Journal of Cellular Biochemistry 94:109–116 (2005)

Role of AKT/PKB Signaling in Fibroblast Growth Factor-1 (FGF-1)-Induced Angiogenesis in the Chicken Chorioallantoic Membrane (CAM)

Reza Forough,[1]* Brian Weylie,[1] Chirag Patel,[1] Sandy Ambrus,[2] Ugra S. Singh,[3] and James Zhu[4]

[1]Department of Medical Physiology and Cardiovascular Research Institute, College of Medicine, The Texas A&M University System Health Science Center, College Station, Texas 77843
[2]Department of Veterinary Pathobiology, College of Veterinary Medicine, The Texas A&M University, College Station, Texas 77843
[3]Division of Molecular Cardiology, Cardiovascular Research Institute, The Texas A&M University System Health Science Center, College of Medicine, Temple, Texas 76504
[4]Department of Poultry Sciences, The Texas A&M University, College Station, Texas 77843

Abstract Transfection of chicken chorioallantoic membranes (CAMs) with a chimeric secreted version of fibroblast growth factor-1 (*sp-FGF-1*) gene construct leads to a significant increase in vascularization. Though FGF-stimulated angiogenesis has been extensively studied, the molecular mechanisms regulating FGF-1-induced angiogenesis are poorly understood in vivo. This study was designed to investigate the role of the AKT (PKB) kinase signaling pathway in mediating sp-FGF-1-induced angiogenesis in the chicken CAM. The involvement of the AKT pathway was demonstrated by up-regulation of AKT1 mRNA expression in sp-FGF-1 compared to vector alone control transfected CAMs as demonstrated by real-time RT-PCR. Western analysis using an antibody specific to the activated AKT (phosphorylated AKT), demonstrated an increase in AKT activity in sp-FGF-1 compared to vector control transfected CAMs. More importantly, the AKT inhibitor ML-9 significantly reduced sp-FGF-1-induced angiogenesis in CAMs. These results indicate that AKT signaling plays a role in FGF-1-stimulated angiogenesis in vivo and the AKT pathway may serve as a therapeutic target for angiogenesis-associated diseases. J. Cell. Biochem. 94: 109–116, 2005.

Key words: angiogenesis; FGF-1; acidic FGF; AKT; PKB; PI3K; ML-9; CAM assay; transfection

Polypeptide growth factors are the most potent stimulators of angiogenesis [Cao et al., 2004; Raab et al., 2004]. Angiogenic activities of fibroblast growth factor-1 (FGF-1) and FGF-2, the two prototypic members of the FGF family, have been widely investigated in health and disease [Thompson et al., 1988; Puumala et al., 1990; Lopez et al., 1998; Sellke and Ruel, 2003]. It is their multi-potent ability to stimulate the different stages of angiogenesis, namely EC migration, proliferation, survival, and capillary tube formation, which underlies the interest for exploring the angiogenic properties of these FGFs. Despite the wide use of FGF-1 as a standard stimulator of angiogenesis, the molecular mechanisms regulating FGF-1-induced angiogenesis remain poorly understood in vivo. It is known that both FGF-1 and FGF-2 initiate signaling immediately after binding to and activating their cognate high affinity cell surface receptor, termed fibroblast growth factor receptor-1 (FGFR1), a member of receptor tyrosine kinases (RTKs) [Werner et al., 1992; Mohammadi et al., 1997]. Among the tyrosine kinase receptor-activated pathways, the AKT cascade has emerged as a critical regulator of cell growth, survival, and migration. AKT, a serine-threonine effector kinase, has been known to be activated by a wide variety of

Grant sponsor: The Texas A&M University System HSC Funds; Grant sponsor: American Heart Association, Texas affiliate; Grant number: 0455045Y.

