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Low-Power Laser Irradiation Promotes Cell Proliferation by Activating PI3K/Akt Pathway

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Low-power laser irradiation (LPLI) can stimulate cell proliferation through a wide network of signals. Akt is an important protein kinase in modulating cell proliferation. In this study, using real-time single-cell analysis, we investigated the activity of Akt and its effects on cell proliferation induced by LPLI in African green monkey SV40-transformed kidney fibroblast cells (COS-7). We utilized a recombinant fluorescence resonance energy transfer (FRET) Akt probe (BKAR) to dynamically detect the activation of Akt after LPLI treatment. Our results show that LPLI induced a gradual and continuous activation of Akt. Moreover, the activation of Akt can be completely abolished by wortmannin, a specific inhibitor of PI3K, suggesting that the activation of Akt caused by LPLI is a PI3K-dependent event. Src family is involved in Akt activation as demonstrated by the part inhibition of Akt activity in samples treated with PP1 (an inhibitor of Src family). In contrast, loading Gö 6983, a PKC inhibitor, did not affect this response. Further experiments performed using GFP-Akt fluorescence imaging and Western blot analysis demonstrate that, the activation of Akt is a multi-step process in response to LPLI, involving membrane recruitment, phosphorylation, and membrane detachment. LPLI promotes cell proliferation through PI3K/Akt activation since the cell viability was significantly inhibited by PI3K inhibitor. All these studies create a concernful conclusion that PI3K/Akt signaling pathway is well involved in LPLI triggered cell proliferation that acts as a time- and dose-dependent manner.

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Low-power laser irradiation (LPLI) is a non-thermal irradiation within the visible to near infrared range of light spectrum which has been used clinically to accelerate wound healing and reduce pain and inflammation in a variety of pathologies (Schindl et al., 1999). In the latest decades, a large body of evidence has showed that He–Ne laser light can stimulate a number of biological processes, including cell growth, proliferation (Yu et al., 1996) and differentiation (Bibikova and Oron, 1993; Conlan et al., 1996). In vitro, the effects on cell proliferation by LPLI have been studied in various cell types including fibroblasts, endothelial cells, skeletal cells, keratinocytes, myoblasts, and other types (Yu et al., 1996; Stadler et al., 2000; Shefer et al., 2002; Jia and Guo, 2004; Stein et al., 2005). However, the mechanism associated with the stimulatory effects of LPLI has not been fully clarified. One classic mechanism involved is that, the laser energy is absorbed by intracellular chromophores and converted to metabolic energy, since cellular ATP levels increase almost twofold after He–Ne laser irradiation (Karu et al., 1995). To further understand the mechanism, it is necessary to identify the signal transduction pathways of cell proliferation stimulated by LPLI. Shefer et al. show that, LPLI specifically activates MAPK/ERK pathway and consequently induces satellite cell proliferation (Ben-Dov et al., 1999; Shefer et al., 2001). Our previous studies have demonstrated that LPLI specifically activates RTK/PKCs signaling pathway to promote cell proliferation (Gao et al., 2006). We have also shown that LPLI triggers a significant activation of ROS/Src pathway (Zhang et al., 2008). Furthermore, Akt can be activated by either Src or PKCs protein kinase (Kassenbrock et al., 2002; Kawakami et al., 2004; Partovian and Simons, 2004; Bentley et al., 2007). Therefore, it is likely that Akt is involved in LPLI-induced cell proliferation. On the other hand, LPLI treatment can increase the level of intracellular ROS generation (Karu, 1999; Alexandratou et al., 2002; Jou et al., 2002; Zhang et al., 2008). The increased intracellular oxidants can mediate the activation of Akt (Ushio-Fukai et al., 1999; Wang et al., 2000). These reports suggest the existence of ROS/Akt signaling pathway during LPLI-induced proliferation. Therefore, the working hypothesis is to explore the functions of Akt in modulating cell proliferation under LPLI treatment.

Serine/threonine protein kinase, Akt, also known as protein kinase B, regulates a variety of cellular processes, such as cell growth, proliferation, and survival (Brazil et al., 2004; Bellacosa et al., 2005). Its activation can be initiated by various extracellular signals that turn on PI3K (Engelman et al., 2006). After stimulus affecting on its respective receptor (Kumar et al., 2007), PI3K is activated and then phosphorylates inositol lipids (Bevan, 2001). This results in the production of PI3,4,5P3 and PI3,4P2 at the plasma membrane (Cantrell, 2001). Akt is recruited to the membrane from the cytosol through specific binding of the N-terminal PH domain to PI3,4,5P3 and/or PI3,4P2 (Andjelkovic et al., 1997; Frech et al., 1997), where it is thought to undergo a conformational change and become activated by phosphorylation of two residues: Thr308 and Ser473 (Alessi et al., 1996; Bayascas and Alessi, 2005; Song et al., 2005). Once activated, Akt translocates to various sites within the cell and phosphorylates a number of "effector" substrates to exert its biological effects (Catalucci and Condorelli, 2006). Akt promotes proliferation through regulating certain cell cycle proteins to drive quiescent cells into cell cycle and to speed up the progression of the cell cycle. This process is accomplished by phosphorylating p21, down-regulating transcription of p27

Abbreviations: BKAR, B kinase activity reporter; CCK-8, Cell Counting Kit-8; CFP and YFP, cyan and yellow fluorescent protein; GFP, green fluorescent protein; EGF, epidermal growth factor; FRET, fluorescence resonance energy transfer; LPLI, low-power laser irradiation; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; RTK, receptor tyrosine kinase.

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and promoting stabilization of cyclin D1 protein via inactivation of GSK3 (Diehl et al., 1998; Sun et al., 1999; Alt et al., 2000; Gesbert et al., 2000; Graff et al., 2000). Akt is then dephosphorylated and inactivated by protein phosphatases such as protein phosphatase 2A (PP2A) (Andjelkovic et al., 1996; Gao et al., 2005).

Fluorescence resonance energy transfer (FRET) is a non-radiative transfer of energy from an excited donor molecule to a suitable acceptor molecule in close proximity. It has become a powerful tool for investigating of molecular events in living cells (Uchiyama et al., 1996; Zhang et al., 2002). To examine Akt signaling in living cells, we used a B kinase activity reporter (BKAR) that generally consists of two different fluorescent proteins flanking a phosphoamino acid-binding domain and a kinase substrate sequence (Kunkel et al., 2005). Phosphorylation of the substrate sequence causes intramolecular complexation by the phosphoamino acid-binding domain, thus, altering FRET between the fluorescent proteins. This provides a means of powerful visualization of kinase signaling in situ in living cells. This cannot be fully elucidated by traditional biophysical or biochemical approaches that can only measure average behavior of a cell population and static spatial information from fixed cells.

