Effects of Wnt5a protein on proliferation and apoptosis in JAR choriocarcinoma cells

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Abstract. Placental choriocarcinoma is a highly malignant tumor of the female reproductive organs. Even with chemotherapy, placental choriocarcinoma has a 20% mortality rate due to its indistinct pathogenesis and the absence of efficient therapeutic methods for this cancer. Wnt proteins and Wnt signaling are involved in a variety of developmental and cellular processes, and aberrant activation of Wnt signaling is linked to carcinogenesis in many types of tissue. In the present study, using immunostaining and reverse transcription PCR, we found that Wnt5a was prominently expressed in human primary cytotrophoblast cells, but absent in the human choriocarcinoma cell line JAR. This implies that there is a previously unknown correlation between Wnt5a and placental choriocarcinoma. Furthermore, we found that the addition of exogenous recombinant Wnt5a protein to the JAR cells repressed their proliferation and induced apoptosis, indicating that Wnt5a has a tumor suppressive effect in placental choriocarcinoma. In conclusion, the results of the present study suggest that there is a correlation between Wnt5a and human placental choriocarcinoma; therefore, Wnt5a has a potential use in the treatment of this malignancy.

Introduction

The first requirements for embryonic development are implantation in the uterus and the formation of hemachorial placenta, in which trophoblast cells enable embryo invasion deep into the stroma through the uterine epithelium (1). This process is strictly regulated in a temporal and spatial pattern. Too shallow or excess invasion may result in pregnancy abnormalities, such as preeclampsia or placental choriocarcinoma (2).

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Placental choriocarcinoma is a malignant cancer of the reproductive system. Even with chemotherapy, it has a mortality rate of 20% due to its indistinct pathogenesis and the absence of efficient therapeutic methods.

Wnts are secreted as glycosylated lipid-modified cysteine-rich proteins (3) and play crucial roles in embryonic development, morphogenesis, cell proliferation, differentiation and migration (4-6). Wnt signaling includes the canonical (Wnt/β-catenin, CTNNB1) and non-canonical (Wnt/Ca^{2+}) pathways (7,8). A less described Wnt pathway is the Wnt-JNK pathway (9). It has been suggested that Wnt5a, which increases intracellular Ca^{2+} levels through the activation of protein kinase C and Ca^{2+}/calmodulin-dependent kinase II, operates through the Wnt/Ca^{2+} signaling pathway (10,11).

In numerous previous studies, increased β-catenin signaling in tumorigenesis was found to be accompanied by elevated transcription of the genes involved in cell proliferation (12). However, the function of non-canonical Wnt/Ca^{2+} signaling in cancer has not been elucidated as thoroughly. Recent studies suggest that Wnt5a is related to the aggressiveness of certain malignancies, such as prostate and colorectal cancer (13,14). However, its exact role during the carcinogenesis of placental choriocarcinoma remains unknown. In this study, we examined the expression of Wnt5a in primary human cytotrophoblast (CTB) cells and in the placental choriocarcinoma cell line JAR. Wnt5a was found to be expressed in CTB cells, but absent in JAR cells. Furthermore, the addition of recombinant Wnt5a protein was found to suppress proliferation and promote serum starvation-induced apoptosis in JAR cells. This suggests that Wnt5a has a tumor suppressive effect on placental choriocarcinoma, which may function through the inhibition of the canonical Wnt/β-catenin signaling pathway.

Materials and methods

Human sample collection and primary CTB cell isolation. Samples of normal first trimester placental tissue were obtained from the Maternal and Child Health Hospital of Shaanxi Province after termination of pregnancy between 8 and 12 weeks of gestation. Official consent was provided by the patients and the project was approved by the ethics committee of Northwest A&F University.

