RNA interference-induced reduction in CD98 expression suppresses cell fusion during syncytialization of human placental BeWo cells

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Abstract **The physiological importance of CD98 surface antigen in regulating placental trophoblast cell fusion has been studied in a cell model of syncytialization (the cytotrophoblast cell line BeWo following increased intracellular cAMP by forskolin treatment) using RNA interference. CD98 protein abundance (determined by Western blot) was decreased by 40–50% following double-stranded small interfering RNA transfection. Cell fusion (determined by quantitative flow cytometry) was similarly inhibited and human chorionic gonadotropin secretion was suppressed. These findings show that CD98 is involved in the process of cell fusion necessary for syncytiotrophoblast formation.**

Keywords: Trophoblast; Cell fusion; Syncytialization; CD98; RNA interference

1. Introduction

During development, cytotrophoblasts fuse with each other to generate a syncytium of trophoblasts (the syncytiotrophoblast) covering the surface of the human placental chorionic villi. The syncytiotrophoblast layer, in direct contact with maternal blood, plays an important role throughout pregnancy since it is the site of numerous distinct placental functions, including nutrient exchange and the synthesis of steroid and peptide hormones required for normal fetal growth and development. The molecular basis of cell fusion in this tissue has remained ill-understood despite the recent discovery of two human endogenous retroviral proteins (HERV-3 [1] and HERV-W (syncytin) [2,3]) and their implication in this process.

CD98 cell surface antigen is an integral membrane protein [4], which is expressed on the cytotrophoblast and particularly on the plasma membrane of the syncytiotrophoblast (functionally [5], immunohistochemically [6]). This molecule has been found to be identical to a fusion regulatory protein-1 (FRP-1) and its expression is necessary for virus-induced cell fusion [7] and for osteoclast formation [8]. Importantly, CD98 is also able to activate specific integrins and thus may be of particular importance in cell adhesion [9,10]. CD98 is thus a multifunctional protein that is also involved in amino acid transport; the heavy chain forms part of a dimeric structure formed with one of a family of light chains [11].

Our recent experiments showed that there is a time-dependent increase in the expression of CD98 mRNA during syncytialization of BeWo cells following forskolin treatment [12], and further that using transient transfection with antisense oligonucleotides to CD98 this molecule is involved in the process of cell fusion necessary for syncytiotrophoblast formation (syncytialization) [13]. We have now applied RNA interference (RNAi) to reduce CD98 expression and thus assess the role of this molecule in the process of syncytialization using BeWo cells as a well-established cellular model of placental trophoblast syncytialization [14].

2. Materials and methods

2.1. BeWo cell culture

Cloned BeWo cells expressing either a fusion protein of green fluorescent protein and human histone H2B (H2B-GFP) or a fusion protein of red fluorescent protein and the mitochondrial targeting sequence from subunit VIII of human cytochrome *c* oxidase (Mit-DsRed2) were maintained at 37 °C as monolayers in Ham's F-12K ($[Ca^{2+}] = 0.92$ mM) medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin in a humidified atmosphere of 5% CO_2 and 95% air [15]. These two cell lines were subcultured by treating with 0.05% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate buffer saline (PBS) containing 0.02% ethylenediaminetetraacetate, mixed at 50% and seeded in 25 cm^2 plastic culture flasks and grown for 2–3 days to the stage of 50% confluence. At 50% confluency, 30 pmol per flask double-stranded small interfering RNA (siRNA) to CD98 (Table 1) was introduced into cells using TransIT-TKO transfection reagent according to the manufacturer's protocol by incubating cells for 12 h. Forskolin or vehicle (dimethyl sulfoxide) was then added to the final concentration of 100 μM, followed by further incubation for indicated times at 37 °C. At the end of the incubation period, cells were more than 95% viable as assessed by trypan blue dye exclusion. Cultures were conducted in triplicate for each set of experiments to assess reproducibility. The conditioned medium was collected and centrifuged at 3000 × *g* at 4 °C for 10 min to remove cellular debris and stored at −70 °C until use.