*Correspondence to: Reza Forough, Department of Medical Physiology and Cardiovascular Research Institute, College of Medicine, The Texas A&M University System Health Science Center, College Station, TX 77843.
E-mail: forough@tamu.edu

Received 27 May 2004; Accepted 20 July 2004
DOI 10.1002/jcb.20274

angiogenic growth factors, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and interleukin-8 (IL-8) [Franke et al., 1995; Tilton et al., 1997; Gibson et al., 1999]. While there are a limited number of studies directed at evaluating the importance of AKT signaling in FGF-2-mediated angiogenesis [Qi et al., 1999; Nakashio et al., 2002], there are virtually no reports on the importance of AKT in mediating FGF-1-stimulated angiogenesis. The chicken chorioallantoic membrane (CAM) offers a great in vivo model for our proposed study because the embryonic chicken egg contains a very large number of FGFR1 molecules, a major factor that led to the successful purification and sequence determination of FGFR1 [Lee et al., 1989]. In addition, chicken FGFR1 is highly homologous to its human counterpart (98% amino acid sequence identity in the kinase region, 86% in the transmembrane region, 92% in the juxtamembrane region, and 80% in the carboxyterminus region) [Lee et al., 1989; Dionne et al., 1990], and can be activated when treated with a small dose of the human FGF-1 ligand [Schumacher et al., 1998]. Due to these salient features, the application of FGF proteins to chicken CAM assay has long been recognized as a classical model for studying angiogenesis in vivo [Auerbach et al., 1991; Ribatti et al., 1999].

In the present study, we explored the FGF-1-initiated and AKT-mediated angiogenesis signaling in vivo using a modified chick CAM assay model where transfection and overexpression of a secreted version of *FGF-1* gene construct has the ability to stimulate an exaggerated angiogenic response [Forough et al., 2003]. Our results indicate that neovascularization in vivo, stimulated by angiogenic growth factor FGF-1, can be reduced by selectively targeting the AKT signaling pathway.

MATERIALS AND METHODS

Constructions of sp-FGF-1 Plasmid

We previously have described construction of the signal-peptide-containing FGF-1 [Partridge et al., 2000]. Briefly, the signal-peptide used corresponds to the published sequence of one of the secretory members of the FGF family known as FGF-4 [Delli Bovi et al., 1987; Taira et al., 1987] growth factor. We chose the FGF-4 signal peptide sequence for our construct because the FGF-4 oncogene, in its native form, encodes a secreted growth factor with 30–40% homology to FGF-1. In addition, polymerase chain reaction (PCR) was used to add a Kozak sequence (CCACCATGG) to the final construct for maximal eukaryotic translational efficiency [Kozak, 2002]. The expression vector pMEXneo containing MSV-LTR and the SV40 polyadenylation site [Martin-Zanca et al., 1989] was chosen for the stable transfection of the sp-FGF-1 construct.

Angiogenesis Assay in Chicken Embryos

Single-comb-White Leghorn fertilized chicken eggs (Poultry Sciences, Texas A&M University, College Station, TX) were incubated at 37°C under ambient atmosphere with constant humidity for 8 days. A rotary tool was used to create a circular opening of 6–8 mm in diameter in the shell over the air sac to expose the CAM.

Direct Gene Transfer

CAMs were exposed as above and a 50 μl mixture containing 10 μg of sp-FGF-1 DNA construct plus 300 μM ML-9 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA) dissolved in 0.1% DMSO (Sigma, Inc., St. Louis, MO) plus 20 μl Lipofectamine 2000 transfection reagent (Invitrogen Life Technologies, Carlsbad, CA) was pipetted onto the CAM. Eggs were paraffin sealed and incubated under constant humidity until day 13. Negative controls were achieved by removing sp-FGF-1 construct from the 50 μl mixture delivered in one set of eggs and by delivering only DMSO plus vector control plus the transfection reagent in another set of eggs. CAMs were collected and digital images were prepared. For vessel counts, the 'point counting' method [Forough et al., 2003] was used.

RNA Preparation

Total cellular RNA was extracted using Totally RNA kit (Ambion, Inc., Austin, TX), an acid guanidinium thiocyanate-phenol-chloroform extraction-based method [Chomczynski and Sacchi, 1987]. Integrity and concentration of RNA samples were determined using an Agilent Bioanalyzer (Agilent Technologies, San Diego, CA).

