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Original Research

Tspan5 inhibits proliferation and migration of JEG-3 cells by inhibiting FAK and AKT phosphorylation

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Summary

Objectives: To study the effect of Tspan5 on the proliferation and migration of trophoblast cells. *Materials and Methods:* The authors silenced the Tspan5 expression in human choriocarcinoma cell line JEG-3 using RNAi, and then conducted the cell proliferation assay and scratch assay to detect the proliferation and migration of JEG-3 cells. *Results:* The present authors found that the JEG-3 cell proliferation and migration activity of interference group (Tspan5/1005) had decreased significantly. Tspan5/1005 moderately decreased FAK and AKT phosphorylation compared to the control group. In this study, the authors found that the proliferation and migration of choriocarcinoma cells were reduced after using RNAi technology to downregulate the expression level of Tspan5 expression. The level of Tspan5 expression was positively correlated with the proliferation and migration of choriocarcinoma cells. *Conclusions:* Tspan5 could play a biological role through the FAK/AKT signaling pathway.

Key words: Tspan5; Human choriocarcinoma cell line JEG-3; Proliferation; Migration; FAK/AKT.

Introduction

After fertilization, a fertilized egg will move towards the uterus and undergo a series of cell divisions. A solid mass of cells known as a morula will form and a cavity will appear, where blastocoelic fluid will begin to accumulate, indicating the formation of the blastocyst. The inner cluster of cells of the blastocyst will develop into an embryo while the outer layer, known as the trophoblast, will absorb nutrients directly from the mother for embryonic growth. Trophoblastic cells are uniquely different from other cells in terms of their biological behavior, development, and histogenesis. They can be divided into two cell layers, the outer layer known as syncytiotrophoblast (ST) and the inner layer known as cytotrophoblast (CT). The ST facilitates the circulation of nutrients between the mother and the fetus while functioning as the endocrine tissue of the placenta [1]. The ST secretes a variety of protein hormones, including hCG [2].

Extravillous cytotrophoblasts (EVCTs) are located at the tip of the anchoring villi. EVCTs are similar to tumor cells in that they can differentiate into a multilayered cell column and invade one-third of the decidua and myometrium. They can also enter the uterine spiral arteries and replace endothelial cells. However, these only occur during the early stages of pregnancy and the invasions gradually stop after the placenta has formed [3]. EVCT invasions are regulated by multiple cytokines and an abnormal invasion can lead to a series of problems. For example, insufficient invasion can lead to miscarriages, preeclampsia, and intrauterine growth restriction, while excessive invasion can lead to gestational trophoblastic tumors [4, 5].

Tetraspanin5 (Tspan5), also known as TM4SF9 or NET-4, is a multiple transmembrane protein containing 268 amino acids that belongs to a transmembrane 4 superfamily (TM4SF), is encoded by the TM4SF gene, and is located in the human gene 4q23. TM4SF consists of 33 members in the human genome and they span the cell membrane four times to form two extracellular loops and one intracellular loop, with the larger extracellular loop containing approximately 120 amino acids that play a significant role in biological behavior [6]. Tspan5 plays an important role in cell development, differentiation, growth and signal transduction linked to cell migration. Studies have found that overexpression of four transmembrane proteins affects the survivability and invasiveness of multiple myeloma (MM) cells [7], and that Tspan5 may affect the metastasis of colorectal cancer [8]. Gao *et al.* [9] discovered that Tspan5 proteins are mainly expressed in the invasive EVCTs and hyperplasia CTs found in the maternal-fetal interface. With an increased proliferation and invasiveness, the expression of Tspan5 proteins in normal villi of early pregnancy, hydatidiform mole, invasive hydatidiform mole, and chorionic carcinoma tissues gradually enhanced. RNA interference (RNAi) was used to downregulate the expres-

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sion of Tspan5 and it was found that the heterogeneous adhesiveness and motility of choriocarcinoma cells were weakened. This indicates that the level of Tspan5 expression has a positive correlation with the strength of invasiveness of trophoblast cells. However, further research is required to determine if there is a correlation between Tspan5 and the proliferation and mobility of trophoblast cells.

