Endothelial Krüppel-like Factor 4 Regulates Angiogenesis and the Notch Signaling Pathway

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Capsule

Background: The transcription factor Krüppel-like Factor 4 (KLF4) is a critical regulator of endothelial cell biology.

Results: Sustained expression of endothelial KLF4 limits tumor growth by creating ineffective angiogenesis.

Conclusion: KLF4 is an upstream regulator of angiogenesis in part by mediating Notch expression and activity.

Significance: KLF4 regulates sprouting angiogenesis and may be a therapeutic target in regulation of tumor angiogenesis.

Abstract

Regulation of endothelial cell biology by the Notch signaling pathway (Notch) is essential to vascular development, homeostasis, and sprouting angiogenesis. While Notch determines cell fate and differentiation in a wide variety of cells, the molecular basis of upstream regulation of Notch remains poorly understood. Our group and others have implicated the Krüppel-like factor family of transcription factors as critical regulators of endothelial function. Here, we show that Krüppel-like factor 4 (KLF4) is a central regulator of sprouting angiogenesis via regulating Notch. Using a murine model in which KLF4 is overexpressed exclusively in the endothelium, we found that sustained expression of KLF4 promotes ineffective angiogenesis leading to diminished tumor growth independent of endothelial cell proliferation or cell cycling effects. These tumors feature increased vessel density yet are hypoperfused, leading to tumor hypoxia. Mechanistically, we show that KLF4 differentially regulates expression of Notch receptors, ligands, and target genes. We also demonstrate that KLF4 limits cleavage-mediated activation of Notch1. Finally, we rescue Notch target gene expression and the KLF4 sprouting angiogenesis phenotype by supplementation of DLL4 recombinant protein. Identification of this hitherto undiscovered role of KLF4 implicates this transcription factor as a critical regulator of Notch, tumor angiogenesis, and sprouting angiogenesis.
Introduction

Formation of a functional vascular network via sprouting angiogenesis occurs by a coordinated series of events including selection of a leading tip cell, sprout elongation and stalk formation, branching, anastomosis, pruning and finally, stabilization of the connected network. The Notch family of ligands, receptors, and transcription factors (Notch) has critical but incompletely understood roles in vascular development (1). Mouse models null for the Notch receptor NOTCH1 and the Notch ligands DLL1, DLL4, & JAG1 are embryonic lethal, demonstrating a failure to remodel the primitive vascular plexus with defective arterial branching and hemorrhage (2, 3, 4, 5).

Tip cells are specialized endothelial cells (ECs) that lead to outgrowth of new sprouting vessels. Studies have shown that increased tip cell expression of the Notch ligand DLL4 suppresses the tip cell phenotype in adjacent endothelial cells resulting in coordinated, effective sprouting and network formation (6). This DLL4-mediated lateral inhibition of DLL4 expression encourages neighboring ECs to become the proliferating stalk cells that make up the lumenized body of the developing vessel. In the absence of DLL4-NOTCH1 signaling, all sprouting ECs become tip cells and an organized, mature vascular network is not formed (7). Dynamic fluctuation of DLL4 expression occurs at the cellular level during normal sprouting angiogenesis in vertebrates and Drosophila melanogaster (8, 9). In mouse models, this dynamic DLL4 expression pattern is partially due to utilization of a VEGF-VEGFR-DLL4-Notch-VEGFR feedback loop (8); however, the molecular mechanisms governing upstream regulation of Notch have otherwise remained elusive.

Krüppel-like factors (KLFs) are increasingly appreciated as key regulators of endothelial cell biology. KLFs are members of the zinc-finger family of transcription factors known to play important roles in the regulation of cellular growth and differentiation (10). Our group has previously shown that Krüppel-like factor 4 (KLF4) is regulated by physiologic stimuli (e.g. shear stress and inflammatory stimuli); and KLF4 regulates key endothelial targets that modulate the inflammatory and coagulant state of the endothelium (11). More recently, using a mouse model of atherothrombosis, we have unequivocally demonstrated that EC KLF4 is an important in vivo regulator of critical endothelial functions (12). However, a role of endothelial KLF4 in neovascularization has not yet been described. Herein, we propose that KLF4 is an upstream regulator of Notch expression and that sustained expression of KLF4 leads to ineffective angiogenesis, resulting in pronounced attenuation of tumor growth.

It is becoming apparent that dual anti-angiogenesis therapy will be required to treat tumors as many become resistant to anti-VEGF therapy alone (13). In recent years, combination anti-tumor therapy using anti-VEGF agents coupled with controlled expression of DLL4 has been an active area of discussion (14); however, the upstream signaling pathways that regulate expression of Notch in blood vessels have remained largely unknown (15). We have discovered that the endothelial transcription factor KLF4 regulates mammalian neovascularization with a phenotype reminiscent of DLL4 blockade (16, 17) suggesting that KLF4-mediated regulation of Notch may be an attractive therapeutic target.

Experimental Procedures

Mice. EC-KLF4 Tg mice are on a C57BL/6 background and were generated in our laboratory using a VE-Cadherin promoter (gift of K. Walsh, Boston University, Boston, Massachusetts, USA) driving the expression of a human KLF4 transgene. Mice were assessed for EC-specific overexpression previously by protein levels (12) and by mRNA copy number analysis herein. All animals and protocols were used in accordance with the Institutional Animal Care and Use Committee of Case Western Reserve University and with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Tumor Inoculation Studies. Subcutaneous injection of 1-2.5x10⁶ pathogen-free B16-F10 melanoma cells into the rear flank was
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Three experiments of n=4-14 animals per group) as previously described (18). Eight to ten days after injection animals were sacrificed and tumors were harvested, weighed and photographed. One hour prior to sacrifice Hypoxyprobe (60 mg/kg; Chemicon; HP2-1000) was administered via intra-peritoneal injection and 5 minutes prior to sacrifice 100 μl FITC-labeled Lycopersicon Esculentum lectin (20 μg/mL; Vector Laboratories; FL1171) was administered intravenously.

