**Laminar Shear Stress Inhibits Endothelial Cell Metabolism via KLF2-Mediated Repression of PFKFB3**

Anuradha Doddaballapur, Katharina M. Michalik, Yosif Manavski, Tina Lucas, Riekelt H. Houtkooper, Xintian You, Wei Chen, Andreas M. Zeiher, Michael Potente, Stefanie Dimmeler, Reinier A. Boon

**Objective**—Cellular metabolism was recently shown to regulate endothelial cell phenotype profoundly. Whether the atheroprotective biomechanical stimulus elicited by laminar shear stress modulates endothelial cell metabolism is not known.

**Approach and Results**—Here, we show that laminar flow exposure reduced glucose uptake and mitochondrial content in endothelial cells. Shear stress–mediated reduction of endothelial metabolism was reversed by silencing the flow-sensitive transcription factor Krüppel-like factor 2 (KLF2). Endothelial-specific deletion of KLF2 in mice induced glucose uptake in endothelial cells of perfused hearts. KLF2 overexpression recapitulates the inhibitory effects on endothelial glycolysis elicited by laminar flow, as measured by Seahorse flux analysis and glucose uptake measurements. RNA sequencing showed that shear stress reduced the expression of key glycolytic enzymes, such as 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3), phosphofructokinase-1, and hexokinase 2 in a KLF2-dependent manner. Moreover, KLF2 represses PFKFB3 promoter activity. PFKFB3 knockdown reduced glycolysis, and overexpression increased glycolysis and partially reversed the KLF2-mediated reduction in glycolysis. Furthermore, PFKFB3 overexpression reversed KLF2-mediated reduction in angiogenic sprouting and network formation.

**Conclusions**—Our data demonstrate that shear stress–mediated repression of endothelial cell metabolism via KLF2 and PFKFB3 controls endothelial cell phenotype. (Arterioscler Thromb Vasc Biol. 2015;35:137-145. DOI: 10.1161/ATVBAHA.114.304277.)

**Key Words:** angiogenesis ■ endothelium ■ glycolysis ■ hemodynamics ■ metabolism ■ shear stress down regulated gene-1 protein, human

Endothelial cells form the inner lining of all blood vessels and not only regulate transport of nutrients to the underlying tissue but also coordinate the formation of new blood vessels, a process termed angiogenesis. Therefore, endothelial cells are highly plastic cells that are capable of switching from a resting quiescent state in normal conduit blood vessels to a highly proliferative and migratory state when angiogenesis takes place. Resting quiescent endothelial cells are termed phalanx cells, whereas migratory angiogenic endothelial cells are referred to as tip cells, which are followed by proliferating so-called stalk cells. Although the mechanisms regulating tip and stalk cell behavior have been extensively studied, relatively little is known about the control of the phalanx state.

Shear stress, the force that laminar blood flow exerts on endothelial cells, is thought to be one of the factors that determine the quiescent state of endothelial cells. This biomechanical stimulus induces the expression of the transcription factor Krüppel-like factor 2 (KLF2), which orchestrates a network of genes that elicit a quiescent endothelial cell phenotype. Among the factors that are upregulated by KLF2 are anti-inflammatory and antithrombotic proteins, whereas proinflammatory and prothrombotic factors are downregulated by KLF2. Although not all effects of shear stress on endothelial cells are mediated by KLF2, KLF2 coordinates approximately half of the gene expression programs evoked by shear stress.

Recent studies have highlighted the importance of cellular metabolism for the control of endothelial cell phenotype. Particularly, it was shown that angiogenic endothelial cells rely heavily on glycolysis for migration and proliferation. The enzyme PFKFB3 is a key regulator of glycolysis in endothelial cells that has been shown to promote angiogenic sprouting. However, how resting endothelial cells control...
their metabolic activity and whether this affects the functional properties of the phalanx phenotype is unclear.

Here, we show that the biomechanical signal shear stress, through the upregulation of KLF2, reduces endothelial metabolic activity by repressing PFKFB3 expression, and thereby maintains a metabolic quiescent phenotype reminiscent of phalanx cells.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
Shear Stress Reduces Endothelial Glucose Uptake and Mitochondrial Content in a KLF2-Dependent Manner
To assess the effects of laminar shear stress on endothelial cell metabolism, human umbilical vein endothelial cells (HUVECs) were exposed to laminar shear stress (20 dynes/cm²) for 72 hours to achieve steady-state quiescence or left under static conditions. Glucose uptake and mitochondrial content analysis revealed that shear stress reduces basal endothelial cell glucose uptake and the relative quantity of mitochondria per endothelial cell (Figure 1A and 1B). These results were substantiated by fluorescence microscopy–based analysis of glucose uptake of individual HUVECs exposed to shear stress, which showed that cellular alignment to the flow direction inversely correlates with glucose uptake (Figure 1A in the online-only Data Supplement). Because the transcription factor KLF2 is known to be responsible for many shear stress–induced effects on endothelial cells, we determined whether reduction in glucose uptake by shear stress is dependent on KLF2. To this end, we transduced HUVECs with a lentiviral short hairpin RNA construct to silence KLF2 and subsequently subjected the cells to laminar shear stress, which showed that KLF2 reduces glucose uptake and the relative quantity of mitochondria per endothelial cell (Figure 1A and 1B) and indeed reduces basal mitochondrial respiration and ATP production (Figure 1A and 1B), but does not affect maximal respiratory capacity or spare respiratory capacity, further corroborating that KLF2 reduces mitochondrial activity, rather than affecting mitochondrial integrity (Figure 2I). Assessment of cellular oxygen consumption revealed that KLF2 overexpression also reduces basal mitochondrial respiration and ATP production (Figure 2J and 2K), but does not affect maximal respiration or spare respiratory capacity, further corroborating that KLF2 does not affect mitochondrial integrity. Because both oxygen consumption and lactate production are lower in KLF2-overexpressing cells, the ratio of oxygen consumption over lactate production rate is not altered by KLF2, arguing against a potential KLF2-mediated shift between aerobic respiration and glycolysis (Figure 1C in the online-only Data Supplement).

