Study of the effect of cAMP signaling pathway on HTR8/SV-neo cell line proliferation,invasion and migration

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Research Article

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Abstract

Pre-eclampsia (PE) is thought to be related to placental dysfunction, particularly poor extravillous trophoblast (EVT) invasion and migration abilities. However, the pathogenic mechanism is not yet fully understood. Here, we used the HTR8/SV-neo cell line as a model for the in vitro functional study of human extravillous trophoblasts. HTR8/SV-neo cells were treated with different concentrations of Forskolin solution (cAMP pathway-specific agonist) to alter intracellular cAMP level. Taking the DMSO group as the control group, firstly, cAMP assay kit was used to detect the cAMP concentration in HTR8/SV-neo cells treated with different concentration of Forskolin, and the cell proliferation function was detected by cell growth curve drawing and the colony formation experiment. The invasiveness and migration of cells were observed by Transwell experiments, and the expression of intracellular EMT-related markers was further evaluated by qPCR and WB experiments. According to our research, the intracellular cAMP level in HTR8/SV-neo cells was increased in a dose-dependently manner, and the proliferation, invasion and migration abilities of HTR8/SV-neo cells were significantly enhanced. Further detection by qPCR and WB experiments proved that the expression of EMT and angiogenesis markers in cells were up-regulated, including MMP2, PECAM, N-cadherin and VE-cadherin. In addition, with the increase of intracellular cAMP level, the phosphorylation level of intracellular MAPK signaling pathway was significantly increased. Thus, these results provide a novel rationale for the treatment of PE patients by improving the poor placental environment.

1 Introduction

Pre-eclampsia (PE) usually occurs in women who are pregnant after 20 weeks suffering from the symptoms such as high blood pressure, proteinuria and so on. It is thought to be related to placental dysfunction[1], particularly poor extravillous trophoblast (EVT) invasion and migration abilities upon uterine spiral arteries, which leads to defective vascular remodeling and placental hypoperfusion[2].

As the embryo implants in the maternal uterus, it gradually develops into the blastocyst stage, while the trophectoderm layer develops into two subtypes: the villous trophoblast (VT) and the extravillous trophoblast (EVT)[3]. EVTs then invade into the maternal endometrium and migrate through the myometrium to the spiral arteries. This causes the uterine spiral arteries physiologically remodeled into large-diameter and low-resistant vessels, which increase the blood supply to fetus, thus promote the growth of the embryo and placenta[4]. However, over-invasive EVTs can lead to multiple pathologic pregnancy including placenta accreta, placenta implantation, uterine perforation, hemorrhagic shock and even death[5]. Therefore, it is supposed that the highly coordinated EVTs are necessary in a healthy utero-placental status.

EVTs have highly tumor-like aggressiveness, including the ability of invasion, migration and proliferation, some scientists even jokingly call them "physiological tumor cells". Recent studies of tumors have shown that cyclic adenosine monophosphate (cAMP), an important mediator of the signaling cascade between cells, is closely linked to the development of tumors. The currently known cAMP signaling pathway

mainly includes cAMP/PKA/CREB and cAMP/Epac/Rap1. The changes of intracellular cAMP levels can induce a variety of physiological and pathological changes, including tissue metabolism, cell proliferation or apoptosis, and tumor cell invasion and migration[6, 7]. In the studies of urothelial bladder carcinoma (UCB), centrosomal protein (CEP)-72 promotes the invasion and migration capacity of UCB cells by enhancing the binding of cAMP response element binding protein (CREB) of the cAMP signaling pathway to the SERPINE1 promoter of serpin family[8]. In esophageal squamous cell carcinoma, lysosomal-associated membrane protein 3(LAMP3) attenuates the phosphorylation level of VASP at the Ser239 site through cAMP-dependent protein kinase A (PKA), thereby enhancing the aggressiveness of esophageal squamous cells (ESCC), and promoting the metastasis of esophageal squamous cell carcinoma[9].

Furthermore, previous studies have shown that cAMP signaling pathway may affect various tumor cell proliferation and migration ability by regulating MAPK signaling pathway in the downstream[10]. The mitogen-activated protein kinase (MAPK) signaling pathway is also a key pathway that regulates a variety of intracellular processes, including the pathway called ERK1/2, JNK1/2/3 and p38-MAPK kinase signaling pathways[11]. However, cAMP signaling pathway has different effects on the MAPK signaling pathway depending on the type of cells. In the studies of clear cell renal cell carcinoma (ccRCC), activation of the intracellular cAMP signaling pathway was found to inhibit ERK phosphorylation in the MAPK signaling pathway in a c-Raf-dependent manner, thereby attenuating the proliferative ability of tumor[12]. However, in prostate cancer, the overexpression of PKA inhibitor proteins (PKIs) was found to block the activation of protein kinase A, causing a continuous increase in intracellular cAMP levels through negative feedback, which in turn activated another signaling pathway, cAMP/Epac/Rap1, and the phosphorylation of downstream MAPK signaling pathways, ultimately promoting the proliferation and migration of cells[13].

Forskolin, a traditional medicine for centuries, is a natural compound extracted from the roots of Indian plants[14], and used as a specific agonist of the cAMP signaling pathway to increase the intracellular cAMP levels. A large number of in vivo and in vitro studies have shown that it inhibited the development of cancer in a certain degree. It binds directly to the catalytic subunit of adenylate cyclase(AC), inducing conformational changes at the ATP site, thereby converting ATP to cAMP and pyrophosphate[15]. It has no effect on the Km value of the ATP reaction, but increases its maximum reaction rate, resulting in potent and rapid synthesis of cAMP[16].

