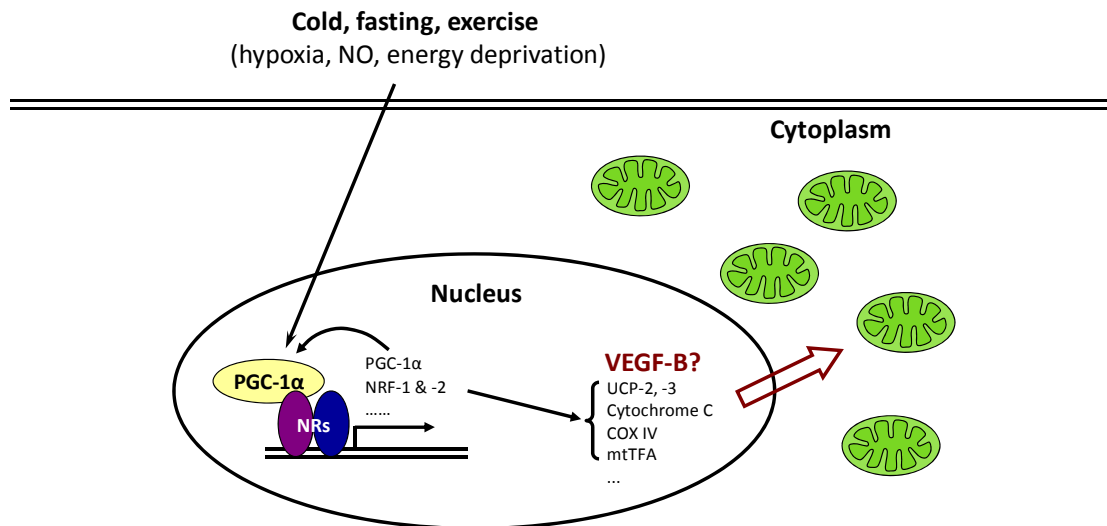


Figure 5. PGC-1 α and mitochondrial biogenesis. Physiological stimuli such as cold, fasting and exercise drive the expression of PGC-1 α , which then upregulates a set of downstream genes through coactivation of a number of transcription factors including PPARs, ERR α and NRFs. There are positive feedback loops for the expression of PGC-1 α and some of the transcription factors. Subsequently, mitochondrial biogenesis is initiated. We wanted to test if VEGF-B is regulated in parallel. NRs, nuclear receptors; UCP, uncoupling protein; COX, cytochrome c oxidase; mtTFA, mitochondrial transcription factor A.

4.2.2 PGC-1 α regulates *Vegfb* through coactivation of ERR α *in vitro*

NO is known to trigger PGC-1 α -mediated mitochondrial biogenesis in cells, which was dependent on cyclic guanosine monophosphate (cGMP)⁹⁹. By applying an NO donor on C2C12 myotubes, we were able to activate this pathway and induce *Vegfb* as well as mitochondrial genes. The expression of *Vegfa* and *Pgf*, however, remained unchanged after the treatment. This differential expression pattern of *Vegfb* vs. *Vegfa/Pgf* is in line with our previous findings in Paper I. But at first look, PGC-1 α failed to regulate *Vegfa* expression, which is seemingly contradictory to a previous finding¹⁷. We focused on chronic effect of NO (days) like Nisoli et al. tested⁹⁹, while Arany et al. tested *Vegfa* expression by PGC-1 α upregulation in a short-term setup (hours)¹⁷. Whether *Vegfa* is upregulated within hours of NO stimulation in the myotubes or not, remains to be tested.

In a time course study, we saw an initial downregulation of *Vegfa* in C2C12 myotubes and the expression returned to basal level after 16 hours of serum deprivation. Again, this finding seemingly does not come in line with what Arany et al. have found¹⁷. This can be explained by: 1) different choice of cell lines: we chose C2C12 myotubes, since its *in vivo* counterpart is a primary source of VEGF-B expression, while Arany et al. chose 10T $\frac{1}{2}$ as a natural VEGF-A expressing cell line. 2) other experimental conditions: serum deprivation alone in comparison to that in combination with hypoxia. Hypoxia does not regulate VEGF-B expression and hence is not the focus of this paper.

ERR α recognizes the consensus DNA sequence AGGTCA¹⁰⁰. Mutation of the putative ERR α binding site "AGGTCC" at 566 base pair upstream of *Vegfb* greatly attenuated, instead of abolishing, the induction of luciferase activity in the reporter assay. ERR α

itself has been shown to bind to specificity protein 1 (Sp1) and to activate thyroid hormone receptor α (TR α) through an Sp1 binding site¹⁰¹; moreover, ERR α has recently been shown to induce the expression of Sp1¹⁰². ERR α has also been shown to activate PPAR α gene expression via direct binding to the PPAR α promoter¹⁰³. PPAR α binds to the same response element as PPAR γ ⁹². In this paper, we also show that PPAR γ , without additional ligands other than the free FA and lipid metabolites presented in the serum, modestly induced the *Vegfb* promoter in the luciferase assay. Taken these findings together, it can at least partially explain the remaining promoter activity with the absence of the functional ERR α binding sites in respective *Vegfb* promoter and intron constructs. Chromatin immunoprecipitation (ChIP) assay could confirm whether PGC-1 α /ERR α transcription complex binds to the putative ERR α response element.

