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Involvement of Connexin 43 in human trophoblast cell fusion and differentiation

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ABSTRACT

The syncytiotrophoblast is the major component of the human placenta involved in feto-maternal exchanges and hormone secretion. The syncytiotrophoblast arises from the fusion of

villous cytotrophoblasts. We recently demonstrated that functional gap junctional intercellular communication (GJIC) is an important prerequisite for syncytiotrophoblast formation and that Connexin 43 (Cx43) is present in both 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 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.

INTRODUCTION

In humans, fetal cytotrophoblasts play a key role in the implantation of embryos 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). Due 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 GH (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 non-proliferative 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. This process is accompanied by a concomitant increase in cellular levels of cAMP, 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: 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 triphosphate 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, 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 demonstrated, that Cx 26, 32, 33, 40 and 45 are not detected in human trophoblast, whereas Cx 43 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), demonstrated the presence of a functional gap junctional inter-trophoblastic communication before trophoblast fusion (Cronier *et al.*, 2001, Cronier *et al.*, 1994). In addition, treatment of CT with heptanol (a non-specific junctional uncoupler blocking all connexin channels) inhibits trophoblastic GJIC leading to a decrease in ST formation suggesting a role for GJIC in trophoblastic fusion (Cronier et al. 1994).

The possibility of non-specific 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), 5 IU/ml of DNase I, 25 mM HEPES, 4.2 mM $MgSO_4$ and 1% (W/V) penicillin/streptomycin (Biochemical industrie) in HBSS. This process was monitored under a light microscope. The first and/or second digestion were 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 to 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, EVT) but not with villous cyto- or syncytiotrophoblast. Briefly, the isolated cells were transferred to culture dishes coated with the monoclonal antibody. After 15 minutes at 37°C, non-adherent cells were recovered by gently rocking the dishes and removing them with a pipette. Cytotrophoblastic cells were diluted to a final concentration of $5x10^5$ 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, non-adherent 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). 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 Connexin 43 were purchased from Biognostik (Gottingen, Germany). The phophorothioate-modified connexin 43 antisense -oligonucleotide is the reverse complement of a target sequence described in (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, four hours before the addition of phosphorothioate-modified antisense. Typically, normal cultured cytotrophoblastic cells were incubated with 10 μM synthetic Connexin 43 antisense oligonucleotide and 10 μM scrambled antisense (control, Biognostik). After 48h 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, USA), as previously described (Alsat *et al*., 1996, Frendo *et al*., 2000b). After washing, samples were mounted in medium with DAPI for nuclear staining (Vector laboratories, Burlingame CA, USA).

To detect Cx43, specimens were fixed for 10 min 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 non-specific 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, USA). 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 secondes 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). The sensitivity of the assay was 2 mU/ml. All values are means ± SEM 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 RT-PCR, essentially as previously described (Frendo *et al*., 2000a), using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystem) 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, 50 mM Tris-HCl (pH8) supplemented with protease inhibitors. The lysates were incubated at 4°C for 10 minutes and then centrifuged for 10 minutes at 10000g to pellet the nuclei. Protein concentration was determined according to Bradford's method (BioRad) 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% SDS, 10% glycerol, 5% ß-mercaptoethanol. They were heated at 95°C for 15 minutes and then loaded on 10% SDS-PAGE 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, USA). Values are presented as mean ± SEM. 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 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 48h in 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). In 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, the desmoplakin staining present at the intercellular boundaries of aggregated cells progressively disappears 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). In 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. In 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.

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), demonstrating 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 to standard conditions (6%). In 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 72h ($p< 0.035$) 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.

