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Current Topic

Effect of 14-3-3 tau protein on differentiation in BeWo choriocarcinoma cells

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ABSTRACT

This study aimed to investigate the location and function of tau isoform of 14-3-3 proteins in human trophoblast. 14-3-3 tau was localized in human cytotrophoblast cells, but not in syncytiotrophoblast cells in both first trimester and term placenta by immunochemistry stain. Forskolin-induced cell fusion (BeWo cells) confirmed that 14-3-3 tau was decreased during trophoblast differentiation. Forskolin-induced differentiation was stimulated by small-interfering (si) RNA induced down-regulation of 14-3-3 tau, contrarily, it was suppressed by plasmid induced upregulation of 14-3-3 tau in BeWo cells. When BeWo cells were treated with 14-3-3 tau siRNA, an increase in protein concentration of cell cycle inhibitor p27kip1 and a decrease in protein concentration of proliferating cell nuclear antigen, as well as activation of the extracellular signal-regulated kinase (ERK) pathway, were also noticed. These findings suggest that 14-3-3 tau might be mediated trophoblast differentiation through cell cycle regulation.

1. Introduction

Disturbed function of trophoblast may lead to a variety of pregnancy-associated pathologies, but functional regulation of trophoblast differentiation and proliferation is not fully understood. Recent studies have suggested that 14-3-3 proteins which are required for signal transduction pathways, cell division and apoptosis [1,2], probably play a key role in trophoblast function.

The 14-3-3 proteins are 30-kDa homodimers and have seven isoforms [3]. Several isoforms of 14-3-3 were considered relevant in functional regulation of trophoblast. For example, 14-3-3 ε was detected in the first trimester human cytotrophoblast and hypoxia altered its subcellular localization, suggesting that 14-3-3 ε played a functional role in cellular responses to reduced oxygen levels [4]. In the preeclamptic placentas, the levels of 14-3-3 ζ were significantly higher than that in normal placentas, preeclampsia was also associated with a weak interactions between 14-3-3 ζ and Bax and a strong interaction between 14-3-3 ζ and protein kinase C-δ (PKC-δ) [5]. Recently, we found a decreased expression of another 14-3-3 protein isoform, 14-3-3 tau, in hypoxia-treated human trophoblast cell line BeWo by proteomic analysis [6], which suggests that 14-3-3 tau plays a role in hypoxia-induced responses in the syncytialization of trophoblast.

The 14-3-3 proteins modulate protein activity through a variety of mechanisms. Binding of 14-3-3 to apoptosis-associated proteins such as BAD and Bcl2 affected their interaction with other proteins [7]. Alternatively, 14-3-3 proteins may act as a scaffold to bring proteins together as in the case of Raf and PKC [8]. Probably the most commonly identified mode of action of 14-3-3 proteins is to sequester proteins into inappropriate cell compartments, thereby inhibiting their function. A corollary to this mechanism is that 14-3-3 frequently binds to phosphorylated proteins. Dephosphorylation of the protein results in release of 14-3-3, releasing the protein which is then translocated it or otherwise activated, an example of which is KSR1 and Raf-1 [9].

In humans, 14-3-3 tau is widely expressed in brain neurons and T cells [10], which is induced by DNA damage and is required for regulation of G_2/M-transition and G_1/S-transition in cells [11,12]. But, there are no data about the potential role of 14-3-3 tau in trophobalst. To further investigate it potential role, we examined the effects of 14-3-3 tau on human trophoblast differentiation.

2. Materials and methods

2.1. Tissue collection

Human placentas were collected from 7 to 40 weeks of gestation (GW). Placentas from 6 to 11 GW were obtained from elective terminations of pregnancies ($n = 6$). Term human placentas (38–40 GW) were obtained from uncomplicated pregnancies after

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elective caesarean section ($n = 6$). Collection and processing of human placentas were approved by the Human Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University and informed consent was obtained from all patients participating in this study. Placental samples were immediately fixed in 4% paraformaldehyde dissolved in PBS, dehydrated, and embedded in paraffin. Sections (4 μm) were placed on poly-L-lysine-coated glass slides.