Spheroid formation assays. Spheroid formation assays were performed as previously described (19). Fifty μL/well of Matrigel diluted 1:1 in cold serum-free DMEM was pipetted into a 96-well plate and incubated at 37°C for ~1 hour. The diluted Matrigel was supplemented with either 0.2μg/mL of recombinant DLL4-Fc (Abcam, ab108557) or IgG-Fc isotype control (Abcam, ab90295). 3x10^4 primary mouse cardiac EC were then seeded in each well and cultured in DMEM containing 20% FBS, 25 μg ECGS, 1% penicillin/streptomycin, and 1% heparin. The cells were allowed to grow for 3-4 days until visible sprouts were observed. The cells were subsequently fixed in 4% paraformaldehyde and imaged (100x) using an inverted photomicroscope. Endothelial structures 0.19 mm or larger were considered sprouts. Quantification was performed using ImageJ (NIH).

Notch activation assays. 96-well plates were coated with 10 μg/mL recombinant DLL4-Fc (Sinobiologicals, 10171-H02H) or IgG-Fc isotype control (Abcam, ab90295) suspended in sterile phosphate buffered saline (PBS), incubated at room temperature for ~6 hours, and aspirated off before EC seeding. The tissue culture plate was rinsed with warmed PBS before 1.5x10^4 UVEC/well were seeded and incubated at 37°C for 16 hours. The cells were rinsed with warmed PBS then lysed and harvested for gene expression analysis. Two wells were pooled for RNA isolation and considered an n=1. Each experiment was performed using an n=3 for each condition and repeated three times. Data was pooled from all experiments and represented as relative gene expression normalized to empty virus (EV) IgG. Statistical significance was determined using a one-way unstacked ANOVA.

Transient Transfection Assays. We assessed Notch activity in transfection experiments using a CSL concatamer reporter (CBX4) and cotransfection of NICD and KLF4 expression plasmids. Notch activation was determined using a Veritas luminometer (Promega) and data is expressed as relative luciferase units (RLU). We determined the effect of KLF4 on activation of Notch target gene activity using a HES1 promoter-reporter construct. We also performed assays in which we adenovirally overexpressed KLF4 and NICD in order to determine patterns of interaction between these transcriptional regulators on endogenous Notch family member and Notch target gene expression. The ICN (described here as NICD) retrovirus was created as previously described (20).

Endothelial Cell Isolation. Murine cardiac EC were isolated as described previously (12). Hearts from 5 mice were pooled, sorted, and purified using Dynal beads (Invitrogen, 110.07) at a concentration of 4x10^6 beads/mL. The first purification round was performed using an anti-PECAM antibody, and the second using an anti-ICAM antibody (BD Pharmigen, 553370 and 553325, respectively). Murine EC were cultured in DMEM supplemented with 20% FBS, 50mg ECGS, 1% penicillin/streptomycin, 0.1% Fungizone, and 1% heparin. EC at passage 1-3 were used for all experiments.

HUVEC were isolated as previously described (22, 23). De-identified umbilical cords were collected from the maternity ward at University Hospitals Case Medical Center with institutional consent. Cords of 10-30 cm of length were used for all studies. Briefly, the umbilical vein was flushed with 1X HBSS to remove blood. The vein was then clamped using a hemostat and filled with 0.2% wt/vol collagenase (Roche, 103586) until the vein was moderately distended. The cord was then massaged gently prior to incubation at room temperature for 30 minutes. The collagenase digest was collected and a final volume of 50 mL was obtained with additional HBSS. The
material was centrifuged at ~1200 rpm for 5 minutes, the supernatant was aspirated, and the pellet resuspended in 10 mL of EBM-2 media (Lonza, CC-3162) with EGM-2 aliquots (Lonza, CC-4176) and seeded on plates coated with 0.1% gelatin. The resulting cell culture was incubated overnight at 37°C with 5% CO₂. For all cell culture past p1, EBM2 media with EGM-2 aliquots was used. All primary HUVEC used were at p1-4 and commercial HUVEC were p1-7.

Cell Culture, Viral Infection, RNA Isolation, and Reverse Transcription. All studies were performed with HUVEC we isolated from umbilical cords, commercially obtained (Lonza) HUVEC, or human aortic endothelial cells. Similar results were obtained from each cell source. Efficacy of adeno- or lenti-viral infection was assessed by quantitative polymerase chain reaction (qPCR) of total cellular RNA isolated from the respective cell source. The adenovirus overexpresses human KLF4 (Ad-K4) while the lentivirus overexpressing KLF4 (Lenti-K4) contains a mouse transgene (pLOVE-KLF4, Addgene plasmid #15950; control EV is pLOVE, Addgene plasmid #15948). Effects on gene expression in all overexpression experiments were assessed 2 days after virus infection. Appropriate empty viral constructs were used as controls. Knockdown of KLF4 was achieved using Dharmacon On-Target siRNA plus (J-005089-09, 9314; K4-/-) and appropriate non-targeting siRNA control (D-001810-01-05; NS). Effects on gene expression were assessed two days after virus infection. Appropriate empty viral constructs were used as controls. Knockdown of KLF4 was achieved using Dharmacon On-Target siRNA plus (J-005089-09, 9314; K4-/-) and appropriate non-targeting siRNA control (D-001810-01-05; NS). Effects on gene expression were assessed two days after virus infection. Appropriate empty viral constructs were used as controls.

Quantitative PCR. All qPCR reagents used were from Light Cycler 480 kits (Roche, 04887-301001 or -352001) using probes from the Roche Universal Probe Library or SYBR green. Gene expression was normalized to SDHA, β-actin, GAPDH, 36B4, or Pecam using the ΔΔCp method and shown as relative fold-change to the respective control. Primer sequences are shown in Table 1.

Copy Number Analysis. To perform absolute quantitation of mKLF4 overexpression after lentiviral infection in HUVEC or of hKLF4 overexpression in EC-K4 Tg tissues, we created a standard curve (copy number vs. Cp) of copy number by performing qPCR using purified amplicon for mouse or human KLF4 derived from their respective EC. We then derived copy number from the line of best fit for the standard curve using the Cp for KLF4 from our cell/tissue samples.

