Involvement of connexin 43 in human trophoblast cell fusion and differentiation

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Summary

The syncytiotrophoblast is the principal component of the human placenta involved in feto-maternal exchanges and hormone secretion. The syncytiotrophoblast arises from the fusion of villous cytotrophoblasts. We recently showed that functional gap junctional intercellular communication (GJIC) is an important prerequisite for syncytiotrophoblast formation and that connexin 43 (Cx43) is present in cytotrophoblasts and in the syncytiotrophoblast. To determine whether Cx43 is directly involved in trophoblast fusion, we used an antisense strategy in primary cultures of human villous cytotrophoblasts that spontaneously differentiate into the syncytiotrophoblast by cell fusion. We assessed the morphological and functional differentiation of trophoblasts by desmoplakin immunostaining, by quantifying hCG (human chorionic gonadotropin) production and by measuring the expression of specific trophoblast genes (hCG and HERV-W). Furthermore, we used the gap-FRAP (fluorescence recovery after photobleaching) method to investigate functional GJIC. Cytotrophoblasts treated with Cx43 antisense aggregated and fused poorly. Furthermore, less HERV-W env mRNA, hCGβ mRNA and hCG secretion were detected in Cx43 antisense-treated cytotrophoblasts than in cells treated with scrambled antisense. Treatment with Cx43 antisense dramatically reduced the percentage of coupled trophoblast cells. Taken together, these results suggest that Cx43 is directly involved in human trophoblast cell-cell communication, fusion and differentiation.

Key words: Cx43, Placenta, Herv-W, hCG, Cell-cell fusion

Introduction

In humans, fetal cytotrophoblasts play a key role in embryo implantation and in placental development. In early pregnancy, mononuclear cytotrophoblasts (CT) proliferate and invade the maternal endometrium to form the anchoring villi. Cytotrophoblasts also differentiate into a multinucleated continuous layer known as the syncytiotrophoblast (ST). This layer, which covers the chorionic villi, is bathed with maternal blood in the intervillous spaces (Benirschke and Kaufmann, 2000). Owing to its position, the syncytiotrophoblast is the site of numerous placental functions including exchanges, metabolism and the synthesis of the steroid and peptide hormones required for fetal growth and development (Eaton and Contractor, 1993; Ogren and Talamentes, 1994). Some of these hormones, such as human chorionic gonadotropin (hCG) (Muyan and Boime, 1997), human placental lactogen (hPL) (Handwerger, 1991) and placental growth hormone (PGH; also called GH variant) (Lacroix et al., 2002) are specific to pregnancy. In situ and in vitro studies have shown that the ST arises from the fusion of mononuclear CTs (Kliman et al., 1986; Richard, 1961). The morphological aspects of this in situ differentiation pathway were recently described in the broader context of continuous trophoblast turnover including the continuous proliferation of CT stem cells, the recruitment of post-mitotic cells to the ST after membrane fusion and progression towards apoptosis (Mayhew, 2001). Isolated mononucleated CTs aggregate and fuse in vitro, forming a nonproliferative multinucleate syncytiotrophoblast that produces pregnancy-specific hormones (Kliman et al., 1986; Malassiné et al., 1990). This in vitro differentiation involves all of the activities of normal CTs during in vivo maturation. The fusion of human CTs is a very important step in the formation of the ST, but remains poorly understood. The process is accompanied by a concomitant increase in cellular levels of cAMP, which is required for the synthesis of numerous trophoblast-specific proteins, and a decrease in basal Ca^{2+} activity (Cronier et al., 1999; Roulier et al., 1994). Several factors are involved in the fusion of trophoblastic cells, including a phosphatidylserine (PS) flip (Adler et al., 1995), a human endogenous retroviral envelope glycoprotein encoded by HERV-W (syncytin) (Blond et al., 2000; Frendo et al., 2003; Mi et al., 2000).

Gap junctions are clusters of transmembrane channels composed of connexin (Cx) hexamers. In general, the effects of Cx expression have been attributed to gap junctional intercellular communication (GJIC) and sharing a common pool of intracellular messengers and metabolites. Gap junctions provide a pathway for the diffusion of ions and small

molecules such as cAMP, cGMP, inositol trisphosphate (IP_3) and Ca^{2+}. Connexins represent a family of closely related membrane proteins, which are encoded by a multigene family that contains at least 20 members in humans. These connexins have different biophysical properties and functional and regulatory characteristics (Willecke et al., 2002). The permeability of junctional channels is finely regulated. This regulation involves the cyclic phosphorylation and dephosphorylation of connexins and changes in intracellular Ca^{2+}, H^+ and cAMP concentrations. In addition, connexin expression varies during differentiation, proliferation and transformation processes and following treatment with biologically active substances such as growth factors and hormones (Bruzzone et al., 1996; Kumar and Gilula, 1996; Lau et al., 1992; Loewenstein, 1981). The exchange of molecules through gap junctions is thought to be involved in the control of cell proliferation, in the control of cell and tissue differentiation, in metabolic cooperation and in spatial compartmentalization during embryonic development (Bani-Yaghoub et al., 1999; Constantin and Cronier, 2000; Lecanda et al., 1998; Loewenstein, 1981; Saez et al., 1993).