A modified method described by Xu *et al* (15) was used to isolate and culture CTB cells. Briefly, human chorionic

villi tissues were digested with 0.25% trypsin for 1 h at 4˚C and with 15 IU/ml DNase I (Sigma, St. Louis, MO, USA; all reagents from Sigma unless otherwise specified) for 15 min at room temperature. The cells were plated at 1 to $2x10^5$ cells/well in 24-well plates coated with collagen I, and cultured in serum-free FD medium containing 1:1 Ham F12 (Gibco, Grand Island, NY, USA) and Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 ng/ml epidermal growth factor, 10 μg/ml insulin, 0.1% BSA, 1.75 mM Hepes and 2 mM glutamine. The cells were then incubated at 37˚C, 5% CO_2 in a humidified incubator in order to yield CTB cells with a high purity, determined as cells positively stained for cytokeratin and gonadotropin releasing hormone (GnRH), and negative for vimentin (Fig. 1). JAR, a human choriocarcinoma cell line, was cultured in DMEM supplemented with 10% fetal bovine serum at 37˚C, 5% CO_2.

Total RNA extraction and semi-quantitative RT-PCR. Total RNA was extracted using TRIzol Reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was dissolved in 20 μl nuclease-free water. RT-PCR was performed according to a coupled one-step procedure using the Access RT-PCR System (Promega, Madison, WI, USA). Briefly, 2 μg of total RNA was reverse transcribed at 37˚C for 1 h, denatured at 94˚C for 2 min and amplified for 30 cycles of denaturation at 94˚C for 30 sec, primer annealing at 55˚C for 30 sec and extension at 72˚C for 45 sec, with a final extension step of 10 min at 72˚C. The amplified products were analyzed by electrophoresis on a 1% agarose gel. Images of the RT-PCR ethidium bromide-stained agarose gels were acquired with a High Performance CCD camera. β-actin was used as an internal control. The sequences of specific primers were as follows: Wnt5a, F: 5' CGAAGACAGGCATCAAAGAA 3', R: 5'-GCAAAGCGGTAGCCATAGTC-3'; β-actin, F: 5'-TGG AATCCTGTGGCATCCATGAAAC-3', R: 5'-TAAAACGCA GCTCAGTAACAGTCCG-3'.

Immunofluorescence. Cells were fixed in 4% paraformaldehyde and blocked with 5% BSA before incubation at 4˚C overnight with goat anti-Wnt5a (AF645; R&D Systems Inc.), rabbit anti-β-catenin (RB-1491-P0; NeoMarkers Biotechnology, Fremont, CA, USA), rat anti-vimentin antibody (MAB2105; R&D Systems Inc.), goat anti-GnRH antibody (sc-21140; Santa Cruz) or goat anti-cytokerin (sc-17091; Santa Cruz). Then, the cells were incubated in corresponding FITC-conjugated secondary antibody (Zhongshan Biotechnology, Beijing, China) at a dilution of 1:100 in PBS for 1 h at 37˚C. Nuclei were stained with 10 μg/ml propidium iodide (PI) for 10 min and viewed under a laser scanning confocal microscope (Leica, Heidelberg, Germany). As a negative control, parallel experiments were performed with cells using pre-immune goat or rabbit serum.

Cell proliferation analysis. JAR cells were plated in 6-well plates 24 h before BrdU incorporation analysis. Recombinant mouse Wnt5a (mWnt5a; 645-WN; R&D Systems Inc.) at concentrations of 0, 100, 200 and 400 ng/ml were added to the medium. To label cells in the S-phase of the cell cycle, 40 μM BrdU (10280879001; Roche, Indianapolis, IN, USA) was added to the medium for 4 h before analysis. The cells were subsequently harvested and fixed in 100% cold methanol for 10 min, washed two times in PBS, incubated in 2 M HCl for 45 min to denature the DNA, washed in PBS again and finally neutralized with 0.1 M $Na_2B_4O_7$ for 30 min. After several washes in PBS, the cells were incubated with anti-BrdU antibody (ZM0013; Zhongshan Biotechnology), then the appropriate FITC-labelled secondary antibody (ZF-0312; Zhongshan Biotechnology) was added, followed by an additional incubation for 45 min at 37˚C. Staining to determine the number of cells was performed by the addition of 10 μg/ml PI followed by incubation for 10 min at room temperature, and was visualized using a confocal microscope (Leica). Cells were quantified by counting the number of BrdU-positive cells as PI-positive cells in 5-8 alternative areas. Each experiment comprised at least three independent cultures.

