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14-3-3 β -Rac1-p21 Activated Kinase Signaling Regulates Akt1-Mediated Cytoskeletal Organization, Lamellipodia Formation and Fibronectin Matrix Assembly

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Abstract

Akt1 belongs to the three-gene Akt family and functions as a serine–threonine kinase regulating phosphorylation of an array of substrates and mediating cellular processes such as cell migration, proliferation, survival, and cell cycle. Our previous studies have established the importance of Akt1 in angiogenesis and absence of Akt1 resulted in impaired integrin activation, adhesion, migration, and extracellular matrix assembly by endothelial cells and fibroblasts. In this study, we identify the downstream signaling pathways activated by Akt1 in the regulation of these cellular events. We demonstrate here that Akt1 is necessary for the growth factor stimulated activation of 14-3-3 β -Rac1-p21 activated kinase (Pak) pathway in endothelial cells and fibroblasts. While activation of Akt1 resulted in translocation of Rac1 to membrane ruffles, enhanced Rac1 activity, Pak1 phosphorylation, and lamellipodia formation, resulting in enhanced adhesion and assembly of fibronectin, inhibition of Akt1 resulted in inhibition of these processes due to impaired Rac1-Pak signaling. Formation of lamellipodia, adhesion, and fibronectin assembly by myristoylated Akt1 expression in NIH 3T3 fibroblasts was inhibited by co-expression with either dominant negative Rac1 or dominant negative Pak1. In contrast, impaired lamellipodia formation, adhesion, and fibronectin assembly by dominant negative-Akt1 expression was rescued by co-expression with either constitutively active-Rac1 or -Pak1. Moreover, previously reported defects in adhesion and extracellular matrix assembly by *Akt1*^{-/-} fibroblasts could be rescued by expression with either active-Rac1 or -Pak1, implying the importance of Rac1-Pak signaling in growth factor stimulated cytoskeletal assembly, lamellipodia formation and cell migration in endothelial cells and fibroblasts downstream of Akt1 activation.

Ability of the cells to migrate is determined by environmental cues, including growth factors, cytokines, and extracellular matrix (Le and Carlier, 2008). Interplay between these molecules results in activation of receptor tyrosine kinases and integrins via outside-in signaling, thus activating an array of intracellular pathways that regulate the ability of cells to migrate via inside-out signaling (Arnaout et al., 2007). Cell migration is essential for embryonic development, inflammation, tissue remodeling, neovascularization, and tumor invasion (Lauffenburger and Horwitz, 1996; Ridley, 2004; Cernuda-Morollon and Ridley, 2006). Regulation of directionality and velocity of cell migration is a complex process that depends upon formation of membrane protrusions termed as lamellipodia at the leading edge of the cell. These protrusions propel forward advancement of the cell, forming adhesion complexes with the migratory substrate and coordinating cytoskeletal dynamics (Lauffenburger and Horwitz, 1996; Ridley, 2004; Prass et al., 2006). Integrins then link the

extracellular matrix with large bundles of intracellular microfilaments that form prominent stress fibers (Calderwood et al., 2000), followed by formation of actin-based structures such as microspikes and membrane ruffles resulting in cell spreading and migration (Jin and Wang, 2007).

Considerably less is known about the mechanism of integrin activation via growth factor-mediated inside-out signaling and how extracellular matrix acts as an insoluble extracellular agent capable of inducing the assembly of these same structures when cells are plated on a matrix. Previous studies have established the association of Akt with actin cytoskeleton and have implicated its importance in the cytoskeletal organization (Guo et al., 2006; Vandermoere et al., 2007). Deficiency of Akt1, the predominant Akt isoform in endothelial cells and fibroblasts, resulted in impaired fibronectin matrix assembly by fibroblasts in vitro (Somanath et al., 2006, 2007) and impaired collagen and laminin assembly in skin and blood vessels in vivo, resulting in loose skin and leaky blood vessels (Chen et al., 2005; Somanath et al., 2008). Our studies also showed that Akt1 is necessary for angiogenesis and vascular maturation in tumor and wound healing models (Chen et al., 2005; Somanath et al., 2008). One of the major reasons we identified for these defects was the impaired inside-out activation of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in *Akt1*^{-/-} vascular cells, thus resulting in impaired adhesion and migration of endothelial cells and fibroblasts on various matrix proteins (Somanath et al., 2007).

Rho family of small GTPases such as RhoA, Rac1, and cdc42 is poised to contribute to the integrin-mediated events that control cytoskeletal changes involved in fibroblast morphology during adhesion, spreading, migration, and extracellular matrix assembly (Linseman and Loucks, 2008). Activation of RhoA stimulates microfilament bundling in serum-starved cells that are already in adherent condition (Linseman and Loucks, 2008). While activated cdc42 controls the extension of actin spikes to form the filopodia, activation of Rac1 triggers growth factor stimulated membrane ruffling and formation of lamellipodia (Linseman and Loucks, 2008). A previous study reports the importance of Akt in the activation of Rho family of GTPase member Rac (Pankov et al., 2005).

Our observation that integrin activation, adhesion, and migration of *Akt1*^{-/-} endothelial cells and fibroblasts on matrix proteins is impaired, we hypothesized that Akt1 is responsible for the growth factor mediated activation of one or more of the Rho family of small GTPase, necessary for the cytoskeletal organization and development of lamellipodia in vascular cells. Hence, in order to prove our hypothesis, we studied the ability of Akt1 to regulate growth factor stimulated lamellipodia formation, actin cytoskeletal assembly, and translocation as well as activation of Rho GTPase in endothelial cells and fibroblasts. Efforts were also made to study its importance in the regulation of adhesion to fibronectin and mediate fibronectin matrix assembly. We find that modulation of Akt1 activity has a profound effect on modulation of 14-3-3 β -Rac1-p21 activated kinase (Pak) signaling, in turn, regulating lamellipodia formation, adhesion to fibronectin and fibronectin assembly. Moreover, the defect in adhesion and fibronectin matrix assembly by *Akt1*^{-/-} fibroblasts was rescued by restoring active Rac1-Pak signaling. Altogether, we provide a novel mechanism regulated by Akt1 in growth factor stimulated endothelial and fibroblast lamellipodia formation and fibronectin matrix assembly.

Materials and Methods

Animals

Akt1^{-/-} mice were generated as previously described (Chen et al., 2001) and were maintained in the 129 R1/C57BL/6 background. We used sex- and age-matched wild-type and *Akt1*^{-/-} littermates for the study. The animals were 8–12 weeks of age at the time of cell

isolation. All the procedures were approved by Cleveland Clinic Institutional Animal Care and Use Committee.

