PLACENTAL GROWTH FACTOR REGULATION BY

SHEAR STRESS AND IRON METABOLISM IN

ENDOTHELIAL CELLS

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Abstract: Arteriogenesis is the complex process of outward enlargement of small arteries and arterioles, also known as the collaterals. Arteriogenesis involves cell proliferation and vasculature remodeling and it is induced by increased fluid shear stress (FSS) after occlusion in major arteries upstream. Many cell types, including endothelial cells, smooth muscle cells, monocytes, and endothelial progenitor cells, have been implicated in this process. Previous studies indicate that placental growth factor (PLGF) plays a key role in arteriogenesis by acting as both a cell proliferation inducer and a chemoattractant. Previously, our group has shown that PLGF is mainly produced by endothelial cells. Furthermore, using *in vitro* and *ex vivo* models, our group was the first to demonstrate that PLGF is upregulated by FSS, and this process is NADPH oxygenase-4 (NOX-4)- and heme oxygenase-1 (HO-1)-dependent. Furthermore, our group discovered iron, a product of HO-1, can upregulate PLGF. In this study, we demonstrate transcription cofactor p300 as a key regulator in PLGF expression. We discovered that FSS upregulates PLGF by increasing transcription, and p300's histone acetyl transferase (HAT) activity is required in this process. This study identifies epigenetic modification as an important aspect in the complex process of arteriogenesis, and provides important insights for future studies and potential pharmaceutical targets of non-invasive treatments for cardiovascular diseases.

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CHAPTER I

REVIEW OF LITERATURE

1. Arteriogenesis

1.1. Definition

Arteriogenesis is the *in situ* enlargement and remodeling of pre-existing arterioles through the growth of endothelial cells (EC) and smooth muscle cells (SMC), which utilizes the pre-existing collateral network to bypass the occluded artery[1]. Although initial vasodilation is involved[2], arteriogenesis is a complex chronic process that leads to arteriole structure change through EC and SMC proliferation[3]. Multiple cell types, cytokines and other factors such as nitric oxide and proteases are involved in arteriogenesis. The observation of collateral growth was first made in human coronary artery disease (CAD) patients by Fulton in 1963[4]. In contrast to angiogenesis, which is regulated mainly by hypoxia, arteriogenesis is regulated by fluid FSS[5-7]. Clinical observations have shown that increased collateral network is protective against adverse cardiac events in CAD patients[8-11]. Collateral network growth is also beneficial for pain management and amputation prevention in peripheral artery disease (PAD) patients[12].

1.1.1. Arteries and arterioles Structure

Arteries are constituted of mainly 2 types of cells, endothelial cells and smooth muscle cells. A single layer of endothelial cells forms the inner lining of the vessel wall and has important functions in inflammation and platelet aggregation. Upon injury, the endothelium senses damage to the blood vessel and triggers blood coagulation. Leukocyte migration is facilitated by decreased adhesion between endothelial cells[13]. Endothelial cells are of special importance in this review for their functions in arteriogenesis, e.g. sensing of fluid FSS and recruitment of monocytes, which will be discussed in following sections. Smooth muscle cells run circularly around the vessel wall and their contraction is responsible for vasoconstriction. Interestingly, large arteries have their own blood vessels within their vessel walls, which are named *vasa vasorum*.

Arterioles are small arteries which only have 1-2 layers of smooth muscle cells. The arteriole diameter can increase substantially[5,14] through proliferation of EC and SMC in association with the

increase in FSS, thus substituting blood flow for occluded arteries[6] **Fig. 1** illustrates the structure of an arteriole.
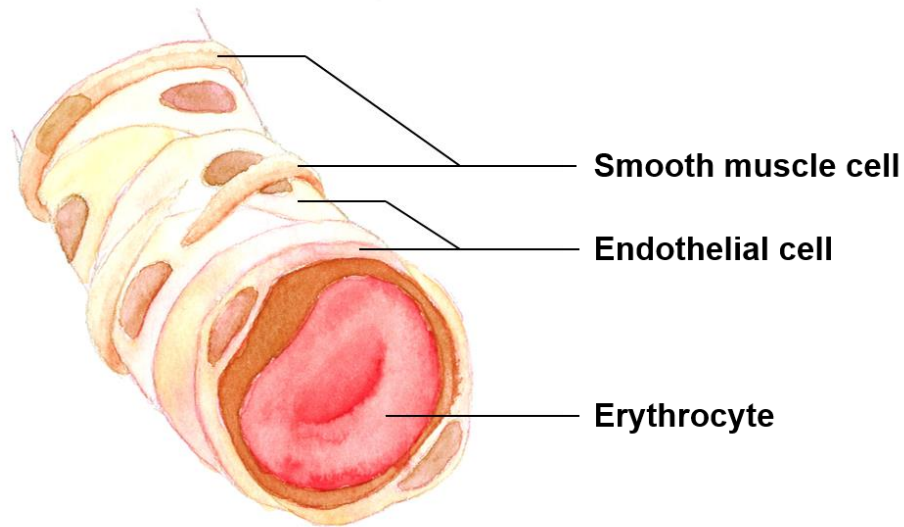

Fig. 1. Structure of arteriole

1.1.2. *Function*

Arteries carry and distribute blood from the heart to the lungs for oxygen exchange or to organs and tissues to provide oxygen and nutrition. The pressure gradient of the systemic circulation (starting from the left ventricle and ending in the right atrium) blood vessels ensures the movement of blood through the body. Individual vessels within the circulatory system are a part of this overall pressure gradient, and pressure drops along their length. Arterioles are the major resistance vessels in the vascular tree with a pressure drop from 93 mm Hg to 37 mm Hg on average. This pressure decline helps blood enter various organs[3].

The overall function of the arterial circulation is to deliver blood to the capillaries. Capillaries are formed by a single layer of endothelial cells, through which gas and nutrient exchange take place[3]. Interestingly, this connection between arteries and veins (later discovered and named capillaries) confounded William Harvey, the physician who first made detailed and systemic observations of the blood circulation, for years.

1.2. Conditions under which arteriogenesis occurs

1.2.1. In response to occlusion

Atherosclerosis. Atherosclerosis is a progressive, degenerative arterial disease characterized by increased thickness of the vessel wall due to formation of atherosclerotic plaque, which is composed of a lipid-rich deposit surrounded by an abnormal growth of smooth muscle cells. Atherosclerosis decreases the lumen of the blood vessel, which increases FSS on the endothelium as the same volume of blood is forced through a narrower opening. As the atherosclerotic plaque size increases, the endothelium covering it may become damaged, exposing the plaque. Platelets can adhere to the collagen-rich plaque and form an intravascular blood clot (thrombus). The continued flow of blood past the thrombus site can dislodge the thrombus, leading to complete occlusion downstream[3,13].

Collateral network growth has been widely described after occlusion caused by atherosclerosis in human patients since 1956[15,16]. Baroldi et al. observed that the average diameter of collateral vessels in atherosclerosis patients was 432 mm, a 1.78-fold increase compared to chronic hypoxemia patients without atherosclerosis[15].

Moyamoya disease. Moyamoya is the Japanese word meaning "a puff of smoke". It is used to describe the extensive collaterals developed at the base of the brain after progressive occlusion of bilateral distal carotid arteries[17]. The cause of Moyamoya disease is still under investigation.

Artificial occlusion in animal models. Many animal models have been established to study arteriogenesis induced by occlusion through surgically implanted occluding devices. Collateral growth has been observed after surgical ligation in pig, dog, rabbit, mouse, and rat[5,18-23]. Typically, in these studies, femoral artery ligation was done in one of the hindlegs, while the other leg was either left un-operated or sham-operated.

1.2.2. In response to exercise

In CAD and PAD patients. Exercise has long been known to be both protective against the development of cardiovascular diseases, and beneficial for rehabilitation in CAD[24,25] and PAD patients[26]. Exercise can also increase coronary collateral flow in CAD patients[27,28] and femoral artery

diameter in PAD patients[29]. Exercise has been shown to promote arteriogenesis in various animal models in both the coronary[30] and peripheral circulation[31,32].

In athletes. It is a well-documented phenomenon that the size of arteries can be promoted by exercise in human athletes[33,34]. Many studies have also shown that exercise can promote arteriogenesis in healthy animals[35-37]. It is believed that increased blood flow induces arteriogenesis following exercise[34,37]. A study in marathon runners demonstrated increased coronary collateral growth[38]. Similarly, elite tennis players were found to have more developed collaterals in their dominant arms.

1.3. *Diabetes and metabolic syndrome affect arteriogenesis*

Diabetes has long been recognized as a major risk factor for the development of cardiovascular disease, and also reduces life expectancy, worsens prognosis, and limits recovery after cardiac events[8,39-41]. The life expectancy in patients with diabetes who experienced myocardial infarction at age 45 is reduced by 20 years, as compared to a 6-10 years reduction in cardiac patients without diabetes[42]. The prevalence of PAD in patients over 40 is more than twice as high in diabetic patients compared to non-diabetics[43].

Both type I and type II diabetes affect arteriogenesis, as shown by clinical[44,45] and animal studies[46,47]. A mouse study by Yan et al. (2009) suggested that type II diabetes affects arteriogenesis more severely than type I[46]. In this study, foot and calf blood flow recovery was reduced more severely (by 78% and 57% respectively) in type II diabetic mice after femoral artery ligation than in type I diabetic mice (58% and 37%). Furthermore, type II diabetes is more clinically relevant to arteriogenesis research, due to its prevalence in the population and its complicated pathology. 12.2% of U.S. adults are estimated to have diabetes, of which 95% are type II[48]. Metabolic syndrome is a major risk factor for type II diabetes[49]. Metabolic syndrome is characterized by visceral obesity, dyslipidemia, hyperglycemia, and hypertension. In metabolic syndrome, a variety of other indicators are also higher than the normal range, but do not meet the criteria for diabetes. These indicators include fasting plasma glucose (FPG), glycated hemoglobin A1C levels (A1C) and performance on

the oral glucose tolerance test (OGTT)[50]. The diagnostic criteria for Type II diabetes are FPG levels > 126 mg/L, A1C levels > 6.5%, and 2-h plasma glucolse (PG) in OGTT $\geq$ 200 mg/L[51]. Metabolic syndrome is a major risk factor for type II diabetes[52].

Many studies in both clinical populations and animal models have given valuable insights into the underlying mechanisms by which diabetes impairs arteriogenesis. Endothelial dysfunction is recognized as a hallmark in diabetes progression, and is characterized by a proinflammatory and prothrombic state, impaired vascular remodeling and vasodilation. Hyperglycemia[53,54], altered arteriole dilation[55,56], NO production[57], neutrophil dysfunction[58] and growth factor related pathways[59-61] have been shown to be related to endothelial dysfunction in diabetes.

1.4. Mechanisms of arteriogenesis

1.4.1. Initial stimulus

Fluid shear stress (FSS). Narrowing of arteries caused by atherosclerosis or experimental occlusion by ligation leads to increased blood flow in the collateral circulation, which increases collateral FSS exponentially[62,63]. FSS has been recognized as the most important factor in inducing adult arteriogenesis, as indicated by various studies. EC proliferation was shown to be increased by FSS *in vitro*[64]. FSS has also been found to induce differentiation of stem cells and progenitor cells to EC[65,66]. EC and SMC proliferation, and collateral artery growth were observed 1 day and 3 days after femoral artery ligation in rabbits[19]. Femoral ligation, as well as creation of a shunt between the artery and vein, increase FSS in collaterals. These two procedures have been shown to induce arteriogenesis in various animal models. In a study in rabbits, the maximum collateral conductance increased by 1.3-fold 2 weeks after femoral ligation compared to the control leg, and doubled after 4 weeks[21]. In a mouse study, the diameter of collaterals showed an increase of 2.1-2.4-fold after occlusion compared to control. In the same study, the authors compared arteriogenesis and angiogenesis and identified arteriogenesis as the most effective mechanism to increase blood flow after occlusion[18]. In a study in pigs, collateral vessel number increased by ~2.4-fold compared to control 2 weeks after femoral

ligation, as observed by MRI[5]. One thing worth noting is, the collaterals and the surrounding tissues are not hypoxic, especially after increased FSS brought by major artery narrowing.

1.1.1. Cell types involved

1.4.1.1. Endothelial cells

In FSS sensing. FSS is the friction between the vessel wall and flowing blood driven by blood pressure. FSS is a fundamental regulating force in the vasculature in homeostasis, remodeling, cardiac development and inflammation. Endothelium, as the barrier between blood flow and tissues, is directly exposed to the change of flow parameters such as pulsatile frequency, direction, and pressure. Various surface proteins, including PECAM-1, integrins, vascular endothelial growth factor receptor-2 (VEGFR-2), and VE-cadherin on EC have been shown to take part in mechanical stress sensing[67-72], which will be discussed later in this review. Upon sensing a change in FSS, a series of changes in morphology, protein phosphorylation/migration, cytoskeletal rearrangement, NO production and gene expression take place in EC[67,73-76]. The endothelium's ability to sense FSS can become impaired in pathological conditions such as diabetes, and this impairment is considered to be the cause of dysfunctional arteriogenesis in diabetic patients by many researchers[56,77].

In producing nitric oxide. Endothelial nitric oxide synthase (eNOS) is a constitutively expressed isoform of nitric oxide synthase (NOS). Like the other 2 types of NOS, neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS), eNOS produces nitric oxide (NO) from the substrate L-arginine[78,79] with several regulating cofactors including BH4 and Fe^{2+}-heme[80]. NO is a small, uncharged molecule which can diffuse through cell membranes freely. NO induces vasodilation by activating soluble guanylyl cyclase (sGC) in SMC to produce cyclic guanosine monophosphate (cGMP). cGMP induces SMC relaxation via Ca^{2+} and K^+ channel phosphorylation by cGMP-dependent protein kinase (PKG)[81]. eNOS knockout in mice causes hypertension and impairs arteriogenesis, suggesting that eNOS is vital in both maintaining normal blood pressure and arteriogenesis[82,83]. However, the role of eNOS in arteriogenesis is not entirely clear. One study by Mees et al suggested that eNOS is necessary for vasodilation but not essential for arteriogenesis while

others suggest otherwise[83-85]. For example, a study in rats done by Lloyd et al. (2001) showed that inhibition of eNOS by L-NAME only blocked arteriogenesis, but not angiogenesis in skeletal muscle after femoral ligation and exercise[85], suggesting that NO is more critical for arteriogenesis than for angiogenesis. More research is needed to address this question.

In recruiting monocytes. Monocytes play a critical role in arteriogenesis by producing cytokines to regulate EC and SMC proliferation and proteases to induce extracellular matrix remodeling. Upon activation, EC surface adhesion molecules are upregulated, and EC release chemotaxins that attract monocytes to the site of altered FSS. Many studies have shown that monocyte chemoattractant protein 1 (MCP-1) is upregulated by FSS in EC, leading to monocyte recruitment[86-91]. Peroxide[92], VEGF-A and placental growth factor (PLGF)[93,94] have also been shown to play a role in FSS-induced monocyte recruitment. Both VEGF-A and PLGF bind to vascular endothelial growth factor receptor-1 (VEGFR-1), which is the only VEGF receptor present on monocytes. Interestingly, PLGF has a stronger chemoattractive effect on monocytes than VEGF-A *in vivo*[92]. PLGF knockdown with siRNA reduces monocyte migration *in vitro*[95]. It is worth noting that our lab's previous studies showed that EC are the major source of PLGF, while SMC highly express VEGF-A[95], and that EC PLGF is upregulated by FSS[73], implicating the monocyte recruiting role of EC PLGF in arteriogenesis.

In growth factor secretion. ECs produce growth factors that serve both autocrine and paracrine mechanisms. Our lab's previous studies showed that PLGF is highly expressed in EC. PLGF has been shown to be a critical factor in arteriogenesis[32,93]. PLGF can induce both monocyte and SMC migration[96]. FSS has also been shown to upregulate endothelial VEGF-A, basic fibroblast growth factor (bFGF), platelet derived growth factor-B (PDGF-B) and TGF-β1[97-99]. Autocrine VEGF-A serves crucial functions in EC survival and proliferation[100,101]. bFGF was shown to play a critical role in SMC migration and proliferation in pathological conditions by various studies[102-104]. PDGF-B has proliferative and chemoattractive effects on both EC and SMC[105-108]. TGF-β1 has a synergistic effect with PDGF on human arterial SMC proliferation, implying it has a function in maintaining the vasculature[109].

1.4.1.2. Smooth muscle cells

Studies suggest that vasodilation controlled by SMC tone is one of the first responses to increased FSS [20,110]. As arteriogenesis proceeds, SMC differentiation and proliferation is essential to collateral development[23]. Coronary SMC proliferation was observed 3 weeks after occlusion in dogs[111]. Studies done previously by our lab and other groups suggest that SMC-secreted VEGF-A may have paracrine regulatory effects on EC functions and proliferation [95,112,113]. Another important function of SMC in arteriogenesis is secretion of matrix metalloproteinases (MMP-2 and MMP-9) for the remodeling of the vasculature[114].

1.4.1.3. Monocytes

Monocytes have been recognized as a critical mediator in arteriogenesis by secreting proteases[115] and arteriogenic cytokines. Clusters of monocytes can be observed gathering at sites of increased FSS within 12 h after occlusion[19]. Monocyte-secreted TNF-α and bFGF have been identified as important factors in inducing arteriogenesis. Increased monocyte production of TNF-α by LPS stimulation leads to significantly increased capillary density in rabbit calf muscles, implying that it affects EC proliferation[116]. Monocyte depletion by 5-fluorouracil (5-FU) markedly impairs arteriogenesis in occluded mouse and rabbit legs, while a quick "rebound" increase of monocyte count after 5-FU administration is discontinued is correlated with recovered arteriogenesis[117]. Knockout mice lacking the MCP-1 receptor chemokine (C-C motif) ligand 2 (CCL-2) also have impaired arteriogenesis, confirming the arteriogenic role of monocytes[118].

1.4.1.4. Progenitor cells

Several types of endothelial progenitor cells (EPC) have been identified, including endothelial colony –forming cells (ECF), pro-angiogenic hematopoietic cells (PHC), late-outgrowth EPC, etc[119]. But the field is new and the definition of endothelial progenitor cells is still under debate[120]. Monocyte-derived EPCs have been shown to help improve arteriogenesis in mouse hid-limb ischemia[121]. In a recent study by Carrabba et. at. (2016), adventitial progenitor cells in bioengineered scaffolds improved arteriogenesis in a mouse femoral artery ligation model[122]. More research on

EPC's arteriogenic capacity may provide important insights to the mechanism of arteriogenesis and prove useful in developing new therapies for cardiovascular diseases.

1.4.2. Growth factors and other regulators involved

1.4.2.1. VEGF-A and VEGFRs

VEGF-A. Vascular endothelial growth factors (VEGF) are a family of growth factors. VEGF-A is the most studied member of this family, and is often referred to simply as VEGF in the literature. VEGF-A is a critical regulating factor in EC proliferation and homeostasis, as it is a ligand for both VEGFR-1 and VEGFR-2. VEGF-A treatment of EC monolayers increases their permeability by regulating adherens junctions[123]. VEGF-A knockout is lethal in mice, implying its critical role in embryonic vasculogenesis. However, its role in arteriogenesis is less clear. A study in mice suggested that VEGF-A has less arteriogenic effect than PLGF. VEGF-A did not show a synergistic effect with PLGF on arteriogenesis, although it showed synergistic effects with PLGF on SMC proliferation[124]. This is in agreement with another study which showed that PLGF was more effective than VEGF-A in inducing arteriogenesis[93].

VEGFR-1 and VEGFR-2. Both VEGFR-1 and VEGFR-2 are tyrosine kinase receptors. Many studies suggest that VEGFR-2 is highly important in regulating angiogenesis. A fifth VEGF family member, VEGF-E, encoded by Orf virus, specifically binds to VEGFR-2[125]. In one study done by Kiba et al. (2003), mice overexpressing VEGF-E showed highly increased angiogenesis (10-fold), while PLGF-2 overexpressing mice only showed 2-3-fold, implying the highly angiogenic effects of VEGFR-2. Inhibition of VEGFRs with ZD4190 in rats only partially impaired angiogenesis, but completely blocked arteriogenesis, suggesting there may be alternative mechanisms for angiogenesis, but that VEGFRs play an essential role in arteriogenesis[32].

1.4.2.2. PLGF

PLGF plays a critical role in FSS-induced arteriogenesis. It has been reported that mice overexpressing PLGF in skin have a hypervascularized phenotype, with an increase in number, branching and size of arterioles, demonstrating PLGF's arteriogenesis-inducing effects[126]. Our lab

was the first to show that PLGF is upregulated by FSS[73]. PLGF KO mice have a normal phenotype under normal physiological conditions, but have impaired arteriogenesis and wound healing. An important function of PLGF in arteriogenesis is monocyte recruitment. PLGF knockdown with siRNA impairs monocyte recruitment *in vitro*[95]. Monocyte recruitment was also found to be reduced in diabetic mice, and was rescued by treatment with an adenovirus expressing PLGF[61]. PLGF also has stronger chemotactic effects on monocytes than VEGF[93]. Our lab has found that Western diet-fed mice, a model for metabolic syndrome and type II diabetes, have reduced PLGF in skeletal muscle.

1.4.2.3.   Proteases

Proteases play an important role in collateral remodeling by degrading the extracellular matrix, which allows for vessel growth in both angiogenesis and arteriogenesis. Clinical observations have shown that certain matrix metalloproteinases (MMP-3 and MMP-9) are upregulated in cardiovascular disease patients with signs of collateral remodeling[127]. Inhibition of MMPs in rabbits after myocardial infarction reduced collateral remodeling, but not angiogenesis[128]. In a recent study, siMMP-10 treated mice were shown to have a reduced number of arterioles and delayed tissue reperfusion after ischemia induced by notexin (a myotoxic and neurotoxic phospholipase discovered in the venom of the Australian tiger snake *Norechis scutatus*). Injection of MMP-10 rescued the phenotype, confirming the importance of MMPs in arteriogenesis[129].

2. PLGF

2.1. Definition

PLGF was first discovered in placental tissue and its cDNA was synthesized by Maglione et al in 1991[130]. There are 4 isoforms of PLGF produced from variant splicing. PLGF-2 and 4 have a heparin binding sequence[131-133]. Functional differences among the isoforms besides heparin binding and ECM association are not clear to our knowledge. PLGF is a growth factor in the VEGF family that specifically induces arteriogenesis.

2.2. Functions

2.2.1. PLGF is a ligand for VEGFR-1.

PLGF in angiogenesis. Unlike VEGF-A, PLGF only binds to VEGFR-1, and thus it targets different cell types and triggers different effects than VEGF-A. Initially, PLGF occupying VEGFR-1 was thought to affect vascular growth by the mechanism of enhancing VEGF-A stimulated angiogenesis through VEGFR-2[134]. However, more recent studies have shown that PLGF binding stimulates phosphorylation of specific VEGFR-1 tyrosine residues and leads to angiogenesis alone[135] or synergistically with VEGF[136]. PLGF KO mice have impaired angiogenesis in retina and heart[136]. Adenovirally overexpressed PLGF has been shown to enhance wound healing[61]. PLGF can also induce tumor angiogenesis by recruiting hematopoietic progenitor cells and macrophages[136]. These studies indicate PLGF is angiogenic.

PLGF in arteriogenesis. It has been well established that increased FSS is the major stimulator for arteriogenesis[73,137]. Our group's published work shows that both PLGF mRNA and protein are upregulated by FSS[73]. PLGF is one of the first growth factors to increase at the onset of collateral growth, as shown by a study published by Prior et. al[20]. In this study, after femoral ligation in rats, PLGF mRNA in the perforating artery was upregulated ~8-fold by day 2, and a 3-fold upregulation of PLGF mRNA still persisted 25 days post ligation. PLGF KO mice have impaired collateral growth after femoral ligation, but the inhibition is abolished with bone marrow transplant or PLGF treatment[124,138]. Recombinant human PLGF (rhPLGF) infusion enhances arteriogenesis after myocardial infarction in mice[139]. Mice overexpressing PLGF in the skin have a hypervascularized skin phenotype with an increase in number, branching and size of arterioles[126]. Altogether, these studies demonstrate that PLGF is a key factor in arteriogenesis.