2.2. Immunohistochemistry

Deparaffinized tissue sections were pretreated with 3% H_2O_2 for 15 min to inhibit endogenous peroxidase activity. The anti 14-3-3 tau polyclonal rabbit antibody (1:200, Santa Cruz Biotechnology, CA) diluted in PBS was applied onto the sections, and these sections were maintained overnight at 4 °C in a humidified chamber. Sections then reacted with a poly-HRP labeled anti-rabbit IgG polymer for 30 min at room temperature. After washing, sections were developed in a staining solution containing DAB for 5 min at room temperature. Sections were examined under a light microscope (Olympus D70, Tokyo, Japan) for satisfactory developed staining. To facilitate cytoplasmic visualization of the immunostained product, hematoxylin was used for counterstaining. As negative immunohistochemical controls, sections were incubated with normal rabbit serum.

2.3. Cell culture

BeWo choriocarcinoma cells were purchased from the China Center for Type Culture Collection (CCTCC, Wuhan, China) and were maintained as monolayers in Kaighn's modification of Ham's F-12 medium (Sigma–Aldrich, St.Louis, USA) supplemented with 10% fetal bovine serum (FBS) under standard culture conditions of 5% CO_2 in air at 37 °C with medium renewal every 2–3 days.

2.4. Induction of differentiation using forskolin

BeWo cells were incubated with 20 μM forskolin or vehicle (dimethyl sulfoxide, DMSO) (Sigma–Aldrich, St.Louis, USA) in Ham's F-12 medium supplemented with 1% FBS at 37 °C for 2–3 days to induce enhanced hCG secretion and cell fusion, which are indicators of cell differentiation. The medium was replaced every day. Following addition of forskolin, cells were harvested at specific time-points for isolation of RNA and protein. BeWo cells were stained with anti E-cadherin monoclonal mouse antibody (1:400, BD biosciences, Mississauga, ON, Canada) along with an FITC-labeled goat anti-mouse IgG (Invitrogen, Carlsbad, CA) to distinguish cell border. The use of E-cadherin immunostaining has been utilized in the past for such assessments of trophoblast fusion [13]. Syncytialization was considered genuine when at least 3 or more nuclei were present in the same cytoplasm. The nuclei were counterstained with DAPI. The number of multinuclear cells in the five areas under microscope which were randomly selected and counted. The data are expressed as the ratio of each control, and the effect of 14-3-3 tau on forskolin-induced cell fusion was evaluated from three independent experiments.

2.5. Regulation of 14-3-3 tau expression in BeWo cells

The BeWo cells with 30% confluency in 12-well culture plates were transfected with a target small-interfering RNA (siRNA, 80 nmol/L) and a Negative control siRNA using Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After treatment for 6 h with siRNA, the medium containing siRNA and transfection reagents was removed, and after washing, the cells were cultured for 24–96 h with fresh culture medium.

To up-regulate 14-3-3 tau, we constructed an expression plasmid using a full-length 14-3-3 tau cds (Invitrogen, Carlsbad, CA) which was cloned into the expression vector pcDNA_HA. The resultant construction was verified by sequencing and was called HA tagged 14-3-3 tau plasmid. The BeWo cells with 80% confluency in 12-well culture plates were transfected with 14-3-3 tau plasmid (1.6 μg/mL)and pcDNA_HA vector (as vector control) using Lipofectamine 2000 according to the manufacturer's instruction. The cells were cultured for 24–72 h, protein and transcript expression determined by Western Blot and RT-PCR, respectively.

2.6. Human chorionic gonadotropin (hCG) secretion

Conditioned media were collected at the time intervals specified and were kept at −80 °C. Protein concentration in culture media was quantified. hCG secretion was determined by measuring its concentrations in the conditioned media using an enzyme immunoassay kit which specifically detects β-chain of hCG. Then β-hCG values were normalized to the protein concentration in culture media.

