The Hedgehog Transcription Factor Gli3 Modulates Angiogenesis

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Rationale: The Gli transcription factors are mediators of Hedgehog (Hh) signaling and have been shown to play critical roles during embryogenesis. Previously, we have demonstrated that the Hh pathway is reactivated by ischemia in adult mammals, and that this pathway can be stimulated for therapeutic benefit; however, the specific roles of the Gli transcription factors during ischemia-induced Hh signaling have not been elucidated.

Objective: To investigate the role of Gli3 in ischemic tissue repair.

Methods and Results: Gli3-haploinsufficient (Gli3<sup>+/−</sup>) mice and their wild-type littermates were physiologically similar in the absence of ischemia; however, histological assessments of capillary density and echocardiographic measurements of left ventricular ejection fractions were reduced in Gli3<sup>+/−</sup> mice compared to wild-type mice after surgically induced myocardial infarction, and fibrosis was increased. Gli3-deficient mice also displayed reduced capillary density after induction of hindlimb ischemia and an impaired angiogenic response to vascular endothelial growth factor in the corneal angiogenesis model. In endothelial cells, adeno-virus-mediated overexpression of Gli3 promoted migration (modified Boyden chamber), small interfering RNA-mediated downregulation of Gli3 delayed tube formation (Matrigel), and Western analyses identified increases in Akt phosphorylation, extracellular signal-regulated kinase (ERK)1/2 activation, and c-Fos expression; however, promoter–reporter assays indicated that Gli3 overexpression does not modulate Gli-dependent transcription. Furthermore, the induction of endothelial cell migration by Gli3 was dependent on Akt and ERK1/2 activation.

Conclusions: Collectively, these observations indicate that Gli3 contributes to vessel growth under both ischemic and nonischemic conditions and provide the first evidence that Gli3 regulates angiogenesis and endothelial cell activity in adult mammals. (Circ Res. 2009;105:818-826.)

Key Words: angiogenesis ■ sonic Hedgehog ■ endothelial cells ■ Gli transcription factors ■ ischemia ■ myocardial infarction
upregulated in ischemic limb muscle and may participate in tissue repair, including myogenesis and angiogenesis. Gli3 is expressed by endothelial cells (ECs) and promotes EC migration and survival in vitro. In vivo, administration of an adenovirus encoding Gli3 increased capillary density and promoted superficial limb perfusion in an animal model of hindlimb ischemia (HLI). After considering these observations, we hypothesized that Gli3 plays a role in ischemia-induced angiogenesis and performed a series of studies to further characterize the role of Gli3 during angiogenesis and ischemic tissue repair.

Methods

Mice
C3HeB/FeJ-Mc1r1/H11006 Gli32/−/− mice (Gli32/−/− mice) were bred with C3HeB/FeJ mice; both mouse strains were obtained from The Jackson Laboratories (Bar Harbor, Me). In vivo experiments were performed with Gli32/−/− mice and their wild-type (WT) littermates. Mice were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee at Northwestern University.

Blood Pressure Measurement
Blood pressure measurements were performed in pretrained, conscious mice via the tail-cuff method (CODA 6 system, Kent Scientific Corporation, Torrington, Conn).

Myocardial Infarction Model and Assessments
Myocardial infarction (MI) was induced in 10- to 12-week-old Gli32/−/− female mice and their WT littermates (8 to 9 animals/group). Left ventricular (LV) ejection fractions were measured echocardiographically 7 ± 1, 14 ± 1, and 28 ± 2 days after MI. Fibrosis and capillary density were evaluated in hearts from mice euthanized on day 28; fibrosis was reported as the ratio of the length of fibrosis to the LV circumference. Capillaries were identified by positive staining for CD31. Surgical, echocardiographic, and histological protocols are summarized in the Online Data Supplement, available at http://circres.ahajournals.org.

HLI Model and Assessments
HLI was performed as previously described21 in 8-week-old male mice (5 to 9 animals/group). Capillary density and the number of smooth muscle–containing vessels were evaluated in sections of tibialis anterior muscles stained for the expression of CD31 and smooth muscle α-actin (αSMA). Surgical and histological protocols are summarized in the Online Data Supplement.

Corneal Angiogenesis Assay
Pellets containing vascular endothelial growth factor (VEGF) or PBS were implanted in the corneas of 6- to 8-week-old female mice as previously described. Eight days later, mice were injected with 50 μL of fluorescent-BS1-Lectin I (Vector Laboratories, Burlingame, Calif) 15 minutes before euthanasia. Angiogenesis was quantified by analyzing BS1-Lectin I fluorescence as described previously. Pellet preparation and the surgical and histological protocols are summarized in the Online Data Supplement.