Although a large number of recent studies have demonstrated that growth factors can induce cell proliferation through activating PI3K/Akt signaling pathway, few studies have been performed during LPLI-induced cell proliferation. In the present study, using fluorescent imaging and Western blot analysis, great efforts have been focused on the investigation of Akt activity in COS-7 cells under LPLI treatment, and the associated mechanism was discussed. Our findings will extend the knowledge about the cellular signaling mechanisms mediating LPLI-induced proliferation.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO (Grand Island, NY). Epidermal growth factor (EGF) (diluted in DMSO) was purchased from PeproTech (Rocky Hill, NJ). Wortmannin was purchased from BIOMOL Research Laboratories, Inc. (Plymouth, PA). 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine (PP1) was purchased from Invitrogen (Carlsbad, CA). Gö 6983 was purchased from Merck (Darmstadt, Germany). Anti-phospho-Akt (Thr308) antibody and anti-Akt antibody were purchased from Cell Signaling (Beverly, MA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Lipofectamine™ Reagent was purchased from Invitrogen. DNA Extraction kit was purchased from Qiagen (Valencia, CA). Other chemicals were mainly from Sigma (St. Louis, MO).

Cell culture and transfection

African green monkey SV40-transformed kidney fibroblast cell line (COS-7) was obtained from Department of Medicine, Zhongshan University. The cells were cultured in DMEM medium supplemented with 15% fetal calf serum, penicillin (100 units/ml), and streptomycin (100 mg/ml) in 5% CO_2 at 37°C in a humidified incubator. Transient transfections were performed with 1 μg of expression vectors using the Lipofectamine™ 2000 reagent according to the manufacturer's instructions in serum-free medium. The serum-free medium was replaced with fresh culture medium after 5 h and incubated for an additional 24 h for expression. The cells were then starved for 24 h in serum-free DMEM before treatment and were examined, according to the protocol, for the following 24 h.

Plasmid DNA of BKAR (a kind gift from Dr. Newton, University of California, San Diego) was used to monitor Akt activity. It consists of mCFP (cyan fluorescent protein), the FHA2 domain of Rad53p, a consensus Akt phosphorylation sequence and mYFP (yellow fluorescent protein) (Kunkel et al., 2005). In the unphosphorylated state, mCFP and mYFP are in a proximity and orientation resulting in FRET. Once phosphorylated by Akt at the threonine within the substrate sequence, the FHA2 domain binds the phosphorylated sequence resulting in a conformational change that decreases the FRET ratio (Fig. 1). Thus the ratio of YFP/CFP increases with the inactivation of Akt (unphosphorylated state), while the ratio of YFP/CFP decreases with the activation of Akt (phosphorylated state). pΔp85 (a kind gift from Dr. Cooper, Harvard Medical School, Boston) (Pap and Cooper, 1998) is a dominant-negative construct of the p85α regulatory subunit of PI3K, making it incapable to binds to the p110 catalytic subunit. So over-expression of Δp85 in cells indicates that it is unable to elicit the protein PI3K activation in vivo. pEGFP-Akt was kindly supplied by Dr. Badger (He et al., 2006).

LPLI treatment and cell viability assays

COS-7 cells were cultured in DMEM supplemented with 15% serum at a density of 4×10^3 cells/well in 96-well microplates. After 24 h serum starvation in DMEM, the cells were divided into four groups and each group was irradiated with He–Ne laser (632.8 nm, 10 mW, 12.74 mW/cm^2, HN-1000, Guangzhou, China) at dose of 0, 0.2, 0.4, 0.8, and 1.2 J/cm^2 or treated with EGF (50 ng/ml). The interval wells were filled with black ink in order to minimize the light scattering. After irradiation, the cells were maintained in serum-free DMEM and the 96-well microplates were returned to the incubator for a further culture at 37°C, 5% CO_2. The irradiation was performed on monolayer cells. In all cases, control (non-irradiated) cells were kept in the same conditions as the treated cells.

Cell viability was assessed with CCK-8 at 1, 2, and 3 days after the laser irradiation, respectively. At the indicated time, CCK-8 was added to the cells and incubated for 1.5 h. OD_{450}, the absorbance value at 450 nm, was read with a 96-well plate reader (DG5032, Hua dong, Nanjing, China). The value is directly proportional to the number of viable cells in a culture medium and the cell proliferation.

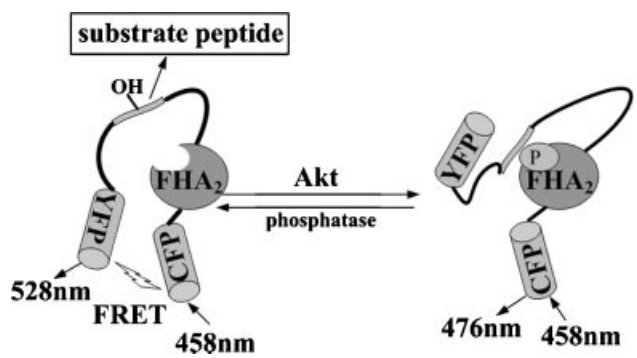

Fig. 1. A cartoon depicting the working of genetic reporter BKAR. BKAR, an Akt kinase activity reporter, consists of mCFP, the FHA2 domain of Rad53p, a consensus Akt phosphorylation sequence and mYFP. In the unphosphorylated state, mCFP and mYFP are in a proximity and orientation resulting in FRET. Once phosphorylated by Akt at the threonine within the substrate sequence, the FHA2 domain binds the phosphorylated sequence resulting in a conformational change that decreases the FRET ratio. Since it does not contain the full Akt sequence, but an Akt substrate, it monitors the activation of endogenous Akt in live cells.

Laser scanning microscopy (LSM) and FRET analysis

FRET was performed on a commercial Laser Scanning Microscope (LSM510/ConfoCor2) combination system (Zeiss, Jena, Germany). For excitation, the 458 nm line of an Ar-Ion Laser was attenuated with an acousto-optical tunable filter (AOTF), reflected by a dichroic mirror (main beam splitter HFT 458 nm), and focused through a Zeiss C-Apochromat 40×, NA 1.3 objective onto the sample. The emission fluorescence was split by a second dichroic mirror (secondary beam splitter NFT 515 nm) into two separate channels: a 470–500 nm bandpass (CFP channel) and a 530 nm longpass (YFP channel), respectively. For intracellular measurement, a single cell was chosen in the LSM image. To quantify the results, images of CFP and YFP emission intensities were processed with Zeiss Rel3.2 image processing software (Zeiss). After background subtraction, the average fluorescence intensity per pixel was calculated.

GFP fluorescence was excited at 488 nm with an argon ion laser and emission was recorded through a 500–550 nm band pass filter. With control experiments, it was confirmed that the bleaching of the probe was negligible for all protocols.

Spectrofluorometric analysis

COS-7 cells transfected with BKAR reporter were grown in DMEM for 24 h. Then, the cells were treated with LPLI (1.2 J/cm^2). After irradiation, the cells were immediately transferred into a quartz cuvette. The quartz cuvette was then placed inside the sample chamber of a luminescence spectrometer (LS55, PerkinElmer, Wellesley, MA). The fluorescence emission spectra from 0 to 30 min after the LPLI treatment were then acquired. The excitation wavelength was 434 centered. The excitation and emission slits were set for 10 and 15 nm, respectively. The scanning speed was 200 nm/sec. The fluorescence emission spectra of cells from same generation were recorded in the similar ways, 0–30 min after EGF treatment. The corresponding background spectra of cell-free culture medium were subtracted.

GFP-Akt translocation assay

COS-7 cells were transfected with pGFP-Akt and with/without Δp85 and then treated with LPLI in the presence or absence of wortmannin. Using the Zeiss LSM 510 confocal microscope, we imaged the distribution pattern of GFP-Akt during LPLI-induced proliferation.

