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Hypoxia stimulates invasion and migration of human cervical cancer cell lines HeLa/SiHa through the Rab11 trafficking of integrin αvβ3/FAK/PI3K pathway-mediated Rac1 activation

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Hypoxia plays a key role in tumour cell survival, invasion, and metastasis. An increasing number of studies have attempted to characterize the tumour response to hypoxia and to identify predictive markers of disease. Here we show that hypoxia increases tumour cell invasion and migration by the modulation of Rab11, an important molecule for vesicular trafficking. In our study, we found that Rab11, together with the activation of Rac1, could stimulate invasion and migration of cervical cancer cell lines HeLa/SiHa in hypoxia. Activation of Rac1 activity by hypoxia seems to be central to carcinoma invasion. We also found that these effects could be related to the integrin αvβ3. In addition, we studied the molecular pathway for this process. Our results showed that in cervical cancer cell lines HeLa/SiHa, Rac1 activation in hypoxia could stimulate invasion and migration, and this process was mediated by integrin αvβ3-mediated FAK and PI3K phosphorylation. Furthermore, hypoxia induced a dramatic increase in αvβ3 integrin surface expression, and this increase is dependent on Rab11. In conclusion, our study might provide a new mechanism for the effect of hypoxia on stimulating cervical carcinoma invasion.

Keywords. Cervical cancer; hypoxia; Rab11; Rac1; αvβ3/FAK/PI3K

1. Introduction

Cervical cancer is a common gynecological cancer, and in recent years the frequency has increased, particularly in younger individuals. It is the second cause for cancer incidence for women (Li 2011), and the main causes of deaths are distant metastasis. In recent years, an increasing number of researchers began to the dig out the important roles of tumour microenvironment in cell invasion and migration, but the mechanisms underlying cervical cancer cell lines invasion and migration are not yet clearly understood. Furthermore, and perhaps more importantly, tumour cells must survive in environmental conditions not present in normal tissue (Brown 1999). One of the most formidable barriers to their survival is hypoxia (Yoon *et al.* 2005). The oxygen tension within many solid tumours is dramatically reduced compared with that in adjacent normal tissue, presumably due to poor vascularization (Brown 1999). Hypoxia provides a strong selective pressure for the survival of the most aggressive and metastatic cells, and they utilize aerobic glycolysis regardless of the oxygen levels, known as the Warburg effect (Chan *et al.* 2012). As some studies demonstrated that hypoxia in primary tumours has been linked to poor prognosis and outcomes, and could promote cancer cell invasion (Bae *et al.* 2015).

In this study, we tested the assumption that hypoxia stimulates the trafficking of specific proteins that are involved in the invasive process. Among them Rab GTPases serve as master regulators in intracellular membrane traffic (Hutagalung and Novick 2011), and Rab11 plays a key role in this progression, as it also has a close relationship with the membrane transport of some proteins that are critical for invasion and migration (Deneka and van der Sluijs 2002; Maxfield and Mcgraw 2004). As a study substantiated recently, Rab11-FIP2 can regulate MMP7 expression in colorectal cancer, which is related to cell invasion and migration, through activating PI3K/Akt signalling pathway (Xu *et al.* 2016). Emery and Ramel (2013) demonstrated that Rab11 promotes cell migration through the control of Rac1 activity. Additionally, Angptl3 could lead to Rac1 activation and increase the motility of podocytes, which is induced through integrin αvβ3 and is mediated by FAK and PI3K phosphorylation (Lin *et al.* 2013). Moreover, Yoon *et al.* (2005) substantiated that hypoxia induced a significant increase in α6β4 surface expression, which could promote carcinoma invasion, and this increase is dependent on Rab11. Hence, we focused our efforts on the hypothesis that hypoxia stimulates invasion and migration of human cervical cancer cell lines through the Rab11 trafficking of integrin αvβ3/FAK/PI3K pathway-mediated Rac1 activation.

2. Materials and methods

2.1 *Cells and reagents*

Human cervical cancer cell lines HeLa/SiHa were purchased from Shanghai Institute of museum collections; Silentfect and Trizol were purchased from Invitrogen, CA, USA; Fetal bovine serum (FBS) from Sijiqing Biotechnology, Hangzhou, China; RPMI-1640 and DMEM media from Hyclone, UT, USA; BCA protein assay kit and SDS-PAGE Gel Kit from Beyotime Biotechnology, Shanghai, China; Rab11 and an actin antibody from Santa Cruz Biotechnology, CA, USA; Rac1 antibody from Bioss Biological, Beijing, China; αvβ3 from ChuanBo Biological Technology, Nanjing, China; FAK antibody, p-FAK antibody, PI3K antibody and p-PI3K antibody rom CST, BSN, USA; Western blot rabbit antimouse antibody from Bioworld, MN, USA; immunofluorescence rabbit antimouse antibody from Vicmed Biological Technology, Xuzhou, China; Matrigel glue from BD, NY, USA; and quantitative real-time PCR assay kit from Invitrogen, CA, USA.

