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Angiogenic-regulatory network revealed by molecular profiling heart tissue following Akt1 induction in endothelial cells

Stephan Schiekofer,

Department of Institute of Clinical Chemistry and Laboratory Medicine, Regensburg University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany & Department of Internal Medicine I, Ulm University, Ulm, Germany

Kurt Belisle,

Department of Internal Medicine I, Ulm University, Ulm, Germany

Gennaro Galasso,

Department of Cardiology, Naples University, Naples, Italy

Jochen G. Schneider,

Department of Internal Medicine, Washington University, St. Louis, USA

Bernhard O. Boehm,

Department of Internal Medicine I, Ulm University, Ulm, Germany

Timo Burster,

Department of Internal Medicine I, Ulm University, Ulm, Germany

Gerd Schmitz, and

Department of Institute of Clinical Chemistry and Laboratory Medicine, Regensburg University, Franz-Josef-Strauss-Allee 11, 93053 Regensburg, Germany

Kenneth Walsh

Whitaker Cardiovascular Institute, Molecular Cardiology, Boston University, School of Medicine, Boston, USA

Abstract

Akt is a pivotal signaling molecule involved in the regulation of angiogenesis. In order to further elucidate the role of Akt1 in blood vessel development, a tetracycline-regulated transgenic system was utilized to conditionally activate Akt1 signaling in endothelial cells to examine transcript expression changes associated with angiogenesis in the heart. Induction of Akt1 over the course of 6 weeks led to a 33% increase in capillary density without affecting overall heart growth. Transcript expression profiles in the hearts were analyzed with an Affymetrix GeneChip Mouse Expression Set 430 2.0, which represents approximately 45,000 cDNAs and ESTs. A total of 248 transcripts were differentially expressed between transgenic and control mice (fold change $>/<1.8$; false discovery rate <0.1 ; $P < 0.01$). A subset of these differentially expressed transcripts included angiogenic growth factors, cytokines, and extracellular matrix proteins. More specifically, these transcripts included VEGF-receptor2, neuropilin-1, and connective tissue growth factor, each of which is implicated in blood vessel growth and the maintenance of vessel wall integrity. Furthermore, these factors may be involved in an autocrine-regulatory feedback system, one believed to promote vessel growth. Knowledge of these and other targets could be used to treat ischemic heart disease, a disease whose broad spectrum of manifestations range from patients with only effort-induced angina without myocardial damage, through stages of myocardial ischemia that are associated with reversible and

irreversible impairment in left ventricular function, to states of irreversible myocardial injury and necrosis resulting in congestive heart failure (CHF).

Keywords

Angiogenesis; AKT; Heart

Introduction

Neovascularization in the chronically ischemic adult heart is a combination of several processes, including angiogenesis and arteriogenesis. Angiogenesis refers to the sprouting of new capillaries from postcapillary venules [1], and in adults, is mainly stimulated by tissue hypoxia via activation of hypoxia-inducible factor (HIF-1 α). Expression of HIF-1 α increases transcription of vascular endothelial growth factor (VEGF), VEGF receptors, neuropilin-1, as well as other target genes [2]. Arteriogenesis refers to the maturation or de novo growth of collateral conduits and production of vessels capable of carrying significant blood flow [3,4]. The primary arteriogenic stimuli are thought to be shear stress and the accumulation of blood-derived mononuclear cells at the sites of arterial narrowing. This accumulation results in the production and subsequent release of angiogenic growth factors including fibroblast growth factors (FGF), platelet derived growth factors (PDGF), and VEGF [3,5]. Therefore, angiogenesis and arteriogenesis contribute to neovascularization in the adult heart, with arteriogenesis being the most critical for significant improvements in myocardial blood flow.

Both collateral growth and angiogenesis are potential targets for gene therapy in the myocardium. As such, induction of collateral growth can be beneficial both as a preventive treatment in severe multivessel arterial occlusive disease and after acute occlusion of the vessel, to improve the function of the conducting vessel [6]. Angiogenesis increases local tissue perfusion and would be a beneficial response after acute myocardial infarction to increase perfusion in the ischemic area and to salvage hibernating myocardium. Furthermore, recent studies have shown that uncoordinated angiogenesis and cardiac hypertrophy can contribute to heart failure. Therefore, a better understanding of the angiogenesis-regulatory network in the heart could provide insights into the mechanisms that contribute to heart failure and potentially provide the basis for future treatments.