Western blot analysis

After different treatments, cells were scraped from the dish, then washed twice with ice-cold phosphate-buffered saline (PBS, pH 7.4), and lysed with ice-cold lysis buffer (50 mmol/L Tris–HCl pH 8.0, 150 mmol/L NaCl, 1% Triton X-100, 100 μg/ml PMSF) for 30 min on ice. The lysates were centrifuged at 12,000 rpm for 5 min at 4°C, and the protein concentration was determined. Equivalent samples were subjected to SDS–PAGE on 12% gel. The proteins were then transferred onto nitrocellulose membranes, and probed with primary antibody: anti-phospho-Akt (Thr308) and anti-Akt at a dilution of 1:1,000, followed by secondary antibodies, goat anti-mouse conjugated to Alexa Fluor 680 for phospho-Akt and goat anti-rabbit conjugated to IRDyeTM800 for Akt. Detection was performed using the LI-COR Odyssey Infrared Imaging System (LI-COR, Inc., Lincoln, NE).

Statistics analysis

All assays were repeated independently for a minimum of three times. Data are represented as mean ± SEM. Statistical analysis was performed with Student's paired *t*-test. Differences were considered statistically significant at $P < 0.05$.

Results

LPLI promotes cell proliferation in dose- and time-dependent manner

To establish a proper laser irradiation dose to induce proliferation, we used CCK-8 to observe cell viability after cells were treated with different doses of laser irradiation. Serum-starved COS-7 cells were treated with LPLI at the dose of 0, 0.2, 0.4, 0.8, 1.2 J/cm^2, respectively, and the cell viabilities were observed 2 days following the irradiation. As shown in Figure 2A, the cell viability significantly increased in LPLI-treated groups (≥0.4 J/cm^2) compared with that of non-treated cells. The data show that, in the range 0.4–1.2 J/cm^2, laser irradiation had a significant promotive effect on cell proliferation and the effect of LPLI on proliferation of COS-7 cells was dose-dependent. Therefore, in our following experiments, we selected 1.2 J/cm^2 as the irradiation dose.

We next analyzed the cell proliferation kinetics induced by LPLI. After 24 h serum starvation, COS-7 cells were irradiated with LPLI (1.2 J/cm^2) and then maintained in serum-free medium. The cell viability was analyzed by CCK-8 at 1, 2, and

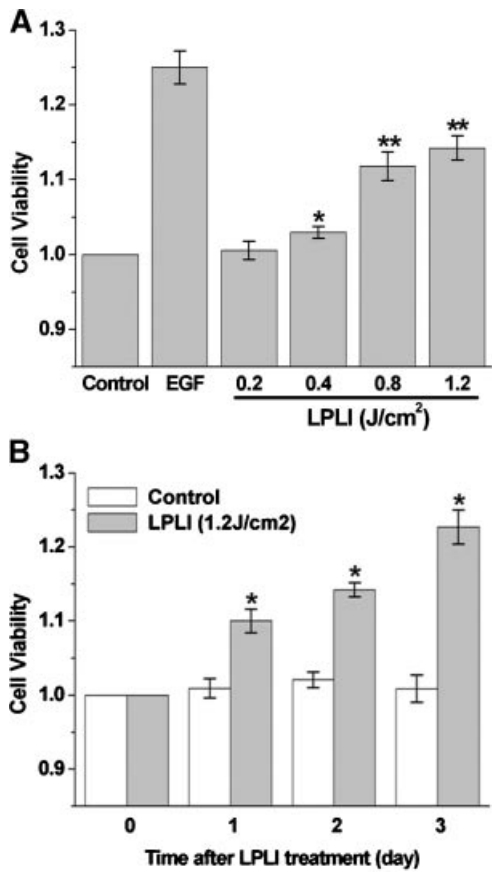

Fig. 2. Dose- and time-dependent proliferation of COS-7 cells induced by LPLI. A: LPLI induces dose-dependent proliferation. COS-7 cells were seeded on 96-well microplates for 24 h in 15% serum containing medium and maintained in serum-free medium for 24 h. Cells were then irradiated with He–Ne laser at dose of 0, 0.2, 0.4, 0.8, and 1.2 J/cm^2, respectively or treated with 50 ng/ml EGF. After irradiation or EGF treatment, cells were maintained in serum-free medium for 2 days and cell viability was assessed by the CCK-8 assay. Error bars are s.e.m. from four independent experiments. *$P < 0.05$; **$P < 0.01$. B: LPLI induces time-dependent proliferation. Cell viability was assessed by the CCK-8 assay at 0, 1, 2, 3 days after 1.2 J/cm^2 LPLI. Error bars are s.e.m. from four independent experiments. *$P < 0.01$.

3 days, respectively. Compared with non-irradiated cells, LPLI-treated cells showed an increase in cell viability, and a positive correlation was observed with post-treatment period (Fig. 2B), indicating that the effect of LPLI on cell proliferation of COS-7 cells is time-dependent.

Real-time monitoring of Akt activation induced by LPLI in single living cell

To explore the mechanism of LPLI promoting proliferation, the effects of LPLI on Akt activation in single living cell were monitored by FRET technique with the plasmid BKAR. After 24 h starvation, COS-7 cells transfected with BKAR were treated with LPLI, and then the real-time CFP, YFP and YFP/CFP fluorescence images were collected with LSM microscopy for 30 min (Fig. 3A). The fluorescence intensities of the CFP and YFP images and their ratio (YFP/CFP) are shown in Figure 3B. The results show that, as the time lapsed, the CFP fluorescence increased, while the YFP fluorescence and the YFP/CFP ratio decreased, during the 30 min observation period post-irradiation. This indicates that, Akt is activated after LPLI treatment and this activation is gradual and continuous in the first 30 min. Similar result was obtained in cells treated with EGF, except for the YFP/CFP ratio decreased more rapidly (Fig. 3C,D), suggesting a higher degree of Akt activation stimulated by EGF. The results also suggest that BKAR is an effectively reporter for reflecting the activation of Akt. To further validate our results, in the control group (No LPLI or EGF), the fluorescence intensities of CFP and YFP, and their ratio (YFP/CFP) remained stable for more than 30 min (Fig. 3C,D), which well excluded the possibility of the spontaneously FRET change induced by scar laser and other factors in our experiment.

Spectrofluorometric analysis, a technique for monitoring the overall profile of FRET fluorescence emission from a group of cells, was recommended to further confirm that the activation of Akt was a common phenomenon in our experimental mode. After 24 h serum deprivation, COS-7 cells transfected with BKAR were treated with LPLI. The emission peak of CFP (476 nm) increased gradually, while that of YFP (527 nm) decreased during the first 30 min post-irradiation (Fig. 3E). Similar results were also obtained in cells treated with EGF (Fig. 3F). These results further confirm that BKAR is a specific reporter for Akt activation, meanwhile, support the results shown in Figure 3A–D that Akt is activated in response to LPLI stimulation.

Since Akt activation indicated by FRET effect of Akt reporter (BKAR) is only determined by the balance between Akt kinases and protein tyrosine phosphatases (PTPs), we next explored whether Akt activation induced by LPLI depends upon an increased level of Akt phosphorylation. Western blot analysis show that the level of Akt phosphorylation at Thr308 was elevated as early as 10 min after LPLI treatment and gradually increased during the next 20 min. Similar results were obtained in EGF-treated cells, with the level of Akt phosphorylation increasing more significantly and rapidly (Fig. 3G). However, Akt phosphorylation decreased at 1.5 h and remained at a low level up to 72 h after LPLI stimulation (Fig. 3H). Taken together, these results suggest that, LPLI effectively promotes Akt activation due to its phosphorylation on Thr308.