2.2.2. ECs are the major producer of PLGF in the vasculature.

Our lab's previous studies made the unexpected discovery that PLGF was highly expressed by 4 EC cell lines (human lung microvascular EC, human umbilical vascular EC, human coronary artery EC, and mouse hemangioendothelioma EC), while very low PLGF expression was found in SMC cell lines[95]. This is in agreement with other studies human cell lines and in mice[136,140-142]. This finding was surprising since EC express very low levels of VEGF-A, as shown by our group and others.

2.2.3. PLGF effects on different cell types

PLGF can induce EC, SMC, neuronal cell and fibroblast proliferation and migration, and is a survival factor for EC and SMC[61,96,141,143-145]. PLGF is also a survival factor for both solid and hematological tumors[146,147]. Thus, methods for its inhibition have been investigated as potential cancer treatments[146,148]. PLGF was shown to function in maintaining hematopoiesis through progenitor cell recruitment[149]. PLGF can recruit both monocytes and macrophage by activating VEGFR-1[92,124]. PLGF is essential for placental development. Decreased serum PLGF levels have been linked to dysfunctional placental vascularization, preeclampsia, and small-for-gestational-age (SGA) newborn infants[150].

2.2.4. PLGF KO mice

PLGF KO mice have an essentially normal phenotype both in fetal development and as adults, but reduced angiogenesis was observed in ovaries and infarcted heart[136]. Reduced wound healing has also been observed in PLGF KO mice[61]. Collateral growth in ischemic limb was impaired in PLGF KO mice, while treatments with bone marrow transplantation or PLGF injection rescued arteriogenesis[61,124,138]. These observations confirm that although PLGF does not appear to be required for embryonic development or normal vascular function, it is essential for vascular remodeling in pathological conditions.

2.2.5. Role of PLGF in preeclampsia and reproduction

Abnormal PLGF/sVEGFR-1 levels are linked to preeclampsia. Preeclampsia is a pregnancy disorder characterized by onset of high blood pressure, and proteinuria[151]. Preeclampsia can lead to eclampsia, and other severe complications, including organ damage and death[151]. Soluble VEGFR-1 in plasma functions as a trap binding circulating PLGF, preventing it from activating cell-associated VEGFR1. The proposed mechanism of preeclampsia is that endothelial dysfunction occurs in preeclampsia patients, leading to hypertension and increased placental oxidative stress[152]. Serum PLGF levels have been demonstrated by many clinical studies to be a good biomarker for diagnosing preeclampsia [153-155]. PLGF treatment in a mouse preeclampsia model attenuated the symptoms[156].

2.2.6. PLGF in COPD

Interestingly, patients with chronic obstructive pulmonary disease (COPD) have been found to have elevated PLGF levels[157,158]. PLGF has been suggested to affect COPD prognosis by increasing neutrophil elastase-induced emphysema[159,160]. Surprisingly, PLGF overexpression in mice was found to be sufficient to induce pulmonary emphysema[161]. Likewise, PLGF KO mice are resistant to elastase-induced emphysema[162]. These studies suggest that PLGF may play an important role in the development of emphysema. However, the mechanism(s) for this effect remain unknown.

2.3. Regulation of PLGF

2.3.1. FSS

FSS is the hydrodynamic force exerted on the endothelium by blood flow. It is represented with the unit dyne/cm^2. 1 dyne is equal to $1 \cdot 10^{-5}$ N. FSS is calculated according to the following equation (**Eq. 1**):

$$\tau = \frac{4\mu Q}{\pi r^3}$$

Eq. 1. τ is FSS. μ is blood viscosity. Q is flow rate. r is the internal radius of the vessel.

Following arterial occlusion, blood flow will increase in the downstream collateral arterioles, thereby increasing the FSS applied on the arteriole endothelium. **Fig. 2** is an illustration of this process.
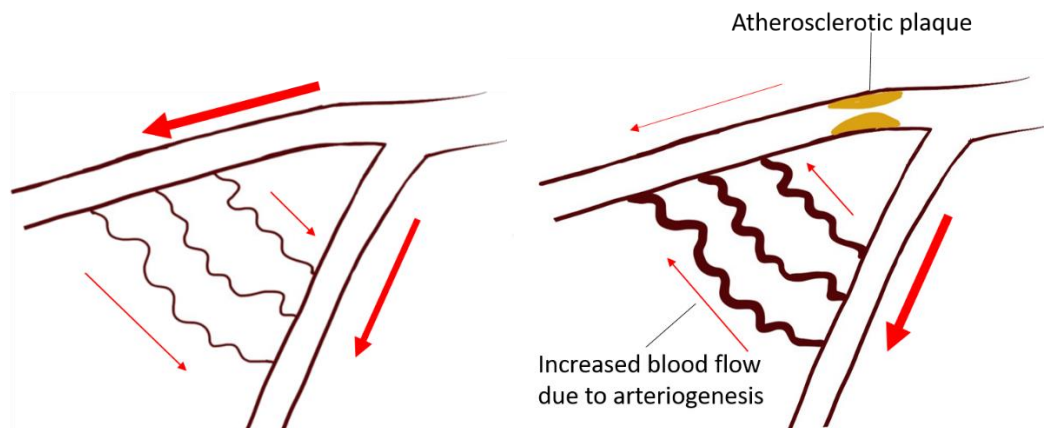

Fig. 2. Arteriogenesis after atherosclerosis

2.3.1.1. Cell sensing of FSS

Integrins. Integrins are cell surface glycoprotein receptors formed by αβ-integrin heterodimers. At least 18 α and 8 β subunits have been discovered in humans, which can combine to form 24 different integrin heterodimers. Integrins connect intracellular actin filaments with extracellular matrix (ECM) components such as fibronectin and collagen across the cell membrane. Therefore, integrins can act as transmitters of mechanical forces. In a study done by Jalali et al., Shc was activated at different levels in EC grown on different ECM protein substrates, including human fibronectin (FN), human vitronectin (VN), rat collagen type I (CL), human laminin (LM), and human fibrinogen (FG)[70]. Shc is involved in many cell functions such as apoptosis, oncogenesis and proliferation via mitogen activated protein kinase (MAPK) and other pathways[163]. Inhibition of integrins by antibodies has been shown to attenuate FSS induced NF-ƙB translocation and ERK2/JNK1 activity in EC, processes which are involved in gene activation and EC migration respectively[164,165]. In an *ex vivo* study with pig coronary arterioles, it was shown that that the integrin-ECM binding antagonist RGD peptide reduced FSS-induced vasodilation in a dose-responsive manner[69]. In the same study, integrin β3 antibody also inhibited FSS-induced vasodilation. Integrins have also been shown to activate the small GTPase Rho and lead to FSS-induced cell alignment[166]. Integrins have also been shown to act as a co-effector for FSS sensing by VEGFR-2. FSS induced VEGFR-2 phosphorylation was blocked by both αvβ3 and β1 antibodies. However, treatment with the VEGFR-2 tyrosine kinase inhibitor SU1498 did not affect activation of Shc by integrins, suggesting that VEGFR-2 is downstream of integrins in FSS sensing[167]. Interestingly, FSS can also upregulate EC integrin α5β1[168]. These studies provide strong evidence that integrins are important FSS mechanotransducers in EC.

VEGFR-2. VEGFR-2 has also been recognized as a FSS mechanosensor in EC. Both a VEGFR-2 kinase inhibitor and siVEGFR-2 treatment reduced FSS induced Akt and eNOS activation in EC[71]. Another study done by Shay-Salit et al. (2002) suggested that VEGFR-2 acts as a co-transducer of FSS with the adherens junction, VE-cadherin and β-catenin[72]. In this study, VEGFR-2 translocation

induced by FSS was abolished in VE-cadherin knockout cells, suggesting that VE-cadherin is a cofactor for VEGFR-2 activation by FSS. Interestingly, in both studies, VEGFR-2 phosphorylation levels increased substantially with short periods of FSS (5-30 min). In the study done by Shay-Salit et al. (2002), VEGFR-2 phosphorylation levels were reduced to less than 0.5-fold of static control after 2h and 4h of FSS, suggesting VEGFR-2's FSS sensing is rapid but transient.

G-proteins. G-proteins are important cellular signal transduction molecules involved in many cellular functions, including FSS sensing. A study done by Ohno et al. showed that the cGMP level increased by 4-fold after shear, which was mediated by the G-protein-coupled K^+ channel. The K^+ channel antagonist tetraethylammonium ion (TEA) and the G-protein inhibitor pertussis toxin were able to block the FSS-induced cGMP increase, confirming G-protein's function in FSS sensing[169]. Using fluorescence resonance energy transfer (FRET), Chachisvilis et al. observed FSS-induced G-protein activation by detecting a conformational change in the G-protein-coupled receptor bradykinin receptor-2 after exposing EC to FSS. Bradykinin is a 9-amino acid-peptide vasodilator that induces NO production in EC. In another study using liposome-bound G-protein, Gudi et al. showed that FSS activated GTPases Ga_q and $G_{\alpha i3}$ in a dose-dependent manner[170]. These studies provide valuable insights into G-protein mediated FSS sensing.

Ion channels. Ion channel activation has been recognized as a major impact of FSS on EC. Ion channel activation is essential for vascular tone regulation by the endothelium. FSSA study done by Olesen et al. (1988) discovered the first FSS-activated K^+ channel in EC. In this study, the authors compared the membrane potential of EC treated with acetylcholine (ACh) and FSS. Atropine, an Ach competitive antagonist, blocked Ach-induced inward K^+ current but did not affect FSS-induced K^+ current, implying the existence of an FSS-sensing K^+ channel[171]. Another study showed that inward rectifier K^+ (IRK) channel activation by FSS was regulated by Ca^{2+} in a dose-dependent manner, and inhibition to Ca2+ channels abolished IRK channel activation by FSS, suggesting the involvement of Ca^{2+} channels in FSS sensing[172]. Many studies have shown that Ca^{2+} influx is an important process in FSS induced signaling in EC. Studies done by Yamamoto et al. showed that in EC, FSS induced Ca^{2+}

influx is mediated by P2X4, a ligand-gated cation channel which is a purinoceptor for ATP[173,174]. P2X4 knockdown with antisense oligonucleotides (AS-oligos) blocked FSS-induced Ca^{2+} influx *in vitro*. An elegant mouse study done by the same group showed that knockout of P2X4 leads to impaired vascular tone and remodeling[175]. These studies show the important role Ca^{2+} channels play in FSS signaling.

2.3.2. Oxidative stress

Our group previously demonstrated that NOX-4 (NADPH oxidase 4) and HO-1 (heme oxygenase 1) are involved in PLGF upregulation in EC by FSS. NOX are the predominant sources of ROS in the vasculature[176]. ROS produced by NOX activate many pathways involved in arteriogeneic signaling[177,178]. Other studies have also shown FSS activates and upregulates NOX[179-181]. Furthermore, NOX have been implicated in collateral growth[182]. NOX-4 is the most abundant NOX isoform in endothelial cells and produces H_2O_2[183]. HO-1 is important in oxidative stress protection and generates three products (carbon monoxide, biliverdin, and ferrous iron) [184]. NOX-4 mRNA, H_2O_2, and HO-1 mRNA levels all increased after FSS treatment, while siRNA knockdown of either NOX-4 or HO-1, and catalase treatment were all able to block FSS-induced PLGF upregulation in EC-SMC cocultures[73]. These results suggest NOX-4 and HO-1 are important mediators PLGF upregulation.

2.3.3. Hypoxia

As an angiogenic growth factor, PLGF has been widely considered to be regulated by hypoxia inducible factor (HIF)[185-187]. Complete HIF is formed by dimerization of the HIF subunits α and β. HIF-β, first cloned in 1991[188], was originally named aryl hydrocarbon receptor nuclear translocator-1 (ARNT-1) for its function in regulating p4501A1 enzyme upon interaction with aryl hydrocarbon receptors[189]. HIF-1β binds with HIF-α and the complete HIF dimer translocates to the nucleus[190]. The three isoforms of HIF-α (HIF-1α, HIF-2α and HIF-3α) arise from three different genes. They have been shown to have overlapping and compensating functions with each other. Upon dimerization, HIF translocates to the cell nucleus and binds to hypoxia response elements (HRE) to regulate transcription of target genes[191-193].

HIF-1β is constituently present in cells, while the three isoforms of HIF-α are constantly degraded by ubiquitination under normoxic conditions[194]. HIF-α degradation by the proteasome is regulated by prolyl hydroxylase domain proteins (PHDs) though hydroxylation of HIFα at proline residues[195]. Hydroxylated HIFα is recognized by Hippel-Lindau (VHL) protein, which initiates ubiquitination[195,196]. Factor inhibiting HIF (FIH) is another regulator of HIF-1α protein. Hydroxylation at Asp823 by FIH inhibits HIF-1α interaction with transcription cofactor p300[197]. Under hypoxic conditions, PHDs and FIH are inhibited due to lack of oxygen as a substrate. PHDs and FIH use iron, 2-oxoglutarate (2-OG), and oxygen as substrates to hydroxylate HIF-1α at different sites. Iron chelators[198], $CoCl_2$[95], and the 2-OG derivative dimethyloxalyl glycine (DMOG)[197] are commonly used to inhibit PHDs and FIH and increase HIFα accumulation and activity.

The role of HIF in regulation of PLGF is somewhat unclear. Tudisco et al. identified several HREs in the second intron of the PLGF gene[199]. In this study, under hypoxic conditions, the HREs were responsive to HIF-1α in human umbilical cord vein endothelial cells (HUVEC), and silencing with siHIF-1α abolished hypoxia-induced PLGF upregulation. Likewise, Gonsalves et al showed that erythropoietin increases PLGF expression in erythroid cells via HIF-1α[200]. However, another study published by Gobble et. al. (2009) demonstrated that PLGF is not regulated by HIF-1α in human trophoblast cells[201].

2.3.4. Epigenetic regulation

DNA methylation. In human lung and colon carcinoma, hypermethylation of the promoter region was linked to reduced PLGF transcription while treatment with demethylating agent 5-Aza-dC restored PLGF transcription and translation[202].

Histone acetylation and transcription cofactor p300. Histone acetylation, a modification to the lysine residues, adds negative charges to histones, decreasing the attraction between histones and DNA and facilitating the transcription complex's access to DNA[203]. Histone acetylation levels are regulated by histone acetyl transferases (HAT) and histone deacetylases (HDAC). In the study by Tudisco et al. mentioned above, histones H3 and H4, which are in close contact with PLGF hypoxia

responsive elements (HREs) located in the second intron, were found to be hyperacetylated under hypoxic conditions[199]. Furthermore, the HAT known as p300 CTB associated factor (PCAF) has been shown to be important in inducing arteriogenesis in a study with PCAF KO mice[204].

Transcription cofactor p300, first discovered as a HAT by Ogryzko et. al. in 1996[205], has drawn our particular attention as a factor that may play an important role in PLGF regulation through modification of histone acetylation. p300 was first discovered as a homologue of the transcription cofactor cAMP-response element-binding protein (CREB)-binding protein (CBP) in studies on adenovirus protein E1A[206,207]. CBP and p300 are structurally and functionally closely related, and therefore are often referred to together as CBP/p300[205]. However, studies overexpressing or inhibiting expression of p300 or CTB show that these proteins do have different functions[208-210]. Both CBP and p300 facilitate gene transcription through histone acetylation at lysine residues[207]. They are also involved in post-translational modification of other proteins by acetylation, such as nuclear factor erythroid-2 (Nrf2)[211] and p53[212]. In addition, p300 also goes through autoacetylation that increases its HAT activity[213,214]. A recent study by Weinert et. al. (2018) shows that histone acetylation regulated by p300 is highly dynamic[215], which is in agreement with our observation that PLGF upregulation occurs quickly upon exposure to FSS.

Several inhibitors are commonly used to inhibit p300 interaction with transcription factors, or its HAT activity. Chetomin, a secondary metabolite of fungus Chaetomium cochliodes[216], inhibits p300's cysteine/histidine rich domain-1 (CH1) through a "zinc ejection" mechanism[217]. Curcumin inhibits p300 by forming covalent bonds with its HAT domain[218,219]. Lys-CoA[220], C646[221], and A485[222] are competitive inhibitors for the HAT pocket of p300.

2.3.5. Transcription factors involved in PLGF regulation

HIF, as mentioned previously, has been implicated in PLGF transcription. Metal transcription factor-1 (MTF-1) has also been shown to regulate PLGF either alone[223] or in coordination with NFκB p65, under hypoxic conditions[224]. Interestingly, p65 has been implicated in the regulation of FSS-induced transcription of PDGF[225] and eNOS[226] in endothelial cells.

2.3.6. Iron

Results so far. HO-1 uses NADPH to degrade heme and produce biliverdin, carbon monoxide (CO) and Fe^{2+}. Previous studies by our group showed that treatment of HCAEC/HCASMC cocultures with biliverdin did not affect PLGF levels. Likewise, a carbon-monoxide releasing molecule (CORM) was unable to upregulate PLGF[227]. However, several forms of iron including hemin (a form of heme), ferric ammonium citrate (FAC) and iron-nitrilotriacetic acid (Fe-NTA) all stimulated PLGF expression in a dose-dependent manner. These results led us to the hypothesis that FSS upregulates PLGF by increasing cellular iron concentration through HO-1 activation. Iron metabolism is discussed in more detail below.

3. Iron metabolism

3.1. Iron absorption

Iron is essential for mammalian health. Iron is required for oxygen transportation, respiration, enzyme activities and gene regulation, among other functions. Dietary iron is 10% heme, while 90% is ferric iron (Fe^{3+}), which is neither soluble in water nor bioavailable. Fe^{3+} is reduced to Fe^{2+} by ferrireductase before being absorbed by the intestinal epithelium. Fe^{2+} is transferred into epithelial cells by the divalent metal transporter 1 (DMT1)[228,229]. Once within cells, Fe^{2+} is oxidized to Fe^{3+} by hephaestin and released back into circulation via ferroportin[230]. Hepcidin, a small peptide hormone, regulates ferroportin exportation of iron by binding to ferroportin and inducing its endocytosis and subsequent lysosomal degradation[231].

3.2. Transferrin

Circulating iron is the most dynamic iron pool contributing to iron homeostasis. After being released into circulation, Fe^{3+} binds to transferrin (Tf). Under normal physiological conditions, only 30%-40% of Tf is iron-loaded. Iron-loaded transferrin is referred to as holotransferrin. Non-loaded Tf is referred to as apotransferrin. Transferrin binds to the transferrin receptor and is endocytosed. The affinity of the transferrin receptor for holotransferrin at pH 7.4 is 700 to 1500-fold higher than its

affinity for apotransferrin[232]. Subsequent to cellular uptake, holotransferrin releases iron in response to the endosomal pH decrease, and is recycled back into circulation as apotransferrin[233,234].

3.3. Ferritin

Fe^{3+} is reduced to Fe^{2+} when it is released into the endosome, due to the endosomal pH decrease, and becomes part of the labile iron pool[234]. Fe^{2+} can be transported from the endosome into the cytoplasm by natural resistance-associated macrophage protein 1 (NRAMP1) in the endosomal membrane. NRAMP1 is in the same protein family as DMT1 and has a similar structure and function[228]. Ferritin then oxidizes cytosolic Fe^{2+} to Fe^{3+} and sequesters it. Iron can be released from ferritin by lysosome degradation[235,236]. Iron release from ferritin with reductive mobilization reactions independent of lysosome degradation has been proposed[232237].

3.4. Iron-containing proteins

Hemoproteins. Iron is of fundamental importance to most aerobic organisms for its characteristic ability to bind and release oxygen when in the heme molecule. In humans, the heme-containing protein hemoglobin accounts for 65% of total body iron. Myoglobin and cytochromes also use heme for oxygen storage and for catalysis[238].

Iron-sulfur (Fe-S) cluster proteins. Iron-sulfur (Fe-S) clusters are also an efficient way of utilizing iron for electron transport. Numerous enzymes contain Fe-S clusters, e.g., mitochondrial respiratory chain complex I, dehydrogenases, aconitases, DNA primases and polymerases[239]. This review will focus more on one particular aconitase, iron responsive element-binding protein 1 (IRP-1), which also acts as an iron sensor.

PHDs. Prolyl hydroxylase domain proteins (PHDs) hydroxylate conserved prolyl residues in HIF-1α, labeling it for proteasomal degradation. Iron is a required element in the PHD active site[240]. Iron deficiency has been shown to affect PHD activity *in vitro*[241]. Iron is provided to PHDs by poly (rC) binding protein 1 (PCBP1). In a study by Nandal et. al. (2011), depletion of iron with deferoxamine (DFO, an iron chelator) and siRNA knockdown of PCBP1 both blocked PHD-2 activity in cell lysates. After adding Fe^{2+}, PHD-2 activity was rescued[241].

3.5. IRPs

Iron responsive element-binding proteins (IRPs) are iron sensors that regulate iron metabolism-related protein expression by binding to iron responsive elements (IRE) on mRNAs of iron-regulated genes, including transferrin receptor 1 (TFR-1), divalent metal transporter 1 (DMT1), and ferritin-H and L. IRP binding can either suppress mRNA translation by binding to the 5'UTR (e.g. ferritin H and L), or can enhance translation by binding to the 3'UTR to prevent mRNA degradation (e.g. TFR-1). Two IRP isoforms have been described, IRP-1 and IRP-2. Iron regulates IRP-1 and IRP-2 differently. In iron replete cells, IRP-1 can acquire a 4Fe-4S cluster, which releases IRP-1 from IRE binding, and transforms it into an aconitase. In contrast, IRP-2 is degraded by the proteasome when cellular iron levels are high[242].

3.6. Iron related conditions

Iron deficiency. Iron deficiency is the most common nutritional disorder worldwide[243]. Iron deficiency can be caused by dietary deficiency, high plasma soluble transferrin levels (sTf)[244], high levels of hepcidin[231], or chronic disease (ammonia of chronic disease, ACD). Oral iron supplementation is recommended by the World Health Organization for iron deficiency management[245].

Iron overload. Besides diet-caused iron overload, 4 types of hereditary hemochromatosis (HFE) have been described that cause hepcidin deficiency and iron overload. The 4 types of mutations are in human hemochromatosis protein (HEF-1), hemojuvelin (HJF)/hepcidin (HAMP), TfR2 and ferroportin 1[246]. Iron depletion by phlebotomy and iron chelators are used clinically to treat iron overload[247].

Sickle cell disease and PLGF. Sickle cell disease (SCD) patients have been shown to have excessive accumulation of iron levels due to blood transfusions[248,249]. Interestingly, SCD patients have also been found to have elevated PLGF levels[250,251]. This suggests a potential role of iron in PLGF regulation, consistent with our lab's findings.

3.7. Iron and the Fenton reaction

Fe^{2+} can catalyze H_2O and O_2 into forming H_2O_2 and other radicals, including $HO\cdot$ and $HOO\cdot$. This process is named the Fenton reaction. It has been shown to cause DNA damage[252] and lipid oxidation[253].

4. Discussion

Arteriogenesis has been regarded as a "natural bypass"[254]. It has the potential to reduce the risk of myocardial infarction brought by CAD[255]. Therefore, it is an important therapeutic target. However, arteriogenesis is a complex process that involves many cell types and regulatory factors. Arteriogenesis can induced by FSS produced by blood being diverted through collaterals from stenosed major arteries, or by the increased blood flow produced by exercise.

PLGF is an important factor in arteriogenesis; however, its regulation by FSS, signaling pathways, and epigenetic mechanisms remains unclear. Our group was the first to show PLGF is upregulated by FSS[73]. These previous studies further determined that NOX-4, HO-1, and iron have important functions in this process. Therefore, one of the key goals of this project was to identify further downstream steps in the pathway by which FSS regulates PLGF. Iron overload in SCD patients is linked to increased PLGF levels[248,250,251], and our previous results show that several forms of iron can upregulate PLGF. However, the mechanism of PLGF upregulation by iron requires further elucidation. Thus, a second major goal of this project was to characterize the mechanism by which iron regulates PLGF.