Chromatin Immunoprecipitation Assays (ChIP). ChIP assays were performed according to manufacturer instructions (Millipore, 17-295). Briefly, HUVEC were infected with EV or an adenovirus overexpressing human KLF4 (Ad-K4) for 48 hours then fixed in 1% paraformaldehyde at 37°C for 10 minutes. The fixed HUVEC were then washed in cold PBS (with protease inhibitors), scraped and resuspended in lysis buffer. DNA was sheared to fragments between ~200-1,000-bp in size. The cell lysate was collected and diluted 10-fold, followed by treatment with protein A-agarose/salmon sperm DNA slurry. The supernatant was then incubated with 5 µg of KLF4 antibody (Sigma, HPA002926) or IgG control overnight at 4°C. The immunoprecipitant was collected by pulldown with protein A-agarose/salmon sperm DNA slurry and washed. DNA-protein crosslinks were reversed with 5M NaCl and DNA was recovered using the Qiagen PCR purification kit (28104). Input and immunoprecipitated products were subjected to qPCR using the primers listed in Table 2. Data is normalized to input DNA and expressed as percentage of input.

Western blotting. Protein analysis was performed using 75-150 µg of denatured whole cell lysate in 8% SDS-PAGE. Rabbit anti-human cleaved NOTCH1 (NICD) (1:500;
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Cell Signaling, 2421) was used with a goat anti-rabbit Alexa-fluor conjugated secondary antibody (1:800; Invitrogen, A-11012) and ECL-plus chemiluminescent detection reagent (Amersham). Densitometry was performed using ImageJ (NIH), and represents data pooled from four separate blots using HUVEC derived from separate umbilical cords.

Matrigel sprout formation assays. Sprout formation assays with HUVEC were performed using growth factor-reduced (GFR) and regular Matrigel (BD Biosciences, 354230 and 354234) as previously described (21). Growth-factor enriched (normal) or growth-factor reduced (GFR) Matrigel was plated in a 12-well plate in triplicate. Using 6x10^5 cells/well, sprouts were allowed to form for ~6-8 hours, then cultures were fixed with 4% paraformaldehyde and imaged using phase contract microscopy. Images were analyzed using ImageJ for total cellularity and tubule length, number of tubules, number of nodes, and tubule: node ratio. Data was pooled from 3 experiments using 3 wells per condition and 3 images per well. Data was analyzed using a one-way unstacked ANOVA and student’s t-test for paired normoxia-hypoxia samples.

Tissue Preparation and Immunohistochemical Analysis. Harvested tumors were carefully partitioned in optimal cutting temperature medium, placed on dry ice, and frozen at -80°C for at least 24 hours prior to sectioning; cryosections were cut into 10 µm slides for immunohistochemical (IHC) analysis. IHC studies were performed for vessel density analysis by staining for PECAM (1:100; BD Pharmigen, 553370) and KLF4 levels (1:50; R&D systems, AF3158). Corresponding Alexa-fluor-conjugated secondary antibodies were used (1:800; Invitrogen, donkey anti-rat, A-21208) and (1:200; Invitrogen, chicken anti-goat, A-21467), respectively. Hypoxyprobe staining was performed using Hypoxyprobe monoclonal antibody conjugated with FITC (1:200; Chemicon, 90529) with corresponding anti-FITC secondary antibody (Chemicon, 90530). Pericyte coverage was assessed using PECAM, Ng-2 (Millipore, AB5320), and laminin (Covance, PRB-432C) antibodies. Assessment of proliferation in tumors was performed by co-staining for 4',6-diamidino-2-phenylindole (DAPI, GBI labs; E19-18) and proliferating cell nuclear antigen (PCNA, Cell Signaling Technology; 13110).

Statistics. All data are reported as the mean ± SEM pooled from all experiments. Analysis between two paired samples was performed using a two-tailed unpaired student’s t-test. Analysis between more than two sample groups was performed using a one-way unstacked ANOVA (Minitab). For all data, statistical significance was defined as p < 0.05 and notated by *.

Results

KLF4 has features characteristic of an angiogenically-active factor. We first sought to determine the role of KLF4 in regulating angiogenesis-associated genes in vitro using overexpression and knockdown experiments and assessing target gene expression by qPCR. These experiments were performed using HUVEC infected with Lenti-K4, achieving ~7 fold overexpression, or KLF4 siRNA (K4 -/-) achieving ~80% knockdown, and appropriate EV and NS controls, respectively (Fig. 1A-B). Overexpression and knockdown of KLF4 was assessed by fold change (Fig. 1A) and copy number analysis (Fig. 1B). Overexpression of KLF4 promotes expression of canonical angiogenesis-associated target genes HIF-1α, VEGFR1, and eNOS, while decreasing VEGFR2 expression levels (Fig. 1C, left panel). Knockdown of KLF4 leads to decreased expression of HIF-1α, VEGFR1, VEGFR2 and eNOS (Fig. 1C, right panel). Overexpression of KLF4 leads to increased VEGFA expression independent of hypoxia (3% O₂) (Fig. 1D, left panel). Knockdown of KLF4 limits VEGFA expression independent of hypoxia (Fig. 1D, right panel). Similar results were obtained in commercially available Lonza HUVEC and human aortic endothelial cells. Endogenous KLF4 expression is induced by hypoxia (Fig. 1E). Briefly, we overexpressed human KLF4 in HUVEC and immunoprecipitated KLF4 bound to target DNA. We designed primers flanking the putative KLF4 binding site
CACCC to determine fold enrichment of KLF4 on target promoters. Limited mechanistic studies (ChIP) demonstrate enrichment of KLF4 at specific CACCC sites in the promoters of VEGFA, VEGFR1 and VEGFR2 (Fig. 1F). These findings suggest that KLF4 expression is sensitive to angiogenic stimuli and that KLF4 plays a direct role in transcriptional regulation of angiogenesis.