We previously showed that Cx26, Cx32, Cx33, Cx40 and Cx45 are not detected in human trophoblast, whereas Cx43 mRNA and protein are present between cytotrophoblastic cells and between cytotrophoblastic cells and the syncytiotrophoblast (Cronier et al., 2002). Furthermore, in vitro studies using fluorescence recovery after photobleaching (gap-FRAP) showed the presence of a functional gap junctional intertrophoblastic communication before trophoblast fusion (Cronier et al., 2001; Cronier et al., 1994). In addition, treatment of CT with heptanol (a nonspecific junctional uncoupler blocking all connexin channels) inhibits trophoblastic GJIC leading to a decrease in ST formation, which suggests a role for GJIC in trophoblastic fusion (Cronier et al., 1994).

The possibility of nonspecific actions for heptanol lead us, using an antisense strategy, to determine the specific functional role for Cx43 in trophoblastic fusion and differentiation. We assessed the morphological and functional differentiation of cultured human villous trophoblasts by desmoplakin immunostaining, by measuring hCG production and by measuring the expression of trophoblast-specific genes (hCG and HERV-W). Furthermore, we used the gap-FRAP method to investigate functional GJIC.

Materials and Methods

Cell culture

Term placentas were obtained after elective cesarean section from healthy mothers who had had uncomplicated pregnancies. Villous tissue was dissected free of membranes, rinsed and minced in Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (HBSS). Cytotrophoblast cells were isolated after trypsin-DNase I digestion and discontinuous Percoll gradient fractionation using a slightly modified version of the method described by Kliman (Alsat et al., 1991; Cronier et al., 1997; Kliman et al., 1986) as previously described (Frendo et al., 2001). Briefly, the villous sample was submitted to sequential enzymatic digestions in a solution containing 0.5% (W/V) trypsin powder (Difco, USA), 5 IU/ml of DNase I, 25 mM HEPES, 4.2 mM $MgSO_4$ and 1% (W/V) penicillin/streptomycin (Biochemical industrie, Israel) in HBSS. This process was monitored under a light microscope. The first and/or second digestion was discarded after light microscopy analysis to eliminate syncytiotrophoblast fragments, and the following four or five sequential digestions were kept. The cells collected during these last digestions were purified on a discontinuous Percoll gradient (5-70% in 5% steps). The cells that sedimented in the middle layer (density 1.048-1.062 g/ml) were further purified using a monoclonal anti-human leukocytic antigen A, B and C antibodies (W6-32HL, Sera Lab, Crawley Down, UK) as previously described (Cronier et al., 2002). This antibody reacts with most cell types (e.g. macrophages, fibroblasts, extra villous trophoblast) but not with villous cyto- or syncytiotrophoblasts. Briefly, the isolated cells were transferred to culture dishes coated with the monoclonal antibody. After 15 minutes at 37°C, nonadherent cells were recovered by gently rocking the dishes and removing them with a pipette. Cytotrophoblastic cells were diluted to a final concentration of 5×10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS). After 4 hours at 37°C in a 5% CO_2 atmosphere, nonadherent cells and syncytial fragments were removed by three washes with culture medium. After 3 hours of culture, 95% of the cells isolated from term placentas were cytotrophoblasts, as determined by cytokeratin 7-positive staining, using a specific monoclonal antibody (dilution 1:200, Dako, USA). Cells were cultured in 2 ml of DMEM supplemented with 25 mM HEPES, 2 mM glutamine, 10% heat-inactivated FCS and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin) at 37°C in a humidified 5% CO_2 95% air atmosphere.

Modulation of gene expression by antisense oligonucleotides

Synthetic antisense oligonucleotides targeting Cx43 were purchased from Biognostik (Gottingen, Germany). The phosphorothioate-modified Cx43 antisense oligonucleotide is the reverse complement of a target sequence described previously (Fishman et al., 1991) (Table 1). The absence of duplex and hairpin formations and the absence of cross-reactivity with related sequences in GenBank were checked. The cells were seeded into 35 mm dishes at a density of 100,000 cells per well, 4 hours before the addition of phosphorothioate-modified antisense. Typically, normal cultured cytotrophoblastic cells were incubated with 10 μM synthetic Cx43 antisense oligonucleotide and 10 μM scrambled antisense (control, Biognostik). After 48 hours of incubation, the cells were harvested and protein and total RNA were extracted.

Immunocytochemistry

To detect desmoplakin, cultured cells were rinsed with PBS, fixed and permeabilized in methanol at –20°C for 25 minutes. A monoclonal anti-desmoplakin antibody (1:400, Sigma-Aldrich, Saint-Quentin Fallavier, France) was then applied, followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (1:400, Jackson Immunoresearch Laboratories, Wet Grove, PA), as previously described (Alsat et al., 1996; Frendo et al., 2000b). After washing, samples were mounted in medium with 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector laboratories, Burlingame, CA).

Table 1. RT-PCR primers and antisense oligonucleotides

Target mRNA	Oligonucleotides
HERV-W	(+) CGGACATCCAAAGTGATACATCCT
	(–) TGATGTATCCAAGACTCCACTCCA
RPLP0	(+) GGCGACCTGGAAGTCCAACT
	(–) CCATCAGCACCACAGCCTTC
hCGβ	(+) GCTACTGCCCCACCATGACC
	(–) ATGGACTCGAAGCGCACATC
CX43 antisense	GCAAGTGTAAACAGCG
Scrambled	GNNNNNNNNNNNNNG

Upper (+) and lower (–) primers used in RT-PCR assays.

