Biological functions of nuclear-localized insulin receptor (IR) on A549 lung cancer cells

Ren Qiu[1, 2], Tian Miao[3], Lin Lin[4], Zhang Wei[1]

[1]*Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China*
[2]*Department of Respiratory, Hei Long Jiang Province Hospital Harbin, Hei Long Jiang, China*
[3]*Hei Long Jiang College of Business and Technology, Hei Long Jiang, China*
[4]*Department of Respiratory and Critical Care Medicine, The Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China*

Abstract

Introduction: Under normal physiological conditions, insulin exhibits a series of important biological functions and roles. 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 receptor (IR) on lung cancer have not been fully revealed.
Material and 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 activities of insulin are closely related to its cell behaviour. Therefore, we used lung cancer cell lines as a model to explore the cellular behaviour and properties of insulin/IR in the current study, and the results showed that the IR could internalize into lung cancer cells, and it can also transport into the cell nuclei under insulin treatment. Further study showed that nuclear-localized IR could promote the proliferation of lung cancer cells.
Conclusion: Taken together, this study shows that IR's nuclear localization is closely related to cell proliferation. This work lays the foundation for further research on the relationship between insulin and the occurrence and development of lung cancer. **(Endokrynol Pol 2022; 73 (1): 121–130)**

Keywords: *insulin; insulin receptor; lung cancer*

Introduction

It is well-known that insulin has important bioactivities [1]. Almost all organs and tissues express insulin receptor (IR); therefore, the biological functions of insulin are extensive, such as lowering blood sugar, reducing lipolysis and increasing protein synthesis [2]. Insulin's biological effects are mediated by IR, which mainly expresses on cell membrane [2]. Insulin receptor is a tetramer composed of two α subunits and two β subunits linked by disulphide bonds, the α subunit of the IR is entirely extracellular and contains the insulin-binding site. The β subunit is composed of three domains: the extracellular domain, the transmembrane domain, and the intracellular domain. The extracellular domain of the IR's β 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 β-subunit. After IR is activated, a series of downstream signal transduction molecules (such as mitogen-activated protein kinases (MAPKs) and 3-phos-

phoinositide kinase [phosphotidylinositide-3-kinase (PI3K) pathways] are activated [3].

These activated signal molecules are then transported to the nucleus to regulate gene transcription [4].

Under normal physiological conditions, insulin exhibited a series of important biological functions [1]. However, an increasing amount of evidence indicates that insulin is closely related to the occurrence and development of tumours (such as regulating tumorigenicity, proliferation, and metastasis) [5, 6]. Scientists have found that insulin receptors are highly expressed in many tumour types [5]. Studies have shown that insulin receptor is a potential anti-tumour therapeutic target [5].

Recent studies have shown that insulin is also closely related to the occurrence and development of lung cancer [6]. For example, it has been reported that insulin promoted the proliferation and migration of lung cancer. Furthermore, previous studies showed that insulin is associated with lung cancer susceptibility [7, 8]. In addition to increasing cell proliferation, insulin could promote cell survival by inhibition of pro-apoptotic

✉ Qiu Ren, Department of Respiratory and Critical Care Medicine, The First Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang, China; e-mail: rqhospital@163.com

cytokines [5]. The traditional view is that the internalized IR will target lysosomal degradation, but a series of recent studies have shown that internalized IR also plays an important biological role.

However, until now, the cellular properties of insulin/insulin receptors on lung cancer have not been fully revealed. In the current study, we used lung cancer cell lines as a model to explore the cellular behaviour and properties of IR under insulin stimulation. We found that the IR internalized into lung cancer cells and was transported into the cell nuclei under insulin stimulation. Further study showed that 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.

Material and methods
Antibodies and reagent
Human recombinant insulin and IG-1 were obtained from Sigma (St. Louis, MO) p-AKT (cat# 4784, 1:1000 dilution), p-IR (cat#3023, 1:500 dilution), and p-IRS1 (1:1000 dilution) antibodies were purchased from Cell Signalling Technology (CST). IR (cat #23413, 1:300), AKT (cat#9272, 1:500 dilution) and IRS1 (cat #2385, 1:1000 dilution) antibodies were purchased from Cell Signalling Technology (CST). Insulin-like growth factor 1 receptor (IGF-1R) *(cat# ab182408, 1:200 dilution)* and IR *(cat# ab277763)* were purchased from Abcam. The 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). Unless otherwise specified, reagents were purchased from Sigma-Aldrich.
Cell culture
A549 cells (lung carcinoma cell line) were 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 from cell samples was extracted, and the 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 three times, the membranes were then blocked with 3% BSA 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 dilution or 1:2000 dilution) was added and incubated for another 2 h at 37°C. The membranes were rinsed three times with TBST. The membranes were detected using a fluorescence imaging system (Bio-Rad).

Laser confocal scanning microscope observation
A549 cells were seeded onto coverslips at 1×105 per well. When A549 cells grew to approximately 50% confluence, the cells were rinsed twice with sterile PBS. Then the 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 three times with PBS, they were incubated with primary antibodies at 4°C for 12 h, after which, they were rinsed three times with PBS, followed by incubation with secondary antibodies at 37°C for 1 h. Hoechst 33342 was used to stain the cell nuclei. Cell samples were observed with a confocal laser scanning microscope (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. The cells were then collected and 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 then analysed 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 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 0.5 h at RT in the dark. The cell samples were then filtered through a 40-μm nylon mesh and analysed by flow cytometry (Becton Dickinson).