**EC KLF4 regulates sprouting angiogenesis in vitro.** Next, we utilized an *in vitro* Matrigel model of sprouting angiogenesis in the context of EC overexpression and knockdown of KLF4 and exposure to hypoxia in order to study the effect of KLF4 on sprout formation. Notably, sustained overexpression of KLF4 markedly increased total cellular area in normal Matrigel culture independently of and synergistically with hypoxia, demonstrating enhanced overall sprout formation (Fig. 2A). Hypoxia, a potent stimulus of neovascularization, increased the number of sprouts and number of nodes in a similar manner to overexpression of KLF4 (Fig. 2B). In GFR Matrigel, KLF4-stimulated increases in cellular area, sprouts and nodes are even more prominent than those induced by hypoxia. (Fig. 2C-D). We observed no difference in sprout: node ratio or average sprout length with either hypoxia or sustained KLF4 expression in GFR Matrigel (Fig. 2C-D). Representative images are shown in Fig. 2E.

Knockdown of KLF4 resulted in decreased cellular area, sprouting and number of nodes in normal and GFR Matrigel (Fig. 3A-D). With knockdown of KLF4 we observed no difference in length per sprout and sprout: node ratio in normal Matrigel. However, in GFR Matrigel, we observed decreased length per sprout and sprout: node ratio with knockdown of KLF4 (Fig. 3C-D). Representative images are shown in Fig. 3E.

Together, these findings indicate that KLF4 expression is responsive to stimuli that induce neovascularization and that altered levels of KLF4 translates to an angiogenic response, particularly in an environment of low oxygen tension. Overall, these data suggest that KLF4 plays a role in neovascularization, warranting *in vivo* study.

**EC KLF4 overexpression inhibits tumor growth by causing ineffective angiogenesis.** To define the *in vivo* role of EC KLF4 in neovascularization, we used mice with endothelial-specific overexpression of human KLF4 driven by the VE-cadherin promoter (EC-K4 Tg). These mice demonstrate enhanced EC-specific KLF4 expression as previously demonstrated (12). Herein we employed a widely used model of *in vivo* angiogenesis, i.e. subcutaneous flank inoculation of B16-F10 melanoma cells (18). In EC-K4 Tg mice, tumor growth at 10 days after inoculation of 1.0x10^6 or 2.5x10^6 B16-F10 melanoma cells is significantly decreased compared to WT mice (Fig. 4A). Representative images (1.0x10^6 cells) are shown in Fig. 4B. The EC-K4 Tg mice demonstrate enhanced EC KLF4 signal in EC-K4 Tg tumors (Fig. 4C), as best illustrated at higher magnification (right panels). To assess for analogous changes in our *in vivo* angiogenesis, we isolated RNA from the vascular rim of the tumors. Tumor tissue from EC-K4 Tg mice shows increased expression of Vegfa and the EC markers Vwf and Pecam, consistent with the increased vessel density documented by IHC (Fig. 4E). In order to identify EC that are exposed to circulating blood, FITC-labeled lectin was infused intravenously prior to animal sacrifice. Post-sectioning, tumors were costained for PECAM using a red-fluorescing secondary antibody. All vessels are stained red and perfused vessels appear green/yellow when assessed by fluorescence microscopy. The ratio of perfused to non-perfused (green/yellow: red) vessels in WT tumors is markedly higher than that in EC-K4 Tg tumors (Fig. 4F). Thus, despite the existence of many more vascular structures in the EC-K4 Tg tumors, there is deficient perfusion. Poor perfusion would be expected to lead to tissue hypoxia and limited growth. Assessment of tumor hypoxia using Hypoxyprobe demonstrates a decrease in the distance...
between the vascular tumor rim and hypoxic areas in EC-K4 Tg tumors to approximately half of that in the WT tumors (Fig. 4G), consistent with impaired perfusion of the EC-K4 Tg tumors. Assessment of melanoma proliferation by staining with proliferating-cell-nuclear-antigen (PCNA) shows that proliferating cells extends fairly deeply towards the tumor center in the WT tumor, but is largely limited to the vascular rim in the EC-K4 Tg tumors (Fig. 4H, tumor edge is to the left in both photographs). These results are consistent with the Hypoxyprobe results. However, in the tumor areas where proliferation was observed, the percentage of PCNA+ cells were similar in WT and EC-K4 Tg tumors (WT 54.7± 7.13%, EC-K4 Tg 45.69± 9.73%). Similarly, we observed no significant difference in tumor proliferation markers Mybl2, Bub1, Plk1,Ccne1, Ccnd1 and Ccbn1 in tumor rim homogenate (Fig. 4I). Finally, as a gross measure of vessel maturation we assessed vessel pericyte coverage. There was no difference in pericyte coverage between WT and EC-K4 Tg tumor vessels as demonstrated by analysis of NG-2 and laminin IHC (Fig. 4J; PECAM:laminin signal is 0.89 arbitrary units of area (AUA) for WT and 0.90 for EC-K4 Tg; PECAM:Ng-2 signal is 1.13 AUA for WT and 1.27 for EC-K4 Tg). Taken together, these data demonstrate that EC-K4 Tg mice have a tumor angiogenesis phenotype of ineffective (hypoperfused) hypervascularity leading to reduced tumor growth.

The role of KLF4 in EC proliferation, apoptosis, migration, metabolism and cell cycling. Despite enhanced vascularity of EC-K4 Tg tumors, we observed no changes with overexpression of KLF4 in EC proliferation, apoptosis (Caspase 3/7 activity), or migration (wound healing “scratch” assay) in in vitro experiments (Fig. 5A-C). We also detected no effect of KLF4 overexpression on EC metabolic rate using a cellular metabolic reduction assay (alamarBlue assay; Fig. 5D). However, with knockdown of KLF4 there is a profound inhibition of proliferation, increase in apoptosis, migration defect and decrease in metabolic rate (Fig. 5E-H). Furthermore, overexpression of KLF4 did not cause appreciable changes in cell cycling by flow cytometry or in expression of a panel of cell cycle-regulating genes with overexpression of KLF4 (Fig. 6A-C).

