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Fusiogenic endogenous-retroviral syncytin-1 exerts anti-apoptotic functions in staurosporine-challenged CHO cells

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Abstract Fusiogenic glycoprotein syncytin-1, expressed in human placenta, is a promising candidate for acquiring a basic knowledge of placental syncytialization. However, its cellular mode of action is unidentified. We investigated whether syncytin-1 may exert influence on apoptotic processes. Therefore, we incubated CHO cells after stable transfection with syncytin-1 (CHO-52) in the presence or absence of staurosporine (STS), a kinase inhibitor well characterized to induce apoptosis. When testing the phenotype of CHO-52 cells, we could demonstrate that the induction of apoptosis by STS was delayed over a period of up to 24 h. Furthermore, the cell death rate was decreased by approx 75% following transfection of syncytin-1 in CHO-52 compared to mock-treated cells. In detail, after 18h of incubation with 500 nM STS, 64 ± 2% of CHO-52 cells were viable compared to 16 ± 1% of CHO-mocks, after 24 h 43 ± 3% vs 5 ± 2%, respectively. CHO-52 cells exhibited a lower expression of active caspase 3 and anti-apoptotic Bcl-2 was found to be increased in CHO-52 cells at baseline and following STS treatment.

Our study provides first evidence that syncytin-1 serves anti-apoptotic function under certain conditions. A lessened activation of caspase 3 and an increased expression of Bcl-2 are possible mechanisms.

Keywords Apoptosis · Bcl-2 · Caspase 3 · CHO · Staurosporine · Syncytin-1

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Introduction

During human pregnancy, the multinuclear syncytiotrophoblast layer performs the main physiological functions of the placenta, such as nutrient supply, gas exchange and hormone production. Briefly, the syncytiotrophoblast is built up by underlying cytotrophoblast cells, proliferation, differentiation and fusion processes being coordinated by a large number of regulatory genes. The process of syncytium formation requires what might be designated as the "syncytin-1 system", a regulatory unit within the human placenta consisting of the fusogenic glycoprotein syncytin-1, its receptor, and, eventually, an intracellular signal molecule, the transcription factor Glial Cells Missing a (GCMa). Syncytin-1 was first described in 2000 [1] as the envelope of the human endogenous retrovirus W family (HERV-W), which is encoded on chromosome 7 (7q21–7q22, *OMIM* 604659). One possible receptor is the dual functioning placental amino acid transporter ASCT2, a sodium-dependent amino acid transporter for neutral amino acids (19q13.3, *OMIM* 109190). The syncytin-1 glycoprotein consists of 518 amino acids, its fusogenic properties have been demonstrated, for example, in transfected cells such as CHO [1] or using antisense strategies [2]. It has also been shown using adenovirus-directed expression of GCMa, that GCMa-driven syncytin gene expression is followed by syncytin-mediated cell fusion in the choriocarcinoma cell lines [3]. Furthermore, our group has described that cAMP agonists enhance the expression of

syncytin-1 whereas hypoxia down regulates syncytin-1 at the transcriptional level [4, 5].

Possible cellular effects of syncytin-1 may include membrane rearrangements or apoptotic behavior. The apoptotic program is highly complex and the cellular events involve the activation of many signalling cascades triggered either by external stress signals or internal organelle-specific initiation events. The three signalling pathways known to induce apoptosis are the cell surface-mediated signal transduction pathway, the mitochondrial-mediated pathway, and the endoplasmic reticulum stress-induced pathway [6]. Apoptosis has a central role in the control of placental cellular homeostasis, and also pathological stages during pregnancy. It is known that malfunctions in placentation and pregnancy are associated with an increased apoptotic rate e.g., preeclampsia, a disorder of late pregnancy characterized by proteinuria and hypertension [7, 8]. The succession of hypoxia and reoxygenation, in particular, leads to apoptotic cell death within the placental syncytiotrophoblast [9]. Placental apoptosis can not, however, be considered detrimental, since changes in membrane composition and stability, occurring in the course of the apoptotic cascade, may contribute to cellular differentiation patterns [10]. Early apoptotic steps, such as the activation of caspase 8, may play a role in differentiation and fusion events in the cytotrophoblast cells [11]. However, whether syncytin-1 exerts its function on membrane rearrangements has to be elucidated further [12].