Cell Maintenance and Transfection/Transduction
Human umbilical vein endothelial cells (HUVECs) (Cambrex Corporation, East Rutherford, NJ) and MS1 cells (CRL-2279; American Type Culture Collection, Manassas, Va) were maintained and transfected/transduced as described in the Online Data Supplement; cell assays were performed 48 to 72 hours after transfection/transduction. The adenovirus encoding β-galactosidase and green fluorescent protein (GFP) (Ad-LacZ) was kindly provided by Dr A. Rosenzweig,23 the adenovirus encoding human Gli3 (Ad-Gli3) was kindly provided by Dr C. M. Fan,23 and the adenovirus encoding a dominant-negative mutant of Akt (DN-Akt) was kindly provided by Ken Walsh.26 Human Gli3 small interfering (si)RNA was purchased from Santa Cruz Biotechnology Inc, and non–Gli3-silencing GFP siRNA (5′-GGCUACGUCCAGGAGCGCAdTdT-3′) was purchased from Dharmacon Inc (Lafayette, Colo). The Gli-BS-luciferase, mutant-Gli-luciferase, and pcDNA3.1-His-human Gli3 plasmids were kindly provided by Dr H. Sasaki.27 Luciferase was assayed with a luciferase assay system (Promega Corporation, Madison, Wis), and β-galactosidase activity was assayed as previously described. For each sample, luciferase activity was normalized to β-galactosidase activity to compensate for differences in transfection efficiency. Each condition was assayed in triplicate, and each experiment was performed at least three times.

Western Blot
Akt and extracellular signal-regulated kinase (ERK)1/2 phosphorylation was evaluated by SDS-PAGE with anti-Akt, anti–phospho-Akt, anti–p42-p44, and anti–phospho-p42-p44 antibodies (Cell Signaling Technology Inc, Danvers, Mass).

Quantitative RT-PCR and Microarray Analyses
Quantitative RT-PCR was performed with RNA isolated from 3×106 cells or from homogenized skeletal or cardiac muscle; RNA microarray analyses were performed with 3 μg of total RNA. Analytic protocols are summarized in the Online Data Supplement, and primer and probe sequences are reported in Online Table I.

Cell Activity Assays
Tube formation was performed in Matrigel-coated (BD Biosciences, San Jose, Calif) plates, and migration was evaluated with a modified Boyden’s chamber (Neuro Probe, Inc, Gaithersburg, Md), as summarized in the Online Data Supplement. Migration under each condition was assayed in triplicate, and each experiment was performed at least three times.
Gli3 mRNA Expression Is Impaired in Gli3\(-/-\) Mice

To verify that Gli3 expression was reduced in Gli3-haploinsufficient (Gli3\(-/-\)) mice and, consequently, that Gli3\(-/-\) mice were suitable for studying the role of Gli3 in adult animals, we compared Gli3 mRNA expression in WT and Gli3\(-/-\) mice. Gli3 mRNA expression was significantly lower (by \(\geq 50\%\)) in Gli3\(-/-\) mice (Online Figure I, A); however, histological examination of skeletal muscle (Online Figure I, B) and heart tissue (Online Figure I, C) harvested from WT and Gli3\(-/-\) mice revealed no apparent structural differences. Similarly, physiological assessments found no significant differences between WT and Gli3\(-/-\) mice in blood pressure, heart rate, LV volume, or LV ejection fraction (Online Figure I, D through G). Thus, Gli3 haploinsufficiency significantly reduced Gli3 expression, but the muscle tissue of Gli3\(-/-\) mice appeared normal, and Gli3\(-/-\) mice displayed no significant cardiovascular functional anomalies at baseline.

Gli3 Contributes to Ischemic Tissue Repair After MI

MI was induced in WT and Gli3\(-/-\) mice. In WT mice, Gli3 expression was 5- to 7-fold greater in the ischemic myocardium than in nonischemic myocardium during the two-week period after MI (Figure 1A). Gli3 was expressed by ECs in the ischemic zone (Figure 1B) and by cardiomyocytes in the border zone of infarcted hearts (Figure 1C), but was not expressed by cardiomyocytes in the absence of infarction (Online Figure II). Echocardiographic measurements of LV ejection fractions indicated that cardiac function was significantly worse in Gli3\(-/-\) mice than in WT mice 14 days (Gli3\(-/-\): 31.9±4.49%; WT: 39.7±5.7%; \(P=0.02\)) and 28 days (Gli3\(-/-\): 27.9±4.5%; WT: 37.6±7.0%; \(P=0.004\)) after MI (Figure 1D and 1E). In hearts harvested 28 days after MI, fibrosis area was \(1.7\pm0.5\)-fold greater (\(P=0.007\)) in Gli3\(-/-\) mice than in WT mice (Figure 1F and 1G), and capillary density was 30.14±11.23% lower in the ischemic region and 28.29±8.49% lower in the ischemic border zone (Figure 1H and 1I).
Gli3 Contributes to Vascular Growth in Ischemic Hindlimbs