Real-Time PCR

For RT, 1 μg of total CAM RNA was incubated with 0.5 μg of random primers and 10 mM each dATP, dGTP, dCTP, dTTP in the presence of M-MLV RT following protocol listed for first strand cDNA synthesis using M-MLV RT

(Invitrogen Life Technologies, Carlsbad, CA). For real-time PCR, each well contained a 50 μl mixture consisting of 3 μl of CAM cDNA made during RT plus 5 μl each of 300 nM forward (5′-AAGGAAGGATGGCTCCACAAA-3′) and reverse primers (5′-CGTTCCTTGTAGCCAATGAATGT-3′) specific to chicken AKT1 (Genbank Accession number: AF039943 plus 25 μl qPCR™ Mastermix Plus for Sybr Green 1 (Eurogentec North America, Inc., Philadelphia, PA) plus 12 μl dH_2O. Background controls were achieved through substitution of CAM cDNA with appropriate amount of dH_2O. For internal controls, we used chicken β-actin specific primers (forward: 5′-CTGATGGTCAGGTCATCACCATT-3′ and reverse: 5′-TAC CCAAGAAAGATGGCTGGAA-3′) (Genbank Accession number L08165) in substitution for the AKT primers.

Differences were measured between sp-FGF-1 treated CAM RNA and vector control CAM RNA samples using ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) as per directions for using Sybr Green. Relative quantification of *AKT1* gene expression was done using the Comparative Ct Method (Applied Biosystems, Foster City, CA). More specifically, the average number of cycles taken to reach the threshold (average Ct value) for sp-FGF-1 with AKT primers was calculated along with the calculation of the average Ct value for the sp-FGF-1 with actin primers samples. The difference between the average Ct values for the two samples was calculated by subtracting the average Ct for actin primers from the average Ct for AKT primers ($\Delta Ct =$ Ctactin − Ctsp-FGF-1) (normalized value). Identical calculations were made to achieve a normalized Ct value for the Vector Control CAM samples. The differences between the Vector Control normalized value and the sp-FGF-1 normalized value were calculated as $\Delta\Delta Ct = \Delta Ct$ vector control − ΔCtsp-FGF-1. Relative changes in mRNA levels were calculated using the formula $2^{\Delta\Delta Ct}$. For real-time PCR studies, we performed three independent experiments and each experiment performed in quadruplicates or triplicates except for one single case done in duplicates.

Western Analysis

For Western analysis, total proteins were extracted from each sample as described [Partridge et al., 1999] and quantified using BCA Protein Assay kit (Pierce, Inc., Rockford, IL). A volume containing 40 μg total protein from each sample was subjected to 10% SDS–PAGE. Subsequently, the proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane (BioRad, Hercules, CA). Membranes were blocked in TBST solution (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Tween-20) containing 5% BSA for 30 min at RT. Incubation with the 1:1,000 dilution of the primary antibody (phospho-AKT Ser 473, catalog #9271S; Cell Signaling Technology, Inc., Beverly, MA) was carried overnight with shaking at 4°C. After several washes in TBST, the membrane was incubated with the secondary antibody conjugated to HRP for 30 min at RT. The membrane was washed in TBST at least three times for 5 min each to remove the nonspecific bindings and was processed for development using SuperSignal® West Pico chemiluminescent kit (Pierce, Inc.) according to the manufacturer's recommendations. Subsequently, the membrane was stripped of the Ab by incubating for 30 min at a 70°C shaking incubator in a stripping buffer consisting of 2% (w/v) SDS, 62.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol. The membrane was washed in TBST wash buffer two times for 10 min each. For normalization, the stripped membrane was reprobed with a commercial antibody recognizing total AKT protein (Catalog #9272; Cell Signaling Technology, Inc.). For quantification, bands corresponding to the phosphorylated AKT and total AKT (internal control) proteins on the X-ray films were scanned with BioRad Gel-Doc 1000 using the Multianalyst version 1.0 program (BioRad, Inc.).

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) and the Student's *t*-test. All results are expressed as means ± SD. $P < 0.05$ was accepted as significant.

RESULTS

sp-FGF-1 Gene Transfection Induces Increased Angiogenesis in the Chick CAM

sp-FGF-1 gene plasmid and the vector alone control plasmid were separately introduced into day 8 CAMs. Quantification of the number of vessels revealed a 40% increase in vascularization in sp-FGF-1 compared to vector alone transfected CAMs when evaluated at day 13

(sp-FGF-1, 99.1 ± 16.9 vessels/2.7 mm^2; vector control, 59.8 ± 11.2 vessels/2.7 mm^2, $n = 12$ eggs per treatment group (Fig. 1) [Ribatti et al., 1999; Forough et al., 2003].