Analysis of apoptosis by flow cytometry
A549 cells were collected. Then the cells were stained with FITC-Annexin V and propidium iodide (PI) using an Annexin V-FITC//PI Apoptosis Detection Kit (BD Biosciences, USA) according to the manufacturer's instruction. After washing, the cell samples were subjected to flow cytometry to detect the apoptotic rate according to the manufacturer's protocols. Calculation of apoptosis rate was performed: lower left quadrant, viable cells (annexin V-FITC and PI negative); lower right quadrant, early apoptotic cells (annexin V-FITC positive and PI negative); upper right quadrant, late apoptosis/necrosis cells (annexin V-FITC and PI positive). Cell apoptosis rate = lower right quadrant + upper right quadrant.

Immunofluorescence
The cells (2×10^4 cells/well) were seeded onto glass coverslips and cultured for 6 h. After washing, the cells were fixed with 4% paraformaldehyde at 37°C for 10 min. After three washes with PBS, the cells were incubated with 1% Triton X-100 for 30 min at RT, after which the cells were blocked with 5% BSA for 2 h at 37°C. The cell samples were then incubated with the primary antibodies at 4°C overnight, following incubation with fluorescently labelled conjugated secondary antibodies for 120 min at 37°C. After washing, cell samples were observed and imaged by using a laser scanning confocal microscope (CLSM).

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 was then centrifuged at 14,000 $\times$ g at 4°C for 0.5 h. The supernatants were then 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 six-well culture plates. Total RNA from cell samples were extracted using an RNeasy Mini kit (Qiagen, Valencia, CA, USA). 1 μg 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'-ATGCGCGGGCAAATTTGATCCCATA-3'; GAPDH forward: 5'-TGGAGTCTACTGGCGTCTT-3', reverse: 5'-TGTCATATTTCTC-GTGGTTCA -3 '.

ELISA

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

Cell transfection

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

Cell migration and invasion assay

1×10^5/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 was 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 were fixed in paraformaldehyde (4%), washed with PBS, and stained with crystal violet. The stained cells were analysed under a light microscope.

The extraction of nuclear/cytoplasmic proteins

Cytoplasmic/nuclear proteins of A549 cells were extracted by using a subcellular protein fractionation kit according to the 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 IR was evaluated by confocal laser scanning microscope (CLSM). As shown in Figure 1A, CLSM analysis showed that IR was mainly expressed in the cell membrane. Furthermore, flow cytometry also showed that A549 cells expressed high levels of IR (Fig. 1B).

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

(knockdown of IGF-1R did not cause significant cell apoptosis) (Fig. 1C).

Internalization of IGF-1 in A549 cells

The internalization of insulin/IGF-1 on A549 cells was checked. The cells were challenged with IGF-1 for different time points. The cell samples were then analysed by CLSM. The results showed that fluorescently labelled insulin was internalized into A549 cells in a time-dependent manner (Fig. 2A). Furthermore, IGF-1's internalization was also evaluated by CLSM (Fig. 2B). In addition, we used insulin antibody (Abcam, cat#181547 at 1/200 dilution) to detect the internalized insulin, and the results showed an identical staining pattern to that of FITC-insulin (see Supplementary Fig. 1).

IR nuclear transport is induced by insulin but not by IGF-1

Here, we studied the intracellular trafficking of IR under insulin stimulation, and the results indicated that IR could internalize into cell cytoplasm in a time-dependent manner. In addition, IR also transported into cell nuclei (Fig. 3A). We also explored the trafficking of IR under IGF-1 treatment, and the results showed that IR could internalize into cell cytoplasm, but IR could not transport into cell nuclei (Fig. 3B). 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. In addition, we also used another non-small cell lung cancer cell line (HCC827) to observe whether IR can also transport into the cell nuclei, and the results indicated that IR transported into the nuclei of CC827 cells (see Supplementary Fig. 2).