Our previous experiments demonstrated that overexpression of Gli3 promotes neovascularization and perfusion in ischemic hindlimb muscle. To determine the role of endogenous Gli3 expression in response to ischemia, we evaluated the vascularity of ischemic hindlimb muscles from WT and Gli3/−/− mice after surgically induced HLI.

Seven and 14 days after HLI, Gli3 mRNA expression in ischemic muscle was approximately 3-fold lower in Gli3/−/− mice than in WT mice (Figure 2A), whereas mRNA expression of Gli2, Gli1, and of the Hh receptor Patched-1 (Pch1) (Figure 2B through 2D) was preserved and did not differ significantly between strains. Seven, 14, and 28 days after HLI, capillary density (Figure 2E and 2F) was significantly lower in tissues harvested from Gli3/−/− mice than from WT mice, with the most significant reduction observed on day 7 (Gli3/−/−: 9.57±2.24 vessels per high-power field [HPF]; WT: 18.56±6.77 vessels/HPF; P=0.0009). The number of vessels that expressed αSMA, a marker for smooth muscle cells, was also significantly lower (P=0.0009) in Gli3/−/− mouse tissues than in tissues from WT mice 14 days after HLI surgery (Figure 2G and 2H); however, the proportion of vessels that expressed αSMA did not differ significantly (Figure 2I), indicating that the development of vessels containing smooth muscle cells, which express Gli3, may not be disproportionately impaired in Gli3/−/− mice.

VEGF-Induced Angiogenesis Is Impaired in Gli3/−/− Mice

The studies described thus far suggest that Gli3 participates in ischemia-induced angiogenesis. To determine whether Gli3 is required for the induction of vessel growth by angiogenic factors, we used the mouse corneal angiogenesis model and compared the growth of vessels toward VEGF-containing pellets implanted in the corneas of Gli3/−/− and WT mice.
VEGF-induced angiogenesis was substantially impaired in Gli3-deficient mice (Figure 3A and 3B); the expression of Gli3 by ECs in the VEGF-induced vasculature was confirmed via double immunofluorescent staining for Gli3 and for expression of the EC marker von Willebrand factor (Figure 3C).

Gli3 Regulates Function and Gene Expression in ECs

The potential influence of Gli3 on EC function was assessed in vitro. Downregulation of Gli3 delayed tube formation (Figure 4A): 8 hours after seeding on Matrigel, total tube length was significantly shorter \( (P=0.008) \) in HUVECs transfected with Gli3 siRNA \((934\pm976 \text{ pixels/HPF})\) than in cells transfected with GFP siRNA \((5261\pm2008 \text{ pixels/HPF})\). Gli3 overexpression via Ad-Gli3 transduction enhanced HUVEC migration (Figure 4B).

mRNA microanalyses of HUVECs transduced with Ad-Gli3 or Ad-LacZ identified several factors upregulated by Gli3 overexpression, including CXC-chemokine ligand (CXCL)1 (also known as growth-regulated oncogene \( \alpha \)), CXCL2 (growth-regulated oncogene \( \beta \)), CXCL5, CC-motif ligand (Ccl)2 (monocyte chemotactic protein 1), interleukin (IL)-8 (CXCL8), and colony-stimulating factor 3 (granulocyte colony-stimulating factor) (Online Table II). Quantitative RT-PCR analyses with 2 different EC lines (HUVECs and MS1 cells) confirmed that Gli3 overexpression upregulated CXCL1, CXCL2, Ccl2, and IL-8 (Figure 4C through 4F; Online Figure III, A through D). Ad-Gli3 transduction also upregulated the expression of platelet-derived, endothelial cell growth factor (PD-ECGF) (thymidine phosphorylase) (Figure 4G; Online Figure III, E); notably, PD-ECGF expression was significantly lower \( (P=0.002) \) in ischemic limb muscle harvested from Gli3 \( ^{-/-} \) mice than in WT ischemic limb muscle on the seventh day after HLI (Figure 4H).

We also investigated whether Gli3 overexpression influenced Gli-dependent transcription in ECs by evaluating luciferase activity in HUVECs transfected with a plasmid coding for Gli-Bs–regulated luciferase expression. Overexpression of Gli3 did not significantly alter luciferase activity (Figure 4I), and similar experiments demonstrated that Gli3 overexpression does not modulate Gli1 or Ptc1 expression in HUVECs (data not shown).