The serine-threonine protein kinase Akt (protein kinase B) is activated by growth factors or cytokines in a phosphatidylinositol-3 kinase (PI3 K)-dependent manner in a variety of systems. Akt functions as an important regulator of physiological and pathological growth processes [7-9]. Akt signaling is also known to preferentially promote cell survival. Akt is involved in many aspects of cellular regulation, including survival, metabolism, and proliferation. VEGF has been shown to mediate cell survival and growth via the Flk1/VEGFR2-PI3 K-Akt pathway [10,11]. In addition to anti-apoptotic effects, VEGF stimulates Akt-mediated eNOS phosphorylation leading to an increase in eNOS activity [12,13]. In vivo studies have also shown that Akt functions as a regulator of vasomotor tone [14].

In order to investigate Akt1 dependant transcript activation during cardiac vessel growth, a tetracycline-regulated transgenic system was utilized, which conditionally expresses an activated form of Akt1 in the endothelium [15,16]. Transgene expression was induced at 8 weeks of age and was maintained for 6 weeks. Induction of Akt1 increased capillary density but did not alter heart size. The effect of increased vascularization of the heart tissue was associated with changes in gene-regulatory programs that are associated with blood vessel growth and maintenance of vessel wall integrity.

Experimental procedures

Materials

Adenovirus vectors containing the gene for β -galactosidase (Ad- β gal) and myr-Akt (Ad-Akt) as previously described [17]. Akt and phospho-Akt (Ser473), extracellular signal-regulated kinase (ERK) and phospho-ERK (Thr202/Tyr204), as well as P-38 and phospho-P38 (Thr180/Tyr182) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for VEGFR-2 and neuropilin-1 were purchased from Santa Cruz Biotechnology, USA. The mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (MEK)-1/2 inhibitor U0126 was obtained from New England Biolabs Inc. (Beverly, MA, USA). The phosphatidylinositol-3 kinase (PI-3 k) inhibitor Wortmannin was purchased from Sigma Chemical Company (St. Louis, MO, USA). The P-38 MAPK inhibitor SB203580 was purchased from Calbiochem (San Diego, CA, USA).

Production of transgenic mouse lines

Generation and phenotypic characterization of Tet-myr-Akt1 and VE-Cadherin-tTA mice was previously described [15,16]. In brief, two lines of transgenic mice (Tet-myr-Akt1 and VE-Cadherin-tTA) were used to generate endothelial-cell-specific inducible Akt1 TG mice (Fig. 1). The Tet-myr-Akt1 TG line harbors a constitutively active form of Akt1 (myr-Akt1) transgene under the control of multimerized tetracycline operator (tetO) sequences. The VE-Cadherin-tTA TG line conditionally expresses tetracycline transactivator (tTA: a fusion protein of tetO-binding domain and VP16 transactivation domain) targeted to endothelial cells using the VE-Cadherin promoter. Treatment of DTG mice with doxycycline (DOX) (0.5 mg/ml in drinking water) results in the repression of myr-Akt1 expression, whereas withdrawal of DOX results in the induction of myr-Akt1 expression. The Tet-myr-Akt1 transgene and the VE-Cadherin-tTA transgene were detected by PCR [15,16].

Tissue preparation and immunohistochemistry

The mice were sacrificed with an overdose of sodium pentobarbital. For total protein or RNA extraction, isolated tissue samples were rinsed in phosphate-buffered saline (PBS) to remove excess blood, snap-frozen in liquid nitrogen, and stored at -80°C until used. For immunohistochemistry, heart samples were imbedded in OCT compound (Miles, Elkhart, Indiana, USA) and snap-frozen in liquid nitrogen. Tissue sections (5 μ m in thickness) were prepared and stained with hematoxylin and eosin (HE) for overall morphology, Masson's trichrome (MT) for detection of fibrosis, and CD31 for detection of microvessels, as previously described [16]. Capillary density within the heart specimen was quantified by choosing ten randomly chosen microscopic fields from three different sections in each sample and the number of CD-31-positive features per high power field (400 $\times$) were counted [17].

Preparation of cRNA for microarray analysis

Hearts of control and DTG mice were homogenized in TRizol (UltraspecTM-II RNA Isolation System, Biotecx, USA) to extract total RNA according to the manufacturer's recommendations. RNA was resuspended in DEPC treated H₂O and further purified using RNATackTmResin (UltraspecTM-II RNA Isolation System, Biotecx, USA) according to the manufacturer's instructions. After purification, an aliquot was electrophoresed in a 2.0% agarose gel and visualized by staining with ethidium bromide to establish the integrity of the RNA. RNA was reverse transcribed. Resulting cDNA was used to generate labeled cRNA by incorporating biotinylated CTP and UTP using the ENZO Bioarray High Yield transcript labeling kit (Affymetrix). cRNA (20 μ g) was fragmented in fragmentation buffer (40 mM Tris (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate) for 35 min at 94°C. Subsequently, samples and hybridization controls were hybridized to Affymetrix GeneChip®

Mouse Expression Set 430 microarrays 2.0 for 16 h at 45°C. The microarrays were stained with streptavidin-phycoerythrin and with anti-streptavidin antibody according to the manufacturer's instructions. The arrays were then scanned at 488 nm using G25000A gene array scanner (Agilent, Palo Alto, Ca). Four independent experimental replicates of each condition for DTG positive mouse hearts 6 weeks after Akt1 induction and control mouse hearts were performed.