It is also important to emphasize that our CAM assay (assay from day 8 to 13) explores the effects of angiogenic factor sp-FGF-1 on a growing vasculature since the CAM is not yet fully differentiated during this period of embryonic development. At the molecular level, a number of well-recognized potent soluble angiogenic factors with characteristic tyrosine kinase receptors, including PDGF-BB, EGF, and IGF-I, mediate their pro-growth and survival signaling cues via AKT activation [Porter and Vaillancourt, 1998; Brazil et al., 2002]. Thus, we hypothesized sp-FGF-1-induced angiogenesis in CAMs was in part mediated through the AKT activation. In order to test this hypothesis, we assessed changes in AKT1 mRNA expression in response to sp-FGF-1 overexpression in CAMs. We should mention that it is also possible that the observed increase in AKT mRNA level in sp-FGF-1 CAMs might have been a secondary effect resulted from the improved vascularization generated by sp-FGF-1 treatment of CAMs.

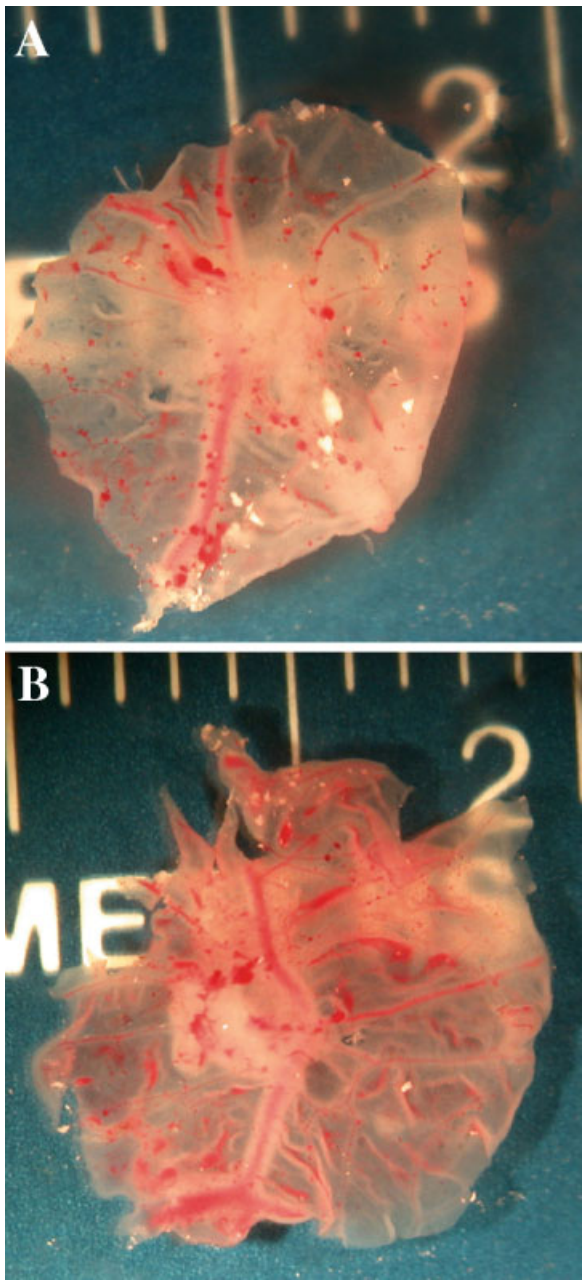

Fig. 1. Direct gene transfer into the chick CAM. At embryonic day 8 CAMs were transfected and returned to incubator for an additional 5 days. At day 13, CAMs were harvested and examined for vascularization: (**A**) vector alone-transfected CAM; (**B**) sp-FGF-1-transfected CAM. Pictures were taken by the aid of a dissection microscope (×20). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Increase in AKT mRNA Expression by *sp-FGF-1* Gene Transfection

To evaluate the role of AKT in sp-FGF-1-driven angiogenesis, real-time RT-PCR was employed to assess the AKT1 mRNA expression between day 13 (5 days post-transfection) chick CAMs transfected with *sp-FGF-1* gene construct and vector alone control. Based on this study, we observed approximately 2.3-fold increase in the level of the AKT mRNA expression for sp-FGF-1 compared to vector control CAMs (Fig. 2).