Our results described in the following chapters show that p300 HAT activity is a key contributor to PLGF upregulation by FSS. Likewise, we provide evidence that histone acetylation is increased by FSS in HCAEC, as measured by mass spectrometry. In future studies, more mass spectrometry analyses can be done to characterize the effect of FSS on histone acetylation levels. Likewise, Western blotting using antibodies for histones with specific acetylated lysine sites can be used to examine the effect of FSS on acetylation levels.

Our results further suggest that a transcription factor is critical in PLGF transcription, and demonstrate that HIF-1α, and HIF-2α are not involved in upregulation of PLGF by FSS. The transcription factor involved in stimulating PLGF transcription in HCAEC following FSS exposure remains undetermined. NFκB is one potential candidate, as it has been shown to be activated by FSS and to interact with the PLGF promoter region. Further studies are necessary to define the key transcription factor(s) involved in PLGF regulation by FSS.

In summary, the results of our study have important implications for our understanding of how arteriogenesis is regulated. Further exploration of the signaling pathways determined in this study may identify novel pharmaceutical targets for effectively inducing arteriogenesis to reduce the risk and burden of CAD.

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CHAPTER II

IRON HOMEOSTASIS AND PLACENTAL GROWTH FACTOR REGULATION

Abstract

Placental growth factor (PLGF) has been shown to have important functions during development and under pathological conditions. PLGF is regarded as an important factor in arteriogenesis, the remodeling and enlargement of smaller arterioles induced by fluid shear stress (FSS) that is produced when blood is diverted from occluded upstream arteries. Our lab's published work indicates that PLGF is upregulated by FSS, and that heme oxygenase-1 (HO-1) is activated by FSS[1]. HO-1 uses heme to produce CO, iron, and biliverdin. Our lab's previous results showed that neither CO nor biliverdin is able to increase PLGF expression in endothelial cells. However, we found that several forms of iron, including hemin, ferric ammonium citrate (FAC), and ferric nitrilotriacetate (Fe-NTA) can upregulate PLGF in endothelial cells (HCAEC)[2]. Interestingly, it has previously been reported that iron overload in sickle cell disease (SCD) patients is correlated with PLGF upregulation in serum[3-5]. Our results are in agreement with those findings. We next asked which signaling pathway(s) mediate this effect of iron on PLGF. Iron is known to influence hypoxia inducible factor (HIF) expression though PHD proteins[6-8], and others have reported a role for HIF in regulation of PLGF[9,10]. Therefore, we hypothesized that iron produced by FSS-activated HO-1 increases intracellular iron to upregulate PLGF via an iron-response protein (IRP)-dependent pathway. We also hypothesized that iron deficiency would impair PLGF expression. In this chapter, we show that iron upregulates PLGF in human coronary artery endothelial cells (HCAEC). However, we were not able to show a role for ferritin or IRPs in FSS-induced PLGF expression. Similarly, dietary-induced iron deficiency did not affect PLGF levels in rat hearts, although it increased transferrin receptor mRNA (~8-fold) as expected. In further experiments, we measured PLGF and HO-1 mRNA in HCAEC treated with FSS, curcumin, and chetomin. Surprisingly, curcumin and chetomin treatments had opposite effects on PLGF and HO-1 mRNA. These results suggest that increased HO-1 mRNA levels alone are not

sufficient to upregulate PLGF. Further studies to explore the dynamic interactions between PLGF regulation and iron metabolism are needed.

Introduction

Placental growth factor (PLGF) has been deemed to be a critical factor in arteriogenesis[11-14], the process of small artery remodeling and enlargement in response to increased fluid shear stress (FSS) after occlusion of a major artery. Our lab's previous work has shown that PLGF upregulation by FSS is dependent on upregulation of NADPH oxidase-4 (NOX4)[1] and heme oxygenase (HO-1)[2]. Our results revealed an interesting link between FSS regulation of PLGF and cellular iron homeostasis. HO-1 breaks down heme and produces iron, biliverdin, and CO. Since both biliverdin and CO act as antioxidants, HO-1 is an important enzyme in the cellular response to oxidative stress[15,16]. In our lab's previous studies, human coronary artery endothelial cells (HCAEC) were treated with iron, biliverdin, or CO, followed by measurement of PLGF levels. Only iron, but not biliverdin or CO, could upregulate PLGF[2]. Upregulation of PLGF by iron has been observed by other researchers[17,18], in agreement with our results. This finding is of clinical interest because iron overload has been linked with elevated PLGF levels in human patients[3,19].

A previously published study by our lab showed that H_2O_2 can upregulate PLGF by increasing its mRNA half-life in human coronary artery smooth muscle cells (HCASMC) [20]. Likewise, the upregulation of PLGF in HCAEC/HCASMC cocultures is mediated by a H_2O_2 – dependent pathway. Transition metals such as iron can donate or accept electrons easily[21-23], and the Fenton reaction describes the phenomenon by which transition metals generate ROS in solution. Therefore, in the present studies, we first tested whether iron-mediated upregulation of PLGF is mediated by reactive oxygen species (ROS) created by the Fenton reaction. The response to iron persisted in the presence of catalase, suggesting that the effect of iron on PLGF is mediated by cellular signaling pathways other than ROS.

We next tested the hypothesis that FSS-induced HO-1 upregulation increases intracellular iron content, which in turn upregulates ferritin though iron responsive element binding protein-1

and/or 2 (IRP1, IRP2). We further hypothesized that this increased ferritin would sequester intracellular free iron, thereby reducing activity of prolyl hydroxylase domain proteins 1, 2, and 3 (PHD1, 2, and 3). Lastly, we anticipated that reduced PHD activity would cause hypoxia inducible factor (HIF) to accumulate and bind to the PLGF promoter region's putative hypoxia responsive element to increase PLGF transcription, as illustrated in **Fig. 1**.

We tested several steps of this hypothesis by assessing ferritin levels in HCAEC following FSS exposure, the effect of IRP-1 and -2 knockdown on upregulation of PLGF by FSS, and the effect of an iron-deficient diet on PLGF expression in rat hearts. Neither ferritin nor IRPs appeared to be involved in regulation of PLGF by FSS. Likewise, the iron-deficient diet failed to reduce PLGF levels in rat hearts. The involvement of HIF in FSS-mediated upregulation of PLGF was also thoroughly investigated, leading us to rule out a role for HIF in this pathway (see chapter 4).

In the course of the HIF experiments, we identified transcription cofactor p300 as a key component of the pathway by which FSS upregulates PLGF. We therefore tested whether p300 also mediates upregulation of PLGF by iron. Our results show that iron upregulates PLGF by a pathway that involves 1) p300 binding to a transcription factor other than HIF and 2) p300 histone acetyltransferase (HAT) activity.

To our knowledge, there have been no studies linking iron homeostasis with arteriogenesis. Our results suggest that p300 plays an important role in this novel signaling pathway. Further elucidation of the mechanism of PLGF upregulation by iron will provide new insights which could lead to novel treatments to improve arteriogenesis.

Materials and Methods

Cell culture. Passage 6 (P6) human coronary artery endothelial cells (HCAEC, Lonza) and human coronary artery smooth muscle cells (HCASMC, Lonza) were used in all experiments. The cells were cultured in matching media (Smooth Muscle Growth Medium-2/SmGM-2 and Microvascular Endothelial Cell Growth Medium-2/EGM-2MV, Lonza) for 3 d to reach confluence before seeding. For monoculture experiments, HCAEC were seeded in 12 well plates and allowed to reach confluence for 24 h before ~18 h of serum restriction. For serum restriction, cells were incubated with 2% FBS medium supplemented with 15 mM HEPES, 30 μg/mL gentamycin, and 15 ng/mL amphotericin-b (Lonza). The 2% FBS medium was a 3:2 mixture of serum-free DMEM (Sigma) and HCAEC or HCASMC medium. For coculture experiments, the cells were seeded on either side of a polyester insert with 0.4 μm pores in a 6-well plate format (Corning Transwell). This allows the two types of cells to come into contact but prevents cell migration. Before seeding, the seeding side was coated with 0.1% gelatin in matching medium for 1 h in a cell culture incubator (37˚C, 5% CO_2). The gelatin coating medium was then aspirated and the inserts were allowed to dry. HCASMC were seeded on the inverted side of the insert at 10,000 cells/cm^2 then incubated to allow attachment for 4-6 h before the inserts were repositioned into a receiving well with 2 mL of SmGM-2. HCASMC were incubated overnight before HCAEC were seeded on the receiving side of the insert at 25,000 cells/cm^2. HCASMC were fed with fresh SmGM-2 when HCAEC were seeded. Co-cultures were incubated at 37˚C, 5% CO_2 for 24 h before 18 h of serum restriction.

Fluid shear stress setup. Fluid shear stress (FSS) was applied to co-cultures with motor-attached (Optimal Engineering Systems, Inc.) cones made of ultra-high molecular weight polyethylene with a 5˚ incline towards the center as described previously by our group and others[1,24,25] (**Fig. 2. A**). The motors were controlled by specialized software, Allegra (Optimal Engineering Systems, Inc.) to turn at different rates simulating pulsatile blood flow. For these

studies, we used a waveform that simulates collateral flow downstream of a coronary artery with 60% stenosis (average FSS 1.24 Pa, **Fig. 2. B**), which is considered clinically critical, as described in previous publications[1,26]. Only HCAEC on the upper side of inserts were directly exposed to FSS, whereas HCASMC were not directly exposed to FSS but were accessible to diffusible mediators produced by HCAEC in response to FSS. The FSS stimulus was applied to the cocultures for 2 h. During FSS exposure, the Transwell plates were kept on plate heaters to maintain cell cultures at 37 ˚C. Media and/or cell lysate was collected 0-24 h after the 2 h-shear stress exposure. The static inserts were covered with parafilm to prevent excessive evaporation and was placed next to FSS exposure inserts for the same duration of treatments as published by our group previously[1].

Animal experiments. Weanling male Sprague-Dawley rats were housed in individual cages in a humidity and temperature controlled room with a 12 h light/dark cycle in the Laboratory Animal Resource Facility at Oklahoma State University. Upon arrival at the facility, rats were fed with control (C) diet (Harlan Teklad) for 3 days to allow acclimation to the facility. C group rats were fed with the C diet at 50 mg Fe/kg body weight, and iron-deficient (ID) group rats were fed with ID diet (Harlan Teklad.) at <5 mg Fe/kg. C and ID groups were allowed *ad libitum* access to diets. Pair-fed (PF) group was fed with the C diet restricted to the average amount consumed by the C group. All groups were allowed *ad libitum* access to deionized water. After 21 days, rats were injected with a ketamine/xylazine cocktail followed with euthanization by exsanguination. Rat tissues were isolated and flash-frozen and stored in -80 °C freezer.

Chemical exposure. Unless otherwise specified, all chemicals were added to both the HCAEC and HCASMC sides of the Transwell insert immediately before FSS exposure. Control groups were treated with same volume of the vehicle. Chemicals used were ferric ammonium citrate (FAC, Sigma), catalase (Sigma), chetomin (Sigma), curcumin (Tocris), A485 (Tocris), and hemin (Sigma).

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siRNA treatments. Pre-designed, lyophilized siRNA (Silencer Select) was purchased from Thermo Fisher and reconstituted with RNAase free H_2O before being stored at -20 ˚C. For cell treatment, a siRNA mix was made with Opti-MEM and Lipofectamine RNAiMAX following the manufacturer's instructions to reach a final siRNA concentration of 5 nM in cell culture media. The siRNA mix was combined with normal culture media and added to cell culture wells/flasks immediately before cells were trypsinized, re-suspended in media, and added to wells/flasks. Cells were incubated with the siRNA mix for 24 h. Knockdown efficacy was determined using real-time qRT-PCR. For siRNA treated HCAEC used in shear stress experiments, HCAEC were seeded in inserts immediately after siRNA treatment and incubated with 2% FBS medium for 18 h before shear stress exposure.

Real-time qPCR. RNA extraction was performed with the RNeasy Mini Kit (Qiagen), or RNeasy Fibrous Tissue Mini Kit (Qiagen). Cells were trypsinized with TrypLE (Gibco) then centrifuged at 5,000 RPM for 5 min to form a pellet. The pellet was lysed with buffer RLT from the RNeasy Mini kit then stored at -80 ˚C until further steps following the manufacturer's instructions. Tissue samples were homogenized with a homogenizer (Bio-Gen) in buffer RLT from the Fibrous Tissue Mini Kit (Qiagen) then RNA extraction was performed following the manufacturer's instructions. The RNA concentration was determined with a Synergy Plate Reader (Biotek) using a Take3 Micro-Volume Plate (Biotek). For cDNA synthesis, reverse transcription was done within 1 h after RNA extraction using the QuantiTect Reverse Transcription Kit (Qiagen). The remaining RNA was stored at -80 ˚C. cDNA was stored at 4 ˚C. For real-time qPCR, 5 or 10 ng of cDNA was used per reaction for cell culture samples, and 40 ng was used for animal tissue samples. A PCR master mix was made using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosystems), and 50 nM primers. Real-time qPCR was performed in an ABI 7500 Fast instrument (Applied Biosystems). Relative mRNA quantification was normalized to β-actin and a reference control sample, then calculated as $2^{-\Delta\Delta Ct}$. For ferritin and PLGF in rat hearts,

mRNA levels were normalized to β-actin, ribosome proteins RPL19, and RSP29. **Table 1** shows

the primer sequences used.

Homo sapiens	*β-actin*	Forward	TGCCGACAGGATGCAGAAG
		Reverse	CTCAGGAGGAGCAATGATCTTGAT
Homo sapiens	*PLGF*	Forward	CCTACGTGGAGCTGACGTTCT
		Reverse	TCCTTTCCGGCTTCA TCTTCT
Homo sapiens	*HIF-1α*	Forward	TCATCCATGTGACCATGAGGAA
		Reverse	GAGAAAAAAGCTTCGCTGTGTGT
Homo sapiens	PHD-2	Forward	CCAGAAGGCAAAGCCCAGTT
		Reverse	CTCGTGCTCTCTCATCTGCAT
Homo sapiens	PHD-3	Forward	GTTCCCTCTCTGGTTGCACG
		Reverse	CAGGAAGTTGTCCAGGTAGCA
Homo sapiens	IRP-1	Forward	GCTTGGGTCAGGTTCGCC
		Reverse	GATCGAAAATGGTAAGCGCCC
Homo sapiens	IRP-2	Forward	GACGCCCCAAAAGCAGGATA
		Reverse	CAGCTTCCAACAAGACCCGT
Homo sapiens	HO-1	Forward	CTGCGTTCCTGCTCAACATC
		Reverse	GGCAGAATCTTGCACTTTGTTG
Rattus rattus	β-actin	Forward	AGTTCGCCATGGATGACGAT
		Reverse	TGCCGGAGCCGTTGTC
Rattus rattus	RPL19	Forward	CGTCCTCCGCTGTGGTAAA
		Reverse	TGGCGATTTCGTTGGTTT
Rattus rattus	RPS29	Forward	GCCAGGGTTCTCGCTCTTG
		Reverse	GGCACATGTTCAGCCCGTAT
Rattus rattus	TfR	Forward	TCGGCTACCTGGGCTATTGT
		Reverse	CCGCCTCTTCCGCTTCA
Rattus rattus	PLGF	Forward	CTGCTGGGAACAACTCAACAGA
		Reverse	GCGGCCCCACACTTCATT

Table 1. Primer sequences

ELISA. Media were collected at certain time points after shear stress exposure and treated

with a cocktail of protease inhibitors (1 mM PMSF, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM

benzamidine-HCl, 1 μg/mL aprotinin, 1 μg/mL pepstatin A) before being stored at -80 ˚C. For

cell lysis, after cells pellets were collected as described in the previous passage, pellets were lysed

with RIPA buffer supplemented with the same cocktail of protease inhibitors. Both PLGF and

HIF-1α ELISA were performed using the respective DuoSet ELISA kits (R&D Systems)

following the manufacturer's instructions. For FAC and catalase treated samples, the concentration of PLGF was expressed in pg/mL to eliminate the difference in protein concentration introduced by adding catalase. For the rest of the experiments, the concentration of PLGF was normalized to total protein as measured with the BCA assay (Pierce).

Western blot. RIPA buffer supplemented with a protease inhibitor cocktail (1 mM PMSF, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM benzamidine-HCl, 1 μg/mL aprotinin, 1 μg/mL pepstatin A) was used for both tissue and cell lysis. Protein concentration was determined using the BCA assay (Pierce).

For protein extraction from cultured cells, media were removed and the cells were rinsed with warm DPBS (Lonza). Cells were dissociated by incubation with TrypLE (Gibco) for 5 min at 37 ˚C. Cold EGM-2MV was then added to the wells to halt trypsinization. Cells were centrifuged at 5,000 RPM for 5 min to form a pellet, then lysed with RIPA buffer on ice for 30-45 min with vortexing every 10 min. The cell lysate was then centrifuged at 8,000 RPM for 15 min, and the supernatant was collected and stored at -80 ˚C. A rabbit-anti human/mouse/rat polyclonal antibody (Abcam) was diluted to 1:2000 for β-actin detection. A rabbit-anti-human monoclonal antibody (Abcam) was diluted to 1:2000 for ferritin detection. An HP-tagged mouse IgGκ binding protein (Santa Cruz) and an HP-tagged donkey-anti-rabbit secondary antibody (Jackson Immuno) were diluted to 1:5,000 and 1: 10,000 respectively to detect primary antibodies. For protein extraction from rat heart tissue, 10-30 mg of rat heart (mainly ventricle tissue) was homogenized with a homogenizer (Bio-Gen) in RIPA buffer. The homogenized tissue was then centrifuged at 8,000 rpm for 15 min. The supernatant was collected, aliquoted, and stored at -80 ˚C. For Western blotting, 10 μg of protein was loaded in each lane of a 12% acrylamide gel.

Samples were mixed with 3x Laemmli buffer supplemented with bromophenol blue. The electrophoresis condition was 100 V for 150 min at room temperature. The transferring condition

was 60 V for 150 min in an ice-cooled chamber. PVDF membrane (Fisher) was used for blotting.

Membranes were blocked with SuperBlock (Fisher) TBS blocking buffer for 10-15 min at room

temperature. A monoclonal mouse-anti-rat PLGF antibody (Santa Cruz) was diluted at 1: 1,000

for PLGF detection. An HP-tagged mouse-anti-mouse secondary antibody (Santa Cruz) was

diluted at 1:5,000 to detect the primary antibody. Primary antibodies were incubated overnight on

a shaker at 4 ˚C. Secondary antibodies were incubated for 1 h on a shaker at room temperature.

West Pico PLUS Chemiluminescent Substrate was used for chemiluminescent detection of

protein bands. Chemiluminescent images were captured with an Amersham Imager 600 series

instrument (GE). Pixel density was analyzed with ImageJ software and normalized to β-actin as a

loading control.

Statistical analysis. Student's t-test was used to detect difference between two groups. One-

way or two-way ANOVA followed by Tukey's or Holm-Sidak t-test were used as recommended

by SigmaStat software to detect differences between multiple treatment groups. All data are

presented as mean ± SE. Statistical significance is indicated as *p<0.05, **p<0.01, ***p<0.0001,

and ****p<0.0001.

Results

Placental growth factor (PLGF) upregulation by fluid shear stress (FSS)s is a rapid reaction in HCAEC. To determine the activation of mRNAs of prolyl hydroxylase domain protein-2 (PHD-2), iron responsive element binding protein-1 (IRP-1). PLGF was upregulated by 3.22-fold ($\pm$0.60 SE, $p<0.01$, N=4) at 2 h post-FSS (**Fig. 3. A**). IRP-1 mRNA was not affected by FSS, while IRP-2 mRNA was upregulated immediately following FSS (**Fig. 3. B and C**). Interestingly, PHD-2 was upregulated immediately following FSS (**Fig. 3. D**) while HIF-1α mRNA was downregulated 4 h post FSS (**Fig. 3. E**). PHD-2 targets Hypoxia inducible protein-1α (HIF-1α), other than HIF-2α, for proteasome degradation[7]. PHD-2 upregulation will lead to reduced HIF-1α protein levels, which is in agreement with our observation that the levels of HIF-1α and PLGF are inversely correlated in HCAEC. Rishi et. al. (2015) have also shown that KO of PHD-1 and PHD-3 improves perfusion after hind-limb ischemia in mice[27].

Iron upregulates PLGF, but not by the Fenton reaction. Our lab's previous results show that three forms of iron (hemin, FAC, and Fe-NTA) can upregulate PLGF[2]. Iron, as a transition metal, can cause a Fenton reaction since it can easily accept or donate electrons, creating reactive oxygen species[21,22]. Our lab previously showed that H_2O_2 can upregulate PLGF in HCASMC[20] and HCAEC[1]. To determine whether PLGF upregulation by iron is due to the Fenton reaction and generation of H_2O_2, HCAEC were treated with catalase and ferric ammonium citrate (FAC) for 6 h. PLGF mRNA was measured with real-time qRT-PCR. Catalase treatment did not affect PLGF upregulation by FAC, indicating that eliminating H_2O_2 did not prevent PLGF upregulation by FAC (**Fig. 4**). This result suggests that iron upregulates PLGF though a different mechanism than the Fenton reaction.

Iron deficiency does not affect PLGF levels in rat hearts. It has been widely documented that iron overload in SCD patients is correlated with increased PLGF levels[3-5,28,29]. Our results confirm that iron can upregulate PLGF. Therefore, we hypothesized that iron deficiency would

have the opposite effect to reduce PLGF. To test this hypothesis, PLGF mRNA and protein levels were measured in iron-deficient (ID) diet-fed rat hearts. The ID diet increased transferrin receptor mRNA substantially, as expected (by 5-6 fold compared to control diet and pair-fed rat hearts). However, PLGF mRNA was not affected by the ID diet (**Fig. 5. A, B, and C**). Furthermore, PLGF protein levels were not affected by the ID diet (**Fig. 5. D and E**). These results suggest that an iron-deficient diet does not affect basal PLGF levels in rat hearts.

Knockdown of IRPs does not affect PLGF levels. Iron-responsive element binding proteins (IRP) are key factors in the regulation of ferritin levels. Both IRP-1 and IRP-2 bind to the 3' UTR iron responsive element (IRE) of ferritin mRNA and prevent ferritin from being translated under normal conditions[30]. When cellular iron levels increase, IRP-1 forms an iron-sulfur cluster and functions as a cellular aconitase[31,32], while IRP-2 is degraded by proteasomes[33]. Both actions release IRP from ferritin mRNA, allowing for ribosome binding and translation to occur. To test the hypothesis that IRP play a role in upregulation of PLGF by iron, we knocked down IRP-1 and IRP-2 in HCAEC using siRNA. Neither IRP-1 nor IRP-2 knockdown affected PLGF mRNA levels in HCAEC (**Fig. 6. A, B, and C**). These results suggest IRP-1 and IRP-2 are not involved in PLGF regulation by iron.

Ferritin is not upregulated by fluid shear stress. Our lab's previous results show that HO-1 is upregulated by FSS. HO-1 breaks down heme to produce iron, biliverdin, and CO. When cellular iron levels increase, more ferritin is produced by increased translation mediated by iron response proteins (IRP). We hypothesized that FSS induced HO-1 upregulation and the resulting increase in cellular iron levels would lead to increased ferritin. To test this hypothesis, we used Western blotting to measure ferritin protein in HCAEC from cocultures that were treated with 2 h FSS. Ferritin levels were measured at 0, 2, 4, 8, 12, and 16 h after the shear exposure. Both the heavy chain and light chain of ferritin were measured. Total ferritin levels were not affected by

FSS (**Fig. 7. A and B**). This result suggests that ferritin is not a component of the pathway by which FSS upregulates PLGF.

PLGF upregulation by FSS is not HIF-dependent. We next tested the hypothesis that the FSS-induced increase in transcription of PLGF mRNA is mediated by HIF. Hypoxia inducible factor (HIF) is widely regarded as one of the most important transcription factors in regulating angiogenesis and arteriogenesis[34]. To characterize the role that HIF plays in regulation of PLGF by FSS in HCAEC, cocultures were treated with 100 μM of DMOG, a prolyl hydroxylase domain protein inhibitor, to increase HIF-1α and HIF-2α. PLGF protein was upregulated by DMOG in HCAEC under both static and FSS conditions, and FSS resulted in an additive increase in PLGF by 1.23-fold (control static 187.80±13.63 pg/mg, control FSS 298.71±27.25 pg/mg, $p < 0.0001$; DMOG static 264.19±16.14 pg/mg, DMOG FSS 369.99±12.90 pg/mg, $p < 0.0001$, N=4. **Fig. 8. A**). This result suggests the induction of HIF may not be the mechanism of PLGF upregulation by FSS, since FSS produced an increase in PLGF even when HIF was already highly induced.