We have tested our hypothesis that overexpression of syncytin-1 may influence cellular apoptotic response under certain conditions, such as challenge with hypoxia or staurosporine (STS), a microbial alkaloid and broad spectrum inhibitor of protein kinases leading to apoptosis in, e.g., trophoblastic cells or CHO cells [13, 14].

Materials and methods

Cell culture

CHO cells (purchased from ATCC) were cultured in RPMI 1640 medium (PAA Laboratories, Coelbe, Germany) supplemented with 10% fetal calf serum and antibiotics (100 IU/mL penicillin, 100 μg/mL streptomycin). Trophoblastic BeWo and JAR cells (both purchased from ATCC) and primary human term trophoblasts were cultured as reported previously [4, 5, 15]. Cells were maintained under standard tissue culture conditions under a humidified atmosphere of 95% air (O_2 tension 130 mmHg), 5% CO_2 at 37°C, or under hypoxic conditions with 1% O_2 (O_2 tension 14 mmHg), 5% CO_2, 94%N_2 (Thermo Forma cell incubator, Forma Scientific, Marietta, OH, USA). To promote expression of syncytin, incubation was performed with or without 100 μM forskolin or 150 μM 8-Br-cAMP [4]. Apoptotic challenge was applied as indicated using 100 nM to 1 μM STS (Sigma, Deisenhofen, Germany) for 3 to 24 h or vehicle.

Stable transfection and protein expression

Generally, CHO cells are widely employed to produce glycosylated, recombinant proteins. We therefore established a CHO cell line stable expressing the syncytin-1 gene product. Using a pSecTag2 vector (Invitrogen, Karlsruhe, Germany) we applied an expression system which includes a leader sequence for protein exocytosis. Before cloning the syncytin-1 cDNA without its natural leader into the expression cassette of the vector, the c-terminal *myc* epitope has been replaced by a human IgG1 (Fc) epitope in a PCR-based step (Fig. 1A). After confirmation of identity, 10 μg purified syncytin-1 plasmid DNA was electroporated into 1×10^7 CHO cells (purchased from ATCC) in 0.8 mL medium at 900 μF and 260 V in an Easyject electroporation unit (Eurogentec, Cologne, Germany). After 24 h cells were challenged with hygromycin B (Invitrogen, 0.4 mg/mL). After 2 wks, surviving clones were tested on their mRNA and protein expression by Real-Time PCR and Western Blot (Fig. 1B). One hygromycin B-resistant clone (CHO-52) was used for further experiments. Control cells (CHO-mocks) were transfected with pSecTag2 vector only.

Flow cytometry, Annexin-V/propidium iodide apoptosis assay and Anti-Bcl-2 staining

Apoptotic cell detection was performed by fluorescence-activated cell sorting (FACS) using fluoresceinisothiocyanate (FITC)-conjugated Annexin-V and propidium

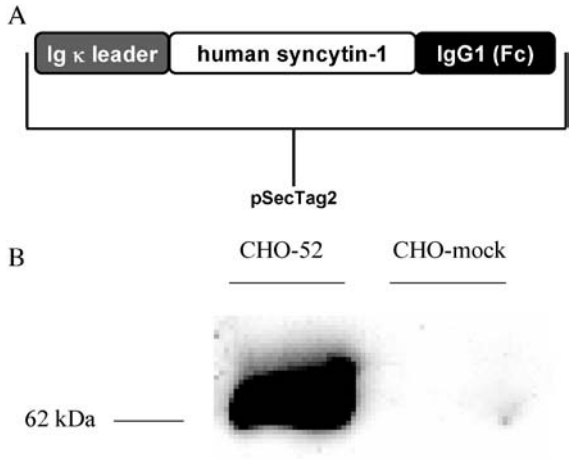

Fig. 1 **A** Scheme of the construct covering the human syncytin-1 sequence (GenBank accession NM_014590). Based on a pSecTag2 vector, the expression plasmid of syncytin-1 lacks the natural syncytin-1 leader. On the c-terminus, the myc epitope of the vector was replaced by a human IgG1 (Fc) tag. **B** After hygromycin-challenge cell clones have been tested for expression of the syncytin-1-IgG1-(Fc) fusion protein by Western blot analysis. The stable transfected clone CHO-52 (*left*) expresses the fusion protein in comparison to CHO-mocks (*right*)