In this study, RNAi was used to silence the expression of Tspan5 found in human choriocarcinoma cell line JEG-3 to investigate the effect that Tspan5 had on the proliferation and mobility of JEG-3 cells and to explore a new option for treating gestational trophoblastic diseases (GTD).

Materials and Methods

Human choriocarcinoma cell line JEG-3, purchased from the Shanghai Institutes for Biological Sciences, Stem Cell Bank, was resurrected and subcultured. RNAi was performed when the cells were in good condition.

Tspan5 siRNA was designed and synthesized. There are a total of 5 siRNA and the fragment sequences are shown in Table 1:

The 2×10^5 JEG-3 cells were placed in a six-well cell culture plate and incubated at 37°C for 24 hours within a 5% CO_2 incubator. This was done in triplicate. Transfection was conducted after confluence of cells per well reached 80-90%. The transfection was done according to instructions for the lipofectamine 3000 reagent. Each well contained 125 pmol of siRNA, 10 μl of P3000 reagent, and 5 μl of lipofectamine 3000 reagent. After transfection, cells were incubated again in a 5% CO_2 incubator at 37°C for 48 hours. Cells were washed three times with 4°C precooled phosphate-buffered saline (PBS), an appropriate amount of RIPA lysis buffer (about 100 μl per well) was added, and a cell scraper was used to scrape cells and reagents to one side after five minutes. The cells were then placed in an ice bath for 30 minutes and repeatedly pipetted. Samples were collected at 4°C and subjected to 12,000 g of centrifugation for 15 minutes. The supernatant, which consists of the total protein solution, was collected. Protein concentration was measured using the Bradford method, prepared SDS-PAGE gel (10% separating gel, 5% stacking gel), calculated the volume of solution containing 40 μg protein samples as the sample volume, electrophoresis, and transmembrane. The sample was then placed in a blocking buffer of 5% skim milk and shaken slowly with the shaker at room temperature for one hour. It was placed at 4°C overnight in the antibody Tspan5 (1/500 dilution) working solution, and the unbound antibody was washed with the 1×TBST at room temperature on the shaking bed five minutes for three times. The secondary antibody was diluted 3,000 times with 1×TBST, incubated at room temperature for about 30 minutes, and the free secondary antibody was washed with 1×TBST. The resulting film was scanned and the quantification of the gel bands were analyzed by computer-assisted software.

The 5×10^4 JEG-3 cells were placed in a 96-well cell culture plate for 24 hours at 37°C within a 5% CO_2 incubator and this was done in triplicate. Transfection was conducted after the confluence of cells per well to reached 80-90%. The transfection was done according to instructions for the Lipofectamine 3000 reagent. Each well contained 125 pmol of siRNA, 10 μl of P3000 reagent, and 5 μl of lipofectamine 3000 reagent. After transfection, cells were incubated again in a 5% CO_2 incubator at 37°C for 48 hours. The culture medium was siphoned off and 100 μl of CCK-8 working solution (containing CCK-8 reagent 10 μl) was added to each well. A blank solution (containing CCK-8 working solution without cells) was also set up and incubated at 37°C in a 5% CO_2 incubator for two hours. The absorbance was measured using a microplate reader at 450 nm.