2.2. Western blot analysis

Total protein (cellular extract) was extracted from cultured cells with PARIS™ according to the manufacturer's protocol and stored at −70 °C until use. The cellular extracts were mixed with Laemmli sample buffer [16] and boiled for 5 min before loading. Samples (20 μg of protein for each lane) were separated under reducing conditions on 12% (w/v) sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. After blocking by incubation in Tris buffer saline (TBS) containing 2% (w/v) bovine serum

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Table 1
Sequence of double-stranded siRNA to CD98

	Sequence	Position
CD98 siRNA1		
Sense	5′-UCUGAAGGAUGCAUCCUCA(TT)-3′	1222–1240
Antisense	3′-(TT)AGACUUCCUACGUAGGAGU-5′	
Scrambled CD98 siRNA1		
Sense	5′-AUCACCUUAAGACGUUCGG(TT)-3′	
Antisense	3′-(TT)UAGUGGAAUUCUGCAAGCC-5′	
CD98 siRNA2		
Sense	5′-GAAUGGUCUGGUGAAGAUC(TT)-3′	586–604
Antisense	3′-(TT)CUUACCAGACCACUUCUAG-5′	
Scrambled CD98 siRNA2		
Sense	5′-GUGUAUAUGGACUGGGCAA(TT)-3′	
Antisense	3′-(TT)CACAUAUACCUGACCCGUU-5′	

Two sets of double-stranded siRNA (designated CD98 siRNA1 and CD98 siRNA2) were designed to target bases (position) of the CD98 coding sequence (Accession No. NM_002394). The control siRNAs (scrambled sequence) for each siRNA were also designed.

albumin (BSA) for 1 h at room temperature, the membrane was soaked overnight at 4 °C in TBS containing CD98 polyclonal antibody (1:200 dilution) and 1% (w/v) BSA. The membrane was rinsed and washed three times for 5 min in TBS containing 0.1% (v/v) Tween 20 (TBS-T), incubated with anti-rabbit IgG peroxidase-linked antibody (1:5000 dilution) in TBS-T for 1 h at room temperature and then rinsed and washed three times for 5 min in TBS-T followed by one wash in TBS for 5 min. Proteins were detected with the ECL detection system. The intensity of band for each sample was quantified using an image documentation and analysis system.

2.3. Cell fusion analysis

The rate of cell fusion (syncytialization) was measured as described previously by flow cytometry [15]. Cells were harvested by trypsinization, fixed and the number of single fluorescent (green or red) positive cells (i.e., non-fused or non-detectably fused cells) and double fluorescent (green and red) positive cells (i.e., detectably fused cells) were counted using a flow cytometer (BD FACSAria, Becton Dickinson, Franklin Lakes, NJ, USA). Twenty thousand cells were analyzed on each sample.

2.4. Human chorionic gonadotropin secretion

Human chorionic gonadotropin (hCG) secretion was determined by measuring its concentrations in the conditioned medium by an immunoassay kit, which specifically detects the β-chain of hCG.

2.5. Protein estimation

Protein concentration of the cell extract was determined by the method of Lowry et al. [17] using bovine serum albumin as a standard.

2.6. Statistical analysis

Differences between groups were analyzed using an ANOVA and results were considered statistically significant at $P < 0.05$.

2.7. Materials

BeWo cells (passage number approximately 40) were kindly provided by Dr. S.L. Greenwood (Academic Unit of Child Health, St. Mary's Hospital, University of Manchester). Double-stranded siRNA was obtained from Takara-bio (Kusatsu, Shiga, Japan) and TransIT-TKO was purchased from Mirus (Madison, WI, USA). PARIS™ was purchased from Ambion (Austin, TX, USA), CD98 (H-300) rabbit polyclonal antibody raised against a human recombinant protein was from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-rabbit IgG, peroxidase-linked antibody and ECL detection system were purchased from Amersham Life Science (Amersham, Buckinghamshire, UK). Forskolin was obtained from Sigma–Aldrich Chemical (St. Louis, Missouri, USA), tissue culture supplements were from Gibco BRL (Rockville, MD, USA) and the hCG enzyme immunoassay kit was from ICN Biomedicals (Irvine, CA, USA). All chemicals were of the highest purity commercially available.

3. Results

In order to determine the effect of transfection with double-stranded siRNA to CD98 on the level of CD98 protein and also on BeWo cell fusion, two different sets of double-stranded siRNA designed against different sequences within CD98