The phenotype of small tumors with high vessel density is reminiscent of that seen with Dll4 blockade (16, 17). In those studies, inhibition of Dll4 activity with either soluble Dll4 or neutralizing anti-Dll4 antibodies led to growth-limiting dysregulation of tumor angiogenesis in multiple solid tumor models. Thus, we decided to investigate the relationship between KLF4 and Notch.

EC KLF4 regulates Notch expression and activity. The similarity of the EC-K4 Tg tumor phenotype to that seen with Dll4 blockade raised the possibility that KLF4 might regulate development of vascular branches during sprouting angiogenesis by regulating Notch. When normalized to Pecam, we observed decreased Dll4 expression in tissue dissected from the EC-K4 Tg tumor vascular rim (Fig. 7A), analogous with the Dll4-inhibition vascular phenotype (16, 17). Furthermore, we observed changes in Notch1, Hey2, and Dll1 expression in this tissue (Fig. 7A). These data lead us to investigate the relationship between KLF4 and Notch.

After ligand binding to receptor, the next phase of canonical Notch activation is mediated by sequential cleavage, nuclear translocation, and binding of the Notch intracellular domain (NICD; the cleaved intracellular fragment of NOTCH1), to a transcriptional complex containing the DNA-binding protein CSL (CBF1, Suppressor of Hairless, Lag-1). Binding of NICD to CSL converts a transcriptionally inhibitory complex into an activating complex (4). Thus, we performed assays in which we virally overexpressed KLF4 and NICD in order to determine independent and combinatorial effects of these transcription factors on Notch family member and Notch target gene expression. In vivo expression of many of the Notch family members appears limited to the arterial beds; however, EC from venous sources cultured in vitro also express Notch and are used to assess Notch function (24, 25). For the following experiments, results were consistent when we used primary or
commercially obtained HUVEC as well as commercially obtained human aortic EC. KLF4 overexpression led to a significant decrease in expression of Notch receptors NOTCH1 and NOTCH4, but did not affect NOTCH2 and NOTCH3 expression (Fig. 7B, left panel). Next, we observed that NICD-enhanced NOTCH1 expression is unaffected by KLF4 overexpression (Fig. 7B, right panel). In regard to Notch ligands, overexpression of KLF4 led to decreased expression of DLL4, increased expression of DLL1, and no change in JAG1 expression (Fig. 7C, left panel). We also observed that NICD-enhanced DLL4 expression is limited by KLF4 (Fig. 7C, right panel). Intriguingly, we observed that DLL1 expression is induced by KLF4 independent of NICD stimulation (Fig. 7C, right panel). Together, these results suggest that KLF4 controls Notch expression in part by direct transcriptional regulation.

KLF4 regulates Notch activity and sprouting angiogenesis through NOTCH1 and DLL4. Next we sought to investigate the role of KLF4 in mediating Notch activity as measured by receptor cleavage. We lentivirally overexpressed KLF4 and used appropriate EV control in primary HUVEC, isolated protein, and assessed Notch activation by western blot using an antibody specific for NOTCH1 NICD. We found that overexpression of KLF4 in HUVEC inhibits accumulation of NICD (Fig. 8A). Thus, sustained expression of KLF4 results in reduction of both DLL4/NOTCH1 expression and NOTCH1 activation (Fig. 7B, 7C and 8A).

We hypothesized that downregulation of DLL4 by KLF4 may be the mechanism by which sustained EC expression of KLF4 results in a sprouting angiogenesis phenotype consistent with that created by direct DLL4 blockade. Dynamic expression of DLL4 has been demonstrated to play a central role in tip vs. stalk cell identity, thus finely tuned regulation of this molecule is an important determinant of vascular network development (8). As a first step in determining whether supplementation with DLL4 protein can ameliorate the effect of sustained KLF4 expression on Notch, we used the recombinant fusion protein DLL4-Fc. Importantly, immobilized DLL4-Fc activates Notch (26). We coated tissue culture plates with either DLL4-Fc or IgG-Fc isotype control and seeded HUVEC with lentivirally-sustained overexpression of KLF4 or EV control. Inhibition of the Notch target genes HES1,
HEY1, and HEY2 by KLF4 is reversed by the presence of immobilized DLL4-Fc (Fig. 8B).

In order to recapitulate our tumor phenotype in vitro, we used EC spheroids made from primary cardiac EC isolated from WT and EC-K4 Tg mice. In 3D culture (Matrigel), EC-K4 Tg spheroids demonstrate enhanced sprout formation that is “rescued”- sprout number is reduced- by addition of DLL4-Fc protein to the Matrigel matrix (Fig. 8C; representative photographs in Fig. 8D). In sum, our data suggests that KLF4 regulates Notch expression and activity. Sustained expression of EC KLF4 in vivo significantly alters vascular network formation and leads to significant physiological consequences, as demonstrated by the tumor angiogenesis model, and is consistent with inhibition of the dynamic expression patterns of the DLL4/Notch1 signaling pathway.

Discussion

The primary roles of the endothelium include regulation of vascular tone, coagulant and inflammatory state, permeability, and control of new blood vessel growth. Over the past several years evidence has accrued that suggests the transcription factor KLF4 plays a significant role in the first four of these five essential functions (11, 12, 27, 28). This study completes the description of KLF4 as a mediator of each of these fundamental processes; herein we show that sustained endothelial-specific overexpression of KLF4 causes a profound dysregulation of tumor sprouting angiogenesis, leading to marked reduction in tumor size. The precise molecular mechanisms by which KLF4 modulates angiogenesis are as yet undetermined; however, our data suggests that interaction with the Notch signaling pathway is an important part of its effect. Notch has a central role in modulating sprouting angiogenesis, with one of the most interesting aspects being control of generation of tip cells in the emerging vascular sprouts. Our data suggests that EC KLF4 regulates EC Dll4 expression, and may thusly be involved in control of tip cell generation. The importance of this interaction is supported by Drosophila experiments demonstrating that Krüppel (Kr) determines the fate of neural tip cells in developing Malpighian tubules, and that Kr expression is controlled by Notch-dependent (Delta) signaling (29). Indeed, Delta/Dll4 has been ascribed central roles in epithelial and endothelial sprouting in Drosophila, zebrafish, and mice (4). Interaction between Notch and KLF4 has been shown in terminal differentiation of epithelial cells in mouse models and in human epithelial cell lines (30-31). Hence, interaction between analogs of Notch and KLF4 may play a phylum-spanning fundamental role in both organ development and cell differentiation.