To detect Cx43, specimens were fixed for 10 minutes in methanol at –20°C. They were then washed three times in PBS and processed using a method similar to that described by Tabb et al. (Tabb et al., 1992). After incubation in a blocking solution consisting of 2% bovine serum albumin and 1% Triton X100 in PBS for 30 minutes at room temperature, specimens were washed three times in PBS and incubated overnight with monoclonal Cx43 primary antibodies (1:200, Transduction Laboratories, Lexington, KY). After five further washes in PBS, FITC-conjugated goat anti-mouse antibodies (1:100) were applied for 45 minutes at room temperature. After washing, samples were mounted in medium with DAPI for nuclear staining. The controls, which consisted of omitting the primary antibody or applying the nonspecific IgG of the same isotype, were all negative.

Gap-FRAP method

The degree of intercellular communication between neighboring cultured trophoblastic cells was determined by measuring the cell-to-cell diffusion of a fluorescent dye (Wade et al., 1986) using an interactive laser cytometer (ACAS 570, Meridian Instruments, Okemos, MI). Briefly, the cells were internally loaded for 10 minutes at room temperature with the membrane-permeant molecule, 6-carboxyfluorescein diacetate (7 μg/ml in 0.25% DMSO; Sigma Chemical Co.). The highly fluorescent and membrane impermeable 6-carboxyfluorescein moiety is released and accumulates within cells. After washing off the excess extracellular fluorogenic ester to prevent further loading, a cell adjacent to other cells was selected and its fluorescence was photobleached by strong laser pulses (488 nm). Digital images of the fluorescent emission excited by weak laser pulses were recorded at regular intervals for 12 minutes (scanning period 2 minutes before and after photobleaching) and stored for subsequent analysis. In each experiment, one labeled, isolated cell was left unbleached as a reference for the loss of fluorescence due to repeated scanning and dye leakage, and an isolated, bleached cell served as a control. When the return of fluorescence followed a fast step-like course, reaching ≥90% of the final steady state within <30 seconds of photobleaching, the diffusion of the dye was neither prevented by the cell membranes nor limited by the presence of gap junctions. We thus assumed that the fusion of cell membranes had been completed and that the cellular elements were part of a true syncytium. When the bleached cells were connected to unbleached contiguous cells by open gap junctions, the fluorescence recovery followed a slow exponential time-course. Therefore, the analysis of the kinetics of fluorescence recovery makes it possible to distinguish between aggregated cytotrophoblastic cells and the syncytiotrophoblast. In our experimental conditions, GJIC was investigated (coupled cells or not) by measuring the percentage of coupled cells in a population of neighboring cells. GJIC was analyzed 2 days after plating. Three different topologies were recognized: contiguous cytotrophoblastic cells, contiguous syncytiotrophoblasts, and contiguous cyto- and syncytiotrophoblasts. During trophoblast differentiation and cell treatments, the percentage of coupled cells was analyzed whatever the topology of the trophoblastic elements (Cronier et al., 1994).

Syncytium formation analysis

Syncytium formation was followed by fixing and immunostaining cells so that the distribution of desmoplakin and nuclei in cells could be observed (Keryer et al., 1998). The staining of desmoplakin present at the intercellular boundaries in aggregated cells progressively disappears as the syncytium is formed (Alsat et al., 1996; Douglas and King, 1990). The nuclei contained in 100 syncytia in a random area near the middle of the slides were counted. Three coverslips were examined for each experimental condition. Results are expressed as number of nuclei per syncytium.

Hormone assay

The concentration of hCG was determined in culture media by use of the chemiluminescent immunoassay analyser ACS-180SE system (Bayer Diagnostics, Germany). The sensitivity of the assay was 2 mU/ml. All values are means±s.e.m. of triplicate determinations.

RNA isolation and analysis

Total RNA was extracted from cultured cells as described by Qiagen (Courtabeuf, France). The total RNA concentration was determined at 260 nm and its integrity was checked in a 1% agarose gel. The relative mRNA levels of the different genes were measured by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), essentially as previously described (Frendo et al., 2000a), using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystem, USA) and the SYBR Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). The nucleotide sequences of the primers used are listed in Table 1. Each sample was analyzed in duplicate and a calibration curve was constructed in parallel for each analysis. The level of transcripts was normalized according to the RPLP0 gene (also known as 36B4), which encodes human acidic ribosomal phosphoprotein P0 as an endogenous RNA control, and each sample was normalized on the basis of its RPLP0 content.

Immunoblot analysis

Cells were washed twice with ice-cold PBS, scraped and lysed at 4°C in a buffer containing 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate and 50 mM Tris-HCl (pH 8) supplemented with protease inhibitors. The lysates were incubated at 4°C for 10 minutes and then centrifuged for 10 minutes at 10,000 ***g*** to pellet the nuclei. Protein concentration was determined according to Bradford's method (BioRad, USA) using bovine serum albumin as the standard. Supernatants were then frozen at –70°C until further analysis. Immunobloting was performed in accordance with standard procedures. Cell lysates (30 μg) were mixed 3:1 (vol:vol) in a 100 mM Tris HCl (pH 6.8) buffer containing 1% sodium dodecyl sulphate (SDS), 10% glycerol, 5% β-mercaptoethanol. They were heated at 95°C for 15 minutes and then loaded on 10% SDS-PAGE (polyacrylamide gel electrophoresis) gels. After transfer onto nitrocellulose membranes, the membranes were incubated in Tris–buffered saline with 5% milk powder and 0.05% Tween 20 overnight at 4°C. Immunostaining was performed in the same buffer with 1% powdered milk. The blots were probed with the following antibodies: a mouse monoclonal anti-connexin 43 (Transduction Laboratories) at 1/1000 dilution and a cytokeratin 7-specific monoclonal antibody (dilution 1:1000, Dako). Finally, blots were developed by using horse-radish peroxidase-conjugated antibodies (Jackson) and an enhanced chemiluminescence kit (Pierce supersignal, Interchim France).