Antibodies

Anti-human panAkt, Akt1, phosphoT308-Akt, phosphoS473-Akt, phospho-GSK3, and phospho-PAK antibodies were purchased from Cell signaling (Boston, MA). Anti-Rac1 antibody was purchased from BD Biosciences (Franklin Lakes, NJ). Anti-14-3-3 antibody and Alexa fluor 585 secondary antibody were purchased from Invitrogen (Carlsbad, CA). Anti-fibronectin and (β -actin antibodies and TRITC-labeled phalloidin were purchased from Sigma (St. Louis, MO).

Cell lines and cell culture

NIH 3T3 fibroblasts were obtained from ATCC (Manassas, VA) and were transfected with myristoylated-Akt1 (mAkt1; constitutively active), DN-Akt1 (Akt1 M¹⁷⁹), 14-3-3 β , CA-Rac1 (Rac1-L⁶¹), DN-Rac1 (Rac1-N¹⁷), CA-Pak1 (PAK1 E⁴²³), DN-Pak1 (Pak1 83–149 or Pak1 R²⁹⁹) using FuGENE 6 transfection reagent as per the manufacturer's protocol. Approximately 70–80% transfection efficiency was obtained in NIH 3T3 cells and 60–70% efficiency was observed in immortalized mouse fibroblasts as observed by GFP staining. Fibroblasts were cultured in DMEM with 10% fetal bovine serum, whereas endothelial cells were cultured in DMEM/F-12, supplemented with L-glutamine, 90 μ g/ml heparin, 50 μ g/ml endothelial cell growth supplement (BD Biosciences), and 10% fetal bovine serum.

Aortic ring assay

We performed aortic ring assays as previously described (Mahabeleshwar et al., 2006). We stimulated vessel sprouting using 60 ng/ml of VEGF. Pictures were taken on different days (4, 6, and 10 days) and analyzed for sprout lengths using Image Pro Plus software.

Isolation of primary endothelial cells and mouse embryonic fibroblasts

The microvascular ECs were isolated from lungs using a previously described protocol (Chen et al., 2005; Mahabeleshwar et al., 2006). Briefly, lungs were minced and digested using a 3 mg/ml collagenase–dispase mixture (Roche Diagnostics, Basel, Switzerland) for 4 h at room temperature. The rest of the procedures were performed as described previously (Chen et al., 2005). Mouse embryonic fibroblasts were collected from 11- to 14-day-old embryos. Fetuses were removed from amniotic sacs and cleared of the heads, viscera, and limbs. Embryos were transferred to a sterile container containing DMEM pre-warmed to 37°C. Embryos were then washed and chopped to 3 mm³ cubes until the washes were clear of blood cells. Cubes were digested using a 3 mg/ml collagenase–dispase mixture for 4 h at room temperature. After enzymatic digestion, the supernatant was collected through a tissue strainer (Fisher Scientific, Pittsburgh, PA). The filtrate was centrifuged and the pellet was washed in PBS. The cells were then resuspended in DMEM culture medium with 10% FBS and plated on plastic.

Cell adhesion assay

Cell adhesion assays were performed as previously described (Somanath et al., 2007). Mouse lung ECs and mouse embryonic fibroblasts, within 1–2 passages, were suspended in serum-free medium (DMEM/F-12) and then stimulated with 20 ng/ml VEGF and FGF, respectively. The cells in suspension were immediately transferred to ligand-coated 12-well plates at 1×10^5 . After incubation at 37°C for 42 min, the wells were washed three times. Cells were then fixed in 70% methanol and stained with 1% toluidine blue in 1% borax, and adherent cells were examined microscopically and quantified.

Analysis of fibronectin assembly

A previously described protocol was used for the analysis of fibronectin assembly (Somanath et al., 2007). Fibroblasts were grown on glass coverslips along with 10 μ g/ml exogenous fibronectin and after 8 h incubation in serum-free medium, the media were removed and the confluent monolayer was fixed in 1% paraformaldehyde then probed with anti-fibronectin antibody overnight at 4°C. The cells were probed with labeled secondary antibody (Alexa Fluor 585, Invitrogen) and mounted using 4',6-Diamidino-2-phenylindole-containing Vectashield (Vector Laboratories, Burlingame, CA). The slides were photographed using a fluorescence microscope.

Immunocytochemistry

The immunofluorescence staining was performed as described previously (Somanath et al., 2007). Briefly, cells grown on coverslips were fixed at different time points with 1% paraformaldehyde in PBS. Cells were permeabilized with 0.1% triton X-100 in PBS. The non-specific staining was blocked with 2% BSA for 1 h at room temperature. Primary antibody diluted at an appropriate concentration was applied and incubated overnight at 4°C. In the case of phalloidin staining the fixed and permeabilized cells on coverslips was incubated with standardized dilution of TRITC-phalloidin for 20 min and washed. After washing with PBS, the slides were incubated with Alexa Fluor-labeled secondary antibody for 2 h at room temperature. The slides were mounted with Vectashield (Vector Laboratories). The images were taken by confocal microscope.

Rac activation assay

Rac activation was assayed using a kit from Cytoskeleton (Denver, CO) as per the manufacturer's protocol. Briefly, the p21-binding domain (PBD) of PAK fused to glutathione S-transferase (GST) in turn conjugated with glutathione-Sepharose 4B beads that were mixed with cell lysates in Rac lysis buffer, and 1 mg of total lysate and were nutated at 4°C for 45 min. Beads were collected by centrifugation and were washed three times with washing buffer [25 mM Tris-HCl (pH 7.6), 1 mM DTT, 30 mM MgCl₂, 40mM NaCl, 1% (v/v) Nonidet P-40] and twice with washing buffer without Nonidet P-40. Proteins were eluted by boiling beads in 2× Laemmli sample buffer for 5 min, separated on a 15% SDS-polyacrylamide gel, and blotted for Rac.

Western blot analysis

Cell lysates were prepared using lysis buffer (20 mM Tris-HCl, pH 7.4; 1% Triton X-100, 3 mM EGTA, 5 mM EDTA, phosphatase inhibitors (10 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 5 mM sodium fluoride, and 10 μ M okadaic acid), protease inhibitor cocktail (Roche Diagnostics) and 1 mM PMSF. SDS-PAGE and Western blotting were performed as described previously (Laemmli, 1970). Densitometry analyses were performed using Kodak Image Station 4000MM.