Gli3 Activates the Akt Pathway and the Mitogen-Activated Protein Kinase–ERK1/2 Pathway

Because Hh signaling is known to activate the Akt and mitogen-activated protein kinase (MAPK)–ERK1/2 pathways in ECs,29–32 we performed experiments to determine whether these pathways are also activated by Gli3 overexpression.

Western blot analyses indicated that ERK1/2 phosphorylation is higher in ECs transduced with Ad-Gli3 than in Ad-LacZ–transduced HUVECs (Figure 5A), and when Gli3 expression was knocked down by transfection with Gli3 siRNA, the level of phosphorylated ERK1/2 declined both in the presence and absence of VEGF (Figure 5B). Gli3-transduced ECs also expressed markedly higher (25 ±3-fold) levels of c-Fos, a downstream target of the MAPK-ERK1/2 pathway, and Gli3-induced c-Fos upregulation was significantly lower in the presence of the MAPK-ERK1/2 inhibitor U0126 (Figure 5C), indicating that ERK1/2 activation contributes to the upregulation of c-Fos expression by Gli3.

Akt phosphorylation was also higher in Ad-Gli3–transduced HUVECs than in HUVECs transduced with Ad-LacZ (Figure 5D). To determine whether Gli3-induced Akt phosphorylation occurred upstream, downstream, or independently of ERK1/2 activation, phosphorylated Akt and ERK1/2 levels were measured in ECs transduced with Ad-Gli3 and DN-Akt (a dominant-negative mutant of Akt) or in Ad-Gli3–transduced ECs cultured in the presence and absence of U0126. Gli3-induced ERK1/2 phosphorylation was impaired in ECs cotransduced with DN-Akt (Figure 5E), but Gli3-induced Akt phosphorylation was not affected by the presence of U0126 (Figure 5F), providing evidence that the activation of ERK1/2 by Gli3 occurs downstream of Akt activation.

Gli3-Induced EC Migration Is Dependent on Both Akt and MAPK-ERK1/2

The role of Akt and ERK1/2 in Gli3-induced EC migration and gene expression was investigated in HUVECs transduced with Ad-Gli3 and DN-Akt or in Ad-Gli3–transduced HUVECs cultured in the presence and absence of U0126. Gli3-induced EC migration declined when cells were cotransduced with DN-Akt or cultured in the presence of U0126.
(Figure 5G), indicating that the enhanced migration associated with Gli3 overexpression is dependent on both Akt and MAPK-ERK1/2 activation. DN-Akt cotransduction, but not U0126 exposure, inhibited Gli3-induced expression of CXCL1 (Figure 5H), CXCL2, IL-8, and Ccl2 (Online Figure IV, A through C), and CXCL1 expression was also impaired in Ad-Gli3/DN-Akt–cotransduced cells cultured with U0126 (Online Figure IV, D), which suggests that Akt, but not ERK1/2, mediates the upregulation of these genes by Gli3. In contrast, PD-ECGF expression in Ad-Gli3–transduced HUVECs was not significantly impaired by DN-Akt cotransduction or by the presence of U0126 (Figure 5I), suggesting that Gli3-induced PD-ECGF expression occurs through an Akt- and ERK-independent mechanism.

**Discussion**

Very recently, we have shown that Gli3, a transcription factor targeted by Shh during Hh signaling, is strongly upregulated in the ischemic tissue of adult mammals and may have a favorable effect on myogenesis and angiogenesis after an ischemic insult.21 The findings reported here confirm our previous results and are the first to indicate that endogenous Gli3 expression contributes to postnatal angiogenesis. Our in vivo experiments demonstrate that Gli3 haploinsufficiency impairs angiogenesis in both the MI and HLI models and in response to VEGF stimulation, and the impairment in angiogenesis worsened functional outcomes in ischemic animals. Collectively, our observations strongly suggest that Gli3 has an important role during angiogenesis in adult mammals and that Gli3 upregulation is required for normal neovascularization during ischemic tissue repair.