Data quantification, normalization, and analysis

Following data acquisition, the scanned images were quantified using MAS 5.0 software (Affymetrix) yielding signal intensity for each probe on the GeneChip. The signal intensities from the probes for each transcript were then used to determine an overall expression level, a detection confidence score, and a present/absent call according to algorithms implemented in MAS 5.0 software. The arrays were then linearly scaled to an average expression level of 500 units on each chip in MAS 5.0. For each transcript, fold change and statistical significance of differential expression were calculated. Fold change was calculated using the average signal from each experimental group. Statistical significance was calculated using a one-factor (time point) ANOVA implemented in the NIA Array Analysis Tool (<http://cgd.rrc.uic.edu/anova/>) using a Bayesian estimate of the variance between replicates. ANOVA *P*-values were corrected for multiple hypothesis testing using the method of Benjamini and Hochberg [18]. Each transcript was annotated based on a complete download of the NetAffx database (Affymetrix, Santa Clara, CA). In addition, automated analysis of related transcripts was performed using Ingenuity Pathways Analysis, a web-delivered application that evaluates biological networks www.ingenuity.com. For this analysis, a set of 248 gene identifiers of transcripts, which displayed a significant change in DTG mice as compared to control mice (fold change ≥ 1.8 ; FDR < 0.1 ; $P < 0.01$) was uploaded as a tab-delimited text file. Each gene identifier (i.e., Focus Gene or transcript) was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base that is derived from information in the scientific literature. These Focus Genes were then used for generating biological networks based upon the identities of the Focus Genes and interactions with genes/proteins reported in the literature. The software then computes a score for each network according to the fit of the Focus Genes that were significantly changed in WT and DTG mice. The score is derived from a *P*-value and indicates the likelihood of the Focus Genes in a network being found together due to random chance. A score of 2 indicates that there is a 1% chance that the Focus Genes are together in a network due to random chance. Therefore, scores of ≥ 2 have $\geq 99\%$ confidence of not being generated by random chance alone.

Biological functions were assigned to each gene network by using the findings that have been extracted from the scientific literature and stored in the Ingenuity Pathways Knowledge Base. The biological functions assigned to each network are ranked according to the significance of that biological function to the network. A Fischer's exact test is used to calculate a *P*-value determining the probability that the biological function assigned to that network is explained by chance alone.

QRT-PCR analysis

Total RNA was isolated and purified from the control and DTG mouse hearts as described above by RNA-TRIZOL extraction (UltraspecTM-II RNA Isolation System, Biotecx, USA). RNA (2 μ g) was treated (30 min at 37°C) with amplification grade DNase 1 (Invitrogen, USA) following phenol/chloroform extraction (1:1). cDNA was produced using Taqman reverse transcription (Invitrogen, USA) kits. Real-time polymerase chain reaction (QRT-PCR) was performed in triplicate on ABI-Prism 7900 QRT-PCR machine using SYBR[®] Green PCR Master Mix according to manufacturer's instruction (1:1, PE-Applied Biosystems, Great Britain). The appropriate primers (200 nM each) (Table 1) were obtained from Integrated DNA Technologies, USA. Primers were designed to be compatible with a single QRT-PCR thermal

profile (95°C for 10 min, and 40 cycles of 95°C for 30 s and 60°C for 1 min) such that multiple transcripts could be analyzed simultaneously. Accumulation of PCR product was monitored in real time (PE-Applied Biosystems), and the crossing threshold (Ct) was determined using the PE-Applied Biosystems software. For each set of primers, a no template control and a no reverse amplification control were included. Postamplification dissociation curves were performed to verify the presence of a single amplification product in the absence of DNA contamination. Fold changes in transcript expression were determined using the Ct method. To standardize the quantitation of the selected transcripts, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) for each sample was quantified by QRT-PCR and the selected transcripts were normalized to GAPDH. Data of QRT-PCR analysis are shown as means $\pm$ S.E. of mean. All data were evaluated with a two-tailed, unpaired Student's *t* test or compared by one-way analysis of variance ($P < 0.01$).