Consequently, understanding the regulatory mechanisms of members of the Notch signaling pathway and KLF4 are of interest and, for both, at a surprisingly nascent stage. In ECs KLF4 expression is regulated by shear stress, physiologic inflammatory mediators, and by some agents with therapeutic potential such as HMG-CoA reductase inhibitors (statins) and resveratrol (11, 12, 33). Herein we add “Notch” and hypoxia to the list of regulators of EC KLF4 expression. While discovery of upstream regulators of Notch is a burgeoning field, much remains to be elucidated. Perhaps particularly pertinent to the current study is an elegant series of experiments by Jakobssen et al. showing that dynamic, likely sequential, changes in levels of each member of a VEGFR-Dll4-Notch signaling circuit occur concurrent with dynamic changes in tip versus stalk cell differentiation during angiogenic sprouting (8). In both in vitro and in vivo experiments we used sustained overexpression of KLF4 to reveal an angiogenic phenotype. It is intriguing to consider that it may be dynamic expression of KLF4 that is required for effective sprouting angiogenesis. In this manuscript we show that KLF4 regulates expression of other angiogenically active factors in addition to Notch and Notch target genes, including VEGFA, VEGFR1 and VEGFR2. While the multifactorial transcriptional effects of KLF4 can make it difficult to attribute its function to a singular target, these results are consistent with those we have found in other physiologically relevant contexts such as the development of
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atherosclerosis (12) and pulmonary artery hypertension (27). In fact, diverse activating or inhibitory transcriptional modulation is likely integral to the homeostatic role EC KLF4 appears to play. The data presented in this manuscript is the first to suggest that KLF4 may be a central player of dynamically interrelated signaling pathways that mediate sprouting angiogenesis.

The function of KLF4 appears highly cell type-specific and context-dependent. We have described a profound effect on tumor angiogenesis; however, global KLF4 knockout has no obvious developmental vascular phenotype (35). Contrastingly, KLF4 is required for terminal differentiation of goblet cells during development, but not in the adult mouse (30, 31). Rowland et al. demonstrated that the role of KLF4 as a tumor-suppressor or –activator is context-dependent, with differential effects on cell proliferation depending upon the presence or absence of p53 (36). Indeed, several laboratories have described increased cell proliferation as a result of KLF4 deficiency, but there is contradictory data on the proliferative effect of KLF4 in B-cells, keratinocyte stem cells, and endothelial progenitor cells (37-40). We found no discernable effect of sustained KLF4 overexpression on HUVEC proliferation, cell cycling, and metabolism. Thus, KLF4-mediated EC proliferation is unlikely to be a primary cause of the increased vascular density in tumors of EC KLF4 Tg mice. Further studies, in context-dependent models, might elucidate how our data correlates to data suggesting that antibody-mediated blockade of EC Dll4 enhances proliferation as well as sprouting in co-culture with skin fibroblasts (17).

There is great interest in the potential of DLL4 blockade as a therapeutic target in tumor angiogenesis (reviewed in 14, 40). Specific targeting of DLL4 may have an advantage over broad inhibition of the Notch signaling pathway via γ-secretase inhibitors since toxicity, gastrointestinal in particular, should be minimized. Clinical trials are underway assessing the effectiveness of anti-DLL4 antibodies in the treatment of advanced solid malignancies. However, there have been reports that chronic DLL4 blockade may actually induce vascular neoplasms in some contexts (41) and speculation that DLL4 blockade may work best as an anti-tumor agent when used in combination with anti-VEGF therapy. It is conceivable that therapeutic agents that regulate EC KLF4 expression may similarly have a beneficial role in limiting tumor angiogenesis. Given our understanding of KLF4 in mediating atherosclerosis, thrombosis, pulmonary arterial hypertension, inflammation, and now, angiogenesis, we suggest that KLF4 is an important regulator of multiple facets endothelial health and function.
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Figure Legends

Table 1: qPCR primer sequences.

Table 2: Chromatin Immunoprecipitation qPCR primer sequences.

Figure 1: KLF4 has angiogenic effects. We designed human- and mouse-specific KLF4 primers in order to compare levels of overexpressed mKLF4 to endogenous human (hKLF4) as well as hKLF4 levels after siRNA-mediated knockdown. Total KLF4 (mKLF4 plus hKLF4) is expressed as fold change from endogenous (hKLF4) alone (A) or by copy number (B). Data shown represents data pooled from HUVEC derived from 4 umbilical cords, normalized to SDHA and presented as mean ± SEM. (C) KLF4 alters EC expression of several genes involved in angiogenesis. HUVEC were transduced with a Lenti-K4 or EV control and assessed by qPCR (C, left panel). In separate experiments, HUVEC were transfected with K4 -/- siRNA or NS control (C, right panel). (D, left panel) Overexpression of KLF4 upregulates VEGFA expression independent of hypoxia (norm, normoxia; hyp, hypoxia). (D, right panel) Knockdown of KLF4 results in decreased VEGFA expression independent of hypoxia. Gene expression is normalized to $\beta$-actin. (E) Commercially obtained EC were plated and exposed to hypoxia (3% O2) for the indicated number of hours, then harvested and assessed for hKLF4 by qPCR. (F) ChIP experiments using an anti-KLF4 antibody versus IgG control showed enrichment of KLF4 at specific CACCC sites in the VEGFA, VEGFR1 and VEGFR2 promoters. Data shown represents data pooled from HUVEC derived from 3 cords (n=3). 18S is the non-template control. * Indicates p<0.05 and data is represented as mean ± SEM. * Indicates p<0.05 by student’s t-test.