Statistical analysis

All the data are presented as means $\pm$ SD. We performed all the analyses using two-sample *t*-tests with a two-tailed distribution and the significance was set at 0.05 levels (marked with an asterisk wherever data are statistically significant).

Results

Akt1 deficiency results in impaired aortic ring sprouting

We had previously shown that Akt1 is necessary for integrin activation in endothelial cells and fibroblasts that in turn regulate endothelial cell as well as fibroblast adhesion and

migration on various matrix proteins (Somanath et al., 2007). We further wanted to study the time course effects of Akt1 deficiency in vascular cells in an ex vivo model and we utilized aortic ring assay which is a frequently used model to study the vascular cell properties such as cell migration and proliferation ex vivo. Aortic rings isolated from WT and *Akt1*^{-/-} mice, implanted in growth factor containing matrigel and allowed to sprout were quantified using Image Pro plus software. Length of cell sprouts from the origin was measured at days 4, 6, and 10 and the data were quantified. Our data show that on the days 4, 6, and 10 that we analyzed, the lengths of sprouts developed from *Akt1*^{-/-} aorta were lesser compared to the WT ($P < 0.02$, $P < 0.0003$, and $P < 0.001$, respectively) implicating that Akt1 is necessary for the serum-induced aortic ring sprouting and vascular cell migration (Fig. 1A,B).

Akt1 is necessary for the growth factor mediated lamellipodia formation in endothelial cells and fibroblasts

An early event that regulates cell spreading as well as migration of endothelial cells and fibroblasts is their ability to effectively form lamellipodia and membrane ruffles at the leading edge that help the cells to migrate with a sense of direction (van et al., 2006). Since we know that Akt1 is necessary for cell spreading and directional migration (Byzova et al., 2000; Somanath et al., 2007), we wanted to determine whether Akt1 is necessary for the endothelial and fibroblast lamellipodia formation in response to growth factors. In order to study this, we first treated serum starved NIH 3T3 fibroblasts with bFGF in the presence and absence of inhibitors of PI3 kinase and Akt (10 μ M LY294002 and 1 μ M SH-5, respectively) and analyzed newly polymerized actin using TRITC-labeled phalloidin. Our data showed that treatment with bFGF in serum starved NIH 3T3 fibroblasts resulted in enhanced lamellipodia and actin stress fiber formation compared to untreated control (Fig. 2A). The number of cells showing lamellipodia in response to bFGF was ~6-fold higher compared to the control (Fig. 2A, right part). However, bFGF stimulated lamellipodia and stress fiber formation was completely blunted by co-incubation with inhibitors of PI3 kinase and Akt (LY294002 and SH-5, respectively), thus suggesting that Akt is necessary for the lamellipodia formation and cell spreading in fibroblasts (Fig. 2A).

Next, we wanted to study whether these effects are specific due to inactivation of Akt1 in these cell types. In order to do this, we transected NIH 3T3 cells with constitutively active (myristoylated) form of Akt1 (mAkt1) or DN-Akt1 (Akt1 K179M) with GFP marker and studied the ability of Akt1 to regulate actin stress fiber formation and lamellipodia formation. We selected the GFP positive cells in each case and looked for actin polymerization. Our data show that cells transfected with mAkt1 exhibited enhanced lamellipodia formation even in the absence of bFGF stimulation with a minimal increase in stress fiber density compared to un-transfected cells (Fig. 2B). When expressed with mAkt1 (active Akt1), we observed ~5-fold increase in the total number of cells showing lamellipodia (Fig. 2B, right part). In contrast, cells transfected with DN-Akt1 and treated with bFGF resulted in impaired lamellipodia formation with a very modest decrease in stress fiber density in most of the cells we analyzed (Fig. 2B). When compared to control cells treated with bFGF, cells expressing DN-Akt1 exhibited ~6-fold decrease in lamellipodia positive cells (Fig. 2B, right part). In order to further confirm our data, we utilized WT and *Akt1*^{-/-} endothelial cells and plated them on fibronectin. Serum starved cells were treated with VEGF (20 ng/ml) for 1 h and analyzed for phalloidin staining. Our data indicated that treatment with VEGF enhanced stress fiber formation as well as lamellipodia formation in WT endothelial cells compared to untreated control (Fig. 2C). In contrast, even though there was only a modest decrease in actin stress fiber formation, lamellipodia formation was significantly impaired in VEGF treated *Akt1*^{-/-} endothelial cells compared to WT (Fig. 2C). Quantitative analyses showed ~2–3-fold decrease in lamellipodia positive endothelial cells lacking Akt1, both in the presence and absence of VEGF, compared to WT (Fig. 2C, right

part). Together, these studies indicate that Akt1 is necessary for fibroblast and endothelial lamellipodia formation in response to bFGF and VEGF, respectively.

Akt1 regulates growth factor mediated activation of Rac1-p21 activated kinase signaling

Actin cytoskeleton assembly upon integrin-matrix interaction and growth factor stimulation is mainly mediated through small Rho-GTPase family members such as Rho, Rac, and cdc42 (Cernuda-Morollon and Ridley, 2006). While Rho regulates actin stress fiber formation, Rac and cdc42 regulates lamellipodia and filopodia formation, respectively (Cernuda-Morollon and Ridley, 2006). While modest changes were observed in stress fiber formation, a more significant inhibition of endothelial and fibroblast lamellipodia formation was observed upon inhibition of Akt1, suggesting that Akt1 regulated lamellipodia formation, cell spreading, and migration may involve activation of Rac and p21 activated kinase (Pak) pathway. Hence we next wanted to determine whether Akt1 could modulate the activity of Rac and Pak in endothelial cells and fibroblasts.

First, we studied the time course of Akt phosphorylation in endothelial cells in response to VEGF. Treatment of serum starved endothelial cells with 20 ng/ml VEGF resulted in enhanced T308 phosphorylation at 5 min after treatment reaching a plateau at 15–30 min (Fig. 3A). Similar increase in phosphorylation of GSK-3, a major substrate of Akt in mammalian cells, was also observed upon treatment with VEGF (Fig. 3A). Using this information, WT and *Akt1*^{-/-} endothelial cells were treated with VEGF for 15 min and the lysates were subjected for Rac1 activity analysis. In WT endothelial cells, treatment with VEGF resulted in increased levels of GTP bound Rac1 (Fig. 3B). In contrast, basal level of active Rac1 was significantly reduced in *Akt1*^{-/-} endothelial cells compared to WT (Fig. 3B). Also, we observed a significant decrease in the VEGF stimulated increase in Rac1-GTP levels in *Akt1*^{-/-} endothelial cells compared to WT (Fig. 3B), thus demonstrating that Akt1 activity is necessary for the activation of Rac1 in endothelial cells.