Western blot analysis

Heart tissue samples obtained from DTG mice after Akt1 induction and control mice without Akt1 induction were removed, snap frozen, and crushed in liquid nitrogen before tissue was homogenized in cold lysis buffer (20 mM Tris-HCL (pH 8.0), 1% NP-40, 150 mM NaCl, 0.5% deoxycholic acid, 1 mM sodium orthovanadate, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 1 mg/ml Pefabloc SC Plus (Roche)). Proteins (50 μ g) were separated by SDS-PAGE on 10% separation gels and transferred to nitrocellulose membranes by semidry transfer. Following transfer to membranes, immunoblot analysis was performed with the indicated antibodies at a 1:200 dilution. This was followed by incubation with a secondary antibody conjugated with horseradish peroxidase (HRP) at a 1:2000 dilution. ECL-PLUS Western Blotting Detection kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) was used to detect bound antibody. The relative changes were normalized to the tubulin signal (purchased from Cambridge, MA, USA) and expressed as percent relative to control.

EPC isolation and cell culture

Circulating human EPCs were cultured according to previously described techniques [19,20]. In brief, mononuclear cells (MNCs) were isolated by Histopaque-1077 (Sigma) density gradient centrifugation at 400g for 30 min. The mononuclear fraction was collected, washed in Dulbecco's phosphate-buffered saline, followed by red cell lysis with ammonium chloride solution (Stemcell Technologies) and purification with 3 washing steps, 2.5×10^6 cells/cm². MNCs were seeded on Fibronectin coated slides (Biocoat®). Cells were cultured in endothelial basal medium-2 (Clonetics) supplemented with EGM-2 Bullet Kit and 20% FCS for 5 days. Before each experiment, cells were placed in endothelial cell basal medium-2 with 0.5% fetal bovine serum (FBS) for 16 h for serum starvation. Experiments were performed by infecting EPCs with adenoviral constructs encoding β -galactosidase (Ad- β gal) and myr-Akt (Ad-Akt) at a multiplicity of infection of 50 for 24 h. In some experiments, EPCs were pretreated with 200 nM Wortmannin (PI-3 K inhibitor), 10 μ M U0126 (MEK-1/2 inhibitor) or 25 μ M SB203580 (P-38 MAPK inhibitor) for 1 h in medium containing 1% FBS. In some experiments, total RNA was extracted, and QRT-PCR for kinase insert domain protein receptor and neuropilin-1 was performed.

Results

Effect of Akt1 overexpression in cardiac endothelial cells

In order to investigate the consequence of endothelial Akt1 overexpression in the heart, transgene expression was induced at 8 weeks of age by removal of DOX from drinking water, and the expression was maintained for 6 weeks until sacrifice. Cross sections of heart tissue were obtained from DTG mice after 6 weeks of Akt1 expression and from control mice without Akt1 expression. Tissue samples were stained with hematoxylin and eosin (HE) to view overall

morphology Masson's trichrome (MT) for detection of fibrosis and CD31 for detection of microvessels (Fig. 2a). Histological examination of the hearts revealed no increase in individual myofiber size and no increase in interstitial fibrosis in DTG mice. There were, however, significant changes in microvessel density, as revealed by CD31 staining for the detection of microvessels when compared to control mice (Fig. 2b). The activation of Akt1 signaling for 6 weeks induced no significant increase in the heart/body weight (HW/BW) ratio of the DTG mice when compared to control mice in which Akt1 was not over expressed (Fig. 2c).

Transcript expression in hearts after endothelial Akt1 induction

To identify genes differentially regulated by Akt1 in the cardiac endothelial cells, we compared the gene expression profiles of DTG and control mice. Four DTG mouse hearts after 6 weeks of Akt1 induction and four control mouse hearts without Akt1 induction were harvested and used for DNA microarray analysis. Statistical analyses revealed that endothelial expression of myr-Akt1 resulted in the differential regulation of 248 transcripts of the ~46,000 transcripts examined (Fig. 3). Compared to control mice, 100 transcripts were upregulated and 148 transcripts were downregulated in DTG mice after 6 weeks of Akt1 induction.

Pathway analysis

An examination of related transcripts was performed using Ingenuity Pathways Analysis as described above. The set of 248 gene transcripts revealed as having a significant change in regulation in DTG mice compared to control mice (fold change $>/<1.8$; FDR < 0.1 , $P < 0.01$) were utilized for the Ingenuity Pathways Analysis. The network shown in Fig. 4 includes a number of angiogenesis related transcripts, such as connective tissue growth factor (Ctgf), kinase insert domain protein receptor (VEGFR-2), and neuropilin-1 (Nrp) (Table 2). A key providing transcript identity for this network and their fold change is provided in Table 3.