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 of the possibility that villous trophoblast 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. Due 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. In 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, prior to 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 (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 demonstrated 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 junction have been implicated in placental development (for 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 junctions are present during cytotrophoblastic cell fusion in the guinea-pig placenta (Firth *et al.*, 1980). In human trophoblast, we have previously demonstrated that Cx 26, 32, 33,40 and 45 are not detected, whereas Cx 43 mRNA and protein are present. Furthermore, using gap-FRAP we have demonstrated the presence of a functional gap junctional inter-trophoblastic 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. A number of lypophilic substances, such as aliphatic alcohols, and compounds isolated from liquorice roots (glycyrrhetinic acid) can uncouple gap junctions 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 inter-trophoblastic communication and trophoblastic cell fusion. Although its exact mechanism of action is not known, heptanol seems to decrease the fluidity of membraneous cholesterol-reach 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, heptanol 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 suspected 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 non-specific action blocking all the connexin channels lead us to develop an antisense strategy. It was demonstrated in this study, that treatment with a scrambled antisense does not affect the functional gap junctional communication and trophoblast differentiation whereas treatment with Cx 43 antisense abolishes gap junctional communication and reduces trophoblast differentiation. These data showed the major implication of Cx 43 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 inter-trophoblastic 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 explained by gene knock-out approaches in mice (Rossant and Cross, 2001). Due 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 demonstrate 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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FIGURE LEGENDS

Figure 1: **Effects of Cx43 antisense on Cx43 protein production in human trophoblasts.**

Panel A: Oligonucleotide uptake by primary cytotrophoblasts. After 4 hours of culture, cytotrophoblasts were incubated with FITC-labeled scrambled oligonucleotide for 1h, 2h, 24h and 48h. 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). (X 600)

Panel 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. (X 1000)

Panel C: Cx43 protein levels in trophoblast cells after treatment with a scrambled antisense (control) or with a specific Cx43 antisense (Cx43 antisense) 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.

Figure 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. In contrast, immunoflurescent staining can be observed at the boundaries between aggregated cytotrophoblasts in cells treated with a specific Cx43 antisense (**b**).

Figure 3: **Cell fusion index.**

Human cytotrophoblasts were incubated with a scrambled antisense (dark columns) or with a specific Cx43 antisense (white columns). After 24h (upper panel), 48h (middle panel) and 72h (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±SEM of three independent experiments.

Figure 4: **Functional intercellular communication measured by means of gap-FRAP method.**

Upper panel: Typical computer-generated images of fluorescence distribution in villous trophoblastic cells cultured in the presence of scrambled antisense for 48h measured during a gap-FRAP experiment. After a prebleach scan, 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. The evolution of fluorescence intensities was measured starting just after photobleaching for 12 min with a scanning period of 2 min. After 12 min (panel C), a fluorescence recovery had occured in area 1, whereas the fluorescence intensity remains weak

in area 2 indicating that cell 2 is not coupled to neighbouring cells. Panel 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 48h 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.

Figure 5: **hCG expression and secretion.**

Panel A: hCGβ mRNA levels were determined by real-time quantitative RT-PCR in cytotrophoblasts treated with an scrambled antisense used as a control (dark columns) or with a specific Cx43 antisense (white columns). These assays were carried out 24h, 48h and 72h after plating. Values are the levels of hCGβ mRNA normalized to the level of PPIA mRNA.

Panel B: levels of hCG secreted into the culture medium at 24h, 48h and 72h of culture in presence of a scrambled antisense (dark columns) or of a specific Cx43 antisense (white columns).

Values are means from three separate dishes ± SEM 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 tables. Graphs are representative of the experiment 1.

