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The effect of RNAi silencing Tspan5 expression on the proliferation and migration of human choriocarcinoma cell line JEG-3

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Summary

Objective: To study the effect of Tspan5 on the proliferation and migration of trophoblast cell, seeking a new target for the treatment of gestational trophoblastic disease. *Materials and Methods:* The authors used RNAi to silence the Tspan5 expression in human choriocarcinoma cell line JEG-3 first, and then used Western blotting to detect the expression of Tspan5, calculating the efficiency of interference. CCK-8 test, colony formation, and scratch assay were used to detect the proliferation and migration of the before and after interference JEG-3 cells. *Results:* Western blotting results showed that Tspan5/NC band gray value was highest and Tspan5/1005 lowest, so the authors chose Tspan5/1005 as an interference group to finish the follow-up experiment. Interference group (Tspan5/1005) clone number of JEG-3 cell was significantly reduced compared with the control group (Tspan5/NC) (148±23 *vs.* 276±31, $p < 0.01$). There was no significant difference between blank group and control group (328±39 *vs.* 276±31, $p > 0.1$). The JEG-3 cell proliferation activity of blank group was the highest, the control group (Tspan5/NC) group's proliferation activity was slightly lower, however there was no significant difference (1.200 ± 0.103 *vs.* 1.185 ± 0.022, $p > 0.05$) between the two. Compared with the control group (Tspan5/NC), the JEG-3 cell proliferation activity of interference group (Tspan5/1005) decreased significantly (1.185 ± 0.022 *vs.* 1.076 ±0.028, $p < 0.01$). In the absolute value of the scratch width, 24 hours after scratching minus the scratch width 0 hours after scratching, the interference group (Tspan5/1005) was significantly less than the control group (Tspan5/NC) (1.27 ± 0.03 cm *vs.* 1.97 ± 0.38 cm, $p < 0.05$). *Conclusion:* In this study, the authors found that the proliferation and migration of choriocarcinoma cells were reduced after using RNAi technology to knockdown Tspan5 expression. The quantity of Tspan5 expression was positively related with proliferation and migration of choriocarcinoma cells.

Key words: Tspan5; Human choriocarcinoma cell line JEG-3; Proliferation; Migration; Transmembrane 4 superfamily; RNA interference.

Introduction

Trophoblast cells are the very peculiar cells, that differ from other cells in their histogenesis, development or biological behavior. After fertilization, fertilized egg moves to the uterus and begins cell division at the same time, and gradually forms into a morula, which continues to divide; a gap occurs between the peripheral cells and the interior cell, and is filled with the liquid, and is then called a blastocyst. The outer layer cells of the blastocyst can absorb nutrients directly from the mother for embryonic development, so called trophoblast. Trophoblast cells can be divided into trophoblasts (CT) and syncytiotrophoblasts (ST). STs participate in gas and nutrient exchange between mother and fetus, while shouldering the endocrine function of placental tissue [1]. ST secretes a variety of protein hormones, including hCG [2].

Extravillous cytotrophoblasts (EVCTs) are located at the tip of anchoring villi (AV) which fix the placenta to uterine wall. EVCTs are similar to tumor cells, which can proliferate to form multilayer cell columns and invade the decidua up to the upper-third of the myometrium. EVCTs also can invade the uterine spiral arteries and replace endothelial cells, but EVCT's invasion only occurs during early pregnancy [3]. This invasion is varied and is regulated by a variety of cytokines and can cause a range of diseases. For example, shallow invasion may lead to spontaneous abortion, pre-eclampsia, and fetal growth restriction [4, 5], and excessive invasion may cause gestational trophoblastic tumor.

Tetraspanin5 (Tspan-5), also known as TM4SF9 or NET-4, is a multiple transmembrane protein containing 268 amino acids, belonging to transmembrane 4 superfamily (TM4SF), encoded by Tm4sf9 gene, which is located at 4q23 in the human gene. TM4SF have total of 33 members in humans, and they are four times across the cell membrane to form two extracellular loops and a cytoplasmic loop, The large extracellular loop consisting of approximately 120 amino acids, plays a central role in biological effects [6]. Tspan-5 plays an important role in cellular signal transduction associated cell development, differentiation, growth, and migration. Studies have found that overexpression of TM4SF will affect the survival and invasion of multiple myeloma (MM) cells [7], and Tspan-5 can affect the metastatic ability of colorectal cancer [8]. Gao *et al.* found that Tspan-5 was mainly expressed in the

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EVCTs and hyperplasia cytotrophoblasts performing maternal-fetal invasion, with a degree of proliferation and invasive enhancement; the degree increases with the proliferation and invasiveness with enhancement. Tspan-5 expression gradually increases in normal villi, hydatidiform mole, invasive mole, and choriocarcinoma [9]. After using RNAi technology to reduce Tspan-5 expression, it was found that choriocarcinoma cell heterogeneity adhesion and motility were decreased [10]. This shows that the Tspan-5 expression quantity is positively correlated with trophoblast invasion ability; however whether Tspan-5 is also associated with trophoblast cell proliferation and migration ability still needs further study.