QRT-PCR was performed to validate the differential expression identified by the microarray analysis of 4 selected transcripts between DTG and control mice (Table 2). This analysis confirmed the upregulation of connective tissue growth factor (Ctgf), kinase insert domain protein receptor (VEGFR-2), neuropilin-1 (Nrp), and the Akt1 transgene.

VEGFR-2 has been shown to contribute to accelerated vessel growth [21]. Immunoblotting for VEGFR-2 in tissue samples obtained from DTG mice, post Akt1 induction, confirmed the upregulation of VEGFR-2 protein compared to control mice (Fig. 5a). Immunoblotting for neuropilin-1, whose overexpression results in increased microvessel density and endothelial cell proliferation [22,23], also detected an increase in the neuropilin-1 protein content in DTG mice after Akt1 induction (Fig. 5b).

Induction of signaling pathways in DTG mouse endothelial cells after Akt induction

The induction of Akt1 for 6 weeks in DTG cardiac endothelial cells resulted in phosphorylation of both Akt (the PI-3 K pathway) and Erk-1/2 (the MAPK pathway) compared to control mouse hearts as detected by immunoblotting (Fig. 6). In contrast, Akt1 induction had no effect on the levels of phosphorylated P-38 MAPK in DTG hearts (Fig. 6).

VEGFR-2 and NRP-1 expression in EPCs is regulated by the PI-3 K and Erk MAPK signaling pathways

In order to investigate the roles of specific signal-transduction pathways on VEGFR-2 and NRP-1 expression in endothelial progenitor cells (EPCs), EPCs were pretreated with the PI-3 K inhibitor Wortmannin, the MEK inhibitor U0126, and the P-38 MAPK inhibitor SB203580 and then induced with β -galactosidase (Ad- β gal) or myrAkt (Ad-Akt). Inhibition of the PI-3 K or MEK pathway blocked VEGFR-2 or NRP-1 mRNA expression as quantified by QRT-

PCR (Table 4). In contrast, inhibition of the P-38 pathways alone had no inhibiting effect on VEGFR-2 or NRP-1 mRNA expression (Table 4).

Discussion

Using a conditional transgenic (TG) system in which Akt signaling can be turned on in endothelial cells, we have shown that transient activation of Akt is associated with an increase in capillary density in the myocardium. These data are consistent with the observations of Sun et al. [15], which explores the role of dominant-active myr-Akt signaling during microvascular remodeling and shows that transient signaling impacts normal physiological microvascular development. We, however, did not see an increase in HW/BW ratio.

Our data also show that activation of Akt signaling within endothelial cells is sufficient to promote angiogenesis within the myocardium. Myocardial ischemia is an important clinical issue, and a number of clinical trials are examining whether angiogenic growth factor supplementation can be used to treat angina. It has long been recognized that Akt signaling within endothelial cells can promote angiogenic cellular responses in vitro and stimulate new blood vessel growth in vivo [24]. Therefore, we utilized the inducible Akt1 transgenic mouse system to elucidate the angiogenic program that accompanies vessel growth in the heart. This analysis identified a number of transcripts that are associated with blood vessel growth and integrity that were differentially regulated between transgenic and control mice.