Table 1. — *Fragment sequences of siRNAs.*

siRNA name	Fragment sequences
Tspan5/464	GUCAGUUGUUGCAUCAAAUTT
	AUUUGAUGCAACAACUGACTT
Tspan5/796	CCAGCUGUAUUUCUUUAUATT
	UAUAAAGAAAUACAGCUGGTT
Tspan5/1005	CCGCAGAAGAUGUCAUCAATT
	UUGAUGACAUCUUCUGCGGTT
Negative control (NC)	UUCUCCGAACGUGUCACGUTT
	ACGUGACACGUUCGGAGAATT
GAPDH positive control	UGACCUCAACUACAUGGUUTT
	AACCAUGUAGUUGAGGUCATT

Several horizontal lines were first drawn on the back of the six-well plates with a marker pen. The 2×10^5 JEG-3 cells were plated 24 hours at 37°C, 5% CO_2 incubator on six-well cell culture plates; the same sample in three wells. Transfection was conducted after confluence of cells per well to reached 80-90%. The transfection was done according to instructions for the lipofectamine 3000 reagent. Each well contained 125 pmol of siRNA, 10 μl of P3000 reagent, and 5 μl of lipofectamine 3000 reagent. After transfection, cells were incubated again in a 5% CO_2 incubator at 37°C for 48 hours. After scratching a center line perpendicular to the marks with sterile pipette tip, the cell culture medium was siphoned off, and washed three times with PBS, 2 ml of serum-free medium was added to each well, the width of the scratch was photographed with a fixed-focus camera at ×4 eyepiece, ×10 objective lens, and recorded as "D0h". The cells were placed back in the 5% CO_2 incubator at 37°C and photographs were taken every 12 hours, with results being recorded as D12h, D24h, and so on. The width of the scratches (cm) were measured and the results were calculated.

The SPSS18 software was used for statistical analysis and the data were presented as mean ± standard deviation (X±S). All measurement data were analyzed by *t*-test and $p < 0.05$ was considered statistically significant.

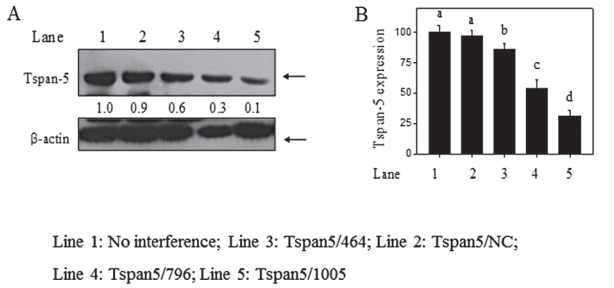
Result

Tspan5/NC band gray value was highest, Tspan5/1005 lowest, the relative expression gray level from low to high was Tspan5/1005 < Tspan5/796 < Tspan5/464 < Tspan5/NC, so Tspan5/1005 was chosen as an interference group to finish follow-up experiments (Figure 1).

Compared to the control group (Tspan5/NC), the cell proliferation activity of interference group (Tspan5/1005) (1.076 ± 0.028) was decreased significantly ($p < 0.01$) (Figure 2).

In the absolute value of the scratch width 24 hours after scratching minus the scratched width 0 hours after scratching, the interference group (Tspan5/1005) (1.27±0.03cm) was significantly less than the control group (Tspan5/NC) (1.97±0.38cm) ($p < 0.05$) (Figure 3).

Homogenized cell lysate from each group were prepared



Line 1: No interference; Line 3: Tspan5/464; Line 2: Tspan5/NC;
Line 4: Tspan5/796; Line 5: Tspan5/1005

Figure 1. — Tspan-5 expression quantity.

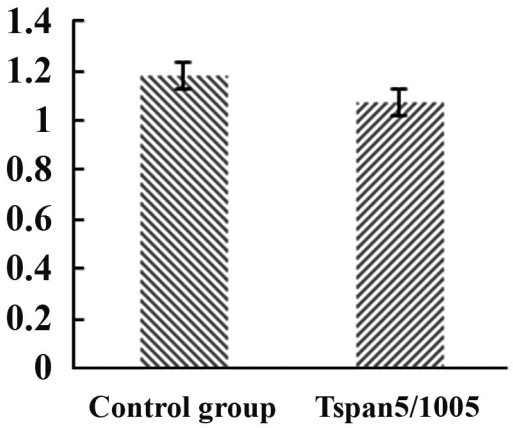
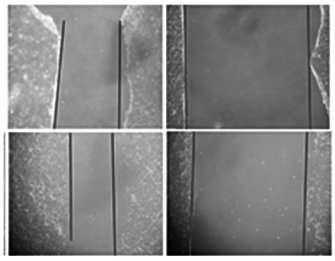


Figure 2. — Cell proliferation assay (OD_{450}).