iodine (PI) double staining as reported previously [16]. Briefly, after stimulation 1×10^6 cells were isolated, and resuspended in 100 μL incubation buffer. Then, Annexin V (Responsif, Erlangen, Germany) and propidium iodide (PI, Sigma) were added. After incubation in the dark at room temperature, 400 μL incubation buffer were added and cells were analyzed by flow cytometry (FACS Calibur, Becton-Dickinson, Heidelberg, Germany). For each cell type, appropriate electronic compensation of the instrument was performed to avoid overlapping of the emission spectra. Bcl-2 was detected using a monoclonal anti-human Bcl-2 antibody and a phycoerythrin (PE) conjugated anti-mouse secondary antibody (Becton-Dickinson) according to a modified protocol. Generally, the first aliquot of cells was used as autofluorescence control. Data collection and analyses out of three to six independent experiments were performed as reported before [17].

SDS-PAGE and Western blot

SDS-PAGE and Western blot were performed as reported previously using sodium desoxycholate and IGEPAL-630 (Sigma Chemicals) as combined detergents [15]. Equal amounts of protein were separated by SDS-PAGE, blotted to a protran nitrocellulose membrane (Schleicher and Schuell Bio Science, Dassel, Germany), and detected with a polyclonal goat anti-human IgG antibody or rabbit anti-active-caspase 3, anti-Bcl-2 or anti-actin antibodies (purchased from Promega, Mannheim, Germany, Sigma or Delta Biolabs, Campbell, CA, USA), followed by a visualization using a horseradish peroxidase-conjugated donkey anti-goat IgG or goat anti-rabbit IgG (Promega and New England Biolabs, Frankfurt, Germany) and exposure to X-ray films (Eastman Kodak, Rochester, NY, USA) using enhanced chemiluminescence (ECL, Amersham Biosciences, Freiburg, Germany). Western blots shown are representative of three separate experiments.

Molecular analysis and real-time PCR

CHO-52 cells were harvested for further molecular analysis. Reverse transcription of 1 μg total RNA after DNAse digestion was performed and gene expression patterns of syncytin-1 and β-actin were measured using Real-time PCR (TaqMan, Applied Biosystems, Darmstadt, Germany) according to methods published earlier [5, 18].

Cell fusion assay

A two-color fluorescence assay was applied for the measurement of cell fusion [19] between primary trophoblasts or between CHO-52 cells as well as controls. Briefly, cells were stained separately with two different dyes, CellTracker Green CMFDA or CellTracker Orange CMTMR (Molecular probes, Leiden, The Netherlands) over 20 min in the dark. Cells were resuspended in serum-free medium, counted (Casy Cell Counter, Scharfe, Reutlingen, Germany) and mixed together at a density of 1×10^5 cells per mL. Thereafter, 1 mL suspension was then seeded into culture slights (Becton Dickinson) for up to 72 h in the presence or absence of 100 μM forskolin or 150 μM 8-Br-cAMP (Sigma). Nuclei were stained with diamidino-2-phenylindole (DAPI, Invitrogen). Microscopy was performed as reported previously [4]. Green and red pictures were composed following visual analysis of a random area of 0.13×0.18 mm. Fusion events between green and red labeled cells were visible by double-fluorescent cytoplasm and nuclei. However, this is not a quantitative method. All fusion experiments were three to six times repeated.

Statistics

Analysis was performed using GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA). Where appropriate, data are expressed as means and SEM of three to six independent experiments performed in triplicates. Statistical significance was calculated using either a Mann–Whitney test or one-way ANOVA, if indicated, and significance was assumed at $p < 0.05$.