Three key factors identified by this analysis include the VEGF receptors, VEGF-2 or neuropilin-1 and the connective tissue growth factor (Ctgf). Neuropilin-1 was originally described as a 130-140-kd cell-surface glycoprotein expressed in the developing *Xenopus laevis* nervous system [25]. Several studies suggest a role for neuropilin-1 in embryological vasculogenesis and angiogenesis [21-23] and it has been shown to be expressed in the skeletal and cardiovascular systems in embryos. Neuropilin knockout mice suffer from insufficient and delayed lethal failure in vascularization, whereas overexpression of Neuropilin-1 in transgenic mice is lethal due to hemorrhaging in the head and neck, excess blood vessel formation, and malformed hearts [26-28]. Neuropilin-1 has also been found to be expressed in adult endothelial cells and in a variety of other tissues including e.g., heart, bone marrow stromal cells, or bone marrow progenitor cells, which supports its proposed role in the regulation and induction of angiogenesis [29,30]. It has recently been shown that VEGF-R inhibitors and/or neuropilin-1 inhibitors blocked VEGF-A-induced differentiation of human bone marrow progenitor cells into cells with endothelial like characteristics and reduced the number of EPCs [29]. Therefore, it can be postulated that VEGF-R and/or neuropilin-1 are involved early in the mechanisms of VEGF-A, which lead to EPC differentiation. Furthermore, this suggests that after Akt1 induction, VEGF-R2 and/or neuropilin-1 upregulation may be a potent method to stimulate differentiation of EPCs, and consequently, to increase the involvement of EPCs in the angiogenic response. Our experiments revealed on the protein level that induction of Akt1 for 6 weeks in DTG cardiac endothelial cells resulted in phosphorylation of both Akt (the PI-3 K pathway) and Erk-1/2 (the MAPK pathway) compared to control mouse hearts, while Akt induction had no effect on the levels of phosphorylated P-38. In vitro experiment with EPCs showed that the inhibition of the PI-3 K or MEK pathway blocked VEGFR-2 or NRP-1 mRNA expression after Akt induction as quantified by QRT-PCR (Table 4), while inhibition of the P-38 pathways alone had no effect on VEGFR-2 or NRP-1 mRNA expression. MAPKs are a family of serine/threonine kinases that comprise 3 major subgroups, namely, extracellular signal-regulated kinase (ERK), p38 MAPK (p38), and c-Jun N-terminal kinases (JNK). It has recently been shown that p38 plays a critical role in the regulation of numbers of EPCs and that down-regulation of p38 increases proliferation and enhances endothelial differentiation in vivo [31]. These findings were supported in our experiments by the induction of EPCs with

Akt, thereby up-regulating VEGFR-2 or NRP-1 mRNA levels, which represent crucial factors for angiogenesis, while inhibition of p38 had no significant inhibiting effect on this increase.

Although connective tissue growth factor (Ctgf), a member of the CCN gene family, was originally identified in conditioned medium from human umbilical vein endothelial cells (HUVEC), attention at that time was focused on its effects on fibroblasts rather than on endothelial cells (ECs) [32]. However, immunohistochemical observations in mouse hearts show that Ctgf is present in the endothelium of the cardiovascular system as well as in highly vascularized structures such as lung suggesting that Ctgf may play a role in angiogenesis during cardiovascular development [33,34]. ECs in, e.g., angiolioma or angioleiomyoma express Ctgf mRNA, whereas those of venous lake or angiosarcoma do not [35], suggesting that benign proliferating ECs express Ctgf. Compelling evidence that Ctgf can regulate EC function is demonstrated by the ability of recombinant Ctgf to promote proliferation, migration, or adhesion of BAECs. Supplementation of Ctgf to monolayers of BAECs results in the formation of a branched network of capillary-like tubes. In addition, antiapoptotic effects are observed by supplementation with Ctgf [36].

The central role of vascular endothelial growth factor (VEGF) in angiogenesis in health and disease makes it attractive both as a therapeutic target for anti-angiogenic drugs and as a pro-angiogenic cytokine for the treatment of ischemic heart disease. Most biological functions of VEGF are mediated via VEGFR2. Neuropilin-1, a non-tyrosine kinase transmembrane molecule, may function as a co-receptor for VEGFR2. Considerable progress has recently been made toward delineating the signal transduction pathways distal to activation of VEGFR2. Activation of the mitogen-activated protein kinase, protein kinase C, and Akt pathways are all strongly implicated in mediating diverse cellular functions of VEGF, including cell survival, proliferation, and angiogenesis. Upregulation of metalloproteinases (e.g., MMP3), as revealed by our microarray experiments, is strongly implicated in VEGF-induced endothelial cell migration.

Myocardial ischemia is one of the most promising targets of gene therapy for angiogenesis. Although several growth factors and delivery approaches have yielded positive results in preclinical studies, first clinical studies have shown little or no real clinical benefit to the patients. It is likely that less than optimal gene therapy approaches have been used so far, and more thorough preclinical studies are needed to establish safe, efficient pro-angiogenic therapy. Therefore, “therapeutic angiogenesis” induced by Akt in endothelial cells leading to a angiogenesis-related network of growth and paracrine factors (e.g., kinase insert domain protein receptor (VEGFR-2), neuropilin-1 (Nrp)⁻¹, or connective tissue growth factor (Ctgf)) as revealed in our study may be an additional alternative approach for the treatment of ischemic heart disease with its broad spectrum from only effort-induced angina without myocardial damage to congestive heart failure (CHF).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Mouse model. Schematic illustration of the tetracycline-regulated transgenic system to conditionally switch Akt1 signaling on or off in the adult composed of Tet-myr-Akt1 and VE-Cadherin-tTA TG mice. In DTG mice, tTA expressed in the heart drives the expression of Akt1 transgene. Administration of doxycycline (DOX) results in the binding of DOX to tTA and repression of Akt1 transgene. Mice were divided into DOX (+) and DOX (-) groups and treated with DOX (0.5 mg/ml) in drinking water or not. Akt1 transcript upregulation was detected in the heart of DTG mice withdrawn from DOX (Table 2)

Fig. 2.