To test the effect of HIF inhibition on PLGF regulation by FSS, we treated cocultures with chrysin (a flavonoid that has been shown to reduce both HIF-1α mRNA and protein[35-37]) before FSS exposure. Chrysin reduced the basal level of PLGF mRNA; however, FSS upregulation of PLGF persisted (1.82 fold, **Fig 8. B**). These results also suggested that HIF-1α and HIF-2α are not likely to mediate the increased transcription of PLGF produced by FSS exposure.

Knockdown (KD) of PHD-2 reduces basal levels of PLGF but did not inhibit PLGF upregulation by FSS. To determine the involvement of PHD-2 in PLGF regulation, we treated HCAEC with siPHD-2 before exposure to FSS. siPHD-2 reduced PLGF protein levels at 12 h in both static and FSS treated HCAEC, but PLGF upregulation by FSS prevailed. PHD-2 inhibition leads to increased HIF-1α (**Fig. 9**). This result is in agreement with our observation that HIF-1α is negatively correlated with PLGF.

Knockdown (KD) of PHD-3 abolishes PLGF upregulation by FSS. To determine the involvement of PHD-3 in PLGF regulation, we treated HCAEC with siPHD-3 before exposure to FSS. siPHD-3 did not affect basal level of PLGF, but inhibited PLGF upregulation by FSS (**Fig. 10. A and B**). PHDs can target many proteins for prolyl-hydroxylation[38]. KD of PHD-2 may affect a target that is involved in PLGF regulation.

PLGF upregulation by hemin is p300 dependent. Other studies in our lab ruled out HIF as a mediator of FSS-induced PLGF expression, but revealed a role of transcription cofactor p300 in this pathway. Therefore, we hypothesized that p300 is also a mediator of iron-induced PLGF expression. To test this hypothesis, cocultures were treated with hemin ± chetomin for 12 h. Chetomin inhibits the interaction between p300 and transcription factors at p300's cysteine/histidine rich domain-1 (CH1). Hemin upregulated PLGF as expected. Chetomin abolished the stimulatory effect of hemin on PLGF transcription, and also sharply reduced basal PLGF mRNA levels (**Fig. 11. A**). A similar trend was seen for PLGF protein levels. (**Fig. 11. B**). In addition to binding to transcription factors, p300 also possesses histone acetyltransferase (HAT) activity, which can modulate gene transcription by altering histone acetylation. To determine whether p300's HAT activity also contributes to upregulation of PLGF by iron, we treated HCAEC with hemin ± A485, a potent and specific p300 HAT inhibitor[39]. A485 treatment affected PLGF levels similarly to chetomin, suggesting that p300 HAT activity is also required for PLGF upregulation by hemin (**Fig. 11. C**). Overall, these results confirm that p300 is critical for both basal and iron-stimulated PLGF transcription.

HO-1 upregulation does not correlate with PLGF upregulation with p300 inhibition. Our lab previously made the observation that HO-1 is upregulated by FSS, and that HO-1 is required for PLGF upregulation by FSS[2]. Curcumin is known to be somewhat nonspecific[40,41], and other groups have reported that curcumin can induce HO-1 expression[42,43]. Since we previously showed a stimulatory role for HO-1 in regulation of PLGF expression, we therefore assessed the effect of

p300 inhibition by curcumin and chetomin on HO-1 expression. Inhibition of p300 by both curcumin and chetomin strongly upregulated HO-1 mRNA (**Fig. 12. A and B**). Nevertheless, PLGF expression was inhibited by both drugs, in contradiction to our previous work showing that HO-1 is required for the FSS-induced increase in PLGF. These results suggest that upregulation of HO-1 mRNA may be necessary, but not sufficient, for upregulation of PLGF.

Discussion

PLGF is regarded as a critical factor in arteriogenesis[11-14]. In this study, we confirmed the positive correlation between iron and PLGF observed in our previous work, and investigated the mechanism for this effect. Contrary to our initial hypothesis, we found that neither ferritin nor IRP-1/IRP-2 contributes to the upregulation of PLGF by iron. In further studies, we tested the importance of p300 in PLGF regulation by treating HCAEC with the p300 inhibitors chetomin, curcumin, and A485. Our results show that iron upregulates PLGF though transcription cofactor p300. Both the transcription factor binding activity and the histone acetyltransferase activity of p300 appear to be required. The key transcription factor(s) that interact with p300 to regulate PLGF expression remain to be identified. However, our other studies have ruled out a role for HIF in this pathway (chapter 3). HIFs are regulated by PHDs at the post-translational level[8]. Under hypoxia conditions, PHDs are inhibited thus HIFs can accumulate. PHD-2 targets HIF-1α more specifically than HIF-2α[44]. KD of PHD-2 will leads to increased HIF-1α. We observed that KD of PHD-2 lead to reduced basal levels of PLGF, but PLGF upregulation persisted. This result corroborates with our observation that reduced HIF-1α leads to increased PLGF levels (Chapter 4), confirming HIF-1α's negative effect on PLGF transcription. Interestingly, siPHD-3 did not affect basal levels of PLGF, but abolished PLGF upregulation by FSS. In a recent study, Stoehr et. al. (2016), employing bioinformatics and mass spectrometry, demonstrated that PHDs can hydroxylate many protein targets[38]. It is possible that other than the HIFs, a protein targeted by PHD-3 is involved in PLGF regulation by FSS. This is a very interesting observation and it is worth further studies.

Our previous studies on FSS showed that upregulation of PLGF by FSS is dependent on HO-1[2]. Therefore, we would expect increased HO-1 levels to result in increased PLGF levels. However, while curcumin and chetomin abolished PLGF upregulation by iron (as well as FSS – see chapter 3), these p300 inhibitors both dramatically increased HO-1 mRNA levels, in

agreement with other researchers' observations[42,45]. We did not assess HO-1 protein levels or HO-1 activity in these studies. Therefore, it is possible that the increase in HO-1 mRNA which we observed following drug treatment did not result in any change in HO-1 activity. This is a limitation of the current study. Nevertheless, we conclude that upregulation of HO-1 mRNA alone is not sufficient to stimulate PLGF expression.

Transcription factors that interact with heme compounds, including nuclear receptor Rev-erbα (NR1D1) and BTB domain and CNC homolog 1 (Bach-1) can potentially be involved in PLGF regulation. Binding with heme enhances gene suppression activities of both NR1D1 and Bach-1, as both are important regulators of heme synthesis[46,47]. Further investigation into NR1D1 and Bach-1 can potentially lead to important links between PLGF regulation and iron metabolism.

It is also possible that the compounds we used are inhibiting HO-1 from inducing PLGF through some other mechanism. Curcumin in particular is known to be quite nonspecific, and targets many proteins and pathways including mTOR[41], HSP70, tubulin, and even β-actin[40]. This nonspecificity makes it difficult to interpret results from curcumin-treated cells. However, we also tested other p300 inhibitors (chetomin, A485) and obtained results similar to those from curcumin, suggesting that the effects of curcumin on PLGF are mediated through inhibition of p300. Chetomin reduced PLGF mRNA more drastically compared to PLGF protein in media at 12 h of treatment. This is probably because the media had been incubated with the HCAEC for 18 h before exposure to chetomin, during which period the PLGF protein in media accumulated, while PLGF mRNA transcription was inhibited by chetomin. We have observed that PLGF mRNA has a relatively short half-life, 1.67 h.

The mechanism by which the p300 inhibitors used in the present study stimulates HO-1 expression remains undefined. HO-1 is regulated by the transcription factor nuclear factor

erythoid 2–related factor 2 (Nrf2). Several studies indicate acetylation of Nrf2 by p300 or the p300 paralog CREB binding protein (CBP) increases its transcription activity[48,49]. It is worth noting that p300 and CBP share 50% homology and work closely together physically and functionally[50]. There are also non-acetylation dependent mechanisms by which p300 activates Nrf2 and increases transcription of Nrf2 target genes. Indeed, curcumin is known to inhibit p300 HAT activity by forming covalent bonds with the p300 acetyltransferase domain though the Michael addition[51]. This observation indicates that curcumin does not activate HO-1 transcription though acetylation of Nrf2. Likewise, another study showed that curcumin activates HO-1 transcription by increasing Nrf2 expression via the MAPK pathway[42].

A very interesting phenomenon has been studied by several research groups that Nrf2 competes with NFkB p65 for interaction with p300[43,52]. Upregulation of HO-1 by heme or overexpression was also found to inhibit p65 target genes expression[53].

As noted above, the transcription factor(s) that interact with p300 to regulate PLGF in response to FSS or iron remain to be identified. One possible candidate is NF-kB. The NF-kB subunit p65 has been implicated in FSS regulation of endothelial nitric oxide synthase (eNOS)[54] and platelet derived growth factor (PDGF)[55], through binding with the shear stress responsive element (SSRE) in the promoters of these genes. Several SSREs are also found in PLGF promoter, and p65 has been shown to regulate PLGF[56]. Thus, we anticipate that p65 is likely to be the transcription factor involved in FSS upregulation of PLGF. Although Nrf2 also interacts with p300, it appears less likely to mediate the effects of FSS and iron on PLGF. Whereas p65 interacts with p300 at its CH1 domain[57], Nrf2 interacts with the CH3 domain of p300[58]. Chetomin also acts at the CH1 domain to inhibit p300 by "stealing" a zinc atom from this domain, but to our knowledge has no effect on the CH3 domain. Therefore, the inhibition of PLGF expression which we observed following chetomin treatment is consistent with a role for p65, but not Nrf2. Furthermore, this could explain the differential effects of p300 inhibitors on PLGF and HO-1

mRNA, since HO-1 is regulated by Nrf2. Studies are currently ongoing to examine the role of p65 in regulation of PLGF by FSS and iron.

Although an association between iron and arteriogenesis has not previously been demonstrated, a link between iron and PLGF has been reported by others. Iron overload due to blood transfusions in sickle cell disease (SCD) has been linked with increased blood PLGF levels, and PLGF levels have been suggested to be an indicator for the severity of iron overload in SCD patients[3,19,29]. We therefore hypothesized that iron deficiency would have the opposite effect, to reduce PLGF levels. We tested this hypothesis in a rat model of diet-induced iron deficiency. Contrary to expectations, we found that iron deficiency did not reduce the basal level of PLGF in rat heart tissue. It is possible that iron plays a role in upregulation of PLGF above basal levels, but that iron is not required in order to maintain normal unstimulated levels of PLGF expression. It is also possible that there are tissue-specific differences in the regulation of PLGF that were not detected in this study, since only heart tissue was examined.

In conclusion, this study revealed the importance of the transcription cofactor p300 in regulation of PLGF by iron. However, further details of the mechanism by which iron upregulates PLGF remain to be discovered. Studies are currently underway to assess the role of p65 in this pathway. Better understanding of this mechanism could lead to new treatments to improve arteriogenesis or to modulate other processes which are mediated by PLGF.

Figures

Fig. 1

FSS → NOX-4↑ → HO-1↑ - → PLGF↑

iron↑   HIF↑

IRP↓   PHDs↓

Ferritin↑ - - → iron↓
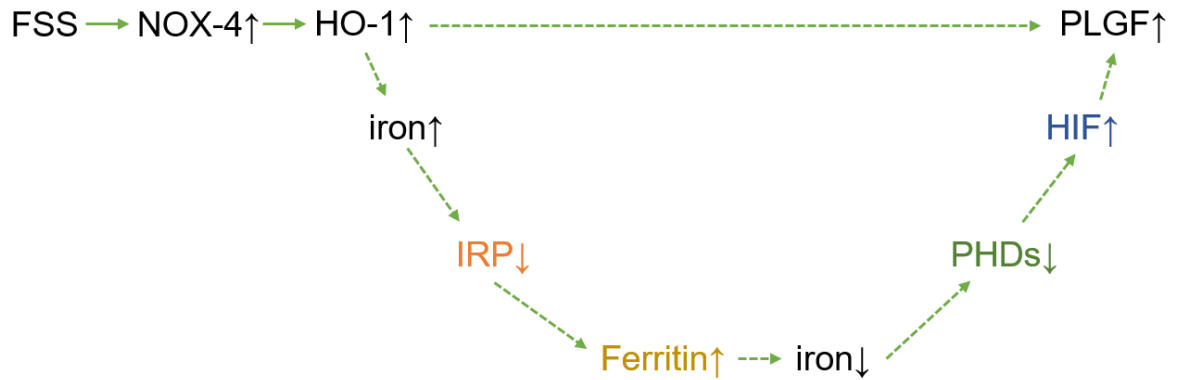
Fig. 1. Proposed pathway of PLGF upregulation by FSS through iron homeostasis.

Heme oxygenase-1 (HO-1) is a downstream target of NADPH oxidase-4 (NOX-4). HO-1

degrades heme to produce iron, CO, and biliverdin, among which iron was shown to upregulate

PLGF in HCAEC. Increased intracellular iron levels reduces the activity of iron responsive

element binding protein (IRP), which releases ferritin mRNA for translation. Increased ferritin

levels in turn sequesters and reduces free iron. Prolyl hydroxylase domain proteins (PHDs)

activities are inhibited under reduced iron conditions, thus hypoxia inducible factor (HIF) can

accumulate, which induces placental growth factor (PLGF) transcription.

Fig. 2
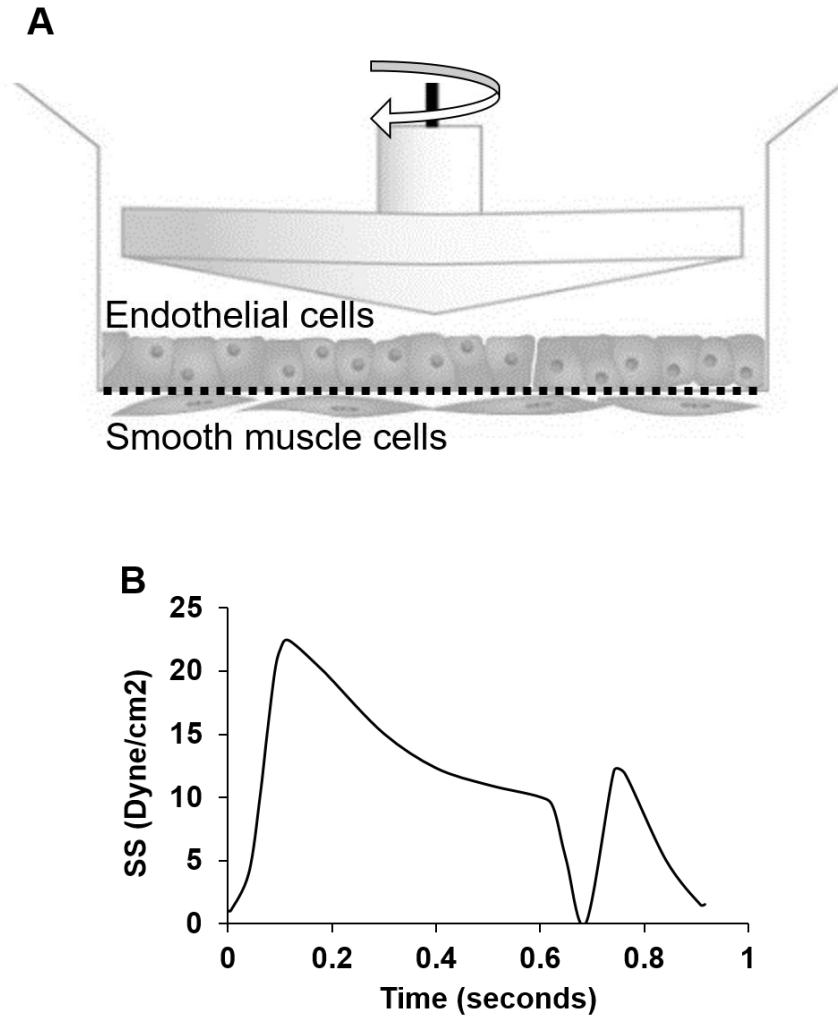
A

Endothelial cells

Smooth muscle cells

B

Fig. 2. **Coculture fluid shear stress (FSS) model. A. Coculture setup.** Human coronary artery endothelial cells (HCAEC) and human coronary artery smooth muscle cells (HCASMC) were seeded on either side of the inserts to simulate artery structure. 4 μM pores in the transwell membrane allow communication between EC and SMC but not cell migration. Shear stress was applied with a polyethylene cone connected to a micromotor. **B. FSS wave form.** Micromotors were controlled by software to turn at certain rates to simulate the shear stress in collateral arteries diverted from a clogged artery with 60% stenosis[1,26].

Fig. 3

D

y-axis: Relative PHD-2 mRNA normalized to β-actin

data points with significance markers: ** at 0, * at 2, * at 6, ** at 8

x-axis: hours after FSS (0, 2, 4, 6, 8)

E

y-axis: Relative HIF-1α mRNA normalized to β-actin

significance: *** brackets

x-axis categories: ctr, siCTR, siHif-1α

legend: □ sta, ■ FSS
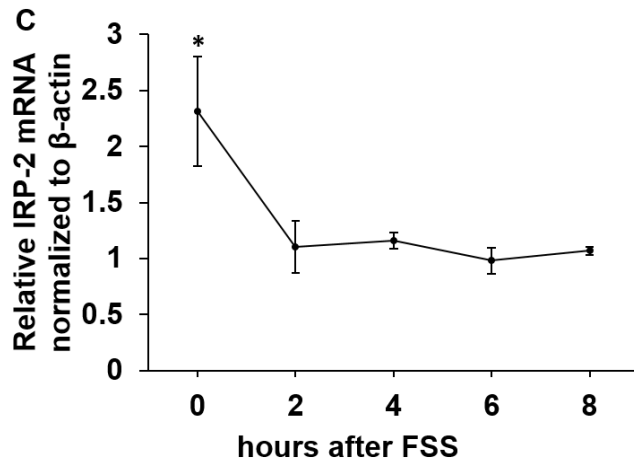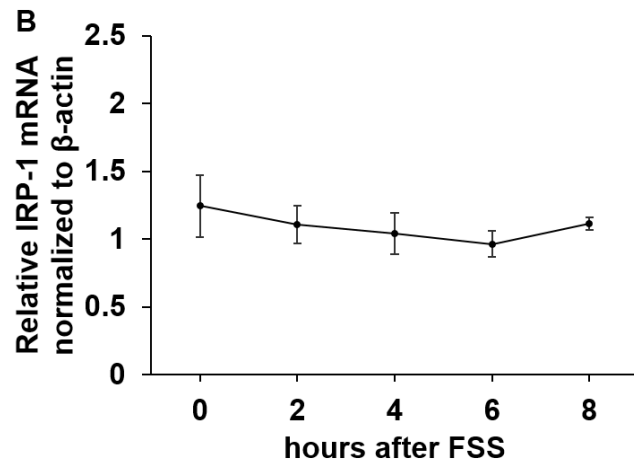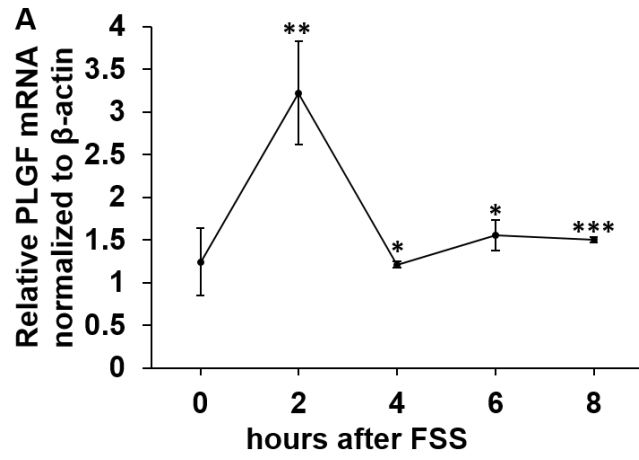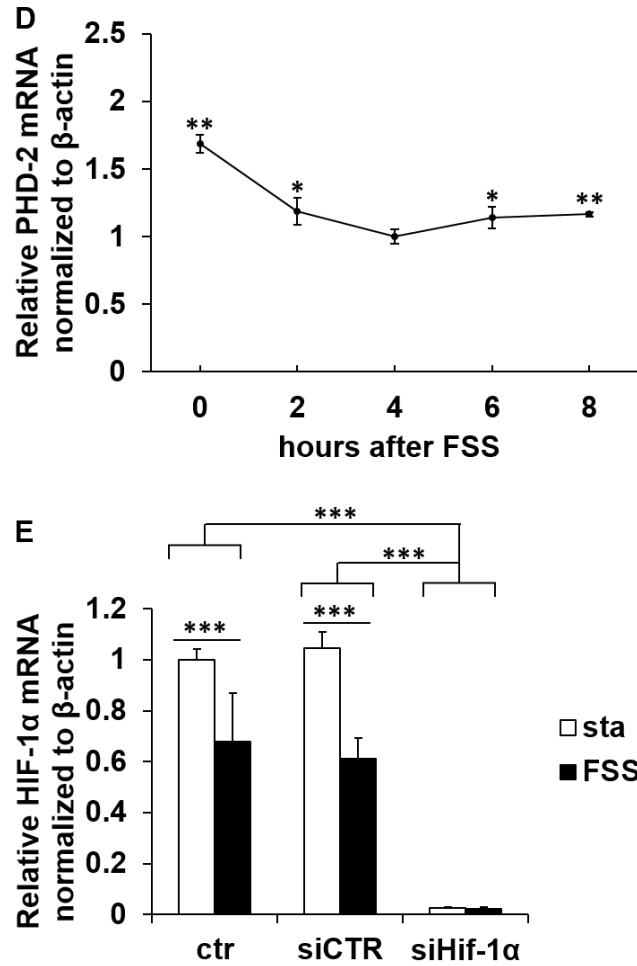
Fig. 3. mRNA levels of PLGF, IRP-1, IRP-2, PHD-2, and HIF-1α after FSS. A-D, asterisks indicate significant differences from static controls (N=3 or 4). E. HCAEC was treated with siHIF-1α before seeding to the inserts. Cocultures were exposed to FSS for 2 h. HCAEC cell lysis was collected 4 h after FSS.

