Cellular property and potential functions of insulin receptor (IR) induced by IGF-1/insulin on lung cancer cells

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Research

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Abstract

Background

Under normal physiological conditions, insulin exhibited a series of important biological functions. However, more and more evidence indicate that insulin is closely related to the occurrence and development of tumors. Recent studies have shown that insulin is also closely related to the occurrence and development of lung cancer. However, until now, the cellular properties of insulin/insulin receptors on lung cancer have not been fully revealed.

Methods

Indirect immunofluorescence, western-blot and other techniques have been used to identify the biological activity of insulin on lung cancer cell lines.

Results

The biological activity of insulin is closely related to its cell behavior. therefore we used lung cancer cell lines as a model to explore the cellular behavior and properties of insulin/IR in the current study, and the results showed that the IR can internalize into lung cancer cells, and it can also transport into the nucleus under insulin treatment. further study showed nuclear-localized IR could promote the proliferation of lung cancer cells. Taken together, this study shows that IR's nuclear localization is closely related to cell proliferation.

Conclusions

This work lays the foundation for further research on relationship between insulin and the occurrence and development of lung cancer.

Background

It is well-known that insulin has important bioactivities\(^1\). Almost all organs and tissues expressed insulin receptor (IR), therefore the biological functions of insulin are very extensive, such as lowering blood sugar, reducing lipolysis and increasing protein synthesis\(^2\). All of insulin's effects are mediated by the IR which expressed on cell membrane of target cells or tissues\(^2\). The insulin receptor (IR) is a tetramer composed of two \(\alpha\) subunits and two \(\beta\) subunits linked by disulfide bonds, the \(\alpha\) subunit of the IR is entirely extracellular and contains the insulin-binding site. \(\beta\) subunit is composed of three domains: extracellular domain, transmembrane domain and intracellular domain. The extracellular domain of the IR's \(\beta\)-subunit exhibited a role in the regulation of insulin receptor protein-tyrosine kinase activity. insulin binding to the
a-subunit can induce IR’s conformational changes, which in turn triggers the kinase activity in the b-subunit, which is the first step in the action of insulin on glucose metabolism. After the IR is activated, a series of downstream signal transduction molecules (such as Mitogen-activated protein kinases (MAPKs) and 3-phosphoinositide kinase (Phosphotidylinositide-3-kinase (PI3K)) pathways) are activated. These activated signal molecules are transported to the nucleus to turn on gene transcription.

Under normal physiological conditions, insulin exhibited a series of important biological functions. However, more and more evidence indicate that insulin is closely related to the occurrence and development of tumors, contributes to tumorigenicity, proliferation and metastasis. Scientists have found that insulin receptors are highly expressed in many tumor types. Studies have shown that insulin receptor is a potential anti-tumor therapeutic target.

Recent studies have shown that insulin is also closely related to the occurrence and development of lung cancer. For example, Jiang et al found that insulin increased the proliferation, migration of lung cancer. Furthermore, insulin is associated with Lung cancer Susceptibility. In addition to increasing cell proliferation, insulin could promote cell survival by inhibition of pro-apoptotic cytokines.

However, until now, the cellular properties of insulin/insulin receptors on lung cancer have not been fully revealed. In addition to insulin, IGF-1 is also a ligand for the insulin receptor, which also can bind and activated IR, although its affinity for IR is very low. In the current study, we used lung cancer cell lines as a model to explore the cellular behavior and properties of IR under insulin/IGF-1 stimulation. We found that the IR can internalize into lung cancer cells, and it can also transport into the nucleus under ligands treatment. Further study showed nuclear-localized IR could promote the proliferation of lung cancer cells. This study laid the foundation for studying the relationship between IR and lung cancer.

**Materials And Methods**

**Antibodies and Regent**

Human recombinant insulin and IGF-1 were obtained from Sigma (St. Louis, MO) p-AKT, p-IR, p-IRS1 antibodies were purchased from Cell Signaling Technology (CST). IR, AKT and IRS1 antibodies were purchased from Cell Signaling Technology (CST). MTT assay kit was obtained from Abcam. Foetal calf serum (FCS) was purchased from Invitrogen (Carlsbad, CA, USA). PVDF membrane was obtained from Beyotime Biotechnology (Shanghai, China). Bovine serum albumin (BSA) and low-fluorescence PVDF membrane were purchased from Bio-Rad. Dulbecco’s modified Eagle’s medium (DMEM) and Fetal bovine serum (FBS) were obtained from Invitrogen (Carlsbad, CA, USA). Unless otherwise specified, reagents were purchased from Sigma-Aldrich.