Clathrin and caveolin are involved in the endocytosis of IR

The above study showed that insulin/insulin receptor could internalize into cell cytoplasm. Here, the endocytic mechanism of IR was explored. The study showed that the endocytic pathway of the same cytokine/receptor is different in different types of cells. In lung cancer cells, 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 also existed. For this, co-localization analysis was conducted to explore 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
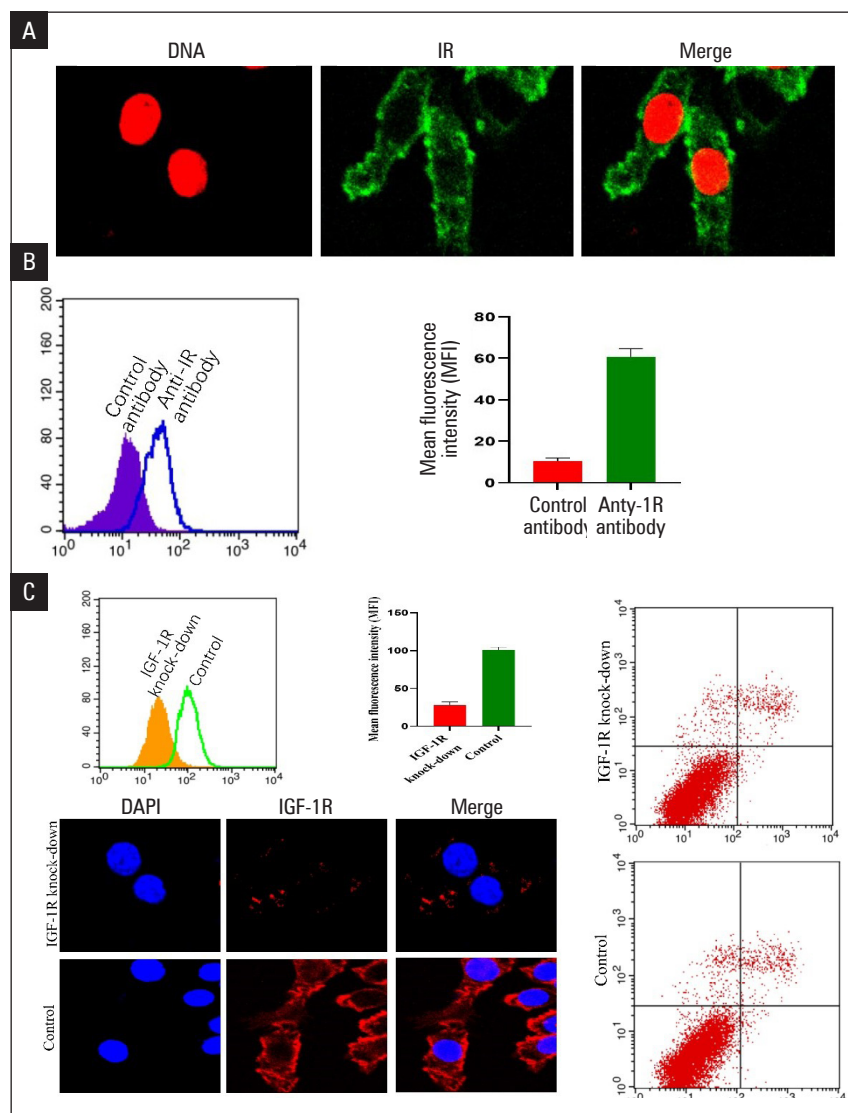
Figure 1. *Detection of insulin receptor (IR) expression by confocal laser scanning microscope (CLSM) (**A**) and flow cytometry (**B**). Insulin-like growth factor 1 receptor (IGF-1R) was knocked down through the use of siRNA technology. Data are expressed as mean ± standard deviation. p < 0.05 was considered to indicate a statistically significant difference (**C**)*

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

In addition, we investigated whether ERK is involved in the internalization of IR; therefore, SHP2 inhibitor and MEK inhibitor were used to treat A549 cells, and the results showed that SHP inhibitor or ERK inhibitor did not significantly affect the internalization of IR (see Supplementary Fig. 3).

The internalized IR localized in different types of endosomes
We further analysed 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 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

are rich in the late endosomes. Colocalization analyses have shown that IR is localized in Rab5/7/11-positive endosome, which provides an explanation for different cytoplasmic localization of IR.

Tyrosine phosphorylation of IR is required for nuclear localization of IR
We tested whether the IR's tyrosine phosphorylation is required for its nuclear localization. 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 significantly inhibited, but its internalization was not affected (Fig. 6). This finding suggests that IR phosphorylation is required for IR's nuclear localization.

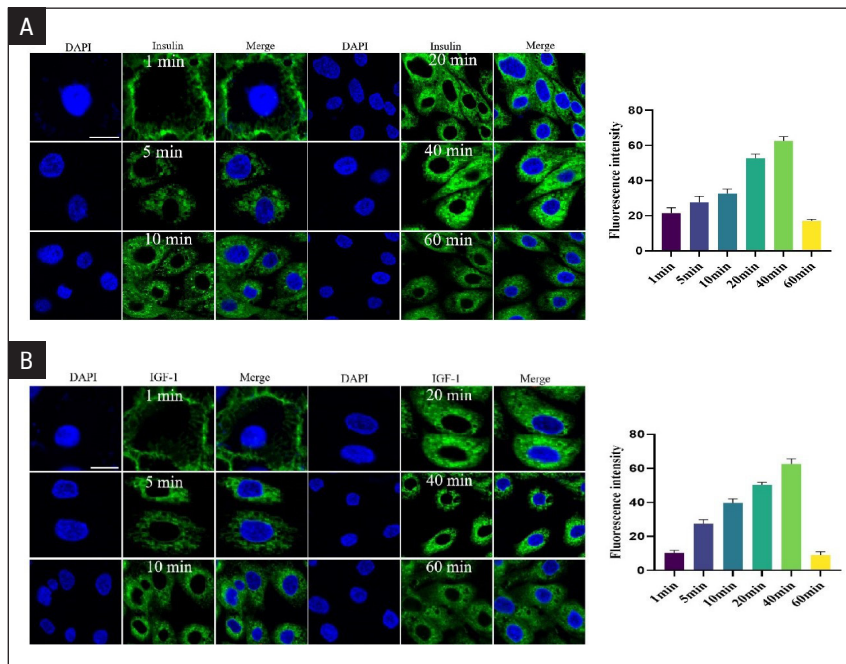Additionally, we explored whether ERK is involved in the nuclear localization of IR; hence, SHP2 inhibitor