Results

We investigated whether overexpression of syncytin-1 in CHO cells may exert effects on apoptosis. Therefore, an appropriate expression system for syncytin-1 was designed. We decided to use a vector with a leading sequence known to signal an excretion of the translated protein and fused a c-terminal human IgG1 (Fc)-tag to facilitate detection as well as purification of the recombinant protein (Fig. 1A). CHO cells were stably transfected with this human syncytin-1 fusion-vector. One suitable clone expressing syncytin-1 (CHO-52) was used for further experimental procedures (Fig. 1B). A clone of mock-transfected CHO cells was used as control in all experiments.

To test for functional relevance and cell fusion processes induced by syncytin-1, a fusion assay of CHO-52 and mock-transfected CHO cells was applied to visualize cell fusion by fluorescence microscopy. Mock-treated cells were found either green-labeled or red-labeled (Fig. 2A), whereas CHO-52 cells overexpressing syncytin-1 exhibited also a double-fluorescence signal of orange color within their cytoplasm, being indicative of cell fusion (Fig. 2B). Comparable findings were made for primary human trophoblasts following treatment with cAMP agonists (Fig. 2C,D).

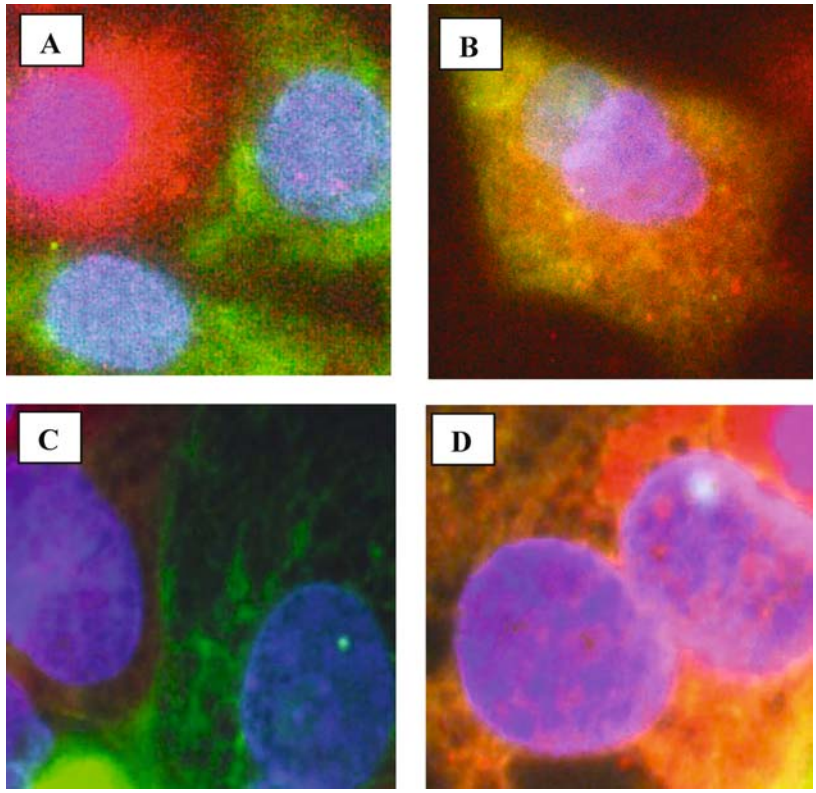

Fig. 2 Two-color cell fusion assay of mock-transfected CHO **A** and syncytin-1-transfected CHO-52 cells **B** both following treatment with cAMP agonists, native trophoblasts **C** and trophoblasts following 72 h of incubation with cAMP agonists **D**, as visualized by fluorescence microscopy. Single mock-treated CHO cells or native trophoblasts are either labeled green or red. Upon fusion a double-fluorescence signal of orange color is detectable in CHO-52 cells or trophoblasts