2.2 *Transfections*

Small interfering RNA that targeting Rab11 and scrambled nonspecific sequence, which served as negative controls were all designed, synthesized and purified by GenePharma (Shanghai, China). Cells were transfected with Silentfect according to the protocol of the manufacturer. Sequence of Rab11siRNA: 5′-GAG CAC CAU UGG AGU AGA GTT-3′. Sequence of negative control: 5′-UUC UCC GAA CGU GUC ACG UTT-3′.

2.3 *Hypoxia experiments*

For experiments that involved hypoxic culture, HeLa cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS), and SiHa cells were grown in 1640 supplemented with 10% heat-inactivated fetal bovine serum; cells were incubated in a hypoxic chamber maintained at 3% O2 for 48 h.

2.4 *Transwell migration assay*

The migration capacities of HeLa and SiHa cells were determined using transwell chambers (8 pm pore size, Corning, NY, USA). 5×10^4 cells were plated into the chamber with 200 mL serum-free medium per well. The outer chambers were filled with 600 mL medium contain-ing 10% FBS. After 12 h, the top surface of the membrane was scrubbed gently with a cotton swab, whereas cells migrated to the undersurface were then fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet prior to undergoing a series of washes. The cells passing to the undersurface of each filter were then photographed and counted (200× magnifications), and the mean number of migrated cells was determined by count-ing five random fields of each well.

2.5 *Matrigel invasion assay*

For invasion assays, each chamber was coated freshly with 50 μg Matrigel (BD Bioscience, NY, USA) before starting the assay, then cells were seeded into the upper trasnwell chamber. Briefly, the bottom chambers were filled with RPMI-1640 and DMEM media with 10% FBS and the top chambers were seeded with 5×10^4 cells that trans-fected with negative control or Rab11 siRNA per well in RPMI-1640 and DMEM media. After incubation for 24 h, the noninvasive cells that remaining on the upper chambers were removed by cotton swabs, and the invaded cells on the lower surface of the filter were fixed and stained followed by microscopic examination. The number of cells in five random optical fields was averaged.

2.6 *Western blotting*

For Western blotting, the total proteins of cancer cells were extracted by lysis buffer containing phosphatase and prote-ase

inhibitors, then centrifuged at 4°C at 12,000*g* for 5 min. The protein concentration was determined by the enhanced bicinchoninic acid (BCA) Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Equal amounts of protein were loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, Shanghai, China) for electrophoresis and then transferred to nitrocellu-lose filter membranes (Millipore, MA, USA). The membrane was blocked in a solution of washing buffer containing 5% non-fat milk for 2 h, and then incubated with the following diluted primary antibodies overnight at 4°C (MMP-2, MMP-9, Rab11, Rac1 1:500 dilution; αvβ3, FAK, p-FAK, PI3K, p-PI3K 1:1000 dilution; and β-actin 1:10,000 dilution). After washed in washing buffer 3 times for 15 min, membrane was incubated for 2 h with secondary antibodies at a 1:10,000 dilution at room temperature and then washed again in washing buffer containing Tween-20. At last, the membrane was visualized using an enhanced chemiluminescence (ECL) reagent (Tanon, Shanghai, China) and analysed by Image J software. The β-actin was used as an internal reference.

2.7 *Quantitative real-time PCR*

Quantitative real-time PCR was performed using ROX's Platinum SYBR Green qPCR per Mix-UDG kit (Invitrogen) on an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems, Foster city, CA). The reactions were performed in a 25 μL volume of a mixture containing 1 μL cDNA template, 12.5 μL SYBR Green qPCR SuperMix-UDG, 0.5 μL of each primer, and 10.5 μL of nuclease-free water. The thermocycling program was as follows: 50°C for 2 min, 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. At the end of each PCR run, a melting curve analysis from 65 to 95°C was applied to all reactions to ensure the specificity of the amplified product. Standard curves were created based on a fivefold dilution series of cDNA (1:5, 1:25, 1:125, 1:625, 1:3125, and 1:15625). The corresponding qRT-PCR efficiencies (*E*) were calculated according to the equation: $E = (10^{[-1/\text{slope}]} - 1 \times 100)$. Each sample was prepared as three biological replicates, and each reaction was analyzed with three technical replications.