Based on the discovery of the role of the cAMP signaling pathway in tumors, and we speculate that the cAMP signaling pathway may also have a certain effect on the proliferation, invasion and migration functions of trophoblasts. Therefore, in our study, forskolin was used to activate the cAMP signaling pathway, and the changes of the EVTs in proliferation, invasion and migration functions were observed to explore whether the cAMP signaling pathway plays a role on EVTs through the MAPK signaling pathway. This is of great importance for understanding embryo implantation, placenta formation and regulation, and the prevention and treatment of pregnancy-related placenta disorders.

2 Materials and Methods

2.1 Cell culture

The human first-trimester placenta trophoblast cell line HTR8/SV-neo (HTR8) was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in DMEM medium contained with 10% FBS in a 5% CO2 humidified atmosphere at 37℃.

2.2 cAMP Assay

Prior to the experiment, HTR8 cells were plated in dishes (5x105 cells) and incubated for 48h in DMEM medium contained different concentration of forskolin. After lysis with RIPA solution on the ice, the cell suspension was centrifuged for 10 min at 600g to remove the insoluble materials. According to manufacturer instructions, cells were distributed into 96-well plates, and incubated with compounds for hours at 37℃. Then read the plate at OD450 nm. The cAMP level in HTR8/SV-neo cells treated with different concentration of forskolin was calculated according to the obtained formula by plotting the standard curve of cAMP.

2.3 Cell growth curve

The cells were allowed to grow in 24-well plates (2x103 cells) and cultured for 4 days. The cells were disaggregated by enzymes and counted every day. Each cell line experiment was performed in triplicates.

2.4 Colony formation assay

The cells were harvested from the DMEM medium contained different concentration of forskolin after incubated for 48h. And seeded into 12-wells plate (5x102 cells). After two weeks, the cell colonies were stained with Giemsa staining solution for 15 minutes after fixation with 4% paraformaldehyde (Biosharp, China) for 30 min. The number of colonies were counted.

2.5 Transwell assay

For Transwell assay, the Matrigel (100μl per filter; BD Biosciences) was added into the upper chambers with 8-μm pores (BD Biosciences). The cells(2x104) treated with different concentration of forskolin for 48 hours were seeded to the upper chambers within the medium containing 10% FBS in the lower chambers. After 24 hours culturing, the invaded cells on the lower membrane were fixed by 4% paraformaldehyde and stained with Giemsa. Select randomly several fields for cells counting

2.6 Transwell migration assay

The cells(2x104) treated with different concentration of forskolin for 48 hours were seeded to the upper chambers (8-μm pores, 24-well plates) within the medium containing 10% FBS in the lower chambers. After 24 hours culturing, the migrated cells on the lower membrane were fixed by 4% paraformaldehyde and stained with Giemsa. Select randomly several fields for cells counting.

2.5 RNA isolation and quantitative real-time PCR

Total RNA was extracted using TRIzol regent (Invitrogen Life Technologies, USA) according to the instruction. Complementary DNA (cDNA) was synthesized with the Prime Script® RT reagent Kit (Takara, Japan). The primers used in this study were listed in Supplementary Table 1. The expression level of gene mRNA was quantified using SYBR® Premix Ex Taq TM II kit (Takara, Japan). Relative expression level was determined by normalizing the expression level of each target to GAPDH, and relative mRNA fold was determined using the 2(-ΔΔCt) method. Samples were run in triplicate.

2.6 Western blot

Total proteins were extracted and purified from the cells treated with different concentration of forskolin for 48h. Then, separate the proteins (30μg) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Beyotime, China) and transfer onto the polyvinylidene fluoride (PVDF, Beyotime, China) membranes. After blocking with 3% bovine serum albumin (BSA, Beyotime, China) in Tris-buffered saline containing 0.1% Tween-20 (TBST), the membranes were incubated with anti-N-Cadherin antibody (1:1000,142158, CST, USA), anti-MMP-2 antibody (1:1000, 97779,Abcam, Cambridge, UK), anti-CDH5 antibody (1:1000, 2158S,CST, USA), anti-PECAM antibody (1:1000, K003627P,Solarbio, China), anti-c-Raf Antibody(1:1000, 9422T,CST, USA),anti-Phospho-c-Raf(1:1000,9421T,CST,USA),anti-MEK1/2-Antibody(1:1000,4694S CST,USA),anti-Phospho-MEK1/2Antibody(1:1000,3958S,CST,USA),Anti-ERK1 + ERK2 antibody(1:1000,Abcam,54230,Cambridge,UK),anti-Phospho-p44/42MAPK(Erk1/2) antibody(1:1000, 4370T,CST, USA) at 4°C overnight. And were incubated with HRP-conjugated anti-rabbit/mouse secondary antibodies the next day. Finally, the specific protein bands were visualized with a chemiluminescent substrate kit (Millipore, USA), and images were captured using a Tanon 4600SF instrument (Tanon, China). The densitometric analyses were performed using Image-J® software (NIH, USA), and all results were expressed as a ratio relative to GAPDH total protein content as needed. The anti-GAPDH antibody (1:20000, 8245,Abcam, Cambridge, UK) was used as the control.

2.7 Statistical analysis

All data collected were analyzed using Graphpad prism (version 7.0 for Windows). The results were presented as the means ± SDs. It is considered that P value < 0.05 was statistically significant.