Next we determined whether Akt1 is necessary for the activation of Rac1-Pak signaling in fibroblasts. To do this, we serum starved NIH 3T3 fibroblasts and treated them with bFGF (20 ng/ml) in the presence and absence of inhibitors of PI3 kinase (10 μ M LY294002; 100 nM Wortmannin), Akt (1 μ M SH-5) and GSK-3 (SB-415286) and the lysates were subjected to Rac1 activity analysis. Our data show that bFGF treatment resulted in enhanced Rac1 activity levels in NIH 3T3 cells (~2–3-fold) compared to DMSO treated controls (Fig. 3C, left part and Fig. 3D). Treatment with inhibitors of PI3 kinase and Akt blunted the increase in Rac1-GTP levels stimulated by bFGF compared to bFGF alone treated cells (Fig. 3C, left part). However, treatment with GSK-3 inhibitor (SB-415286) did not inhibit bFGF mediated Rac1 activation (Fig. 3C, left part and Fig. 3D).

P21 activated kinase (Pak) is a downstream target of Rac and cdc42 in endothelial cells and fibroblasts (Fryer and Field, 2005). Depending upon the localization of activated Rac and cdc42, its function in these cells may vary (ten Klooster et al., 2006). Since Rac1 activity is regulated by Akt1, we determined whether inhibition of PI3 kinase, Akt or GSK-3 will have any effect on Pak activation. As expected, we observed that phosphorylation of Pak1 and Pak2 was enhanced in bFGF treated NIH 3T3 fibroblasts compared to untreated control (Fig. 3C, left part). As observed in the case of Rac1 activation, activation of Pak1/2 by bFGF was also blunted by treatment with PI3 kinase (LY294002, Wortmannin) and Akt (SH-5) inhibitors, but not with GSK-3 inhibitor (SB-415286) (Fig. 3C, left part).

We next determined whether specific activation or inhibition of Akt1 in NIH 3T3 fibroblasts would have an effect on modulating Rac1 and Pak1/2 activation. To do this, NIH 3T3 cells were transfected with either mAkt1 (active) or DN-Akt1 (inactive) and lysates were prepared for Rac1 activity assay and Pak1/2 phosphorylation. As expected, we observed

enhanced Rac1 activity as well as Pak1/2 phosphorylation upon mAkt1 expression, where as expression of DN-Akt1 resulted in decreased levels of Rac1-GTP and Pak1/2 phosphorylation (Fig. 3C, right part).

To further confirm these results, we utilized WT and *Akt1*^{-/-} fibroblasts and serum-starved cells were treated with bFGF in a time course study. Lysates were subjected to Rac1-GTP pull down assay and activation status of Rac1 was compared between WT and *Akt1*^{-/-} cells. Our data indicated that basal level of Rac1 activity was lesser in *Akt1*^{-/-} fibroblasts (Fig. 3E), similar to our observation in *Akt1*^{-/-} endothelial cells (Fig. 3B). In WT fibroblasts, bFGF stimulated Rac1 activity reaching a maximum in 15–30 min. In contrast, in *Akt1*^{-/-} fibroblasts, even though Rac1 activity was enhanced upon 15 min stimulation with bFGF, at all the time points studied, Rac1 activity was significantly lesser in *Akt1*^{-/-} fibroblasts compared to WT (Fig. 3E).

Since Akt1 activation was correlated with the activation of Rac1 and Pak signaling in vascular cells, we next wanted to determine whether both Akt1 and Rac1 share the same localization in these cells. To check this, we probed NIH 3T3 fibroblasts with Rac1 and Akt1 antibodies and analyzed whether these two molecules co-localize in vivo. We observed that under normal growth conditions in vitro, both Akt1 and Rac1 co-localize in fibroblasts and were present both in the peri-nuclear space in the cytoplasm and in the membrane ruffles in the lamellipodia (Fig. 3F). Altogether, these results indicate an association between Akt1 and Rac1-Pak signaling in vascular cells.

Akt1 regulates localization of Rac1 to lamellipodia and augments membrane ruffling during spreading of fibroblasts on fibronectin

Studies so far demonstrated that Akt1 activity is necessary for the activation of Rac1-Pak signaling in vascular cells. Rac1-Pak signaling has been implicated to regulate spreading, and migration of endothelial cells and fibroblasts (Wojciak-Stothard et al., 2006). A major step in mediating these effects is proper localization of Rac1 into the membrane ruffles from the cytoplasm (Chittenden et al., 2006; Lanahan et al., 2006). Inability to properly localize Rac1 in the cell membrane has been shown to have defects in cell spreading and cell migration (Lanahan et al., 2006). Hence, we wanted to study whether Akt1 can regulate the migration of Rac1 from the cytoplasm to the membrane ruffles during spreading of fibroblasts on fibronectin. Data show that in normal conditions Rac1 is localized on the cell membrane of NIH 3T3 cells (Fig. 4A). NIH 3T3 cells expressing DN-Akt1 however showed inhibition of Rac1 migration to membrane (Fig. 4A). In contrast, expression of mAkt1 as well as 14-3-3 β , an adapter protein that has previously been implicated to activate Rac1-Pak signaling and integrin activation (Bialkowska et al., 2003), resulted in localization of Rac1 to membrane ruffles even in the absence of stimulation (Fig. 4A) thus suggesting that Akt1 is upstream of 14-3-3-Rac-Pak signaling in fibroblasts.

Changes in localization in Rac1 have direct implications of actin polymerization and lamellipodia formation (Samayawardhena et al., 2007). Hence, we next wanted to study the effect of Akt1 regulated Rac1 localization and activation on actin polymerization and spreading. Treatment with bFGF resulted in increased actin stress fiber formation and membrane ruffling in NIH 3T3 cells compared to untreated control (Fig. 4B). While expression of NIH 3T3 cells with DN-Akt1 resulted in increased stress fiber formation upon bFGF treatment, bFGF stimulated lamellipodia formation as well as spreading was inhibited in DN-Akt1 expressing cells (Fig. 4B,C). In contrast, expression of NIH 3T3 cells with mAkt1 exhibited enhanced lamellipodia formation and cell spreading even in the absence of bFGF stimulation, with no significant increase in actin stress fiber formation (Fig. 4B,C). Similarly, expression of NIH 3T3 cells with 14-3-3 β , active Rac1 as well as active Pak1 resulted in enhanced lamellipodia formation and cell spreading akin to that observed in the

case of mAkt1 expression (Fig. 4C). Interestingly, expression of active Rac1 resulted in specific enhancement in lamellipodia formation but not stress fiber formation in serum starved cells, expression of active Pak1 resulted in enhanced stress fiber formation as well as lamellipodia extension (Fig. 4C).