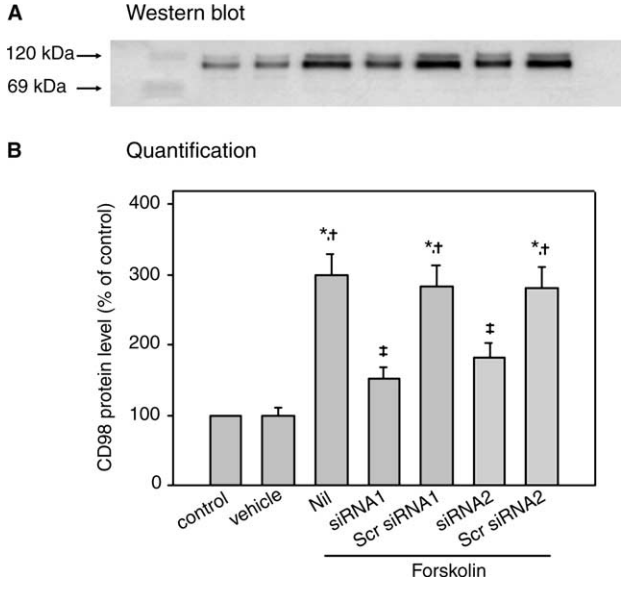

Fig. 1. Effect of double-stranded siRNA to CD98 on CD98 protein. BeWo cells expressing either H2B-GFP or Mit-DsRed2 were mixed, cultured and transfected without or with double-stranded siRNAs or corresponding scrambled siRNAs. CD98 protein levels in the cellular extracts before (control) and after a further 36 h treatment with 100 μM forskolin or with vehicle were analyzed as described in Section 2. (A) Western blot (under reducing conditions). The results presented are from a single representative experiment. (B) Quantification of CD98 protein levels. The intensity of each band was quantified by using an image documentation and analysis system. Data represent means ± S.D. of four separate experiments, expressed as percentage of control (i.e., values without culture). *Significantly different from control. †Significantly different from values cultured with vehicle alone. ‡Significantly different from values cultured with forskolin alone. siRNA1, CD98 siRNA1; siRNA2, CD98 siRNA2; Scr siRNA, scrambled siRNA.