3 Results

3.1 cAMP levels were upregulated by Forskolin in HTR8/SV-neo cells

We treated HTR8/SV-neo cells with 0, 10, 50 and 100 μM forskolin solutions for 48 hours. As shown in Fig. 1, determined by cAMP assay, the intracellular cAMP levels are low in wild-type HTR8/SV-neo. Furthermore, the cAMP levels were increased in a dose-dependent manner.

3.2 cAMP signaling pathway enhanced HTR8/SV-neo cells proliferation

In order to detect the effect of the cAMP signaling pathway on the proliferative capacity of EVT, HTR8/SV-neo cells were incubated with forskolin solution as above. As compared with the control group, the results showed that cells treated with 10μM and 50μM concentration significantly increased cell proliferation for 72 and 96 hours respectively, while the cells with 100 μM concentration seemed nearly to the untreated group (Fig. 2A,B). In addition, the results from the colony formation assay revealed that 10 μM concentration, followed by 50 μM, increased HTR8/SV-neo cell proliferation (Fig. 2C). These results were analyzed as shown in Fig. 2D. Thus, we used 10μM and 50μM concentration of forskolin in subsequent experiments.

3.3 cAMP signaling pathway enhanced HTR8/SV-neo cells invasion and migration

In concordance with the cell growth and proliferation, we investigated whether cAMP signaling pathway promote or suppress HTR8 cells invasion and migration. The transwell assay revealed a significant increase in the invasion rate in cells treated with 10μM and 50μM concentration compared to the control group, especially with 50μM(Fig. 3A,B). The transwell migration assay indicated that HTR8/SV-neo cells with higher cAMP levels exhibited a significantly more extensive migration ability compared with control group as shown in Fig. 3C.

3.4 The cAMP signaling pathway increased the expression of mRNA and protein levels of N-cadherin, CDH5, MMP2 and PECAM.

To investigate the molecular mechanism associated with cAMP signaling pathway upon the cell proliferation, invasion and migration, we employed RT-PCR and western blot to assess the ability of epithelial mesenchymal Transition, EMT. Thus, the expression of mRNA and protein levels of N-cadherin, CDH5[17], MMP2[18] and PECAM were measured as major EMT markers, which are closely related to cell migration and invasion[19, 20]. With intracellular cAMP levels increased, mRNA expression levels of N-cadherin, CDH5, MMP2 and PECAM were significantly higher than in controls (Fig. 4A). As shown in Fig. 4B, the expression of N-cadherin, CDH5 and MMP2 protein were significantly enhanced than in the control group. Overall, cAMP signaling pathway may induce HTR8/SV-neo cells the ability of EMT by increasing N-cadherin, CDH5, MMP2 levels.

3.5 The cAMP signaling pathway promoted the phosphorylation of the MAPK/ERK signaling pathway protein

It was reported that the cAMP signaling pathway was associated with MAPK signaling pathway, in trophoblasts, thus the phosphorylated levels of MAPK/ERK were evaluated in HTR8/SV-neo cells treated with different concentration forskolin. It was shown that with the intracellular cAMP upregulated, the

MAPK/ERK phosphorylation was significantly increased, including c-raf259, MEK1/2 and ERK1/2(Fig. 5A-F).

Previous studies revealed that cAMP signaling pathway enhanced the ability of growth, proliferation, invasion and migration, especially at 50μM concentration. To confirm whether the cAMP signaling pathway regulated trophoblasts via MAPK/ERK signaling pathway, a specific ERK inhibitor, U0126 was used to block this pathway. Therefore, we examined the phosphorylation of MAPK/ERK signaling pathway by using forskolin at 50μM concentration or the MAPK/ERK pathway inhibitor U0126 or a combination. When HTR8/SV-neo cells were treated with U0126 alone, the phosphorylation of ERK protein in the cells was suppressed. When HTR8/SV-neo cells were treated with 50μM concentration forskolin alone, the phosphorylation of ERK protein in the cells was significantly enhanced. However, when the cells were treated again with 50μM forskolin after inhibited by U0126, the phosphorylation of ERK remained inactivated and was even more inhibited(Fig. 5G,H).In conclusion, cAMP signaling pathway may promote proliferation, invasion and migration of HTR8/SV-neo cells through phosphorylation of the MAPK/ERK pathway.

4 Discussion

Pre-eclampsia is a complication of pregnancy which is discussed hotly because of its high morbidity, mortality and increasingly risk of chronic diseases in postpartum such as diabetes, hypertension and cardiovascular diseases. However, the pathogenesis of pre-eclampsia is poorly understood and there is no effective treatment other than childbirth in current[1]. Many studies have shown that after the embryo implanted into the endometrium in physical, the ability of EVT cells to proliferate, invade and migrate gradually increased. On the contrary, when the pre-eclampsia occurs, the invasion and migration capacity of EVT cells appear to impaired apparently, which leads to insufficient remodeling of the uterine spiral artery, resulting in low placental blood perfusion and superficial placental implantation in the uterus[21]. Thus, the proliferation, invasion and migration functions of EVT cells are thought to be essential for pregnancy[22].