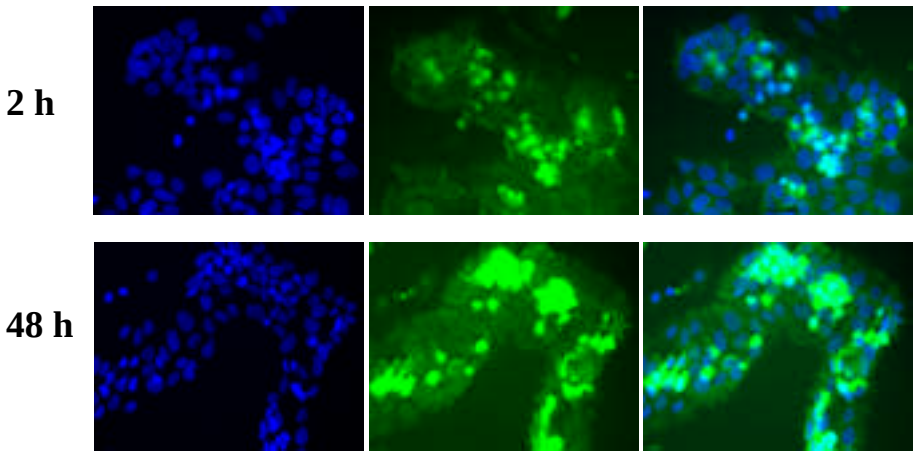
TABLE 1. RT-PCR primers and antisense oligonucleotides

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

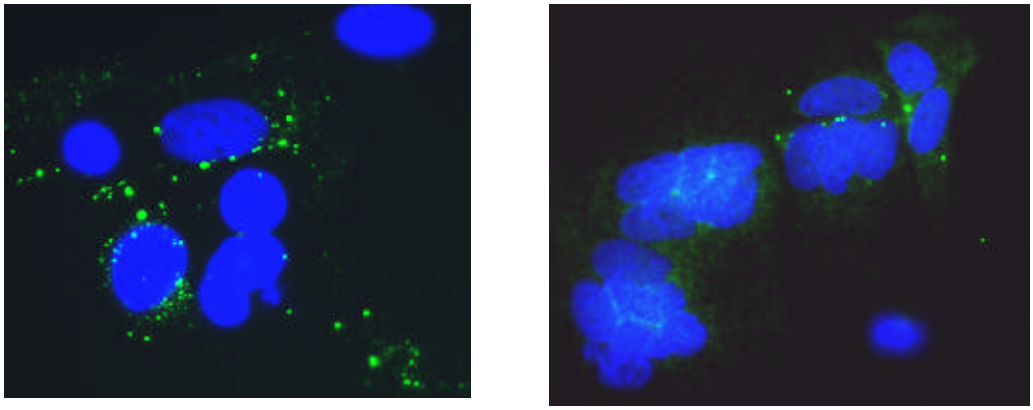
Upper (+) and lower (-) primers used in RT-PCR assays.

Figure 1

A


2 h
48 h
DAPI
FITC
DAPI+FITC

B


Control
Cx43 Antisense

C

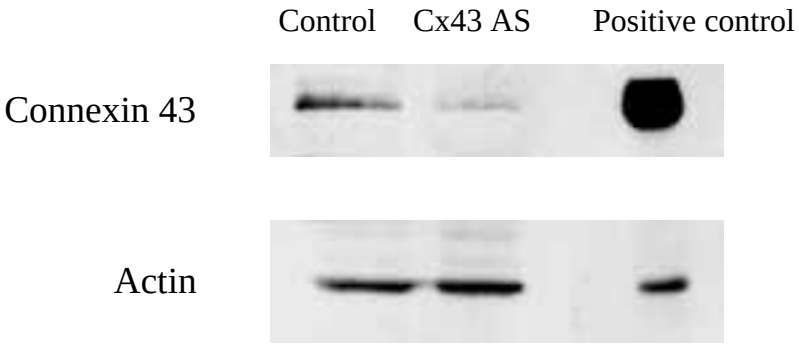
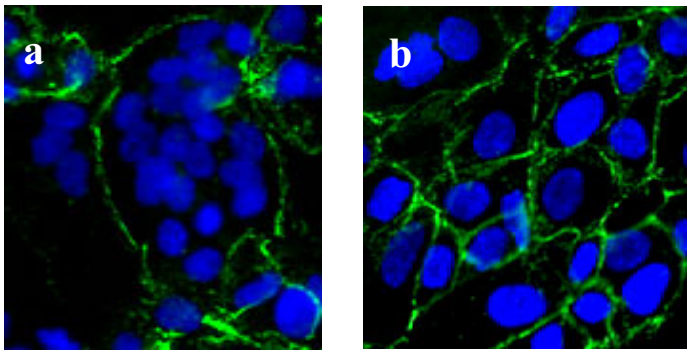