Figure 2. *A. The internalization dynamics of insulin on A549 cells; B. The internalization dynamics of insulin-like growth factor 1 receptor (IGF-1R) on A549 cells. Data are expressed as the mean ± standard deviation. p < 0.05 was considered to indicate a statistically significant difference*
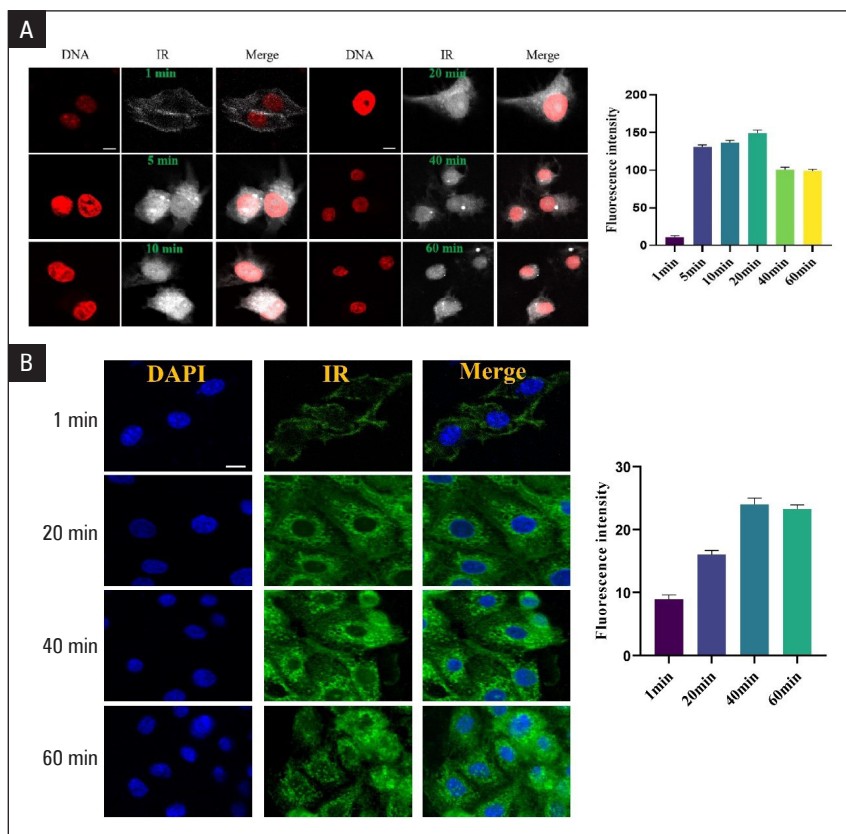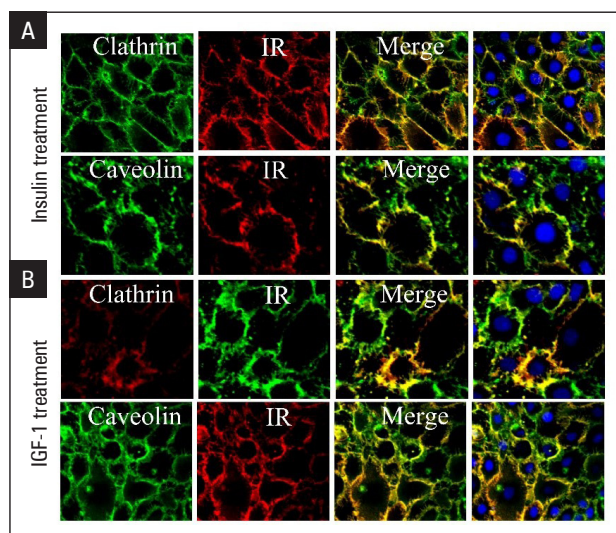

Figure 3. *A. The internalization dynamics of insulin receptor (IR) under insulin stimulation; B. The internalization dynamics under insulin-like growth factor 1 receptor (IGF-1R) treatment. Data are expressed as the mean ± standard deviation. p < 0.05 was considered to indicate a statistically significant difference*

Figure 4. *A. Both clathrin and caveolin were involved in the insulin receptors (IR's) endocytosis under insulin stimulation; **B.** Both clathrin and caveolin were involved in the IR's endocytosis under insulin-like growth factor 1 receptor (IGF-1R) treatment. p < 0.05 was considered to indicate a statistically significant difference*

and MEK inhibitor were used to treat A549 cells, and the results indicated that SHP inhibitor or ERK inhibitor did not significantly affect the nuclear localization of IR (see Supplementary Figure 4).

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 [9]. 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 analysed 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 from 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). Nup358 knock-down does not affect the proliferation of lung cancer cells and does not lead to the apoptosis of lung cancer cells (see Supplementary File — Fig. 5).