4.2.3 Differential regulation of *Vegfb* and *Vegfa*

VEGF-A-mediated angiogenesis is vital for tissue remodeling in response to exercise training^{104,105} and cold adaptation¹⁹. In response to these physiological stimuli, PGC-1 α regulates mitochondrial genes, *Vegfb* as well as *Vegfa*^{17,18,20} through co-activation of ERR α . These findings point to a co-regulation of VEGF-A and VEGF-B in parallel with mitochondrial biogenesis. This suggestion is, however, contradictory from what we found in Paper I, where *Vegfb* has a tight correlation with mitochondrial gene cluster but not *Vegfa*.

After a single bout of intensive exercise, *Vegfa* mRNA needs to be downregulated to basal level after the initial peak to avoid exceeded angiogenic response¹⁰⁶. VEGF-B, on the other hand, modulates physiological function other than growth of the endothelium, which may partially explain why modest *Vegfb* upregulation can only be observed after long-term endurance training⁸⁴. Furthermore, mitochondrial biogenesis is a nutrient- and energy-demanding and hence time-consuming biological process, with which VEGF-B expression should be synchronized. We believe this differential regulation of VEGF-B and VEGF-A is crucial for tissue remodeling in response to various physiological stimuli. It is known that VEGF-B is not regulated by hypoxia⁵⁷. One hypothesis is that under hypoxic conditions, certain transcription factors, other than ERR α , regulate *Vegfa* expression when coactivated by PGC-1 α . Variation in *Erra* expression pattern in response to hypoxia and/or nutrient deprivation could also play a key role in the putative differential regulation of the two VEGFs (Figure 6).

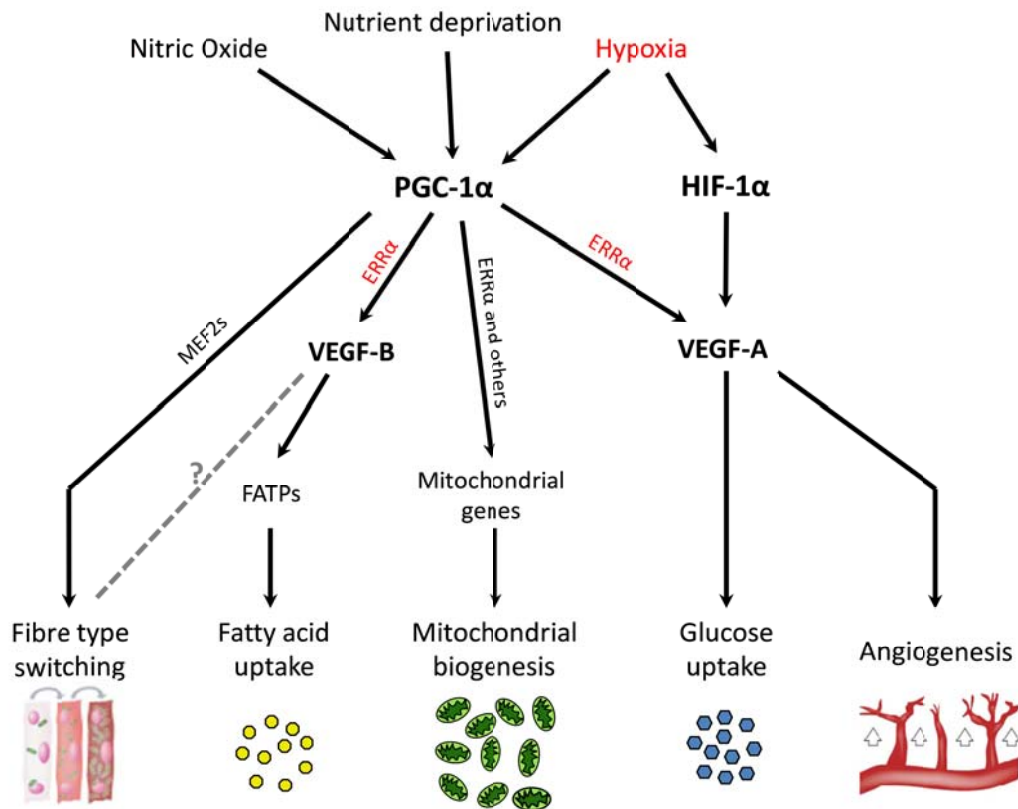


Figure 6. A schematic illustration of the differential regulation of VEGF-B and VEGF-A in the context of tissue remodeling. The unknown components underlying the differential regulation of the VEGFs by hypoxia are marked in red. The gray dotted line indicates a possible link between VEGF-B expression and muscle fibre type switching described in Paper II. Referenced and modified from Arany et al. 2010¹⁷. MEF2, myocyte enhancer-binding factor 2.