**Cell culture**

A549 cell (lung carcinoma cell line) was obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/ml
penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine.

**Western-blot**

Total protein was extracted and protein concentration was measured using a BCA protein assay kit (Thermo Scientific Pierce). The protein samples were then subjected to SDS-PAGE (4–12%), and transferred into a low-fluorescence PVDF membrane. After washing the membrane for three times, the membranes were blocked with 3% for 2 h at RT. The low-fluorescence PVDF membranes were incubated with primary antibody for 12 h at 4°C. After three washes in TBS-0.1% Tween-20, AlexaFluor555/488-conjugated secondary antibody (1: 3000 dilutions or 1:2000) were added and incubated for another 2 h at 37°C for 2h. the membranes were rinsed for three times with TBST, and were detected using a fluorescence imaging system (Bio-Rad).

**Laser confocal scanning microscope observation**

A549 cells were seeded onto coverslips at 1 × 10^5 per well. When A549 cells grew to approximately 50% confluence, the cells are used to perform the following experiments. The cells were rinsed twice with sterile PBS. Then, cells were fixed with 4% PFA and permeabilized with 0.4% Triton X-100 for 0.5 h. After washing twice with PBS, the cells samples were blocked with 10% donkey serum in PBS for 2 h at 37°C. After washing cells for three times with PBS, cell samples were incubated with primary antibodies at 4°C for 12 h, after which, cells were rinsed three times with PBS, followed by incubating with secondary antibodies at 37°C for 1 h. Hoechst 33342 was used to stained the cell nuclei. Imaging was performed with an Olympus CLSM.

**Flow cytometry**

The cells were cultured in serum-free DMEM for 12 h. The cells were then treated with insulin/IGF-1 at the indicated time points, after which, the cells were collected, washed three times with ice cold PBS to remove the culture medium. Then, the cells were fixed with ice-cold 70% ethanol at 4°C for 12 h. After blocking with 5% BSA for 2 h, the cells were incubated primary antibodies, followed by incubation with a second antibody conjugated to Alexa Fluor 488 or Alexa Fluor 555. The cell samples were analyzed by Flow cytometry (Becton Dickinson). All experiments were performed in triplicate.

**Cell proliferation assay (MTT Assay)**

The A549 cells were seeded in a 96-well plate at 2×10^4 cells per well, and incubated for 48 h. After washing, MTT solution (10 µL, 5 mg/mL) was added to each well and incubated for 4 h at 37°C. The medium was then discarded. The reaction was then stopped by dissolving the cells with 100 mL of dimethyl sulfoxide (DMSO). The absorbance was detected at a wavelength of 490 nm by a microplate reader (MultiSkan FC, Thermo Scientific).

**Cell cycle analysis by Flow Cytometry**

A single cell suspension of A549 cells was prepared and centrifuged at 1000 rpm for 8 min. the supernatant was removed. The cells were washed twice with PBS. The cells were then fixed with 70%
alcohol at 4°C for 1 h. The cells were collected by centrifugation, after which, the supernatant was removed. The cell pellets were resuspended with RNase A in PBS (30 ug/ml) and incubated at 37°C for 1 h. Subsequently, cell samples were incubated with PI for 05 h at RT in the dark. The cell samples were then filtered through a 40 µm nylon mesh, and analyzed by Flow cytometry (Becton Dickinson).

**Co-IP**

A549 cells were lysed in CHAPS lysis buffer (120 mM NaCl, 0.3% CHAPS, 1 mM EDTA, 40 mM HEPES (pH 7.5) at 4°C for 4 h. Cell lysate were then centrifuged at 14,000 × g at 4°C for 0.5 h, after which, the supernatants were collected and mixed with the indicated antibodies and rotated at 4°C for 12 h. The supernatants were mixed with the indicated antibodies overnight at 4°C with rotation, and then incubated with Protein A/G-PLUS Agarose beads for another 5 h at 4°C. The beads were then washed five times with CHAPS buffer and subjected to western blotting.