In this study, RNAi was used to silence the expression of Tspan-5 in human choriocarcinoma cell line JEG-3, while detecting the JEG-3 cell proliferation and migration before and after interference, in order to study whether Tspan-5 can affect the proliferation and migration of trophoblasts in order to seek a new target for the treatment of gestational trophoblastic disease.

Materials and Methods

Human choriocarcinoma JEG-3 cell line, was resurrected and subcultured. RNA interference was starting when the cells in good condition.

Tspan5 siRNA was designed and synthesized in a total of five siRNA fragment sequences as follows:

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

The 2×10^5 JEG-3 cells were plated for 24 hours at 37°C, 5% CO_2 incubator in a six-well cell culture plate, and the same occupied three wells. Observation of cells per well reached 80-90% confluence beginning transfection. The transfection was done according to the instructions of lipofectamine 3000. Each well contained siRNA 125 pmol, P3000 reagent 10μl, and lipofectamin 3000 reagent 5μl. After transfection, cells were placed at 37°C, 5% CO_2 incubator and incubated for 48 hours. Cells were washed three times with 4°C precooled phosphate buffer saline (PBS), adding an appropriate amount of RIPA lysis buffer (about 100 μl per well) five minutes later using a cell scraper to scrape cells and reagents to one side, ice bathed for 30 minutes, and pipetting repeatedly. Samples were collected at 4°C, 12,000 g centrifugation for 15 minutes; the supernatant was then collected, which was the total protein solution. Protein concentration was measured using the Bradford method, prepared in SDS-PAGE gel (10% separating gel, 5% stacking gel), calculating the volume of solution containing 40 μg protein samples as the sample volume, electrophoresis, and transmembrane. Placed in blocking solution of 5% skim milk, it was shakened slowly for one hour at room temperature. Placed at 4°C overnight in the antibody Tspan5 (diluted 500-fold) working solution, the unbound antibody was washed with 1×TBST at room temperature on the shaking bed three times, each for about five minutes. The secondary antibody was diluted 3,000 times with 1×TBST, incubated at room temperature for about 30 minutes, and free secondary antibody was washed with 1×TBST. Chemiluminescence, scanning, Image-j image processing software was used to analyze the optical density of the target band.

Logarithmic growth phase JEG-3 cells were fetched, cell surface was washed with PBS and digested with 0.25% trypsin, and pipetted into single cells. The cell suspension was diluted to an appropriate cell density (300 cells per dish), seeded in a Petri dish, while adding 10% FBS culture medium of 2 ml, and gently shaken, and adding the pre-configured siRNA/lipofectamin complex (containing siRNA 125 pmol, P3000 reagent 10μl, and lipofectamin 3000 reagent 5 μl). Placed at 37°C in a 5% CO_2 incubator for one week, the culture was terminated when there was a visible colony. After discarding the supernatant, it was gently washed twice with PBS, adding pure methanol 5 ml and fixed for 15 minutes, then discarded. Giemsa dye staining was added for 20 minutes, washed three times with PBS, and dried. Transparented with a grid affixed to the bottom of the dish, visual counting of the number of clones was performed.

The 5×10^4 JEG-3 cells were plated for 24 hours at 37°C in a 5% CO_2 incubator in 96-well cell culture plate, and the same sample occupied three wells. Observation of cells per well was performed until 80-90% of the beginning confluence transfection was reached. The transfection was done according to the instructions of lipofectamine 3000. Each well contained siRNA 5 pmol, P3000 reagent 0.4μl, and lipofectamin 3000 reagent 0.2μl. After transfecting, the cells were placed at 37°C in a 5% CO_2 incubator for 48 hours. After siphoning off the culture medium, 100μl of CCK-8 working solution was added to each well (containing CCK-8 reagent 10 μl), while setting a blank hole (with only CCK-8 working solution without cells), back in 37°C in 5% CO_2 incubator for two hours. The absorbance was measured using a microplate reader at 450nm.