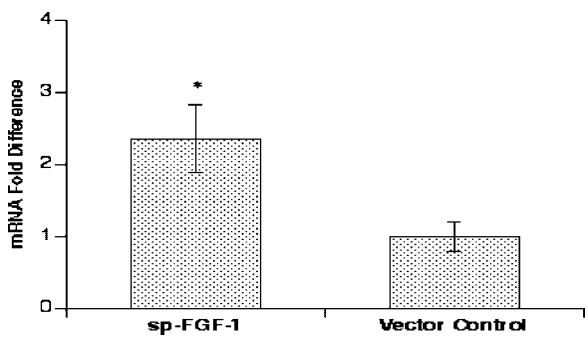

Fig. 2. Evaluation of the AKT1 mRNA expression in sp-FGF-1 and vector alone transfected CAMs by RT-real-time PCR. Total RNA isolated from day 13 transfected CAMs were reverse transcribed to cDNAs. Real-time SYBR-Green-based PCR was used to amplify chicken AKT1 and β-actin (internal control) in generated cDNAs for vector control and sp-FGF-1 transfected CAMs. Graph presentation depicts the relative changes in AKT mRNA expression in sp-FGF-1 and vector control transfected CAMs. AKT mRNA levels were normalized to actin mRNA levels in each condition. Bars, SD; $*P < 0.05$, Student's *t*-test.

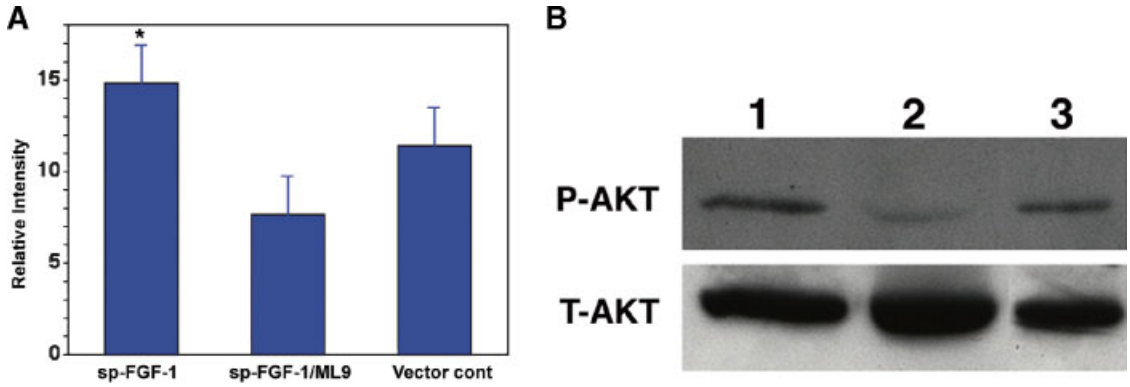

Fig. 3. Evaluation of the AKT phosphorylation in sp-FGF-1, sp-FGF-1 plus treatment with ML-9, and vector alone transfected CAMs by Western analysis using phospho-AKT antibody. Total protein extracts were harvested from day 13 CAMs that were transfected 5 days earlier. **Panel A**: For densitometric analysis, phospho-AKT levels were normalized to total-AKT levels in each condition. Values are means ± SD for three independent experiments. *Significantly different ($P < 0.05$; Student's *t*-test) from sp-FGF-1 plus ML-9 and vector control. **Panel B**: A representative Western blot is shown; (**lane 1**) sp-FGF-1, (**lane 2**) sp-FGF-1 plus ML-9, (**lane 3**) vector alone. P-AKT refers to phospho-AKT and T-AKT refers to total-AKT. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

These results suggested a possible role for AKT signal transduction cascade in regulating the molecular mechanism of angiogenesis stimulated by polypeptide growth factor FGF-1.