Similar to the tumor cells, EVT cells exhibit high proliferation, aggressiveness, and migration features[23]. In the first trimester, EVTs, together with natural killer cells and macrophages, invade the maternal endometrium under the role of matrix metalloproteinases, gradually migrating to the uterine spiral arteries, invading the blood vessels, replacing the endothelial cells[24], which physiologically reconstituted the uterine spiral arteries into a high-drain, low-resistance type to increase the blood flow for fetus[4]. HTR8/SV-neo cell lines are widely used to study the adhesion, migration and invasion functions of trophoblasts and are generated by transfecting human primary trophoblasts with SV40-containing large T antigens[25]. Therefore, it is important to use HTR8/SV-neo cell lines as an in vitro model of human extravillous trophoblasts to observe changes in proliferation, invasion and migration functions of HTR8/SV-neo cell lines and explore possible potential mechanisms.

Forskolin is a specific activator of cAMP signaling pathway and our study also found that intracellular cAMP levels increased dose-dependently with increasing forskolin concentrations. Due to the poor solubility of forskolin in water, it requires up to 3% by mass of DMSO solution to dissolve it, whereas in most intact mammalian cell experiments, it can only tolerate up to 1% concentration of DMSO solution[16]. In this experiment, the cells were treated with different concentration of forskolin, which was dissolved with DMSO solution in necessity. In order to exclude the possible influence of DMSO solution on the experimental results, a toxic solution to cells in a certain concentration, we added the largest amount of DMSO solution in control group, compared to the highest DMSO concentration in the experimental group. As a result, in the control group, in 25μl DMSO solution mixed in 3ml culture medium, HTR8/SV-neo cells were cultured. In addition, the high hydrophobicity of forskolin indicates that there is a slow rate of dissociation, which keeps a certain drug prolonging effect, and means it can be effectively achieved applying forskolin to treat cells for 48h and 10min.

By treating HTR8/SV-neo cells with different concentrations of forskolin solution, we found that the proliferation, invasion and migration ability of HTR8/SV-neo cells also increased significantly within increasing intracellular cAMP levels, but there was no significant difference between 10μM and 50μM concentration. At 100μM concentration, the ability of cells to proliferate did not change significantly compared to controls. We speculate that intracellular cAMP may improve the proliferative properties of cells within a certain concentration range, because studies have shown that elevated cAMP levels can promote cell proliferation in some physical cells or tumors, and some data suggest that cAMP may cause cell growth cycle arrest or even apoptosis[12, 26]. When it exceeds a certain level, the proliferation of cells can be decreased or stopped or even prone to apoptosis, such as in tumor.

The cAMP/PKA/CREB signaling pathway and MAPK signaling pathway are two classical pathways that regulate cell growth and metabolism, and have been extensively studied in a variety of tumor cells and trophoblasts[27]. In trophoblasts, the cAMP/PKA/CREB signaling pathway has been found to be involved in the regulation of trophoblast fusion function[28, 29]. Therefore, we further investigated the specific mechanism of the cAMP signaling pathway and the MAPK signaling pathway in regulating trophoblasts. In our study, we found that when the cAMP level was increased in cells, the phosphorylation levels of c-Raf259, MEK1/2 and ERK1/2 in the MAPK signaling pathway were significantly increased. Previous studies in lung cancer cells found that after activation of the MAPK signaling pathway, the phosphorylation level of c-Raf259 protein was reduced, while the phosphorylation level of c-Raf338 protein was significantly increased, the growth of lung cancer cells was inhibited, and the functions of angiogenesis and epithelium-mesenchyme transformation were downregulated[30]. And in our experiments, the results are as the same. Therefore, we believe that EVT cells may promote cell proliferation and invasion abilities through activation of the phosphorylation level of Ser259 locus of c-raf protein in the MAPK signaling pathway.

5 Conclusion

this study showed that the cAMP signaling pathway promote the EVT cells to proliferate, invade and migrate, by activating the MAPK signaling pathway by enhancing the phosphorylation of c-Raf259, MEK1/2 and ERK1/2 proteins, and promote the epithelial-to-mesenchymal transformation. The cAMP signaling pathway is involved in the regulation of a wide range of fundamental life processes in cells and in the occurrence and development of tumor cells. Based on the similarity of EVTs to tumor cells in the first trimester, we investigated whether the cAMP signaling pathway is also involved in regulating EVT cell functions, helping us to understand functions in EVT cells better and providing directions for the treatment of trophoblast-related pregnancy disorders. There is currently no effective treatment for PE other than termination of pregnancy, despite extensive researches into the condition. To improve the perinatal safety of PE patients, some anticoagulants are currently used to improve placental perfusion. The results of this trial suggest that we may also be able to use certain drugs to improve placental cell function, thereby improving perinatal outcomes and the poor prognosis of PE patients.

Declarations

Acknowledgments

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Data Availability

The date used to support the results of the study are included in the published paper.

Conflicts of Interest

The authors of this paper have no potential conflicts of interest.

Authors, Contributions and Consent to participate

Yuanhua Huang and Yanlin Ma were responsible for the conception and design of the study and the experiments, while data collection were performed by Jiaoqi Mei and Mengyi Song,Yanhong Yi and ChaoSun designed the experiments and drafted the manuscript. The final version of the manuscript was approved by all authors.

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Ethics approval

The Medical Ethics Committee of First Affiliated Hospital of Hainan Medical University reviewed and approved our project for implementation, confirming its compliance with all relevant laws and regulations.

Consent for publication

All authors have consented to the publication of this ariticle.

Availability of data and material

The data is transparent and can be made publicly for publication.

Code availability

Not applicable.

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Tables

Table 1
qPCR primer sequences.