KLF2 Reduces Endothelial Metabolic Activity
Overexpression of KLF2 in endothelial cells recapitulates many aspects of shear stress stimulation, including induction of cellular quiescence.4,12 Lentiviral overexpression of KLF2 (Figure 2A) in endothelial cells mimics the induction of KLF2 after shear stress stimulation (Figure 1C) and indeed reduces glucose uptake (Figure 2B). Furthermore, using a transwell assay to model glucose availability to underlying tissue in vitro, we measured an increase in available glucose underneath endothelial monolayers that overexpress KLF2 (Figure 2C), indicating that the reduction of glucose consumption mediated by KLF2 results in more bioavailability of glucose underneath the endothelium. Using Seahorse Flux analysis, we determined that the extracellular acidification rate, an indicator of lactate production, is also lower in KLF2-overexpressing endothelial cells (Figure 2D). Not only basal acidification rate but also glucose-induced glycolysis and maximal glycolytic capacity were reduced in KLF2-transduced cells when compared with mock-transduced control cells (Figure 2E).

Next, we determined whether mitochondrial content and function are also regulated by KLF2. KLF2 overexpression reduces mitochondrial content (Figure 2F), metabolic activity (measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Figure 2G), and ATP levels (Figure 2H), which is similar to the effects of shear stress on endothelial cells (Figure 1). Importantly, mitochondrial membrane potential is significantly enhanced after KLF2 overexpression, suggesting that KLF2 reduces mitochondrial activity, rather than affecting mitochondrial integrity (Figure 2I). Assessment of cellular oxygen consumption revealed that KLF2 overexpression also reduces basal mitochondrial respiration and ATP production (Figure 2J and 2K), but does not affect maximal respiration or spare respiratory capacity, further corroborating that KLF2 reduces mitochondrial activity. Because both oxygen consumption and lactate production are lower in KLF2-overexpressing cells, the ratio of oxygen consumption over lactate production rate is not altered by KLF2, arguing against a potential KLF2-mediated shift between aerobic respiration and glycolysis (Figure 1C in the online-only Data Supplement).

KLF2 Does Not Induce Senescence or Apoptosis
To exclude the possibility that the KLF2-mediated reduction in metabolic activity is because of induction of senescence, we analyzed proliferation (Figure 3A), acidic β-galactosidase activity (Figure 3B), and p21 expression (Figure 3C) in KLF2-transduced and mock control cells. Whereas KLF2 slightly reduces the number of cells in G2/M-phase, KLF2 overexpression reduced β-galactosidase activity and p21 expression, indicating that reduction in proliferation by KLF2 is not because of senescence. Furthermore, KLF2 reduces apoptosis, as measured by caspase 3/7 activation (Figure 3D) and annexin V staining (Figure 3D in the online-only Data Supplement).

KLF2-Mediated Suppression of Metabolic Activity Is Not Mediated via AMPK or Nitric Oxide
To delineate how KLF2 reduces endothelial cell metabolic activity, we first performed phospho-kinase proteome profiling (Figure IIA in the online-only Data Supplement), which

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<th>Nonstandard Abbreviations and Acronyms</th>
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<td>HUVEC</td>
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<td>KLF2</td>
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showed that KLF2 overexpression reduces phosphorylation of the 5′ adenosine monophosphate–activated protein kinase α 1 subunit (Figure IIB in the online-only Data Supplement). However, silencing 5′ adenosine monophosphate–activated protein kinase α 1 (Figure IIC in the online-only Data Supplement) did not recapitulate any of the metabolic effects observed after KLF2 overexpression (Figure IID–IIG in the online-only Data Supplement). Conversely, KLF2 overexpression induces endothelial nitric oxide synthase expression and phosphorylation (Figure IIA in the online-only Data Supplement). Because nitric oxide has been shown to inhibit mitochondrial respiration, increased nitric oxide production could potentially account for the inhibition of mitochondrial activity by KLF2. However, inhibition of nitric oxide synthesis did not affect the inhibitory effect of KLF2 on respiration or glycolysis (Figure IIH and III in the online-only Data Supplement).