Increase in AKT Phosphorylation by *sp-FGF-1* Gene Transfection

Activation of Akt kinase signaling involves its phosphorylation at Ser473 and Thr308 [Alessi et al., 1996; Yang et al., 2002]. To evaluate changes in AKT activation in response to FGF overexpresion, Western analyses using a commercial antibody with the ability to recognize phosphorylated (active) form of the AKT were performed on total protein extracts prepared from day 13 sp-FGF-1 and vector transfected CAMs. Based on this study, a significant increase in Ser473 phosphorylation of the 60 kD AKT was observed in sp-FGF-1 compared to vector alone transfectants (Fig. 3).

In order to demonstrate the selectivity of the reaction, transfected CAMs were treated with 300 μM ML-9, a selective inhibitor of AKT activity [Smith et al., 2000; Steinle et al., 2002]. ML-9 treatment of the CAMs at the time of transfection at day 8 resulted in reduction of AKT activity at day 13 in sp-FGF-1 transfected CAMs (Fig. 3). Taken together, these data demonstrate the involvement of AKT signaling in sp-FGF-1 CAMs.

AKT Inhibitor ML-9 Selectively Inhibits Vascularization in sp-GF-1 CAMs

To evaluate the role of the AKT signaling pathway in the regulation of FGF-1-stimulted angiogenesis, CAMs were treated with AKT inhibitor ML-9 at the time of *sp-FGF-1* gene transfection at day 8. We initially did a dose response curve study (n = 3 eggs per drug concentration) and determined that a dose of 300 μM ML-9 inhibited sp-FGF-1-induced angiogenesis with no apparent gross side effects in CAMs. We repeated the experiment (n = 4) and have shown that the blockade of the AKT pathway using ML-9 inhibitor resulted in a significant reduction in angiogenesis in sp-FGF-1 CAMs (Fig. 4).

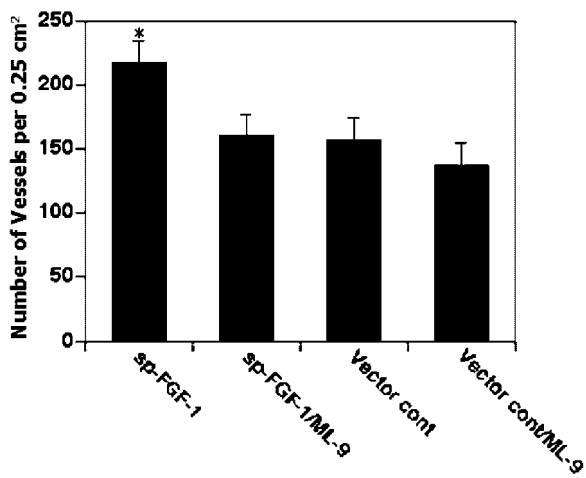

Fig. 4. Effect of AKT inhibitor ML-9 on CAM vascularization. Direct sp-FGF-1 or vector alone gene delivery to day 8 CAM was accomplished as described in the presence or absence of AKT inhibitor ML-9. On day 13, 5 days following the plasmid gene delivery, the CAM tissues underneath the air sac were excised and their blood vessels (four randomly selected sites per egg and n = 4 in each group) counted in a blind manner. Bars, SD; *$P < 0.05$; Student's *t*-test.

The data from the blocking experiments support the importance of the AKT signaling in the in vivo induction of angiogenesis by the angiogenic growth factor FGF-1.

DISCUSSION

This study establishes the importance of the AKT signaling pathway in the regulation of angiogenesis stimulated by overexpression of a secretory version of FGF-1. This conclusion was reached based on the following observations: (1) CAMs transfected with *sp-FGF-1* gene constructs exhibited increased levels of mRNA synthesis and activity of the AKT molecule, (2) the AKT inhibitor ML-9 significantly reduced sp-FGF-1-stimulated angiogenesis in CAMs.