Name	Sequences
GAPDH-F	5’-CGAGATCCCTCCAAAATCAA-3’
GAPDH-R	5’-TGTGGTCATGAGTCCTTCCA-3’
MMP2-F	5’-ATGACAGCTGCACCACTGAG-3’
MMP2-R	5’-ATTTGTTGCCCAGGAAAGTG-3’
N-Cadherin-F	5’-GCCCAAGACAAAGAGACCCA-3’
N-Cadherin-R	5’-ACCCAGTCTCTCTTCTGCCT-3’
CDH5-F	5’-AGCCCTACCAGCCCAAAGTG-3’
CDH5-R	5’-TGATGTTGGCCGTGTTATCGTG-3’
PECAM1-F	5’-CTCAGACGTGCAGTACACGGA-3’
PECAM1-R	5’-AGGGAGCCTTCCGTTCTAGAGT-3’

Supplementary Table 1

Supplementary Table 1 is not available with this version

Figures

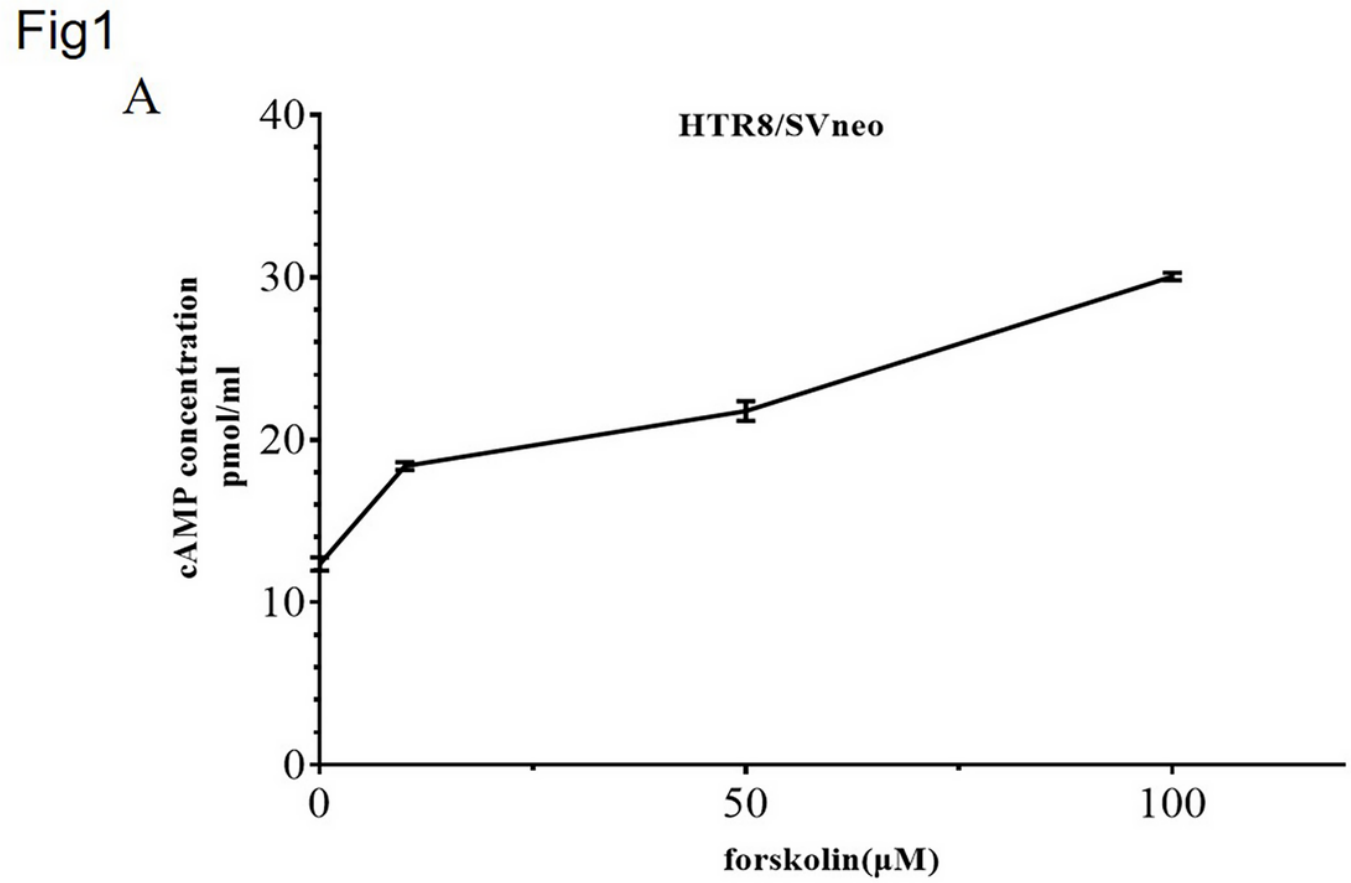

Figure 1

cAMP levels were upregulated by Forskolin in HTR8/SV-neo cells(A).HTR8/SV-neo cells were treated with 0, 10, 50 and 100μM Forskolin for 48h, Dose-dependent increase in cAMP levels. The data are presents as the mean ± SD obtained form at least three independent experiments.