4.3 VEGF-B mediates insulin resistance in MCK-PGC-1α TG mice

4.3.1 Randle's cycle, muscular lipid accumulation and insulin resistance

Substrate competition for respiration in animal tissues has been known for almost a century⁷⁶. In 1963, Randle proposed a “Glucose Fatty Acid Cycle” theory, also known as the Randle cycle, which is a metabolic process involving substrate competition of glucose and FA in respiratory oxidation⁷⁵. FA oxidation inhibits glucose catabolism and stimulates gluconeogenesis and glucose storage, while glucose oxidation inhibits FA oxidation and promotes storage for both fuel substrates⁷⁶. In the original publication, Randle already hypothesized an important role of the cycle in the pathogenesis of insulin resistance and T2D, even though the inferred underlying molecular mechanism was later proven wrong⁷⁵.

Lipid accumulation in skeletal muscle and liver may be a result of increased uptake and/or synthesis of FA when total energy intake exceeds the storage capacity of the adipose tissue. Acquired or inherited mitochondrial dysfunction may also lead to muscular lipid accumulation⁷⁷. This pathological accumulation will in turn promote development of insulin resistance and T2D.

The molecular mechanisms underlying the lipid-induced insulin resistance has been studied intensively for the past two decades although still not fully understood. Early observations revealed a negative correlation of insulin resistance with plasma FA concentrations and intramyocellular lipid content¹⁰⁷. Lipid infusion studies pointed to the theory that defects in glucose transport, but not impaired glycolysis as hypothesized by Randle, was underlying the insulin resistance induced by high plasma FA concentrations¹⁰⁷. Glucose transporter 4 (GLUT4) is highly expressed in adipose tissue and skeletal muscle¹⁰⁸. It is acutely translocated to the cell membrane upon stimulation of insulin signaling and increase cellular glucose uptake. This translocation was found to be compromised in T2D patients¹⁰⁹.

Later studies indicated a role of diacylglycerol, a metabolite from triglyceride, in lipid-induced insulin resistance. In both mice and human subjects, it was shown that insulin resistance was associated with high intramyocellular diacylglycerol but not triglyceride accumulation¹⁰⁷. Now the common consensus is that diacylglycerol-induced malfunction in insulin signaling and hence insulin resistance is via activation of novel protein kinase C (PKC) serine-threonine kinases¹⁰⁷. Inflammatory signals in WAT¹¹⁰ and impaired GLUT4 translocation induced by muscular lipid droplet accumulation¹¹¹ have also been linked with lipid-induced insulin resistance.

4.3.2 The paradox of PGC-1 α overexpression and insulin resistance

Choi et al. have identified a paradoxical effect of increased expression of PGC-1 α on muscle mitochondrial function and insulin-stimulated muscle glucose metabolism¹¹². They originally hypothesized that muscular PGC-1 α overexpression would prevent against HFD-induced insulin resistance in mice, since two microarray studies have implicated decreased PGC-1 α expression in T2D patient samples¹¹². But paradoxically, they found decreased insulin sensitivity in the muscle creatine kinase PGC-1 α transgenic (MCK-PGC-1 α TG) mice compared to WT mice when fed a HFD.

In Paper I, we showed an increased cardiac glucose uptake capacity in *Vegfb*^{-/-} mice. This was the first indication that VEGF-B deficiency could be beneficial in insulin resistance. Our recent study has further shown that VEGF-B inactivation in various diabetes animal models reduced lipid accumulation in muscle and halted the development of T2D⁴⁹. Based on these findings, we hypothesized that in HFD-fed MCK-PGC-1 α TG mice, lipid accumulation exceeds mitochondrial β -oxidation capacity due to VEGF-B hyperactivity, and hence promotes insulin resistance. We were then interested to test *Vegfb* inactivation in the context of HFD-induced insulin resistance in MCK-PGC-1 α TG mice.

4.3.3 *Vegfb*: the missing link which solves the paradox

We crossed *Vegfb*^{-/-} with MCK-PGC-1 α TG mice to create the MCK-PGC-1 α TG // *Vegfb*^{-/-} strain. After 15 weeks on a HFD, insulin resistance and glucose intolerance were ameliorated in these mice. The levels of plasma glucose, insulin and triglyceride were also normalized, or nearly-normalized, to lean WT levels. These phenotypes could be attributed by decreased lipid accumulation in the MCK-PGC-1 α TG // *Vegfb*^{-/-} muscle compared to the MCK-PGC-1 α TG counterpart.