Cell growth curve. For the analysis of cell growth, JAR cells were plated at $1x10^4$ in each of the 24-well plates and cultured in medium containing 0.5% FBS, with or without mWnt5a treatment. Viable cells (JAR and JAR-mWnt5a) were counted from days 1-7 using a hemocytomer. The results were obtained from three independent cultures.

Apoptosis analysis. Twenty-four hours after mWnt5a treatment, cells were harvested by trypsin and stained using an Annexin V FITC Apoptosis Detection kit (Biosea Biotechnology Co. Ltd., Beijing, China) according to the manufacturer's protocol. Stained cells were immediately analyzed by flow cytomtery (FACSCalibur; BioRad, USA). Apoptotic cells with exposed phosphatidylserine bound to Annexin V-FITC. Each experiment was repeated at least three times.

Statistical analysis. Values were presented as the mean ± SEM. The data were analyzed using one-way ANOVA as appropriate. P-values <0.05 were considered statistically significant.

Results

Enrichment of CTB cells in vitro. More than 30 human samples were acquired, and isolated CTB cells were assessed as cells positively stained for cytokeratin and GnRH, and negative for vimentin (Fig. 1).

Abnormal expression of Wnt5a in human choriocarcinoma cell line JAR. JAR cells were selected as a model of human placental choriocarcinoma, and established from a trophoblastic tumor of the placenta of a 24-year-old Caucasian woman (16). The expression level of Wnt5a in both the CTB and JAR cells was analyzed by RT-PCR and immunofluorescence. Wnt5a was found to be expressed in CTB cells, but was not detected in JAR cells (Fig. 2). This indicates that Wnt5a had an unknown effect on placental choriocarcinoma.

Effects of Wnt5a on the proliferation and growth of JAR cells. The proliferation of the JAR cells was investigated through BrdU incorporation after treatment with various concentrations of mWnt5a. mWnt5a at a concentration of 100 ng/ml was found to decrease the proliferation of JAR significantly compared to control (Fig. 3), while 200 ng/ml mWnt5a had an even greater inhibitory effect on JAR proliferation. However,