**KLF2 and Shear Stress Inhibit PFKFB3 Expression**

To gain a broad unbiased insight into the regulation of gene expression by shear stress, HUVECs were subjected to laminar shear stress for 72 hours or kept under static control conditions, and we performed next-generation sequencing with RNA isolated from these cells (RNAseq). These experiments...
Figure 2. Krüppel-like factor 2 (KLF2) overexpression reduces endothelial metabolic activity. A, Total cell mRNA was harvested from mock- and KLF2-transduced human umbilical vein endothelial cells (HUVECs), KLF2 mRNA expression levels relative to ribosomal phosphoprotein, large P0 subunit (RP0), and α-tubulin as a loading control. B, Representative flow cytometry histogram overlay (top) and quantification of 2-NBD-glucose uptake (bottom) in HUVECs on KLF2 lentiviral transduction compared with mock-transduced control measured by flow cytometry analysis of 2-NBD-glucose fluorescence (n=6). C, Graphical representation of glucose transwell setup (top) and ratio of 2-NBDG to tetramethylrhodamine (TMR)-Dextran fluorescence (bottom), as measured in the compartment underneath the cells, to represent glucose transport through mock- and KLF2-transduced cells on fibronectin-coated transwell inserts (n=8). D, Extracellular acidification rate (ECAR) profile showing glycolytic function in mock- and KLF2-transduced cells. Vertical lines indicate the time of addition of glucose (10 mM), oligomycin (3 μmol/L), and 2-deoxy-D-glucose (100 μmol/L). E, Quantification of glycolytic function parameters from D, values are normalized to DNA content (n=3). F, Mitochondrial DNA content in KLF2-transduced cells vs mock-transduced control measured by quantitative real-time polymerase chain reaction (PCR) of mitochondrial encoded gene ND1 and nuclear encoded gene RPL0. Relative levels were normalized to median values (n=3). G, Mitochondrial activity in KLF2-transduced cells vs mock-transduced control measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction. Absorbance levels at 550 nm levels were normalized to median values (n=6). H, ATP produced in KLF2-transduced cells vs mock-transduced control analyzed by quantification of luminescence signal from triplicate wells of each condition (n=3). I, Mitochondrial membrane potential in KLF2-transduced cells vs mock-transduced controls assessed by flow cytometry analysis of TMRM fluorescence (n=3). J, Oxygen consumption rate (OCR) profile showing mitochondrial respiration function in mock- and KLF2-transduced cells. Vertical lines indicate the time of addition of oligomycin (3 μmol/L), Carbonyl cyanide 4-trifluoromethoxy-phenylhydrazine (FCCP, 1 μmol/L), antimycin A (1.5 μmol/L), and rotenone (3 μmol/L). K, Quantification of mitochondrial respiration function parameters from I, values are normalized to DNA content (n=3). In all graphs, data represent mean±SEM *P<0.05, **P<0.01, ***P<0.001.
showed that shear stress stimulation downregulates the expression of many genes involved in glycolysis (Figure 4A), including the genes encoding hexokinase, phosphofructokinase, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase (PFKFB3). Hexokinase converts glucose into glucose 6-phosphate, phosphofructokinase converts fructose 6-phosphate into fructose 1,6-bisphosphate, and PFKFB converts 6-phosphate into fructose 2,6-bisphosphate, a strong allosteric activator of phosphofructokinase (Figure 4A). Because the inhibition of expression of these proteins could explain the inhibitory effects of KLF2 on glycolysis and metabolic activity, we next focused on the regulation of expression of these proteins.

To substantiate the observations from our RNAseq experiments (Figure 4A), we confirmed the downregulation of hexokinase-2, PFKFB3, and phosphofructokinase platelet isofrom (PFK1) mRNA expression by quantitative real-time polymerase chain reaction after 72-hour laminar shear stress stimulation (Figure 4B). KLF2 overexpression similarly inhibited the expression of hexokinase-2, PFKFB3, and PFK1 mRNA when compared with mock-transduced cells, as measured by quantitative real-time polymerase chain reaction (Figure 4C). Western blotting confirmed that hexokinase-2, PFKFB3, and PFK1 are also significantly inhibited by KLF2 on protein level (Figure 4D–4F). Next, we determined whether the inhibition of PFKFB3 and PFK1 expression by shear stress is dependent on KLF2. To this end, we exposed HUVECs transduced with shKLF2 lentivirus or control lentivirus to shear stress for 72 hours or kept the cells under static conditions and measured the expression of PFKFB3 and PFK1 (Figure 4G).

These experiments show that in the absence of KLF2, shear stress stimulation does not repress the expression of PFKFB3 and PFK1. Because KLF2 can either activate or repress transcription by binding to specificity protein 1/KLF sites, we examined the PFK1 and PFKFB3 promoters for consensus specificity protein 1/KLF sites and identified a potential KLF2 binding site 14 bp upstream of the PFKFB3 transcriptional start site (Figure III in the online-only Data Supplement). We then placed the 300 bp conserved part upstream of the PFKFB3 promoter in front of a luciferase transporter construct and measured luciferase activity after transfection into HUVECs that were either mock or KLF2 transduced (Figure 4H). KLF2 overexpression markedly reduced PFKFB3 promoter activity and mutation of the specificity protein 1/KLF site in the PFKFB3 promoter abolished the KLF2-mediated repression of promoter activity. Together, these data indicate that shear stress represses the expression of key glycolytic enzymes, in particular, PFKFB3 via direct inhibition of promoter activity by KLF2.

KLF2 Controls Endothelial Cell Glycolysis and Angiogenic Phenotype Partly via PFKFB3 Inhibition

PFKFB3 was recently shown to be an important regulator of endothelial cell glycolysis,9 and we therefore hypothesized that inhibition of PFKFB3 could be the underlying mechanism by which KLF2 reduces endothelial cell glycolysis. First, we set out to confirm the role of PFKFB3 in endothelial cell glycolysis. To this end, we transfected HUVECs with control small interfering RNA or 2 distinct small interfering RNAs targeting PFKFB3, which both significantly reduced PFKFB3 levels (Figure 5A). Seahorse Flux analysis of these cells showed that robust knockdown of PFKFB3 in HUVECs significantly reduces acidification rate in the presence of glucose (glycolysis) and also reduces maximal glycolytic capacity of HUVECs (Figure 5B and 5C). Interestingly, glycolytic reserve capacity (lactate production in the absence of glucose and mitochondrial function) and cellular oxygen consumption (indicative of mitochondrial activity) are not affected by PFKFB3 depletion (Figure 5B–5D), indicating that PFKFB3 preferentially controls glycolysis.

Next, we tested whether a reduction in PFKFB3 levels is the cause of KLF2-mediated inhibition of glycolysis. Hence, we overexpressed KLF2 in combination with lentiviral overexpression of PFKFB3 in endothelial cells, resulting in PFKFB3 levels in KLF2 overexpressing cells that are comparable with mock-transduced HUVECs (Figure IV in the online-only Data Supplement). Seahorse Flux analysis showed that PFKFB3 overexpression in KLF2 overexpressing cells augmented glycolysis but did not affect basal extracellular acidification rate (in the absence of glucose) (Figure 5E–5H). Consistent with PFKFB3 depletion experiments, overexpression of PFKFB3 did not affect glycolytic reserve capacity or oxygen consumption (Figure 5I and 5J). These experiments suggest that KLF2 reduces endothelial cell glycolytic function, in part, via repression of PFKFB3 expression.