Figure 2: Overexpression of KLF4 regulates sprouting angiogenesis in vitro. (A) KLF4 promotes EC contribution to sprout formation (cellular area) in a Matrigel model of sprouting
angiogenesis (norm, normoxia; hyp, hypoxia). (B) KLF4 overexpression causes an increase in total number of sprouts and number of sprout nodes, but does not alter individual sprout length or sprout:node ratio compared to EV HUVEC. (C) KLF4 promotes EC contribution to sprout formation (cellular area) in GFR Matrigel. (D) KLF4 overexpression causes an increase in total number of sprouts and number of sprout nodes, but does not alter individual sprout length or sprout:node ratio in GFR Matrigel. (E) Representative images (40x) of HUVEC seeded and grown in Matrigel for ~6-8 hours. Data is representative of 9 wells per condition repeated three times (n=3). *p<0.05 by ANOVA; **p<0.05 by student’s t-test for paired normoxia-hypoxia samples, i.e. EV normoxia vs. EV hypoxia). Data is presented as mean ± SEM and represents HUVEC derived from 4-6 cords (n=4-6). Images were quantified using ImageJ.

Figure 3: Knockdown of KLF4 regulates sprouting angiogenesis in vitro.
(A) Decreased expression of KLF4 inhibits EC contribution to sprout formation. (B) Knockdown of KLF4 causes decreased number of sprouts and nodes, but length/sprout and sprout:node ratio remains unchanged compared to NS HUVEC. (C) KLF4 deficiency inhibits EC contribution to sprout formation in GFR Matrigel. (D) Knockdown of KLF4 decreased number of sprouts per field, length per sprout, number of nodes and sprout:node ratio in GFR Matrigel. (E) Representative images (40X) of HUVEC seeded and grown in Matrigel for ~6-8 hours. Data is representative of 9 wells per condition repeated three times (n=3). *p<0.05 by ANOVA; **p<0.05 by student’s t-test for paired normoxia-hypoxia samples, i.e. NS normoxia vs. NS hypoxia). Data is presented as mean ± SEM and represents HUVEC derived from 4-6 cords (n=4-6). Images were quantified using ImageJ.

Figure 4: Sustained KLF4 overexpression causes ineffective sprouting angiogenesis. (A) EC-K4 Tg tumors are significantly smaller than WT (1x10^6 cells, left panel; 2.5x10^6 cells, right panel). (B) B16-F10 tumors were harvested 14 days after subcutaneous flank inoculation with 1x10^6 melanoma cells. Tumors from 1 of 5 experiments (n=4-12 animals per group; 1x10^6 cells) are shown. (C) IHC analysis demonstrates enhanced EC KLF4 signal (red, KLF4; green, PECAM; 400x). The vascular tumor rim was partitioned from harvested tumors and subjected to gene expression analysis. Sustained overexpression of human KLF4 was expressed as total KLF4 (mKLF4 plus hKLF4) and represented as fold change compared to WT. (D) PECAM staining demonstrates significantly increased numbers of vessels in EC-K4 Tg tumors (100x, left; 200x, right). Quantification was performed on representative sections from 3-4 tumors per group (20-32 fields/tumor) and normalized to WT vessel density. (E) EC-K4 Tg tumor rim homogenate has increased expression of angiogenesis-associated gene targets. WT and EC-K4 Tg tumor rim was harvested and gene expression was assessed and normalized to 36B4. (F) Vessels in EC-K4 Tg tumors are hypoperfused compared to WT tumor vessels. All vessels are stained red (PECAM), and only perfused vessels are labeled with FITC-lectin (green). Complete sections (7-8 fields) of each tumor with 4 tumors (850-900 vessels) per group were analyzed p<0.025, 200x). (G) Intraperitoneal injection of Hypoxyprobe 1 hr. prior to tumor harvest results in labeling of proteins modified by exposure to a hypoxic environment. Distance from vascular structures of the tumor rim (PECAM, green) to hypoxic regions (red, outlined in white) is decreased in EC-K4 Tg tumors (n=8 sections per group; AU, arbitrary units). (H) We observed no gross difference melanoma proliferation between WT and EC-K4 Tg tumors. Proliferation was assessed using IHC and staining for proliferating cell nuclear antigen (PCNA, green) and DAPI (blue) (100x). Quantification of PCNA staining is shown by the ratio of PCNA-positive cells to total number of cells (DAPI+) per field. (p=NS; 5-10 sections per tumor and 4 tumors were analyzed). * indicates location of outer tumor surface. (I) The vascular tumor rim was partitioned and subjected to gene expression analysis. Data is normalized to 36b4 and represents 4 tumors per group (n=4) presented as mean ± SEM. (J) No gross difference in pericyte coverage between WT and EC KLF4 Tg tumor vessels was observed. Assessment of pericyte coverage was performed by IHC.

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using PECAM (green) and NG-2 or laminin (red) staining. Representative photographs with PECAM and laminin co-staining are shown (200×; p=NS, 6-7 sections per tumor and 3-4 tumors per group were analyzed). Data is presented as mean ± SEM.