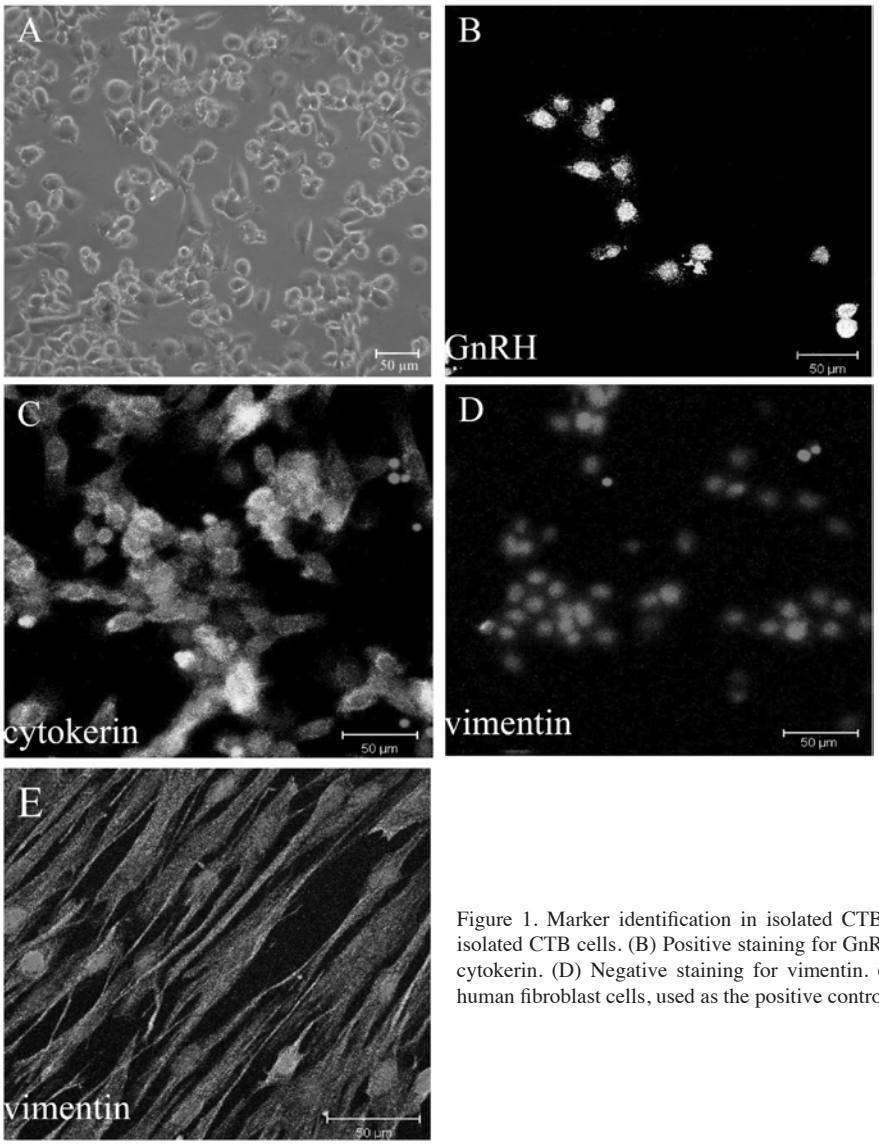

Figure 1. Marker identification in isolated CTB cells. (A) Morphology of isolated CTB cells. (B) Positive staining for GnRH. (C) Positive staining for cytokerin. (D) Negative staining for vimentin. (E) Vimentin expression in human fibroblast cells, used as the positive control. Bars, 50 μm.

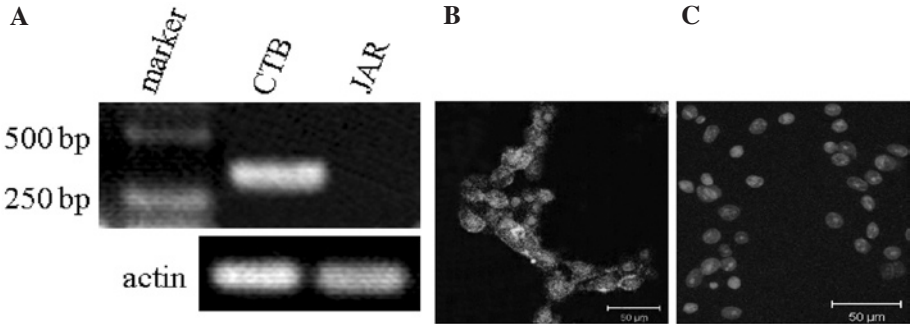

Figure 2. Wnt5a expression in JAR cells, but not in primary CTB cells. (A) Analysis of Wnt5a expression in CTB and JAR cells by PCR electrophoresis. (B) Positive staining of Wnt5a in primary CTB cells. (C) Negative staining of Wnt5a in JAR cells. Bars, 50 μm.

the differences observed between the effects of 200 and 400 ng/ml mWnt5a were not significant (Fig. 3).

To examine the effect of mWnt5a on cell growth, JAR cells were cultured in DMEM containing 0.5% FBS and 100 ng/ml mWnt5a for 7 days. The result indicated that mWnt5a suppressed the cell growth of JAR (Fig. 4). These results taken together suggest a possible anti-tumor function of Wnt5a.

Effects of Wnt5a on the apoptosis of JAR cells and the localization of β-catenin. In order to study the role of Wnt5a in serum starvation-induced apoptosis, JAR cells were cultured in DMEM containing 0.5% FBS and 100 ng/ml mWnt5a for 24 h. Wnt5a was found to markedly increase serum starvation-induced apoptosis in JAR cells compared to the control group without Wnt5a treatment (Fig. 5). This further supports