KLF2 has been proposed to repress angiogenic behavior of endothelial cells, via its ability to induce endothelial cell...
Figure 4. Shear stress represses glycolytic gene expression in a Krüppel-like factor 2 (KLF2)–dependent manner. A, RNA sequencing profiling of shear stress–stimulated human umbilical vein endothelial cells (HUVECs; 20 dyn/cm² for 72 hours) and static controls showing expression levels and regulation of genes in the glycolytic pathway. B, mRNA expression of hexokinase-2 (HK2), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3 (PFKFB3), and phosphofructokinase-1 (PFK1) normalized to GAPDH expression in shear stress–stimulated HUVECs (20 dyn/cm² for 72 hours) vs static controls, fold repression by shear stress stimulation is indicated (n ≥ 6). C, mRNA expression of HK2, PFKFB3, and PFK1 normalized to ribosomal phosphoprotein, large P0 subunit expression in KLF2-transduced HUVECs vs mock-transduced controls, fold repression by KLF2 is indicated (n ≥ 3). D–F, Total cell protein lysates from mock- and KLF2-transduced cells were analyzed by Western blot and probed against antibodies for (D) HK2 (n=4), (E) PFKFB3 (n=4), (F) PFK1 (n=8), and α-tubulin, which served as a loading control. Representative blot images are shown here and respective protein bands were quantified and normalized to α-tubulin signal. G, mRNA expression of PFKFB3 and PFK1 normalized to GAPDH expression from HUVECs after lentiviral-mediated shRNA knockdown of KLF2 and flow exposure (20 dyn/cm² for 72 hours; n=3). H, Luciferase promoter activity assessed 24 hours after transfection of luciferase plasmid with PFKFB3 promoter region bearing wild-type (WT) KLF2 binding site or mutated (mut) version of KLF2 binding site. Data are represented as a ratio of firefly luciferase and Renilla (control) activity (n=6). In all graphs, data represent mean±SEM. *P<0.05, **P<0.01, ***P<0.001.
Figure 5. 6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase-3 (PFKFB3) is required for endothelial glycolysis and partially reverses Krüppel-like factor 2 (KLF2)–mediated repression of endothelial glycolytic function. A, PFKFB3 mRNA levels (top) were measured by quantitative real-time polymerase chain reaction in human umbilical vein endothelial cells (HUVECs) at 48 hours after transfection with control siRNA (siNeg) or 2 different siRNA directed against PFKFB3 (siPFKFB3; 1 and 2; n=3). Representative Western blot analysis (bottom) of total cell protein lysates from HUVECs after 48 hours transfection with siNeg or siPFKFB3; 1 and 2, probed against antibodies for PFKFB3 and α-tubulin, which served as a loading control. B, Extracellular acidification rate (ECAR) profile showing glycolytic function in HUVECs transected with siNeg or siPFKFB3 at 48 hours after transfection. Vertical lines indicate the time of addition of glucose (10 mmol/L), oligomycin (3 μmol/L) and 2-deoxy-D glucose (100 mmol/L; n≥3). C, Quantification of glycolytic function parameters from B (n≥3). D, Basal Oxygen consumption rate (OCR) levels, all values normalized to DNA content measured by Seahorse flux analysis (n=7). E, Representative ECAR profile showing glycolytic function in HUVECs transduced with control, PFKFB3, KLF2, or KLF2 and PFKFB3. Vertical lines indicate the time of addition of glucose (10 mmol/L), oligomycin (3 μmol/L), and 2-deoxy-D glucose (100 mmol/L). F–J, Quantification of glycolytic function parameters (F) basal ECAR, (G) glycolysis, (H) maximum glycolytic capacity, (I) glycolytic reserve, and (J) Basal OCR levels normalized to DNA content measured by Seahorse flux analysis (n=7). In all graphs, data represent mean±SEM *P<0.05, **P<0.01, ***P<0.001.
quiescence,\textsuperscript{15} and PFKFB3 is known to be important for angiogenesis.\textsuperscript{9} We, therefore, determined whether KLF2 reduces endothelial angiogenic function via reduction of PFKFB3. An endothelial spheroid sprouting assay, which allows endothelial cell outgrowth into a 3-dimensional (3D) matrix, showed that KLF2 indeed reduces endothelial cell sprouting (Figure 6A). Interestingly, PFKFB3 overexpression significantly induced sprouting of KLF2 overexpressing endothelial cells. These results were confirmed in a second angiogenesis assay, where endothelial cells form a 2D vessel network (Figure 6B). Here, KLF2 likewise reduced network formation, whereas PFKFB3 overexpression significantly reversed KLF2-mediated inhibition of endothelial network formation. Conversely, endothelial cell–specific deletion of KLF2 induces sprouting angiogenesis in an aortic ring outgrowth assay, which is dependent on PFKFB3, as shown by treatment with the PFKFB3 inhibitor 3-PO (3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one; Figure 6C). Together, these experiments show that KLF2 represses endothelial cell metabolism, in part, through transcriptional inhibition of PFKFB3, thereby inducing a metabolic quiescent endothelial cell phenotype (Figure 6D).

**Discussion**

Laminar blood flow induces a quiescent endothelial cell phenotype. It has recently been shown that endothelial cell metabolism, and in particular glycolysis, is a key determinant of endothelial cell phenotype.\textsuperscript{7} Here, we show that fluid shear stress induces endothelial cell metabolic quiescence, via induction of the key transcriptional regulator KLF2. Laminar flow reduces the expression of several genes involved in glucose metabolism and, in particular, PFKFB3 via KLF2-mediated repression of its promoter activity. These results provide a mechanism by which a biomechanical stimulus reduces endothelial cell metabolic activity, thereby inducing a quiescent phalanx cell-like phenotype.