Control
Cx43 AS
Positive control
Connexin 43
Actin

figure2



Control Cx43 Antisense

Figure 3

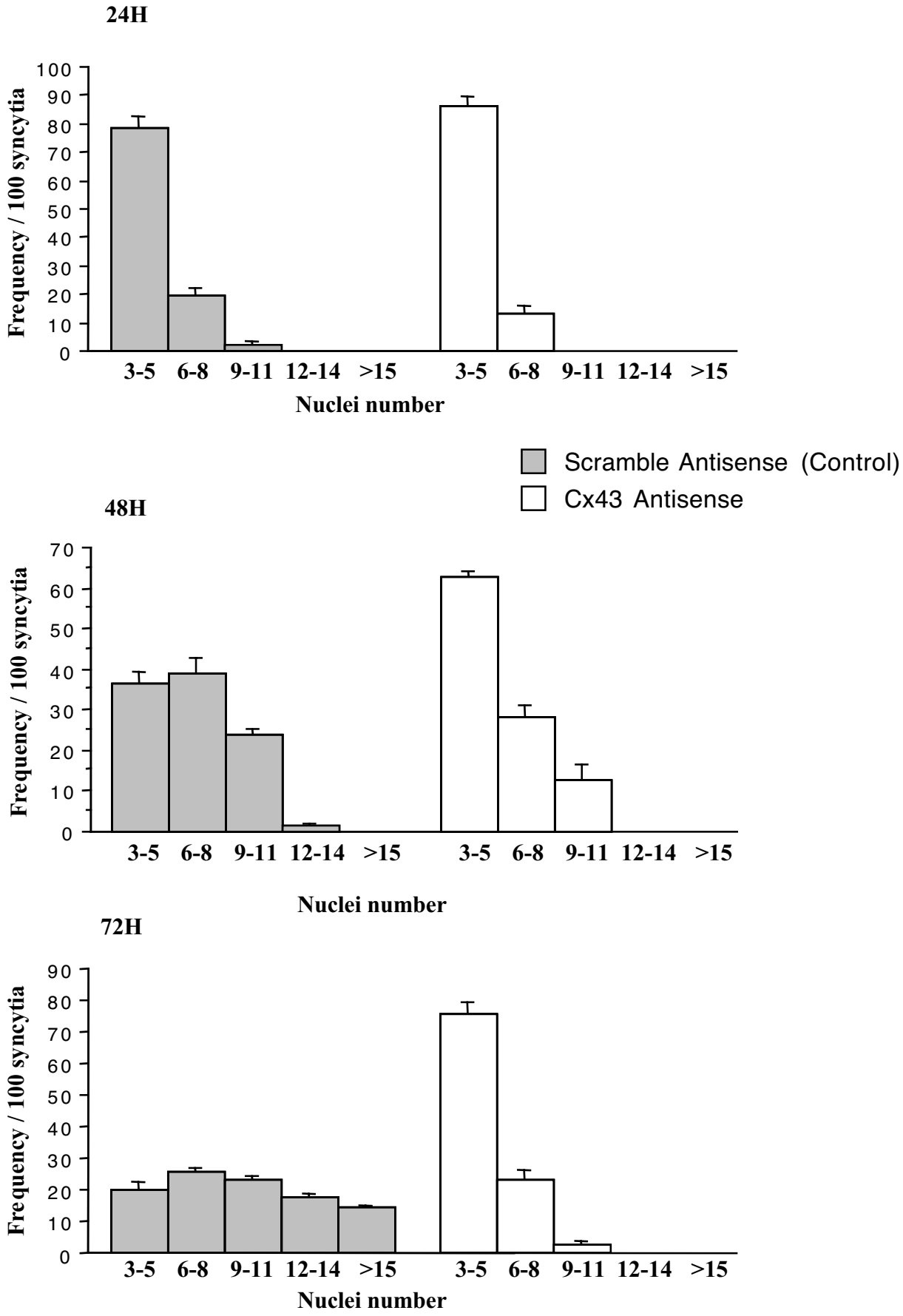

24H
Frequency / 100 syncytia
100
90
80
70
60
50
40
30
20
10
0
3-5
6-8
9-11
12-14
>15
3-5
6-8
9-11
12-14
>15
Nuclei number
Scramble Antisense (Control)
Cx43 Antisense
48H