Fig. 4
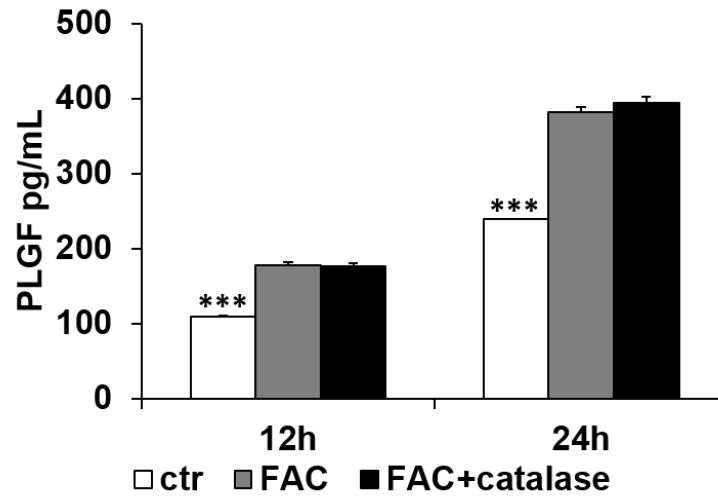
Fig. 4. Iron does not upregulate PLGF though the Fenton reaction. Cocultures were exposed to ferric ammonium citrate (FAC, 100 μg/mL) and catalase (500 U/mL). Media were sampled at 12 and 24 h. PLGF concentration was measured using ELISA. (N=4)

Fig. 5

A

B

C

D

E
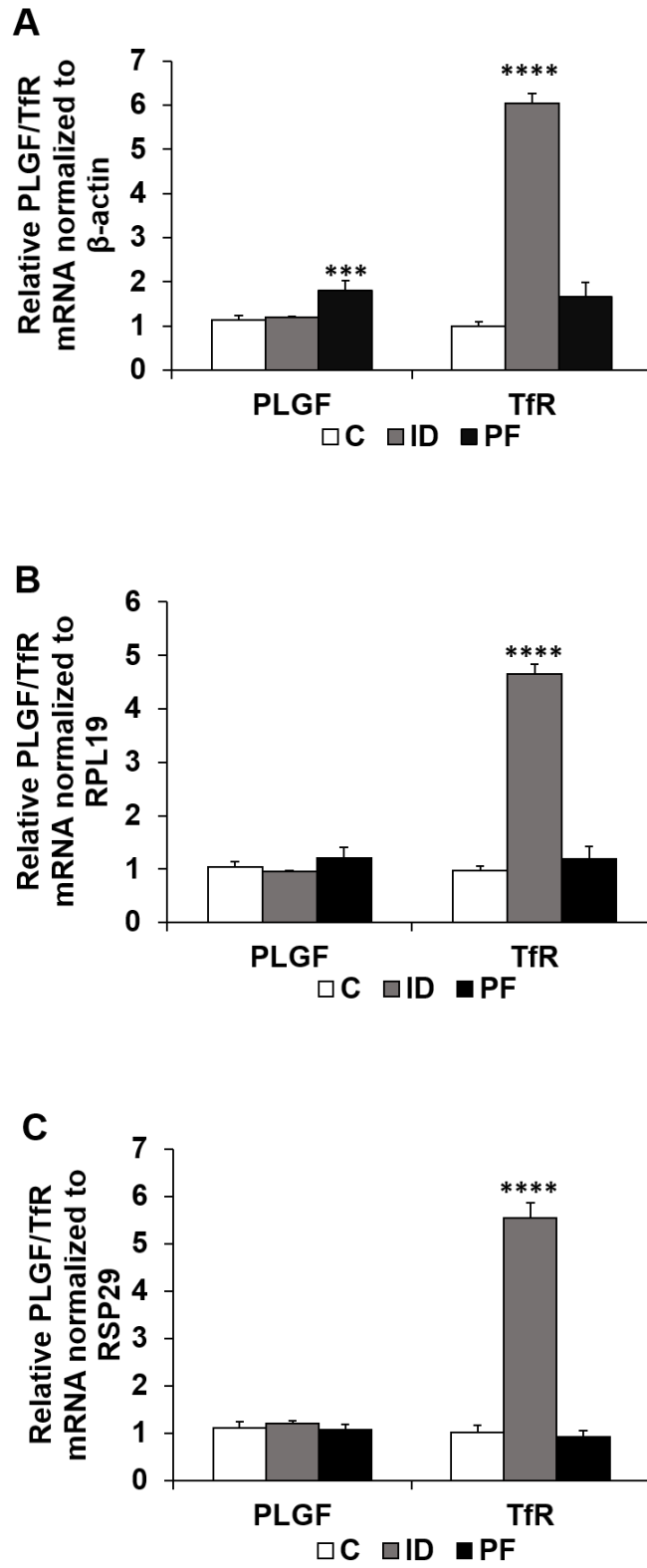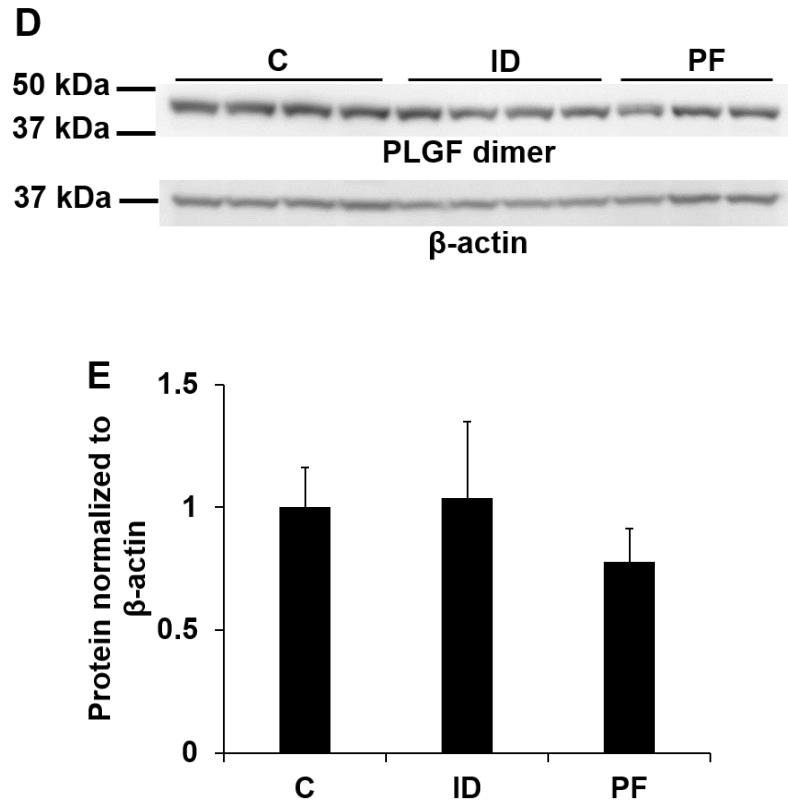
Fig. 5. Iron deficiency (ID) increases transferrin receptor (TfR) mRNA, but does not reduce PLGF mRNA in rat hearts. A, B, and C. TfR mRNA levels were upregulated by ID diet. Three internal controls (β-actin and ribosome proteins RPL19 and RSP29) were used to quantify TfR mRNA. Rats were fed the control (C), iron-deficient (ID), and pair-fed (PF) diets for 21 d. **D, E. The ID diet does not decrease PLGF protein in rat hearts.** PLGF protein was measured in tissue extracts by Western blotting. Since PLGF in samples was heavily dimerized, the dimer was used to quantify PLGF. (N=11, 12, and 7 for C, ID, and PF respectively.)

Fig. 6
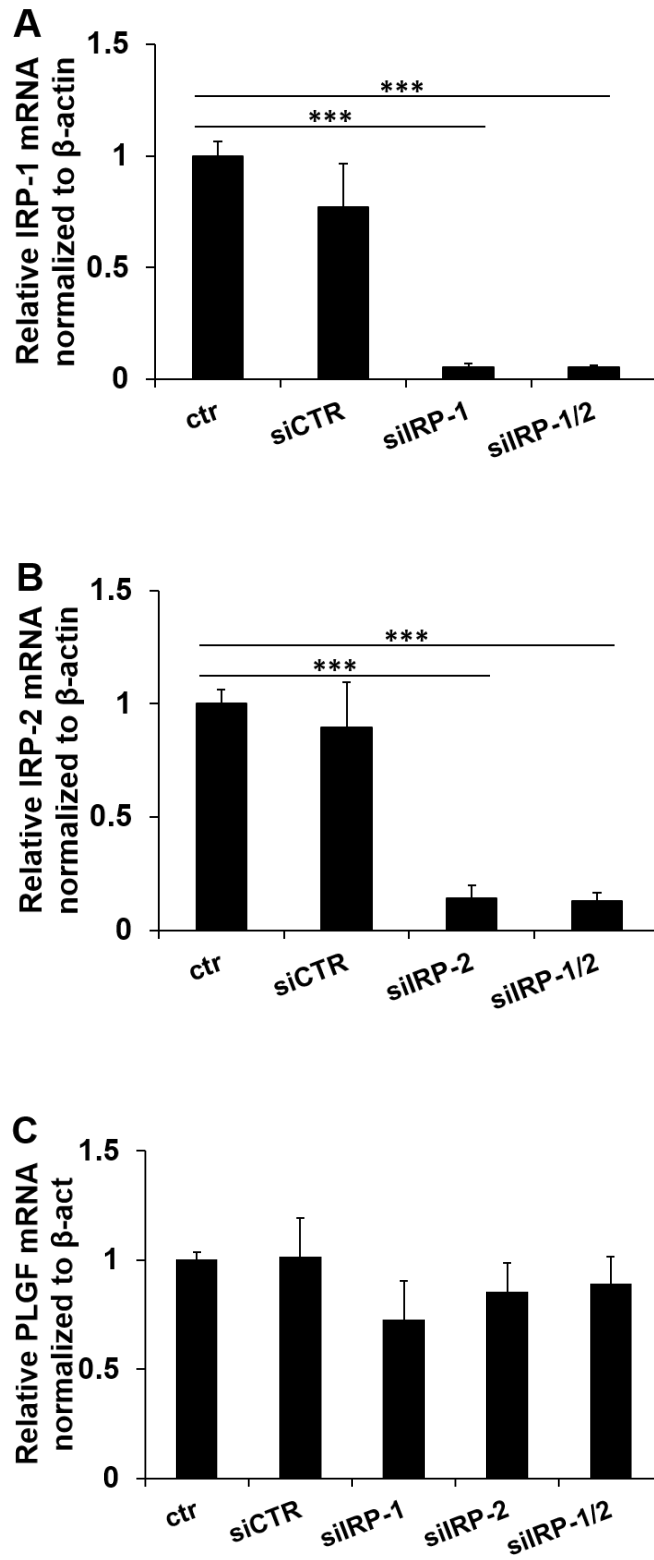
Fig. 6. PLGF transcription is not affected by iron responsive element binding protein (IRP) 1 and 2 knockdown. A, B. siRNA treatments successfully knocked down IRP-1 and IRP-2 mRNA. HCAEC was exposed to siRNA for 24 h, after which cell lysate was collected. **C. Knockdown of IRP-1 and/or IRP-2 does not affect transcription of PLGF.** The same samples shown in A and B were analyzed for PLGF mRNA levels (N=3).

Fig. 7
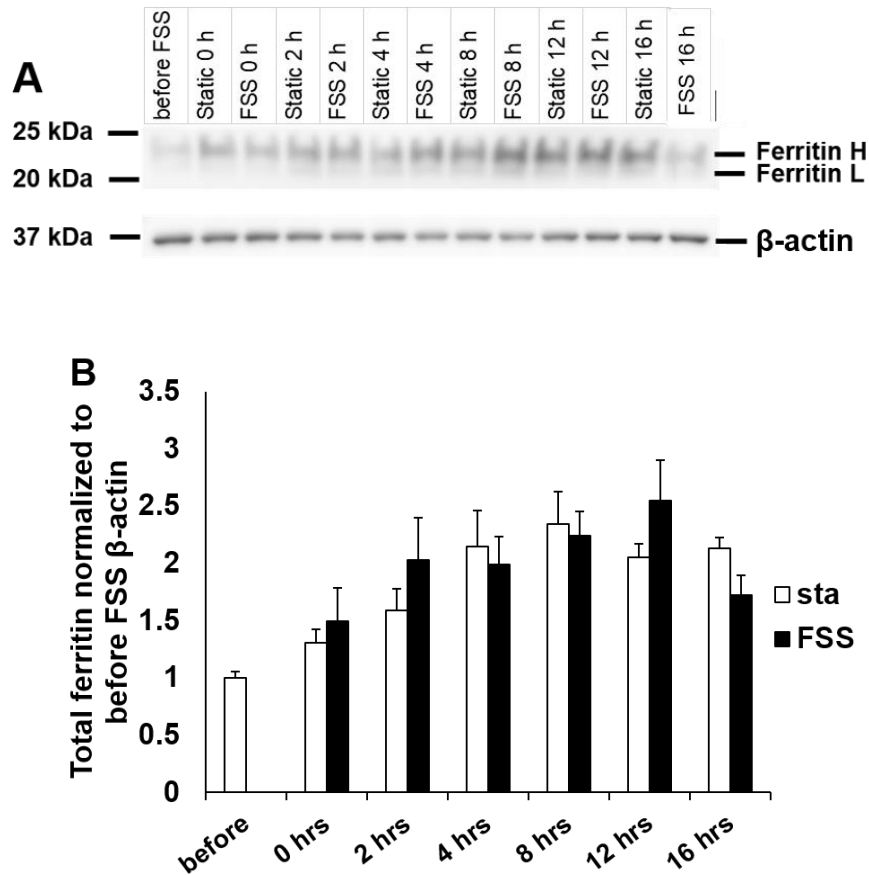
Fig. 7. Fluid shear stress (FSS) does not upregulate ferritin. Cocultres were exposed to 2 h of FSS. Ferritin levels in HCAEC were measured with Western Blot (WB). Ferritin heavy chain (ferritin H) and ferritin light chain (ferritin L) were both detected by the same antibody and their pixel densities were combined during analysis. **A. Typical image of ferritin WB.** Lanes: 1. before FSS, 2-13: post FSS/static exposure: 2. Static 0 h; 3. FSS 0 h; 4. Static 2h; 5. FSS 2h; 6. Static 4h; 7. FSS 4h; 8. Static 8 h; 9. FSS 8 h; 10. Static 12 h; 11. FSS 12h; 12. Static 16h; 13. FSS 16 h. **B. Pixel density analysis of ferritin WB.**

Fig. 8
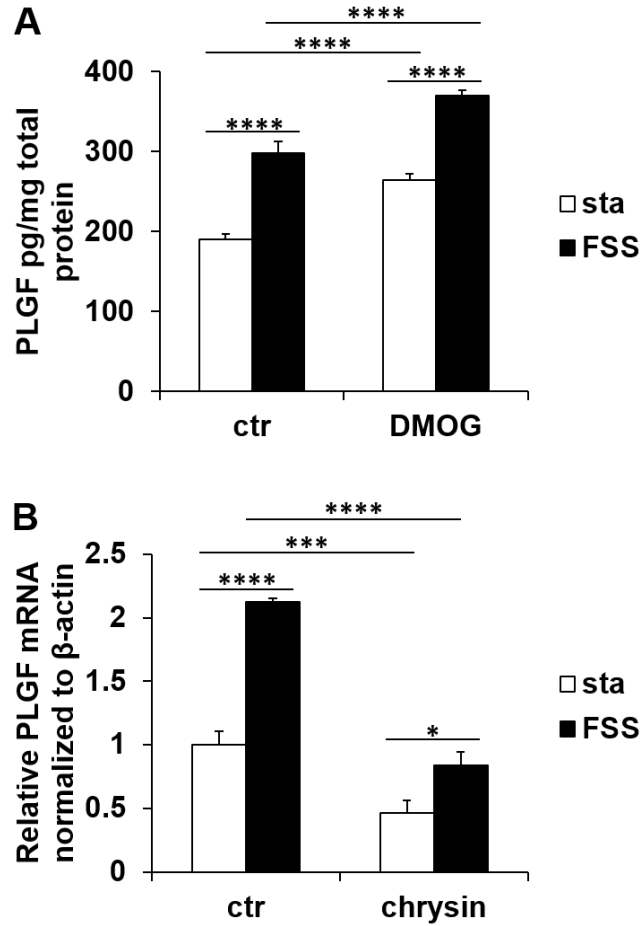
Fig. 8. PLGF upregulation by FSS is not HIF-dependent. A. Induction of HIF does not interrupt PLGF upregulation by FSS. DMOG (100 μM) was added to cocultures immediately before FSS exposure. HCAEC media were collected 12 h post FSS. PLGF in media was measure with ELISA and normalized to total protein (N=4). **B. Reduction of HIF does not interrupt PLGF upregulation by FSS.** Cocultures were treated with chrysin (30 μM) immediately before exposure to FSS. Cell lysis was collected 4 h post FSS. Chrysin was reported to decrease both HIF-1α mRNA and protein.
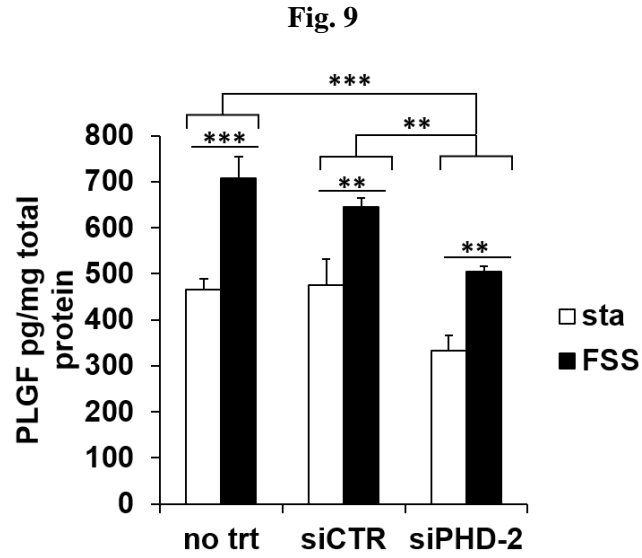
Fig. 9

Fig. 9. Knockdown of prolyl hydroxylase domain protein-2 (PHD-2) reduces basal levels of PLGF but does not affect PLGF upregulation by FSS. HCAEC were treated with siPHD-2 before seeding to inserts. Cell media was collected at 12 h after FSS. PLGF levels in media were analyzed with ELISA and normalized to total protein measured by BCA assay (N=4).

Fig. 10
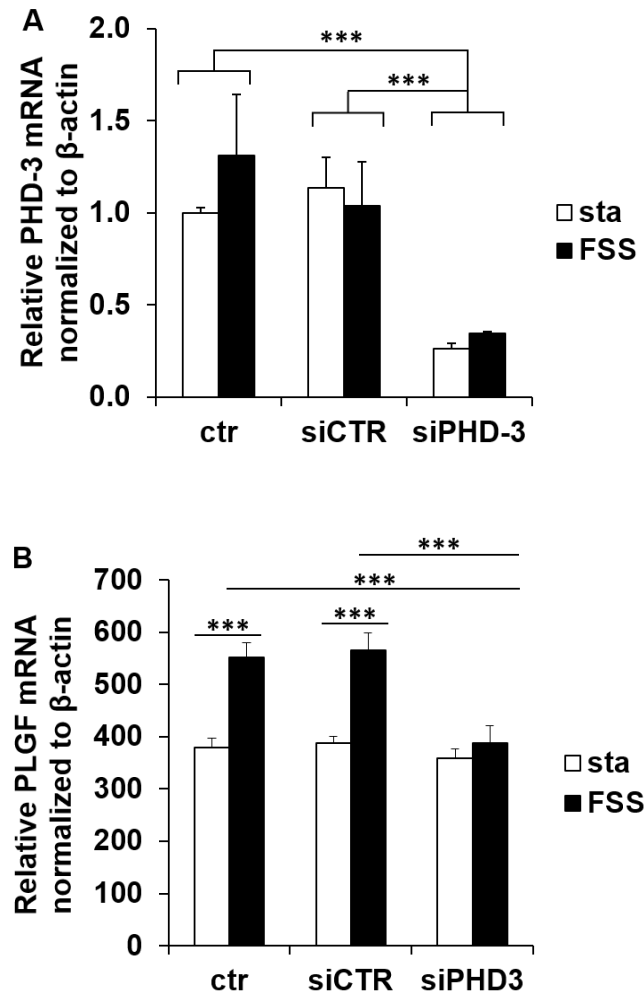
Fig. 10. Knockdown of prolyl hydroxylase domain protein-3 (PHD-3) abolishes PLGF upregulation by FSS. HCAEC were treated with siPHD-3 before seeding to inserts. Cell lysis was collected 4 h after FSS (N=4). **A.** siPHD-3 successfully knocked down PHD-3 mRNA. **B.** siPHD-3 abolished PLGF upregulation by FSS.

Fig. 11
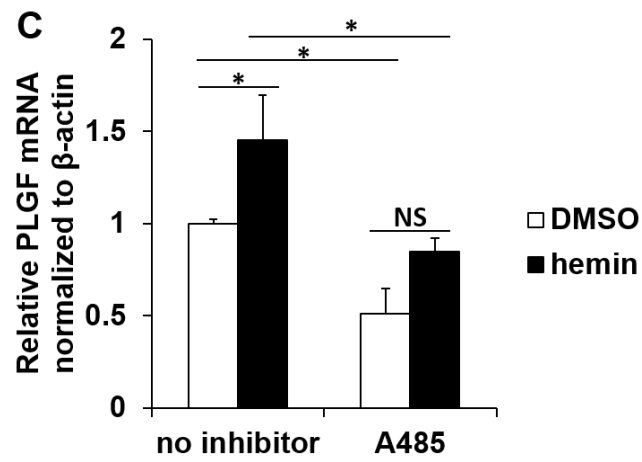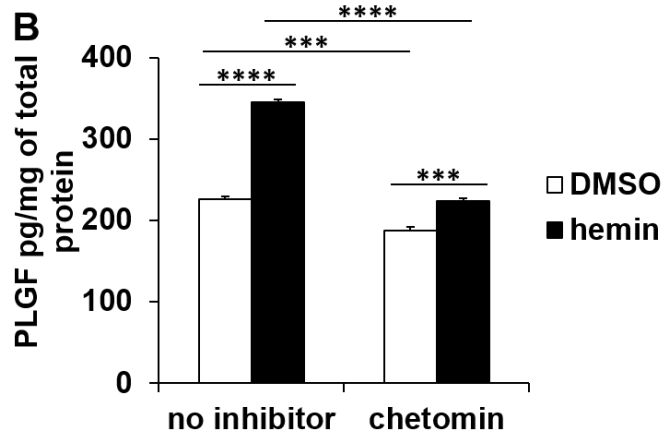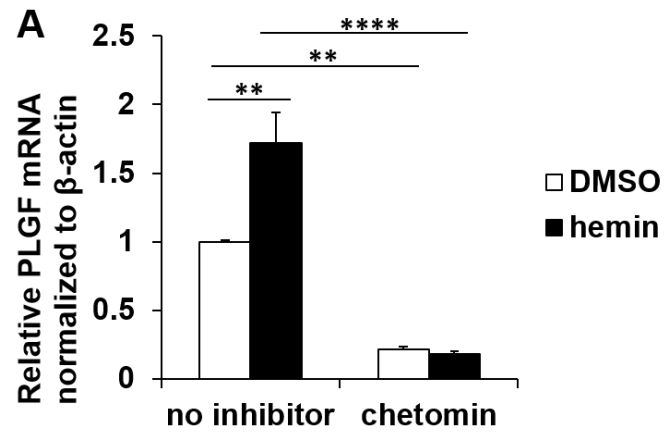
Fig. 11. PLGF upregulation by iron is p300-dependent. A. Chetomin abolished the upregulation of PLGF mRNA expression by hemin and sharply reduced basal PLGF mRNA levels. Inhibition of the interaction between the p300 CH1 domain and transcription factors by chetomin drastically reduced PLGF expression under basal levels and inhibited hemin-stimulated PLGF upregulation. Chetomin interrupts p300-transcription factor binding by a zinc ejection mechanism. (N=3)**. B. Chetomin inhibits PLGF protein upregulation by hemin.** HCAEC were exposed to hemin (10 µM) and/or chetomin (30 nM) for 12 h (N=3). **C. A485 reduced PLGF mRNA and inhibited its upregulation by hemin.** HCAEC was exposed to hemin (10 µM) and/or p300 HAT inhibitor A485 (20 nM) for 6 h (N=4).