Signal propagation of Akt from plasma membrane to nucleus

It is well known that the translocation of Akt from cytosol to plasma membrane is crucial for Akt activation (Andjelkovic et al., 1997; Frech et al., 1997). We therefore examined the subcellular location of Akt in response to LPLI. A GFP-Akt plasmid was transfected into COS-7 cells and the intracellular localization of GFP-Akt was monitored by confocal microscopy. To exclude the potential effects due to over-expression of GFP-Akt under our experimental conditions, we examined distribution of GFP-Akt in cells without any treatment. GFP-Akt remained uniformly distributed throughout the cell for over 30 min (Fig. 4A, upper). Upon LPLI stimulation, as shown in Figure 4A (lower), a significant portion of GFP-Akt translocated from cytosol to plasma membrane at 5 min and this migration lasted for more than 30 min. Interestingly, at about 10 min post-irradiation, we found that GFP-Akt started to return into cytosol and nucleus, and a great deal of GFP-Akt reached nucleus at 30 min after LPLI. Line-scan plots of these GFP-Akt images emphasized the subcellular location changes of Akt (Fig. 4B). These results strongly indicate that the activation of Akt occurs on the plasma membrane and this activation lasts for at least 30 min. The activated Akt disengages from the membrane and diffuses through the cytosol and nucleus to function throughout the cell after LPLI treatment.

LPLI induces Akt activation through a PI3K-dependent pathway

Akt is a downstream target of PI3K during growth factors stimulation (Engelman et al., 2006). Therefore, we sought to determine whether Akt activation was dependent on PI3K activity in respond to LPLI stimulation. After 24 h serum starvation, COS-7 cells transfected with BKAR were treated with LPLI. Wortmannin was added 10 min later. A representative temporal sequence of the pseudocolor images for the ratio of YFP/CFP fluorescence are shown in Figure 5A. The quantitative analysis for the ratio of YFP/CFP fluorescence intensities is shown in Figure 5B. The ratio of YFP/CFP remained stable without treatment, and started to decrease immediately once the LPLI treatment was initiated. The decreased trend of the ratio was reversed in response to wortmannin exposure. The ratio resumed to at least its pre-LPLI treatment level. In control cells, the ratio of YFP/CFP fluorescence intensities remained unchanged over 30 min (Fig. 5B). These results indicate that wortmannin could completely abolish the activation of Akt induced by LPLI. This suggests that, LPLI-induced Akt activation is a PI3K-dependent event.

Subsequently, we tested the effects of Δp85 (a dominant-negative mutant of PI3K) on the activation of Akt under LPLI treatment. As shown in Figure 5C, from cells co-transfected with pΔp85 and BKAR, we found that there was a slight increase of the YFP/CFP ratio, indicating that over-expression of Δp85 completely blocks the activation of Akt induced by LPLI. This also confirms the finding shown in Figure 5A,B that LPLI induces Akt activation through a PI3K-dependent pathway.

To investigate the relationship between Akt membrane translocation and PI3K activity under LPLI treatment, COS-7 cells were either transfected with pGFP-Akt and subjected to wortmannin treatment or co-transfected with pGFP-Akt and pΔp85 before LPLI treatment. Both protocols resulted in complete inhibition of GFP-Akt membrane location as the fluorescence remained uniformly distributed throughout the cells during the whole observation period (Fig. 5D), indicating that Akt membrane translocation is dependent on PI3K activity in response to LPLI stimulation.

Akt activation induced by LPLI is partially inhibited by PP1, but not by Gö 6983

Our recent work prove that PKCs and Src can be activated by LPLI (Gao et al., 2006; Zhang et al., 2008) and several PKC isoforms and Src family have been shown to lie upstream of Akt (Aeder et al., 2004; Kawakami et al., 2004; Partovian and Simons, 2004; Thamilselvan et al., 2007). To investigate whether PKCs and Src contribute to Akt activation, we treated COS-7 cells with PKCs inhibitor (Gö 6983) or Src inhibitor (PP1) prior

