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Response of HEK293 and CHO Cells Overexpressing Fusiogenic Syncytin-1 to Mitochondrion-Mediated Apoptosis Induced by Antimycin A

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ABSTRACT

Apoptosis is essential for the regulation of cellular homeostasis in the placenta and is also involved in the pathophysiology of pregnancy-related diseases such as pre-eclampsia and intrauterine growth restriction (IUGR). Syncytin-1, a fusiogenic glycoprotein of endogenous-retroviral origin expressed in human trophoblasts, facilitates placental syncytium formation and is found reduced in pre-eclamptic placentas. We focus here on the mitochondrial apoptotic pathway and investigate whether the overexpression of syncytin-1 in HEK293-52 (human embryonic kidney cells) and CHO-52 cells influences the apoptotic response to the mitochondrial inhibitor antimycin A (AA). After the induction of apoptosis by 5 μM AA and incubation for up to 36 h in the absence of serum, the mean apoptotic rate was reduced by 15–30% in syncytin-1 transfected cells compared with mock-transfectants. After 12 h of challenge with AA we found lower cytochrome *c* levels in the cytoplasmic protein fraction and higher amounts in the mitochondrial fraction in syncytin-1 transfectants compared with mock-transfectants. We observed a decreased Mitotracker Red staining of mitochondria following AA challenge for 24 h in mock-treated CHO cells, in particular, compared with syncytin-1 transfectants. Moreover, we found a reduced activation of caspase 9 in syncytin-1 transfected HEK293-52 cells after 48 h of apoptotic challenge compared to mock-transfectants. However, a high expression of anti-apoptotic Bcl-x_L was found in both cell types. Using syncytin-1 transfected HEK293-52 cells and CHO-52 cells, we provide initial evidence that syncytin-1 may exert its anti-apoptotic function at the mitochondrial level. A reduced release of cytochrome *c* followed by a diminished activation of caspase 9 is a possible mechanism. J. Cell. Biochem. 105: 766–775, 2008.

KEY WORDS: APOPTOSIS; BCL-X_L; CASPASE 9; CHO CELLS; CYTOCHROME *C*; HEK293 CELLS; PLACENTA; SYNCYTIN-1

In early human pregnancy, cytotrophoblast cells fuse and form the multinuclear syncytiotrophoblast layer which fulfils the nutritive functions of the placenta [Cross, 2006]. Syncytin-1, a glycoprotein originally derived from the human endogenous retrovirus (HERV)-W, is involved in cellular differentiation processes and the cell–cell fusion of cytotrophoblasts [Mi et al., 2000]. Syncytin-1 is encoded on chromosome 7 (7q21–7q22, *OMIM* 604659) and consists of 518 amino acids. Among the regulators of syncytin-1 expression are cAMP and glial cells missing a (GCMa) transcription factor [Knerr et al., 2005]. Severe hypoxia down-regulates syncytin-1 at the transcriptional level [Knerr et al., 2003]. This is associated with the development and severity of pregnancy related disorders such as pre-eclampsia, characterized by onset of hypertension and proteinuria, or with intrauterine growth restriction (IUGR) [Knerr et al., 2002; Soleymanlou et al., 2007; Langbein et al., 2008]. Using a model of ex vivo perfused placental cotyledons and BeWo choriocarcinoma cells, we have shown that syncytin-1 gene expression is down-regulated by severe hypoxia [Knerr et al., 2003].

Apoptosis is essential for the regulation of cellular homeostasis in the human placenta at all stages of intrauterine development, although the regulatory network is not yet understood in detail. Apoptosis is also involved in the pathophysiology of pre-eclampsia and IUGR, together with hypoxia and placental oxidative stress [Straszewski-Chavez et al., 2005]. In pre-eclampsia, excess cell death of trophoblasts, most likely induced by hypoxia, leads to cellular shedding followed by a systemic maternal inflammatory response and endothelial cell injury [Soleymanlou et al., 2007].

In principle, apoptosis may be initiated extrinsically by the surface-mediated death receptor pathway, or intrinsically by the mitochondrial pathway. The release of cytochrome *c*, or other mitochondrial proteins, is followed by the assembly of the apoptosome,

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requiring Apaf-1 and dATP, followed by a rapid activation of caspase 9 and downstream effector caspases and cell death [Cain et al., 2002; Arden and Betenbaugh, 2004].

We have previously shown that fusiogenic syncytin-1 is capable of executing anti-apoptotic effects in staurosporine (STS)-challenged CHO-52 (Chinese hamster ovary) cells [Knerr et al., 2007]. STS is a broad spectrum protein kinase inhibitor. We have tested the effects of mitochondrial metabolic inhibitor antimycin A (AA) in two different syncytin-1 transfected cell lines, HEK293-52 (human embryonic kidney cells) and CHO-52 cells, to address the question as to whether syncytin-1 may serve an anti-apoptotic function. AA is a potent inhibitor of mitochondrial electron transfer at complex III and reduces the mitochondrial membrane potential and ATP production. Moreover, AA leads to an increase in caspase activities indicative of cell damage and apoptotic cell death [Kaushal et al., 1997].

After demonstrating cell–cell fusion and hence the function of syncytin-1 overexpressing cells using confocal laser scanning microscopy, we tested the effects of AA on syncytin-1 transfected cells, mock-transfectants and trophoblastic BeWo choriocarcinoma cells. Serum is an effective anti-apoptotic agent following metabolic stress and nutrient depletion [Arden and Betenbaugh, 2004; Chowdhury et al., 2007]. We therefore performed the apoptotic challenge in the absence of serum.

MATERIALS AND METHODS

CELL CULTURE

CHO cells and HEK293 cells (purchased from ATCC/LGC, Wesel, Germany) were cultured in RPMI 1640 medium (PAA Laboratories, Coelbe, Germany) supplemented with 10% fetal calf serum (FCS) and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin). BeWo cells (purchased from DSMZ, Braunschweig, Germany) were cultivated in Dulbecco's modified Eagle medium (Gibco, Karlsruhe, Germany) as described [Knerr et al., 2003]. Cells were maintained under standard tissue culture conditions at 37°C in a humidified 95% air-5%CO_2 incubator (Forma Scientific, Marietta, OH). Apoptosis was induced by 1–25 μM AA (Antimycin A1, from *Streptomyces* sp., Sigma–Aldrich, Seelze, Germany) for 6–60 h with serum deprivation as indicated; we used 5 μM AA for 12, 24, and 36 h as standard challenge. All experiments were performed in duplicates and were repeated at least three times.