First with a marker pen several horizontal lines were painted on the back of 6six-well plates. The 2×10^5 JEG-3 cells were plated for 24 hours at 37°C in a 5% CO_2 incubator in six-well cell culture plate, and the same sample occupied three wells. Observation of cells per well was performed until 80-90% confluence beginning transfection was reached. The transfection was done according to the instructions of lipofectamine 3000. Each well contained siRNA 125 pmol, P3000 reagent 10μl, and lipofectamine 3000 reagent 5μl. After transfection, cells were placed at 37°C in a 5% CO_2 incubator for 48 hours. A center line was scratched perpendicular to the marks with sterile pipette tip, while siphoning off the cell culture medium, washed three times with PBS, and added serum-free medium 2 ml to each well. The scratch width was photographed with fixed focus camera at ×4 eyepiece and ×10 objective lens, and recorded as "D0h". The cells were placed back to 37°C in a 5% CO_2 incubator, and photographs were taken once every 12 hours, and recorded the results as D12h and D24h. The scratch width was measured in cm and the results were calculated.

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

Table 1. — *Tspan-5 expression quantity gray value of different siRNA interferences.*

	Tspan5/NC	Tspan5/464	Tspan5/796	Tspan5/1005
Tspan-5	11150.78	8656.76	5701.93	3192.45
beta-actin	38562.64	34328.85	35452.15	35955.42
Tspan5/beta-actin	0.29	0.25	0.16	0.09

This result show that Tspan5/NC band gray value was highest and Tspan5/1005 was lowest. It indicates that Tspan5/1005 has the strongest interference, so Tspan5/1005 was chosen as an interference group to conclude the follow-up experiments.

Table 2. — *Comparison of the blank and control groups during cell proliferation and migration.*

	Plate colony formation assay (Clone number)	Cell proliferation assay (OD450)	Scratch assay outcome (cm) $\lvert W_{24h}—W_{0h}\rvert$
Blank group	328±39	1.200±0.103	2.82±0.78
Control group (Tspan5/NC)	276±31	1.185±0.022	1.97±0.38
p value	0.15	0.786	0.165

W_{0h}: scratch width at 0h; W_{24h}: scratch width at 24 hours after interference. $\lvert W_{24h}—W_{0h}\rvert$: the absolute value of W_{24h} subtract W_{0h}
From Table 2, it is seen that there are no significant differences between blank and control groups according to clone number, cell proliferation activity, and the absolute value of the scratch width 24 hours after scratching minus the scratch width 0 hours after scratching. These outcomes indicate that interference technology did not affect the cell's proliferation and migration of JEG-3.

Table 3. — *Comparison of the interference and control group in cell proliferation and migration.*

	Plate clony formation assay (Clone number)	Cell proliferation assay (OD450)	Scratch assay outcome (cm) $\lvert W_{24h}—W_{0h}\rvert$
Interference group (Tspan5/1005)	148±23	1.076±0.028	1.27±0.03
Control group (Tspan5/NC)	276±31	1.185±0.022	1.97±0.38
P velue	0.005	0.001	0.034

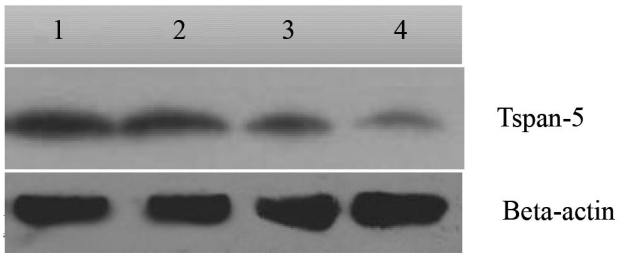
W_{0h}: Scratch width at 0h; W_{24h}: scratch width at 24 hours after interence. $\lvert W_{24h}—W_{0h}\rvert$: the absolute value of W_{24h} subtract W_{0h}
Table 3 shows that the clone number, cell proliferation activity, and the absolute value of the scratch width 24 hours after scratching minus the scratch width 0 hours after scratching of interference group are all \reduced, compared with the control group. Therefore the proliferation and migration of choriocarcinoma cells were reduced after silencing Tspan-5 expression.

Results

Western blotting showed that Tspan5/NC band gray value was highest, Tspan5/1005 was lowest, and the relative expression gray level from low to high was Tspan5/1005 <Tspan5/796 <Tspan5/464 <Tspan5/NC, therefore the authors chose Tspan5/1005 as an interference group to conclude the follow-up experiment (Table 1 and Figure 1).