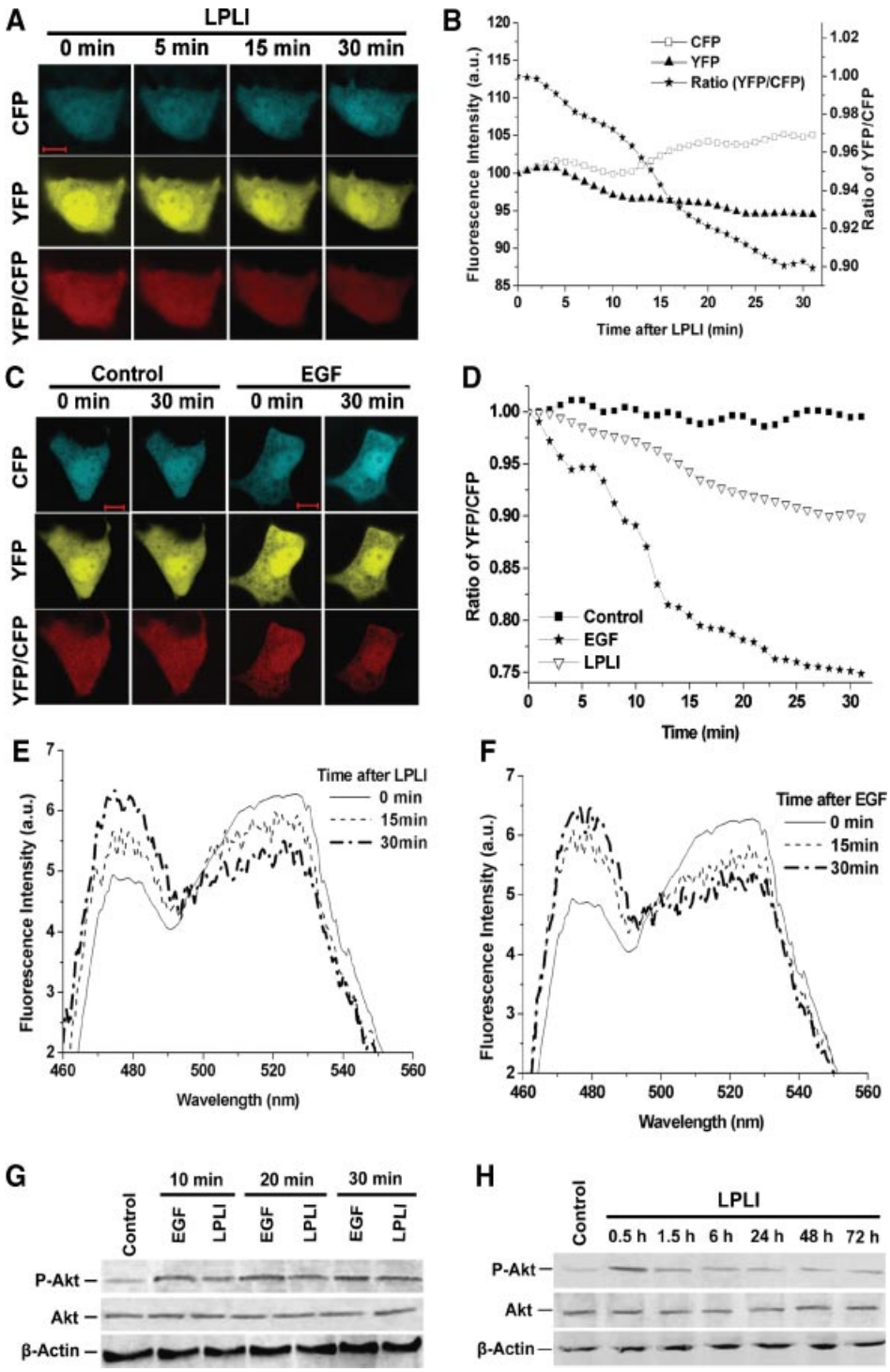

Fig. 3. Real-time monitoring of Akt activation induced by LPLI. COS-7 cells were starved for 24 h and then treated with 1.2 J/cm^2 LPLI or 50 ng/ml EGF. A–D: Single-cell imaging analysis of COS-7 cells transfected with BKAR in different conditions. The fluorescence images of CFP and YFP channels excited by Ar-Ion laser (458 nm) and YFP/CFP ratio were recorded with LSM microscope. The decreased ratio of YFP/CFP indicates the activation of Akt. A: Representative fluorescence images of CFP, YFP and YFP/CFP ratio of cells treated with LPLI. B: Quantitative analysis of CFP and YFP intensities and YFP/CFP ratio corresponding to the images in A. The CFP and YFP intensities at the first time point are normalized to 100, and the YFP/CFP ratio is normalized to 1. C: Representative fluorescence images of CFP, YFP and YFP/CFP ratio of EGF-treated cells and control cells. D: Comparison of YFP/CFP ratios of control cells or cells treated with LPLI or EGF. The YFP/CFP ratios at the first time point are normalized to 1. Results represent 1 of 4 replicates. Scale bar: 10 μm. E,F: Spectrofluorometric analysis of Akt activation induced by LPLI or EGF in living cells expressing BKAR. The cells were excited at the excitation wavelength of CFP (434 ± 5 nm), resulting in a CFP emission peak (476 nm) and YFP emission peak (528 nm) caused by FRET from CFP. And the fluorescence emission spectra were obtained by luminescence spectrometer. E: The emission spectra of BKAR after LPLI treatment. F: The emission spectra of BKAR after EGF treatment. G,H: Representative Western blot analysis for Akt phosphorylation (G) with 30 min after either LPLI or EGF treatment and (H) up to 72 h after LPLI. COS-7 cells were collected at the indicated time after EGF and LPLI treatment, and were analyzed for Akt phosphorylation by Western blotting. Akt and β-actin was used as a loading control. Results represent one of three replicates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

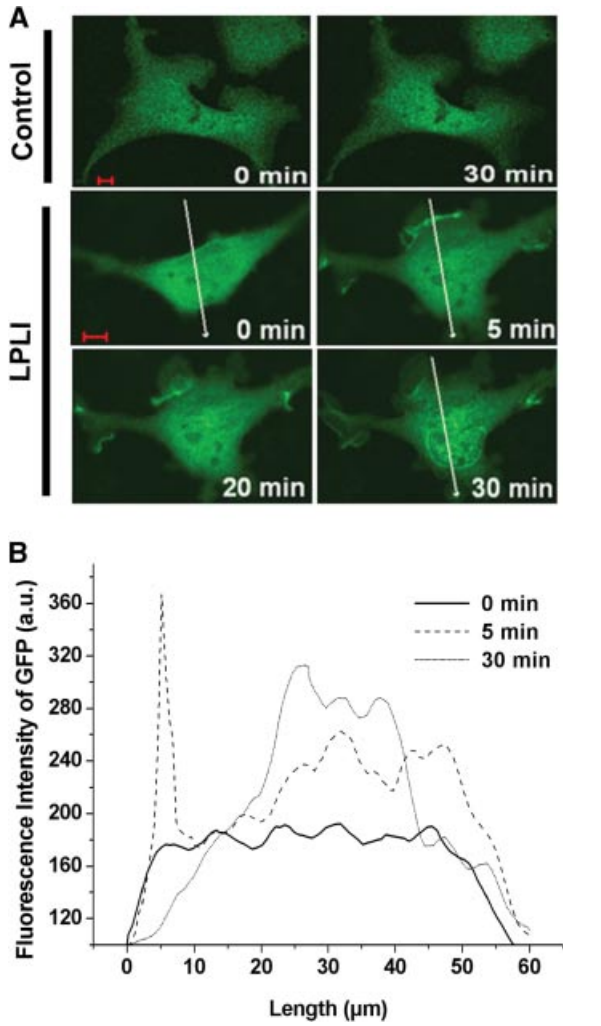

Fig. 4. Dynamics of GFP-Akt distribution after LPLI stimulation. After 24 h serum starvation, COS-7 cells transfected with GFP-Akt were stimulated with 1.2 J/cm^2 LPLI and time-lapse imaged. A: Confocal imaging temporal sequences of GFP-Akt distribution in control and LPLI-treated cells. Scale bar: 10 μm. B: Along the white lines in A, GFP fluorescence intensity is plotted. The width of cell along the white line is normalized to the width of cell at 0 min. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