STABLE TRANSFECTION AND MOLECULAR ANALYSIS, REAL-TIME PCR

We have established both a CHO cell line (CHO-52) and a HEK293 cell line (HEK293-52) which are stably expressing the syncytin-1 gene product (GenBank accession NM_014590). Briefly, 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 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. After confirmation of identity, 10 μg purified syncytin-1 plasmid DNA was electroporated into 1×10^7 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 of culture cells were incubated with hygromycin B (0.2 mg/ml, Invitrogen). After two weeks, surviving clones were tested for their mRNA and protein expression by real-time PCR and Western blot as reported previously [Knerr et al., 2007]. A hygromycin B-resistant clone (designated as CHO-52, HEK293-52) was used for further experiments. Control cells (CHO mocks, HEK293 mocks) were transfected with pSecTag2 vector only.

Syncytin-1 transfected cells were harvested for further molecular analysis to clarify syncytin-1 gene activity. Reverse transcription of 1 μg total RNA after DNAse digestion was performed and gene expression of syncytin-1 was measured using real-time PCR (Applied Biosystems, Darmstadt, Germany) according to methods published earlier [Knerr et al., 2003, 2004]. We calculated the syncytin-1 copy number per cell by comparing the signal generated by the various cell types to that generated by an external plasmid standard containing the syncytin-1 sequence as reported elsewhere in detail [Hammond et al., 2007].

FLOW CYTOMETRY, ANNEXIN V/PROPIDIUM IODIDE (PI) APOPTOSIS ASSAY, TRYPAN BLUE ASSAY

Generally, apoptotic cell detection was performed by fluorescence-activated cell sorting (FACS) using fluoresceinisothiocyanate (FITC)-conjugated annexin V and PI double staining [Knerr et al., 2007]. Briefly, after stimulation 5×10^5 cells were isolated and resuspended in 100 μl incubation buffer. Then, annexin V and PI (Sigma–Aldrich) were added. After incubation in the dark at room temperature, 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 and the first aliquot of cells was used as autofluorescence control.

In addition, we tested cell viability by Trypan blue dye exclusion. In brief, cells were harvested and resuspended in PBS buffer at an appropriate cell density. An aliquot of the cell suspension (10 μL) was mixed with 0.4% (w/v) Trypan blue and mounted immediately in a counting chamber using a Nikon TMS microscope (Langen, Germany). Each experiment was repeated at least three times.

PROTEIN EXTRACTION

Cells were lysed in chilled RIPA buffer and the insoluble material was cleared by centrifugation at 2,000*g* at 4°C for 10 min as reported previously [Klaffenbach et al., 2005]. Cytochrome *c* determination in cytosolic and mitochondrial fractions was done using a protein extraction procedure published in detail elsewhere [Liu et al., 1996]. Briefly, cells were harvested by centrifugation (500*g* at 4°C for 15 min). The pellet was washed with ice-cold PBS and resuspended in HEPES buffer supplemented with the protease inhibitors aprotinin, leupeptin, pepstatin, and PMSF. After incubation on ice for 15 min, cells were broken by 10 strokes through a 25G-needle (Becton-Dickinson). After centrifugation at 2,000*g* at 4°C for 5 min, the supernatant (cytoplasmic protein fraction) and the pellet (mitochondrial protein fraction) were separated. Protein concentrations were determined by the Bradford protein assay (Bio-Rad, Hercules, CA) and equal amounts of protein were analyzed.

SDS–PAGE AND WESTERN BLOT ANALYSIS

SDS–PAGE and Western blot analysis were performed as reported previously using sodium deoxycholate and IGEPAL-630 (Sigma–Aldrich) as combined detergents [Meissner et al., 2005; Knerr et al., 2007]. Equal amounts of protein (25 μg of total protein, 10 μg of cytoplasmic and mitochondrial protein) were separated by 10–15% SDS–PAGE, blotted to a protran nitrocellulose membrane (Schleicher and Schuell Bio Science, Dassel, Germany), and detected with monoclonal anti-cytochrome *c* (purchased from BD Biosciences, Heidelberg, Germany), polyclonal anti-cleaved-caspase 9 (Asp315 and Asp330, Cell Signaling, Danvers, MA), monoclonal anti-Bcl-x_L (R&D Systems, Minneapolis, MN), polyclonal anti-VDAC/porin (Cell Signaling), or polyclonal anti-β-actin antibodies (Santa Cruz Biotechnology, Heidelberg, Germany). This was followed by a visualization using a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Promega, Madison, WI and Cell Signaling) and exposure to X-ray films (Eastman Kodak, Rochester, NY) using enhanced chemiluminescence (ECL, Amersham Biosciences, Freiburg, Germany). Densitometric scanning for signal quantification was performed with AIDA software 4.15 (Raytest, Straubenhardt, Germany) according to the manufacturer's instructions. In principle, protein amounts were calculated based on calibration curves obtained with total, cytoplasmic and mitochondrial protein fractions. Western blots shown are representative of three separate experiments.

FLUORESCENCE IMMUNOCYTOCHEMISTRY

Syncytin-1 transfected cells and mock-transfected cells were fixed with 3.7% paraformaldehyde, permeabilized with acetone and washed twice with PBS buffer. Thereafter, cells were prepared for microscopy by staining with 500 nM of a mitochondria-specific dye, MitoTracker Red CMXRos (Molecular Probes, Eugene, OR), for 15 min at 37°C in PBS buffer. Fixed cells were counterstained with 1 μg/ml of DAPI for 2 min (Calbiochem, San Diego, CA), and placed on microscope slides. All preparations were embedded in Mowiol containing antibleaching DABCO. Visual evaluation was performed using a BX60 microscope (Olympus) with a 40× (oil) objective, and the software and camera system F-View II-Kamera (Soft Imaging System) were applied as reported previously [Knerr et al., 2005].