Left: Control group
Right: Tspan5/1005
Upper, Lower were 0h & 24h after interference respectively

Figure 3. — Scratches' width at 0 and 24 hours after interference.

for Western blotting of expression levels of phosphorylated AKT (p-AKT) and p-FAK. The authors also found that Tspan5/1005 moderately decreased FAK and AKT phosphorylation compared to the control group (Figure 4).

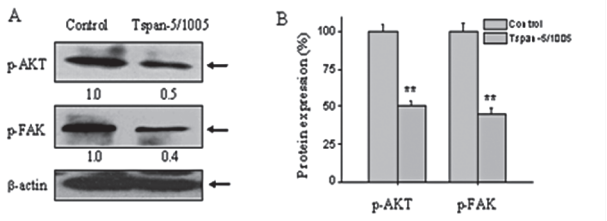


Figure 4. — Tspan5/NC bands density.

The results also showed that the density of the Tspan5/NC bands was high while the Tspan5/1005 was low, and the expression level in ascending order of each protein was as follows: Tspan5/1005 < Tspan5/796 < Tspan5/464 < Tspan5/NC. The Tspan5/1005 was used as the interference group in the following experiments as it had the highest interference efficiency.

Compared with the group of Tspan5/NC, the cell proliferation activity of Tspan5/1005 had significantly decreased, with the difference being statistically significant ($p < 0.01$).

Based on the absolute values of subtracting the width at the time when the scratch was created from the width of the scratch after 24 hours, the Tspan5/1005 had a lower value than the one from Tspan5/NC with a statistical significance of $p < 0.05$. The expression of p-AKT and p-FAK in Tspan5/1005 was also significantly lower than that in the control group.

Discussion

GTD, which belongs to a group of diseases that stems from placental trophoblasts, includes hydatidiform mole, invasive hydatidiform mole, choriocarcinoma, and placental site trophoblastic tumor, of which the last three are also collectively known as gestational trophoblastic neoplasia (GTN). Choriocarcinoma is highly malignant with metastasis occurring early and extensively. It had a mortality rate of nearly to 100% before the advent of chemotherapy drugs[10, 11]. Although post-chemotherapy prognosis has seen great improvement over the years, relapses or metastases still occur due to chemotherapy resistance. Although the pathogenesis of choriocarcinoma is currently unclear, its ongoing study can serve as an important guide in clinical treatments.

TM4SF is widely distributed in almost all animal cells and tissues except red blood cells and its extracellular loops can interact with other proteins to form protein-protein complex structures that play an important role in the regulation of cell development, activation, proliferation, and adhesion [12]. TM4SF can raise the cell signal on the cell membrane into the protein-protein complex structure and induce intracellular signaling molecules (such as PI4K and PKC) to move closer to the integrins, thereby linking the

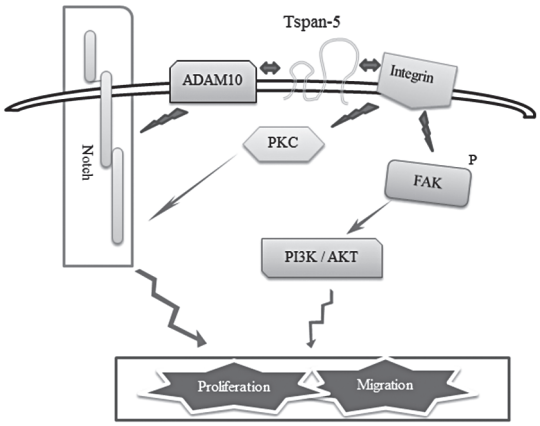


Figure 5. — Proposed signalling pathway.