We next wanted to study whether specific activation of Rac1 or Pak in NIH 3T3 cells would rescue to impaired cell spreading in Akt1 inactive cells. In order to do this, we co-transfected NIH 3T3 cells with various combinations of active and inactive variants of Akt1, 14-3-3 β , Rac1, and Pak and studied the effects on actin assembly. Our results show that defective actin assembly in NIH 3T3 cells with the expression of DN-Akt1 could be rescued by expression with active Rac1 or active Pak, but not by expression with 14-3-3 β (Fig. 4D). In contrast, enhanced lamellipodia formation in NIH 3T3 cells with the expression of mAkt1 is inhibited by co-expression with DN-Rac1 or DN-Pak (Fig. 4D). Collectively, these data demonstrate that both Rac1 and Pak is downstream of Akt1 activation, but 14-3-3 β can regulate cell spreading only in the presence of basal level of Akt1 activity.

Akt1 regulated fibroblast adhesion to fibronectin involves Rac1–14-3-3 β -PAK signaling

Engagement of cellular integrins to the extracellular matrix proteins is an essential step in the regulation of cell attachment and spreading (Mammoto et al., 2008). Our previous studies show that Akt1 is necessary for the activation of integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ in endothelial cells and fibroblasts and impaired integrin activation in *Akt1*^{-/-} fibroblasts resulted in impaired adhesion and migration on fibronectin (Somanath et al., 2007). Since we know that Rac1-Pak signaling, that regulates cytoskeletal changes and integrin activation (Mammoto et al., 2008) is impaired in *Akt1*^{-/-} fibroblasts, we wanted to determine whether specific activation of Rac1 or Pak in the absence of Akt1 activity would rescue the defect in adhesion to fibronectin. To this end, we transfected NIH 3T3 fibroblasts with control vector, 14-3-3 β and active and inactive variants of Akt1, Rac1, and Pak1 and analyzed for adhesion to BSA (non-integrin ligand control) and fibronectin. Expression of any of the abovementioned plasmids did not have any effect on fibroblast adhesion to BSA (Fig. 5A). However, while expression of mAkt1, 14-3-3 β , active Rac1 CA-Rac1 (Rac1-L⁶¹), and active Pak1 (PAK1 E⁴²³) resulted in enhanced adhesion of fibroblasts to fibronectin ($P=0.0005$, $P=0.0001$, $P=0.001$, and $P=0.0004$, respectively), expression of DN-Akt1, DN-Rac1 (Rac1-N¹⁷) and DN-Pak1 (Pak1 83–149 or Pak1 R²⁹⁹) resulted in significant inhibition in adhesion to fibronectin ($P=0.0003$, $P=0.0005$, and $P=0.0004$, respectively) compared to control vector transfected cells (Fig. 5B,C). NIH 3T3 fibroblasts co-transfected with mAkt1 and DN-Rac1 or DN-Pak1 showed impaired adhesion to fibronectin ($P=0.0005$ and $P=0.0006$, respectively) compared to mAkt1 expressing cells (Fig. 5D,E). In contrast, cells expressing DN-Akt1 and active Rac1 or active Pak1 exhibited enhanced adhesion to fibronectin ($P=0.0004$ and $P=0.0001$, respectively) compared to DN-Akt1 expressing cells (Fig. 5D,F). However, co-transfection of DN-Akt1 with 14-3-3 β in NIH 3T3 cells did not show a significant difference in adhesion to fibronectin ($P=0.8$) compared to DN-Akt1 expressing cells (Fig. 5D,F). Together data show that Rac1-Pak signaling is downstream of Akt1 activation in the regulation of fibroblasts adhesion to fibronectin.

Akt1 regulated fibronectin assembly by fibroblasts involves Rac1–14-3-3 β -PAK signaling

To the other end, the ability of the fibroblasts to effectively mediate extracellular matrix assembly is relied upon integrin activation, attachment to matrix protein and its ability to migrate (Somanath et al., 2007; Banno and Ginsberg, 2008). We previously showed that impaired adhesion and migration of *Akt1*^{-/-} fibroblasts resulted in impaired fibronectin matrix assembly in vitro (Somanath et al., 2007) and in vivo (Chen et al., 2005; Somanath et al., 2008). Since impaired adhesion of NIH fibroblasts with DN-Akt1 could be rescued by co-transfections with active Rac1 or active Pak1, we sought to determine whether impaired

fibronectin matrix assembly by DN-Akt1 expressing NIH 3T3 fibroblasts could be rescued by co-transfection with active Rac1 or active Pak1. First we transfected NIH 3T3 fibroblasts with control vector, 14-3-3 β and active and inactive variants of Akt1, Rac1, and Pak1 and analyzed for its ability to assemble exogenously supplied fibronectin. While NIH 3T3 fibroblasts expressing mAkt1, 14-3-3 β , active Rac1, and Active Pak1 exhibited enhanced fibronectin matrix assembly ($P = 0.0001$, $P = 0.0001$, $P = 0.0002$, and $P = 0.0003$, respectively), expression of DN-Akt1, DN-Rac1, and DN-Pak1 resulted in significant inhibition ($P = 0.0004$, $P = 0.0002$, and $P = 0.003$, respectively) compared to control vector transfected cells (Fig. 6A,B). NIH 3T3 fibroblasts co-transfected with mAkt1 and DN-Rac1 or DN-Pak1 showed impaired fibronectin matrix assembly ($P = 0.0005$ and $P = 0.0006$, respectively) compared to mAkt1 expressing cells (Fig. 6C,D). In contrast, cells expressing DN-Akt1 and active Rac1 or active Pak1 exhibited enhanced fibronectin assembly ($P = 0.006$ and $P = 0.009$, respectively) compared to DN-Akt1 expressing cells (Fig. 6C,E). However, co-transfection of DN-Akt1 with 14-3-3 β in NIH 3T3 cells did not show a significant difference in fibronectin assembly ($P = 0.13$) compared to DN-Akt1 expressing cells (Fig. 6C,E). Together data show that Akt1 and Rac1-Pak signaling association is necessary for the regulation of fibronectin assembly by fibroblasts.