CELL FUSION ASSAY AND IMMUNOFLUORESCENCE FOR LASER CONFOCAL SCANNING MICROSCOPY

A two-color fluorescence assay was applied for the detection of cell–cell fusion between HEK293-52 and CHO-52 cells as reported earlier [Borges et al., 2003; Knerr et al., 2007] and adapted to confocal laser scanning microscopy. After incubation for 72 or 96 h with medium containing 10% FCS in the presence of forskolin, 4×10^4 cells/ml were stained separately with two different dyes, CellTracker Green CMFDA or CellTracker Orange CMTMR (dilution 1:1,000, Molecular Probes, Leiden, The Netherlands). Following incubation, fusion events between green and red labeled cells were visible by double-fluorescent yellowish cytoplasm. Nuclei were stained with Sytox blue (dilution 1:1,000, Molecular Probes). Laser confocal scanning microscopy was performed as published elsewhere [Aigner et al., 2002]. These experiments were repeated at least three times.

LABORATORY ANALYSIS OF LACTIC ACID

Cell culture supernatants were collected in special vials (Glucose FH/1.3, Sarstedt, Numbrecht, Germany) and centrifuged at 500*g* for 10 min. Lactic acid concentration was then determined enzymatically using the Cobas Integra biochemical analyzer (Roche Diagnostics, Mannheim, Germany).

STATISTICS

Data were analyzed using Prism software 4.0 (Graph Pad San Diego, CA) and SPSS 14.0 (SPSS Inc., Chicago, IL). Values were calculated as mean ± SEM of three to six independent experiments performed in duplicates if not otherwise stated. Statistical significance was calculated using Mann–Whitney test or one-way ANOVA, if applicable. A P-value <0.05 was considered significant.

RESULTS

We first performed real-time PCR to obtain quantitative data on the initial number of syncytin-1 copies per cell in the various cell lines. We found syncytin-1 expression in comparable quantities in HEK293-52, CHO-52 and BeWo choriocarcinoma cells, but not in mock-transfectants (Fig. 1A). Secondly, we adapted a cell fusion assay for confocal laser scanning microscopy to demonstrate that syncytin-1 is functional in the transfected cell lines (clone 52). Mock-treated HEK293 cells and CHO cells were found to be either green-labeled or red-labeled (Fig. 1B/I, 1B/III), whereas HEK293-52 cells and CHO-52 cells overexpressing syncytin-1 demonstrated an additional double-fluorescence signal of yellowish color within their cytoplasm and accumulated nuclei. This is indicative of intercellular fusion after overexpression of syncytin-1 (Fig. 1B/II, 1B/IV). Cell fusion was occasionally visible and only occurred in syncytin-1 transfectants.

Next, we tested the viability rates of HEK293-52 and CHO-52 cells compared with mock-treated cells and trophoblastic BeWo cells following AA challenge. Following 24 h of culture in the presence or absence of 5 μM AA along with serum deprivation, the mean viability rate of trophoblastic BeWo cells was almost within the range of the viability rate of syncytin-1 transfected CHO-52 and HEK293-52 cells (Fig. 2A,B), as demonstrated using FACS analysis and Trypan blue staining. In syncytin-1 transfected HEK293-52 cells and CHO-52 the mean viability rate following challenge with 5 μM AA for 24 h was 15% and 24% higher, respectively, than in mock-transfectants (Fig. 2A,B).

There was a time-dependent increase of stainability with annexin V/PI following incubation with AA and serum withdrawal for both syncytin-1 transfected cells and mock-transfected controls ($P < 0.001$). However, in syncytin-1 transfected CHO-52 cells apoptotic response was reduced by up to 30% after 36 h of culture (Fig. 3). Moreover, there was a dose-dependent increase of cell mortality, as measured by stainability with annexin V/PI, in the range of 1–25 μM AA for each incubation time (data not shown). Differences in apoptotic response between syncytin-1 transfected cells and mock-transfectants were predominantly found up to 36 h of culture with AA (Fig. 4).