Statistical tests

Statistical analysis was performed using the StatView F-4.5 software package (Abacus Concepts, Inc., Berkeley, CA). Values are presented as mean±s.e.m. Significant differences were identified using Mann-Whitney analysis for hormonal secretions and ANOVA for antisense studies; $P<0.05$ was considered significant.

Results

Effect of Cx43 antisense on Cx43 protein production in human trophoblasts

We used an antisense strategy to study the role of Cx43 in human trophoblast cell fusion. First, we confirmed that the oligonucleotide was efficiently taken up by cytotrophoblast

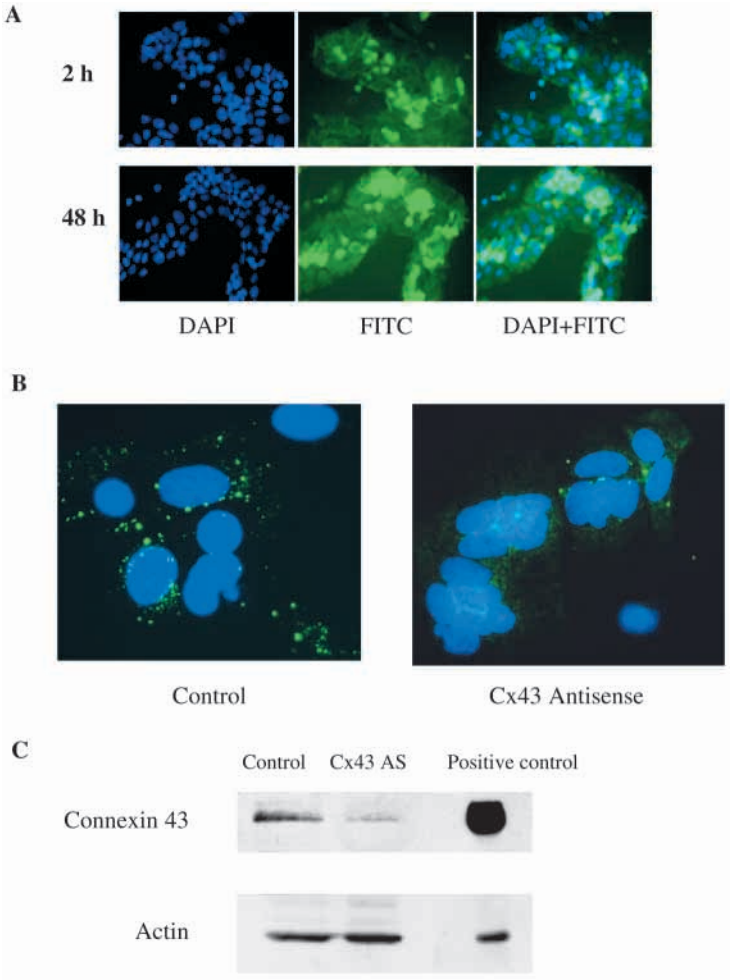


Fig. 1. Effects of Cx43 antisense on Cx43 protein production in human trophoblasts. (A) Oligonucleotide uptake by primary cytotrophoblasts. After 4 hours of culture, cytotrophoblasts were incubated with FITC-labeled scrambled oligonucleotide for 1 hours, 2 hours, 24 hours and 48 hours. At each time-point, cells were washed three times in PBS, fixed and analyzed by fluorescence microscopy. Nuclei were stained in blue by DAPI. Intensely fluorescent green cells have internalized the scrambled antisense oligonucleotide (FITC) (×600). (B) Immunodetection of Cx43 in cytotrophoblast cells isolated from normal placentas. Cells were treated for 48 hours with a scrambled (control) or a specific Cx43 antisense (Cx43 antisense). Cell nuclei were labeled with DAPI (blue immunofluorescence). In the control, Cx43 punctuate immunofluorescence (IF) can be observed around nuclei and at the borders with neighboring trophoblastic cells. In cells treated with Cx43 antisense, the level of IF is largely decreased (×1000). (C) Cx43 protein levels in trophoblast cells after treatment with a scrambled antisense (control) or with a specific Cx43 antisense (Cx43 AS) were determined by western blotting using a mouse anti-Cx43 monoclonal antibody. An anti-actin monoclonal antibody was used as a standard. Proteins obtained from rat brain lysate were used as a positive control. One representative experiment out of the three performed is shown.

cells by adding FITC-conjugated oligonucleotide to the cell culture. Oligonucleotides were taken up from the first hour by primary cells (20% of cells were labeled) and the proportion of labeled cells then increased progressively with time (26% at 2 hours, 35% at 24 hours and 41% at 48 hours) (Fig. 1A). We then evaluated the efficiency and the specificity of Cx43 antisense treatment to block the production of the Cx43 protein. When term cytotrophoblastic cells were cultured for 48 hours in the presence of a scrambled antisense (control), punctate immunostaining could be observed at the borders of adjacent cells (Fig. 1B) as previously described (Cronier et al., 2002). The addition of Cx43 antisense to the culture medium greatly decreased Cx43 immunostaining. We confirmed that the amount of Cx43 protein had indeed decreased by western blot analysis. The amount of Cx43 protein was clearly lower in Cx43 antisense-treated cells than in scrambled antisense-treated control cells (Fig. 1C). By contrast, no difference in actin levels was observed between Cx43 antisense-treated cells and control cells.