IR nuclear localization is associated with cell proliferation of A549

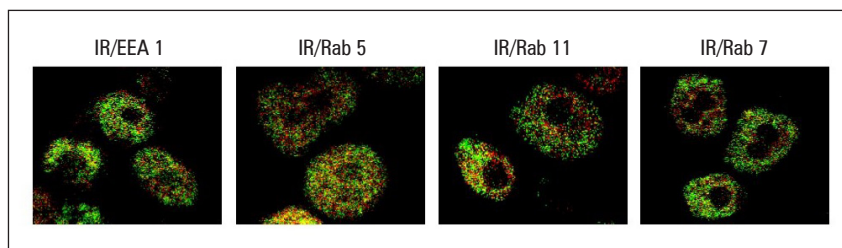To explore the functions or roles of nuclear-localized IGF-1R, a model of the non-nuclear-localized IR was

Figure 5. *The insulin receptor (IR) transported into different types of endosomes under insulin-like growth factor 1 receptor (IGF-1R) treatment. p < 0.05 was considered to indicate a statistically significant difference*
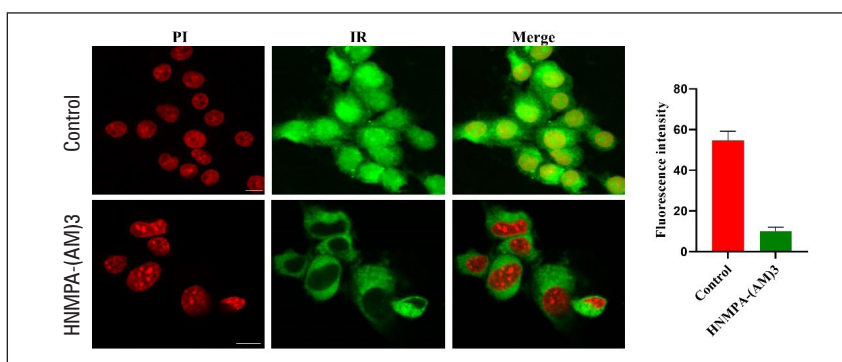

Figure 6. *Tyrosine phosphorylation of insulin receptor (IR) is required for IR's nuclear localization. The experimental process has been described in detail in the materials and methods section. p < 0.05 was considered to indicate a statistically significant difference*
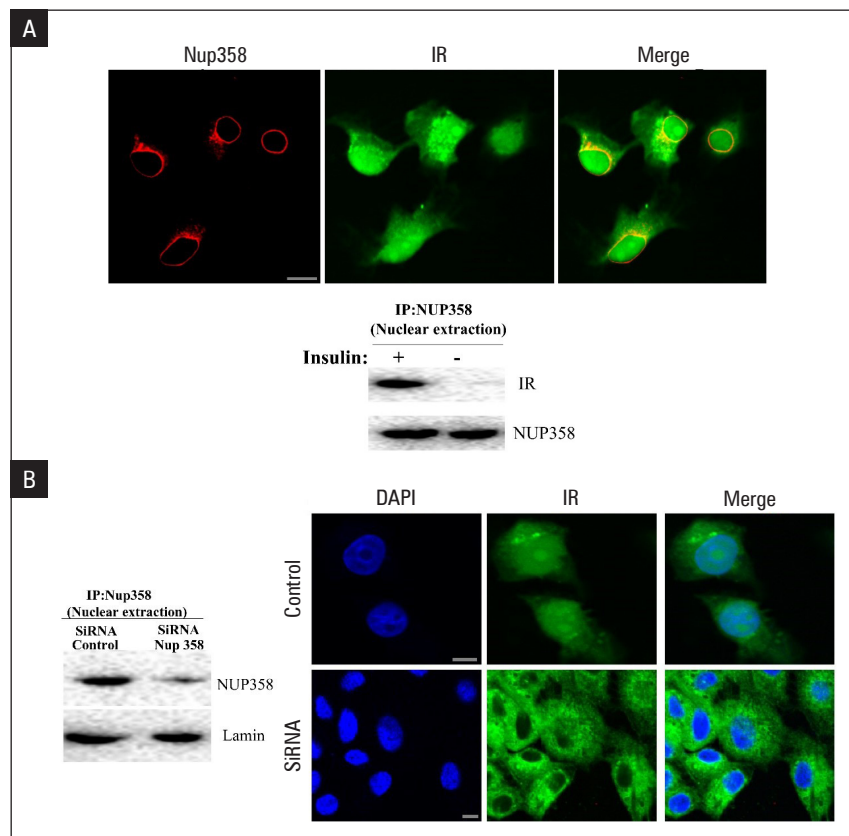
Figure 7. A. *Detection of colocalization between insulin receptor (IR) and Nup358;* **B.** *Analysis of interactions between IR and Uup358 by IP-WB.* **C.** *Nup358 knock-down inhibited nuclear localization of IR. The experimental process has been described in detail in the materials and methods section. p < 0.05 was considered to indicate a statistically significant difference*

established by knocking down Nup358 (as shown in Fig. 7). MTT experiments were used to evaluate the functions of nuclear-localized IR. As shown in Figure 8A, the A549 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 (leptomycin 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 analyse the effect of nuclear-localized IR on A549 cell proliferation, the cell cycle was determined by flow cytometry, and the results showed that the proportion of cells in the S phase was significantly increased compared to the control (Fig. 8C). In addition, Ki67 expression was also enhanced compared to control (Fig. 8D).