Frequency / 100 syncytia
70
60
50
40
30
20
10
0
3-5
6-8
9-11
12-14
>15
3-5
6-8
9-11
12-14
>15
Nuclei number
72H
Frequency / 100 syncytia
90
80
70
60
50
40
30
20
10
0
3-5
6-8
9-11
12-14
>15
3-5
6-8
9-11
12-14
>15
Nuclei number

Figure 4

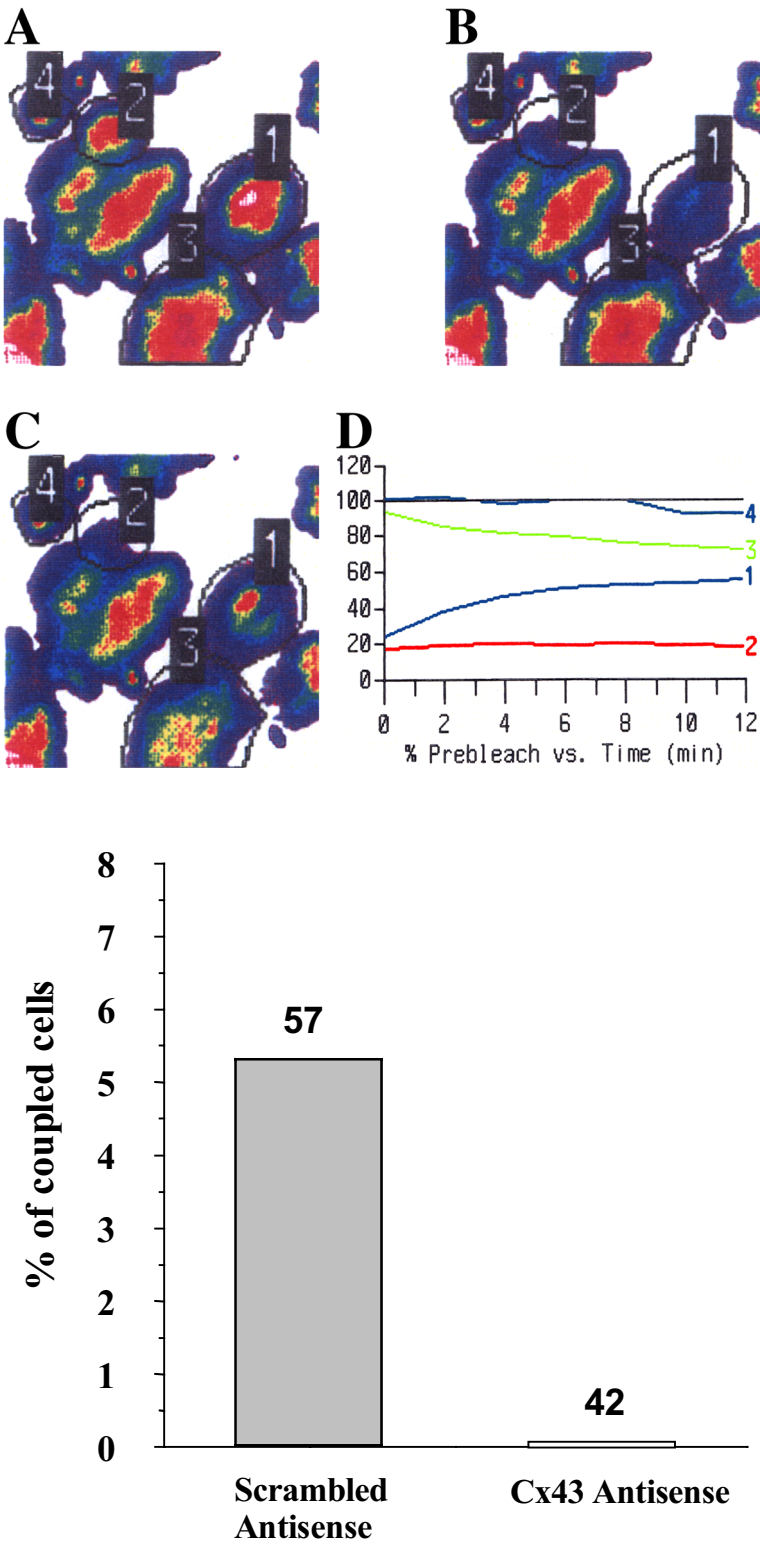
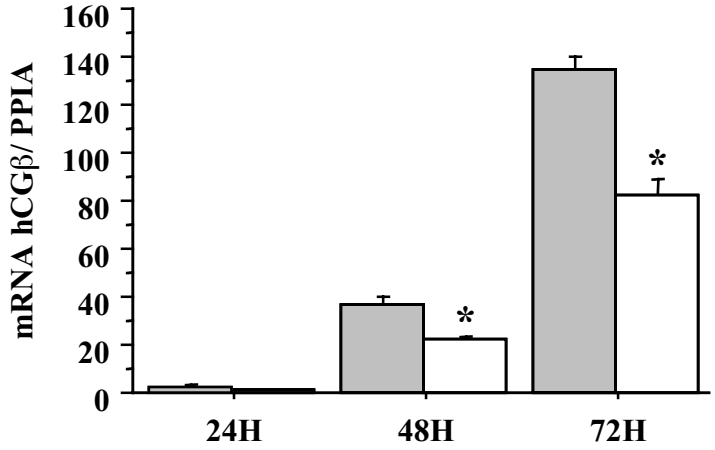