We analyzed if the expression of syncytin-1 alters the phenotype of the transfected cells. Under normoxic cell culture conditions no significant differences between the vitality rates of CHO-52 cells and mock-treated cells were found. The rate of living cells was on average 94 ± 1% for mock-treated cells compared with 93 ± 1% for CHO-52 cells. In addition, under normoxic conditions the number of apoptotic cells was not different between both groups. However, under severe hypoxia (1% O_2, 48 h) syncytin-1 transfected CHO-52 cells seem to be more resistant to the lack of O_2 than the mock-transfected controls (82 ± 3% for CHO-52 vs 73 ± 0% for CHO-mocks, $p < 0.05$). This result was independent from a possible stimulation of the cells with forskolin or 8-Br-cAMP, known to induce endogenous syncytin-1 expression and, hence, syncytialization. BeWo and JAr cells, besides trophoblasts, exhibited a comparable vitality rate, on average of 82 ± 2% under normoxia and 81 ± 5% under hypoxia. In addition, stimulation of cAMP pathways to induce cell differentiation did not affect apoptotic response significantly (data not shown).

We then tested STS as a well-established inducer of apoptosis on its effects in all cells. Investigating the apoptotic behavior of trophoblastic cells in response to STS, we detected that the STS challenge applied to our CHO cell model led to a viability rate of barely 1–7% in the native trophoblastic cells. Next, we used STS to test for a change of phenotype in syncytin-1-transfected CHO-52 cells. FACS analysis revealed significant differences for the rate of apoptotic and necrotic cells between CHO-mocks and CHO-52 cells overexpressing syncytin-1 following STS challenge. Up to the longer incubation periods of 12 to 24 h and also at higher STS concentrations of 500 nM to 1 μM, CHO-52 cells were significantly more resistant to the pro-apoptotic stimulus than CHO-mocks (Fig. 3), $p < 0.05$ to < 0.01, respectively. In detail, after 12 h of treatment with 500 nM STS, 69 ± 2% of CHO-52 cells were viable compared with 34 ± 8% of CHO-mocks and following 1 μM STS 42 ± 8% of the CHO-52 vs 25 ± 7% of the controls. After 18 h of incubation with 500 nM STS 64 ± 2% of CHO-52 cells were viable compared to 16 ± 1% of CHO-mocks. At a higher STS concentration of 1 μM for 18 h, 29 ± 1% of the syncytin-1-transfected CHO-52 cells were viable compared with 8 ± 1% of the CHO-mocks. Choosing a longer incubation period of 24 h with 500 nM STS 43 ± 3% of CHO-52 cells were still viable compared with 5 ± 2% of mock-treated cells, and following 24 h of treatment with 1 μM STS 27 ± 2% of CHO-52 cells were alive compared with 5 ± 1% of mock-treated controls.

Next, we tested the activation of executioner caspase 3 in response to the STS challenge. In general, active executioner caspase 3 was found to be reduced by Western blot analysis in CHO-52 cells incubated with, e.g., 500 nM STS for 6 to 12 h compared with CHO-mocks (Fig. 4). This phenomenon was attenuated for higher STS concentrations at longer incubation periods (24 h, 1 μM).