2.8 *Immunofluorescence*

Cells were plated at low density and incubated overnight at atmosphere with 5% CO2 at 37°C in either normoxia or hypoxia. Firstly, medium was removed, followed with 3 washes of 1xPBS. Subsequently, the cells were fixed immediately with 4% paraformaldehyde for 20 min. Then washed monolayer 3 times with PBS, permeabilize cells with 0.1% Triton X-100 made in PBS solution for 10 min. Then washed monolayer 3 times with PBS, block with 2% BSA for 45 min, and removed the cover glass and add 100 μL of the diluted primary antibody to each well, and incubated at 4°C overnight. Then washed monolayer 3 times with PBS, and added 100 μL of the diluted secondary antibody to each well, and incubated at 37°C for 1 h. Then wash monolayer 3 times with PBS, and added 50 μL DEPI to each well for 5 min at RT. Finally, cells were covered with a Coplin jar with aluminum foil to protect the slides from the light and examined using an immunofluorescence microscope.

2.9 *Statistical analysis*

All experiments were carried out at least three times. Data were expressed as mean ± standard deviation (SD). Statistical analyses were performed by independent samples t-test and one-way ANOVA using the software SPSS version 16.0 (SPSS Inc, IL, USA). $P < 0.05$ was used to determine the statistical significance when interpreting the results ($^*P < 0.05$).

3. Results

3.1 *Hypoxia stimulates migration and invasion*

Metastasis is the principal cause of cancer death, and cell migration and invasion are the hallmarks of cancer metastasis (Shi *et al.* 2016). It is acknowledged the extracellular matrix degradation induced by MMPs is required for cell invasion. The migration and invasion capacities of cervical cancer cell lines were confirmed by Transwell assay. And the expressions of MMP-2, MMP-9, migration and invasion related proteins, were tested by Western blot analysis. The results showed that approximately 48 h after cells cultured under hypoxia and normoxia, the number of cells penetrated through chambers in hypoxia group was remarkably increased compared with that in normoxia both in migration and Matrigel invasion assay (figure 1A and B). Meanwhile, Western blot assay also showed that the expressions of MMP-2, MMP-9 proteins dramatically increased in hypoxia group compared with that in normoxia (figure 1C and D).

3.2 *Rab11 is involved in hypoxia-stimulated invasion and migration*

Tumour cells have the ability to survive autonomously in stressful microenvironments such as hypoxia, and it is known as the Warburg effect (Chan *et al.* 2012). To assess the hypothesis that Rab11 is involved in hypoxia-stimulated invasion and migration, cells were transfected with either a

Rab11-siRNA or a negative control-siRNA. Results showed that the number of cells transfected with Rab11-siRNA was striking decreased compared with cells without transfection. However, cells that transfected with negative control-siRNA showed no difference in invasion and migration (figure 2A and B). Meanwhile, the expression of Rab11 and Rab11 mRNA, were significantly increased when cells cultured in hypoxia for 48 h (figure 2E, G and I). And the expression was decreased when cells transfected with Rab11-siRNA in hypoxia for 48 h (figure 2F, H and J), so were the MMP-2 and MMP-9 (figure 2C and D). The results were consistent with the research studied by Jayachandran *et al.* (2007) in lung cancer. These data argue for the involvement of Rab11 in invasion and migration, an involvement that is amplified in hypoxia.

3.3 *Hypoxia stimulates invasion and migration by a mechanism that involves activation of αvβ3/FAK/PI3K through a Rab11-dependent pathway*

The preceding data supported that Rab11 was involved in hypoxia-stimulated invasion; however, the potential mechanism was not demonstrated. The key question was the identification of surface proteins of which expression was regulated by Rab11 and that function in invasion. Finally, we concentrated on the αvβ3 integrin as potential targets of Rab11 trafficking for several reasons. On one hand, as reported recently, the pharmacologic concentration of melatonin significantly inhibited the migration and invasion of human U251 glioma cells under hypoxia, which accompanied with decreased expression of αvβ3 integrin (Xu *et al.* 2015). On the other, another study demonstrated that Rab-coupling protein promotes cell migration in ovarian cancer through the stimulation of αvβ3 integrin (Caswell *et al.* 2008). Besides, to explore the mechanism underlying the promotion of Rab11 on cell migration and invasion in hypoxia, changes in FAK and PI3K activities were studied due to their important role in the formation of focal adhesions, which is a key process in cell migration and invasion. Activation of FAK and PI3K is indicated by the increase in their phosphorylation (Avraham *et al.* 2000). Therefore, the expression of αvβ3 integrin, and the phosphorylation state of FAK and PI3K, was tested by Western blot analysis. We then evaluated the relative phosphorylation level of FAK and PI3K to the total protein level respectively. Results showed