Effects of Cx43 antisense on the morphological differentiation of trophoblasts

In vitro, purified mononuclear cytotrophoblasts isolated from normal human term placentas aggregate and then fuse, forming the multinucleated syncytiotrophoblast. The fusion and differentiation of isolated human cytotrophoblast cells have been monitored by staining cells with anti-desmoplakin antibodies to reveal cell boundaries (Alsat et al., 1996; Douglas and King, 1990). Indeed, in our experiments the desmoplakin staining present at the intercellular boundaries of aggregated cells progressively disappeared as the syncytiotrophoblast is formed. After 72 hours of culture, most mononuclear cytotrophoblasts had differentiated into syncytiotrophoblasts, as illustrated by the gathering of numerous nuclei in a large cytoplasmic mass a (Fig. 2a). By contrast, cytotrophoblasts treated for 48 hours with 10 μM Cx43 antisense aggregated but did not fuse or fused poorly. Syncytiotrophoblasts were rare, as indicated by the persistence of desmoplakin immunostaining at the intercellular boundaries of aggregated cells (Fig. 2b).

To assess further the direct involvement of Cx43 in cell fusion and syncytium formation, we estimated the number of DAPI-stained nuclei per syncytium. During the first 3 days of culture, the number of nuclei per syncytium increased (Fig. 3). For instance, after 72 hours of culture, 33% of syncytia observed contained more than 12 nuclei. By contrast, in the presence of Cx43 antisense, ST formation was impaired; only small syncytia with three to six nuclei were observed. At 72 hours, no syncytia with more than nine nuclei were observed, illustrating that Cx43 antisense disrupts cell-cell fusion.

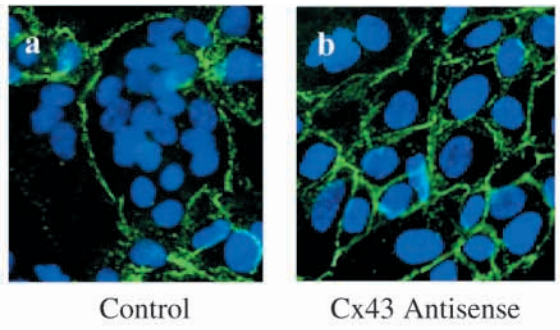


Fig. 2. Effects of Cx43 antisense on the morphological differentiation of trophoblasts. Cytotrophoblasts isolated from human placentas were cultured in the presence of a scrambled antisense (Control) or with a specific Cx43 antisense (Cx43 antisense). After 72 hours of culture, the cells were fixed, immunostained with anti-desmoplakin monoclonal antibody and counterstained with DAPI. Large syncytia were observed in control cells (a) as immunofluorescent staining disappeared when cells have fused to form the syncytiotrophoblast. By contrast, immunoflurescent staining can be observed at the boundaries between aggregated cytotrophoblasts in cells treated with a specific Cx43 antisense (b).

Functional analysis of the effects of Cx43 antisense on cell-cell communication

We used the gap-FRAP method to study the effect of Cx43 antisense on GJIC. GJIC was previously analyzed between cultured contiguous trophoblastic cells (Cronier et al., 1997), illustrating that dye could diffuse between cytotrophoblastic cells, between cyto- and syncytiotrophoblasts and between syncytiotrophoblasts. In our study, the presence of scrambled antisense in the culture medium for 48 hours did not significantly affect the trophoblastic cell-to-cell communication (5.3% of coupled cells) compared with standard conditions (6%). By contrast, the presence of Cx43 antisense in the culture medium dramatically reduced the percentage of coupled trophoblastic cells (Fig. 4).

Effects of Cx43 antisense on gene expression and hormonal secretion

As previously reported, the formation of the syncytiotrophoblast by the fusion of cytotrophoblasts is associated with significant increases in hCGβ mRNA and hCG secretion. Cells treated with Cx43 antisense contained less hCGβ mRNA and secreted less hCG into the culture medium at 48 hours (P<0.018) and 72 hours (P<0.035) than cells treated with the scrambled antisense (Fig. 5).

We recently showed that the expression of HERV-W env, which is also called syncytin, increases during ST formation (Frendo et al., 2003) and is directly involved in the trophoblastic fusion process (Blond et al., 2000; Frendo et al., 2003). Thus, we used real-time quantitative RT-PCR to determine the levels of syncytin mRNA in cytotrophoblasts. HERV-W env mRNA levels were significantly lower (33% decrease after 48 hours of treatment; P<0.019) in Cx43 antisense-treated cells than in control cells treated with a scrambled antisense at 48 hours, which is the time-point at which HERV-W is maximally expressed.