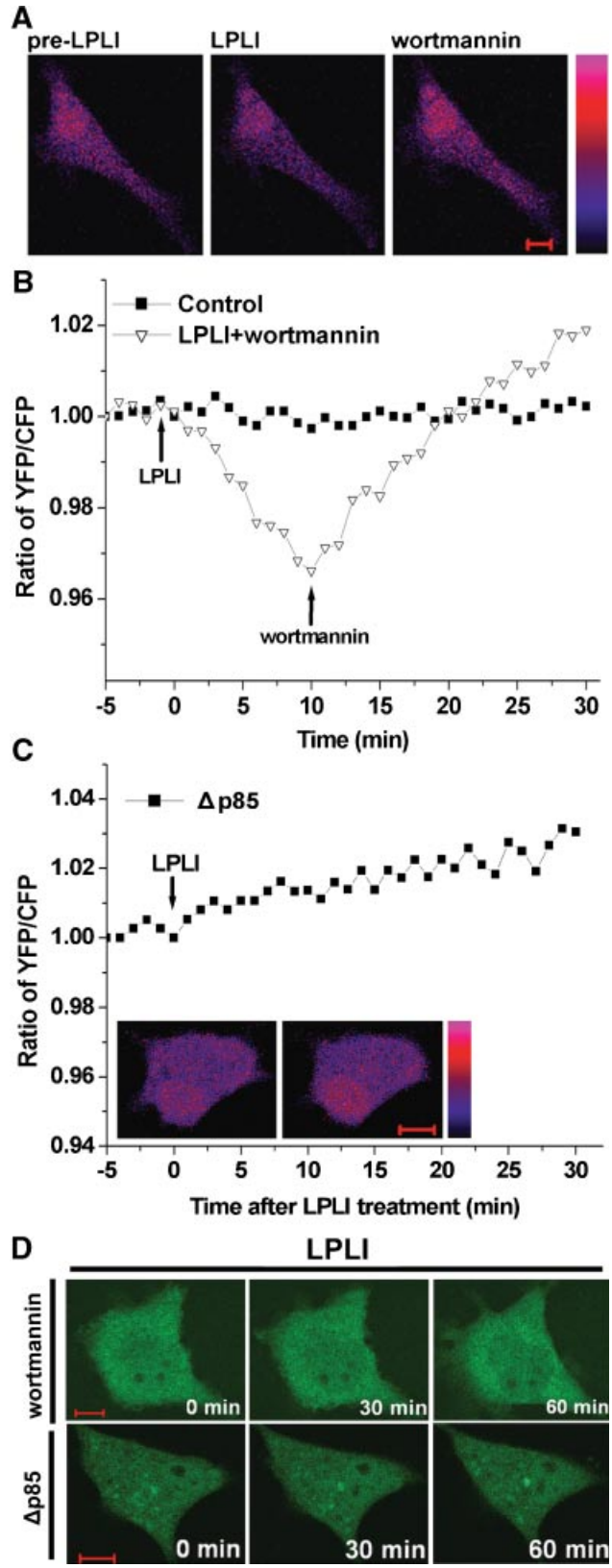

Fig. 5. LPLI induces the activation of Akt through a PI3K-dependent pathway. A,B: Effects of wortmannin on the activation of Akt induced by LPLI. COS-7 cells transfected with BKAR were serum starved for 24 h and then treated with 1.2 J/cm^2 LPLI, 10 min after stimulation, cells were subjected to wortmannin. A: Representative pseudocolor images of YFP/CFP ratio in COS-7 cells at various time during the cause of LPLI followed by wortmannin treatment. Scale bar: 10 μm. B: The quantitative time course of YFP/CFP ratio corresponding to the images in A. C: The quantitative time course of YFP/CFP ratio in COS-7 cells co-transfected with pΔp85 (a dominant-negative mutant of PI3K) and BKAR after 1.2 J/cm^2 LPLI treatment. The inset shows pseudocolor images of YFP/CFP ratio in COS-7 cells before and after LPLI treatment. D: Real-time monitoring of GFP-Akt distribution in cells co-transfected with pGFP-Akt and pΔp85 (upper) or transfected with pGFP-Akt in the presence of wortmannin (10^{-6} M) (bottom) after 1.2 J/cm^2 LPLI treatment. Results represent one of three replicates. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

to LPLI stimulation. As established in the current work that wortmannin could completely inhibit Akt activation in cells treated with LPLI (Fig. 5A,B), we selected wortmannin as a positive control for the subsequent experiment. A representative temporal sequence of the pseudocolor images of YFP/CFP ratio are shown in Figure 6A. Figure 6B is the quantitative analysis of the corresponding YFP/CFP ratio. As shown in these figures, YFP/CFP ratio was significantly reduced by the LPLI treatment, while adding Gö 6983 had minimal effect on the ratio. With PP1, the ratio was also reduced, but only to a less extent. These results indicate that PKCs have no effect on the activation of Akt induced by LPLI. Src is involved in LPLI-induced Akt activation, but not necessarily as a prerequisite. This is because PP1 could not completely block Akt activation like what wortmannin did. These results were also confirmed by the Western blot analysis. As shown in Figure 6C,D, Gö 6983 had minimal effect on the level of Akt phosphorylation in cells treated with LPLI, while PP1 showed a moderate inhibitive effect. All these results strongly indicate that Src, but not PKCs, is involved in the activation of Akt induced by LPLI.

Blockade of PI3K/Akt signaling pathway inhibits cell proliferation induced by LPLI

The results in the present study show that, Akt activity was enhanced during LPLI-induced cell proliferation. The following experiments were performed to examine the cell viability when Akt activation was inhibited by wortmannin. COS-7 cells after 24 h starvation were treated with wortmannin, and the cell viability was analyzed using CCK-8 at various time points after