Impaired adhesion to fibronectin and matrix assembly by *Akt1*^{-/-} fibroblasts are rescued by expression with constitutively active Rac1 or PAK1

From our previous studies, we know that both adhesion to fibronectin and fibronectin assembly are impaired in *Akt1*^{-/-} fibroblasts compared to WT (Somanath et al., 2008). Hence to further confirm our hypothesis and to establish the relationship between Akt1 and 14-3-3 β -Rac1-Pak signaling in the regulation of integrin activation, cell adhesion to fibronectin and in the regulation of fibronectin assembly, we utilized *Akt1*^{-/-} fibroblasts and studied whether expression with active Rac1 or active Pak1 can rescue the defects. As we expected from our previous experiments, *Akt1*^{-/-} fibroblasts transfected with active Rac1 or active Pak1 ($P = 0.007$, $P = 0.02$, and $P = 0.002$, respectively), but not 14-3-3 β ($P = 0.75$), rescued the defects in *Akt1*^{-/-} fibroblasts to adhere to fibronectin (Fig. 7A,B). Similarly, *Akt1*^{-/-} fibroblasts transfected with active Rac1 or active Pak1 ($P = 0.004$, $P = 0.002$, and $P = 0.008$, respectively), but not 14-3-3 β ($P = 0.67$), also rescued the impaired fibronectin assembly by *Akt1*^{-/-} fibroblasts (Fig. 7C,D). These results suggest that impaired activity of Rac1 and Pak is responsible for impaired response to matrix proteins and impaired fibronectin matrix assembly by *Akt1*^{-/-} fibroblasts.

Discussion

In this study, we identified the molecular mechanisms underlying the Akt1 regulated inside-out activation of integrins and modulation of adhesion, lamellipodia formation and fibronectin matrix assembly. Matrigel assay for WT and *Akt1*^{-/-} aortic rings revealed that in the absence of Akt1, aortic ring sprouting, analyzed at various time intervals, is significantly impaired. While treatment of serum starved NIH 3T3 fibroblasts with bFGF resulted in enhanced stress fiber, filopodia and lamellipodia formation, these effects were inhibited by treatment with PI3 kinase and Akt inhibitors. While expression of constitutively active mAkt1 (myristoylated Akt1) resulted in enhanced lamellipodia formation with modest changes in stress fiber formation, expression of dominant negative Akt1 (DN-Akt1) resulted in impaired lamellipodia formation in the presence of bFGF with no significant changes in stress fiber density. Studies using WT and *Akt1*^{-/-} mouse lung endothelial cells revealed that lamellipodia formation in response to treatment with VEGF that observed in WT endothelial cells is blunted in *Akt1*^{-/-} endothelial cells. Further analysis of WT and *Akt1*^{-/-} endothelial cells indicated impaired activation of Rac1 in *Akt1*^{-/-} endothelial cells compared to WT. Treatment of NIH 3T3 fibroblasts with bFGF showed enhanced activation

of Rac1 and Pak1/2 phosphorylation, which was blunted by treatment with PI3 kinase and Akt inhibitors, but not with GSK-3 inhibitor. Moreover, expression of NIH 3T3 cells with mAkt1 resulted in enhanced Rac1 activity and Pak1/2 phosphorylation. In contrast, expression with DN-Akt1 resulted in impaired activation of Rac1 and decreased phosphorylation of Pak1/2. While expression of 14-3-3 β , active forms of Akt1, Rac1 or Pak1 showed enhanced lamellipodia formation, adhesion and fibronectin assembly by NIH 3T3 cells, these effects stimulated by bFGF were significantly inhibited by the expression of DN-Akt1, DN-Rac1, or DN-Pak1. Moreover, defects in adhesion and fibronectin assembly by *Akt1* fibroblasts were rescued by expression with either active Rac1 or active Pak1, but not with 14-3-3 β . Altogether, our study shows a direct association between Akt1 and 14-3-3-Rac1-Pak signaling in endothelial cells and fibroblasts.

Among the three isoforms of Akt in vascular cells, Akt1 was identified to be the major isoform accounting for more than 60% of the total Akt activity (Chen et al., 2005; Somanath et al., 2008). Deficiency of Akt1 resulted in impaired inside-out activation of $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins endothelial cells and fibroblasts resulting in impaired adhesion to extracellular matrix and migration as well as assembly of fibronectin matrix in vitro (Somanath et al., 2007). Whereas *Akt1*^{-/-} mice exhibited loose and under-developed skin, marked by reduced collagen assembly (Chen et al., 2005; Somanath et al., 2008) blood vessels developed in tumors and cutaneous wounds exhibited immatured and leaky blood vessels with poorly developed basement membrane, mainly due to reduced expression of laminin and collagen in blood vessels (Chen et al., 2005; Somanath et al., 2008). Our previous studies as well as the aortic ring sprouting assay revealed that Akt1 is necessary for the directional migration of endothelial cells and fibroblasts.

Rho family of small GTPases such as Rho, Rac and cdc42 is known to play an important role in the regulation of directional migration of vascular cells (Mammoto et al., 2008). P21 activated kinase (Pak), a family of seven isoforms, is a major downstream target for Rac and cdc42 that has implicated in the regulation of cytoskeletal organization and formation of filopodia and lamellipodia (Kumar et al., 2006). Pak1 and Pak2 are the two major isoforms of Pak in endothelial cells and fibroblasts (Zeng et al., 2000; Rhee and Grinnell, 2006). While RhoA and RhoB are involved in actin stress fiber formation, Rac and cdc42, depending upon their localization activate Pak and regulate the formation of lamellipodia and filopodia (Mammoto et al., 2008). Previous reports key into the importance of Akt in the regulation of Rac1 and Pak1 activity (Zhou et al., 2003; Fryer and Field, 2005; Pankov et al., 2005).