At the time Choi's study was published, the "PGC-1 α /ERR α — VEGF-B — fatty acid uptake" regulatory axis has not been revealed. If taken this regulatory axis into account, the findings by Choi et al. are not paradoxical anymore: under a normal diet, not only mitochondrial biogenesis is upregulated by the PGC-1 α overexpression in the muscle but VEGFB-mediated FA uptake is upregulated in parallel; when the two arms, mitochondria and FA uptake, are both induced, the balance of β -oxidation will still be even (Figure 7). When MCK-PGC-1 α TG mice are fed a HFD, similarly as in WT mice, insulin sensitivity will decrease due to lipid accumulation in the muscle exceeding the β -oxidation capacity in the mitochondria. But when VEGF-B is absence in the muscle, even under a double challenge of HFD and PGC-1 α overexpression, FA uptake still matches the capacity of mitochondrial β -oxidation. This balance will in turn attributes to the ameliorated insulin sensitivity.

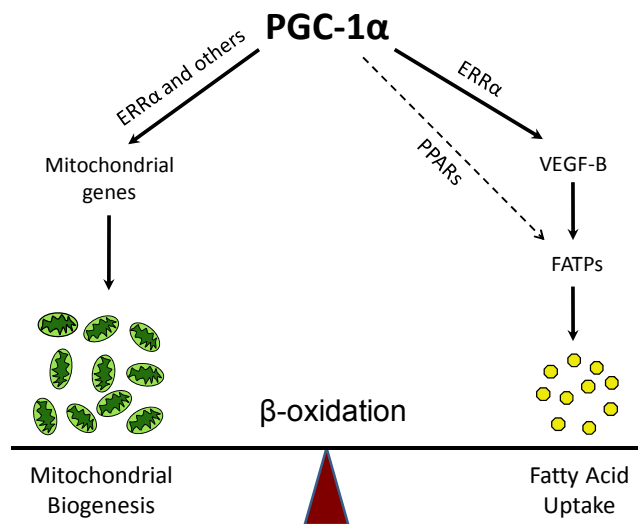


Figure 7. PGC-1 α balances the two arms of β -oxidation (same as Figure 6a in Paper II).

4.4 Conclusion

In conclusion, we elucidated a new "PGC-1 α /ERR α — VEGF-B — fatty acid uptake" regulatory axis. Adding VEGF-B in the knowledge of the complex metabolic regulatory network leads to a better understanding of the balance of β -oxidation and lipid homeostasis in the body. Genetic deletion of *Vegfb* in MCK-PGC-1 α TG mice reduced muscular lipid accumulation but did not influence postprandial blood glucose or glucose tolerance under normal diet (ND). Under a HFD however, deletion of *Vegfb* under the muscle-specific PGC-1 α transgene normalized postprandial blood glucose, drastically reduced plasma triglyceride and insulin, as well as restored glucose tolerance and insulin sensitivity. These improved metabolic characteristics are likely attributed to reduced muscular lipid accumulation. Our findings provided new evidence showing the vital role of VEGF-B in HFD-induced insulin resistance and pathogenesis of T2D. Targeting VEGF-B as a therapeutic approach in treating insulin resistance and development of T2D has promising clinical implications.

5 FUTURE PERSPECTIVES

5.1 Role of VEGF-B in other contexts

Mitochondrial biogenesis is induced upon various physiological stimuli, such as in skeletal muscle after exercise and in brown adipose tissue after cold exposure⁹³. It is then logical to explore the role of VEGF-B upon these stimuli. The cell type that has the highest mitochondrial content in mammals is the acid-secreting parietal cell (PC) in the stomach mucosa¹¹³. Since VEGF-B is also highly expressed in PC, we have investigated whether it has a role in this type of cells. The role of VEGF-B in tumorigenesis is also discussed here.

5.1.1 Role of VEGF-B in endurance running

Exercise training is known to induce mitochondrial biogenesis⁹³. We hypothesized that *Vegfb* is upregulated after long-term endurance training together with mitochondrial genes and *Vegfb*^{-/-} mice have reduced running capacity. After training for 28 days (10 m/min, 48 min per day), we could not detect any difference in running capacity between WT and *Vegfb*^{-/-} mice (unpublished data). In quadriceps, when comparing trained WT mice to sedated littermates, *Vegfb* was downregulated together with *Hadh*, the gene coding for a key β -oxidation enzyme, hydroxyacyl-coenzyme A dehydrogenase (unpublished data). This result indicates that the running speed was insufficient to induce an endurance training stimulus, since the muscle is known to primarily utilize glucose in long-term low-intensity running or high-intensity sprint⁷⁶. *Vegfb* was found to be upregulated following a long-term endurance running protocol in mice⁸⁴. Therefore, optimizations of our current running protocol are needed for further testing of our hypothesis.