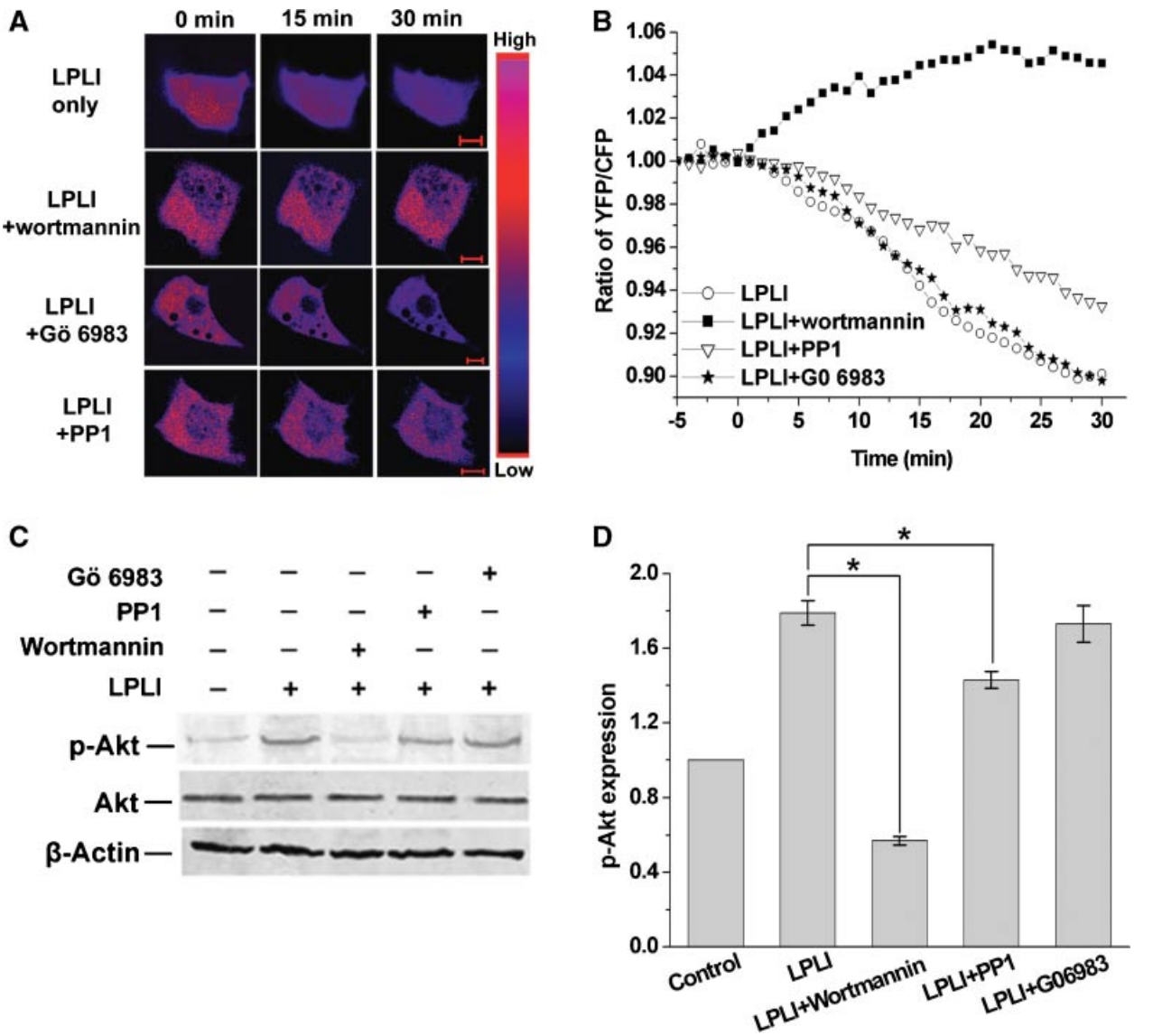

Fig. 6. Role of PI3K, Src and PKC in LPLI-induced Akt activation. A,B: Effects of wortmannin, Gö 6983 and PP1 on Akt activation indicated by the FRET of BKAR. A: Representative pseudocolor imaging series of YFP/CFP ratio at the indicated times after 1.2 J/cm^2 LPLI in the presence or absence of wortmannin, PP1 and Gö 6983, respectively. B: The time courses of YFP/CFP ratio corresponding to the images in A. Results represent one of three replicates. Scale bar: 10 μm. C,D: Effects of wortmannin, PP1 and Gö 6983 on Akt phosphorylation induced by LPLI. C: COS-7 cells were collected at 15 min after 1.2 J/cm^2 LPLI treatment in the presence or absence of wortmannin, PP1 and Gö 6983, and were analyzed by Western blotting for Akt phosphorylation. Akt and β-actin were used as loading controls. D: The levels of endogenous Akt phosphorylation were quantified by densitometry (with an LAS-1000 image analyzer). Data are from three independent experiments and presented as average ± SE. $^*P < 0.01$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

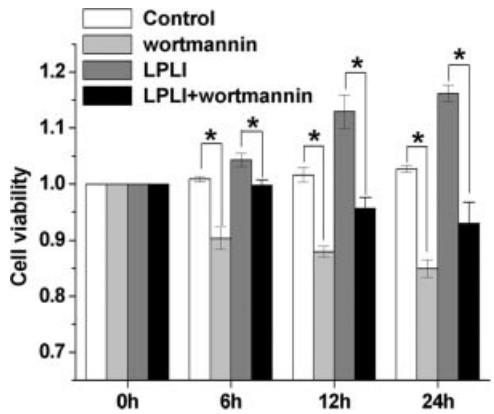

Fig. 7. Effects of wortmannin on LPLI-induced cell proliferation. Cell viability was assessed by the CCK-8 assay at 0, 6, 12, and 24 h after 1.2 J/cm^2 LPLI in the presence or absence of wortmannin. Error bars are s.e.m. from four independent experiments. $^*P < 0.01$.

the irradiation. As shown in Figure 7, compared to that of the non-treated cells, significant decrease of cell viability was observed in cells treated with wortmannin. The viability of cells treated with LPLI in conjunction with wortmannin was also decreased compared to that of cells with/without LPLI treatment. These results suggest that PI3K/Akt signal pathway plays a crucial role in cell proliferation induced by LPLI.

Discussion

In the present study, a novel link between LPLI stimulation and cell proliferation is identified. Our results contribute to the general idea that LPLI promotes cell proliferation through PI3K/Akt signaling pathway.

Akt serine/threonine protein kinases are critical for the regulation of fundamental cellular processes including cell proliferation, differentiation, cell shape, adhesion, migration, and survival (Brazil et al., 2004; Bellacosa et al., 2005). Therefore, we explored the activity of Akt involved in LPLI-induced cell proliferation. In the present study, significant activation of Akt was observed in cells treated with LPLI. We have demonstrated, via four distinctive evidences, that LPLI promotes cell proliferation by activating PI3K/Akt signaling pathway and conducted preliminary work to explore its cross-talk with ROS/Src or RTKs/PKCs pathway: (1) LPLI could induce Akt activation and the activation occurs on plasma membrane (Figs. 3 and 4). (2) The activation of Akt triggered by LPLI is in a PI3K-dependent manner (Fig. 5). (3) Src family partially participates in the activation of Akt caused by LPLI, while PKCs have no contribution (Fig. 6). (4) Blockade of Akt activation by wortmannin negatively affects cell proliferation under LPLI treatment (Figs. 2 and 7).

In order to investigate the activity of Akt under LPLI treatment, we used three different physical–chemical methods. Firstly, we used single-cell FRET analysis, which is a powerful technique that can provide insight into the spatial and temporal dynamics of protein kinase activity using reporter plasmid in vivo. Using this method, we show that LPLI induced gradual and continuous activation of Akt compared to the intense and rapid activation caused by EGF (Fig. 3A–D). The second approach, spectrofluorometric analysis further confirmed the above result on multi-cell and statistical level (Fig. 3E,F). Finally, we used Western blot analysis, a traditional method, to detect the phosphorylation of Akt after LPLI treatment. The level of Thr308 phosphorylation was observed to increase in cells treated with LPLI compared to that of control cells (Fig. 3G). These results strongly suggest that Akt can be effectively activated by phosphorylation in response to LPLI stimulation.

Recent studies indicate that Akt activation induced by growth factors occurs on plasma membrane (Watton and Downward, 1999). Once activated, Akt migrates to subcellular organelles, including nuclei, mitochondria, and other cytosolic locations, and then phosphorylates a number of "effector" substrates throughout the cell. Therefore, the regulation of specific cellular functions is exerted by Akt at the level of the plasma membrane, nucleus, mitochondria, and cytosol in multi-protein complexes (Catalucci and Condorelli, 2006). Akt is then dephosphorylated and inactivated by protein phosphatases (Andjelkovic et al., 1996; Gao et al., 2005). To investigate this intracellular process, we employed a live-cell in situ fluorescent imaging to examine the cellular distribution of Akt after LPLI treatment. As shown in Figure 4, after LPLI treatment, GFP-Akt translocated from cytosol to plasma at 5 min, and then returned to cytosol and nucleus at 10 min post-irradiation. Interestingly, the migration from cytosol to plasma membrane lasted for at least 30 min, indicating the continuous accumulation of activated Akt during this time period after LPLI treatment. Based on our results, it is reasonable to suggest the events involved in LPLI-induced Akt activation as following: Akt is activated on the plasma membrane and maintains its activity during the migration from plasma into cytosol and nucleus, presumably by maintaining its phosphorylation status (Fig. 3G). Akt-mediated phosphorylation sustained in the cytosol and nucleus for 10–30 min after LPLI stimulation, which might be due to a large amount of activated Akt continuously translocating from plasma membrane to cytosol and nucleus, although protein phosphatase simultaneously hydrolyzed some phosphorylated Akt in cells.

How does LPLI activate Akt? One possible mechanism is that LPLI induces ligand-free dimerization and transactivation of RTKs which are in the "right energetic state" to accept the laser energy, leading to their auto-phosphorylation and activation (Karu, 1999; Shefer et al., 2001). Activated RTKs can activate PI3K (Araki et al., 1994; Bruning et al., 1997), resulting in an increase in PI (3, 4, 5) P3 at the plasma membrane (Cantrell, 2001). The binding of PI (3, 4, 5) P3 to the PH domain anchors Akt on the plasma membrane and allows its phosphorylation and activation by PDK1 and other kinases (Alessi et al., 1996, 1997; Andjelkovic et al., 1997; Alessi, 2001; Mora et al., 2004; Dong and Liu, 2005; Sarbassov et al., 2005). In our experiments, wortmannin and Δp85 completely inhibited Akt phosphorylation and activation induced by LPLI (Fig. 5), suggesting this activation of Akt is dependent on PI3K activity. Therefore, we prefer that RTKs/PI3K/Akt pathway mediates Akt activation under LPLI treatment.