**RT-PCR**

A549 cells were seeded at a density of 1.5 × 10^5 cells/well into 6-well culture plates. Total RNA from cell samples were extracted using a RNeasy Mini kit (Qiagen, Valencia, CA, USA). 1µg of total RNA was reverse transcribed into cDNA with ReverTra Ace (TOYOBO, Osaka, Japan) according to the manufacturer's instruction. RT-PCR was performed using a Transcriptor One-Step RT-PCR Kit using the following primer: IGF-1R primers forward: 5'-GGAGTTGTATTTGCCATCACCAGGG-3', reverse: 5'-ATGCGCGGGCAAATTTGTGATCCATCACCAGGG-3'; GAPDH forward: 5'-TGGAGTCTACTGGCGTCTT-3', reverse: 5'-TGTCATATTTCCTCGTGGTTCA - 3'.

**ELISA**

ELISA Kit was used to detect phosphorylated signal molecules according to the manufacturer's instructions.

**Cell transfection**

The A549 cell were transfected with siRNAs against Nup358 and Rab5 (Dharmacon, Pittsburgh, PA, USA) and siRNA control used lipofectamine 3000 according to the manufactures' instruction. RT-PCR and Western blot were used to evaluate the knockdown efficiency and specificity of siRNA.

**Cell migration and invasion assay**

1x10^5 /well of A549 cells were seeded into the upper chamber of the Transwell chamber (Corning) with or without insulin for 24 h. A total of 500 µl DMEM containing 10% FBS were added into the lower chambers. After culturing for 48 h, the non-migrated cells in the upper chamber were removed. The cells in the lower chamber was fixed in paraformaldehyde (4%), washed by PBS and stained with crystal violet. The stained cells were analyzed under a light microscope.

**The extraction of Nuclear/cytoplasmic proteins**
Cytoplasmic/Nuclear proteins of A549 cells were obtained by using a subcellular protein fractionation kit according to manufacturer’s instruction (Thermo Fisher Scientific Inc).

**Statistical analysis**

Data are expressed as the mean ± standard deviation. Statistical analysis was carried out using SPSS (version 20.0; SPSS Inc., Chicago, IL, USA). P < 0.05 was considered to indicate a statistically significant difference.

**Results**

**Detection of IGF-1R expression**

The expression pattern of IGF-1R was evaluated by CLSM (Confocal Laser scanning microscope). As we can see in the Fig. 1A, CLSM analysis showed IR was mainly in the cell membrane, and a small number of insulin receptors were expressed in the cytoplasm (Fig. 1B). Furthermore, Flow cytometry also showed that A549 cell expressed high level of IGF-1R.

In addition, we knocked down IGF-1R through the SiRNA method, and the results showed that the expression level of IGF-1R was significantly reduced (knocking down IGF-1R did not cause significant cell apoptosis) (Fig. 1C).

**Internalization of IGF-1 in A549 cell**

The internalization of insulin/IGF-1 on A549 cell was checked. The cells were challenged with IGF-1 for different time points. The cell samples were then analyzed by Confocal Laser Scanning Microscope (CLSM). The results showed that fluorescently labeled-insulin was internalized into A549 cell in a time-dependent manner (Figure.2A). Additionally, IGF-1’s internalization was also evaluated CLSM (Figure. 2B).

**Intracellular trafficking of IR under insulin but not IGF-1 treatment**

Here, we studied the intracellular trafficking of IR under insulin stimulation, and the results indicated that IR could internalize in to cell cytoplasm in a time-dependent manner under insulin treatment. In addition, IR also transported into cell nuclei (Fig. 3A). In addition, we also explored the trafficking of IR under IGF-1 treatment, and results showed that IR could internalize into cell cytoplasm, but IR could not transport into
cell nuclei (Fig. 3B). Western-Blot also confirmed this result. These results suggested that the nuclear localization of IR is specifically induced by insulin but not IGF-1. These results suggested that IR’s nuclear localization may exhibit important biological activities in A549 cell nuclei.