Fig. 12
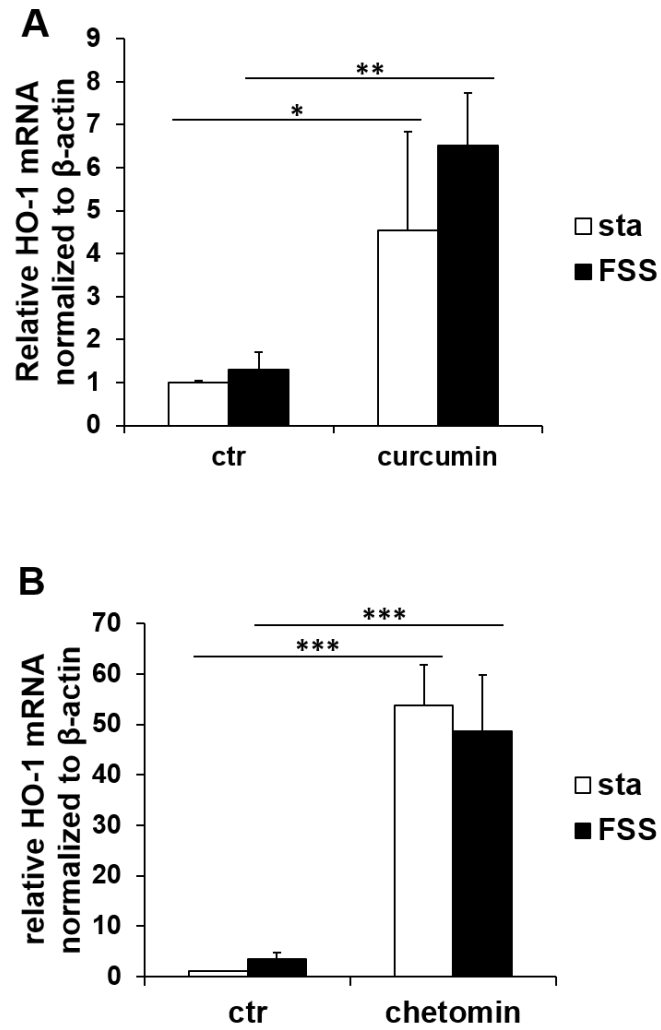

Fig. 12. Heme oxygenase-1 (HO-1) mRNA is sharply increased by treatment with p300 histone acetyl transferase (HAT) inhibitors. Cocultures were treated with curcumin (25 μM) and A485 (20 nM) immediately before exposure to FSS. Cell lysate was collected 4 h post FSS exposure. **(A. N=3; B. N=4)**

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CHAPTER III

FLUID SHEAR STRESS UPREGULATES PLACENTAL GROWTH FACTOR IN

ENDOTHELIAL CELLS VIA TRANSCRIPTION COFACTOR p300

Abstract

Coronary artery disease (CAD) is the leading cause of death worldwide[1]. In CAD, atherosclerosis leads to coronary artery stenosis (narrowing) and results in lack of blood flow to the affected tissue. When the artery becomes nearly or totally occluded, myocardial infarction (MI) occurs, potentially resulting in death. However, in CAD, vessel stenosis also diverts blood flow to surrounding smaller blood vessels, which make up the collateral network. These vessels represent an alternate pathway for blood flow which bypasses the stenosed or occluded coronary artery. Increased fluid shear stress (FSS) in the collateral network has been observed to induce a volume and diameter increase in these collateral blood vessels via endothelial cell (HCAEC) and smooth muscle cell (HCASMC) proliferation[2]. This process of vessel enlargement is called arteriogenesis. By maintaining blood flow to the myocardium, arteriogenesis is protective against the negative consequences of CAD[3-6]. Since current treatments for CAD (coronary artery bypass grafting, balloon angioplasty, stent implantation) are invasive and/or can fail over time, it would be beneficial to find non-invasive treatments to enhance arteriogenesis in human patients. Placental growth factor (PLGF), a member in the vascular endothelial growth factor (VEGF) family, is critical in arteriogenesis. In this study, we explored the mechanism of FSS upregulation of PLGF in HCAEC. Using a HCAEC-HCASMC coculture model to simulate FSS in small arteries, we discovered that p300, a transcription cofactor and histone acetyl transferase (HAT), plays a central role in PLGF upregulation by FSS. p300 is involved in regulation of many genes through acetylation of histones[7] and other regulatory proteins, including p53[8] and nuclear factor-2 (Nrf-2)[9]. We found that inhibition of p300 HAT activity abolished FSS-induced upregulation of PLGF, but did not decrease basal levels of PLGF. Conversely, activation of p300 HAT activity upregulated PLGF. We conclude that FSS upregulates PLGF via p300 HAT activity.

Keywords: collaterals, shear stress, p300, histone acetylation

Introduction

Coronary artery disease (CAD) is the leading cause of death worldwide[10]. CAD causes more morbidity and mortality in high income countries than lower income countries. However, a global shift of increasingly higher mortality caused by CAD has been observed and is predicted to continue[10]. This shift is largely associated to lifestyle changes and obesity[11,12]. Common clinical interventions for coronary artery occlusion caused by CAD usually involve coronary artery bypass grafting (CABG) or percutaneous coronary intervention (PCI), stent placement[13]. CABG is a major surgical procedure which requires the chest to be opened. While stent placement is less invasive, restenosis is common and the vessel often requires further intervention[14]. Moreover, the effectiveness of stenting has recently been called into question, in a controversial placebo-controlled study of the effect of stent placement on chest pain and exercise time[15]. Moveover, CABG and PCI surgeries cost more than $20,000 and $30,000 respectively. At 5 years, the accumulated costs are $56,000 to almost $61,000, as reviewed in 2010[16]. It will be vastly beneficial for patients and greatly reduce the financial burden of treatment if effective non-invasive treatments for CAD can be developed.

The existence of collateral coronary circulation in healthy human hearts had long been disputed since the first observation made in the 1700s[17,18]. In 1963[19], Fulton reported coronary anastomoses were ubiquitous in both healthy and pathological human hearts. More importantly, the collateral coronary circulation was shown to be functional in patients without CAD[20]. Arteriogenesis, the complex remodeling and enlargement of pre-existing collateral vessels upon occlusion, has been shown to reduce major cardiac events[21] and to improve survival and prognosis after percutaneous intervention[22,23]. Although the human coronary collateral network was previously considered to be relatively limited compared to many other mammals such as hamster, dog, and cat[24], recent studies with improved technology suggest that human coronary collateral vessels are also abundant[20,25]. Moreover, a study with 210 patients showed that as little

as 24% fractional collateral flow was sufficient to prevent ischemia[26]. Arteriogenesis is induced

by fluid shear stress (FSS) as shown in many animal models ranging from canine to primate[27-31].

Similarly, in human, the extensiveness of collateral vessels is correlated with the severity of

CAD[32,33], which is determined by reduction of the diameter of coronary arteries[34]. Exercise is

well known to be beneficial for cardiac function including increasing stroke volume, artery

caliber, and myocardial contractility[35,36]. Exercise can also induce collateral growth as observed

in animal models[37-39], as well as in human. A clinical trial conducted in 2016 reported 4 weeks of

high-intensity or moderate-intensity exercise in patients with stable CAD increased coronary

collateral flow index (CFI) by ~40%[40]. Marathon runners were known to have more developed

collateral circulation[41]. These studies and observations confirm FSS as the driving force of

arteriogenesis.

Placental growth factor (PLGF) has been shown by many studies to be a critical factor in the

regulation of arteriogenesis[42-44]. Our lab was the first one to demonstrate that PLGF is mainly

produced by endothelial cells as a secretory protein[45]. Furthermore, our group was the first to

show that PLGF is upregulated by FSS[44,46]. Using a coculture model and *ex vivo* experiments with

mouse mesenteric arterioles, our lab has shown that FSS upregulates PLGF in human coronary

artery endothelial cells (HCAEC), and that this process is NADPH oxidase-4 (NOX-4)

dependent[46]. However, further details of the pathway by which FSS upregulates PLGF remain to

be discovered.

In this study, using the coculture FSS model previously established in our lab, we

demonstrate that FSS induces PLGF expression in HCAEC via a p300-dependent pathway.

Initially, we hypothesized that PLGF regulation in HCAEC by hypoxia and FSS would be

mediated by hypoxia inducible factor (HIF); however, we found that HIF plays no role in this

pathway (an unexpected finding considering that HIF is a well-recognized angiogenic

transcription factor). Once HIF was ruled out as the effector of PLGF upregulation by FSS, our

attention was drawn to p300, a transcription cofactor that numerous transcription factors (including HIF) interact with closely for many genes' regulation. Through its histone acetyltransferase activity, which increases histone acetylation at the promoter region of target genes, p300 has been implicated as an effector in the upregulation of numerous genes, including vascular endothelial growth factor (VEGF) expression under hypoxia[47], and FSS activation of eNOS[48]. Acetyl groups add negative charge to the histone, thereby decrease interaction between the histone and DNA. Using p300 specific HAT inhibitors (curcumin[49] and A485[50]), and a specific p300 HAT activator (CTB[51]), we were able to show that FSS upregulates PLGF by increasing PLGF gene transcription via p300 HAT activity, as illustrated in **Fig. 1**. Epigenetic modifications have been recognized as a dynamic process involved in both short-term and long-term gene expression regulations. Our group is the first to show that p300 is important in PLGF regulation in endothelial cells, which signifies epigenetics as a new aspect of arteriogenesis research.

Materials and methods

Cell culture. Passage 6 (P6) human coronary artery endothelial cells (HCAEC, Lonza) and human coronary artery smooth muscle cells (HCASMC, Lonza) were used in all experiments. The cells were cultured in cell type-specific media (Smooth Muscle Growth Medium-2/SmGM-2 or Microvascular Endothelial Cell Growth Medium-2/EGM-2MV, Lonza) for 3 d to reach confluence before subculturing into plates for experiments. TrypLE Express (Invitrogen) was used to disperse cells for subculturing according to manufacturer's protocol. The cells were seeded on opposite sides of polyester inserts with 0.4 μm pores in a 6-well plate format (Transwell, Corning) to mimic the vascular wall. This allows the two types of cells to come into contact and to exchange signaling mediators, but prevents cell migration and allows gene and protein expression in the two cell types to be analyzed separately. Before seeding, the side to be seeded was coated with 0.1% gelatin in cell type-specific medium and incubated for 1 hr in a cell culture incubator (37°C, 5% CO_2). The gelatin coating medium was then aspirated and the inserts were allowed to dry. The inserts were inverted and HCASMC were seeded on the reverse side of the insert at 10,000 cells/cm^2. Inserts were then incubated in the inverted position for 4-6 h to allow attachment, before being placed right side up into a receiving well containing 2 mL of SmGM-2 and incubated overnight. The following day, HCAEC were seeded on the top side of the insert at 25,000 cells/cm^2 in EGM-2MV. HCASMC were also fed with fresh SmGM-2 at this time. The co-cultures were then incubated at 37°C, 5% CO_2 for a further 24 h. Prior to experimental treatments, cells were serum-restricted for 18 h by incubation with 2% FBS media supplemented with 15 mM HEPES, 30 μg/mL gentamycin, and 15 ng/mL amphotericin-b (Lonza). Serum-restriction medium was prepared as a 3:2 mixture of serum-free DMEM and cell type-specific medium).

Fluid shear stress setup. Fluid shear stress (FSS) was applied to co-cultures with motor-attached (Optimal Engineering Systems, Inc.) cones made of ultra-high molecular weight

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polyethylene with a 5˚ incline towards the center as described previously by our group and others[46,52,53] (**Fig. 2. A**). The motors were controlled by specialized software, Allegra (Optimal Engineering Systems, Inc.) to turn at different rates simulating pulsatile blood flow. For these studies, we used a waveform that simulates collateral flow downstream of a coronary artery with 60% stenosis (average FSS 1.24 Pa, **Fig. 2. B**), which is considered clinically critical, as described in previous publications[46,54]. Only HCAEC on the upper side of inserts were directly exposed to FSS, whereas HCASMC were not directly exposed to FSS but were accessible to diffusible mediators produced by HCAEC in response to FSS. The FSS stimulus was applied to the cocultures for 2 h. During FSS exposure, the Transwell plates were kept on plate heaters to maintain cell cultures at 37 ˚C. Media and/or cell lysate was collected 0-24 h after the 2 h-shear stress exposure. The static inserts were covered with parafilm to prevent excessive evaporation and was placed next to FSS exposure inserts for the same duration of treatments as published by our group previously[46].

Experimental treatments. Unless otherwise specified, all experimental treatments were added to both the upper (HCAEC) and lower (HCASMC) media reservoirs of the Transwell plate immediately before FSS exposure. Control groups were treated with the same volume of the vehicle. Experimental treatments included actinomycin D (Cayman Chemical), chetomin (Sigma-Aldrich), dimethyloxaloylglycine (DMOG; Cayman Chemical), chrysin (Sigma-Aldrich), deferoxamine (DFX; Sigma-Aldrich), curcumin (Tocris), and A485 (Tocris).

siRNA treatment. Pre-designed, lyophilized siRNA (Silencer Select) was purchased from Thermo Fisher and reconstituted with RNAase-free H_2O before being stored at -20 ˚C. For transfection, a siRNA mixture was made with Opti-MEM and Lipofectamine RNAiMAX following the manufacturer's instructions. The siRNA mixture was combined with normal culture media and added to fresh cell culture wells/flasks to reach a final siRNA concentration of 5 nM. The cells to be treated were then trypsinized, re-suspended in media, and added to the wells/flasks

containing the siRNA mixture. Cells were incubated with the siRNA mixture for 24 h. Knockdown efficacy was determined using real-time qRT-PCR as described below. For siRNA treated HCAEC to be used in shear stress experiments, HCAEC were seeded on the inserts immediately after siRNA treatment and incubated with 2% FBS medium for 18 h before FSS exposure.

Hypoxia treatment. For hypoxia studies, HCAEC were seeded in 12-well plates immediately after siRNA treatment. Cells were allowed to attach overnight. A hypoxia chamber (Stem Cell Technologies) was flushed and filled with 1% O_2, 5% CO_2, and 94% N_2 after the 12 well plates were placed inside. The hypoxia chamber was then placed in a cell culture incubator for 18 h before media were collected.

PLGF mRNA half-life. For assessment of PLGF mRNA half-life, HCAEC were seeded in 12-well plates and allowed to reach confluency. Actinomycin D (10 μg/mL) was added to the media to inhibit transcription. Cell lysate was collected at 0, 1, 3, 6, and 9 h post-actinomycin D treatment. Real-time qRT-PCR was used to quantify PLGF mRNA as described below. Half-life was calculated as follows: $t_{\frac{1}{2}} = \frac{\ln(2)}{\lambda}$. λ represents the decay constant.

Real-time qRT-PCR. RNA extraction was performed with the RNeasy Mini Kit (Qiagen). At the conclusion of experiments, cells were trypsinized with TrypLE (Gibco) and the resulting cell suspension was centrifuged at 5,000 RPM for 5 min to form a pellet. The pellet was lysed with buffer RLT from the RNeasy Mini kit, then stored at -80 °C until RNA was extracted following the manufacturer's instructions. The RNA concentration was determined using a Synergy Plate Reader (Biotek) and Take3 Micro-Volume Plate (Biotek). For cDNA synthesis, reverse transcription was done within an hour after RNA extraction using the QuantiTect Reverse Transcription Kit (Qiagen). The remaining RNA was stored at -80 °C and cDNA was stored at 4 °C. For real-time qPCR, 5 or 10 ng of cDNA was used per reaction. A master mix was made

using PerfeCTa SYBR Green FastMix, Low ROX (Quanta Biosystems) and 50 nM primers

(Invitrogen). Real-time qPCR was performed in an ABI 7500 Fast instrument (Applied

Biosystems). Relative mRNA quantification was normalized to β-actin as a reference, then

calculated as $2^{-\Delta\Delta Ct}$ of control samples. **Table 1** shows the primer sequences used.

Species	Target		Sequence 5'-3'
Homo sapiens	*β-actin*	Forward	TGCCGACAGGATGCAGAAG
		Reverse	CTCAGGAGGAGCAATGATCTTGAT
Homo sapiens	*PLGF*	Forward	CCTACGTGGAGCTGACGTTCT
		Reverse	TCCTTTCCGGCTTCA TCTTCT
Homo sapiens	*HIF-1α*	Forward	TCATCCATGTGACCATGAGGAA
		Reverse	GAGAAAAAAGCTTCGCTGTGTGT
Homo sapiens	*HIF-2α*	Forward	CCCTGGATAGCAAGACCTTCCT
		Reverse	CTCCTCAGGGTGGTAACCAATC

Table. 1. Primer sequences

ELISA. Media were collected at multiple time points after FSS exposure and treated with a

cocktail of protease inhibitors (1 mM PMSF, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM

benzamidine-HCl, 1 μg/mL aprotinin, 1 μg/mL pepstatin A) before the samples were stored at -80

˚C. For cell lysis, cell pellets were collected as described above, then lysed with RIPA buffer

supplemented with the same cocktail of protease inhibitors. Both PLGF and HIF-1α ELISA were

performed using the respective DuoSet ELISA kit following manufacturer's instructions (R&D

Systems). The concentration of PLGF was normalized to total protein as measured by the BCA

assay (Pierce).

Results

FSS upregulates both PLGF mRNA and protein. To assess the effect of FSS on PLGF regulation in HCAEC, the cocultures were exposed to 2 h of FSS at an average intensity of 1.24 Pa to simulate flow in collaterals downstream of an artery with 60% stenosis, as described by previous publications[46,54]. After FSS exposure, the cocultures were returned to the cell culture incubator. Media (for PLGF protein analysis) and cell lysate (for PLGF mRNA analysis) was then collected at several time points post-FSS exposure. Real-time qRT-PCR results showed that at 4 h post FSS, PLGF mRNA was upregulated to ~1.4-fold of the level in static controls (1.67±0.27, $p < 0.05$, N=4, **Fig. 3. A**). Likewise, PLGF protein in HCAEC media was upregulated to ~1.4-fold of the static control value at both 12 and 24 h post-FSS (12 h, static 359.83±16.71 pg/mg, FSS 501.00±43.01 pg/mg, $p < 0.05$; 24 h, static 511.03±17.99 pg/mg, FSS 680.05±54.42 pg/mg, $p < 0.05$, N=4. **Fig. 3. B**). These results confirm our lab's previous observation that FSS upregulates PLGF in HCAEC[55].

Actinomycin D inhibits PLGF upregulation by FSS. To determine whether the increase in PLGF mRNA after FSS was due to increased transcription, or to increased mRNA half-life (as observed by our lab's previous work in HCASMC48), the PLGF mRNA half-life was measured. Actinomycin D was added to HCAEC monocultures to abolish transcription. Samples of cell lysate were then collected at several time points and PLGF mRNA levels were assessed with qRT-PCR. Based on these data, we calculated that the half-life of PLGF mRNA in HCAEC is 1.67 h (**Fig. 4. A**). In contrast, β-actin mRNA levels did not decrease after actinomycin D treatment at the time points analyzed (**Fig. 4. C**), while total RNA concentration was reduced by actinomycin D in a time-dependent manner (**Fig. 4. B**). This suggests β-actin as a good reference gene in HCAEC. When transcription was reduced to ~0.1-fold with actinomycin D in cocultures, PLGF mRNA levels were greatly reduced (to ~10%), and exposure of the cocultures to FSS did

not upregulate PLGF mRNA (**Fig. 4. D**). These results indicate that FSS upregulates PLGF

mRNA in HCAEC by increasing transcription.

PLGF upregulation by FSS is not HIF-dependent. We next tested the effect of chetomin,

another small molecule HIF inhibitor. Chetomin, a fungal secondary metabolite produced by

Chaetomium cochliodes[56], has been shown to be a specific and potent HIF inhibitor which acts by

interrupting the interaction between HIF and its transcription cofactor p300[57,58]. Cocultures were

treated with 30 nM of chetomin immediately before exposure to FSS. In contrast to the chrysin

results, chetomin reduced PLGF under both static and FSS conditions (**Fig. 5. A**). Therefore, our

results using small molecule activators and inhibitors of HIF were not entirely conclusive.

Furthermore, although DMOG and chrysin are commonly used to manipulate HIF, they have

been known to have off-target effects[59-62].

To more specifically assess the involvement of HIF in FSS upregulation of PLGF, we used

siRNA to knock down (KD) HIF-1α and HIF-2α in HCAEC before FSS exposure. KD was

successful at reducing both mRNA (<10% remaining) and protein levels (lower than detection

threshold after DMOG treatment) as measured by real-time qPCR (**Fig. 5. B and C**) and ELISA

(**Fig. 5. D**) respectively, whereas a control siRNA had no effect on HIF levels (**Fig. 5. E. and 5.

F**). In agreement with our results from the DMOG and chrysin experiments above, KD of HIF-

1α, HIF-2α, or both did not affect PLGF upregulation by FSS (**Fig. 5. E and F**). We therefore

concluded that PLGF upregulation by FSS is not HIF-1α or HIF-2α-dependent.

PLGF upregulation by hypoxia is not HIF-dependent. Our group has previously shown that

PLGF is upregulated by hypoxia in HCAEC[45]. After determining that PLGF upregulation by FSS

is not dependent on HIF-1α/HIF-2α KD, we questioned whether PLGF upregulation by hypoxia

in HCAEC is HIF-dependent. After KD of HIF-1α/HIF-2α by siRNA, HCAEC monocultures

were exposed to either hypoxia (1% O_2), 100 μM of DMOG, or 100 μg/mL of deferoxamine

(DFX) for 18 h, followed by media collection. Similar to our previously reported results, hypoxia increased PLGF. Surprisingly, HIF knockdown had opposite effects than expected. Under normoxic conditions, KD of either HIF-1α or both HIF-1α and HIF-2α increased PLGF protein. Under hypoxic conditions, the effect of the knockdown on PLGF was even further increased (to 6.89 and 6.93-fold of control) (**Fig. 6. A**). DMOG and DFX treatments showed similar effects for both PLGF mRNA and protein, which were both greatly increased by HIF-1α KD, but not affected by HIF-2α KD alone (**Fig. 6. B**). These results indicate that neither HIF-1α nor HIF-2α are involved in PLGF regulation by hypoxia in HCAEC. However, as we observed with FSS treatment, chetomin reduced PLGF under both normoxia and hypoxia conditions (**Fig. 6. C**). Curcumin, a specific inhibitor to p300 HAT activity, also reduced PLGF under both normoxia and hypoxia conditions (**Fig. 6. C**).

FSS increases PLGF transcription via transcription cofactor p300. As discussed above, our experiments with small molecule HIF activators/inhibitors were not entirely consistent, but further experiments with siRNA conclusively ruled out a role for HIF in upregulation of PLGF by either FSS or hypoxia. Therefore, the ability of chetomin to abolish upregulation of PLGF by FSS and hypoxia drew our attention to the transcription cofactor and histone acetyl transferase (HAT) p300[7]. To determine whether p300 HAT activity is involved in FSS-induced upregulation of PLGF, we treated cocultures with the p300 HAT-specific inhibitors curcumin and A485 prior to FSS exposure. Inhibition of p300 HAT by curcumin and A485 abolished PLGF upregulation by FSS, but did not affect the basal PLGF level (**Fig. 7. A and B**). To assess if p300 activation could induce PLGF, we then treated HCAEC with a p300 HAT activator, N-(4-Chloro-3-trifluoromethyl-phenyl)-2-ethoxy-benzamide (CTB). CTB upregulated PLGF at both 20 and 40 μM (**Fig. 7. C**). Altogether, these results strongly suggest that FSS upregulates PLGF through p300 HAT.

Nuclear factor κ B (NFκB) is involved in PLGF regulation. NFκB p65 has been shown to interact with PLGF promoter in human embryonic kidney cell line HEK293[63]. Furthermore, p65 has been implicated in upregulation of two important FSS-induced proteins in endothelial cells by interacting with the shear stress response element (SSRE): endothelial nitric oxide synthase (eNOS)[55], and platelet derived growth factor (PDGF)[64]. To determine the involvement of p65 in PLGF regulation, we treated HCAEC with TPCA-1[65,66], an inhibitor to inhibitor of IκB kinase-α (IKK-α) and IKK-β. IKK-α and IKK-β phosphorylase inhibitor of κB (IκB). Upon phosphorylation, IκB undergoes ubiquitination and releases NFκB subunits p65 and p50 allowing nuclear transportation. By inhibiting IKK-α or IKK-β phosphorylation, NFκB activity is inhibited.

TPCA-1 reduced PLGF protein in HCAEC media in a dose-responsive manner at 18 h of treatment (r^2=0.687, p<0.001, N=4), suggesting p65 is involved in PLGF regulation (**Fig. 8**).

We have also tried siRNA against p65, but both batches of siRNA sent by the manufacture (Fisher) did not succeed in p65 mRNA KD. Further experiments to specifically knock down p65 will provide meaningful insight in involvement of p65 in PLGF regulation.

Discussion

Although PLGF has been recognized as an important arteriogenesis inducing factor[43,67], it has been studied much less by researchers compared to VEGF-A. On Google Scholar, a search for "PLGF" yields 29,700 results, while "VEGF" yields 1,630,000 results. Unlike angiogenesis (capillary growth), which is mainly driven by hypoxia, arteriogenesis is driven by fluid shear stress (FSS) produced by blood moving through collaterals after being diverted from clogged major arteries[68]. It is worth noting that the collaterals that are receiving this diverted blood from blocked arteries are not hypoxic themselves. Therefore, we surmised that FSS would be a key stimulus for PLGF expression. Our previously published study confirmed this hypothesis by showing that PLGF is upregulated by FSS in human coronary artery endothelial cells (HCAEC) through a NADPH oxidase-4 (NOX4)-dependent mechanism[46]. However, the other specific components of this signaling pathway remain unclear.