The AKT signaling pathway has been implicated in regulation of cell survival/anti-apoptosis and proliferation in response to cytokines and cell adhesion molecules [Downward, 1998]. The mechanism of AKT activation by the tyrosine kinase receptors initiate by the conversion of phosphatidylinositol (4,5)-phosphate (PIP2) into phosphatidylinositol (3,4,5)-phosphate (PIP3) mediated by the enzymatic activity of the PI3K. Upon formation, the membrane-bound PIP3 binds to the pleckstrin homology (PH) domain of the AKT kinase. The membrane-associated AKT undergoes activation after undergoing phosphorylations on specific serine and threonine residues. Activated AKT dissociates from the membrane and continues the signaling processes initiated by extracellular growth factor(s) through phosphorylation of the downstream cytosolic substrates [Krauss, 2001]. Among the multiple signaling pathways downstream of AKT, cascades leading to angiogenesis are of major importance [Shiojima and Walsh, 2002]. For example, abrogation of the *AKT* gene expression and activity diminishes the VEGF-stimulated angiogenesis and the survival of ECs, respectively [Jiang et al., 2000]. In addition, similar shifts in the extent of angiogenesis occur as a result of overproduction and knocking out of the PI3K, the major upstream regulator of AKT, which functions immediately upstream of AKT [Sengupta et al., 2003; Chen et al., 2004].

EC migration, survival, growth, and differentiation are the fundamental steps of angiogenesis. AKT is known to influence all the above cellular functions. Our study specifically indicates for the first time that AKT is a regulator of FGF-1-stimulated angiogenesis presumably through influencing these cellular functions. Although there is not much reported on the role of AKT in regulating angiogenesis stimulated by FGF-1, a handful number of publications have presented data indicating a central role for AKT signaling in the processes leading to angiogenesis initially stimulated by FGF-2 [Qi et al., 1999; Zubilewicz et al., 2001; Nakashio et al., 2002]. Based on these studies, it appears that AKT activation in response to FGF-2 treatment mainly signals growth/survival of sprouting ECs. Overexpression of FGF-1 in non-endothelial NIH 3T3 cell line also functions through the AKT signaling to extend the life-span of the host cells when challenged with a pro-apoptotic dose of a synthetic retinoid [Wan et al., 2001]. These finding suggest that AKT activation in response to FGF-1 and FGF-2 mainly serves to sustain EC survival/proliferation during angiogenesis. Therefore, if AKT is the critical regulator of the signal transduction pathways responsible for the survival function of FGF-1, one can develop better pharmacological therapeutic interventions to selectively target molecular events determining growth or death of an EC.

We also realize that there seems to be an imbalance between the relative expression of AKT mRNA and protein in sp-FGF-1-transfected CAMs. While the exact cause of this observation is unclear, it is not unusual for certain genes to exhibit disparities between mRNA and protein expression levels. For example, mRNA species containing longer 5′ untranslated regions (5′-UTL) with one or more AUG codons positioned upstream of the translational initiation sites are generally subjected to a tighter translational control. Translation of socs-1 mRNA, which is tightly regulated by two upstream AUGs is an example of such mRNA species that are translationally regulated [Gregorieff et al., 2000]. Subsequently, we examined a stretch of 465 nucleotides corresponding to 5′-UTL of chicken AKT1 and identified two upstream AUGs in this long 5′-UTL. Therefore, it is tempting to speculate that AKT1 mRNA possesses the same characteristics (long 5′-UTL and upstream AUG codons) as certain mRNA species that are known to be tightly regulated at the translational level.

The immunoincompetent nature of embryos characterized by "take" of xenografts as well

as its technical simplicity and cost-effectiveness add to the versatility of the proposed chick CAM model for studying the AKT signaling in different contexts [Forough et al., 2003]. For example, xenograft implantations of human solid tumor cells on the described chick embryo can be easily performed to study the role of AKT signaling in tumor angiogenesis. Alternatively, one can use the proposed direct gene transfer model into chick CAM to study the role of AKT signaling in angiogenic events stimulated by other known angiogenic growth factors, cytokines, chemokines, and cell adhesion molecules.

In summary, this study demonstrates the AKT pathway as a regulator of FGF-1-stimulated angiogenesis in vivo. Thus, selective blockade of AKT signaling through the use of proper inhibitor(s) may serve as anti-angiogenic therapies to reduce neovascularization in those clinical settings where unwanted angiogenesis is not desirable. Conversely, selective activation of the AKT signaling may serve as pro-angiogenic therapies when reparative angiogenesis is the objective in order to improve ischemic heart and limb functions by restoring sufficient blood circulation.

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