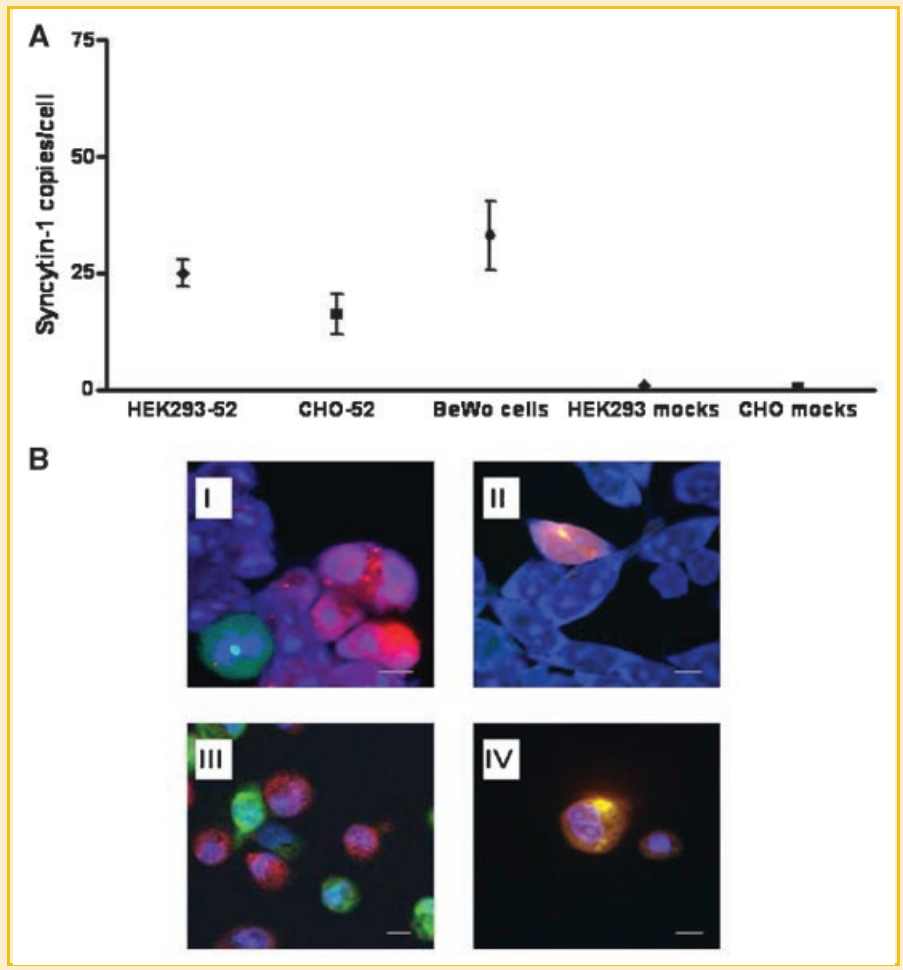

Fig. 1. A: Syncytin-1 copy number per cell for syncytin-1 transfected HEK293-52 and CHO-52 cells and BeWo choriocarcinoma cells compared to mock-transfected HEK293 cells and CHO cells. Data are given as mean and SEM of three different experiments. B: Two-color fluorescence assay of mock-treated HEK293 cells (I) and syncytin-1 transfected HEK293-52 cells (II) both following 72 h treatment with cAMP agonists, of mock-treated CHO cells (III) and syncytin-1 transfected CHO-52 cells after 96 h of incubation with cAMP agonists (IV), as visualized by laser confocal scanning microscopy. Nuclei were stained with Sytox blue. Single mock-treated cells are either labeled with red or green dyes. Upon cell fusion a double-fluorescence signal of orange color is detectable in HEK293-52 and in CHO-52 cells. Scale bars: 10 μm.

Subsequently, we visualized mitochondria using MitoTracker Red, a mitochondrion specific dye that accumulates in a membrane potential-dependent way. Staining of mitochondria in control cells was homogenous, indicative of actively respiring mitochondria; the mitochondria appeared almost evenly distributed within the cell, particularly around the nuclei (Fig. 5/I, III, V). In striking contrast, mitochondria in cells treated with AA, for example, 5 μM for 24 h, revealed a decrease of the fluorescent intensity, especially CHO mocks (Fig. 5/II). Fluorescence intensity of MitoTracker Red in CHO-52 cells following AA challenge was slightly higher than in mock-transfectants, and comparable to the signal obtained in trophoblastic BeWo cells (Fig. 5/IV,VI).

In addition, we focused on the putative mechanisms of increased resistance of syncytin-1 transfected cells to AA under particular circumstances. We investigated the expression of cytochrome *c* in our cell culture model in the presence or absence of apoptotic stimuli. We separately investigated cytoplasmic and mitochondrial cell extracts to determine the cytochrome *c* release from the mitochondria into the cytosol. Following 12 h of culture in the presence of 5 μM AA, for example, we found a release of cytochrome *c* from mitochondria into cytosol in mock-treated HEK293 cells and in CHO cells, which was considerable lower in the syncytin-1 transfected cells (Figs. 6A and 7A). We found much higher levels of cytochrome *c* in the mitochondria than in the cytosol, and tentatively higher levels of mitochondrial cytochrome *c* in syncytin-1 transfectants compared to mock-transfected cells (Figs. 6B and 7B).

We then tested the activation of caspase 9 in response to the AA challenge. As detected by Western blot analysis, caspase 9 was found reduced in syncytin-1 transfected HEK293-52 cells compared with HEK293 mocks following incubation with, for example, 10 μM AA for up to 48 h (Fig. 8). After testing several approaches on CHO cells, we conclude that the caspase 9 antibodies used possibly did not work in CHO cells as they are from hamster.

Moreover, we focused on anti-apoptotic Bcl-x_L. Both CHO mocks and CHO-52 cells exhibited a high expression of anti-apoptotic Bcl-x_L for up to 60 h in the presence of 5 μM AA and absence of serum (Fig. 9). HEK293 and HEK-52 cells also showed comparable results,

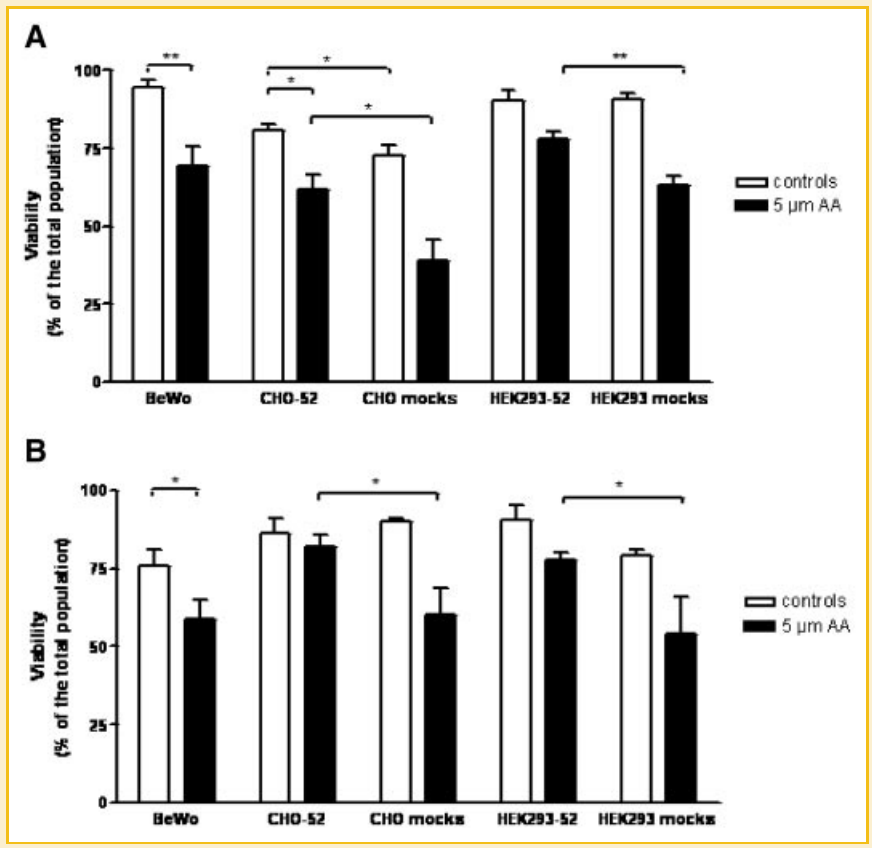

Fig. 2. Viability rates (mean ± SEM) in BeWo choriocarcinoma cells, syncytin-1 transfected CHO-52 and HEK293-52 cells compared with mock-transfected controls (CHO mocks, HEK293 mocks) following an apoptotic stimulus with 5 μM AA for 24 h and serum deprivation. Flow Cytometry after staining with annexin V-FITC and PI (A) or Trypan blue staining (B) were performed following incubation for 24 h in the presence (black bars) or absence of AA (white bars). $^{*}P < 0.05$, $^{**}P < 0.01$.