In this study, using the coculture-FSS model with HCAEC and human coronary artery smooth muscle cells (HCASMC), we discovered that FSS upregulates PLGF through transcription cofactor p300. This finding is in agreement with studies that have shown that p300 is activated by FSS[48,69] and is important for FSS upregulation of endothelial nitric oxide synthase (eNOS)[48]. One mechanism by which p300 facilitates transcription is through its histone acetyl transferase (HAT) activity. Histone acetylation and deacetylation are highly dynamic processes. On average, the acetylation sites regulated by p300 have a turnover rate of less than 30 min[70]. This could explain the rapid increase in PLGF mRNA after FSS which has been observed by our lab. Increased PLGF mRNA levels are observed immediately after FSS exposure, and peak at 4 h post FSS. The increase of PLGF protein in media is observed at 8 h post FSS, and peaks at 24 h post FSS[46]. Mass spectrometry or Western Blot analysis of histone acetylation using acetylation specific antibodies could further confirm the role of p300 HAT activity in PLGF upregulation by

FSS. p300 is known to acetylate many lysine residues of histones: H2AK5, H2BK5, H2BK12, H2BK15, H2BK20, H3K14, H3K18, H3K23, H4K5, H4K8, and H4K12[71], and H3K56[72].

P300 also interacts with numerous transcription factors to regulate gene transcription. Chetomin treatment blocked the effect of FSS on PLGF mRNA and also decreased PLGF levels under static conditions, suggesting that a transcription factor interacting with p300 is important for PLGF expression. Chetomin inhibits p300 cysteine histidine rich domain-1 (CH1) by "stealing" the zinc atoms important for protein-protein interaction[57]. We initially hypothesized that hypoxia inducible factor (HIF) was involved in FSS upregulation of PLGF. Many angiogenic growth factors are regulated by hypoxia, including PLGF[45,73]. Surprisingly, knockdown (KD) of HIF-1α and/or HIF-2α did not affect PLGF expression under static or FSS conditions. Even more surprisingly, HIF KD had no effect on PLGF under hypoxia or hypoxia mimicking conditions (DMOG and DFX). Rather than inhibiting PLGF, KD of HIF-1α increased PLGF protein by ~2-fold under both normoxic and hypoxic conditions. These results indicate that neither HIF-1α nor HIF-2α is the transcription factor regulating PLGF gene expression under conditions of FSS or hypoxia.

Aside from HIF, p300 interacts with many other transcription factors at its CH1 domain[74]. Among these, nuclear factor κB (NFκB) subunit p65[75] appears to be a likely candidate for regulating PLGF expression. HIF-1α and p65 have been shown to compete for p300 binding[76]. This competition could explain the upregulation of PLGF which we observed following HIF-1α KD, by allowing more p65 to bind to p300. p65 is known to be involved in endothelial nitric oxide synthase (eNOS)[55] and platelet derived growth factor (PDGF)[64] upregulation by FSS. In these studies, p65 was shown to interact with the shear stress responsive element (SSRE; sequence 5'-3' GAGACC) found in the promoter regions of eNOS and PDGF. p65 is also known to be involved in PLGF transcription under hypoxic conditions[63]. We identified two SSREs in the PLGF promoter region, located at positions 3116-3122 and 4431-4437. In future studies,

chromatin immunoprecipitation (ChIP) assay or electrophoretic mobility shift assay (EMSA) can be used to study the interaction between p65 and the PLGF SSRE after exposure to FSS.

Our results show that FSS upregulates PLGF transcription by increasing p300 activity. The mechanism by which FSS activates p300 in our system remains to be determined. However, p300 phosphorylation at Ser1834 by Akt is known to increases both its interaction with p65[77] and its HAT activity[78]. Furthermore, FSS upregulates p300 phosphorylation via Akt[79], and FSS has been shown to upregulate PLGF via Akt in human syncytiotrophoblasts[80]. Therefore, FSS-Akt-p300 is a promising possible pathway for PLGF upregulation that can be explored in future studies.

In conclusion, this study showed that p300 and NFκB as direct effectors of FSS-mediated PLGF regulation. Further investigation of this pathway has the potential to lead to development of non-invasive treatments for stenosis by identifying novel pharmaceutical targets.

Fig. 1
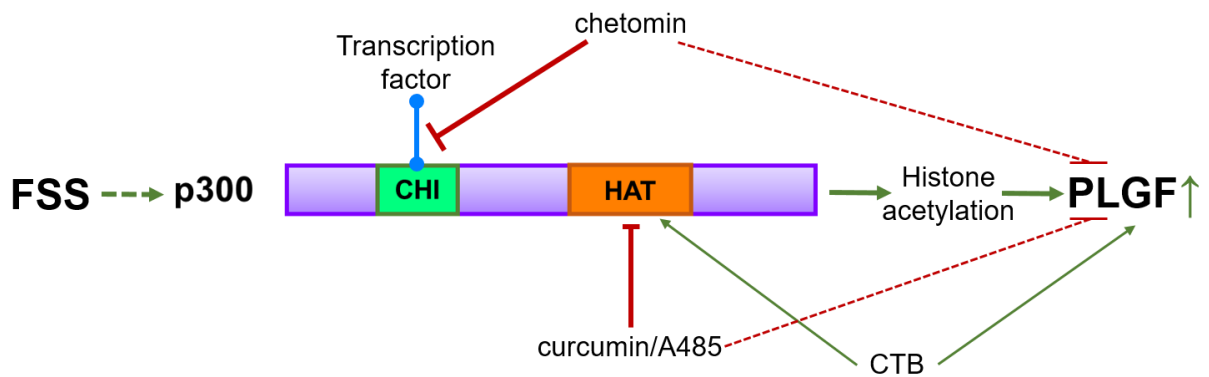
Fig. 1. Proposed pathway of placental growth factor (PLGF) upregulation by fluid shear stress (FSS) through transcription cofactor p300. FSS activates p300, which interacts with a transcription factor to increases histone acetylation at PLGF's promoter region. Chetomin inhibits p300 cysteine/histidine rich domain-1 (CH1) interaction with transcription factors by removing zinc ions located at CH1[81]. Curcumin and A485 are p300 HAT inhibitors[50,82]. CTB is a p300 HAT activator[51].

Fig. 2
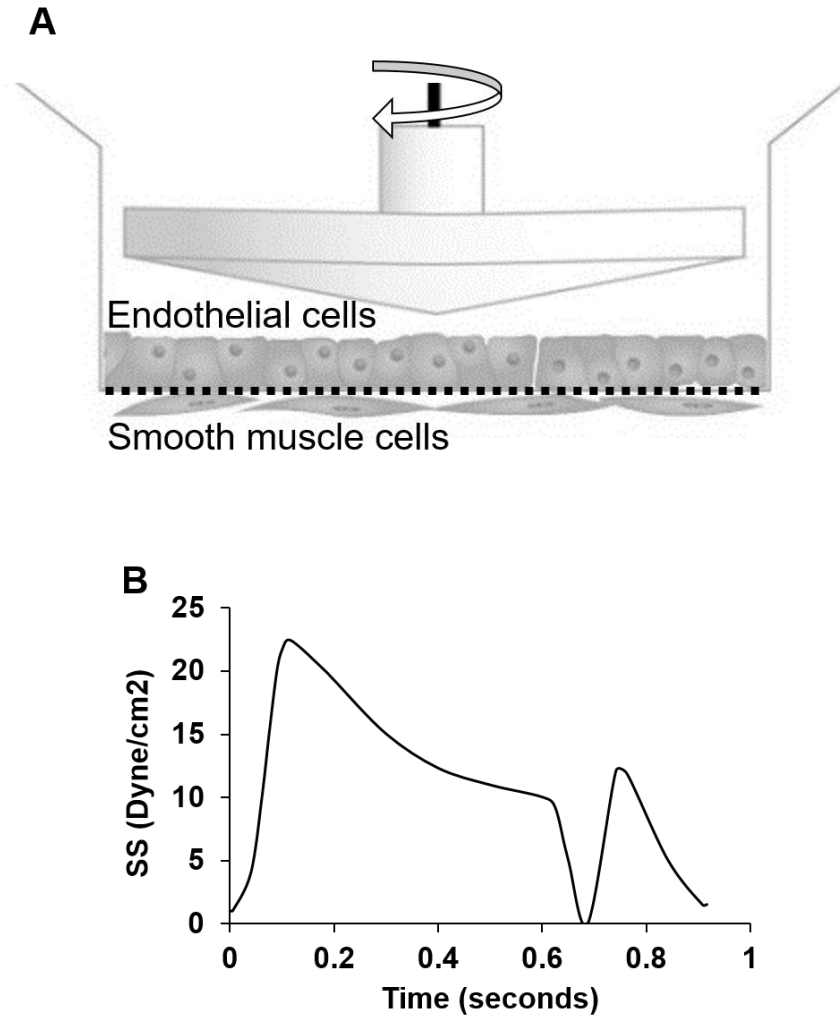
Fig. 2. Coculture fluid shear stress (FSS) model. A. Coculture setup. Human coronary artery endothelial cells (HCAEC) and human coronary artery smooth muscle cells (HCASMC) were seeded on either side of the inserts to simulate artery structure. 4 μM pores in the transwell membrane allow communication between EC and SMC but not cell migration. Shear stress was applied with a polyethylene cone connected to a micromotor. **B. FSS wave form.** Micromotors were controlled by software to turn at certain rates to simulate the shear stress in collateral arteries diverted from a clogged artery with 60% stenosis[46,54].

Fig. 3
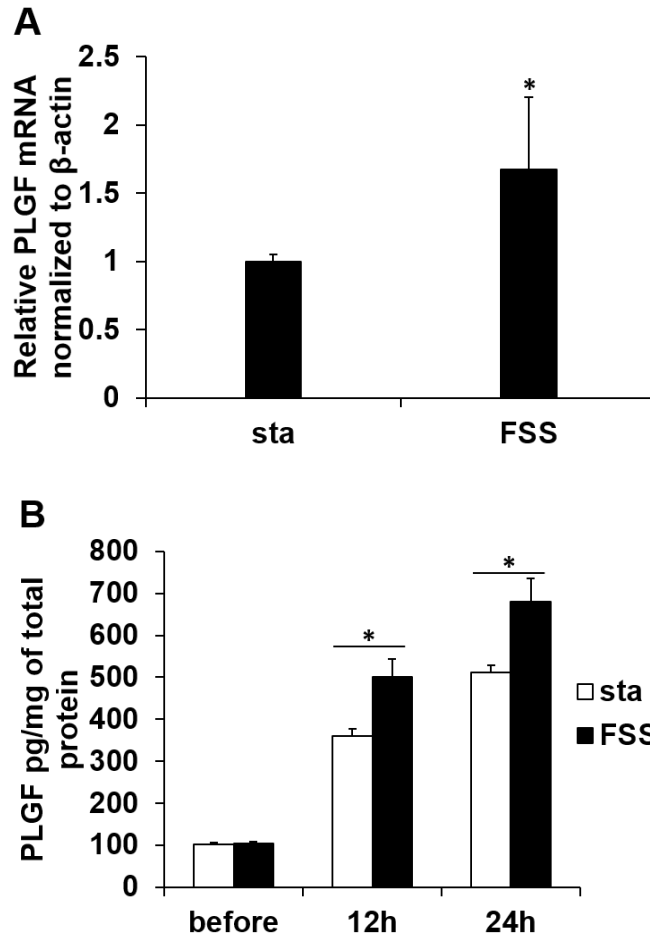
Fig. 3. Fluid shear stress (FSS) upregulated PLGF mRNA and protein. A. Shear stress increased PLGF mRNA in EC. Cocultures were exposed to shear stress for 2 h and incubated under 5% CO_2, 37 °C for 4 h before sample lysis. Relative mRNA levels were calculated using the $\Delta\Delta$Ct method. (Mean±SE, N=4, *p<0.05) **B. Shear stress increased PLGF protein in EC media.** Cocultures were exposed to shear stress for 2 h and incubated under 5% CO_2, 37 °C for 12 or 24 h. PLGF levels were normalized to total protein. (N=4)

Fig. 4

A

half-life: ln(2)/0.415 =<u>**1.67 hrs**</u>
$y = 1.1423e^{-0.415x}$

x-axis: actinomycin D treatment hrs
y-axis: Relative PLGF mRNA normalized to β-actin

B

$y = -137.998x - 5.518$
$r^2 = 0.66$, $p < 0.001$

x-axis: hours of actinomycin D treatment
y-axis: RNA ng/µL

C

x-axis: actinomycin D treatment h
y-axis: β-actin C_T
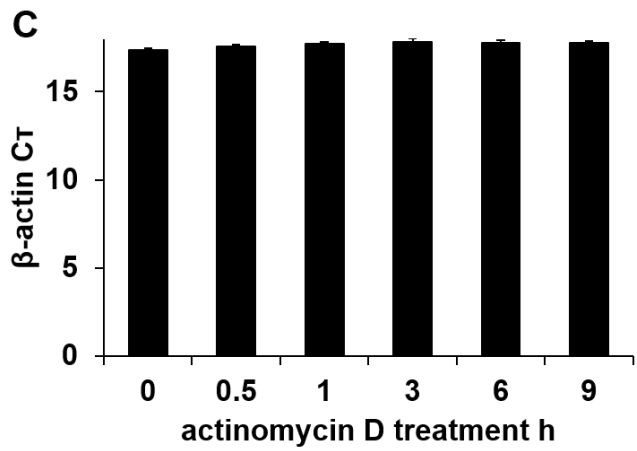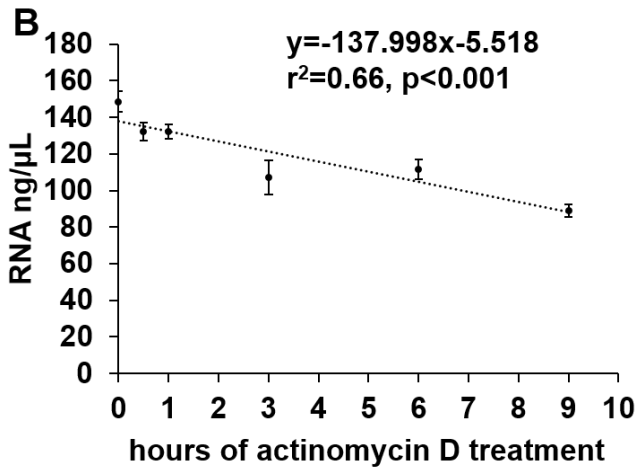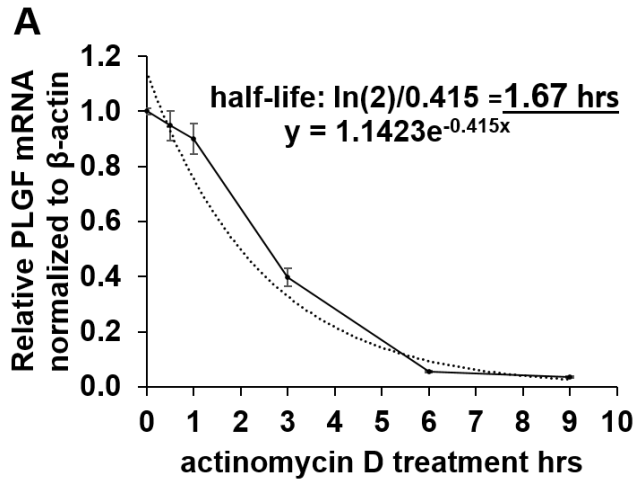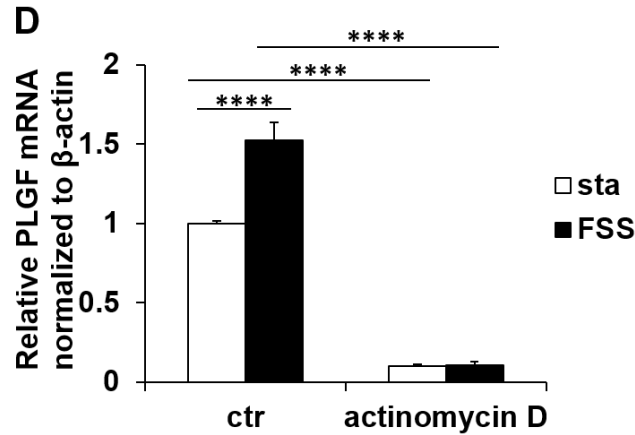
Fig. 4. Shear stress upregulates PLGF by increasing transcription. A. PLGF's half-life was 1.67 h in EC. Actinomycin D inhibits mRNA production by binding to DNA during transcription initiation. EC cell lysis was collected 0, 0.5, 1, 3, 6 h after actinomycin D was added. (Mean±SE, N=4) **B. Total RNA concentration reduction after actinomycin D treatment.** Total HCAEC RNA concentration was reduced by actinomycin D in a time-dependent manner. **C. β-actin mRNA is a good indicator of total mRNA. β**-actin threshold cycle number was measured with 10 ng cDNA/reaction for HCAEC treated with actinomycin D (10 μg/mL) for 0, 0.5, 3, 6, and 9 h. **D. Actinomycin D treatment abolished shear stress upregulation of PLGF.** When transcription was inhibited by actinomycin D, FSS upregulation of PLGF mRNA was abolished. Cell lysis was collected 4 h post shear stress. This indicates shear stress does not upregulate PLGF mRNA by increasing its half-life (N=4).
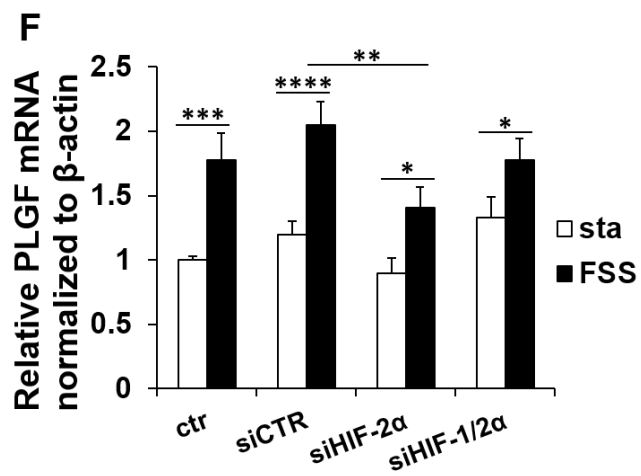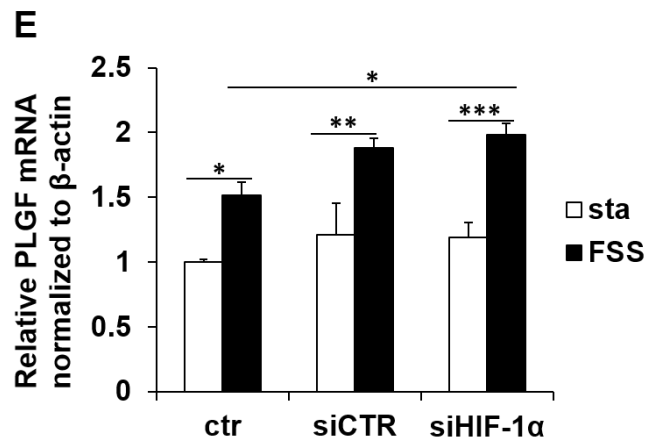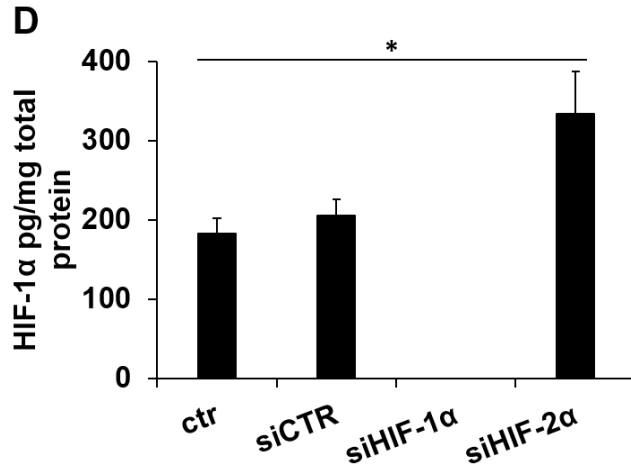
Fig. 5
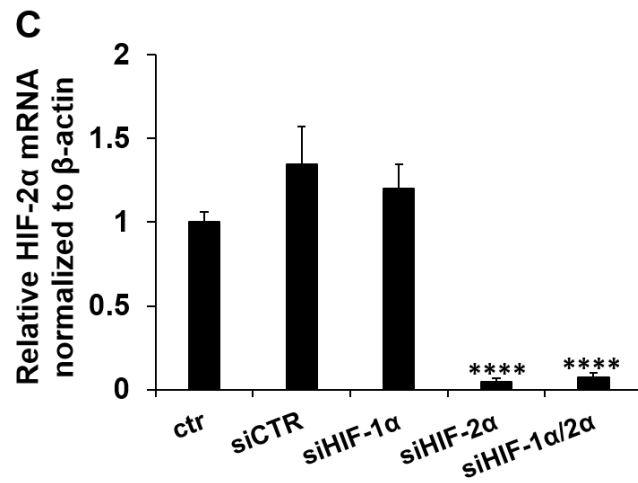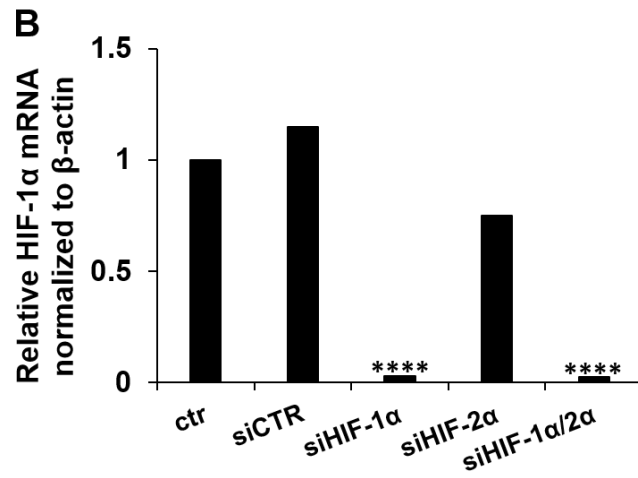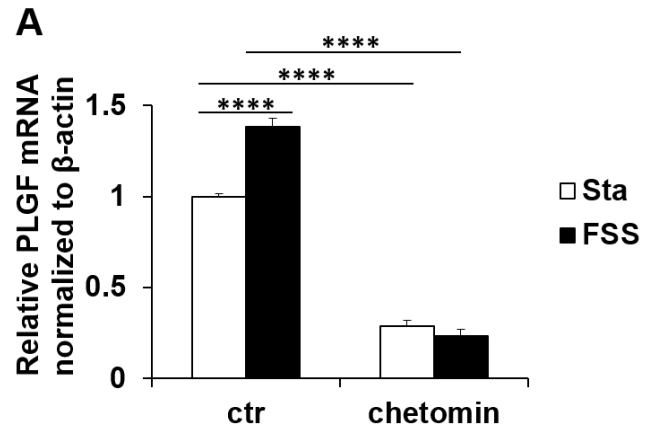
Fig. 5. Transription cofactor p300 is important for PLGF regulation. A. Binding capacity of p300's CH1 domain is critical for PLGF transcription. Cocultures were treated with chetomin (30 μM) immediately before exposure to FSS. Interruption between p300 CH1 domain and transcription factor by chetomin drastically reduced PLGF expression. Chetomin interrupts p300-transcription factor binding by a zinc ejection mechanism. (N=4) **B and C. siRNA treatments successfully knocked down HIF-1α and HIF-2α mRNA.** HCAEC was exposed to siRNA for 24 h. Afterwards, cells were replenished with fresh media and incubated for 24 h before cell lysis was collected. HIF-1α and HIF-2α mRNA levels were measured by real-time qPCR (N=3). **D. siRNA treatment successfully knocked down HIF-1α protein in HCAEC.** HCAEC was exposed to siRNA for 24 h then replenished with fresh media with 100 μM of DMOG for 6 h of treatment. Cellular HIF-1α was measured with ELISA and normalized total protein (N=3). **E and F. Knockdown (KD) of HIF-1α and/or HIF-2α does not interrupt FSS upregulation of PLGF.** HCAEC was treated with siHIF-1α, and/or siHIF-2α before being seeded in inserts. Cell lysis was collected 4 h post FSS (N=3 and 5).
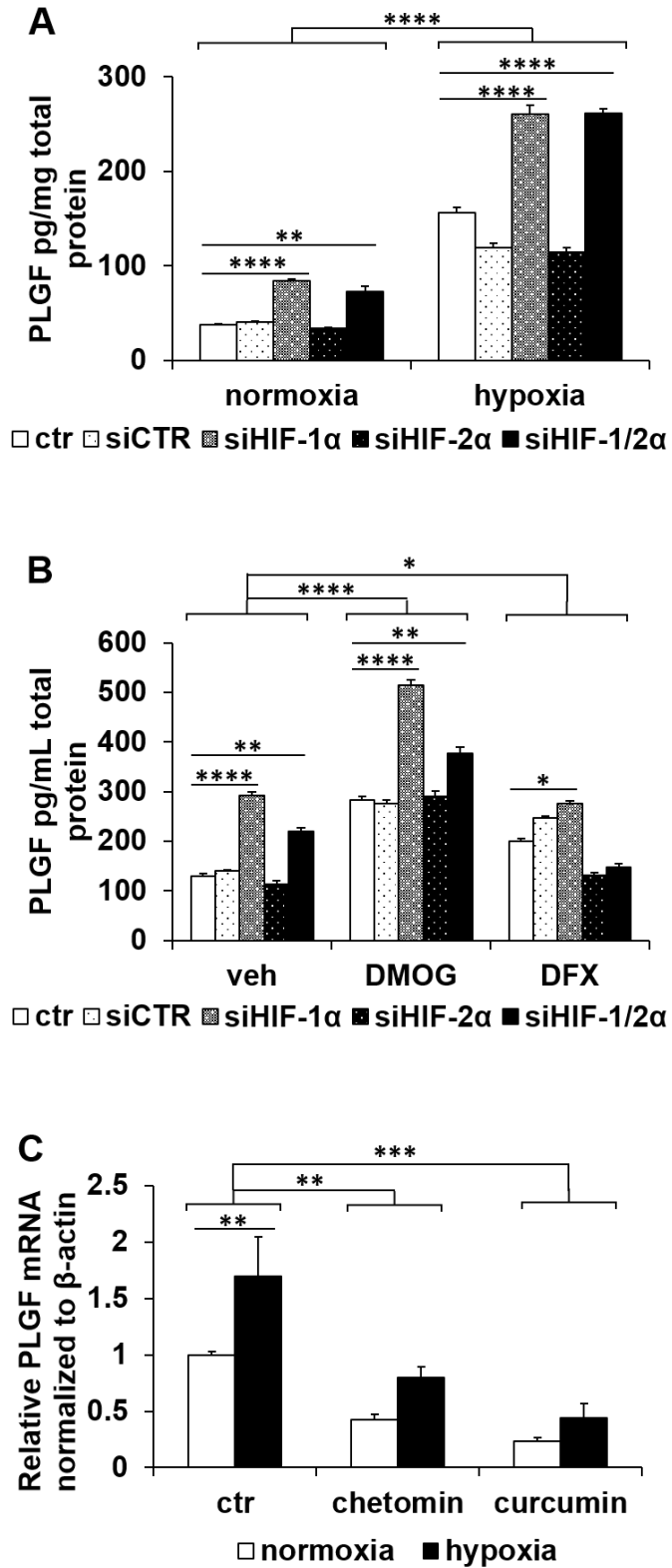
Fig. 6