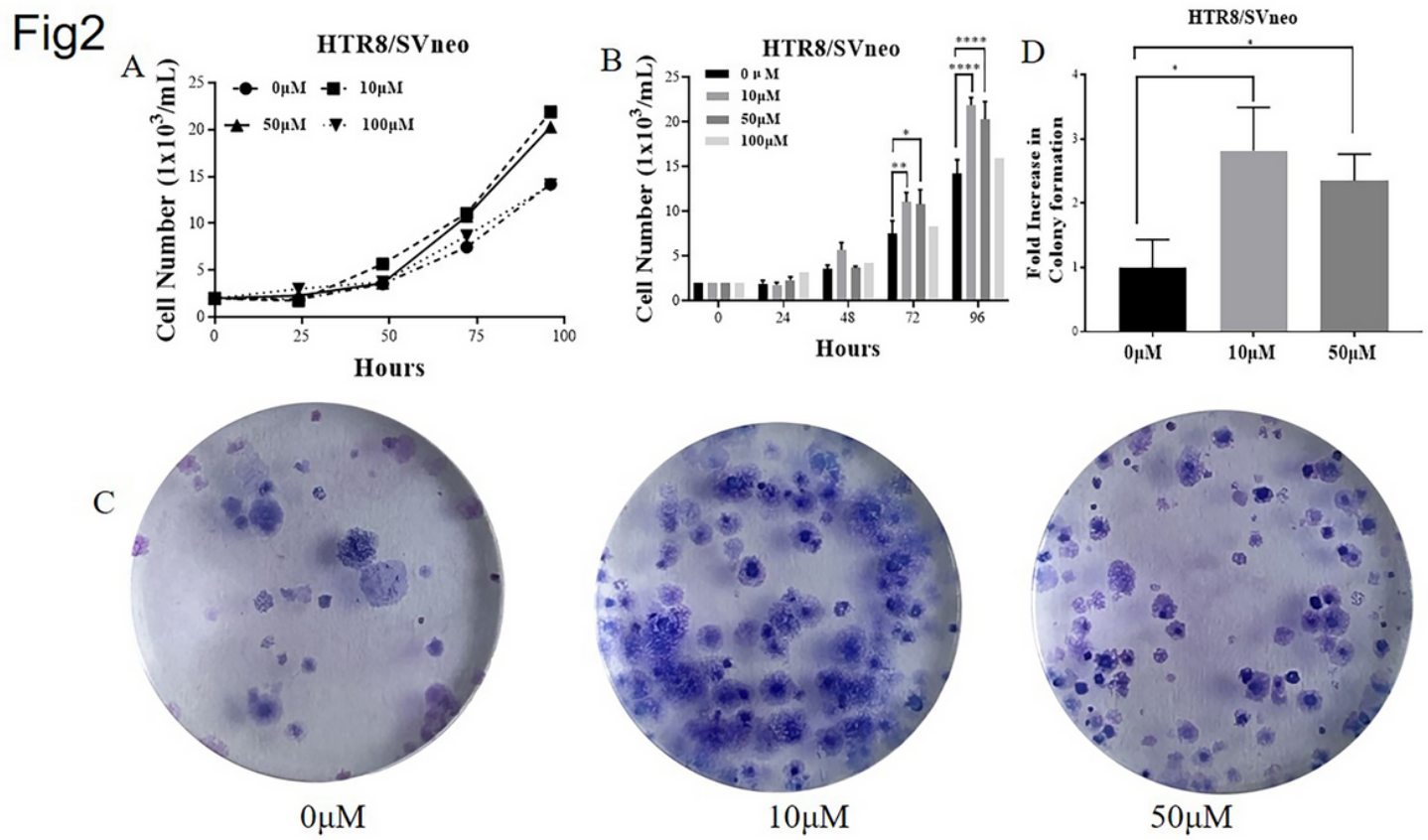

Figure 2

cAMP signaling pathway enhances HTR8/SV-neo cells proliferation. When HTR8/SV-neo cells were treated with 0μM, 10μM, 50μM and 100μM Forskolin, (A,B)Growth curve assays and (C, D) Colony-forming assays showed that the proliferation ability of HTR8/SV-neo cells treated with 10μM and 50μM Forskolin were higher than the control. Error bars represent the mean ± SD of three independent experiments. *p<0.05, **p<0.01, ***p < 0.001, ****p < 0.0001.