5.1.2 Role of VEGF-B in cold adaptation

Mitochondrial biogenesis plays a key role in brown fat activation during cold adaptation in mice⁹³. To test the role of *Vegfb* in cold adaptation, we subjected mice to 4°C for 28 days. We found no difference in body temperature adaptation between WT and *Vegfb*^{-/-} mice, but surprisingly, there was a delayed body weight increase in the *Vegfb*^{-/-} mice compared to WT littermates (unpublished data). This finding indicates that *Vegfb*-mediated FA uptake in the brown adipocytes could be a rate-limiting step for brown fat development upon cold stimulation. Further analyses of the tissue need to be performed for testing this hypothesis.

5.1.3 The most “energetic” cell type in the body

The acid-producing PC in the stomach mucosa is one of the cell types with the highest level of energy consumption. This type of cells are filled with mitochondria and can develop a proton gradient of more than one million-fold across the membrane of the secretory canaliculus. Similarly to brown adipocytes, cardiac- and skeletal myocytes, which all have high level of VEGF-B expression and mitochondrial content, PCs also mainly use fatty acids as energy source¹¹³.

A study has found that *Vegfb* was among a number of genes that were not previously reported to be expressed in PC¹¹³. *Vegfb* has even been identified as a PC-specific marker¹¹⁴. Upon *Helicobacter pylori* (*Hp*) infection, *Vegfb* expression was highly upregulated¹¹³. These findings indicate potential roles of *Vegfb* in gastric-acid secretion and pathogenesis of stomach ulcer and gastric cancer, which are known to be closely linked with *Hp* infection^{115,116}.

The apical membrane of the parietal cell contains an H^+/K^+ -ATPase, which is the primary H^+ pump and also a marker for PC¹¹⁴. Our unpublished data have both shown that VEGF-B and H^+/K^+ -ATPase are co-localized and restrictedly expressed in PCs in the stomach mucosa. Another study also confirmed this co-localization¹¹⁴. We could not detect any difference in the vascular networks between WT and *Vegfb*^{-/-} gastric mucosae. Based on this observation, we then hypothesized that in the gastric mucosa, *Vegfb* is highly expressed in PC to coordinate FA uptake and mitochondrial β -oxidation, rather than regulating or maintaining the vasculature. Further examinations of gastric-acid secretion and experimental gastric ulcer induction in *Vegfb*^{-/-} mice will help us to have more insights to the potential role of VEGF-B in gastric mucosa.

5.1.4 Role of VEGF-B in tumorigenesis

Angiogenesis is a crucial step during tumor progression. The roles of the VEGFs in tumorigenesis were studied rather intensively throughout the years². VEGF-A, was first identified as a growth factor secreted by tumor cells⁴. It is upregulated in many, if not all, human solid tumors¹⁴. These findings marked the starting point of the following intensive research on anti-VEGF-A treatments of solid tumors¹¹⁷. It was shown that PlGF expression correlates with disease progression and patient survival and may be used as a prognostic indicator for colorectal cancer¹¹⁸. Implication of PlGF being a therapeutic target in Ewing's sarcoma was proposed¹¹⁹. Anti-PlGF treatment of various kinds of tumors was proposed followed by some controversial results¹²⁰⁻¹²³. Since sVEGFR1 can sequester VEGF-A, -B and PlGF, sVEGFR1-based traps have been developed, along with VEGFR1 inhibitors and antibodies, as anti-angiogenesis agents⁸⁰. Gene transfer of sVEGFR1 has recently been proposed as a new anti-angiogenic cancer therapy¹²⁴. Both VEGF-C and -D were linked to tumor malignancy and lymphatic metastasis in cancers since the beginning of their discovery^{1,2}.

The freely-diffusible longer VEGF-B isoform, VEGF-B₁₈₆, was shown to be upregulated in mouse and human tumor cell lines and primary tumors⁵⁵. But in contrast to other VEGFs, very few studies have explored the role of VEGF-B in the context of tumorigenesis. Among them, one study by Albrecht et al. is noteworthy. They have investigated the function of VEGF-B in tumorigenesis by the generation of two mouse models of pancreatic cancer. Beta-cell-specific overexpression of VEGF-B in the pancreatic islet carcinoma mouse model retarded tumor growth. Conversely, the same mouse model deficient for *Vegfb* presented with larger tumors. These results led to the questioning of indiscriminate blocking of VEGF signaling in anti-angiogenesis therapies¹²⁵.