It has been suggested that factors that induce endothelial tip cell phenotype, such as hypoxia-inducible factor 1-alpha activation and vascular endothelial growth factor or fibroblast growth factor stimulation induce glycolysis in endothelial cells, dependent on PFKFB3.\textsuperscript{5,16} Because KLF2 is known to inhibit hypoxia-inducible factor 1-alpha\textsuperscript{15} and vascular endothelial growth factor signaling,\textsuperscript{17} these mechanisms may contribute to the effects of KLF2 on glycolysis. Furthermore, KLF2 heterozygous mice, which would putatively display increased PFKFB3 expression, show an increase in capillary density\textsuperscript{13} that is perhaps driven by an increase in glycolysis.

It has recently been shown that PFKFB3-induced glycolysis is required for proper angiogenic behavior in vitro and in vivo,\textsuperscript{9,11} and that transient chemical inhibition of PFKFB3 blocks pathological angiogenesis.\textsuperscript{10} How PFKFB3 expression is physiologically regulated in endothelial cells has not been described before. Furthermore, shear stress and KLF2 also regulate the expression of several other proteins involved in glycolysis, including hexokinase-2 and PFK1. The inhibition of expression of these proteins could additionally contribute to the metabolically quiescent phenotype elicited by shear stress or KLF2 overexpression in endothelial cells. In fact, PFKFB3 overexpression alone does induce endothelial sprouting and tube formation activity but does not fully alleviate
the KLF2-mediated inhibition of endothelial cell activation (Figures 5 and 6). Induction of hexokinase-2 and PFK1 is probably not required to overcome the quiescent state induced by KLF2 fully because these 2 proteins do not seem to be required for endothelial glycolysis (Figure V in the online-only Data Supplement). The role of PFKFB3 in shear stress–induced endothelial phenotype in vivo also remains to be established.

Next to repression of glycolytic activity, KLF2 and shear stress also reduce mitochondrial content and activity. Even though endothelial cells do not rely on oxidative phosphorylation for generating ATP,9 mitochondria are known to induce oxidative stress, regulate calcium signaling, and control apoptosis.18 Interestingly, KLF2 and shear stress are known to reduce oxidative stress in endothelial cells19 and inhibit apoptosis (Figure 3). The reduction of mitochondrial content and increase in membrane potential by KLF2 could contribute to these antiapoptotic and atheroprotective cellular effects of shear stress. Even though KLF2 and shear stress both repress mitochondrial metabolism, the shear stress–mediated repression seems to be independent of KLF2 (data not shown), indicating that other shear stress–induced signaling pathways repress mitochondrial content and activity.

The function of the endothelium is highly dynamic. On hypoxia, endothelial cells need to migrate and proliferate to form new blood vessels rapidly, but under homeostatic resting conditions the endothelium needs to maintain a quiescent state. In this resting state, endothelial cells need to facilitate proper transfer of nutrients and oxygen to the underlying tissues. Indeed, KLF2 reduces metabolic activity of endothelial cells, which results in increased levels of glucose underneath the endothelial cells in our transwell experiments (Figure 2C). It is tempting to speculate that the shear stress–mediated reduction in endothelial cell glucose consumption ensures a maximal delivery of nutrients and oxygen to the adjacent tissues. Furthermore, the present data provide a molecular mechanism for the well-known and important switch of endothelial cell activation and angiogenesis back to a quiescent functional endothelium once blood flow is established.

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**Significance**

Laminar shear stress regulates endothelial cell phenotype by inducing quiescence, but whether metabolic changes contribute to this process was not known. This study show that laminar shear stress affects endothelial cell glycolytic metabolism, which controls angiogenic behavior of endothelial cells. These findings provide mechanistic insight into how the switch between the resting and the angiogenic state of endothelial cells is regulated by physiological stimuli.
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Supplementary figure I.

(A) 2-NBD-Glucose uptake by HUVECs on a μ-slide Y-shaped (Ibidi) exposed to 20dyn/cm² shear stress for 72 hours was analyzed by flow cytometry. 10 images each from high shear and low shear region were analyzed for 2-NBD-Glucose fluorescence and cell alignment. *p<0.05 by Pearson’s correlation.

(B) Total RNA from hearts of WT and EC-KO mice were isolated and analyzed for KLF2 mRNA expression normalized to RPLP0 mRNA. (n≥4)

(C) Ratio of basal ECAR and basal respiration OCR as measured by Seahorse XFe analyzer in mock- and KLF2-transduced HUVECs. (n=3).

(D) Apoptosis as measured by Annexin V staining by flow cytometry in mock- and KLF2 transduced HUVECs. (n=5).

In all graphs, data represent mean ± S.E.M *p<0.05, ***p<0.001 (See also figure 1 and 2)
**A** Phospho-kinase proteome profile

Ratio Lenti-KLF2/Mock (log2)

- AMPKa1 (T174)
- HSP60
- B-Catenin
- c-Jun (S63)
- JNK pan (T183/Y185 T221/Y223)
- STAT6 (Y641)
- AMPKα2 (T172)
- STAT5a (Y694)
- STAT2 (Y698)
- Fgr (Y412)
- p38α (T180/Y182)
- Gsk-2 (T68)
- Lyn (Y397)
- Lck (Y394)
- FAK (Y397)
- STAT5b (Y699)
- HSP27 (S73/S82)
- AMPKα1 (Y106)
- MAPK (Y188)
- EGFR (Y1062)
- EGF R (Y1084)
- Jnk (Y181)
- ERK (S88)
- PI3K (Y1173)
- Akt (S473)
- CREB (Y133)
- p300 (S46)
- PRAS40 (T246)
- p300 (S175)
- TOR (S2448)
- Yes (Y426)
- PDGF (Y705)
- GSK (Y689)
- PKC (T136)
- Akt (T32)
- Akt (T308)
- STAT3 (Y705)
- RSK1/2/3 (S350/S358/S377)
- p70 S6 Kinase (Y421/S424)
- PLCγ (Y783)
- PLC-γ1 (Y783)
- PLC-g1 (Y783)
- eNOS (S1177)
- WNK1 (T60)
- p70 S6 Kinase (Y388)
- ERK1/2 (T200/Y204 T185/Y187)
- PYK2 (Y402)
- STAT3 (T272)
- SREBPs (S1177)

**B** p-AMPKα1 (T174) levels

- Mock
- KLF2

**C** AMPKα1 knockdown

- siNeg
- siAMPKα1 1
- siAMPKα1 2

**D** Glucose uptake

- 2-NBDG (MFI)

**E** Glycolytic function

- Basal ECAR
- Glycolysis
- Glycolytic capacity
- Glycolytic reserve

**F** Mitochondrial DNA content

- Relative DNA content (mtDNA vs. nDNA)

**G** Basal OCR

- siNeg
- siAMPKα1 1
- siAMPKα1 2

**H** Basal ECAR

- KLF2
- L-NAME

**I** Basal OCR

- KLF2
- L-NAME
Supplementary figure II.