Nuclear-localized IR increased the nuclear retention of signalling molecule

We further explored the potential mechanism by which the nuclear-localized IR promotes cell proliferation. Because the biological activity of insulin/IR is achieved by IR-mediated signalling, we explored the action mechanism of the of nuclear-localized insulin/IR from the perspective of IR-mediated signalling. As indicated in Figure 9A, the results showed that the activation of

p-ERK1/2 was significantly prolonged and increased compared to the non-nuclear-localized IR group, suggesting that nuclear-localized IR still has the ability to activate signalling molecules. However, other insulin-induced signalling pathways were not affected (Fig. 9B). This may be one of the potential functions of nuclear-localized IR.

Discussion

Many studies have shown that insulin is closely related to the occurrence and development of lung cancer [8]. IR is often overexpressed in malignant cells, and a high level of IR expression is indicative of poor survival in non-small cell lung cancer [7]. In the current study, lung cancer cells were used as a model to study the cellular behaviour 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, A549 cells were used as a model to explore the cell characteristics and behaviour of insu-
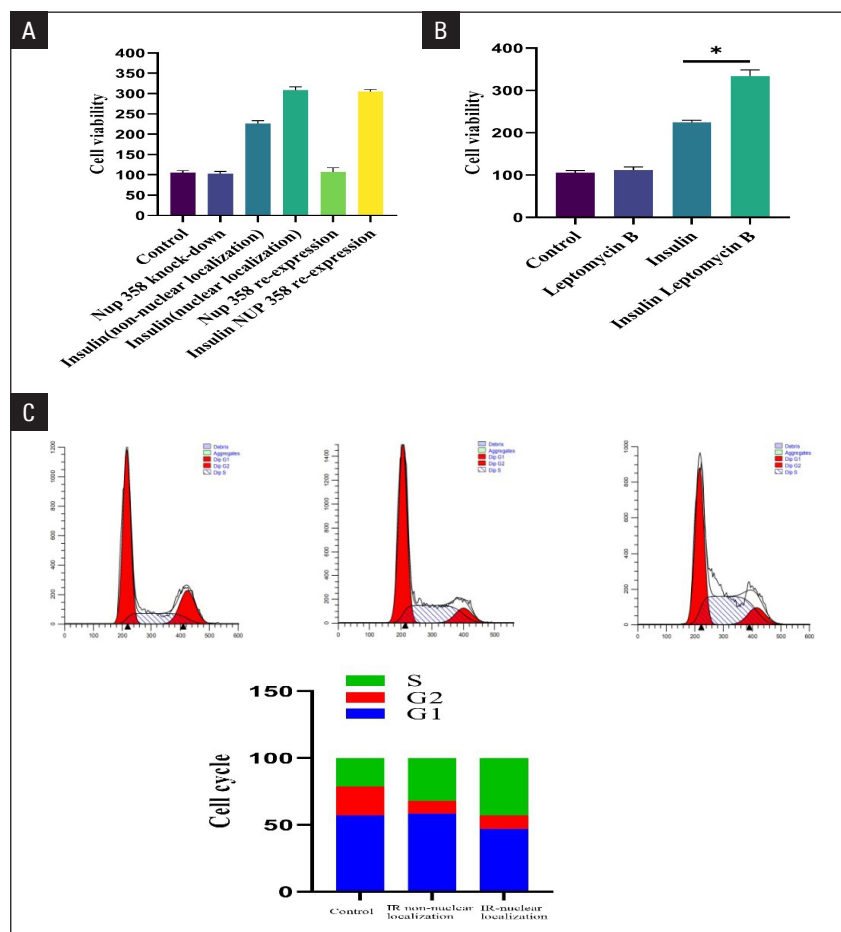
Figure 8. *A. Nuclear-localized insulin receptor (IR) is closely related to the proliferation of A549 cells; **B.** The proliferation ability of cells was also enhanced by increasing the IR's residence time in the cell nuclei; **C.** The nuclear localization of IR was associated with cell cycle; **D.** Ki67 expression was enhanced. Data are expressed as the mean ± standard deviation. p < 0.05 was considered to indicate a statistically significant difference*

lin/IGF-1. Studies have shown that insulin can bind to IR and IGF-1R. IGF-1R is a tyrosine kinase (TK) receptor, which shares 60-70% homology with the IR [10–13]. IGFs (IGF-I and IGF-II) have a variety of bioactivities, such as cell growth, cell differentiation, and cell survival. On the other hand, insulin's structure is similar to that of IGF-1, which mainly mediates anabolic biological functions, such as regulation of glucose and amino acid transport [14]. In general, IGFs have long-term action to regulate the cell fates, whereas insulin mainly regulates metabolic activity. Because A549 cells expressed both IGF-1R and IR, in order to prevent the influence of IGF-1R, we knocked down the expression of IGF-1R by the SiRNA method.

IR's endocytosis is the first step in the process of IR's nuclear localization. Previous studies have shown that the endocytic pathway of cytokine is mainly mediated by clathrin-dependent endocytosis or caveolin-mediated endocytosis [15, 16]. Additionally, the non-clathrin- and caveolin-dependent pathways also exist. In the current study, we found that clathrin-me-

diated 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. Because 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 and Rab4) [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 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 that should be solved in the future.