Our previous work demonstrated that Gli3 is expressed in the ECs of ischemic skeletal muscle.21 The present studies extended our earlier findings by identifying Gli3 expression in cardiac ECs after MI, and we also showed that in vitro Gli3 expression regulates EC migration and tube formation. Other reports have identified similarities between the mechanisms involved in angiogenesis and axonal guidance,33–35 which is also dependent on Gli3, and may lead to speculation about whether the regulation of EC activity by Gli3 could be considered analogous to the Gli3-dependent migration of olfactory neurons.36,37
Gli3 overexpression strongly upregulated the expression of several proangiogenic factors in ECs, including IL-8 and the CXCR2 ligands CXCL1 and CXCL2. IL-8 promotes EC migration and tube formation, whereas CXCR2 ligands have been associated with the mobilization of hematopoietic stem cells and with endothelial progenitor cell recruitment, both of which contribute to ischemia-induced vascular regeneration. Gli3 overexpression in ECs also upregulated the expression of PD-ECGF, a strong proangiogenic factor that has been shown to promote neovascularization in MI and HLI models; furthermore, PD-ECGF expression in ischemic skeletal muscle was significantly lower in Gli3−/− mice than in WT mice. Thus, the impaired angiogenesis observed in Gli3−/− mice may evolve through a variety of mechanisms, including altered gene expression and EC activity or impaired stem cell mobilization and recruitment.

The transcriptional target of Gli3 and the potential cofactors that contribute to Gli3-mediated transcription have yet to be identified. The results from our gene–reporter assays suggest that Gli3 overexpression does not modulate Gli-dependent transcription in ECs, and the expression of Gli1 and Ptc1 mRNA were also unchanged. Gli3 overexpression enhanced ERK1/2 and Akt activity in ECs, but these effects may have occurred indirectly through the Gli3-induced upregulation of IL-8 and/or PD-ECGF. IL-8 has been shown to promote ERK1/2 phosphorylation in ECs, whereas PD-ECGF promotes Akt phosphorylation in U937 cells and Ccl2 and CXCL3 expression in HUVECs. Future investigations are warranted to further characterize these mechanisms.

In conclusion, our observations indicate that Gli3 contributes to neovascularization under both ischemic and nons ischemic conditions and provide the first evidence that Gli3 contributes to angiogenesis in adult mammals. Thus, Gli3
may be a suitable therapeutic target for clinical conditions that require modulation of angiogenesis.

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Disclosures
None.

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MATERIALS AND METHODS

Myocardial infarction (MI) model and assessments
MI was surgically induced in 10- to 12-week-old Gli3+/– female mice and their WT littermates. Experimental groups included 8-9 animals; Mice were anesthetized with intraperitoneal avertin (125 mg/kg), orally intubated with a 22G IV catheter, and artificially ventilated with a respirator (NEMI Scientific, Inc., Framingham, MA, USA). Surgical procedures were performed under a dissecting microscope. A small skin incision and a left intercostal thoracotomy were performed at the fourth intercostal space, and the ribs were retracted with 6-0 polypropylene sutures to open the chest and expose the heart, then the pericardium was opened, and the left-anterior descending branch (LAD) of the left coronary artery was ligated with 8-0 polypropylene sutures distal to the bifurcation between the LAD and the diagonal branch. Positive end-expiratory pressure was applied to fully inflate the lung, then the ribs, minor and greater pectoral muscles, and skin were closed with 6-0 polypropylene sutures.

Left-ventricular ejection fractions were measured 7±1, 14±1, and 28±2 days after MI on a high-resolution echocardiographic system equipped with a 30-MHz mechanical transducer (VEVO700, VisualSonics Inc., Toronto, Ontario, Canada). Mice were anchored to a warming platform in a supine position, limbs were taped to the echocardiograph electrodes, and chests were shaved and cleaned with a chemical hair
remover to minimize ultrasound attenuation. Aquasonic 100 gel (Parker Laboratories, Inc., Fairfield, NJ, USA), from which all air bubbles had been expelled, was applied to the thorax to optimize the visibility of the cardiac chambers. Ejection fractions were evaluated by planimetry as recommended.\(^1\) Two-dimensional, parasternal long-axis and short-axis views were acquired, and the endocardial area of each frame was calculated by tracing the endocardial limits in the long-axis view, then the minimal and maximal areas were used to determine the left-ventricular end-systolic (ESV) and end-diastolic (EDV) volumes, respectively. The system software uses a formula based on a cylindrical-hemiellipsoid model (volume=\(8.\text{area}^2/3\pi/\text{length}\)).\(^2\) The left-ventricular ejection fraction was derived from the following formula: \((\text{EDV- ESV})/\text{EDV} \times 100\).

Hearts were harvested from mice sacrificed 28 days after MI, incubated overnight in 30\% sucrose, embedded in OCT compound (Sakura Finetek U.S.A., Inc., Torrance, CA, USA), snap-frozen in liquid nitrogen, and cut into 5-\(\mu\)m sections, then the sections were stained via the Masson-trichrome protocol. Fibrosis area was assessed with a computerized digital image analysis system (Image Pro, version 4.5; Media Cybernetics, Inc., Bethesda, MD, USA) and reported as the ratio of the length of fibrosis to the left-ventricular circumference. Capillary density was evaluated in sections stained and analyzed as described for the HLI model (below).