(A) Phospho-kinase array profile showing phosphorylation levels of proteins in KLF2-transduced HUVEC versus mock-transduced controls. Code in parenthesis indicates site of phosphorylation and those highlighted in red represent proteins with significant changes in levels as analyzed by two way ANOVA. (n=4)

(B) Phosphorylated-AMPKa1 (174 levels) measured in mock- and KLF2 transduced cell lysates. Final sample concentration was calculated using corrected absorbance values against standard curve concentration. (n=3).

(C) AMPKa1 mRNA levels were measured by real time qPCR in HUVECs at 48 hours after transfection with control siRNA (siNeg) or two different siRNA directed against AMPKa1 (siAMPKa1; 1 and 2), expression normalized to RPLP0 mRNA (n=3). 48 hours after transfection with siNeg or siAMPKa1, HUVECs were analyzed for (D) Glucose uptake by flow cytometry analysis of 2-NBD-Glucose fluorescence (E) ECAR levels normalized to DNA content to quantify glycolytic function (F) Mitochondrial DNA content measured by quantitative real time PCR of mitochondrial encoded gene ND1 and nuclear encoded RPLP0. Relative levels were normalized to median values (G) Basal OCR levels normalized to DNA content measured by Seahorse flux analysis. (n=3). Mock-and KLF2-transduced HUVECs treated with 1mM L-NAME (Nω-nitro-L-arginine-methyl ester hydrochloride) for 4 hours were analyzed for (H) Basal ECAR levels and (I) basal OCR by Seahorse XF flux analysis (n=4). In all graphs, data represent mean ± S.E.M *p<0.05, **p<0.01, ***p<0.001
Supplementary figure III.

(A) Graphical representation showing evolutionary conserved regions in PFKFB3 genome (adapted from rVISTA 2.0, http://rvista.dcode.org/) (B) Enlarged region from (A) within red box showing conserved Sp1/KLF binding sites on PFKFB3 promoter (B) Partial sequence of PFKFB3 promoter constructs wildtype (WT) Sp1/KLF binding site and mutated (mut) binding site (highlighted in red) cloned into firefly luciferase reporter plasmids. (See also figure 4)
Supplementary figure IV.

(A) Total cell mRNA was analyzed for expression of KLF2 and PFKFB3 by real time qPCR in HUVECs transduced with mock, KLF2, PFKFB3 or KLF2 and PFKFB3 lentivirus. All expression levels were normalized to RPLP0 mRNA (n=6). In all graphs, data represent mean ± S.E.M ***p<0.001 (See also figure 5)
Supplementary figure V

A. HK2 knockdown

B. Basal OCR

C. Glycolytic function

D. PFK1 knockdown

E. Basal OCR

F. Glycolytic function
Supplementary figure V.
(A) HK2 mRNA levels were measured by real time qPCR in HUVECs at 48 hours after transfection with control siRNA (siNeg) or two different siRNA directed against HK2 (siHK2; 1 and 2), expression normalized to RPLP0 mRNA (n=3). 48 hours after transfection with siNeg or siHK2, HUVECs were analyzed for (B) basal OCR and (C) ECAR levels to quantify glycolytic function as measured by Seahorse flux analysis, both normalized to DNA content (n=3).
(D) PFK1 mRNA levels were measured by real time qPCR in HUVECs at 48 hours after transfection with control siRNA (siNeg) or three different siRNA directed against PFK1 (siPFK1; 1, 2 and 3), expression normalized to RPLP0 mRNA (n=3). 48 hours after transfection with siNeg or siPFK1, HUVECs were analyzed for (B) basal OCR and (C) ECAR levels to quantify glycolytic function as measured by Seahorse flux analysis, both normalized to DNA content (n=3). In all graphs, data represent mean ± S.E.M *p<0.05, **p<0.01, ***p<0.001.
Material and methods

Cell culture, transfection and lentivirus
Pooled Human Umbilical Vein Endothelial Cells (HUVEC) were purchased from Lonza (Verviers, Belgium) and cultured in EBM (Lonza) supplemented with 10% FBS (Invitrogen) and EGM-SingleQuots (Lonza). Cells were grown at 37°C and 5% CO2 and cell number was determined by NucleoCounter (ChemoMetec A/S). Lipofectamine RNAiMax (Life Technologies) was used to transfect 60-70% confluent HUVECs with 70nM siRNA (Sigma) against PFKFB3, AMPKα1, HK2 and PFK1 according to manufacturer’s instructions. Universal negative control siRNA (Sigma) was used as control. Cells were treated Nω-nitro-L-arginine-methyl ester hydrochloride (L-NAME, 1mM, Sigma). Long term overexpression of KLF2 and shRNA mediated silencing of KLF2 was done as previously described. PFKFB3 cDNA was obtained in pENTR221 (Source Bioscience) and shuttled into plenti4 vector (Invitrogen). Lentiviral particles were generated as previously described.

Shear stress
1x10^5 HUVECs were plated overnight on µ-Slides I (Ibidi) and exposed to laminar flow at a shear stress of 20 dynes/cm² for 72 hours controlled by an Ibidi perfusion system. HUVECs seeded on µ-Slides I (Ibidi) were used as static controls.