Figure 3A. The internalization dynamics of IR under insulin treatment. B. The internalization dynamics of under IGF-1 treatment. Data are expressed as the mean ± standard deviation. Data are expressed as the mean ± standard deviation. P < 0.05 was considered to indicate a statistically significant difference.

Clathrin and caveolin are involved in IR’s Endocytosis

The above study has showed that IGF-1/IGF-1R could internalize into cell cytoplasm. Here, the endocytic mechanism of IR was explored. Study has shown that the endocytic pathway of the same cytokine/receptor is different in different types of cells. In lung cancer cell, the endocytic pathway of the insulin/IR remains unclear. Clathrin-dependent endocytosis or caveolin-mediated endocytosis is involved in the endocytosis of cytokine/growth factor. Additionally, the non-clathrin- and caveolin-dependent pathway was also existed. For this, co-localization analysis was conducted to study the IR’s endocytic pathway. The co-localization signals between caveolin/IR and clathrin/IR were detected under insulin treatment, which suggested that both clathrin and caveolin were involved in the IR’s endocytosis (Fig. 4A).

In addition to insulin, we also analyzed the endocytic mechanism of IR under the stimulation of IGF-1 (Fig. 4B).

Figure 4. A. Both clathrin and caveolin were involved in the IR’s endocytosis under insulin treatment. B. Both clathrin and caveolin were involved in the IR’s endocytosis under IGF-1 treatment. P < 0.05 was considered to indicate a statistically significant difference.

The internalized IR localized in different types of endosomes

We further analyzed which types of endosomes IR enter into by CLSM. The colocalization signal of IR and EEA1 (early endosome marker) could be detected, which indicated that IR enters into the early endosome (Fig. 5). It is well-known that the recycling endosome is rich in Rab4 and Rab11, whereas Rab7 and Rab9-positive endosome is rich in the late endosomes. Colocalization analyses showed that IR was localized in Rab5/7/11-positive endosome, which provides an explanation for different cytoplasmic localization of IR (Different types of endosomes could transport IR to different destinations).

Figure 5. A. The IR transported into different types of endosomes under IGF-1 treatment. P < 0.05 was considered to indicate a statistically significant difference.

Tyrosine phosphorylation of IR is required for nuclear localization of IR

We test whether the IR’s nuclear localization is associated with IR's tyrosine phosphorylation. We used HNMPA-(AM)3 (Insulin receptor tyrosine kinase inhibitor) to treat the cells, and the results showed that the
nuclear localization of IR was inhibited, but its internalization has not been affected (Fig. 6). This finding suggests that IR phosphorylation is required for IR's nuclear localization.

Figure 6. Tyrosine phosphorylation of IR is required for IR's nuclear localization. The experimental process has been described in detail in the materials and methods section. P < 0.05 was considered to indicate a statistically significant difference.

**Nup358 is involved in IR's nuclear localization**

Crossing the nuclear membrane is the most important step in the process of IR's nuclear translocation. Previous studies have shown that NUP358 plays an important role in the nuclear transport process of IGF-1R. IGF-1R has a similar structure to IR, and both IGF-1 and insulin can bind IGF-1R. Similarly, IGF-1 and insulin can also bind to IR. Therefore, we analyzed whether NUP358 is involved in IR's nuclear translocation. As shown in Fig. 7A, the colocalization analyses indicated that IR can interact with Nup358, and the results form IP-WB also indicated that NUP358 interacted with IR.

In order to further determine the role of NUP358 in IR's nuclear transport, NUP358 was knocked down using the SiRNA method (Fig. 7B). The results showed that the nuclear localization of IR was significantly reduced. This further confirmed that NUP358 was involved in the nuclear localization of IR (Fig. 7C).

Figure 7. A. Detection of colocalization between IR and UNP358. B. Analysis of interactions between IR and UNP358 by IP-WB.C. NUP358 Knocking down inhibited IR's nuclear localization. The experimental process has been described in detail in the materials and methods section. P < 0.05 was considered to indicate a statistically significant difference.