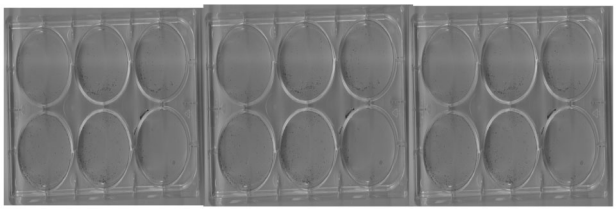
Interference group (Tspan5/1005) clone number of JEG-3 cell was significantly reduced compared with the control group (Tspan5/NC) (148 ± 23 *vs.* 276 ± 31, $p < 0.01$). There was no significant difference between blank and control groups (328 ± 39 *vs.* 276 ± 31, $p > 0.1$) (Tables 2 and 3, Figure 2)

The JEG-3 cell proliferation activity of blank group was the highest and the control group's (Tspan5/NC) prolifera-



Line 1: Tspan5/NC Line 3: Tspan5/796
Line 2: Tspan5/464 Line 4: Tspan5/1005

Figure 1. — Western blotting detects Tspan-5 expression quantity. Tspan5/NC band gray value is highest and Tspan5/1005 the lowest, and the relative expression gray level from low to high is Tspan5/1005 <Tspan5/796 <Tspan5/464 <Tspan5/NC.



Left : Blank group
Mid : Control group (Tspan5/NC)
Right : Interference group (Tspan5/1005)

Figure 2. — Plate colony formation assay.
Interference group (Tspan5/1005) clone number (148 ± 23) is significantly reduced ($p < 0.01$), compared with the control group (Tspan5/NC) (276 ± 31). There is no significant difference between blank group (328±39) and control group ($p > 0.1$)

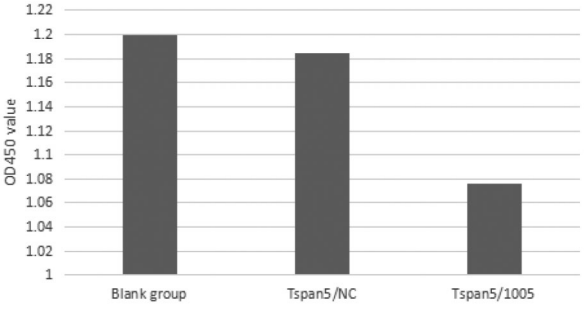


Figure 3. — CCK-8 cell proliferation assay.
The cell proliferation activity of blank group (1.200 ± 0.103) is the highest and the control group (Tspan5/NC) cell proliferation activity (1.185 ± 0.022) is slightly lower; however there is no significant difference ($p > 0.05$) between the two. Compared with the control group (Tspan5/NC), the cell proliferation activity of interference group (Tspan5/1005) (1.076 ± 0.028) decreased significantly ($p < 0.01$)

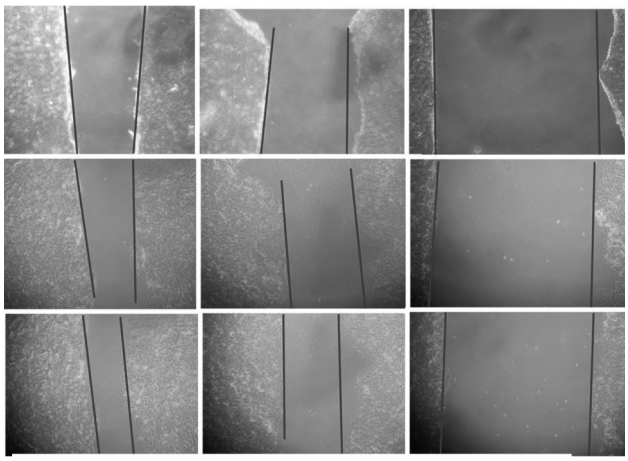


Figure 4. — Scratch cell migration assay.
In absolute value of the scratch width 24 hours after scratching minus the scratch width 0 hours after scratching, the interference group (Tspan5/1005) (1.27 ± 0.03 cm) is significantly less than the control group (Tspan5/NC) (1.97 ± 0.38cm) ($p < 0.05$)

tion activity was slightly lower; however there was no significant difference (1.200 ± 0.103 *vs.* 1.185 ± 0.022, $p > 0.05$) between the two. Compared with the control group (Tspan5/NC), the JEG-3 cell proliferation activity of interference group (Tspan5/1005) decreased significantly (1.185 ± 0.022 *vs.* 1.076 ± 0.028, $p < 0.01$) (Tables 2 and 3, Figure 3)