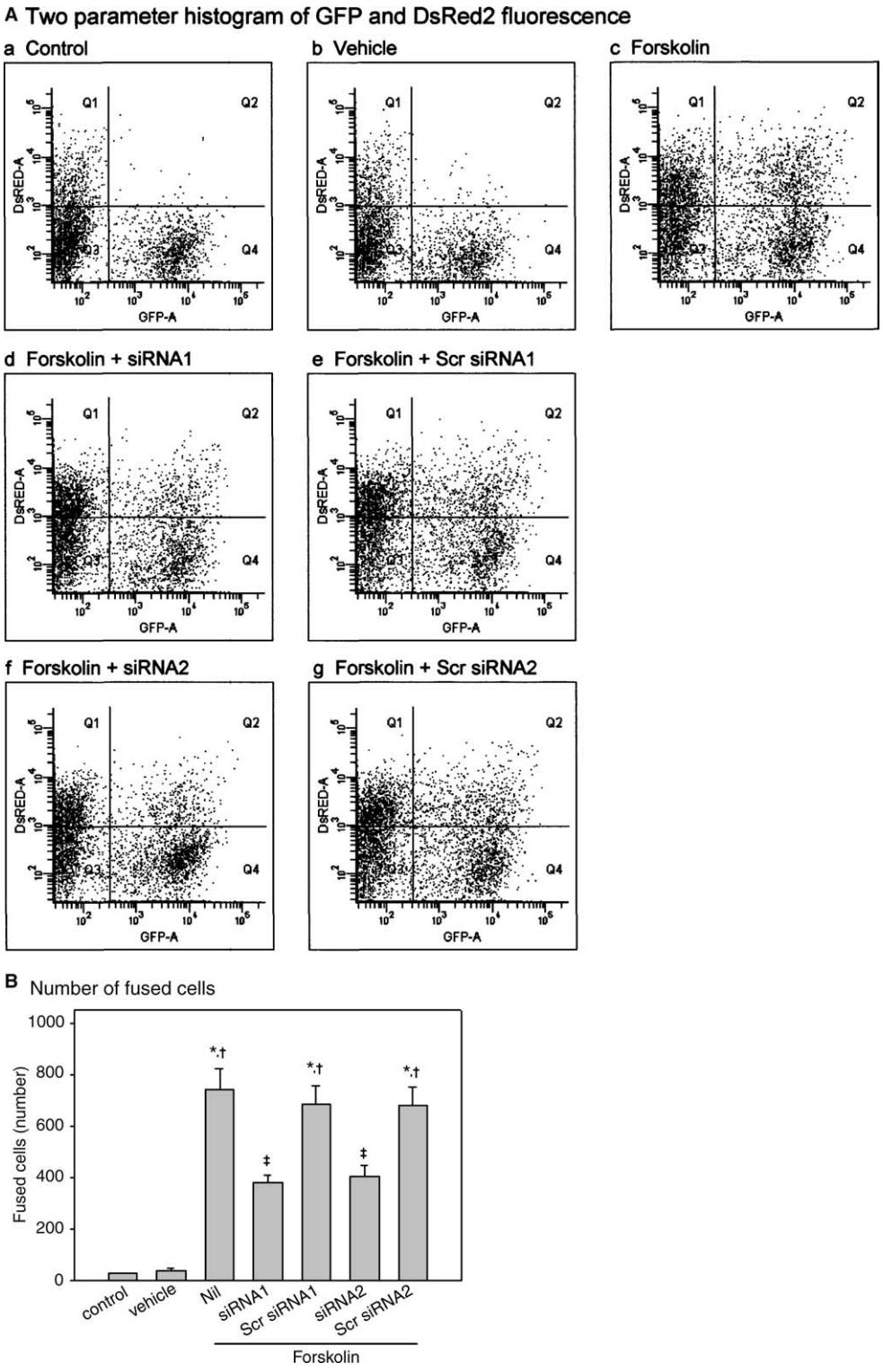

Fig. 2. Effect of double-stranded siRNA to CD98 on cell fusion. BeWo cells expressing either H2B-GFP or Mit-DsRed2 were mixed, cultured and transfected without or with double-stranded siRNAs or corresponding scrambled siRNAs. Cells were further cultured with 100 μM forskolin or with vehicle for 36 h. Cell fusion was analyzed on a flow cytometer as described in Section 2. (A) Two parameter histogram of GFP and DsRed2 fluorescence analyzed on a flow cytometer. Cells in the quadrant (Q) 2 simultaneously express both H2B-GFP and Mit-DsRed2, thus fused cells. (a) Cells before forskolin treatment (control). (b) Cells following 36 h culture without forskolin (vehicle). (c) Cells following 36 h culture with forskolin. (d) Cells transfected with CD98 siRNA1 following 36 h culture with forskolin. (e) Cells transfected with scrambled CD98 siRNA1 following 36 h culture with forskolin. (f) Cells transfected with CD98 siRNA2 following 36 h culture with forskolin. (g) Cells transfected with scrambled CD98 siRNA2 following 36 h culture with forskolin. (B) Number of fused cells. Data represent means ± S.D. of four separate experiments. Scr siRNA, scrambled siRNA.

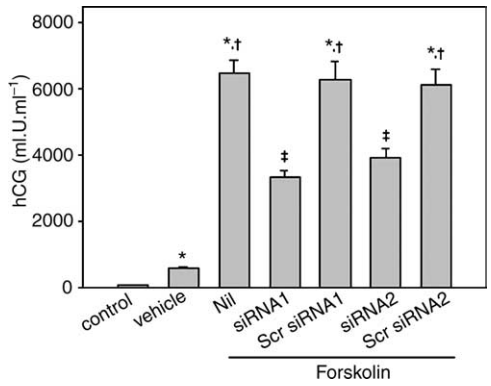

Fig. 3. Effect of double-stranded siRNA to CD98 on hCG secretion. BeWo cells expressing either H2B-GFP or Mit-DsRed2 were mixed, cultured and transfected without or with double-stranded siRNAs or corresponding scrambled siRNAs. hCG concentrations in the conditioned medium before (control) and after further 36 h treatment with 100 μM forskolin or with vehicle were analyzed as described in Section 2. Values are means ± S.D. of four separate experiments with triplicate assay. *Significantly different from control. †Significantly different from values cultured with vehicle alone. ‡Significantly different from values cultured with forskolin alone. Scr siRNA, scrambled siRNA.

mRNA (Table 1) were introduced into the cells. BeWo cells were then cultured in the presence or absence of forskolin and CD98 protein levels in the cellular extract were analyzed by Western blotting (Fig. 1). As expected, under reducing conditions a band was found at 75–80 kDa [6]. In cells not transfected with double-stranded siRNA, forskolin addition produced a marked (2.9-fold) increase in CD98 protein compared to either pre-treatment, or to vehicle-treated cells. Transfection with either set of double-stranded siRNA before forskolin treatment suppressed forskolin-induced stimulation of CD98 protein level significantly. In contrast, scrambled double-stranded siRNAs to CD98 showed no significant effect on forskolin-induced stimulation of CD98 protein quantity.