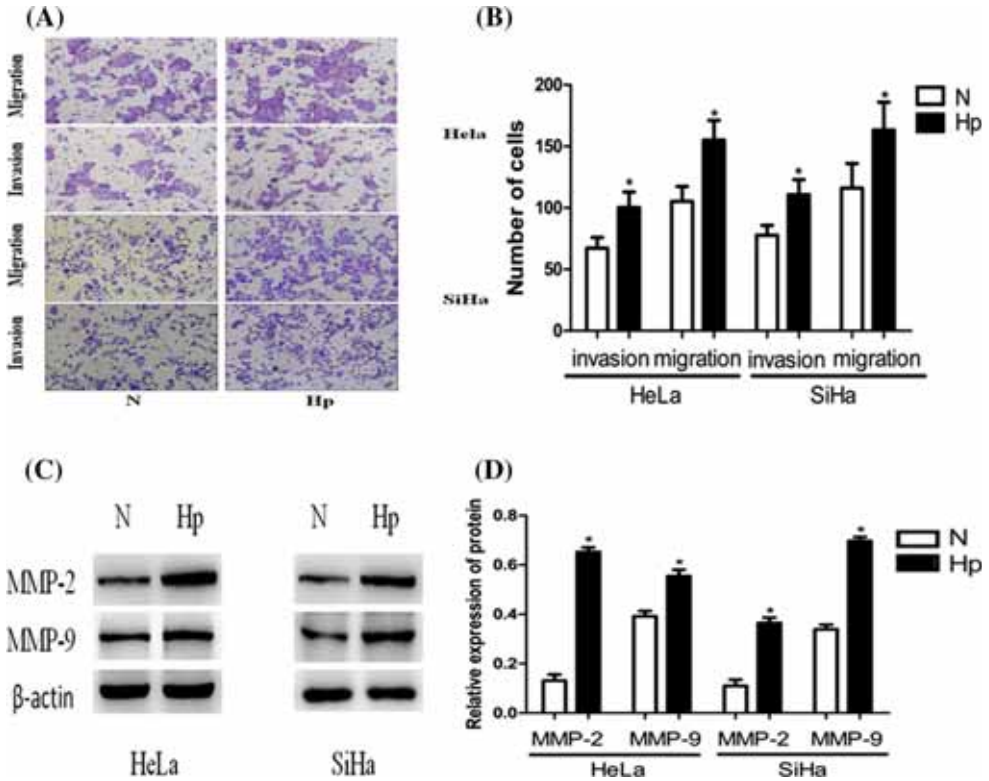

Figure 1. (**A**) Transwell migration assay and Matrigel invasion assay showed that the migration and invasion capacity of cells cultured in hypoxia were enhanced. (**B**) Number of cells in hypoxia and normoxia were evaluated and the number of cells in hypoxia was increased. (**C**) The expressions of MMP-2, MMP-9 proteins were measured by Western blotting. (**D**) The levels of MMP-2, MMP-9 in hypoxia were increased, while that in normoxia were significantly decreased. Results were expressed as Mean ± SD of three independent experiments (*$P < 0.05$)