extracellular integrin alpha-chain and PKC to form TM4SF-PKC-integrin structures [13, 14], so that the invasion of trophoblast cells can be regulated. Studies have shown that PKC is a key factor in the modulation of the Notch signaling pathway and it can enhance its activity when Notch is activated. This can lead to an increase in Snail expression and a decrease in E-cadherin expression, thus promoting tumor cell infiltration and metastasis [15-18]. Therefore, TM4SF is able to modulate the activity of the Notch signaling pathway by forming the TM4SF-PKC-integrin structure and further regulate the invasiveness of tumor cells. Through immunoprecipitation, researchers have also found that Tspan5 and a disintegrin and metalloproteinase (MMP) domain-containing protein 10 (ADAM10) interact via their extracellular regions. Tspan5 can promote ADAM10 maturation [19, 20] and the extracellular domain of the Notch receptor was catalyzed and Notch was activated, and eventually acts through the activated Notch signaling pathway [21]. Tspan5 is also a type transmembrane protein that belongs to one of the old, evolutionarily conservative transmembrane 4 superfamily (TM4SF). This protein family is widely found in a variety of organisms [22-24] and it plays a vital biological role in cell growth, development, migration, and differentiation [25].

In this study, the RNAi technique was used to silence and downregulate the expression of the Tspan5 gene found in the choriocarcinoma cell line JEG-3. The colony forming test, cell proliferation assay, and scratch assay were conducted to detect cell growth, proliferation, and migration. Results showed that the ability of JEG-3 cells to grow, proliferate, and migrate had decreased after interference. This suggests that a decrease in Tspan5 expression can suppress the growth, proliferation, and migration of choriocarcinoma cells.

Unfettered proliferation and migration are important biological characteristics of malignant tumor cells and are also the leading causes of tumor development and growth. This signifies that tumor cells are able to continually metastasize and invade normal tissues and organs in surrounding areas. Additionally, tumor cells are able to metastasize distantly, which signifies they can access nutrients and damage normal tissue structure, leading to a deterioration of bodily functions, loss of body parts and organs, or even death. The results from the present research prove two points. First, the expression level of Tspan5 is positively correlated with the proliferation and migration of choriocarcinoma cells, and it is expected to be a marker of GTD. Second, the proliferation and migration of choriocarcinoma cells can be reduced if the expression level of Tspan5 is downregulated, which signifies that there is a potential treatment for GTD in the future. Cytoplasmic protein tyrosine kinase (FAK) plays an important role in cell proliferation, survival, and migration, and is associated with integrin-mediated signal transductions [26]. Integrin-mediated activation of FAK leads to the phosphorylation of tyrosine 397 (Tyr397), which creates a PI3K binding site [27]. Previous studies have shown that downstream PI3K/AKT signaling pathway can be activated by integrins or growth-factor stimulation via FAK, resulting in the activation or overexpression of pre-metastatic proteins, such as matrix MMPs, urokinase-type plasminogen activator (uPA), and vascular endothelial growth factor (VEGF) [26, 28]. After the Tspan5 was silenced, the expression levels of FAK and AKT fell significantly (Figure 4), indicating that Tspan5 could have some biological effects through the FAK/AKTFAK/AKT signaling pathway (Figure 5). In conclusion, Tspan5 has the ability to influence the migration of cancers, such as multiple myeloma [7], colorectal cancer [8], and choriocarcinoma [29]. The present authors detected the inhibitory effects of Tspan5 on JEG-3 cells in order to create reliable in vitro data. The results showed that Tspan5 inhibited the growth of JEG-3 cells by suppressing the phosphorylation of FAK and AKT.

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