In our study, we observed that both Rac1 and Akt1 share same localization in fibroblasts. Akt1 activity was also correlated with the formation of lamellipodia, but not with the formation of stress fibers or filopodia. Also, we observed impaired lamellipodia and directional migration in *Akt1*^{-/-} endothelial cells and fibroblasts on fibronectin. Hence, we hypothesized that Akt1 regulates cytoskeletal dynamics and migration mainly involving Rac1 and Pak signaling. Our study showed that expression of NIH 3T3 cells with mAkt1 results in migration of Rac1 to the membrane ruffles and enhanced Rac1-GTP levels as well as Pak phosphorylation. In contrast, DN-Akt1 inhibited these effects. Similar results observed in *Akt1*^{-/-} endothelial cells exhibiting impaired activation of Rac1 in response to VEGF indicate a strong correlation between Akt1 and Rac1-Pak signaling in vascular cells.

Localization of Rac1 is mainly mediated through interaction with 14-3-3 proteins (Chahdi and Sorokin, 2008). Protein 14-3-3 is a homo-dimeric adapter protein that exists in various isoforms, that binds to pre-phosphorylated substrates (Bridges and Moorhead, 2005). Many of the Akt substrates such as GSK-3, FoxO, and cRaf are known to interact with 14-3-3 (Woods and Rena, 2002; Wilker and Yaffe, 2004; Lee and Lozano, 2006). Our study

indicated that expression of NIH 3T3 cells with 14-3-3 β results in Rac1 localization to lamellipodia thus enhancing lamellipodia formation, adhesion to fibronectin and fibronectin matrix assembly. However, these effects by 14-3-3 β are inhibited by co-expression with DN-Akt1, suggesting that Akt1 activity is necessary for the activation of Rac1 and its interaction with 14-3-3 for its proper localization to the membrane ruffles. Even though DN-Akt1 inhibited lamellipodia formation in NIH 3T3 cells while spreading on fibronectin, co-expression with active-Rac1 or active Pak1 resulted in enhanced lamellipodia formation. In contrast, enhanced lamellipodia formation by mAkt1 was blocked by co-expression with DN-Rac1 or DN-Pak1, thus indicating that 14-3-3-Rac1-Pak signaling is downstream of Akt1 activation in the regulation of lamellipodia formation.

Similar results were also observed in the ability of NIH 3T3 cells to adhere to fibronectin and mediate fibronectin assembly. While mAkt1, active Rac1, and active Pak1 resulted in enhanced adhesion and fibronectin assembly by NIH 3T3 cells, dominant negative forms of Akt1, Rac1, and Pak1 inhibited these effects. While co-expression of active-Rac1 and -Pak1 expression rescued the defects in adhesion and fibronectin assembly by DN-Akt1 expression, co-expression with DN-Rac1 or DN-Pak1 inhibited enhanced adhesion and fibronectin assembly regulated by mAkt1. Moreover, restoration of active-Rac1 or -Pak1 in immortalized *Akt1*^{-/-} fibroblasts rescued the defects in adhesion and fibronectin assembly, thus confirming our hypothesis that Akt1 involves 14-3-3-Rac1-Pak signaling in the regulation of cytoskeletal organization, integrin activation, cellular adhesion, and fibronectin matrix assembly.

In conclusion, we identify 14-3-3, Rac1, and Pak as novel partners in the pathway of Akt1 regulated integrin activation, adhesion, and extracellular matrix assembly by vascular cells. Activity levels of Rac1 and Pak are impaired in *Akt1*^{-/-} endothelial cells and fibroblasts and restoration of activity of Rac1 or Pak, but not 14-3-3 alone, in *Akt1*^{-/-} fibroblasts rescued the defects in adhesion and fibronectin assembly. Multiple mechanisms have been reported by many researchers for the activation of Rac1-Pak signaling in vascular cells (Fryer and Field, 2005). Importance of 14-3-3 in the activation of Rac1 and cdc42 has been reported in CHO cells (Bialkowska et al., 2003). A possible mechanism would be phosphorylation of Rac1 associated guanine exchange nucleotide factor (GEF) by Akt1 that augments its interaction with 14-3-3 and forms a complex with Rac1, thereby regulating its translocation to membrane ruffles followed by the activation of Pak. Also, Pak has also been shown to be directly regulated by Akt1 via phosphorylation at Ser21 thus regulating its interaction with adapter protein Nck (Zhou et al., 2003). The study is currently underway in the lab to elucidate the molecular mechanisms responsible for Akt1 mediated activation of Rac1-Pak signaling in vascular cells.

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Fig. 1.

Absence of Akt1 results in impaired aortic ring sprouting. Aortic rings were made from WT and *Akt1*^{-/-} mice and were implanted in matrigel and supplemented with culture medium. Pictures of cell sprouts from aorta were made on days 4, 6, and 10 after implantation and the length of aortic sprouts was quantified using ImagePro plus software (Scale bar: 200 μ m). A: Photograph of day 10 aortic sprouts from WT and *Akt1*^{-/-} mice. B: Bar graph showing quantification of length of aortic ring sprouts from WT and *Akt1*^{-/-} mice on day 4,6, and 10. Values are expressed as mean $\pm$ SEM.

Fig. 2.

Akt1 regulates the growth factor mediated lamellipodia formation in endothelial cells and fibroblasts. A: NIH 3T3 fibroblasts were serum starved overnight and treated with bFGF in the presence and absence of PI3 kinase inhibitor (10 μ M LY294002) or Akt inhibitor (1 μ M SH-5). Cells were fixed after 4 h and subjected for TRITC-phalloidin staining. Pictures show actin stress fibers and lamellipodia in NIH 3T3 fibroblasts treated with bFGF and inhibitors. The number of cells showing lamellipodia in each of these treatments is shown in the right part. B: NIH 3T3 fibroblasts were transfected with mAkt1 or DN-Akt1 plated on to glass coverslips and left overnight followed by fixing and staining with TRITC-phalloidin. Same field showing GFP expression is shown in the right parts. The number of cells

showing lamellipodia in NIH 3T3 cells expressing mAkt1 and DN-Akt1 in the presence and absence of FGF is shown in the right part. C: WT and *Akt1*^{-/-} lung endothelial cells plated on fibronectin on coverslips were serum starved overnight and incubated with VEGF for 1 h. Cells were then fixed, permeabilized using 0.1% triton X-100 in PBS and subjected for phalloidin staining. The number of cells showing lamellipodia in WT and *Akt1*^{-/-} endothelial cells in the presence and absence of VEGF is shown in the right part (Scale bar: 10 μ m).

Fig. 3.