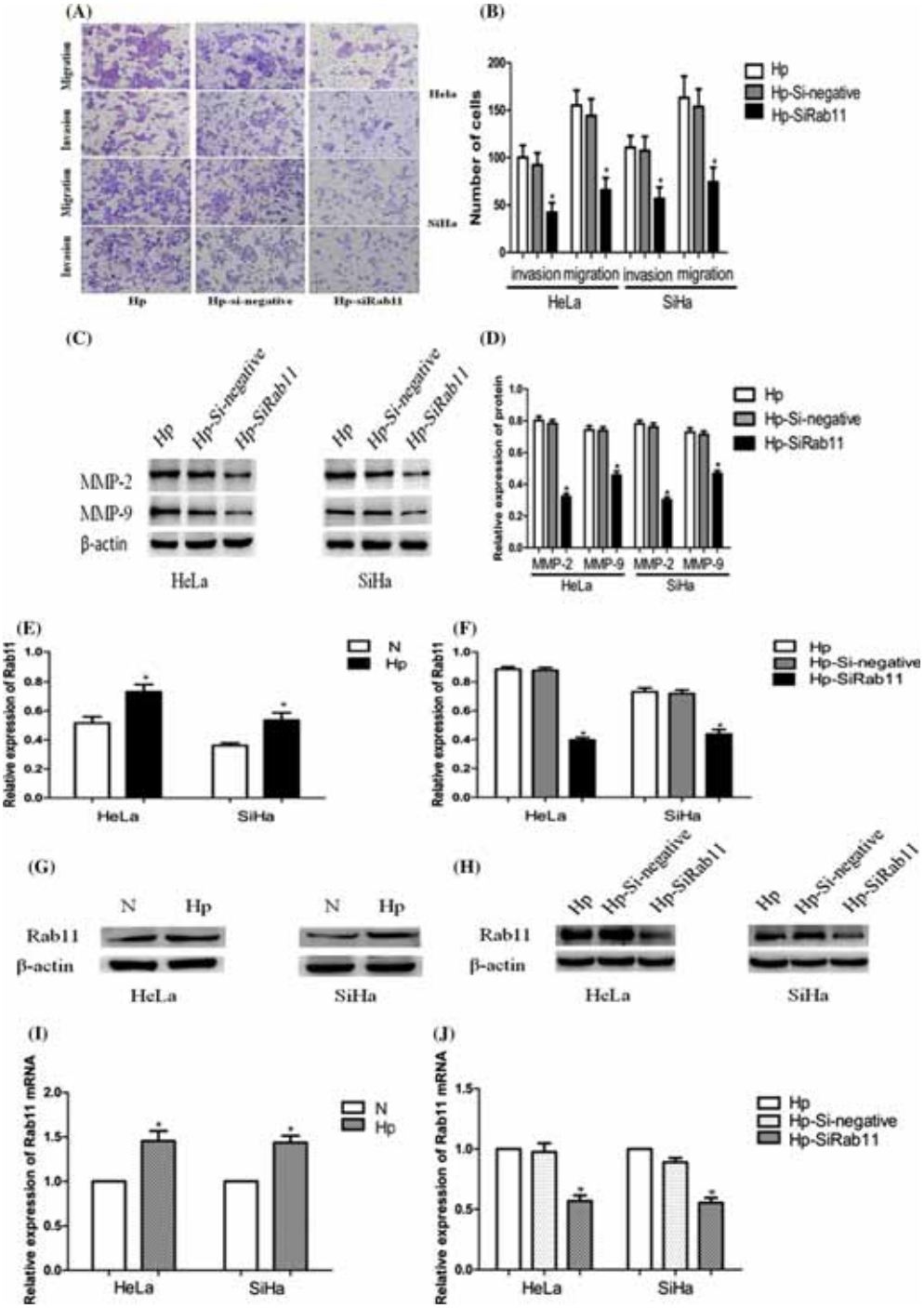

Figure 2. (**A**) Transwell migration assay and Matrigel invasion assay showed that the migration and invasion capacity of cells transfected with Rab11-siRNA in hypoxia for 48 h was dramatically diminished. (**B**) Number of cells in three groups were evaluated and the number of cells transfected with Rab11-siRNA was decreased. (**C**) The expressions of MMP-2, MMP-9 (**G**–**H**) and Rab11 proteins were measured by Western blotting. The relative expression of Rab11 of HeLa cell of Hp, Hp-si-negative and Hp-siRab11 are 0.881 ± 0.017, 0.874 ± 0.019, and 0.397 ± 0.018. The relative expression of Rab11 of SiHa cell of Hp, Hp-si-negative and Hp-siRab11 are 0.731 ± 0.027, 0.719 ± 0.025, and 0.438 ± 0.032. (**D**) The levels of MMP-2, MMP-9 and (**E**–**F**) Rab11 was increased in hypoxia, while that in cells transfected with Rab11-siRNA were significantly decreased. (**I**–**J**) The expression of Rab11 mRNA was measured by real-time-qPCR. It was increased in hypoxia, while that in cells transfected with Rab11-siRNA were striking decreased. Results were expressed as Mean ± SD of three independent experiments (*$P < 0.05$)

that αvβ3 integrin, phosphorylation and activation of FAK and PI3K were stimulated by hypoxia conditions (figure 3A–C). Compared with cells without transfection and those transfected with negative control-siRNA, a significant decrease of αvβ3 integrin was observed under hypoxia in cells transfected with Rab11-siRNA, and so were the phosphorylation level of FAK and PI3K (figure 3D–F). These data suggested that Rab11 may promote cervical cancer cell migration and invasion by the increase of αvβ3 integrin, p-FAK, and p-PI3K under hypoxia.

3.4 *Hypoxia increases the expression and affects the location of Rac1*

Rac1 is a member of the Rho family of small GTPases that control signalling pathways regulating actin cytoskeleton, cell cycle, and other cellular processes (Pulgar *et al.* 2007). Rac1 could be regulated by Rab11 to promote cell migration (Emery and Ramel 2013). However, its role in migration and invasion of cervical cancer cells is still poorly understood. Hence, in this study, the expression of Rac1 was assessed by Western blot analysis, and its location was tested by Immunofluorescence. Results showed that Rac1 was over expressed in hypoxia (figure 4A and C). Compared with cells without transfection and those transfected with negative control-siRNA, a significant decrease of Rac1 was observed under hypoxia in cells transfected with Rab11-siRNA (figure 4B and D). The distribution of red fluorescence intensity concentrated mainly in the nucleus in cells under normoxia while that distributed both in nucleus, plasma membrane and cytoplasm in cells under hypoxia (figure 4E and F). Furthermore, compared with cells without transfection and those transfected with negative control-siRNA, the distribution of red fluorescence intensity decreased in plasma membrane and cytoplasm in cells transfected with Rab11-siRNA under hypoxia (figure 4G and H).