**IR nuclear localization is associated with cell proliferation of A549**

To explore the function or role of nuclear-localized IGF-1R, a model of the non-nuclear-localized IR was established by knocking down Nup358 (NUP 358-knock-down does not affect the proliferation of lung cancer cells, and did not lead to the apoptosis of lung cancer cells (Data not shown)), and the results indicated that IR's nuclear localization was significantly reduced compared to control (Figure.7C), but IR's internalization was not affected. Then MTT experiments were used to evaluate the role of nuclear-localized IR. As shown in Fig. 8A, the 549-cell proliferation ability was reduced compared to the control. In order to further evaluate the role of nuclear-localized IR, we used a nuclear export inhibitor (Leptomyocin B, which can increase the residence time of IR in cell nuclei), and the results showed that the cell's proliferation ability was also increased (Fig. 8B). To further analyze the effect of nuclear-localized IR on A549 cell proliferation, cell cycle was determined by Flow cytometry, and the results showed that the proportion of cells in S phase was significantly increased compared to the control (Fig. 8C). In addition, Ki67 expression was also enhanced compared to control (Fig. 8D).

Figure 8. A. Nuclear-localized IR is closely related to the proliferation of A549 cells. B. Cell's proliferation ability was also enhanced by increasing the IR's residence time in the cell nuclei. C. IR's nuclear
localization was associated with cell cycle. D. Ki67 expression was enhanced. Data are expressed as the mean ± standard deviation. P < 0.05 was considered to indicate a statistically significant difference.

**Nuclear-localized IGF-1R increased the nuclear retention of signaling molecule**

We further explored the potential mechanism by which the nuclear-localized IR promote cell proliferation. Since the biological activity of insulin/IR is achieved by IR-mediated signal transduction, we explored the mechanism of the action of nuclear-localized insulin/IR from the perspective of IR-mediated signaling. As indicated in Fig. 9A, the results showed that the activation of p-ERK1/2 was significantly prolonged and increased compared to the non-nuclear-localized IR group, which suggested that nuclear-localized IR still has the ability to trigger downstream signals. But, insulin-induced other signaling pathways was not affected (Fig. 9B). This may be one of the potential effects of nuclear-localized IR.

**Figure 9. A.** The nuclear localization of IR is closely related to the activation of ERK1/2. The experiment process has been described in detail in the materials and methods section. Data are expressed as the mean ± standard deviation. P < 0.05 was considered to indicate a statistically significant difference.

**Discussion**

Many studies have shown that insulin is also closely related to the occurrence and development of lung cancer. IR are often overexpressed in malignant cells, and a high level of IR expression is indicative of poor survival in nonsmall cell lung cancer. In the current study, we use lung cancer cells as a model to study the cellular behavior of insulin/IR on lung cancer cell lines. In addition, we also explored the biological property of IGF-1 in lung cancer cell lines. We found that insulin/IR was internalized into the A549 cells, and IR was partially localized in the cell nuclei, further research found that the nuclear localization of IR was closely related to A549 cell proliferation.

In the current study, we use A549 cells as a model to study the cell characteristics and behavior of insulin/IGF-1. Studies have shown that insulin can not only bind to IR but also IGF-1R (insulin-like growth factor receptor). IGF-1R is a tyrosine kinase (TK) receptor, which shares 60–70% homology with the IR. IGFs (IGF-I and IGF-II) has a variety of bioactivities, such as cell growth, cell differentiation, and cell survival. On the other hand, insulin's structure is similar to IGF, which mainly mediates anabolic biological functions, such as regulation of glucose and amino acid transport. In general, IGFs has long-term action to regulate the cell fates, whereas insulin mainly regulates metabolic activity. Because A549 cell expressed both IGF-1R and IR, in order to prevent the influence of IGF-1R, we used SiRNA to knock down the expression of IGF-1R receptor, and then carried out related experiments, although insulin shows a low affinity for IGF-1R. Similarly, IGF-1 also shows the low affinity with IR.

IR's endocytosis is first step in the process of IR's nuclear localization. Previous studies have showed that the endocytic pathway of cytokine/cytokine mainly mediated by Clathrin-dependent endocytosis or Caveolin-mediated endocytosis. Additionally, the non-clathrin- and caveolin-dependent pathway is
also existed. In the current study, we found that Clathrin-mediated endocytosis plays a more important role in the process of IR internalization. After IR's endocytosis, the second important step is the IR's cytoplasmic trafficking. Since IR is a biological macromolecule, the cytoplasmic transport of IR requires the participation of endosomes. We found that the internalized IR transported into different types of endosomes (such as EEA1, Rab4 and Rab7)\textsuperscript{17,18}. Of course, the endosomes alone cannot complete the IR's nuclear localization. The cytoplasmic trafficking of insulin/IR additionally requires cofactors (such as motor proteins, microtubules and microfilaments). Previous studies have shown that cytoplasmic organelles and microtubules all play important biological roles in the process of nuclear localization of EGFR. Therefore, the nuclear localization of IR is a complex biological process. There are many important scientific issues to be solved in the future.