There are other potential mechanisms may contribute to the activation of Akt induced by LPLI. It is possible that LPLI activates Akt through ROS. LPLI has been demonstrated to increase the level of intracellular ROS generation (Matsui et al., 2007; Zhang et al., 2008). With LPLI treatment, light is absorbed by endogenous photosensitizers (porphyrins or cytochromes) that dominantly locate at plasma membrane, mitochondria or lysomes. The photosensitizers activation results in ROS ($^1O_2$, O_2^-, and H_2O_2) production (Lavi et al., 2003). Intracellular oxidants can mediate the activation of Akt (Ushio-Fukai et al., 1999; Wang et al., 2000). Our previous work prove that ROS production induced by LPLI can promote the activation of Src (Zhang et al., 2008) and there are several evidences indicate that Src family is an upstream activator of Akt (Kassenbrock et al., 2002; Bentley et al., 2007). Jiang and Qiu (2003) provide that Src directly regulates Akt activity by phosphorylating Tyr315 and Tyr326 in the activation loop of Akt. In recent studies, there are

two major opinions on the activation of Akt mediated by Src. One opinion is that the activation of Akt by Src is a PI3K-dependent signaling pathway (Liu et al., 1998). Others show pressure-induced colon cancer cell adhesion via Src-dependent PI3K/Akt pathways (Thamilselvan et al., 2007). Our results show that Src family inhibitor partially inhibited the activation of Akt induced by LPLI (Fig. 6), suggesting that Src also contributes to Akt activation during LPLI-induced cell proliferation. Furthermore, LPLI-induced Akt activation was completely blocked by wortmannin (Fig. 5), suggesting that Src regulated Akt activation is PI3K dependent. All these studies come to a conclusion that LPLI-induced Akt activation is dominated by the RTKs/PI3K/Akt signaling pathway. There is a secondary signaling pathway, ROS/Src/Akt, which is dependent on PI3K in response to LPLI.

Previous study shows that LPLI can activate RTKs/PKCs in a diacylglycerol (DG)-dependent manner in human lung adenocarcinoma cells (ASTC-α-1) (Gao et al., 2006). Therefore, the possibility of PKCs involved in LPLI mediated RTKs/PI3K/Akt signaling pathway was investigated. The results indicate that PKCs have no effect on Akt activation in cells treated with LPLI (Fig. 6). There are two possible interpretations for this phenomenon: either PKCs are not involved, or the effects of various PKC isoforms counteract and cancel each other. Recent researches reveal that various members of PKC family can either increase or decrease Akt activity. PKCα can up-regulate the activity of Akt by directly stimulating phosphorylation of Ser473 in endothelial cells (Partovian and Simons, 2004). PKCβII can also directly phosphorylate and activate Akt in mast cells (Kawakami et al., 2004). PKCη can modulate the activation of Akt in glioblastoma cells, supporting glioblastoma proliferation (Aeder et al., 2004). By contrast, treating cell with PKCβ inhibitor LY 379196 causes Akt phosphorylation at Ser473 (Wen et al., 2003). The activation of PKCζ results in phosphorylation of Thr34 in the PH domain of Akt and limits its activation in response to insulin (Powell et al., 2003). c-PKCs down-regulate Akt activity upon steel factor (SF) stimulation (Edling et al., 2007).

In accordance with our previous observations and other researches (Shefer et al., 2001; Gao et al., 2006; Tuby et al., 2007; Zhang et al., 2008), we found that LPLI enhanced cell viability and promoted long-term proliferation of cells cultured on serum-free medium (Fig. 2). We also found that Akt activation plays a key role in LPLI-induced cell proliferation as wortmannin and Δp85 effectively decreased the viability of cells treated with LPLI (Fig. 7). Akt promotes proliferation through regulating some cell cycle proteins, such as p21 and the related family members p27/Kip1 (p27) and p57/Kip2, to inhibit cell cycle arrest (Sun et al., 1999; Gesbert et al., 2000; Graff et al., 2000). Akt can promote translation and stabilization of cyclin D1 protein by phosphorylation and inactivation glycogen synthase kinase 3 (GSK3) (Diehl et al., 1998; Muise-Helmericks et al., 1998; Alt et al., 2000).

Akt drives quiescent cells into cell cycle and speed up the progression of the cell cycle, thereby promoting cell proliferation. However, the time period of Akt activation (0.5 h) is not consistent with that of cell proliferation (2–3 days) (Figs. 2 and 3G,H). With Western blotting, it is showed that, Akt phosphorylation decreased at 1.5 h after LPLI, and remained low up to 72 h. It is possible that, Akt activation induced by LPLI within 30 min drives quiescent cells into at least one round of proliferation which lasts for approximately 24 h post-irradiation. The relatively prolonged effect of LPLI on the proliferation of cells may be attributed to growth factors secreted by the cells in response to the irradiation. Recent studies show that LPLI can up-regulate the expression of growth factors, such as vascular endothelial growth factor (VEGF) (Tuby et al., 2006), basic fibroblast growth factor (bFGF), insulin like growth factor-1 (IGF-1) (Saygun et al., 2008) and transforming growth factor-beta (TGF-beta), etc. (Safavi et al., 2008). Further studies are necessary to pinpoint the mechanisms that LPLI triggered long duration of cell proliferation.

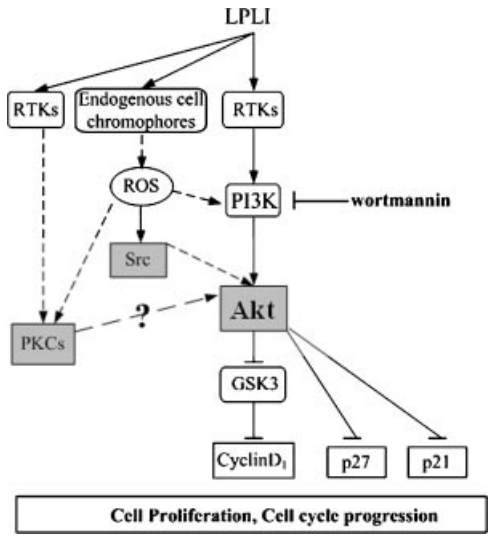

Fig. 8. A model of the signaling pathways of Akt activation induced by LPLI. Broken lines mean that the downstream changes produced by the upstream do not be proved clearly. Real lines mean that the downstream effects induced by the upstream have been proved already.

For the first time, we conclude that LPLI can stimulate Akt activation, which is mediated by PI3K, and the activation of PI3K/Akt signaling pathway is crucial in promoting cell proliferation induced by LPLI. Our results also demonstrate that, with LPLI stimulation, Akt is initially activated on plasma membrane, and then translocates to cytosol and nucleus to function its biological effects. Src partially participates in the activation of Akt, while PKCs show no effect. By combining the results on the presented and previously reported studies, the signaling pathways related to Akt induced by LPLI can be summarized in Figure 8. The delineation of the signaling pathways involved in LPLI-induced proliferation will provide insight into establishing the therapeutic potential of LPLI for indications such as muscle injury, wound healing or vascular regeneration.

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