In addition to the cytoplasmic transport process, how IR crosses the nuclear membrane is also an important scientific issue. We found that Nup358 plays an important role in the nuclear transport of IR. Simi-
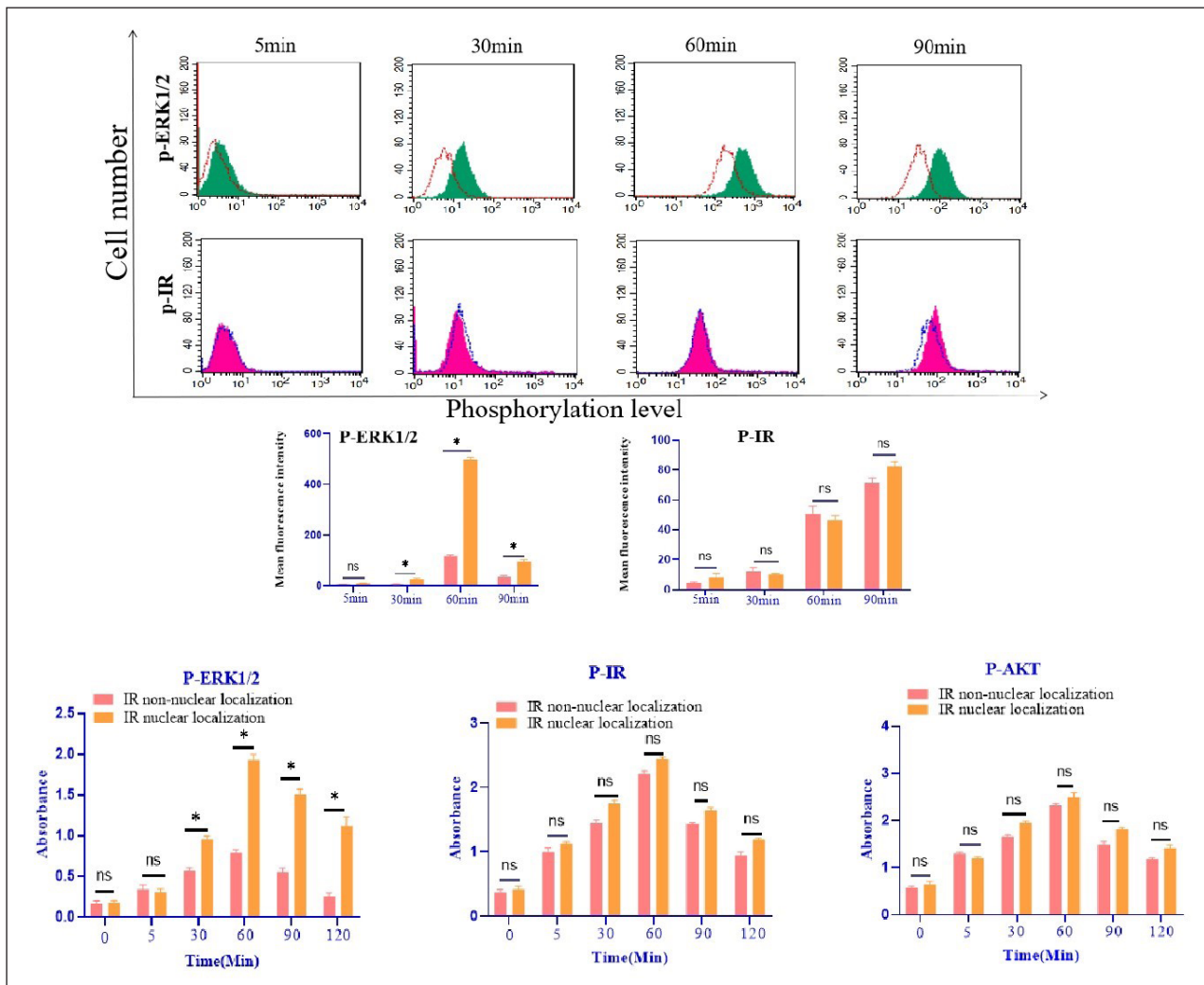
Figure 9. *The nuclear localization of insulin receptor (IR) is closely related to the activation of ERK1/2. The experiment process has been described in detail in the materials and methods section. Other insulin-induced signalling pathways were not affected. Data are expressed as the mean ± standard deviation. p < 0.05 was considered to indicate a statistically significant difference*

larly, previous studies have shown that IGF-1R entered the cell nuclei under Nup358 mediation.

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

Conclusions

Taken together, the current work showed that the nuclear-localized IR has important biological functions. In the

current study, we analysed the biological characteristics of IR in a cell model of lung cancer and found that IR could not only internalize into the cytoplasm; more importantly, we also found that IR transported into cell nuclei of A549 cells 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 the relationship between insulin and the occurrence and development of lung cancer.