**HLI model and assessments**

HLI was performed as previously described\(^3\) in 8-week-old male mice; each experimental group included 5 to 9 animals. Briefly, the left femoral artery was resected from the proximal end of the femoral artery to the distal portion of the saphenous vein,
then the femoral artery and all side-branches were dissected and excised, and the overlying skin was closed with sutures. Tibialis anterior muscles were harvested from mice sacrificed at the indicated time points, fixed in methanol, paraffin-embedded, and cut into 5-µm sections. Capillary density and the number of smooth-muscle containing vessels were evaluated in sections stained for the expression of CD31, an EC-specific marker, and smooth-muscle α-actin (αSMA). Capillaries were identified by positive staining for CD31, and smooth muscle-containing vessels were identified by positive staining for αSMA. Sections were viewed at 40× magnification, and vessels were counted in 20 high-power fields (HPFs) per section.

**Corneal angiogenesis assay**

Pellets were prepared as previously described. Briefly, 10 µg of vascular endothelial growth factor (VEGF) protein (BioVision Inc., Mountain View, CA, USA) diluted in 10 µL sterile phosphate-buffered saline (PBS) was mixed with 5 mg sucrose octasulfate-aluminum complex (Sigma-Aldrich Co., St. Louis, MO, USA), and 10 µL of 12% hydron in ethanol was added. The suspension was deposited on a 400-µm nylon mesh (Sefar America Inc., Depew, NY, USA), then both sides of the mesh were covered with a thin layer of hydron and allowed to dry.

6- to 8-week old female mice were anesthetized by intraperitoneal injection of 125 mg/kg Avertin, then pellets were implanted in the corneas as previously described. Eight days later, mice were injected with 50 µL fluorescien-BS1-Lectin I (Vector Laboratories, Burlingame, CA, USA) 15 minutes before sacrifice. For analysis of BS1-
Lectin I fluorescence, eyes were harvested and fixed with 1% paraformaldehyde, then the corneas were excised and prepared for fluorescent microscopy; angiogenesis was quantified as described previously.\textsuperscript{5} For immunostaining with anti-von Willebrand factor (vWF) and anti-Gli3 antibodies, eyes were fixed in 100% methanol and embedded in paraffin.

**Histology and immunostaining**

Gli3 protein was identified with anti-human Gli3 antibodies (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA), ECs were identified with rat anti-CD31 antibodies (BD Pharmingen Inc, San Diego, CA, USA) or sheep anti-vWF antibodies (AbD Serotec, Raleigh, NC, USA), smooth muscle cells were identified with anti-αSMA antibodies (Sigma-Aldrich Co.), and skeletal or cardiac muscle fibers were identified with anti-desmin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and anti-Connexin 43 antibodies (Sigma-Aldrich Co.). For immunofluorescent analyses, primary antibodies were resolved with Alexa-Fluor–conjugated secondary antibodies (Invitrogen Corporation, Carlsbad, CA, USA) and nuclei were counterstained with DAPI (1/5000). For immunohistochemical analyses, primary antibodies were sequentially stained with biotin-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) and streptavidin-HRP complex (Signet Laboratories, Dedham, MA, USA), then the stain was developed with an AEC Substrate Kit (Vector Laboratories); tissues were counterstained with hematoxylin.

**Cell culture**
Human umbilical-vein endothelial cells (HUVECs) (Cambrex Corporation, East Rutherford, NJ, USA) were maintained in endothelial growth medium-2 (EGM™-2) (Cambrex Corporation) and starved in EBM-2 medium containing 1% fetal bovine serum (FBS) for 24 hours before cell assays were performed. MS1 cells (CRL-2279; ATCC, Manassas, VA, USA) were maintained in 10% FBS-containing Dulbecco's Modified Eagle Medium (DMEM) (Mediatech, Inc., Manassas, VA, USA) and starved in DMEM containing 0.5% FBS for 24 hours before cell assays were performed.

Adenovirus transduction

The adenovirus encoding β-galactosidase and green-fluorescent protein (Ad-LacZ) was kindly provided by Dr A. Rosenzweig,6 the adenovirus encoding human Gli3 (Ad-Gli3) was kindly provided by Dr C.M. Fan,6 and the adenovirus encoding a dominant negative mutant of Akt (DN-Akt) was kindly provided by Ken Walsh.7 Adenoviruses were amplified to high titer in 293 HEK cells then purified via cesium chloride–gradient ultracentrifugation. Viral titers were determined by performing a plaque assay with 293 HEK cells and expressed in plaque-forming units (PFU). Multiplicity of infection (MOI) was defined as the ratio of PFU to the number of cells. HUVECs and MS1 were transduced overnight with an MOI of 200. Transduction was verified via green-fluorescent protein (GFP) expression, and cell assays were performed 48-72 hours after transduction.