but at a lower expression level compared with CHO and CHO-52 cells (data not shown).

Last, we measured lactic acid concentrations in the cell culture supernatant. Although there was a time- and dose-dependent increase of lactate levels ($P < 0.0001$), we found no significant differences between cells overexpressing syncytin-1 and mock-transfected cells. Basal concentrations ranged from 2.09 ± 0.09 to 2.36 ± 0.11 mmol/L, respectively. Following apoptotic challenge with up to 10 μM AA, lactate concentrations ranged from 5.33 ± 0.18 mmol/L in the syncytin-1 transfected cells to 6.01 ± 0.13 mmol/L in the mock transfectants after 24 h, and from 13.33 ± 0.69 to 15.23 ± 0.12 mmol/L after 36 h, respectively.

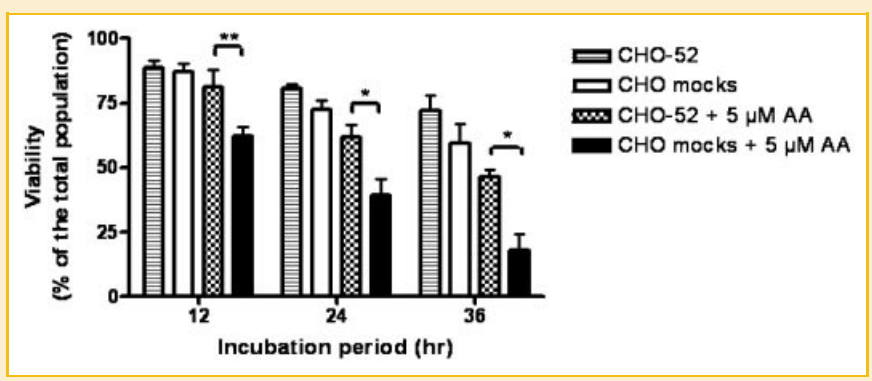

Fig. 3. Viability rates (mean ± SEM) in the syncytin-1 transfected clone CHO-52 and mock-transfected cells following an apoptotic stimulus with 5 μM AA. Flow Cytometry was performed after staining with annexin V-FITC and PI following incubation for 12, 24, or 36 h in the presence or absence of AA as indicated. $^{*}P < 0.05$; $^{**}P < 0.01$. Key: Hatched bars: CHO-52 cells overexpressing syncytin-1, controls, White bars: CHO mocks, controls, Checkered bars: CHO-52 cells overexpressing syncytin-1 following 5 μM AA for 12, 24, or 36 h as indicated, Black bars: CHO mocks following 5 μM AA for 12, 24, or 36 h as indicated.

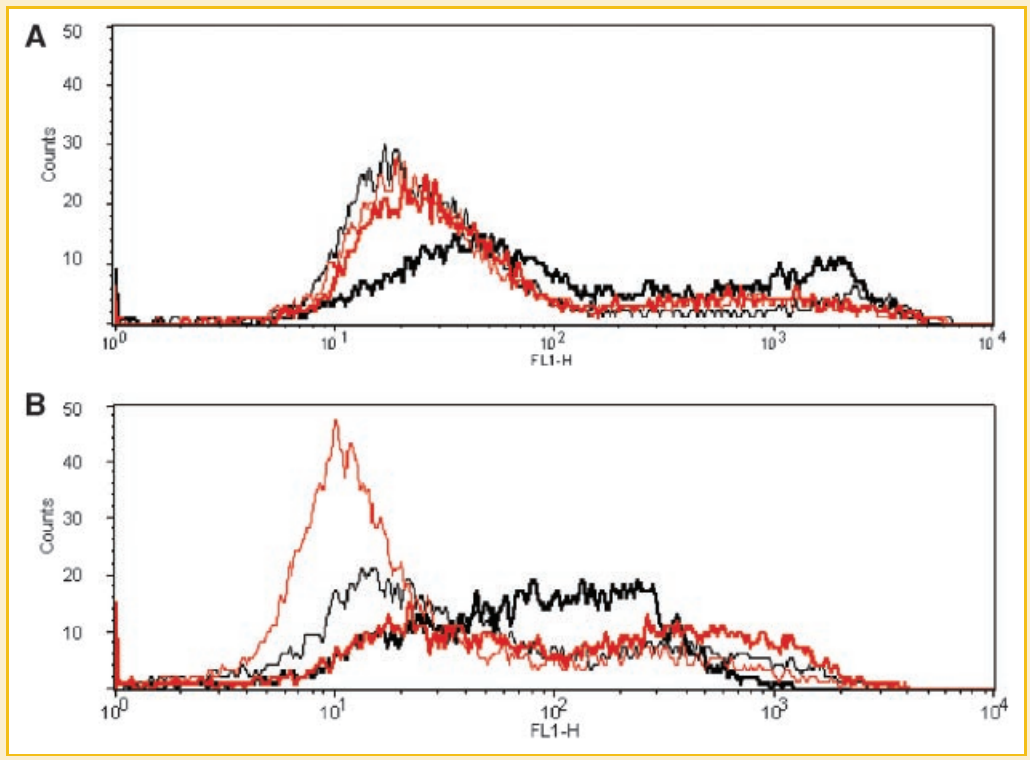

Fig. 4. Apoptosis in response to AA in the syncytin-1 clone CHO-52 and mock-treated controls. Flow Cytometry profiles following staining with annexin V-FITC and PI, the x-axis represents fluorescence intensity (FL1-H), the y-axis cell counts. Key: Thin dark line: CHO mock, controls, Thin red line: CHO-52 overexpressing syncytin-1, controls, Thick dark line: CHO mock, following 10 μM AA for 24 h (A) or 5 μM AA for 36 h (B) Thick red line: CHO-52 overexpressing syncytin-1 following 10 μM AA for 24 h (A) or 5 μM AA for 36 h (B) A representative FACS analysis out of three independent experiments is shown.