References

1. Hosawi SB, Humphries JD, Coward RJ, et al. Global proteomic analysis of insulin receptor interactors in glomerular podocytes. Wellcome Open Res. 2020; 5: 202, doi: 10.12688/wellcomeopenres.16072.1, indexed in Pubmed: 33458251.
2. Lee J, Pilch PF. The insulin receptor: structure, function, and signaling. Am J Physiol. 1994; 266(2 Pt 1): C319–C334, doi: 10.1152/ajpcell.1994.266.2.C319, indexed in Pubmed: 8141246.
3. Hall C, Yu H, Choi E. Insulin receptor endocytosis in the pathophysiology of insulin resistance. Exp Mol Med. 2020; 52(6): 911–920, doi: 10.1038/s12276-020-0456-3, indexed in Pubmed: 32576931.

4. Hancock ML, Meyer RC, Mistry M, et al. Insulin Receptor Associates with Promoters Genome-wide and Regulates Gene Expression. Cell. 2019; 177(3): 722–736.e22, doi: 10.1016/j.cell.2019.02.030, indexed in Pubmed: 30955890.

5. Frisch CM, Zimmermann K, Zilleßen P, et al. Non-small cell lung cancer cell survival crucially depends on functional insulin receptors. Endocr Relat Cancer. 2015; 22(4): 609–621, doi: 10.1530/ERC-14-0581, indexed in Pubmed: 26113601.

6. Argirion I, Weinstein SJ, Männistö S, et al. Serum Insulin, Glucose, Indices of Insulin Resistance, and Risk of Lung Cancer. Cancer Epidemiol Biomarkers Prev. 2017; 26(10): 1519–1524, doi: 10.1158/1055-9965. EPI-17-0293, indexed in Pubmed: 28698186.

7. Ho GYF, Zheng SL, Cushman M, et al. Associations of Insulin and IGFBP-3 with Lung Cancer Susceptibility in Current Smokers. J Natl Cancer Inst. 2016; 108(7), doi: 10.1093/jnci/djw012, indexed in Pubmed: 27071409.

8. Vigneri R, Goldfine ID, Frittitta L. Insulin, insulin receptors, and cancer. J Endocrinol Invest. 2016; 39(12): 1365–1376, doi: 10.1007/s40618-016-0508-7, indexed in Pubmed: 27368923.

9. Packham S, Warsito D, Lin Y, et al. Nuclear translocation of IGF-1R via p150(Glued) and an importin-β/RanBP2-dependent pathway in cancer cells. Oncogene. 2015; 34(17): 2227–2238, doi: 10.1038/onc.2014.165, indexed in Pubmed: 24909165.

10. Lawrence MC, McKern NM, Ward CW. Insulin receptor structure and its implications for the IGF-1 receptor. Curr Opin Struct Biol. 2007; 17(6): 699–705, doi: 10.1016/j.sbi.2007.07.007, indexed in Pubmed: 17851071.

11. Buck E, Gokhale PC, Koujak S, et al. Compensatory insulin receptor (IR) activation on inhibition of insulin-like growth factor-1 receptor (IGF-1R): rationale for cotargeting IGF-1R and IR in cancer. Mol Cancer Ther.

2010; 9(10): 2652–2664, doi: 10.1158/1535-7163.MCT-10-0318, indexed in Pubmed: 20924128.

12. Ulanet DB, Ludwig DL, Kahn CR, et al. Insulin receptor functionally enhances multistage tumor progression and conveys intrinsic resistance to IGF-1R targeted therapy. Proc Natl Acad Sci U S A. 2010; 107(24): 10791–10798, doi: 10.1073/pnas.0914076107, indexed in Pubmed: 20457905.

13. Lawrence MC, McKern NM, Ward CW. Insulin receptor structure and its implications for the IGF-1 receptor. Curr Opin Struct Biol. 2007; 17(6): 699–705, doi: 10.1016/j.sbi.2007.07.007, indexed in Pubmed: 17851071.

14. Vigneri PG, Tirrò E, Pennisi MS, et al. The Insulin/IGF System in Colorectal Cancer Development and Resistance to Therapy. Front Oncol. 2015; 5: 230, doi: 10.3389/fonc.2015.00230, indexed in Pubmed: 26528439.

15. Veiga E, Cossart P. The role of clathrin-dependent endocytosis in bacterial internalization. Trends Cell Biol. 2006; 16(10): 499–504, doi: 10.1016/j. tcb.2006.08.005, indexed in Pubmed: 16962776.

16. Sui ZH, Xu H, Wang H, et al. Intracellular Trafficking Pathways of : From Clathrin- and Caveolin-Mediated Endocytosis to Endosome and Lysosome. Front Cell Infect Microbiol. 2017; 7: 400, doi: 10.3389/fcimb.2017.00400, indexed in Pubmed: 28932708.

17. Vonderheit A, Helenius A. Rab7 associates with early endosomes to mediate sorting and transport of Semliki forest virus to late endosomes. PLoS Biol. 2005; 3(7): e233, doi: 10.1371/journal.pbio.0030233, indexed in Pubmed: 15954801.

18. McCaffrey M, Bielli A, Cantalupo G, et al. Rab4 affects both recycling and degradative endosomal trafficking. FEBS Letters. 2001; 495(1-2): 21–30, doi: 10.1016/s0014-5793(01)02359-6, indexed in Pubmed: 11322941.