In order to investigate the functional role of the VEGF-B during tumorigenesis, we generated tumor cells that overexpress either the soluble or the heparin-binding isoform of VEGF-B. We observed that cells overexpressing VEGF-B₁₆₇ gave rise to tumors that grow at similar kinetics as cells with endogenous VEGF-B levels. In contrast, tumors originated from cells with VEGF-B₁₈₆ overexpression grow significantly faster. These tumors have also an elevated capacity to recruit macrophages. In another tumor model, cells with *Vegfb* deletion attracted macrophages less efficiently to the tumor site compared to the cells with endogenous VEGF-B levels. However, the growth rate of the tumor xenografts in nude mice is similar. These findings indicate that VEGF-B is directly or indirectly involved in attracting macrophages to the tumor site. Tumor associated macrophages play an important role in promoting tumor progression, e.g. in the processes of neo-angiogenesis and/or immuno-suppression. The tumor promotion function of macrophages has also become a target for cancer therapy¹²⁶. Blocking VEGF-B function by e.g. an antibody-based approach might interfere with the tumor promoting capacity of tumor associated macrophages.

Most cancer cells are known to predominantly produce energy by a high rate of glycolysis rather than oxidation of pyruvate in mitochondria as in most normal cells. This is known as the “Warburg Effect”, which helps promoting the fast-growth of cancer cells¹²⁷⁻¹²⁹. In Paper I, we have demonstrated a role of VEGF-B in FA uptake. In the cell, glucose and FA competes for substrates in metabolic processes, known as the Randle’s cycle^{75,76} (described in Section 4.3.1). Taken all aforementioned knowledge together, it is not difficult to infer that VEGF-B can inhibit tumor growth by downregulation of glycolysis through inducing FA uptake. Careful selection of tumor models is crucial for testing this hypothesis due to the complex nature of metabolism in cancerous tissues.

5.2 The significance of our research

5.2.1 Is *VEGFB* a thrifty gene?

In *C. elegans*, a lower animal devoid of a vascular system, a family of four receptor tyrosine kinases that are structurally related to VEGFRs were identified¹³⁰. This evolutionary legacy is a historical reproduction of the beginning of VEGFRs’ evolution. Although it is difficult to investigate the exact evolution paths of each individual VEGF, possible history of VEGF evolution can still be cautiously hypothesized based on a phylogenetic tree of VEGF/platelet-derived growth factors (PDGF) family members (Figure 8).

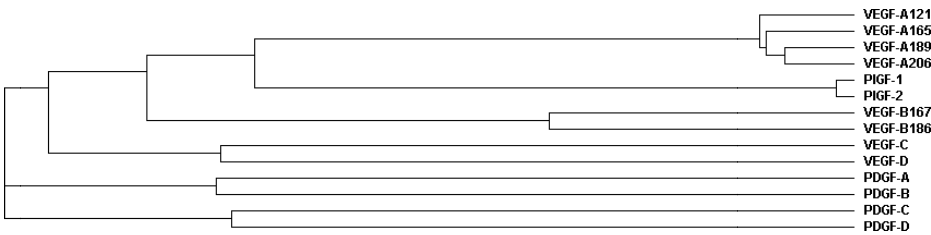


Figure 8. Phylogenetic relationship among the human VEGF/PDGF family members generated with a multiple sequence alignment tool, Clustal Omega¹³¹. Splice variants of VEGF-A, PIGF and VEGF-B are also shown.

Genome duplications, recombinations as well as genetic mutations create opportunities for speciation and evolution to occur, whereas natural selection pressures keep vital gene functions preserved. VEGF-B is highly conserved in mammals (Figure 9). This is an indication that VEGF-B has unique and important roles preserved under selection pressures. *VEGFB* promoter could be derived from a mismatched genome recombination which introduced regulatory elements from a mitochondrial gene. Further genome duplications and mutations could have separated VEGF-B from VEGF-A and PIGF early in the VEGF evolution history. At a certain time point, an ancient VEGF that had similar physiological function to the modern VEGF-B could have evolved, which allowed the organism to make better use of FA to match the respiratory oxidation capacity in the mitochondria. This evolutionary selective advantage could have kept mammalian VEGF-B highly conserved ever since.