Histological analysis. (a) Heart sections were stained with hematoxylin and eosin (HE) for overall morphology (top), Masson's trichrome (MT) for detection of fibrosis and CD31 for detection of microvessels (bottom) in control DTG mice and in Akt1 induced DTG mice at time point 2 (6 weeks after Akt1 induction in heart endothelial cells). (b) Heart sections from DTG mice were stained with CD31 and CD31 positive cells were counted 10 times per microscope field to evaluate the microvessel density in control DTG mice and in Akt1 induced DTG mice at 6 weeks after Akt1 induction in heart endothelial cells. Data are shown as the means $\pm$ SD. (c) Heart weight/body weight ratio (HW/BW) in control DTG and in Akt1 induced DTG mice at time point 2 (6 weeks after Akt1 induction in heart endothelial cells)

Fig. 3. Clustergram. Vertical axis of the clustergram, control and DTG mice included in the study; horizontal axis of the clustergram, the 248 most significantly differentially expressed transcripts (fold change ≥ 1.8 ; FDR < 0.1 ; $P < 0.01$). For each transcript, there is a thin colored band, which visually describes the fold change of expression of that transcript in the sample. Red bands represent overexpressed transcripts; blue bands underexpressed transcripts; and white bands transcripts showing approximately equal expression. Transcript expression profiles in the hearts of control and DTG mice were analyzed 6 weeks after Akt1 induction in DTG mice and in control DTG mice without induction and were examined for statistically

significant differences in expression resulting in the differential regulation of 248 transcripts whose expression has significantly changed of the ~46,000 transcripts examined

Fig. 4.

Pathway analysis. Network constructed from differentially regulated transcripts comparing microarray data from mouse hearts after transgene induction and microarray data from mouse hearts without transgene induction. Shaded genes were identified as differentially expressed by the extent of shading that is indicative of the magnitude of regulation. Red shading indicates upregulation in induced DTG mice compared to non-induced DTG control mice, whereas green shading indicates downregulation. Node shapes indicate function and diamond designates enzyme, square, growth factor, triangle, kinase, and circle, other. In Fig. 4 the merged networks of a network with a network score of 15 ($P < 0.01$) and a network with a network score of 8 ($P < 0.01$) are shown. Yellow shading indicates the shared node shapes in the merged networks

Fig. 5. Immunoblotting for VEGFR-2 and neuropilin-1. Western immunoblots with the indicated antibodies were performed with heart tissue from control mice (=C) and DTG mice induced with Akt1 (=DTG). Representative immunoblots for VEGFR-2 (**a**, left panel) and neuropilin-1 (**b**, left panel) are shown. Quantitative analysis of relative changes for VEGFR-2 (**a**, right panel) and neuropilin-1 (**b**, right panel) as indicated. VEGFR-2 and neuropilin-1 signals were normalized to the tubulin signals and expressed as percent relative to control

Fig. 6. Intracellular signaling pathways. Hearts from control mice (=C) without Akt1 induction and DTG mice induced with Akt1 (=DTG) were harvested for Western blot analysis as described above and immunoblotting for the indicated signaling intermediates was performed

Table 1

Primers for QRT-PCR

Transcript name	5'-Forward primer-3'	5'-Reverse primer-3'	Accession code	Transcript symbol
Connective tissue growth factor (mouse)	TCAAGCTGCCTGGGAAATG	GTCTGGGGCCAAATGTGTCTTC	NM010217	Ctgf
Glyceraldehyde-3-phosphate dehydrogenase (mouse)	ACTCCACTCACGGGCAAAATTCA	GGCCTCACCCCAATTGATG	NM001001303	Gapdh
Glyceraldehyde-3-phosphate dehydrogenase (human)	ACAGCCTCAAGATCATCAGCAA	CCATCACGCCACAGTTTCC	NM002046	Gapdh
Kinase insert domain protein receptor (mouse)	GACCTGGCAGCACGAAACAT	ACCACACATCGCTCTGAAATTGT	NM010612	VEGFR-2
Kinase insert domain protein receptor (human)	CTGTCTCAGTGACAAACCCATACC	CGACTTTTGTGTGACCGCTTCA	NM002253	VEGFR-2
Neuropilin-1 (mouse)	CCGAGAAAAACAAGGTGTTTCATG	CGTCCGAAGCTCAGGTGTGT	NM008737	Nrp1
Neuropilin-1 (human)	CCTTCTGCCACTGGGAACAT	CGTCAGCTTTGGGAAATAGATGAAGT	NM003873	Nrp1
Thymoma viral proto-oncogene 1 (mouse)	CCTTCCTTACGGGCCCTCAA	ACACAAATCTCCGCACCATAGAA	NM009652	Akt1