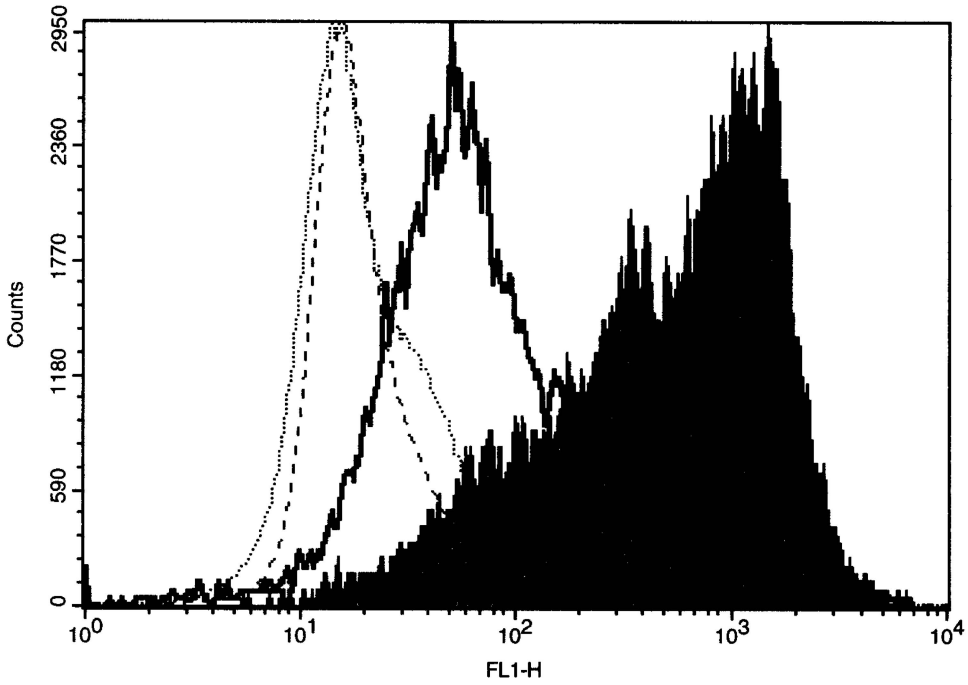

Fig. 3 Apoptosis in response to STS in the syncytin-1 clone CHO-52 and controls. Flow cytometry profiles following staining with Annexin V-FITC and PI, the *x*-axis represents fluorescence intensity (FL1-H), the *y*-axis cell counts. Key: Dark line (*thinly dotted*): CHO-mock controls. Dark line (*intermittent line*): CHO-52 overexpressing syncytin-1,controls. Thick dark line (*drawn through, white area under the curve*): CHO-52 overexpressing syncytin-1 following 500 nM STS for 18 h. Thin dark line (*drawn through, black area under the curve*): CHO-mocks following 500 nM STS for 18 h

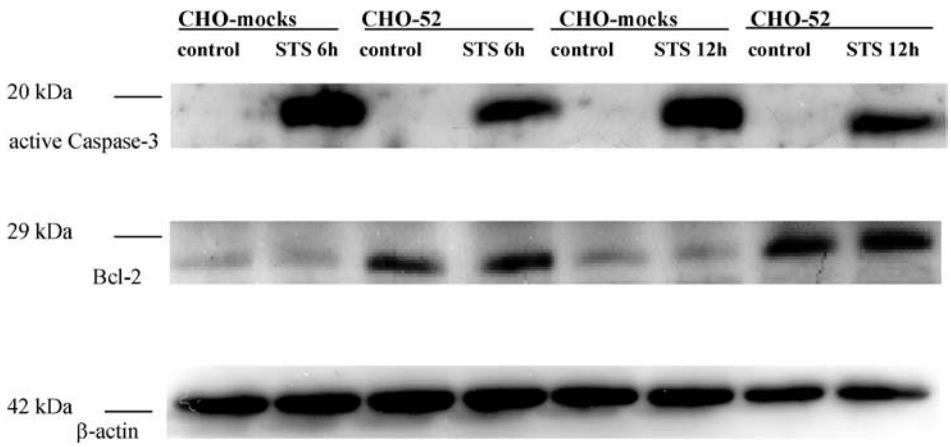

Fig. 4 Mock-transfected CHO and CHO-52 cells overexpressing syncytin-1 were analyzed for active caspase 3 (*upper panel*) and Bcl-2 (*middle*) following incubation with 500 mM STS for 6 and 12 h using Western blot analysis. Beta-actin is used as a loading control (*lower panel*)

Eventually, we investigated the expression of Bcl-2 in our cell culture model in the presence or absence of apoptotic stimuli. In CHO-52 cells overexpressing syncytin-1 Bcl-2 levels, as detected by immunoblot analysis, were higher than in mock-treated controls both at baseline and over a period of up to 24 h of STS challenge (Fig. 4). In addition, anti-Bcl-2 staining revealed higher fluorescence intensity for native CHO-52 cells, as shown by FACS, compared to CHO-mocks (Fig. 5A). Following STS challenge, such as 500 nM for 18 h, CHO-52 cells still exhibited a higher fluorescence signal for Bcl-2 than CHO-mocks (Fig. 5B).