4. Discussion

Hypoxia is considered to be a major feature of the tumour microenvironment, and it is a potential contributor to the enhanced tumorigenicity, which is accompanied by the activation of a subset of proteases that contribute to metastasis (Carnero and Lleonart 2016). Rab proteins act as the key players in performing a variety of cellular functions, such as growth, protein trafficking, and different signalling pathways (Bhuin and Roy 2015). Some studies have indicated that recycling members (Rab11a, Rab11b, Rab25/Rab11c as well as their effectors) exert an important role in cancers of multiple lineages (Mosesson *et al.* 2008). However, less is known about how hypoxia stimulates the migration and invasion of cervical cancer cell lines. In regard to this, the data reported here reveal that Rab11 played a crucial role in cervical cancer development in hypoxia.

Integrins, consisting of two non-covalently bound transmembrane α and β subunits, are an important molecular family involved in tumour angiogenesis. The blockade of integrin signalling has been demonstrated to be efficient to inhibit tumour growth, angiogenesis, and metastasis. As demonstrated in the introduction, Rab11 could increase the expression of integrin α6β4 in hypoxia promote carcinoma invasion (Yoon *et al.* 2005), and Rab11 can promote invasion and migration of colorectal cancer cell through activating PI3K/Akt signalling pathway (Xu *et al.* 2016). In addition, angptl3 could increase the motility of podocytes, which is induced through integrin αvβ3 and is mediated by FAK and PI3K phosphorylation (Lin *et al.* 2013). And among all the integrins, αvβ3 seems to be the most important one during tumour angiogenesis (Liu *et al.* 2008). Expression of αvβ3 integrin is related to the invasion and metastasis of some tumour types, including glioma, breast cancer, and melanoma (Dang *et al.* 2006; Knowles *et al.* 2013). To sum up, we found that Rab11 could stimulate carcinoma invasion, which could be related to integrins, and some have been verified such as α6β4 and αvβ1. But some still remain uncertain, such as αvβ3, and in view of above of researches, we decided to study the connection between αvβ3 integrin and Rab11. The results showed that the expression of both increased in hypoxia when compared to normoxia. Besides, a significant decrease of αvβ3 integrin and Rab11 were observed under hypoxia in cells transfected with Rab11-siRNA, while there were no differences in cells transfected with negative control-siRNA. These results showed that hypoxia increased the expression αvβ3 integrin and could be regulated by Rab11.

We then explored the possible signal pathway by which Rab11 regulated to stimulate invasion and migration. Focal adhesion kinase (FAK) is an intracellular nonreceptor tyrosine kinase and an important modulator of integrin-dependent focal cell contacts (Lin *et al.* 2013). The tumour cells must adhere to the extracellular matrix, which can facilitate the cell adhesion, movement and migration by promoting extracellular matrix signal transduction dependent on the activity of PTK kinase. And the pathway mediated by FAK is one of the most important. Hosphoinositide3-kinase (PI3K) is a classic downstream target of FAK, of which the phosphorylation is important to its activation (Choi *et al.* 2004). So we focused on the FAK/PI3K pathway, and the results showed that the expression of p-FAK and p-PI3K increased in hypoxia when compared to normoxia. Furthermore, a significant decrease of p-FAK and p-PI3K was observed under hypoxia in cells transfected with Rab11-siRNA, while there were no differences in cells transfected with negative control-siRNA. These results indicated that FAK/PI3K could be possible signal pathway regulated by Rab11 in hypoxia.

Rac1 is a Rho-family small GTP-ase, and it is involved in the regulation of critical cellular functions, such as

transcription control, cell cycle, and organization of actin cytoskeleton (Pulgar *et al.* 2007). Recent studies show that Rac1 is overexpressed in many tumours such as colorectal cancer, gastric cancer and lung cancer, which is closely related to cancer cell invasion and metastasis (Whaley-Connell *et al.* 2007). Some researchers showed that both hypoxia and Rab11 have close connections with Rac1. Binker found that hypoxia-reoxygenation increase invasiveness of PANC-1 cells through PI3K-dependent activation of Rac1 (Binker *et al.* 2010). Yang *et al.* (2014) found that Melatonin could suppress hypoxia-induced migration of HUVECs via inhibition of ERK/Rac1 activation. Emery and Ramel (2013) demonstrated that Rab11 promotes cell migration through the control of Rac1 activity. Besides, another study demonstrated that Rab5a was expressed at a high level in cervical cancer tissues. Silencing of Rab5a expression significantly decreased cancer cell motility and invasiveness through integrin-mediated signaling pathway, accompanied with down-regulation of Rac1 (Liu *et al.* 2011). Studies have shown that FAK phosphorylation enables PI3K to promote the activation of Rac1 through the direct interaction between its lipid products and guanine nucleotide exchange factor of Rac1, and finally promote cell migration (Reiske *et al.* 1999). Another study demonstrated that angptl3 could lead to Rac1 activation and increase the motility of podocytes, which is induced through integrin αvβ3 and is mediated by FAK and PI3K phosphorylation (Lin *et al.* 2013). In view of the above researches, we know that hypoxia, Rab11, αvβ3 and Rac1 play important roles in the development of tumour. Hypoxia, Rab11, and αvβ3 could serve as regulators to Rac1 in some tumours. So we suggested a hypothesis that Rab11 may contribute to Rac1 expression by αVβ3/FAK/PI3K in hypoxia in cervical cancer. And the results above suggested that the expression of Rac1 increased in hypoxia when compared to normoxia. In addition, silencing of Rab11 expression by Rab11siRNA