Cone plate viscometer setup
500,000 HUVECs were seeded overnight on 6cm dishes and were subjected to a fluid shear stress of 12 dyn/cm² for 48 hours in a cone plate viscometer as previously described. HUVECs maintained under static conditions served as controls.

In-vitro Glucose up-take
HUVECs were incubated with 100µM of 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG, Life Technologies) for 1hour at 37°C and 2-NBDG fluorescence was measured in the FITC channel (FL-1) using a FACS Canto II device (BD Biosciences).

Glucose uptake imaging
1x10^5 HUVECs were plated overnight on µ-slide y shaped (Ibidi) and exposed to laminar flow at a shear stress of 20 dynes/cm² for 72 hours controlled by an Ibidi perfusion system. Slides were incubated with 1mM of 2-NBDG (Life Technologies) for 30 min at 37°C and confocal micrographs were imaged on LSM 780, Axio Observer (Carl Zeiss) with a LD LCI Plan-Apochromat 25x/0.8 Imm Korr DIC M27 objective. 10 images each were analyzed from straight channel high shear region and branched low shear regions for 2-NBDG mean fluorescence and cell alignment using NIH ImageJ digital image analysis software.

Glucose transwell assay
Mock and KLF2 transduced cells were seeded on fibronectin coated 24-well ThinCert transwell PET inserts (Greiner bio-one). Cells were washed twice in Hank’s BSS (GE healthcare) and incubated with 100µM 2-NBDG (Life Technologies) and 10µM 20kDa -Dextran (TMR-Dextran, Sigma), which served as an internal control, in Hank’s BSS for 1 hour at 37°C. TMR-dextran fluorescence (Ex 525nm and Em 580-640nm) and 2-NBDG fluorescence (Ex 490nm and Em 510-570nm) were measured in duplicates from Hank’s BSS in the compartment underneath the cells using a GloMax®-Multi+ Microplate Multimode Reader with Instinct®
RNA isolation and Real Time Quantitative Polymerase Chain Reaction

Total RNA was isolated from cultured cells using miRNeasy kits (Qiagen) according to the manufacturer’s instruction. cDNA was synthesized from 0.5-1µg RNA using MuLV reverse transcriptase (Life Technologies) and random hexamers (Thermo Scientific). Real time quantitative PCR was carried out using Fast SYBR Green (Applied Biosystems) in a StepOnePlus machine (Applied Biosystems). Gene expression was normalized to RPLP0 or GAPDH. Primer sequences are listed in table 1.

Mitochondrial DNA content measurement

Total DNA was isolated from cells using DNeasy Blood and Tissue kits (Qiagen) as per manufacturer’s instruction. 30ng DNA was analyzed by real time qPCR for mitochondrial encoded gene ND1 and nuclear encoded gene RPLP0. The ratio of the relative levels of mitochondrial ND1 DNA and nuclear RPLP0 DNA was used to express mitochondrial DNA content.

Mice experiments

All mice experiments were carried out in accordance with the principles of laboratory animal care as well as according to the German national laws. The studies have been approved by the local ethical committee (Regierungspräsidium Darmstadt, Hessen). Cdh5-CreERT2 mice 5 and KLF2 flox/flox 6 were described previously and kindly provided by Dr. Adams (Münster, Germany) and Dr. Sebzda (Vanderbilt University, TN), resp. Animals were administered with seven injections of 2 mg tamoxifen base each (Sigma) intraperitoneally over a period of two weeks and sacrificed in the third week.

Ex-vivo glucose up-take in mice hearts

Mice hearts were subjected to Langendorff mediated perfusion and digestion as previously described 7 along with 50µM 2-NBDG. The digest was incubated with 200µM 2-NBDG and 5ul CD31-APC antibody (BD Pharmingen) for 30 min at 37°C and analyzed for CD31+ endothelial cell population and 2-NBDG fluorescence using a FACS Canto II device.

Measurement of cellular metabolism

HUVECs were seeded overnight at 6x10^4 cells per well on fibronectin (Sigma) coated Seahorse XF96 polystyrene tissue culture plates (Seahorse Bioscience). The plate was incubated in unbuffered DMEM assay medium (Sigma) for 1 hour in a non-CO2 incubator at 37°C before measuring in an XF® 96 extracellular flux analyzer (Seahorse Bioscience). Both OCR (oxygen consumption rate) and ECAR (extracellular acidification rate) were measured over 4 min periods with a mixing of 2 min in each cycle, with five cycles in total. Inhibitors and activators were used at the following concentrations: Glucose (10mM), Oligomycin (3µM), 2-DG (100mM), FCCP (1µM), Antimycin A (1.5µM) and Rotenone (3µM). Cellular DNA content using DAPI (Roche) was measured on a microplate reader (TECAN) and the data is represented as OCR or ECAR normalized to DNA content (RFU). Each measurement was averaged from triplicate wells.

Mitochondrial activity measurement
Cells were incubated with 0.5mg/ml of MTT (Dimethylthiazol-zyl-diphenyltetrazoliumbromide) for 4 hours at 37°C. After a wash with PBS, cells were lysed 30 min at room temperature and absorbance of the cleared lysate was photo metrically measured at 550nm.

**ATP assay**

Cell titer Glo luminescent cell viability assay kit (Promega) was used to quantify ATP levels in cells. 3x10⁴ cells in 100µl culture media were incubated with 100µl Cell-Titer-Glo Reagent (Promega) for 10 min at room temperature. Luminescence signal was measured with FlourChem M system (Proteinsimple) and signal normalized to background media control. All conditions were assayed in triplicates.

**Mitochondrial membrane potential measurement**

Mitochondrial membrane potential in HUVECs was assessed using MitoPT® TMRM Assay Kit (Immunochemistry Technologies LLC). Cells were incubated with 100nM TMRM for 20 min at 37°C and the orange red fluorescence (FL2) in cells was analyzed using FACS Canto II device (BD Biosciences).