2.7. Cell proliferation assay

To determine the effect of 14-3-3 tau suppression on BeWo cells proliferation, we used a tetrazolium reagent, 2-(4-indophenyl)-3- (4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-1, Cell Counting Kit, Beyotime, Beijing, China). This assay is based on the measurement of the formazan dye that is liberated after the cleavage of this reagent by mitochondrial dehydrogenase activity in viable cells. BeWo cells were seeded in 96-well culture plates in the media supplemented with 10% FBS, transfected with 14-3-3 tau or negative control siRNA for 48 h–96 h. The cells were then incubated with WST-1 reagent for 1 h at 37 °C. The staining intensity in the medium was measured by determining the absorbance at 450 nm, and the data were expressed as ratios of the control value.

2.8. RNA extraction and semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cell culture dishes using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cDNA was synthesized from 5 μg total RNA by reverse transcription kit (Takara, Dalian, China) as described in manufacturer's instructions. Relative transcript abundance of 14-3-3 tau, syncytin, β-hCG was analyzed by semi-quantitative RT-PCR using β-actin (ACTB) as an internal standard. The primers, expected sizes of the PCR products and amplified conditions used in the subsequent RT-PCR were shown in Table 1. The amount of template cDNA and the number of cycles were determined experimentally so that quantitative comparison could be made during the exponential phase of the amplification process for both target and reference gene. PCR products were separated on a 2% agarose gel. Gels were stained with ethidium bromide. A single band for each gene was observed at the expected size. The intensity of either the target or β-actin band for each sample was quantified using a gel documentation and analysis system and the ratio of the two was used as a normalized value for expression of each target gene. All assays were conducted in triplicate.

2.9. Western blot analysis

Cell lysates were prepared by radioimmunoprecipitation assay (RIPA) buffer. Protein samples were resolved by polyacrylamide gel electrophoresis on SDS −12% polyacrylamide gels and electrotransferred to PVDF membranes. After blocking in PBS/Tween-20 with 5% dry milk for 1 h at room temperature, membranes were incubated with specific primary antibodies overnight at 4 °C. The following antibodies were used in this study: the anti 14-3-3 tau polyclonal rabbit antibody (1:800, Santa Cruz Biotechnology, CA), the anti PCNA monoclonal mouse antibody (1:400, Santa Cruz Biotechnology, CA), the anti p27kip1 polyclonal rabbit antibody (1:1000, Cell Signal, US), the anti phospho-ERK1/2 monoclonal mouse antibody and the anti-ERK1/2 polyclonal rabbit antibody (1:1000, KangChen-Biotech, China). The immunoreactive bands were detected by a chemiluminescence detection kit (Tiangen, Beijing, China) after incubation with horseradish peroxidase-labeled mouse or rabbit IgG antibody (1:10,000, Jackson, USA). The membrane was re-probed with anti-β-actin (ACTB)(1:10,000; KangChen-Biotech, China) as a loading control. All blotting experiments were repeated at least three times, and representative data are shown.

2.10. Statistical analysis

Data were presented as mean ± S.D. of at least three independent experiments performed in triplicate. Differences between groups were analyzed using an ANOVA and a P-value less than 0.05 was considered to be statistically significant.

Table 1
Details of the primers employed in the study.

Name of gene	Primer sequence	Annealing temperature (°C)	Amplicon length (bp)	Cycle
β-hCG	F 5-GTGAACCCCGTGGTCTCCTA-3 R 5-CTTTGAGGAAGAGGAGTCCTGG-3	60	141	25
14-3-3 tau	F 5-AGCCAATGCAACTAATCCAGAGAG-3 R 5-AAGCCGTTTTAGCCAGCGTG-3	60	269	30
syncytin	F 5-AGGAGCTTCGAAACACTGGA-3 R 5-GTGAGCTAAGTTGCAAGCCC-3	56	494	22
β-actin	F 5–AACTCCATCATGAAGTGTGACG-3 R 5–GATCCACATCTGCTGGAAGG-3	60	234	25

All the primers were synthesized by ShengGong, Shanghai, China.