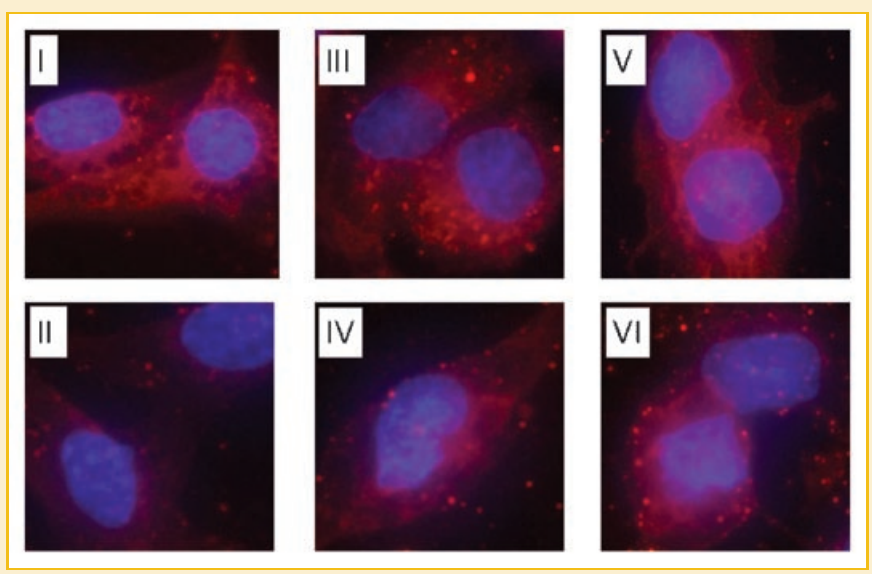

Fig. 5. Mitochondria were stained in red using MitoTracker Red and nuclei were stained with DAPI. The results are representative from three separate experiments. Incubation with 5 μM AA for 24 h resulted in a slight decrease in MitoTracker Red fluorescence in mock transfectants compared with syncytin-1 transfected cells. Key: CHO mocks in the absence (I) and presence (II) of 5 μM AA for 24 h, CHO-52 overexpressing syncytin-1 in the absence (III) and presence (IV) of 5 μM AA for 24 h, BeWo choriocarcinoma cells in the absence (V) and presence (VI) of 5 μM AA for 24 h. The results are representative from three separate experiments.