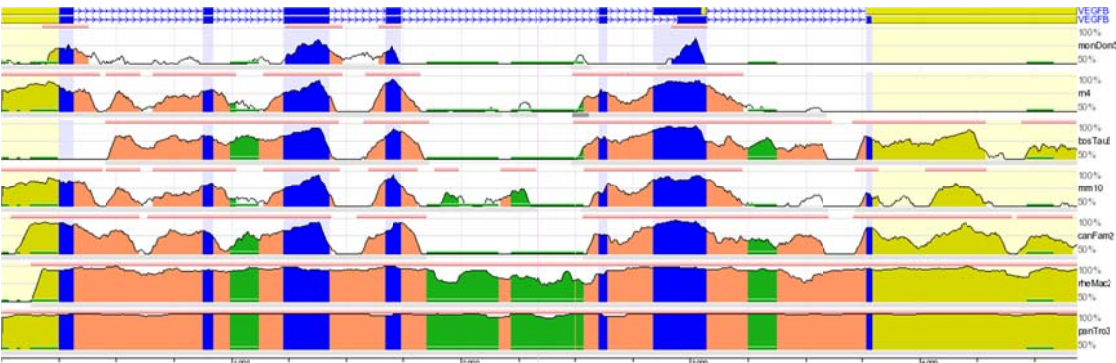


Figure 9, Homology analysis of mammalian VEGF-B genomic sequences by ECR browser¹³². The two mRNA splice variants of human (*Homo sapiens*) VEGF-B are shown on the top. Percentage of sequence homology in each species compared to the human sequence is shown under. Only the ranges of 50% to 100% are shown. From top to bottom: opossum (*Monodelphis domestica*), rat (*Rattus norvegicus*), cow (*Bos taurus*), mouse (*Mus musculus*), dog (*Canis familiaris*), rhesus monkey (*Macaca mulatta*) and chimpanzee (*Pan troglodytes*). Blue: exon sequences; pink: intron sequences; green: repetitive sequences; yellow: sequences in untranslated regions.

Comparing to the evolution timeline of modern humans, easy food access, proper housing and clothing availability for the majority of people in the world has a relatively short history. For millions of years, evolution has favored the individuals with genes to store as much energy as possible in the preparation of temporary food shortages. Energy intake and expenditure for modern human beings have changed dramatically during the past a few hundreds, especially the past a few dozens of years. Relatively long reproductive cycle and life span have made human's evolution impossible to

catch up with the pace of changing life style. This phenomenon was well summarized in the early 1960s by Neel, who proposed the “thrifty gene hypothesis”¹³³. *VEGFB*, hypothesized as a selection advantage throughout most of the mammalian evolutionary history, has now become an unwanted shortcoming that promotes metabolic disorders in modern humans. It is rational to hypothesize that *VEGFB* is one of the thrift genes that promotes lipid storage in heart and skeletal muscle as energy reservoirs.

5.2.2 Insulin resistance and adiposity

Adipose tissue exerts pivotal roles in energy storage and metabolism in animals. Obesity has become one of the prevailing health threats worldwide in modern societies. Severe forms of obesity are linked to reduced mobility, increased risks of metabolic syndrome, T2D, cardiovascular disease and other medical complications. These facts promoted the impression in the general public that the fat tissue is a “bad tissue”.

Lipodystrophy is a medical condition characterized by abnormal or degenerative conditions of the adipose tissue. Some forms of familial lipodystrophies are linked to deficiency in the genes that are crucial in adipocyte differentiation. Patients with these types of lipodystrophy usually have little or no adipose tissue in the body and develop severe hepatic steatosis and diabetes, since the excess lipid all goes to the liver and skeletal muscle. Without proper treatment, these patients hardly live through teen^{134,135}. These findings showed strong evidences proving that adipose tissue is indispensable in whole-body fat metabolism.

In Paper I, we demonstrated an increased cardiac glucose uptake capacity despite an elevated body fat content in *Vegfb*^{-/-} mice. In other words, when VEGF-B is absent, insulin sensitivity is decoupled with adiposity. This finding is in line with the hypothesis that ectopic muscular lipid accumulation, as a consequence of adiposity, but not excess body fat *per se*, is linked to the development of insulin resistance and diabetes¹³⁶.

5.2.3 Current treatments for insulin resistance and T2D

Already in the early 20th century, regular exercise was proposed to be an important approach for managing T2D¹³⁷. Increasing energy expenditure by exercising results in reduced muscular lipotoxicity, which in turn ameliorate insulin resistance in T2D¹³⁶. Medical treatments need to be developed due to the difficulty of implementing life style interventions in patients.

Insulin has been used in treatment of diabetes for a relatively long period of time. Unlike insulin-synthesis/secretion-impaired type 1 diabetes, relatively large doses are necessary to overcome the insulin resistance in treatment of T2D. Insulin treatment is also known to induce weight gain and is associated with the risk of hypoglycemia¹³⁸.

PPAR γ is expressed predominantly in the adipose tissue and plays vital role in adipogenesis¹³⁹. It is a ligand-dependent nuclear receptor that binds a variety of fatty

acids and lipid metabolites. The lipid-sensing nature of PPAR γ has led to the development of synthetic PPAR γ agonists as anti-diabetic drugs^{140,141}. These compounds reduce plasma glucose and triglyceride levels, accompanied with a rise in high-density lipoprotein (HDL) cholesterol through activation of PPAR γ ¹⁴⁰. One group of these compounds, thiazolidinediones (TZD), was shown to improve insulin sensitivity through reduction of intramyocellular diacylglycerol content¹⁴². Although once being a favored anti-diabetes drug, some of the TZD were revoked from the market due to side effects such as cardiac and hepatic complications^{138,143}.