Akt1 is necessary for the growth factor mediated activation of Rac1-p21 activated kinase signaling in vascular cells. A: Mouse lung endothelial cells were treated with VEGF (20 ng/ml) and the lysates prepared at different time points (5, 15, and 30 min) were subjected for western analysis for pT30 Akt and pS9/21 GSK-3. B: Lysates prepared from WT and *Akt1*^{-/-} lung endothelial cells in the presence and absence of VEGF were subjected for Rac activity assay. Bar graph show GTP-bound Rac1 levels in WT and *Akt1*^{-/-} endothelial cells in the presence and absence of VEGF treatment. C: Serum starved NIH 3T3 fibroblasts were treated with bFGF in the presence and absence of PI3 kinase inhibitors (100 nM Wortmannin or 10 μ M LY294002), Akt inhibitor (1 μ M SH-5) or GSK-3 inhibitor (10 μ M

SB-415286) or transfected with mAkt1 or DN-Akt1 followed by cell lysis. Lysates were subjected for Rac activation assay and western analysis for phospho-Pak, phospho-Akt, panAkt, and β -actin. D: Densitometry analysis of the multiple samples ($n = 3$) in the presence and absence of bFGF and inhibitors as explained above. E: Serum starved WT and *Akt1*^{-/-} fibroblasts were treated with bFGF at various time intervals (0, 5, 10, 15, 30, and 60 min.) and Rac1 activity assay was performed. A densitometry analysis of ratio between active Rac1 and total Rac1 in these samples is shown below the respective lane. F: NIH 3T3 fibroblasts showing co-localization of Rac1 and Akt1 in the cytoplasm and membrane ruffles (Scale bar: 10 μ m).

Fig. 4.

Akt1 regulates localization of Rac1 to lamellipodia and augments membrane ruffling during spreading of fibroblasts onto fibronectin. NIH 3T3 fibroblasts treated with bFGF were allowed to spread on coverslips for 45 min and were fixed and permeabilized followed by staining with Rac1 antibody or phalloidin. A: Pictures showing Rac1 localization in NIH 3T3 fibroblasts transfected with mAkt1, DN-Akt1, and 14-3-3 β . B: Pictures showing phalloidin staining in NIH 3T3 fibroblasts transfected with mAkt1 and DN-Akt1 and treated with vehicle (PBS) or 20 ng/ml bFGF. C: Photographs of NIH 3T3 fibroblasts transfected with mAkt1, DN-Akt1, 14-3-3 β , CA-Rac, and CA-Pak showing phalloidin staining. D:

Photographs of NIH 3T3 fibroblasts transfected with DN-Akt1 along with CA-Rac or CA-Pak and mAkt1 along with DN-Rac or DN-Pak with phalloidin staining (Scale bar: 10 μ m).

Fig. 5.

Akt1 involves Rac1-14-3-3β-PAK signaling to regulate fibroblast adhesion to fibronectin. NIH 3T3 fibroblasts transfected with various Akt, Rac, and Pak variants were serum starved and subjected for adhesion assay on BSA or fibronectin in the presence of 20 ng/ml bFGF. A: Bar graph showing number of NIH 3T3 cells adhered to BSA. B: Pictures of Akt, 14-3-3, Rac, or Pak transfected NIH 3T3 cells on fibronectin (Scale bar: 50 μm). C: Bar graph showing number of Akt, 14-3-3, Rac, or Pak transfected NIH 3T3 cells adhered to fibronectin. D: Pictures of NIH 3T3 cells transfected with mAkt1 in combination with DN-Rac or DN-Pak and DN-Akt1 in combination with 14-3-3, CA-Rac or CA-Pak adhered to fibronectin. E: Bar graph showing number of NIH 3T3 cells transfected with mAkt1 in combination with DN-Rac or DN-Pak adhered to fibronectin. F: Bar graph showing number of NIH 3T3 cells and DN-Akt1 in combination with 14-3-3, CA-Rac or CA-Pak adhered to fibronectin (Scale bar: 50 μm).

Fig. 6.

Akt1 involves Rac1-14-3-3 β -PAK signaling to regulate fibronectin assembly by fibroblasts. NIH 3T3 fibroblasts transfected with Akt, Rac, and Pak variants were subjected for fibronectin assembly. A: Pictures showing fibronectin assembly by NIH 3T3 fibroblasts transfected with Akt, 14-3-3, Rac, or Pak variants. B: Bar graph showing the area of fibronectin fibrils assembled by NIH 3T3 fibroblasts transfected with Akt, 14-3-3, Rac, or Pak variants. C: Pictures showing fibronectin assembly by NIH 3T3 cells transfected with mAkt1 in combination with DN-Rac or DN-Pak and DN-Akt1 in combination with 14-3-3, CA-Rac or CA-Pak. D: Bar graph showing area positive for fibronectin fibrils assembled by NIH 3T3 cells transfected with mAkt1 in combination with DN-Rac or DN-Pak. E: Bar graph showing area positive for fibronectin fibrils assembled by NIH 3T3 cells transfected with DN-Akt1 in combination with 14-3-3, CA-Rac, or CA-Pak (Scale bar: 20 μ m).

Fig. 7.

Impaired adhesion to fibronectin and matrix assembly by *Akt1*^{-/-} fibroblasts is rescued by expression with constitutively active Rac or PAK. WT fibroblasts were transfected with control vector or DN-Akt1 and *Akt1*^{-/-} fibroblasts were transfected with 14-3-3, CA-Rac, or CA-Pak and were subjected for adhesion assay and fibronectin assembly assay. A: Pictures of WT fibroblasts transfected with control vector or DN-Akt1 and *Akt1*^{-/-} fibroblasts transfected with 14-3-3, CA-Rac, or CA-Pak adhered on to fibronectin (Scale bar: 10 μ m). B: Bar graph showing quantification of WT fibroblasts transfected with control vector or DN-Akt1 and *Akt1*^{-/-} fibroblasts transfected with 14-3-3, CA-Rac, or CA-Pak adhered on to fibronectin (Scale bar: 10 μ m). C: Pictures of WT fibroblasts transfected with control vector

or DN-Akt1 and *Akt1*^{-/-} fibroblasts transfected with 14-3-3, CA-Rac, or CA-Pak showing fibronectin assembly (Scale bar: 20 μ m). D: Bar graph showing quantification of the area positive for fibronectin fibrils assembled by WT fibroblasts transfected with control vector or DN-Akt1 and *Akt1*^{-/-} fibroblasts transfected with 14-3-3, CA-Rac, or CA-Pak (Scale bar: 20 μ m; *compared to WT cells; **compared to *Akt1*^{-/-} cells).