Figure 5: The role of KLF4 in EC proliferation, apoptosis, migration and metabolism. (A) Lentiviral overexpression of KLF4 does not affect EC proliferation as assessed by cell count quantification over a 3 day incubation period (n=4 independent experiments, 12 total wells per group). (B) Sustained overexpression of KLF4 does not alter Caspase 3/7-activity under either basal conditions or with hypoxia stimulation (n=5 independent preps per group). (C) Overexpression of KLF4 does not alter EC migration after “wound induction” (scratch assay, n=3 independent experiments, nine total wells per group). (D) Metabolism (alamarBlue assay, n=3-4 independent experiments, 18-24 total wells per group) is unchanged with overexpression of KLF4 alamarBlue assay measures redox changes of the growth medium, indicative of metabolic activity. Data is presented as mean ± SEM. (E) Knockdown of KLF4 inhibits EC proliferation. *p<0.05 by student’s t-test for paired samples, i.e. Day 1 NS vs. Day 1 K4 -/- . (F) Knockdown of KLF4 increases Caspase 3/7 activity. *p<0.05 by ANOVA; **p<0.05 by student’s t-test for paired normoxia-hypoxia samples, i.e. K4 -/- normoxia vs. K4 -/- hypoxia (G) Cell migration is inhibited with knockdown of KLF4. *p<0.05 by ANOVA (H) Metabolism is decreased with knockdown of KLF4 *p<0.05 by student’s t-test. Data is presented as mean ± SEM and represents HUVEC derived from 4-6 cords (n=4-6).

Figure 6: KLF4 overexpression does not effect EC cell cycling. (A) Overexpression of KLF4 does not affect EC cell cycle regulation as shown by flow cytometric analysis using BrdU and 7-AAD. One representative dot plot per group is shown. (B) Quantification for R2 (G0/1 transition), R3 (S phase), R4 (G2/M transition), and R5 (apoptosis) are shown as pooled data (n=2 independent experiments, 3 EC preps per experiment). (C) Overexpression of KLF4 in HUVEC does not alter expression of 18 cell-cycle associated genes. Data is presented as mean ± SEM from 3 independent experiments, normalized to expression in control (EV) samples. * Indicates p<0.05.

Figure 7: KLF4 regulates Notch expression. (A) KLF4 differentially regulates Notch family member expression in EC-K4 Tg tumors. Tumor rim homogenate was harvested and assessed for candidate gene expression. Data is normalized to PECAM, presented as mean ± SEM and is pooled from 3-5 tumors per group (n=3-5). KLF4 regulates Notch receptor (B, left panel), ligand (C, left panel), and target gene (D, left panel) mRNA expression levels in HUVEC. Control samples are normalized to 1 and fold change values are expressed relative to respective control. Data is presented as mean ± SEM, represents HUVEC derived from 4-6 cords (n=4-6). (B, C, D-right panels) KLF4 differentially regulates Notch family member mRNA expression after Notch activation by NICD. Endogenous gene expression was assessed after infection with KLF4- or NICD-expressing virus and respective control virus. (For F-H, commercially-available HUVEC were used, n=3-4 independent experiments, *p<0.05.) (F) KLF4 inhibits CSL-mediated Notch activation. A CBX4 reporter plasmid (concatemer with four CSL binding sites), KLF4 expression plasmid, and NICD-expressing virus were transfected into HUVEC and CBX4 activation was expressed as relative luciferase units (RLU). (G) KLF4 inhibits Notch-mediated transcription of target gene HES1. HUVEC were transfected with a Hes1 promoter-reporter construct and KLF4 expression plasmid, NICD or both. (H) Notch activation via NICD decreases KLF4 promoter activity (RLU). HUVEC were transfected with a KLF4 promoter-reporter construct and NICD expression plasmids. (J) KLF4 is enriched on the CACCC sites on the promoters of NOTCH1, DLL4 and HES1 as assessed by ChIP in HUVEC. Data is derived from 3 separate cords and is normalized to input DNA. 18S is the non-targeting DNA control. *p<0.05 by student’s t-test.
**Figure 8: KLF4 negatively regulates Notch activation through NOTCH1 and DLL4.** (A) Overexpression of KLF4 decreases production of NICD, the cleaved intracellular fragment of Notch1, in HUVEC. NICD signal is normalized to β-actin. γ-secretase inhibitor and VEGFA treatment were used as negative and positive controls, respectively. One representative blot is shown. Densitometry analysis represents data pooled from four independent experiments (n=4). (B) Inhibition of Notch target genes HES1, HEY1, and HEY2 by sustained KLF4 overexpression is reversed by supplementation of cultures with DLL4 recombinant protein. Data is presented as mean ± SEM and represents HUVEC derived from 4 cords (n=4). (C) Enhanced sprout formation in primary EC-K4 Tg spheroids is reversed by addition of DLL4 recombinant protein (EC were derived from 5 mice and pooled (n=5), sprouts from 50 spheroids were counted per condition, *p<0.05 by ANOVA). Representative images (4x) of primary cardiac EC after growing 3-4 days in diluted Matrigel are shown in (D).

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### Table 1

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Figure 1

Endothelial Krüppel-like Factor 4 regulates angiogenesis

A. Total KLF4 (fold change)
B. Fold Change
C. VEGFA Expression
D. VEGFA Expression
E. KLF4/AdiP expression
F. % of Input
Figure 2

*Endothelial Krüppel-like Factor 4 regulates angiogenesis*

E V normoxia        Lenti-K4 normoxia        EV hypoxia        Lenti-K4 hypoxia
Figure 3

Endothelial Krüppel-like Factor 4 regulates angiogenesis
Endothelial Krüppel-like Factor 4 regulates angiogenesis
Endothelial Krüppel-like Factor 4 regulates angiogenesis
Figure 6

Endothelial Krüppel-like Factor 4 regulates angiogenesis
Figure 7

Endothelial Krüppel-like Factor 4 regulates angiogenesis

A

B

C

D

E

F

G

H

J
Figure 8

Endothelial Krüppel-like Factor 4 regulates angiogenesis

A

B

C

D

Endothelial Krüppel-like Factor 4 regulates angiogenesis

HES1

Hey1

Hey2

Sprout/spheroid
Endothelial Kruppel-like Factor 4 Regulates Angiogenesis and the Notch Signaling Pathway
Andrew T. Hale, Hongmei Tian, Ejike Anih, Fernando O. Recio III, Mohammad A. Shatat, Trent Johnson, Xudong Liao, Diana L. Ramirez-Bergeron, Aaron Proweller, Masakazu Ishikawa and Anne Hamik

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