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) was significantly less than the control group (Tspan5/NC) (1.27 ± 0.03 cm *vs.* 1.97 ± 0.38 cm, $p < 0.05$) (Tables 2 and 3, Figure 4)

Discussion

Gestational trophoblastic disease (GTD) is a group of diseases derived from placental trophoblasts, including mole, invasive mole, choriocarcinoma, and placental site trophoblastic tumor (PSTT). While invasive mole, choriocarcinoma, and placental site trophoblastic tumor are collectively referred to as gestational trophoblastic tumor (GTNs). Choriocarcinoma, with the highest degree of malignancy, early and extensive metastasis, secondary from non-molar and molar pregnancies, is one of the common gynecological malignancies, before the advent of chemotherapy, with a mortality rate of more than 90% [11]. Despite the fact that prognosis of choriocarcinoma has made great progress over the years with the application of combination chemotherapy, there are still some high-risk patients because resistance cannot be alleviated or recurrence and metastasis can still occur after remission. At present, the pathogenesis of choriocarcinoma it is not clear; its pathogenesis research clearly has an important guiding significance for clinical treatment.

TM4SF is widely distributed in almost all animal cells and tissues except in red blood cells; the large extracellular loops which may interact with other proteins to form protein - protein complex, plays an important role in the biological effects [12]. TM4SF can recruit cell signaling at the cell membrane into the protein - protein complexes, it can induce intracellular signaling molecules (such as PI4K, PKC, etc.) close to integrins as well, thereby directly connecting the extracellular integrin α chain and PKC, forming TM4SF-PKC-integrin complex [13, 14], and thus involved in the regulation of trophoblast invasion. One study found that PKCζ was a key factor modulating Notch signaling pathway, could enhance Notch activity when it is activated, and then can increase the expression of Snail so that E-cadherin expression decreases, while promoting tumor cell invasion and metastasis [15-18]. Therefore, TM4SF can modulate the activity of the Notch signaling pathway by forming TM4SF-PKC-integrin complexes, while further adjusting the invasiveness of tumor cells. In addition, some scholars have found that interactions exist between Tspan-5 and A disintegrin and metalloprotease 10 (ADAM10) with their extracellular region by co-immunoprecipitation technique. Tspan-5 can promote ADAM10 maturity [19, 20], catalyze extracellular domain of Notch receptor shedding, and activate Notch, and finally plays a role by activating Notch signaling channels [21]. Tspan5 is one of the transmembrane protein belonging to the ancient, evolutionarily conserved relative transmembrane 4 superfamily (TM4SF); the family is widely present in a variety of biology [22-25], and plays an important biological role in cell growth, development, migration, and differentiation [26].

In this study, RNAi technology was directly used to silence Tspan-5 gene in choriocarcinoma cell line JEG-3, decrease the expression of Tspan-5, and then detect the growth, proliferation, migration capacity after interference by plate colony formation assay, and CCK-8 cell proliferation assay and scratch assay. The present authors found that after the interference JEG-3 cell's growth, proliferation and migration were reduced. Tspan-5 affected the growth, proliferation, and migration of choriocarcinoma cell, the choriocarcinoma cell's growth, proliferation and migration were suppressed after Tspan-5 expression decreased.

Unlimited proliferation and migration of malignant tumor cells is mainly due to the development of cancer, which is the important biological characteristics of tumor cells. Because tumor cells have the ability of a relatively indefinite proliferation, cause the tumor to continue to grow and then increase oppression to normal tissues and organs surrounding the tumor; meanwhile cancer cells have the ability of local invasion and distant metastasis; tumor cell invasion can surrounding or organize at a distant while acquiring nutrients and destroying normal tissue structure. Thus the body's normal function is damaged, lost, or even

results in death.

Research confirms that the expression levels of Tspan-5 affects the proliferation and migration of choriocarcinoma cell, which at least shows two aspects: first, the expression of Tspan-5 is positively correlated with the proliferation and migration of choriocarcinoma cell, the larger Tspan-5's expression, the higher degree of malignancy; thus Tspan-5 is expected to become the marker of GTD. Second, if we can attempt to reduce the expression level of Tspan-5, we can therefore also reduce the proliferation and migration ability of choriocarcinoma cells, which provides a new target for future treatment of GTD.

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