Plasmid transfection
The Gli-BS luciferase, mutant Gli luciferase, and pcDNA3.1-His-human Gli3 plasmids were kindly provided by Dr H. Sasaki. HUVECs were transfected using JetPEI™-HUVEC (Polyplus-transfection Inc., New York, NY, USA) according to the manufacturer’s instructions. Briefly, HUVECs were seeded on a 6-well plate (2×10^5 cells/well) and co-transfected 1 day later with 3 plasmids: 1) 0.6 µg Gli-BS luciferase or mutant Gli luciferase, 2) 0.6 µg pcDNA3.1-His-human Gli3 or pcDNA3, and 3) 0.15 µg pCMVnLacZ. Luciferase was assayed with a luciferase-assay system (Promega Corporation, Madison, WI, USA), and β-galactosidase activity was assayed as previously described. For each sample, luciferase activity was normalized to β-galactosidase activity to compensate for differences in transfection efficiency. Each condition was assayed in triplicate, and each experiment was performed at least three times.

**siRNA transfection**

HUVECs were transfected with 180 nmol/L human Gli3 siRNA (Santa Cruz Biotechnology, Inc.) or non-Gli3–silencing GFP siRNA (5'-GGCUACGUCCAGGAGCGCAdTdT-3') (Dharmacon, Inc., Lafayette, CO, USA) by using TransFast™ transfection reagent (Promega Corporation) according to the manufacturer’s instructions; the efficiency and specificity of siRNA transfection were verified via real-time reverse-transcription polymerase chain reaction (RT-PCR). Cell assays were performed 48 hours after transfection.

**Quantitative RT-PCR and microarray analyses**
RNA was isolated by using RNA STAT-60 (TEL-TEST, Inc., Friendswood, TX, USA) as instructed by the manufacturer from 3×10^5 cells or from skeletal or cardiac muscle that had been snap-frozen in liquid nitrogen and homogenized. For quantitative RT-PCR analyses, total RNA was reverse transcribed with a Taqman cDNA Synthesis Kit (Applied Biosystems, Foster City, CA, USA), and amplification was performed on a Taqman 7500 (Applied Biosystems); primer and probe sequences are reported in Supplemental Table 1. The relative expression of each mRNA was calculated by the comparative threshold cycle method and normalized to 18S rRNA expression. RNA microarray analyses were performed with 3 µg total RNA by using the human Angiogenesis Oligo GEArray (SA Biosciences Corporation, Frederick, MD, USA) according to the manufacturer's instructions.

**Tube-formation assay**

25×10^3 HUVECs were seeded in each well of a 48-well plate coated with 120 µL Matrigel™ (BD Biosciences, San Jose, CA, USA). Tube formation was evaluated by phase-contrast microscopy 8 and 24 hours later.

**Migration assay**

Cell migration was evaluated with a modified Boyden’s chamber (Neuro Probe, Inc., Gaithersburg, MD, USA). Briefly, a polycarbonate filter (5-µm pore size) (GE Infrastructure, Fairfield, CN, USA) was coated with a solution containing 2.5 µg/mL vitronectin (Sigma-Aldrich Co.) and 0.15% gelatin (Sigma-Aldrich Co.) and inserted between the chambers, then 5×10^4 cells per well were seeded in the upper chamber,
and the lower chamber was filled with EBM-2 medium containing 1% FBS. Cells were incubated for 8 hours at 37°C then viewed under 20× magnification, and the number of cells that had migrated to the lower chamber were counted in 3 HPFs per well; migration was reported as the mean number of migrated cells per HPF. Each condition was assayed in triplicate and each experiment was performed at least three times.
REFERENCES


7. Skurk C, Maatz H, Kim HS, Yang J, Abid MR, Aird WC, Walsh K. The Akt-regulated forkhead transcription factor FOXO3a controls endothelial cell viability