Fig. 2 shows the effect of transfection with double-stranded siRNAs to CD98 on the rate of cell fusion analyzed by flow cytometry. The number of cells expressing combined green and red fluorescence (quadrant (Q) 2), that is detectably fused cells, was clearly increased following forskolin treatment (compare Fig. 2A,a or b with c). At 36 h in the presence of forskolin, approximately 15% of the fluorescence-positive cells were fused (Fig. 2A,c), while in the absence of forskolin the value was about 1% (Fig. 2A,b). In double-stranded siRNA transfected cells (Fig. 2A,d, f), the number of fused cells was approximately 50–55% of that in non-transfected cells while in cells transfected with scrambled double-stranded siRNAs (Fig. 2A,e, g), there was no significant effect on cell fusion. Forskolin-induced hCG secretion, a marker of syncytialization in BeWo cells, was suppressed in cells transfected with double-stranded siRNAs compared to untreated cells or cells transfected with scrambled double-stranded siRNAs (Fig. 3).

4. Discussion

The major objectives of this study were to develop and utilize siRNA to directly investigate the importance of CD98 in trophoblast cell fusion. For experimental reasons, we have used a model human choriocarcinoma cell line which is considered to have many of the properties of freshly isolated human trophoblast [14]. That CD98 is involved in cytotrophoblast cell fusion is suggested by the following evidence; (1) CD98 protein level is increased following cell fusion; (2) when CD98 protein expression is inhibited by transfection with double-stranded siRNAs to CD98, cell fusion and hCG secretion are both suppressed; (3) scrambled double-stranded siRNAs to CD98 have no significant effect on forskolin-induced stimulation of cell fusion and hCG secretion. In contrast to our earlier work with anti-sense oligonucleotides, the siRNA experiments reported here give important mechanistic insight into how CD98 function may be controlled biologically. Thus, the functional experiments (Figs. 2 and 3) show that the stability of the mRNA encoding CD98 must directly alter the level of surface expression of this protein, since recent work shows that it is the recruitment of activated CD98 to the cell membrane that is integral to the normal process of syncytialization (data not shown). Additionally, since in the absence of forskolin stimulation the slow rate of spontaneous BeWo cell fusion was unaltered by siRNA to CD98 (data not shown), we can conclude from these new experiments using the siRNA technique that the steady state level of CD98 mRNA must be sufficient to regulate fusion under stimulated but not under resting conditions when other factors (such as CD98 trafficking to the cell surface) must be rate limiting. Using immunohistochemistry, we have previously shown that CD98 immunoreactivity is prominent particularly at the basal surface (in direct contact with the underlying cytotrophoblast) of the syncytiotrophoblast of the human placenta [13]. The fact that CD98 immunoreactivity is detected in human chorionic villous trophoblast in situ as well as in a choriocarcinoma cell line (BeWo) in vitro (Fig. 1) suggests that the functional results in the BeWo model system described here will have relevance to understanding syncytialization in normal placental development.

Syncytialization is an unusual biological process that occurs in only a few human lineages (e.g., in the development of myotubes, osteoclasts, syncytiotrophoblasts), and its mechanism remains to be elucidated. In addition to CD98, two molecules are currently considered to play an important role in syncytialization. HERV-3 is an endogenous retroviral gene product expressed in syncytiotrophoblasts and circumstantial evidence suggests that it may play a role in cytotrophoblast differentiation [1]; however, it is probably not essential since 1% of the Caucasian population lack any functional protein [18]. Syncytin, a protein encoded by an envelope gene of the recently identified human endogenous retrovirus-W (HERV-W) [19], may mediate placental cytotrophoblast fusion in vivo, and thus be important in human placental morphogenesis [2,20,21]. In primary culture of isolated cytotrophoblast cells, the transcript levels of syncytin increase with the differentiation and fusion of cytotrophoblasts into syncytiotrophoblasts [22]. Immunohistochemical analysis showed syncytin (HERV-W) expression restricted to placenta [3], however, there is little consensus regarding its localization in villous trophoblast [20,23]. Syncytin is mis-expressed in pre-eclampsia where syncytiotrophoblast formation is defective and syncytin is anomalously distributed on the apical surface of syncytiotrophoblast [24]. The viral homolog, ERV-W, is also a highly fusogenic membrane glycoprotein that can induce syncytium formation upon interaction with the type D mammalian retrovirus receptor [3] – an amino acid transporter B^0 (ASCT2) [25,26]. The relative

abundance of the mRNAs of syncytin and its receptor – amino acid transport system B^0 – changes reciprocally in BeWo cells during forskolin-induced syncytialization [27].

Our current studies are directed to developing and utilizing siRNAs to these possible molecules for further analysis of the molecular basis of trophoblast syncytialization.

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