Fig. 6. Upregulation of PLGF by hypoxia is not HIF-dependent. A. KD of HIFs does not reduce PLGF upregulation by hypoxia. HCAEC was exposed to siRNA for 24 h then replenished with fresh media with 2% FBS then kept in 1% oxygen. HCAEC media were collected after 18 h of hypoxia exposure. PLGF in media was measured with ELISA and normalized to total protein (N=4). **B. KD of HIF-1α and/or HIF-2α do not reduce PLGF upregulation by DMOG or DFX.** HCAEC was exposed to siRNA for 24 h, then replenished with fresh 2% FBS media with 100 µM of DMOG or 100 µM DFX. HCAEC media were collected after 18 h of hypoxia. PLGF in media was measured with ELISA and normalized to total protein (N=4). **C. p300 is important for PLGF mRNA expression.** Chetomin (30 nM), and curcumin (25 µM) were added to HCAEC before exposure to hypoxia. Cell lysis was collected after 18 h of hypoxia. Curcumin is a specific inhibitor to p300 histone acetyl transferase (HAT) activity (N=3).
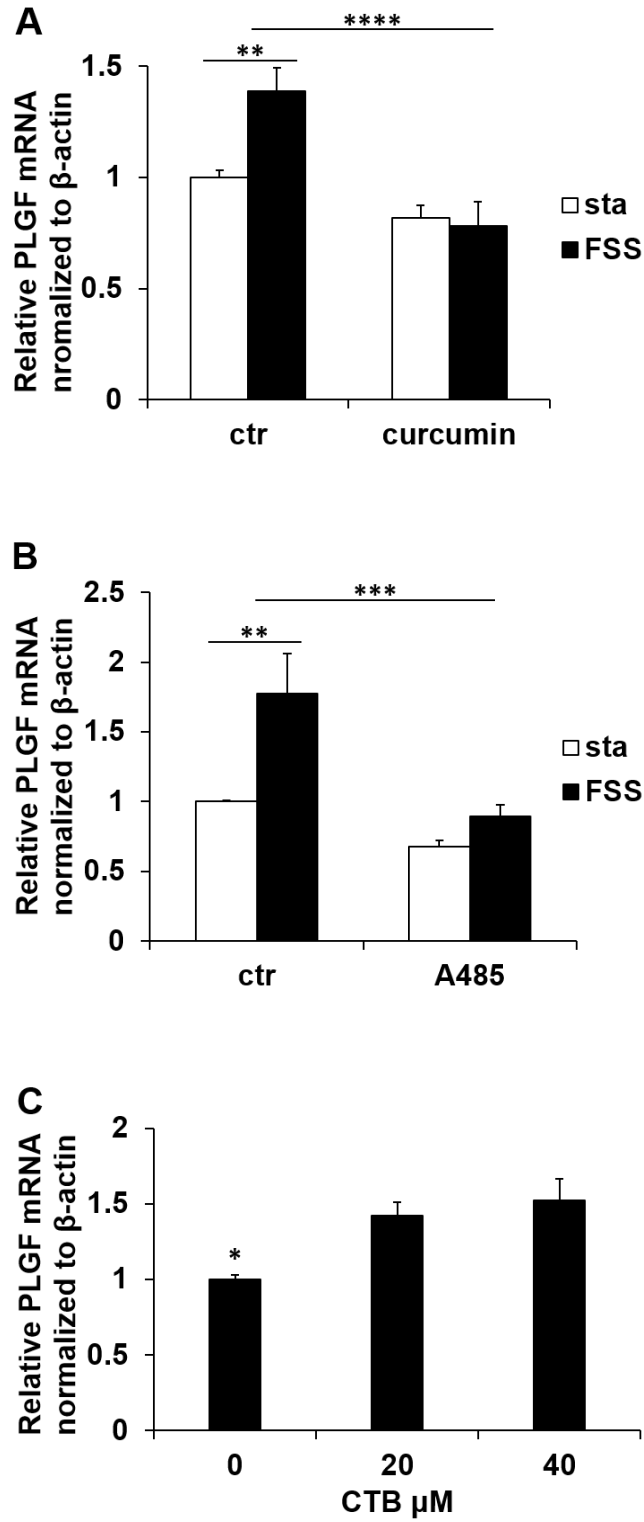
Fig. 7

Fig. 7. Upregulation of PLGF by FSS is p300 histone acetyl transferase (HAT) activity dependent. A and B. Inhibition of p300 HAT does not affect basal level of PLGF but

abolishes PLGF upregulation by FSS. Cocultures were treated with 25 µM of curcumin **(A)**, or 20 nM of A485 **(B)** immediately before exposure to FSS. Cell lysis was collected 4 h post FSS (N=5 for both). **C. Activation of p300 HAT upregulates PLGF.** HCAEC was treated with 20 µM or 40 µM of CTB for 6 h (N=4).
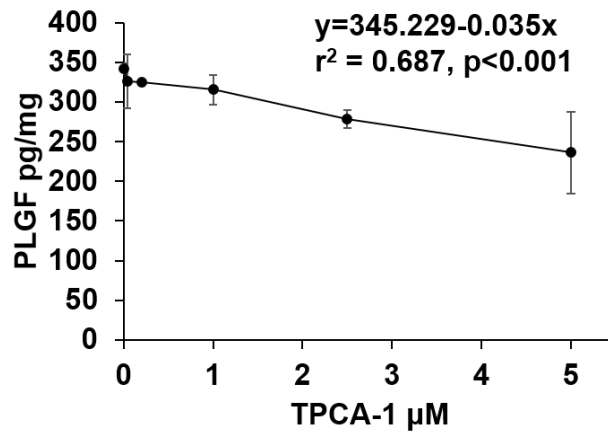
Fig. 8

Fig. 8. Inhibition to NFκB reduces PLGF. HACEC was treated with TPCA-1 at 40 nM, 200 nM, 1 μM, 2.5 μM, 5 μM, or vehicle DMSO for 18 h. PLGF levels in media were, analyzed with ELISA and normalized to total protein in media with BCA assay (N=4).

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CHAPTER IV

DISCUSSION

Arteriogenesis is the process of outward remolding of pre-existing arterioles upon exposure to increased fluid shear stress (FSS)[1]. Placental growth factor (PLGF) is a key factor in inducing arteriogenesis[2]. Our group's previously published work demonstrated that PLGF is upregulated by FSS and that this process is NADPH oxidase-4 (NOX-4) and heme oxygenase-1 (HO-1) dependent[3]. To better understand the mechanism of PLGF upregulation by FSS, we employed an *in vitro* coculture model to simulate the structure of arteriole and its exposure to FSS. Two key discoveries were made in this study. First, FSS upregulates placental growth factor through increased transcription. Second, transcription cofactor p300 plays a central role in PLGF upregulation by FSS and by iron.

Coronary artery disease (CAD) is the leading cause of mortality both in the United States and worldwide[4]. The advancement of treatments has greatly contributed to the reduction of CAD caused mortality (more than 50% age-adjusted reduction by the year 2000 compared to 1980[5]). However, CAD remains a tremendous burden to our society despite advanced pharmaceutical and surgical interventions. The total direct medical cost of CAD is projected to be \$918 billion in the US in 2030[6]. Improved collateral circulation has been shown to reduce the risk of mortality and major cardiac events caused by coronary artery disease[7-9]. Better understanding of the mechanism of arteriogenesis may provide pharmaceutical targets for which potential treatments to improve collateral growth can be developed.

Iron overload is correlated with increased serum PLGF levels in sickle cell disease (SCD) patients due to blood transfusion[10-13]. Our results show several forms of iron, including hemin (a type of heme), ferric ammonium citrate (FAC), and ferric nitrilotriacetate (FeNTA) can upregulate PLGF in HCAEC and that this process is not due to Fenton reaction. Some studies suggest ferric iron can also cause Fenton reaction by being initially reduced by superoxides[14,15]. Interestingly, hemin, FAC, and FeNTA all contain iron (3^+), while ferrous iron (2^+) did not upregulate PLGF. Our results show p300 is also important in PLGF upregulation by iron, but the

mechanism remains unclear. We also discovered dietary-induced iron deficiency in rats does not reduce PLGF mRNA or protein in the hearts. Further studies on PLGF upregulation by iron is an interesting topic and can potentially lead to iron-related therapy for arteriogenesis.

Many studies have shown that p300 is activated by FSS[16-18], which coincides with our discovery that p300 activation is essential for PLGF upregulation by FSS. In addition to the regulation of PLGF, p300 has also been shown to be important for the regulation of many genes involved in arteriogenesis, including endothelial nitric oxide synthase (eNOS)[16], vascular endothelial growth factor (VEGF)[19,20], and monocyte chemoattractant protein-1 (MCP-1)[21]. Interestingly, p300-CBP–associated factor (PCAF), another transcription cofactor/HAT, was also shown to be a key factor in arteriogenesis in a study by Bastiaansen et al (2013)[22]. Analysis of histone acetylation levels using Western blot for p300's targets histone H3K9, H3K18, and H3K27[23,24] after exposure to FSS could provide further insights into the role of p300 in PLGF regulation.

Heme, as an important metabolite, is constantly being synthesized in many types of cells including endothelial cells[25,26]. Supplementing erythrocytes with ferric iron increases heme synthesis[27,28]. Transcription factors nuclear receptor Rev-erbα (NR1D1), and BTB domain and CNC homolog 1 (Bach-1) respond to heme[29,30]. It is plausible that NR1D1 or Bach-1 is involved in PLGF upregulation by hemin, FAC, and FeNTA.

Nuclear factor κ-B (NFκB) p65 was shown to regulate the transcription of PLGF[31], and it is activated by FSS[32]. We discovered several shear stress responsive elements (SSRE), which are recognized by p65, in the PLGF promoter region. p65 also interacts with p300 at its CH1 domain[33,34]. Interestingly, it has been suggested that p65 and HIF-1α antagonize each other for binding with p300[35], which is in agreement with our results that knockdown of HIF-1α upregulated PLGF. Furthermore, protein kinase A (PKA) is an activator of p65[33], and p300[36,37].

Further analysis of p65 could greatly further our understanding of PLGF regulation. Knockdown of p65 using siRNA can be used to determine the role of p65 in PLGF regulation. Western blot for phosphorylated p65 (Ser 536[38]) after exposure to FSS can be used to show if p65 is activated by FSS.

We determined that hypoxia inducible factor (HIF)-1α, HIF-2α, and Metal transcription factor-1 (MTF-1) are highly unlikely to be the transcription factor involved in PLGF transcription in human coronary artery endothelial cells (HCAEC). Activation of MTF-1 by zinc, a heavy metal did not lead to upregulation of PLGF, despite literature showing MTF-1 is involved in PLGF regulation in fibroblasts under hypoxia conditions[39,40]. Another heavy metal, cadmium, at high concentration (200 µM) upregulated PLGF mRNA. However, MTF-1 is a more responsive to zinc than to cadmium[41]. Cd is also highly cytotoxic to endothelial cells, causing apoptosis at as low as 5 µM[42], suggesting the upregulation of PLGF by Cd may be through non-specific mechanisms other than activation of MTF-1.

It is possible that another transcription factor is involved in PLGF regulation. p300's CH1 domain has been shown to interact with many transcription factors including signal transducer and activator of transcription 2 (STAT-2), retinoid X receptor (RXR), and hepatocyte nuclear factor-4 (HNF-4), as reviewed by Chen et al (2011)[43]. Analysis of binding sequences of these transcription factors may reveal potential interactions with the PLGF promoter region.

In this study, we emphasized histone acetylation as a new perspective in PLGF regulation. We demonstrated that iron, HIF-1α, and p300 can be developed as potential pharmaceutical targets for improving arteriogenesis.

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APPENDICES

THE FOLLOWING APPENDIX CONTAINS DATA THAT WERE NOT FURTHER
PURSUED BUT ARE STILL MEANINGFUL.

Metal transcription factor-1 (MTF-1) activation does not upregulate PLGF. MTF-1 is activated by heavy metals including zinc, cadmium, and copper. Zinc also induces interaction between MTF-1 and p300[1]. Studies have linked MTF-1 with PLGF transcription regulation[2,3]. We were interested to know if MTF-1 is the transcription factor involved in PLGF upregulation by FSS. Thus, we treated HCAEC with $ZnSO_4$ and $CdSO_4$, then measured PLGF mRNA levels. At 6 h, PLGF mRNA was not affected by $ZnSO_4$ at 100, 200, or 400 µM. $CdSO_4$ did not affect PLGF mRNA levels at 50 and 100 µM. Only 200 µM of CdSO4 increased PLGF mRNA by 1.63-fold ($\pm$0.253 SE, $p<0.05$) whereas in literatures 50 and 80 showed effect on MTF-1 nuclear translocation[4] and metallothionein expression[5] respectively. Zinc is a strong activator of MTF-1's transcription activity[5,6]. This result suggests MTF-1 is not involved in PLGF transcription regulation in HCAEC.
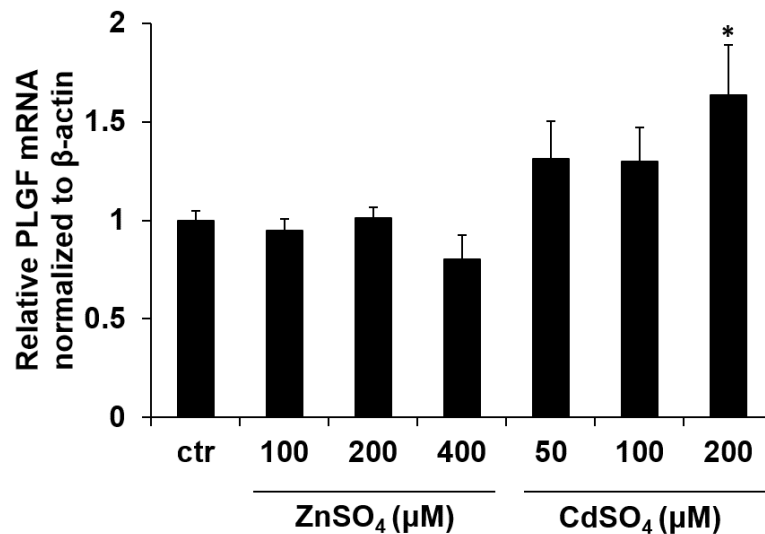

Fig. 1. Activation of metal transcription factor-1 (MTF-1) does not induce PLGF.

HACEC was treated with ZnSO4, or CdSO4, or vehicle autoclaved ddH_2O for 6 h (N=4).

Citrate synthase substrate and product do not affect PLGF levels. Our results show DMOG

increased PLGF basal levels, but did not affect FSS upregulation of PLGF. DMOG not only

inhibits PHDs, it affects enzymes that use 2-oxoglutarate (2-OG) as a substrate. DMOG was also

shown to inhibit mitochondria[7]. Acetyl-CoA levels are directly correlated with p300 histone

acetyl transferase (HAT) activity, because p300 has a low affinity for acetyl-CoA binding[8]. To

determine if citrate synthase, an important enzyme in TCA cycle acetyl-coA production, is

involved in PLGF regulation, we treated HCAEC with citrate synthase upstream substrate acetate

(in the form of sodium acetate)[9], and product citrate (in the form of sodium citrate). Sodium

EDTA was used as a control for citrate's chelating activity. Neither sodium acetate nor sodium

citrate affected PLGF mRNA levels, suggesting citrate synthase may not play an important role in
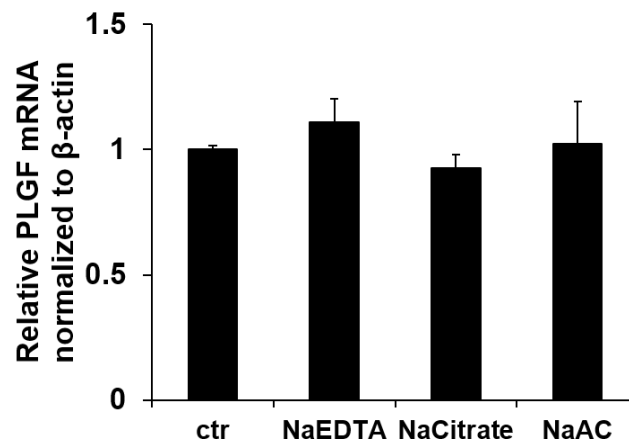
PLGF regulation.

Fig. 2. Citrate synthase substrate and product do not affect PLGF levels. HACEC was

treated with sodium EDTA (NaEDTA, 600 μM), trisodium citrate (NaCitrate, 400 μM), disodium

acetate (NaAC, 600 μM), or vehicle ddH₂O for 6 h. NaEDTA was used as control for citrate's

chelating activity (N=5).

AMPK activation does not upregulate PLGF. AMPK is activated by Ca^2/calmodulin-dependent protein kinase kinase (CaMKK)[10,11]. CaMKK is regulated by intracellular Ca^2 levels[12]. FSS increases intracellular Ca^2 levels by activating calcium channel[13,14]. To determine if AMPK activation can upregulate PLGF, we treated HCAEC with AICAR, an analog of AMP, and an activator of AMPK. AICAR did not affect PLGF mRNA levels, suggesting AMPK may not be involved in PLGF regulation.
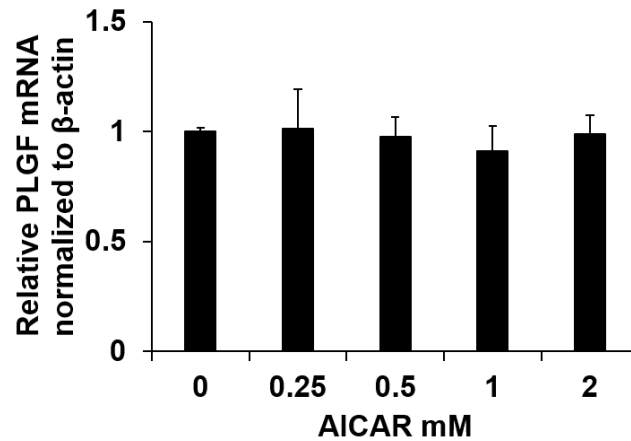

Fig. 3. Activation of AMPK does not affect PLGF. AICAR is an analog of AMP, which activates AMPK. HACEC were treated with AICAR at 0.25, 0.5, 1, and 2 mM for 6 h (N=3).

Histone acetyl transferase (HAT) and histone deacetylase (HDAC) inhibitors affect PLGF mRNA in HCAEC. We showed that p300 HAT activity is critical in PLGF upregulation by FSS. We were curious to know if HAT and HDAC inhibitors could affect PLGF levels. We treated HCAEC with HAT inhibitor anacardic acid (AA), and HDAC inhibitor trichonstatin A (TA). Similar to p300 HAT inhibitors curcumin and A485, AA did not affect basal level of PLGF mRNA, but abolished PLGF upregulation by FSS. show similar effect. However, to our surprise, TA reduced PLGF and abolished PLGF upregulation by FSS. TA inhibits many classes of HDAC, it also inhibits NADPH oxidase-4 (NOX-4)[15]. Our group has previously shown that PLGF upregulation by FSS is NOX-4 dependent. This could explain why TA treatment reduced PLGF.
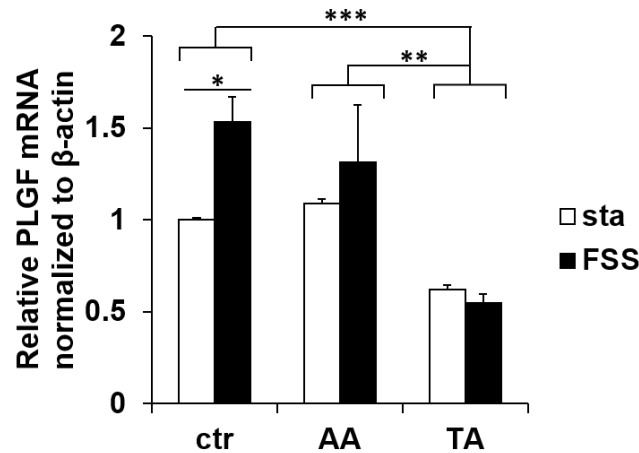

Fig. 4. Histone acetyl transferase (HAT) and histone deacetylase (HDAC) inhibitors affect PLGF mRNA in HCAEC. HCAEC-HCASMC cocultures were treated with HAT inhibitor anacardic acid (25 μm) and trichonstatin-A (300 nM) before exposure to FSS. Cell lysis was collected 4 h after FSS (N=3).

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