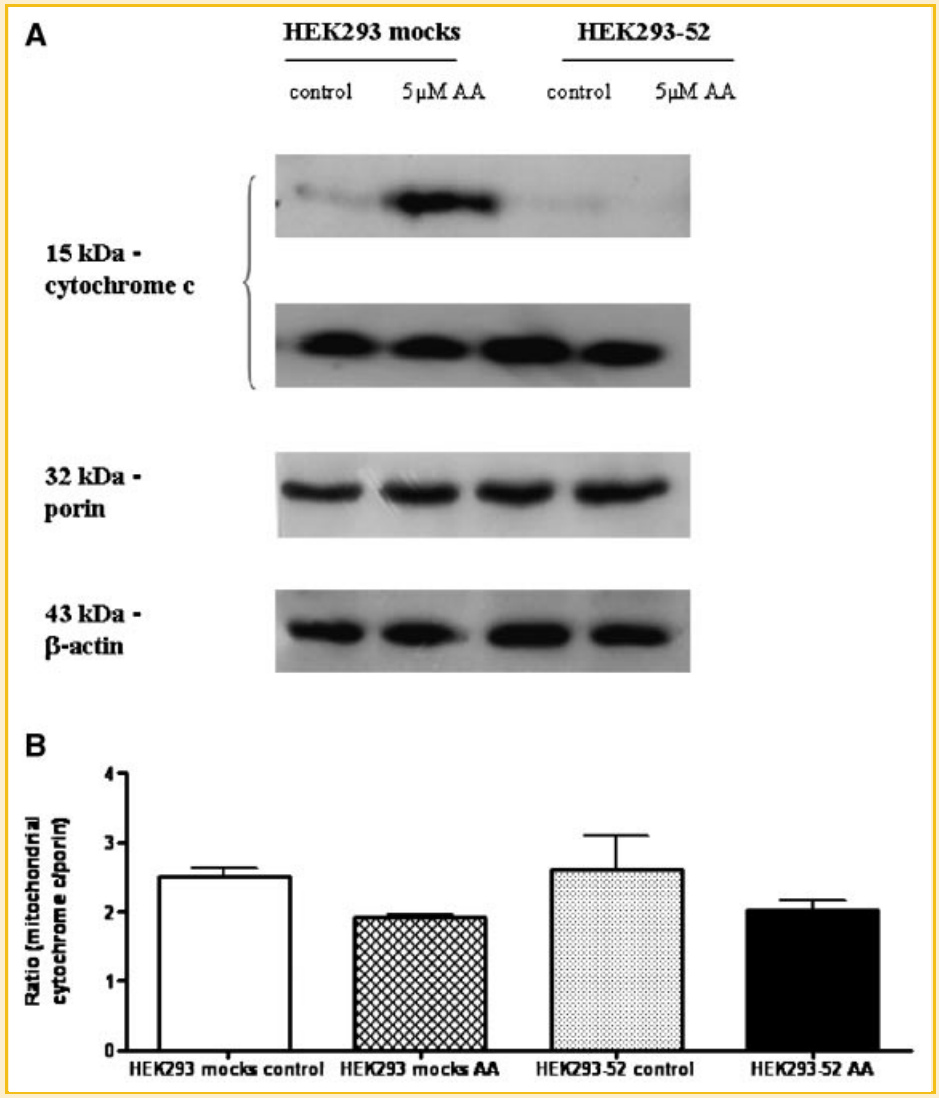

Fig. 6. A: Cytochrome *c* release as determined by Western blot analysis using cytoplasmic and mitochondrial protein. Mock-transfected HEK293 cells and HEK293-52 cells overexpressing syncytin-1 were analyzed after 12 h of culture in the presence or absence of 5 μM AA. We used cytoplasmic protein (upper panel) and mitochondrial protein (middle) to analyze for cytochrome *c*, VDAC/porin as a mitochondrial marker (lower panel), β-actin as a cytoplasmic marker (lowest panel); 10 μg of protein was loaded in each well. A representative Western blot analysis out of three independent experiments is shown. B: Corresponding densitometric analysis of mitochondrial cytochrome *c*. The results are shown as bars (±SEM) of three separate experiments.

DISCUSSION

This study has demonstrated that fusiogenic syncytin-1 may exert anti-apoptotic functions at the mitochondrial level. Basically, it has been stated that apoptosis in trophoblastic cells is under control of the mitochondrion [Perkins et al., 2002]. We have provided evidence that fusiogenic syncytin-1 exerts anti-apoptotic properties together with a lower expression of active caspase 3 in syncytin-1 transfected CHO-52 cells [Knerr et al., 2007], and now focused on the mitochondrial apoptotic pathway. AA is interesting because it initiates apoptotic cell death directly in the mitochondrial respiratory chain. Besides disrupting cellular metabolic function, AA is also capable of inhibiting the hypoxia-response element, HRE [Maeda et al., 2006]. Viability rates of CHO cells treated for 24 h with 5 μM AA in the presence of serum are approx 50–80%, as previously described [Jeong et al., 2004]. We demonstrate that the apoptotic rate following AA challenge and serum deprivation in cells overexpressing syncytin-1 is reduced by 15–30% compared with mock-transfectants. There was also a slight decrease of the MitoTracker Red fluorescent intensity in mock-treated CHO cells compared with syncytin-1 transfectants. It is of interest to see that MitoTracker Red fluorescent intensity of syncytin-1 transfectants following AA challenge was comparable to the signal of trophoblastic BeWo cells that physiologically express syncytin-1. We found lower levels of cytochrome *c* in the cytosol along with higher levels in the mitochondria in syncytin-1 transfected cells compared with mocks after 12 h of AA challenge, indicating that overexpression of syncytin-1 may, in certain circumstances, be associated with the prevention of cytochrome *c* release. Moreover, we found a reduced activation of initiator caspase 9 after AA

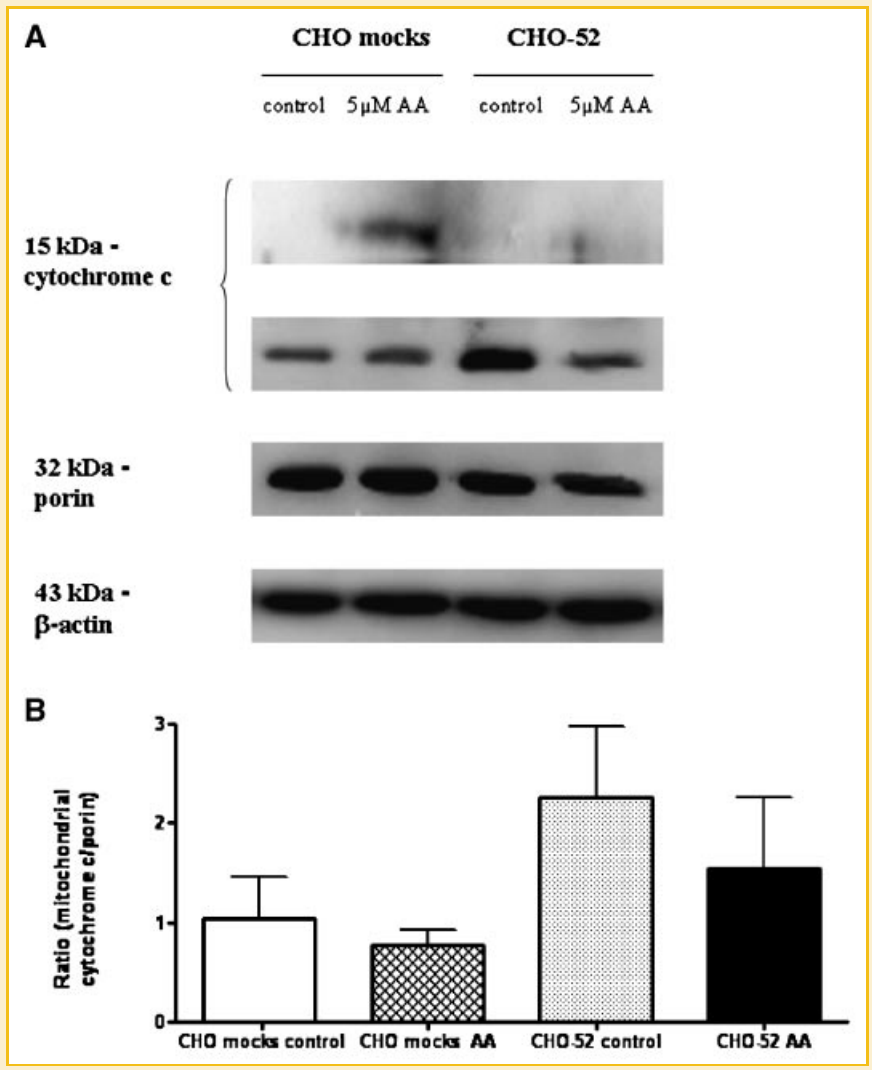

Fig. 7. A: Cytochrome *c* release as determined by Western blot analysis using cytoplasmic and mitochondrial protein. Mock-transfected CHO cells and CHO-52 cells overexpressing syncytin-1 were analyzed after 12 h of culture in the presence or absence of 5 μM AA. We used cytoplasmic protein (upper panel) and mitochondrial protein (middle) to analyze for cytochrome c, VDAC/porin as a mitochondrial marker (lower panel), β-actin as a cytoplasmic marker (lowest panel); 10 μg of protein was loaded in each well. A representative Western blot analysis out of three independent experiments is shown. B: Corresponding densitometric analysis of mitochondrial cytochrome *c*. The results are shown as bars (±SEM) of three separate experiments.