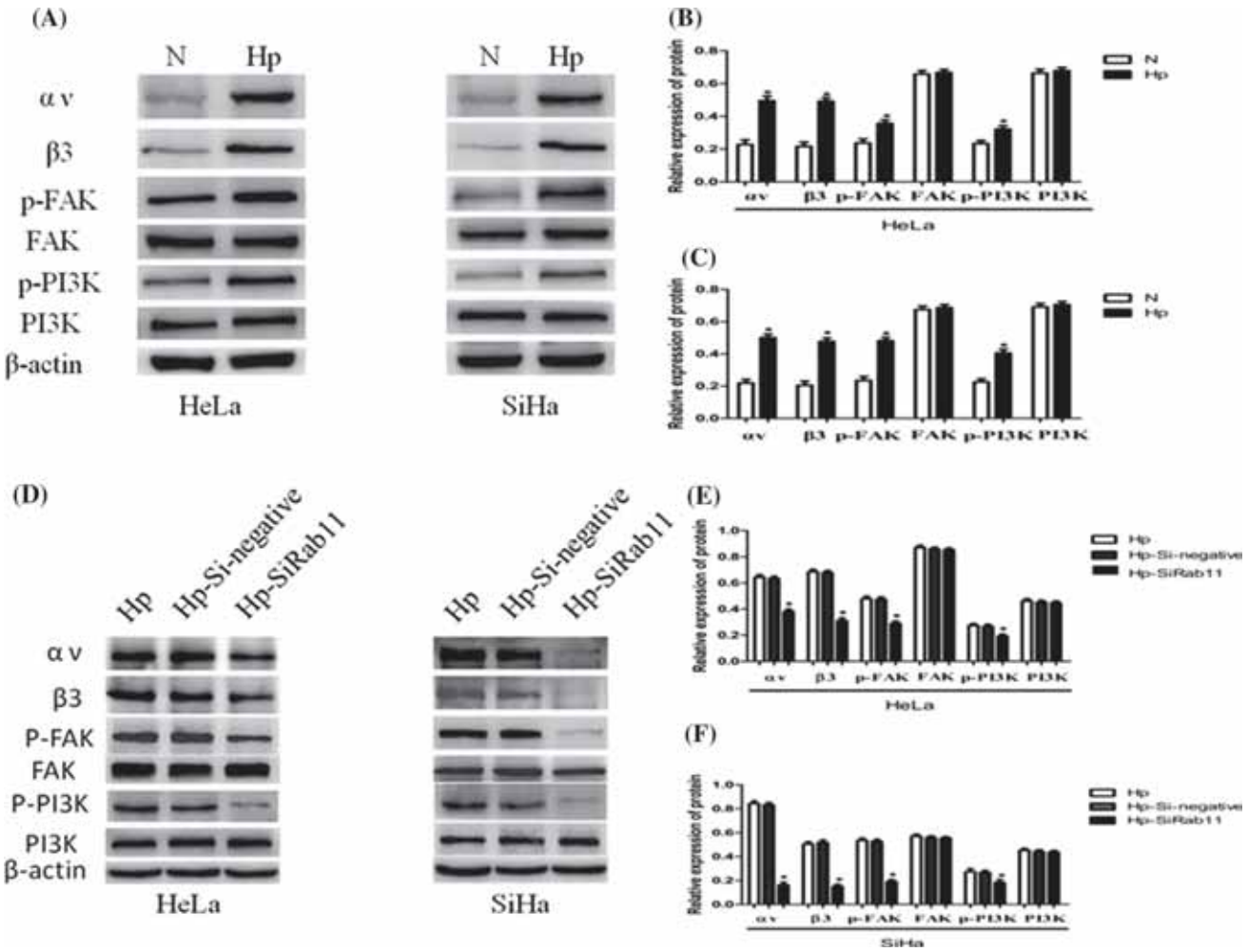

Figure 3. (**A**, **D**) The expressions of proteins were measured by Western blotting. (**B**, **C**) Proteins were over expressed in cells cultured in hypoxia for 48 h, (**E**, **F**) and the expressions were decreased when cells transfected with Rab11-siRNA. Results were expressed as Mean ± SD of three independent experiments (*$P < 0.05$)