Discussion

Our data provides the first evidence that syncytin-1 overexpression in CHO cells may alter apoptotic responses following cell death stimuli. Using FACS and Western blot analysis, we could demonstrate that the apoptosis induced by STS, for example, was reduced in CHO-52 cells overexpressing syncytin-1 compared to mock-transfected cells. Caspase 3 as executioner caspase was determined since it enables apoptotic cell death irreversibly. Comparing between 3–24 h of incubation and between a final concentration of 100 nM–1 μM STS, we found striking effects at 18 h treatment with 500 nM STS, respectively. Under these conditions, CHO-52 cells on average were four times more resistant than mock-treated controls, and exhibited an increased expression of Bcl-2.

We are certainly aware of the limitations of our study. However, we chose the CHO cell line for reasons of transfection efficiency, cell fusion following syncytin-1 overexpression [1] and retaining of the machinery for the apoptosis cascade. Since trophoblastic cells exhibited a comparable

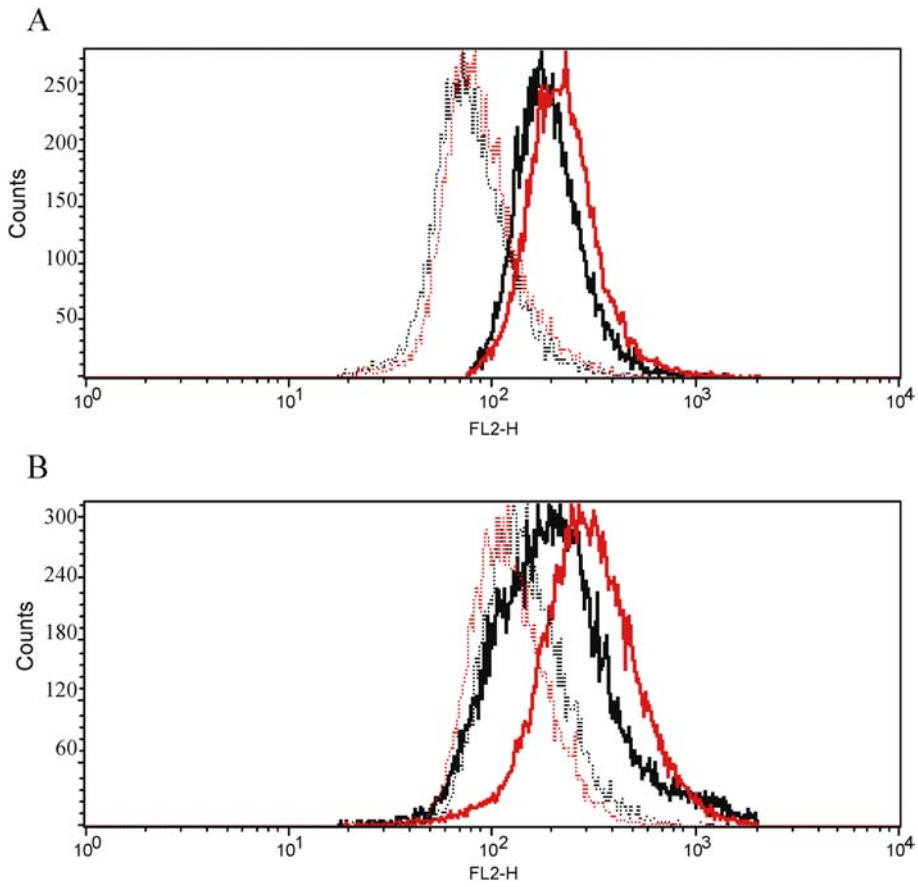

Fig. 5 Flow cytometric analysis of Bcl-2 expression in the syncytin-1 clone CHO-52 and CHO-mocks. Cell debris was removed from the analysis by gating on the viable population. The x-axis represents fluorescence intensity of PE (FL2-H), the y-axis cell counts. Cells were incubated in absence of apoptotic stimuli **A** or 18 h with 500 nM STS **B**. Key: Dark line (*dotted*): CHO-mocks, autofluorescence control. Red line (*dotted*): CHO-52 overexpressing syncytin-1, autofluorescence control. Dark line (*drawn through*): CHO-mocks, control **A**, and following 500 nM STS for 18 h **B**. Red line (*drawn through*): CHO-52 overexpressing syncytin-1, control **A**, and following 500 nM STS for 18 h **B**

vitality rate compared with CHO-mocks, along with a similar apoptotic response following the incubation with STS, we conclude that our cell culture model CHO-52 is appropriate for further studies on syncytin-1. However, STS is polyfunctional like many inhibitors used for studying programmed death phenomena. Furthermore, cellular bioenergetic processes, as discussed by [20], have not been studied here.