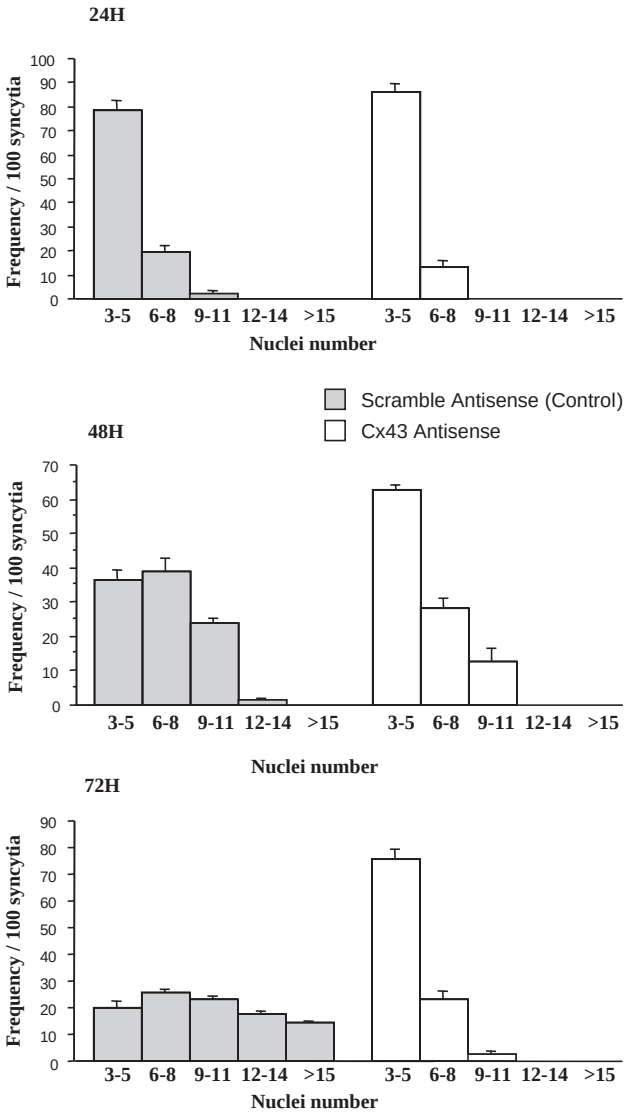


Fig. 3. Cell fusion index. Human cytotrophoblasts were incubated with a scrambled antisense (dark columns) or with a specific Cx43 antisense (white columns). After 24 hours (upper panel), 48 hours (middle panel) and 72 hours (lower panel), the cells were fixed, immunostained with anti-desmoplakin monoclonal antibody and counterstained with DAPI. One hundred syncytia were scored after staining and the nuclei were counted in each syncytium. Data show the distribution of syncytia as a function of the number of nuclei per syncytium. The figure illustrates the mean±s.e.m. of three independent experiments.

Discussion

In this study, we show for the first time that Cx43 antisense impairs human trophoblast cell-cell communication, human trophoblast fusion and differentiation as established on morphological and functional criteria. This suggests that Cx43 plays an important role in these processes.

We used an in vitro model of cultured villous trophoblastic cells that has been used to study certain aspects of the dynamic processes that occur during villous differentiation (Kliman et al., 1986). To rule out the possibility that villous trophoblast

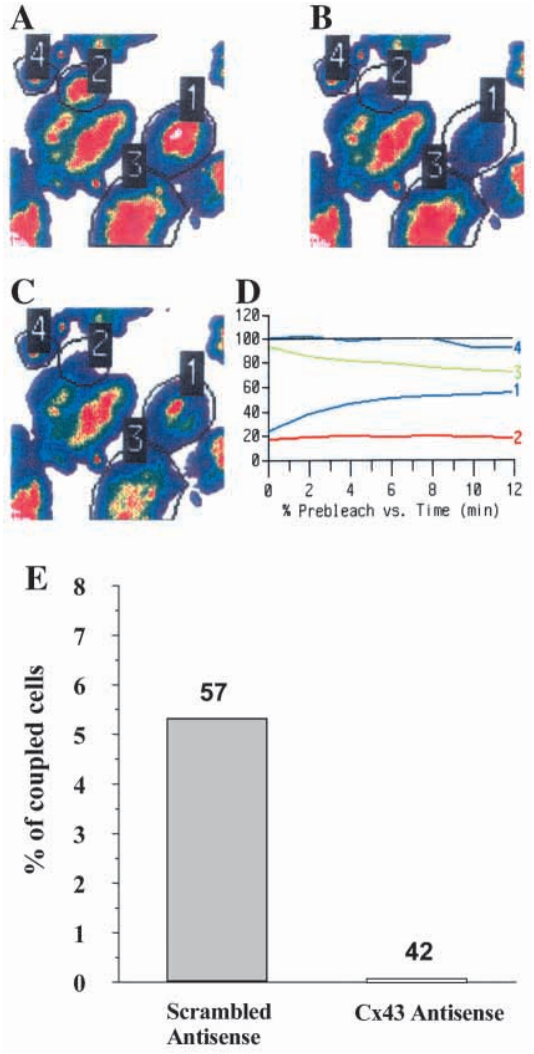


Fig. 4. Functional intercellular communication measured by the gap-FRAP method. Upper four panels: typical computer-generated images of fluorescence distribution in villous trophoblastic cells cultured in the presence of scrambled antisense for 48 hours measured during a gap-FRAP experiment. After a prebleach scan (A), the fluorescent dye was photobleached in some selected cells (polygons 1 and 2) by means of a strong laser illumination. Isolated cells (polygon 4) kept unbleached served as a control for the spontaneous fading of fluorescent emission (B). The evolution of fluorescence intensities was measured starting just after photobleaching for 12 minutes with a scanning period of 2 minutes. After 12 minutes (C), a fluorescence recovery had occured in area 1, whereas the fluorescence intensity remained weak in area 2, indicating that cell 2 is not coupled to neighboring cells. D represents curves of fluorescence evolution in selected cells: fluorescence recovery in cell 1 follows a closely exponential time-course, reflecting the presence of open gap junctional channels. Note the low decrease of fluorescence intensity in the control unbleached cell (4) due to repeated scanning. Lower panel: percentage of coupled cells between villous trophoblastic cells after 48 hours of culture in the presence of scrambled antisense (dark column) or Cx43 antisense (white column). Coupled cells were characterized by an exponential time-course of fluorescence recovery from neighboring cells into a photobleached test cell. Functional communication was measured between cytotrophoblastic cells, between cyto- and syncytiotrophoblasts and between syncytiotrophoblasts. The number of intercellular contacts analyzed is indicated on top of the bars.