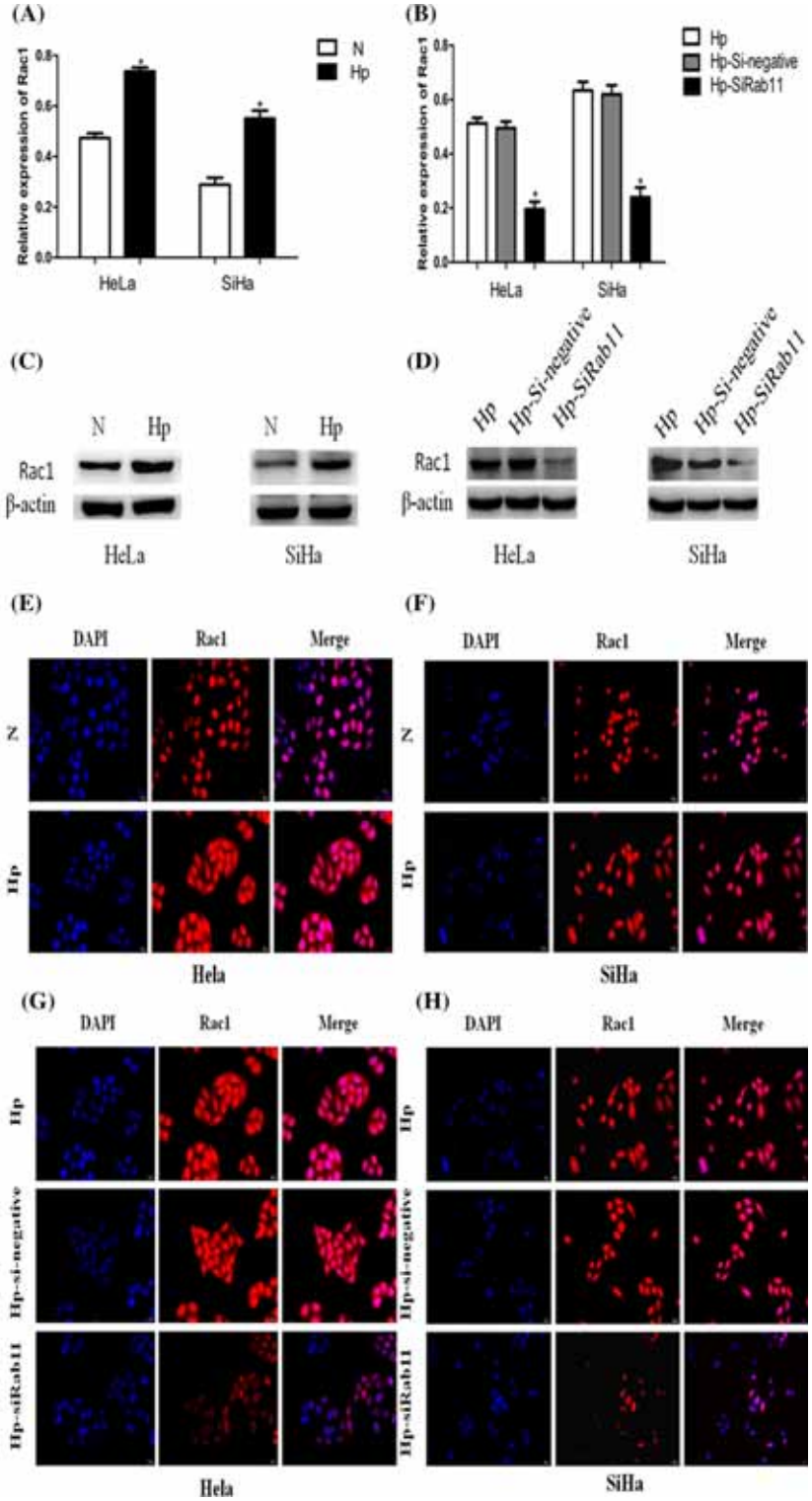

Figure 4. (**A**) Rac1 was over expressed in cells cultured in hypoxia for 48 h, (**B**) and its expression was decreased when cells transfected with Rab11-siRNA in hypoxia. (**C**, **D**) The expression of Rac1 were measured by Western blotting. (**E**–**H**) The location of Rac1 was tested by immunofluorescence. Blue fluorescence represents the nucleus, and red fluorescence represents Rac1 protein. Results were expressed as Mean ± SD of three independent experiments (*$P < 0.05$)

decreased the expression of Rac1, while there were no differences in cells transfected with negative control-siRNA.

In conclusion, this study showed that the invasion and migration capacity of cervical cancer cell lines in hypoxia could be inhibited by silencing the expression of Rab11, which indicated that Rab11 gene might be a potential target for molecular therapy of cervical cancer. And activation of Rac1 by αvβ3/FAK/PI3K could be the potential mechanism.

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