Whether or not syncytin-1 induces cell fusion more directly as exogenous retroviruses or whether there are more indirect effects on membrane composition, is under discussion [12]. Membrane rearrangement during syncytium formation has been linked to the activity of caspases and phosphatidylserine flip as elements of the apoptotic cascade [13] and is therefore, a candidate for the mode of syncytin-1 function. It has been described recently that the *N*-terminal 124 amino acids of the surface subunit of syncytin-1 function as the minimal receptor-binding domain and facilitate interaction with ASCT2 [21]. However, syncytin-1 cannot be regarded as the exclusive mediator for cell fusion and syncytium formation in mammalian placentogenesis since it is derived from a HERV lacking in other mammals, but it can be considered representative of an evolutionary strategy which might have led to the benefits of deep implantation in human pregnancy. Placental apoptosis is essential for controlling cellular homeostasis throughout human gestation, but may be increased in pre-eclampsia or intrauterine growth restriction [10]. Anerobic milieu conditions are an apoptotic challenge, but also a common feature during early placentogenesis. We described recently that trophoblastic cells exhibit considerable survival following hypoxia [16]. The pathophysiology of hypoxia-related placental disturbances, such as the down-regulation of syncytin-1 in pre-eclamptic placentas must therefore still be clarified [18].

Among the anti-apoptotic mechanisms within the placenta are Bcl-2, epidermal growth factor (EGF), hepatocyte growth factor (HGF) and β-catenin [22, 23]. Several members of the Bcl-2 family regulate permeabilization of mitochondrial membranes, a decisive feature of early apoptosis. It has been shown, for example, that the presence of Bcl-2 blocks the activation of caspase 3 [6]. Arresting the apoptotic cascade in fusing trophoblasts could be, therefore, due to inhibitor activity at the mitochondrial level [10]. This is in line with our finding that Bcl-2 expression is higher in CHO-52 cells overexpressing syncytin-1 than in controls, especially following apoptotic stimuli such as STS. Bcl-2 was described formerly as a cellular mechanism attenuating apoptotic response in CHO cells incubated with STS for 6 h, respectively [24].

Taken together, this study provides first evidence that syncytin-1 may serve an anti-apoptotic function in CHO cells

under certain conditions. Future work will seek to elucidate the effects of syncytin-1 on apoptotic signalling pathways along with cellular syncytialization.

Conclusion

In this study we investigated whether overexpression of syncytin-1 in CHO cells may exert effects on apoptosis under distinctive conditions. Therefore, we incubated CHO cells after stable transfection with syncytin-1 (CHO-52) in the presence or absence of staurosporine (STS), a kinase inhibitor of microbial origin well characterized to induce apoptosis. When testing the phenotype of CHO-52 cells, we could demonstrate that CHO-52 cells overexpressing syncytin-1 perform cell fusion. Following the induction of apoptosis by STS, apoptotic response was delayed over a period of up to 24 h. Furthermore, the cell death rate was decreased by approx 75% following transfection of syncytin-1 in CHO-52 compared to mock-treated cells. CHO-52 cells exhibited a lower expression of active caspase 3 and anti-apoptotic Bcl-2 was found to be increased in CHO-52 cells at baseline and following STS treatment. Our study provides first evidence that syncytin-1 serves anti-apoptotic function under certain conditions. A lessened activation of caspase 3 and an increased expression of Bcl-2 are possible mechanisms. However, the physiological effects of syncytin-1 on apoptosis during placental development and in pathophysiological scenarios need to be tested in the near future.

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