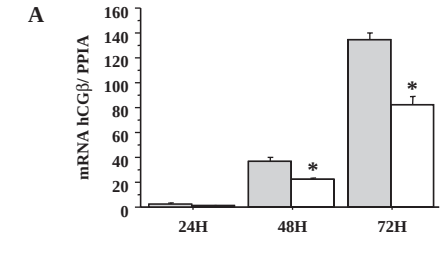
cells were contaminated by other cells containing Cx43 protein and transcripts, such as endothelial and mesenchymal cells, we added an additional purification step with a monoclonal anti-human leukocytic antigen A, B and C. Furthermore, we thoroughly washed cultured trophoblastic cells after cellular adherence to eliminate syncytiotrophoblast fragments (Cronier et al., 1997; Guilbert et al., 2002). Following this procedure, 95% of cultured cells are positive for cytokeratin 7 immunostaining (a specific marker of trophoblasts). As previously described, syncytiotrophoblast formation is associated with significant increases in αhCG mRNA, βhCG mRNA, leptin and PGH mRNA levels (Frendo et al., 2000b).

Few human cell types can fuse together and differentiate into multinucleated syncytia. This process is involved in the formation of myotubes (Constantin and Cronier, 2000; Mege et al., 1994; Wakelam, 1985), osteoclasts (Ilvesaro et al., 2000; Zambonin Zallone et al., 1984) and the syncytiotrophoblast (Midgley et al., 1963). Although they share a common morphological differentiation process, the three cell types that are able to differentiate into a syncytium differ notably. Owing to its position, the syncytiotrophoblast maintains a strong polarity with an apical microvillous membrane both in situ and in vitro and is primarily engaged in absorption, exchanges and endocrine functions. By contrast, myotubes do not exhibit morphological polarity. The myoblast-myotube transition first requires the withdrawal of myoblasts from the cell to G_0, whereas only the highly differentiated cells from the large pool of cytotrophoblastic cells in the G_0 phase actually fuse with the syncytiotrophoblast (Huppertz et al., 1998). Unlike the syncytiotrophoblast, osteoclasts display strong locomotor activity.

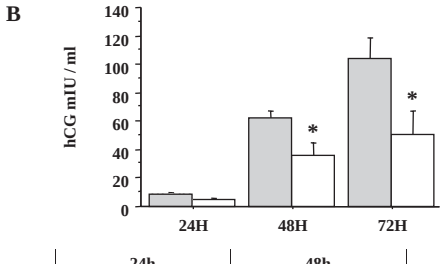
The cell-cell fusion process involved in syncytiotrophoblast formation is poorly understood. One membrane event thought to be involved in fusion is the phosphatidylserine (PS) flip. Phosphatidylserine is a phospholipid that is normally confined to the inner layer of the plasma membrane. However, before fusion, it translocates to the outer layer and facilitates intermembrane fusion (PS flip). Adler et al. (Adler et al., 1995) have shown that incubation with an anti-PS antibody inhibits the forskolin-induced syncytial fusion of choriocarcinoma cells. According to Huppertz and colleagues (Huppertz et al., 1998), this PS flip is a consequence of the activation of an initiator caspase (e.g. caspase 8), suggesting that the molecular machinery of early apoptosis is involved in the fusion process.

Other studies have suggested that human endogenous retroviruses play an important role (Blond et al., 2000; Mi et al., 2000). Indeed, the production of recombinant syncytin (a glycoprotein encoded by Env-W retrovirus) in a variety of cell types induces the formation of giant syncytia. Furthermore, the fusion of a human trophoblast cell line expressing endogenous syncytin is inhibited by an antisyncytin antiserum. Syncytin is highly expressed in normal human trophoblasts and recently we showed using the same antisense strategy that syncytin is involved in human trophoblast cell fusion and differentiation (Frendo et al., 2003).

In this study, we show that Cx43 expression is also involved in cell fusion. Gap junctions have been implicated in placental development (for a review, see Winterhager et al., 2000). Ultrastructural studies have detected gap junctions between the trophoblastic layers in placentas (de Virgiliis et al., 1980; Firth et al., 1980; Malassiné and Leiser, 1984). Furthermore, gap



experiment	24h		48h		72h	
	Control	CX43-AS	Control	CX43-AS	Control	CX43-AS
1	2.5±0.5	1.3±0.3	36.4±3.8	22±1.5	135±5	82±7
2	27±2	12.5±1.5	252.3±6.1	150.6±6.9	1095.5±95	716±16
3	4±1	1.5±0.5	28.3±2	17.3±2.4	104.5±5.5	57.5±2.5



experiment	24h		48h		72h	
	Control	CX43-AS	Control	CX43-AS	Control	CX43-AS
1	8.6±1.3	4.6±1.5	61.8±4.9	35±8.4	103±14.9	50.5±16.6
2	104.6±8.5	61.1±2.9	602±11.3	301±10.3	1496±34.1	777.5±17
3	12±1.5	3.7±0.7	43.7±2.1	19.1±1.7	70.5±1.9	30.4±2.3