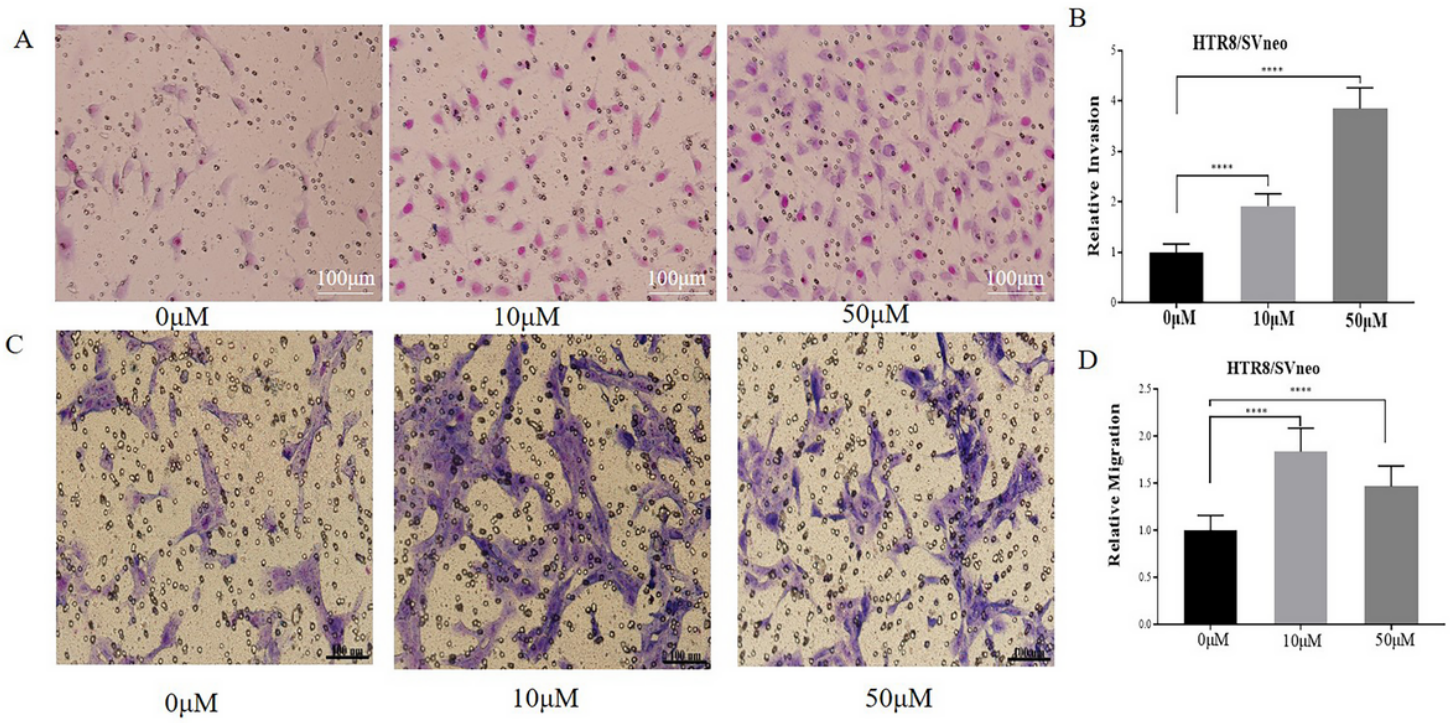

Figure 3

cAMP signaling pathway enhances HTR8/SV-neo cells invasion and migration. (A, B) In the cells treated with 10μM, 50μM Forskolin, the invasion ability was significantly enhanced compared to the control, and the migration ability (C, D). Error bars represent the mean ± SD of three independent experiments. ***p < 0.001, ****p < 0.0001.