Table 2
Microarray and QRT-PCR data in transcripts of interest

Transcript name	Data	FC DTG/Control
Connective tissue growth factor	Microarray data	+2.7 [*]
	QRT-PCR data	+3.7 [*]
Kinase insert domain protein receptor	Microarray data	+2.7 [*]
	QRT-PCR data	+2.2 ^{**}
Neuropilin-1	Microarray data	+1.9 [*]
	QRT-PCR data	+2.2 ^{**}
Thymoma viral proto-oncogene 1	Microarray data	+3.9 [*]
	QRT-PCR data	+2.9 [*]

All analysis values represent SEM performed in triplicates. Values represent fold change (FC) comparing DTG mice after 6 weeks of Akt1 induction and control mice without Akt1 induction

*
 $P < 0.01$

**
 $P < 0.05$

Table 3Pathways analysis network for DTG mice after 6 weeks of induction with Akt1 compared to control mice ($P < 0.01$)

Transcript symbol	Transcript name	Fold change
AKT1	v-akt murine thymoma viral oncogene homolog 1	+3.9
ALAS2	Aminolevulinate, delta-, synthase 2 (sideroblastic/hypochromic anemia)	+3.7
CAMK2A	Calcium/calmodulin-dependent protein kinase (CaM kinase) II alpha	-2.8
CAMK2D	Calcium/calmodulin-dependent protein kinase (CaM kinase) II delta	-2.4
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	-2.0
CTGF	Connective tissue growth factor	+2.7
DNAJA	DnaJ (Hsp40) homolog, subfamily A, member 1	-2.2
ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1 (avian)	-2.3
IFNGR2	Interferon gamma receptor 2 (interferon gamma transducer 1)	-1.8
HSPCA	Heat shock 90 kDa protein 1, alpha	-2.9
HSPCB	Heat shock 90 kDa protein 1, beta	-2.1
KDR	Kinase insert domain receptor (a type III receptor tyrosine kinase)	+2.7
KRT18	Keratin 18	+3.1
MMP3	Matrix metalloproteinase 3 (stromelysin 1, progelatinase)	+9.2
NRP1	Neuropilin 1	+1.9
PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	-1.9
RSN	Restin (Reed-Steinberg cell-expressed intermediate filament-associated protein)	-2.0
SERPINA3	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3	+4.9
SERPINH1	Serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	-3.9
SNCA	Synuclein, alpha (non A4 component of amyloid precursor)	+3.7

Table 4
Inhibition of Akt-induced kinase insert domain protein receptor and neuropilin-1 mRNA expression by signaling inhibitors

Transcript name	Infection with	After infection	+Wortmannin	+U0126	+SB203580	+Wortmannin/SB203580
Kinase insert domain protein receptor	Ad- β gal	+1.7	+1.2	-1.1	+1.7	+1.3
	Ad-Akt	+3.2*	+1.6	+1.4	+2.3*	+1.5
Neuropilin-1	Ad- β gal	+1.5	+1.1	+1.6	+1.7	+1.3
	Ad-Akt	+2.6*	+1.3	+1.6	+2.4*	+1.1

EPCs were treated with 200 nM Wortmannin (a phosphatidylinositol-3 kinase inhibitor), 10 μ M U0126 (a mitogen-activated protein kinase [MAPK]/extracellular signal-regulated kinase [MEK]/Erk-1/2 inhibitor), or 25 μ M SB203580 (a P-38 MAPK inhibitor). One hour later, EPCs were infected with β -galactosidase (Ad- β gal) or myr-Akt (Ad-Akt), and the mixture was incubated for 24 h. Total RNA was then harvested for analysis of VEGFR-2- and NRP-1 mRNA expression. In order to standardize the quantitation of kinase insert domain protein receptor and neuropilin-1, glyceraldehydes-3-phosphate dehydrogenase (GAPDH) from each sample was quantified by QRT-PCR and kinase insert domain protein receptor and neuropilin-1 were normalized to GAPDH. Values represent fold change (FC) comparing kinase insert domain protein receptor and neuropilin-1 after infection with β -galactosidase (Ad- β gal) or myr-Akt (Ad-Akt) and coinubation with the indicated inhibitors compared to control. All analysis values represent SEM performed in triplicates

* $P < 0.05$