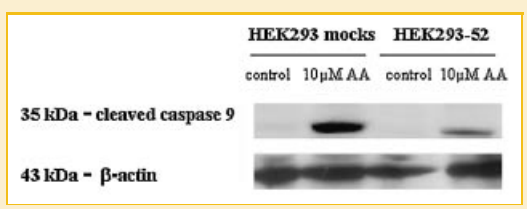

Fig. 8. Mock-transfected HEK293 cells and HEK293-52 cells overexpressing syncytin-1 were analyzed by Immunoblotting for cleaved caspase 9 (upper panel) in the presence or absence of 10 μM AA for 48 h. Beta-actin was used as a loading control (lower panel); 25 μg of total protein was loaded in each well. A representative Western blot analysis out of three independent experiments is shown.

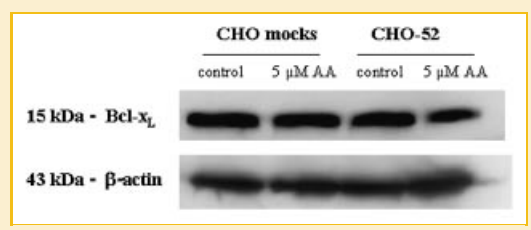

Fig. 9. Mock-transfected CHO cells and CHO-52 cells overexpressing syncytin-1 were analyzed by Immunoblotting for Bcl-x_L (upper panel) in the presence or absence of 5 μM AA for up to 60 h. Beta-actin was used as a loading control (lower panel); 25 μg of total protein was loaded in each well. A representative Western blot analysis out of three independent experiments is shown.

treatment, but only in HEK293-52 cells. There are two possible explanations for this finding. Either syncytin-1 exerts its anti-apoptotic effects in a different path in the two different cell lines, as the regulation of apoptosis under normal or stress conditions varies considerably between cell types [Green and Kroemer, 2004; Schwarz et al., 2007]. Or, despite our exhaustive efforts the caspase 9 antibodies could not work in the hamster CHO cells in the same way as in the human HEK293 cells. Caspase 9 was investigated because it had been previously described that trophoblastic cells exhibit increased cleaved caspase 9 and caspase 3 under hypoxic conditions resembling pre-eclampsia [Perkins et al., 2002]. It is of interest that Apaf-1 and caspase 9 are only moderately expressed in human placenta in the first trimester of gestation, as is caspase 3 [De Falco et al., 2004]. Basically, caspases may play a major role in trophoblast syncytial fusion. Caspase 8, for example, may facilitate cytoskeletal and membrane changes, and consequently the fusion of cytotrophoblast cells to form a syncytiotrophoblast layer [Black et al., 2004].

Moreover, expression of anti-apoptotic Bcl-x_L was found to be high in both syncytin-1 transfected and mock-transfected cells. Bcl-x_L expression prevents cytochrome *c* redistribution and mitochondrial swelling in response to agents, such as AA, that inhibit oxidative phosphorylation [Van der Heiden et al., 1997]. An increased Bcl-2 production in syncytin-1 transfected CHO-52 cells has been previously described [Knerr et al., 2007]. Lactic acid concentrations in the cell culture supernatant were not indicative of an altered production in syncytin-1 transfected and mock-transfected cells, so further studies on mitochondrial function and membrane potential are required.

It is of interest that syncytin-1 was originally the envelope protein of HERV-W. An earlier report [Mallet et al., 2004] describes the preservation of its open reading frame in humans and also in apes, which is indicative of the involvement of this endogenous retroviral glycoprotein in hominoid placental physiology. It would seem that viruses have evolved strategies to protect infected cells from apoptotic clearance. For example, the human T-cell leukemia virus type 1 (HTLV-1), an exogenous retrovirus, is capable of regulating programmed cell death, particularly in the development of neoplasia and neurological abnormalities. Its Tax protein may protect cells from apoptosis induced by serum deprivation by preventing cytochrome *c* release from the mitochondria and cAMP-response element binding protein (CREB) activation [Trevisan et al., 2006]. It is interesting that the syncytin-1 signaling pathway and its transcription factor GCMa are up-regulated by cAMP-driven pathways [Knerr et al., 2005].

Alterations of syncytin-1, along with reduced fusiogenic and anti-apoptotic capacity, may play a role in the placental origin of pre-eclampsia. An altered apoptotic pattern in placental tissues from early gestation on, may eventually lead to an abnormal release of trophoblast material into the maternal blood and systemic symptoms such as pre-eclampsia [Huppertz, 2008]. In addition, it has been described that the number of cytochrome *c* oxidase positive mitochondria is decreased in trophoblasts of pre-eclamptic placentas, indicative of mitochondrial dysfunction the course of the disease [Matsubara et al., 1997].

At this point, we can only speculate on how syncytin-1 may affect mitochondrial apoptosis. We assume that in the course of cell–cell fusion processes, altered nuclear to cytoplasm-ratios and plasma membrane to nucleus-ratios, and affected mitochondrial membranes can alter cellular apoptotic response. Moreover, syncytin-1 could also have a direct effect, for example, by physically binding to other proteins that belong to cytoskeleton pathways or signal transduction. This phenomenon has been described for the HTLV-1 Tax protein which prevents apoptosis directly [Trevisan et al., 2006]. Finally, we should consider the effect of the transcription factor GCMa which is regulated at different levels such as at transcription, protein turnover and subcellular localization and which basically functions as a key regulator of cellular differentiation in trophoblastic cells [Schubert et al., 2008].

In conclusion our results, although not obtained in a physiological setting infer that under certain circumstances, syncytin-1 attenuates the mitochondrial apoptotic response. The precise mechanism is, however, as yet unclear.

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