As of 2010, metformin (a biguanide) and glibenclamide (a sulfonylurea) are the only two oral antidiabetics in the World Health Organization Model List of Essential Medicines¹⁴⁴. Metformin is the most commonly prescribed anti-diabetic drug worldwide. It improves hyperglycemia primarily by suppressing hepatic glucose production¹⁴⁵. Glibenclamide, on the other hand, acts on pancreatic β -cells and stimulates insulin release. The combination oral administration of the two drugs was also proven to be more effective than monotherapy of either drug¹⁴⁶.

Glucagon-like peptide-1 (GLP-1) belongs to a group of gastrointestinal hormones, the incretin hormone family. It is used as an anti-diabetic drug for its beneficial effects in blood glucose, β -cell function and insulin sensitivity. It is secreted upon the presence of nutrients in the lumen of the small intestine and stimulates insulin secretion in pancreatic β -cells through activation of G protein-coupled receptors¹⁴⁷. GLP-1 has gastrointestinal side effects like nausea and diarrhea¹⁴⁸. Not all patients receive GLP-1 can be expected to reach normoglycemia¹⁴⁷.

5.2.4 VEGF-B as the new therapeutic target

The hypothesis that enhancing fat burning will reduce adiposity has led to studies on manipulations of FA oxidation¹⁴⁹⁻¹⁵². However, a recent study has shown that acute or chronic upregulation of mitochondrial FA oxidation has no net effect on whole-body energy expenditure or adiposity¹⁵³. Ironically, to some extent, this has already been predicted by Randle in 1960s^{75,76}. The physiological balance of FA and glucose oxidation has made it difficult to achieve long-term metabolic benefits by altering only a few molecular pathways within the complex metabolic regulatory network.

Current treatments for T2D are mainly focused on reducing plasma glucose and triglyceride levels, as well as stimulate insulin secretion, without concentrating on the root of the disease, which is lipid-induced insulin resistance. Our studies of VEGF-B, however, indicate that manipulation of net energy input into the system by altering tissue FA uptake may be the effective way out for treating insulin resistance induced by muscular lipid accumulation. Combinations of the above mentioned therapies might become the “ultimate treatment” for metabolic disorders such as obesity, insulin resistance and T2D.

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Note: some parts of the acknowledgements are written in my mother tongue with simplified English translations. People are acknowledged in the rough timely order of appearance.

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还有唐婧，你就 miscellaneous 了，没有分类哈

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ONE MORE THING . . .

The title of this sub-chapter is a small tribute to Steve Jobs, former Apple CEO, for his characteristic and usually entertaining “one more thing...” sections in Stevenotes.

Quoting from his famous speech at Stanford University in 2005:
“You’ve got to find what you love”; “Stay hungry, stay foolish”.

Although it is obvious to some readers, I should still emphasize that this sub-chapter is NOT a scientific manuscript. This is special thanks for the people who made it possible and documentation for what have been done.

Data security and network communications in an academic research environment

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There are three inevitable things in life: tax, death and hard drive failure.

We’ve built a file and mailing list server. It’s based on a Mac Mini early 2009 model with OS X Lion Server 10.7.5. An external hard drive is used for primary data storage. The data and boot drive are both daily and incrementally backed up to a Drobo 2 Gen disk array using Time Machine for multi-level data redundancy. The headless server is managed remotely through native VNC support by Mac OS X. All the network communication ports are open to the local department ethernet while KI-VPN connection must be established if accessing from outside of the department.

The file server supports AFP for Macs and SMB for Windows PCs. AFP works seamlessly with Mac computers. But since Apple has replaced SMB with an in-house version of SMBX starting in Lion due to changes in SMB licencing, SMB support for Windows-based computers became poor. The Offline Files function under various versions of Windows became incompatible after upgrading to Lion from Snow Leopard. It is probably due to the changes in Oplocks capability in SMBX. SMB authentication became slower as well. These changes cause problems with opening and saving Microsoft Office and Adobe PDF files. A quick trial under Mountain Lion indicates that the kernel codes of SMBX haven’t been improved to fix the aforementioned bugs.

Each user has his/her own user account and folder with full accessibility. A user can only read data from other users in the same group, while there’s no data accessibility across the groups. A common guest account and temporary folders are also available for easy data exchange.

The mailing list server is based on a series of Gmail addresses and the default Mail program in Lion. An email is sent to a Gmail address, the Mail program will then redirect, rather than forward, according to a set of rules to the corresponding recipients. There is a master address for sending to all the research groups. This address points to the addresses for each group, and then the each group address points to individual recipients. The user addresses and groups are maintained using Address Book in Lion. A combination of groups or specialized groups of addresses like “Cell Culture” or “Starget” was realized by setting up corresponding rules in Mail.

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