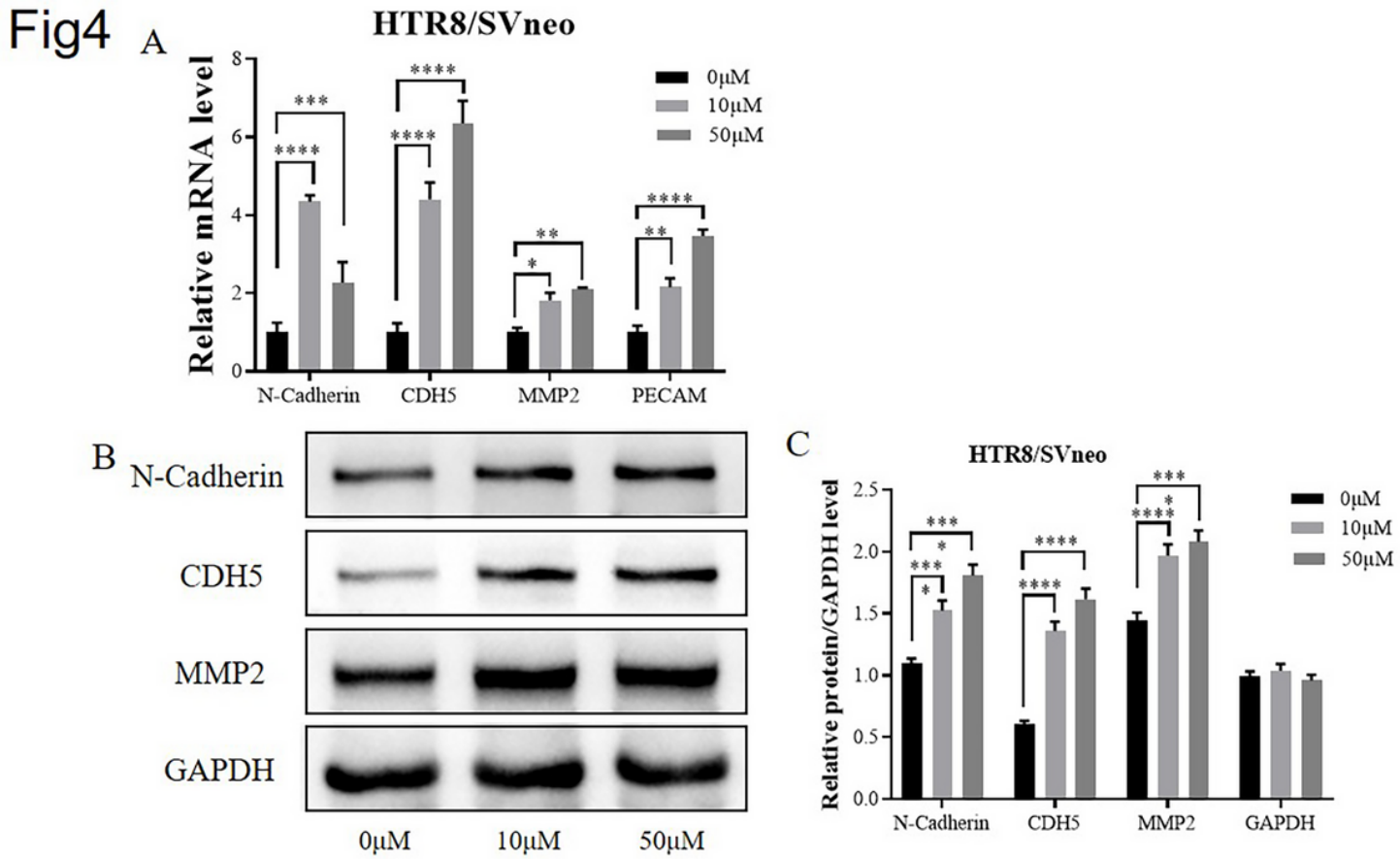

Figure 4

cAMP signaling pathway regulated the expression of the marker proteins related to invasion and migration. (A) The mRNAs expression of N-Cadherin, CDH5, MMP2, PECAM were detected by real-time PCR in HTR8/SV-neo cells treated with 10μM, 50μM Forsklin. (B) The proteins expression of N-Cadherin, CDH5, MMP2, PECAM were detected by western blot in HTR8/SV-neo cells treated with 10μM, 50μM Forskolin. Protein levels were quantified by densitometry analysis using Image J software. *p<0.05, **p<0.01, ***p < 0.001, ****p < 0.0001.

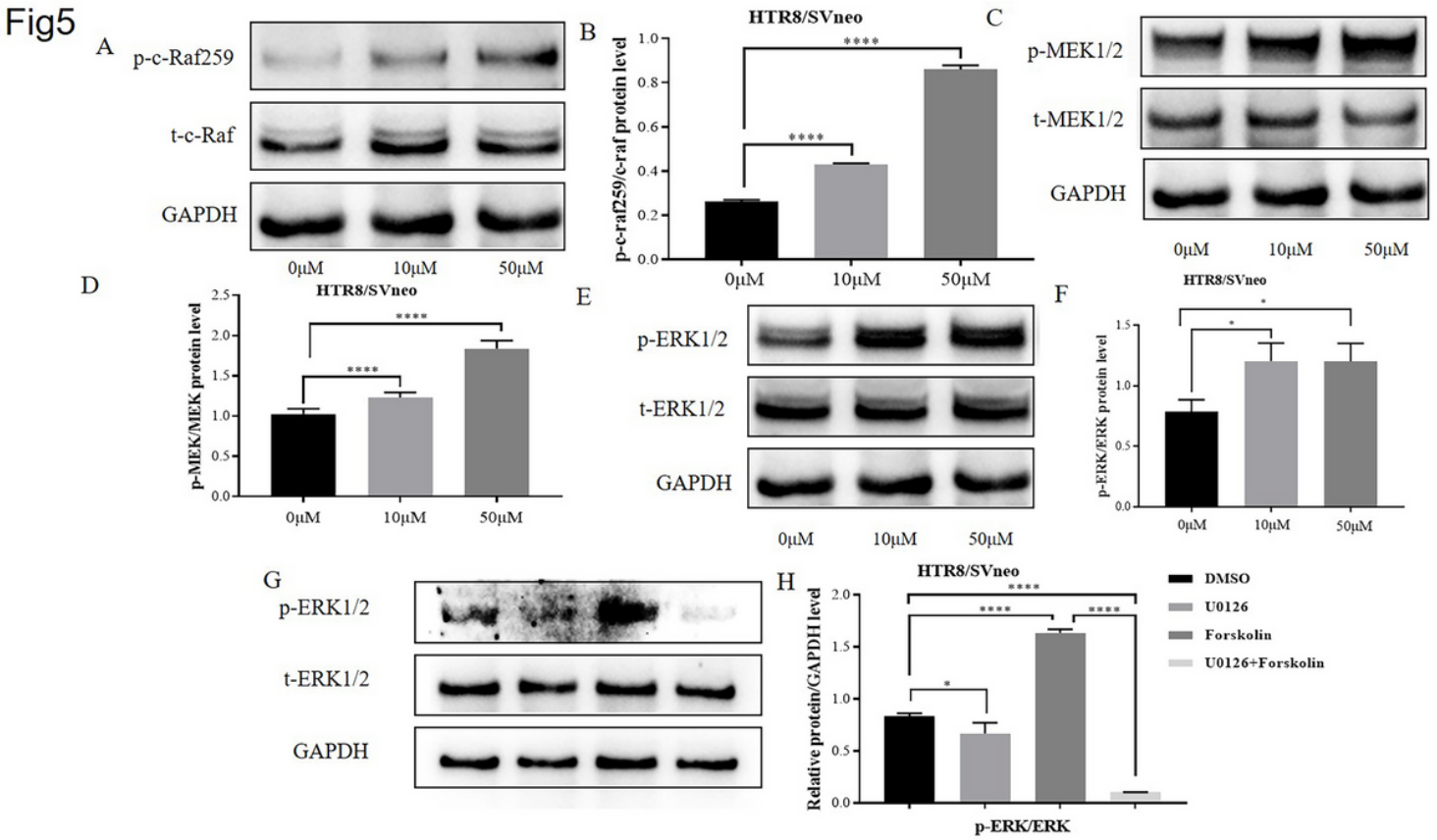

Figure 5

The cAMP signaling pathway regulated the phosphorylation of MAPK/ERK signaling pathway. (A-F) Protein levels of total and phosphorylated c-raf259, MEK and ERK were significantly promoted in 10μM, 50μM Forskolin-treated cells than in the control group. (G,H)The ratio of the expression level of p-ERK and ERK protein was decreased by using U0126, an inhibitor of the MAPK/ERK signaling pathway. *p<0.05, ****p < 0.0001.