Fig. 5. hCG expression and secretion. (A) hCGβ mRNA levels were determined by real-time quantitative RT-PCR in cytotrophoblasts treated with a scrambled antisense used as a control (dark columns) or with a specific Cx43 antisense (white columns). These assays were carried out 24 hours, 48 hours and 72 hours after plating. Values are the levels of hCGβ mRNA normalized to the level of PPIA (cyclophilin A) mRNA. (B) Amounts of hCG secreted into the culture medium at 24 hours, 48 hours and 72 hours of culture in the presence of a scrambled antisense (dark columns) or of a specific Cx43 antisense (white columns). Values are means from three separate dishes±s.e.m. and the figure illustrates one representative experiment from the three performed. **P*<0.05. Values of βhCG mRNA and hCG secretion in the three independent experiments are shown in the tables. Graphs are representative of experiment 1.

junctions are present during cytotrophoblastic cell fusion in the guinea-pig placenta (Firth et al., 1980). In human trophoblast, we have previously shown that Cx26, Cx32, Cx33, Cx40 and Cx45 are not detected, whereas Cx43 mRNA and protein are present. Furthermore, using gap-FRAP we have shown the presence of a functional gap junctional intertrophoblastic communication preceeding trophoblastic fusion. The low occurrence of coupled cells observed (5.3% after 2 days of culture) argues for a brief duration or paucity of this gap junctional communication. This is in accordance with the fact that in trophoblast primary cultures, gap junctions are only observed in a low number of cells with transmission electron microscopy (Cronier et al., 1994).

The role of gap junctions in differentiation can be studied by chemically inhibiting gap junctional communication, by using an antisense oligonucleotide approach or by overexpressing connexin genes. Several lypophilic substances, such as aliphatic alcohols, and compounds isolated from liquorice roots (glycyrrhetinic acid) can uncouple gap junctional channels. Long-term incubation with heptanol considerably reduces the degree of fusion of cultured myoblasts (Constantin and Cronier, 2000; Mege et al., 1994) and preliminary studies showed that heptanol reversibly decreases gap junctional intertrophoblastic communication and trophoblastic cell fusion. Although its exact mechanism of action is not known, heptanol seems to decrease the fluidity of membranous cholesterol-rich domains (Johnston et al., 1980; Takens-Kwak et al., 1992) leading to a decrease of the open probability of junctional channels. Furthermore, in cultured neonatal rat cardiomyocytes, heptanol does not decrease the number of gap junctional channels, and in pancreatic acinar cells, there is a cessation of GJIC, although gap junctions remain structurally intact (Chanson et al., 1989). Nevertheless, heptanol have been implicated in other biological processes. For example, it could modulate the activity of nicotinic acetylcholine receptor channels in cultured rat myotubes (Murrell et al., 1991) and the Ca^{2+}-activated K^+ channel expressed in *Xenopus* oocyte (Chu and Treistman, 1997). Furthermore, heptanol and octanol were thought to affect some of the initiating responses of intracellular calcium elevation (Venance et al., 1998). The possibility of a non-uncoupling action for heptanol and its nonspecific action blocking all the connexin channels led us to develop an antisense strategy. It was shown in this study that treatment with a scrambled antisense does not affect the functional gap junctional communication and trophoblast differentiation, whereas treatment with Cx43 antisense abolishes gap junctional communication and reduces trophoblast differentiation. These data showed the major implication of Cx43 expression in gap junctional communication and cytotrophoblastic cell-cell fusion.

The main effects of Cx expression have been attributed to gap junctional communication. The existence of cell-to-cell channels allows the exchange of second messengers between aggregated trophoblastic cells, and this exchange may regulate the fusion process. The nature of the messengers involved in the intertrophoblastic gap junctional communication needs to be addressed in the near future. It is conceivable that Ca^{2+}, IP_3 and cAMP are exchanged, thus controlling various cellular effectors involved in fusion and in the transcription of syncytiotrophoblast-specific genes (Aronow et al., 2001; Keryer et al., 1998). These intercellular messengers may also crosstalk with GJIC, as gap junction channels are regulated by cAMP and Ca^{2+}. Thus, fusion may be correlated with a concomitant increase in cellular levels of cAMP (Roulier et al., 1994) and with a decrease in basal Ca^{2+} activity (Cronier et al., 1999).

In humans, data concerning GJIC and trophoblast differentiation have been obtained in vitro, and recently the principles of placental development have been explained by gene knockout approaches in mice (Rossant and Cross, 2001). Owing to the striking diversity in placental structure and endocrine function, we must be careful when extrapolating findings regarding placental development from one species to another. In mice, Cx26 and Cx31 deficiencies cause placental alterations (Gabriel et al., 1998; Plum et al., 2001), whereas in the human placenta, Cx26 and Cx31 are not expressed.

In conclusion, cell fusion is the limiting factor in human villous trophoblast differentiation and studies are still required to improve our understanding of the various factors directly

involved in human trophoblast fusion and differentiation. In this study, we show for the first time that Cx43 is one of the componants involved in these processes. Pathological models, such as cytotrophoblasts isolated from T21-affected placentas (Frendo et al., 2001) and in which cell fusion and syncytiotrophoblast formation are defectuous (Frendo et al., 2000b), should help to further our understanding of the cell-cell fusion process.

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