**Proliferation assay**

Cells were incubated with 10mM BrdU for 45 min and subsequent staining was performed using BrdU Flow Kit (BD Pharmingen) as per manufacturer’s instruction. Cells were stained with 2.5µl of anti-BrdU-V450 for 20min and 10µl of 7-AAD for 10min, both at room temperature and analyzed using FACS Canto II device (BD Biosciences).

**Caspase 3/7 activity**

Apo-ONE Homogeneous Caspase-3/7 assay kit (Promega) was used as per manufacturer’s instruction and Caspase 3/7 activity was analyzed by measuring fluorescence at with an excitation of 490nm and emission of 510-570nm using a GloMax®-Multi+ Microplate Multimode Reader with Instinct® (Promega). Mock transduced cells treated with 200nM of staurosporine for 4 hours were used as a positive control.

**Apoptosis measurement**

Mock- and KLF2 transduced cells were stained with Annexin V V450 (BD Biosciences) and 7-AAD (BD Biosciences) in binding buffer (BD Biosciences) for 15 min at room temperature as per manufacturer’s instructions. Apoptosis was quantified by flow cytometry analysis on a FACS Canto II device (BD Biosciences).

**Senescence staining**

Acidic β-gal staining mixture was added onto cells for 24 hours as per manufacturer’s instruction (Cell signaling). Cells were imaged on a bright field microscope (Axiovert 100, Zeiss) and 5 image fields were analyzed per condition for positive β-gal staining using digital image analysis software (AxioVision Rel. 4.8, Carl Zeiss).

**Phospho kinase proteome profiling**

5x10⁶ mock- and KLF2- transduced cells were processed using Human Phospho-Kinase Array Kits (Proteome Profiler™ R&D Systems, Minneapolis, MN) according to manufacturer’s instructions. The phosphorylation level of proteins was analyzed by quantification of membrane profile panels by NIH ImageJ digital image analysis software with a Dot Blot Analyzer macro
plugin. The signal values were subtracted from negative control values (PBS) and further normalized to whole membrane signal intensity.

**AMPK\(\alpha\)1 phosphorylation measurement**

2x10^7 mock- and KLF2- transduced cells were analyzed using DuoSet IC Human Phospho AMPK\(\alpha\)1 (T174) elisa kits (R&D Systems) according to manufacturer’s instruction. Absorbance was measured at 450nm using a GloMax®-Multi+ Microplate Multimode Reader with Instinct® (Promega) and wavelength correction was set to 560nm. Concentration was determined from a standard curve performed along with the samples.

**RNA sequencing**

For RNA sequencing, 0.5μg total RNA isolated from static HUVEC and shear stress exposed HUVEC were used. Sequencing libraries were prepared as described previously^6. The deep sequencing data was mapped against the reference genome GRCH37 by applying tophat2. Afterwards the transcript abundance was calculated by Cufflinks v2.2 based on the Ensemble annotation. The sequence data have been deposited in the NCBI GEO database under accession number GSE54384.

**Western blotting**

HUVECs were lysed in RIPA buffer (Sigma) supplemented with protease and phosphatase inhibitors (Roche) for 20min on ice. Western blot analysis was performed as described previously by using antibodies against PFKFB3, HK2, PFKP (1:1000, Cell signaling), KLF2^9 and Tubulin (1:2000, Thermo Scientific). All protein levels were normalized to tubulin signal.

**Luciferase assay**

Luciferase promoter reporter experiments were performed as described^10. Specifically, the region bearing the putative KLF2 binding site in PFKFB3 promoter or a mutated version of the KLF2 binding site (see Figure S3) were cloned into firefly luciferase reporter plasmid pGL4.10 according to manufacturer’s instructions (Promega). Cells were co-transfected with luciferase plasmid and pGL4 Renilla plasmid (Promega) as control for transfection efficiency by electroporation using Neon transfection system (Invitrogen). The activity of Luciferase and Renilla was assessed after 24 hours with the Dual Luciferase reporter assay system (Promega)

**Tube formation assay**

HUVECs were cultured at a density of 1x10^5 on Matrigel basement membrane matrix (BD) and tube formation was analyzed after 24 hours. Cumulative tube length was quantified from microscopic images taken from 5 random fields for every condition. Quantification was carried out by digital image analysis software (AxioVision Rel. 4.8, Carl Zeiss)

**Spheroid based angiogenic assay**

Endothelial cell spheroids were generated as previously described^11. Cumulative length of sprouts was quantified from 10 spheroids for every condition. Quantification was carried out by digital image analysis software (AxioVision Rel. 4.8, Carl Zeiss)

**Aortic Ring Outgrowth Assay**

Aortae were isolated from WT (KLF2^fl/fl) and EC-KO (Cdh5-CreERT2;KLF2^fl/fl) mice and cultured as previously described^12. Briefly, cleaned 1 mm long aortic tissue was embedded in rat
tail collagen type I gel (1mg/ml, Millipore) in a 96 well plate and cultured in DMEM/F-12 medium (Life technologies) containing 2.5% FBS and treated with or without 3-PO (50µM, Merck Millipore). Endothelial sprouts were allowed to develop over 7 days, which were fixed with 4% PFA and stained with biotin Isolectin B4 (Vector laboratories) and streptavidin AlexaFluor 488 (Molecular Probes). Photomicrographs of sprouts from aortic rings were taken with Axio Observer.Z1 microscope (Zeiss) and cumulative outgrowth length from 3 rings per condition was quantified using NIH Image J digital image analysis software.

Statistical analysis
Data are expressed as mean ± S.E.M. GraphPad Prism 5 software was used to assess statistical significance by student t-test or Mann-Whitney U test when comparing two groups or analysis of variance (ANOVA) followed by Bonferroni’s correction or Kruskal-Wallis test with Dunns correction for multiple comparisons. Statistical significance was defined as follows: *p<0.05, **p<0.01, ***p<0.001.

Table 1. Oligonucleotide primer sequence list

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<th>Target gene</th>
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References