A
B
C
D
4
2
1
3
120
100
80
60
40
20
0
0
2
4
6
8
10
12
% Prebleach vs. Time (min)
% of coupled cells
8
7
6
5
4
3
2
1
0
57
42
Scrambled Antisense
Cx43 Antisense

Figure 5

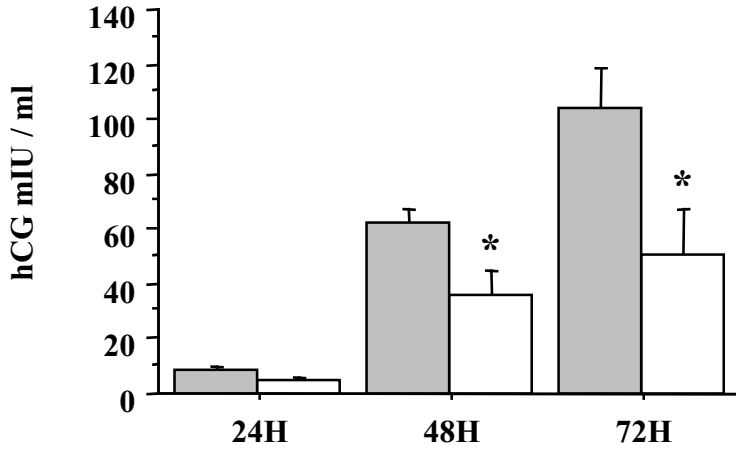
A **hCGβ mRNA**



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

Scramble Antisense (Control)
Cx43 Antisense

B **hCG secretion**



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