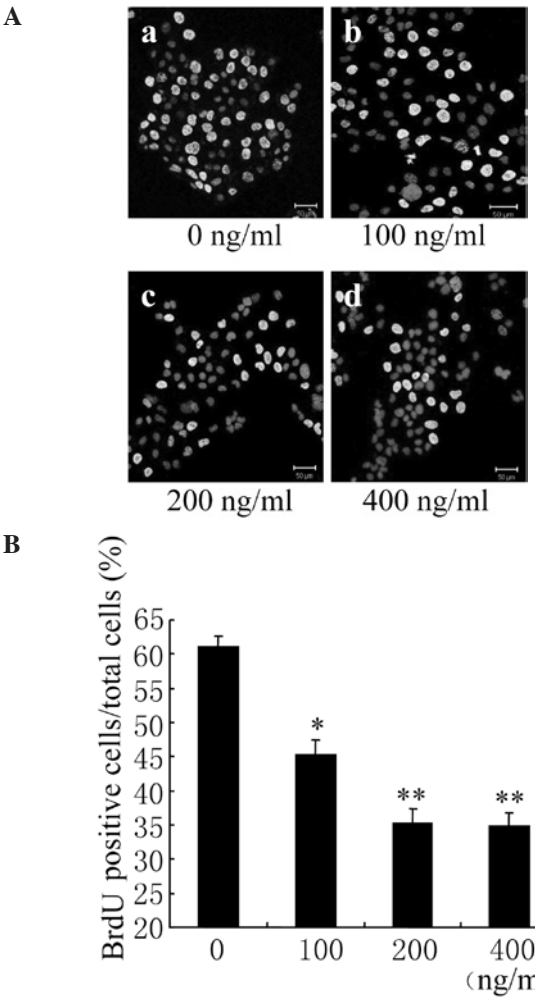

Figure 3. Effect of mWnt5a proteins on the proliferation of JAR cells. (A) Effect of recombinant mWnt5a at concentrations of (a) 0 ng/ml, (b) 100 ng/ml, (c) 200 ng/ml and (d) 400 ng/ml on the proliferation of JAR cells. (B) Quantitative measurement of the percentage of BrdU-positive cells. Analyses were performed three times in independent experiments, and cells were counted in five fields per case. *P<0.05, **P<0.01.

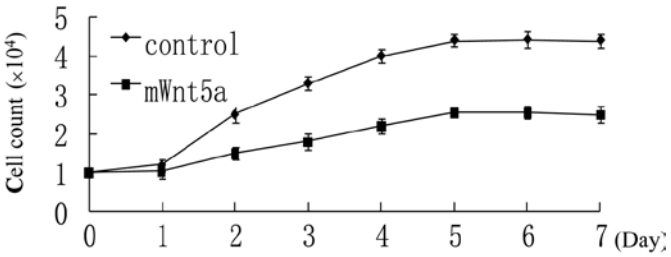

Figure 4. Effect of mWnt5a proteins on the growth of JAR cells. Viable cells were counted from days 1-7. JAR cells without Wnt5a treatment were used as a control. The number of viable cells was higher in JAR-mWnt5a than in JAR cells from days 2-6 (P<0.05).

our hypothesis that the carcinogenesis of placental choriocarcinoma may be correlated with the loss function of Wnt5a during pregnancy. Moreover, LiCl, a well-known glycogen synthase kinase-3 inhibitor, was found to promote the accumulation of β-catenin in the cytoplasm and nuclei of the JAR cells compared to the control cells (Fig. 6A and B). mWnt5a inhibited this LiCl-induced β-catenin accumulation (Fig. 6C). This suggests that Wnt5a may antagonize the canonical Wnt signaling pathway in JAR cells.

Discussion

The results of the present study suggest that Wnt5a suppresses the carcinogenesis of placental choriocarcinoma by decreasing the proliferation and promoting the apoptosis of human choriocarcinoma cells, possibly through the inhibition of the canonical Wnt signaling pathway.

The effects of Wnt5a on the modulation of cellular activity during vertebrate development have been thoroughly demonstrated (17). Recent studies indicate that Wnt5a may play a critical role in tumorigenesis, though this finding is controversial (13,18,19). In prostate and lung cancer, Wnt5a promoted tumor aggression (13,18), while in thyroid carcinoma it suppressed tumor activity (19). The exact function of Wnt5a during the carcinogenesis of placental choriocarcinoma hasnot previously been elucidated.

In the present study, we first observed the lack of Wnt5a expression in JAR cells compared to CTB cells, and hypothesized that the abnormal expression of Wnt5a during pregnancy contributes to the carcinogenesis of placental choriocarcinoma. To support this hypothesis, we examined whether Wnt5a treatment affects apoptosis and proliferation in JAR cells.