**Supplemental Figure 1: Gli3 mRNA expression is significantly lower in Gli3+/− mice than in WT mice.** (A) Total RNA was isolated from the skeletal muscle of Gli3+/− mice and their WT littermates, then Gli3 mRNA expression was evaluated by quantitative RT-PCR and normalized to 18S rRNA expression. ***p≤0.001. (B) Skeletal muscle cross sections from WT and Gli3+/− mice were stained with hematoxylin and eosin (H & E) (upper panels); skeletal muscle longitudinal sections from WT and Gli3+/− mice were stained with desmin (red) to identify skeletal muscle and with DAPI (blue) to identify nuclei (lower panels). (C) Heart cross sections from WT and Gli3+/− mice were stained with H & E (upper panels), with desmin (red) to identify cardiac muscle and DAPI (blue) to identify nuclei, (middle panels), or with Connexin 43 (red) to identify cardiac muscle and DAPI (blue) to identify nuclei (lower panels). (D) Blood pressure of WT and Gli3+/− mice was measured by the tail-cuff method. (E) Heart rates of WT and Gli3+/− mice were measured via electrocardiography and (F) LV volumes (EDV: end-diastolic volume; ESV: end-systolic volume; SV: stroke volume) and (G) LV ejection fractions of WT and Gli3+/− mice were measured via echocardiography.

**Supplemental Figure 2: Gli3 is overexpressed in the infarcted heart.** Cross-sections of heart tissue harvested from uninjured mice (left panel) and from the ischemic border zone of mice with surgically induced MI (right panel) were triple stained with anti-Gli3 antibodies (red), with anti-α-sarcomeric actin antibodies (green) to identify cardiomyocytes, and with DAPI (blue) to identify nuclei.
Supplemental Figure 3: Gli3 regulates gene expression in murine ECs. MS1 cells were transduced with adenoviral vectors coding for Gli3 expression (AdGli3) or control adenoviral vectors coding for LacZ expression (AdLacZ). (A) CXCL1, (B) CXCL2, (C) Ccl2, (D) IL-8, and (E) PD-ECGF mRNA expression were evaluated via quantitative RT-PCR and normalized to 18S rRNA expression.

Supplemental Figure 4: Gli3-induced chemokine expression is dependent on Akt phosphorylation. (A-C) AdLacZ– and AdGli3–transduced HUVECs were incubated with or without 10 µmol/L U0126 for 24 hours or co-transduced with DN-Akt. The mRNA expression of (A) CXCL2, (B) IL8, and (C) Ccl2 was evaluated via quantitative RT-PCR and normalized to 18S rRNA expression. (D) AdLacZ– and AdGli3–transduced HUVECs were incubated without U0126 or were co-transduced with DN-Akt and incubated with 10 µmol/L U0126 for 24 hours; CXCL1 mRNA expression was evaluated via quantitative RT-PCR and normalized to 18S rRNA expression. ***p≤0.001.
Supplemental Table 1. Primers and probes used for quantitative RT-PCR analyses.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer or Probe</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>18S</td>
<td>Forward primer</td>
<td>5’-CGGGTCGGGGAGTGGGT-3’</td>
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<tr>
<td></td>
<td>Reverse primer</td>
<td>5’-GAAACGGCTACCACATCCAAG-3’</td>
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<tr>
<td></td>
<td>Probe</td>
<td>5’- Cal Fluor- TTTGCGCGCCTGCTGCCTT-BHQ-3’</td>
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<tr>
<td>β-actin</td>
<td>Forward primer</td>
<td>5’-GGAGGAGCTGGAAGCAGCAGCC-3’</td>
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<td>(human)</td>
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<td>5’-GCTGTGCTAGTGTCGCCTGCTG-3’</td>
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<td></td>
<td>Probe</td>
<td>None (Syber green was used)</td>
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<td>Gli1</td>
<td>Forward primer</td>
<td>5’-GCTTGATGAAGGACCTTGAG-3’</td>
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<td></td>
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<td>5’-Fam- ACTCTCCACGCTGGCGCCT-BHQ-3’</td>
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<td>Patched-1</td>
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<td>Gene</td>
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<td>Reverse primer (human)</td>
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<td>CXCL1</td>
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<td>CXCL2</td>
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<td>IL8</td>
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<td>PD-ECGF (mouse)</td>
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CXCL: CXC chemokine ligand; PD-ECGF: platelet-derived endothelial cell growth factor; CCL2: chemokine (C-C motif) ligand 2.
**Supplemental Table 2. Pro-angiogenic genes upregulated by Gli3 over-expression**

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<tr>
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<td>Chemokine (C-C motif) ligand 2</td>
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<td>CSF3</td>
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<td>Chemokine (C-X-C motif) ligand 1</td>
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<tr>
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<td>Chemokine (C-X-C motif) ligand 2</td>
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<td></td>
<td>Chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)</td>
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<td>IL6</td>
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<td>Interleukin 8</td>
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<td>PLAU</td>
<td>Plasminogen activator urokinase</td>
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*Ratio of mRNA expression in cells transfected with adenoviral vectors coding for Gli3 or LacZ (Ad-Gli3:Ad-LacZ).