In addition to the cytoplasmic transport process, how IR crosses the nuclear membrane is also an important scientific issue. Based on previous studies, we found that NUP358 plays an important role in the nuclear transport of IR. Similarly, previous studies have shown that IGF-1R also enters into the nucleus under the mediation of NUP358.

We asked what are the important physiological functions of nuclear-localized IR? We found that nuclear-localized IR could increase the activation of ERK1/2. This is probably an important reason for nuclear-localized IR can promote the proliferation of A549 cell. It is well-known that Insulin/IR can activate many signal transduction pathways, among which, the activated ERK1/2 can regulate gene expression and promote cell growth. Of course, this may not be the only mechanism by which IR plays a role in the cell nuclei. Nuclear-localized IR may have a series of important biological activities. These scientific issues require further exploration.

Taken together, the current work showed that the nuclear-localized IR has important biological functions. In the current study, we analyzed the biological characteristics of IR in a cell model of lung cancer and found that IR could not only be internalized into the cytoplasm under the mediation of insulin and IGF-1. In addition, we also found that IR can be localized in the nucleus of A549 cell under insulin stimulation. This study shows that IR's nuclear localization is closely related to cell proliferation. This work lays the foundation for further research on relationship between insulin and the occurrence and development of lung cancer.

**Declarations**

**Ethics approval and consent to participate**

*Not applicable.*

**Consent for publication**

*Not applicable.*
Availability of data and materials

Please contact the correspondence author for the data request.

Competing interests

The authors declare that they have no financial conflicts of interest.

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Authors’ contributions

Qiu Ren, Miao Tian, Lin Lin, li juan Zhang, jia wen Zhang, Wen Luo, Wei Zhang performed the experiments and drafted the manuscript, Qiu Ren and Wei Zhang conducted the study. Miao Tian, Lin Lin collected and analyzed the data. All authors read and approved the final manuscript.

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Figures
Figure 1

A. Detection of IR expression by CLSM (A) and flow cytometry (B). C. IGF-1R was knock-down through the use of siRNA technology. Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.
Figure 2

A. The internalization dynamics of insulin on A549 cells. B. The internalization dynamics of IGF-1 on A549 cells. Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.
Figure 3

A. The internalization dynamics of IR under insulin treatment. B. The internalization dynamics of under IGF-1 treatment. Data are expressed as the mean ± standard deviation. Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.
Figure 4

A. Both clathrin and caveolin were involved in the IR’s endocytosis under insulin treatment. B. Both clathrin and caveolin were involved in the IR’s endocytosis under IGF-1 treatment. P<0.05 was considered to indicate a statistically significant difference.
Figure 5

A. The IR transported into different types of endosomes under IGF-1 treatment. $P<0.05$ was considered to indicate a statistically significant difference.

Figure 6

Tyrosine phosphorylation of IR is required for IR’s nuclear localization. The experimental process has been described in detail in the materials and methods section. $P<0.05$ was considered to indicate a statistically significant difference.
Figure 7

A. Detection of colocalization between IR and UNP358. B. Analysis of interactions between IR and UNP358 by IP-WB. C. NUP358 Knocking down inhibited IR's nuclear localization. The experimental process has been described in detail in the materials and methods section. P<0.05 was considered to indicate a statistically significant difference.
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A. Nuclear-localized IR is closely related to the proliferation of A549 cells. B. Cell's proliferation ability was also enhanced by increasing the IR's residence time in the cell nuclei. C. IR's nuclear localization was associated with cell cycle. D. Ki67 expression was enhanced. Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.
Figure 9

A. The nuclear localization of IR is closely related to the activation of ERK1/2. The experiment process has been described in detail in the materials and methods section. Data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.