The role of Wnt5a in proliferation is complex. Wnt5a has been found to promote the proliferation of human glioblastoma (20) and pancreatic cancer cells *in vitro* (21), but to suppress the proliferation of B cells (22) and colorectal cancer cells (23). Based on these studies, the effects of Wnt5a on cell proliferation are dependent on cell type. The results of the present study revealed that recombinant mWnt5a protein inhibited the proliferation of JAR cells. This suggests that Wnt5a has tumor suppressor activity in human placental choriocarcinomas.

Although rapid progress has been made in elucidating the proliferative effects of Wnt5a, relatively little is known regarding its apoptotic or anti-apoptotic functions. Several recent studies have indicated that Wnt5a has anti-apoptotic action in specific cells, such as dermal fibroblasts and lung fibroblasts (24,25). In this study, we investigated whether Wnt5a causes the apoptosis of JAR cells. Unexpectedly, mWnt5a was observed to significantly promote serum starvation-induced apoptosis in the JAR cells. Since the deregulation of apoptosis has been found to be involved in cancer initiation (26), we speculate that Wnt5a may act as a tumor suppressor in human placental choriocarcinomas by stimulating the apoptosis and inhibiting the proliferation of the choriocarcinoma cells.

It is generally believed that Wnt5a acts though the non-canonical Wnt/Ca^{2+} signaling pathway (10,11). However, a few investigations have demonstrated that non-canonical Wnt ligands inhibit canonical Wnt signaling in transformed cell lines, *Xenopus* embryos and mouse embryos (27,28). Wnt5a has been shown to induce Siah2, a member of an E3 ubiquitin ligase complex that targets β-catenin for degradation, and Wnt5a-deficient mice exhibited increased levels of β-catenin in the distal hind limb (28). Wnt5a also inhibited canonical Wnt signaling downstream of β-catenin stabilization through the calcium-dependent activation of Nemo-like kinase (29), as well as other calcium-independent mechanisms (30). Generally, the

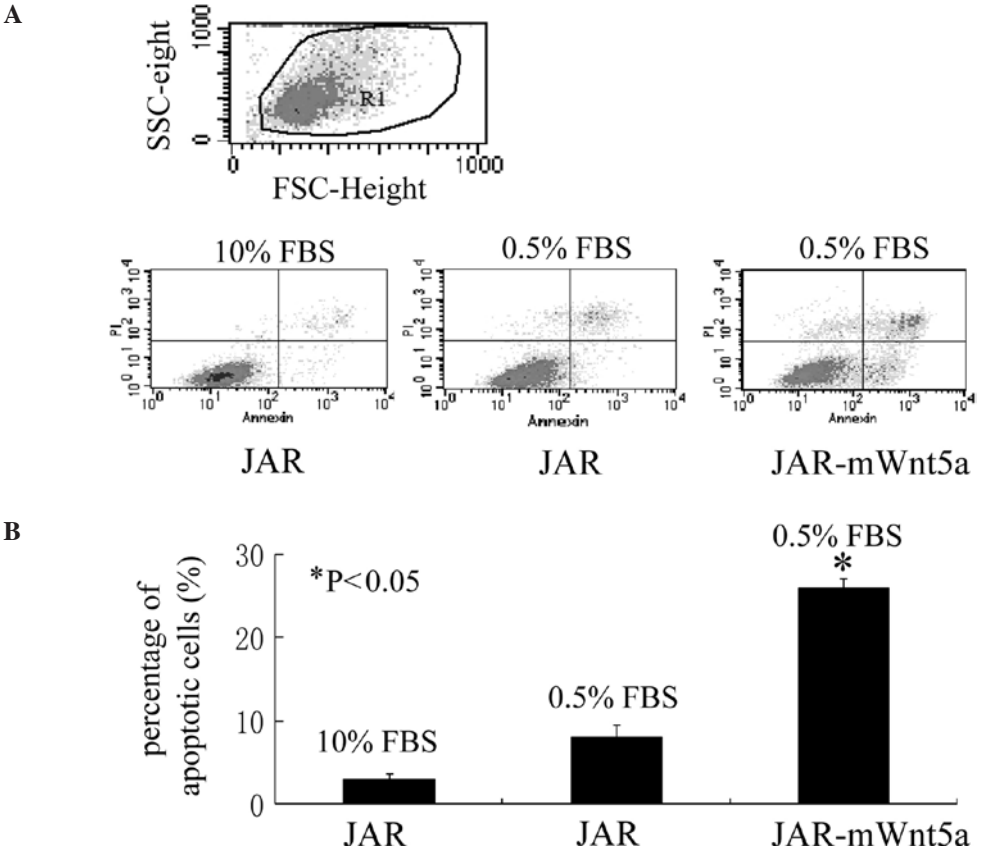

Figure 5. Effect of mWnt5a proteins on the apoptosis of JAR cells. The percentage of Annexin V-positive cells in the serum-starvation culture was examined to evaluate the effect of Wnt5a on the apoptosis of JAR cells. (A) Representative results of flow cytometric analysis of JAR cells in 10% FBS, 0.5% FBS, and 0.5% FBS with mWnt5a. (B) Quantitative measurement of the percentage of Annexin V-positive cells. Analyses were performed three times in independent experiments, and 10,000 cells were counted in each case. *P<0.05.

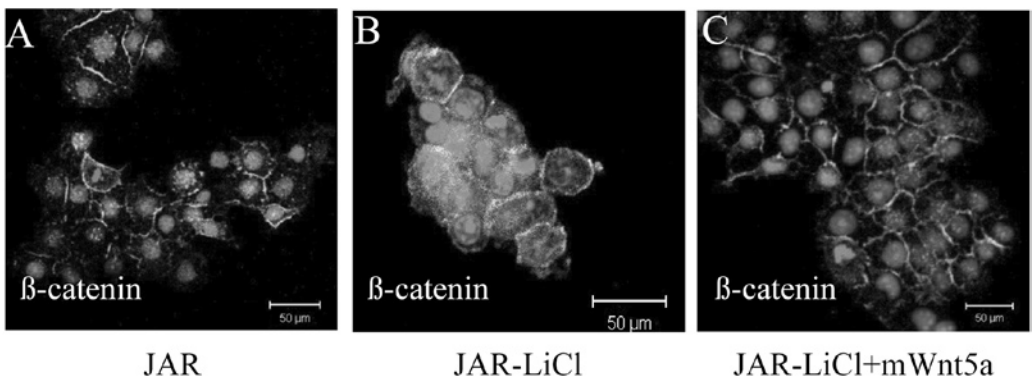

Figure 6. mWnt5a-induced inhibition of canonical Wnt/β-catenin signaling activated by LiCl in JAR cells. (A) β-catenin located in the membrane of JAR cells. (B) β-catenin translocated to the cytoplasm and nucleus of JAR cells after stimulation with 25 mM LiCl for 24 h. (C) β-catenin accumulation in the membrane of JAR cells after stimulation with 25 mM LiCl and 100 ng/ml mWnt5a for 24 h. Bars, 50 μm.

canonical Wnt pathway is considered activated when β-catenin is found expressed in the nuclei (31). Thus, although we did not examine changes in Ca^{2+} levels or the activation of PKC and CaMKII in JAR cells in our experiments, the absence of β-catenin in the cytoplasm and nucleus after Wnt5a treatment indicates that Wnt5a may suppress the activation of the canonical Wnt/β-catenin pathway in JAR cells (32). Whether Wnt5a dependent non-canonical Wnt/Ca^{2+} signaling is involved in Wnt5a regulation during the carcinogenesis of human placental choriocarcinoma requires further investigation.

In conclusion, in the present study, mWnt5a proteins not only inhibited cell growth, but also stimulated the apoptosis of human choriocarcinoma cells. This suggests that Wnt5a has a tumor suppressor effect on placental choriocarcinoma. The findings provide novel insight into the roles of Wnt signaling, and suggest the potential application of Wnt5a in the treatment of human placental choriocarcinomas. However, the exact molecular mechanisms of the effects of Wnt5a on the proliferation and apoptosis of JAR cells remain unclear, and require further investigation.

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