

Review

Involvement of gap junctional communication and connexin expression in trophoblast differentiation of the human placenta

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Summary. Gap junctional intercellular communication (GJIC) permits coordinated cellular activities during development and differentiation processes, and its dysfunction or mutation of connexin genes have been implicated in pathologies. In the human placenta, two distinct differentiation pathways of cytotrophoblastic cell coexist leading to a double model: fusion phenotype (villous trophoblast) and proliferative/invasive phenotype (extravillous trophoblast). This review focuses on current knowledge on the connexin expression and the implication of GJIC in trophoblastic differentiation. Experimental evidence obtained in human placenta demonstrates the involvement of connexin 43-gap junctions in the trophoblastic fusion process and of a connexin switch during the spatially and temporally controlled proliferation/invasion process.

Key words: Gap junction, Connexin, GJIC, Differentiation, Human trophoblast

Introduction

In mammals, the blastocyst defines with the maternal organism, by an implantation process, a structure which allows the development of the embryo and the foetus by exchanging ions, metabolites and waste: the placenta. In addition, the endocrine and immunological functions of the placenta are essential for the development of pregnancy and fetal growth. The close relationship between the development of the placenta and the foetus is illustrated by the well established correlation between the placental weight and the fetal weight throughout pregnancy. Therefore, it is

important to understand placental growth and differentiation. Gap junctions and gap junctional intercellular communication (GJIC) are known to be involved in differentiation process of numerous tissues. Therefore, in this review we will study the involvement of GJIC and connexin expression in human trophoblast differentiation. We briefly summarize the role of trophoblast in human placental development before focusing on the role of GJIC in this process.

The cytotrophoblastic cell: the key cell of human placenta

In human, the formation of the placenta after implantation is the result of a complex series of interactions between fetal trophoblast and maternal cells in the decidua of the uterus (hemomonochorial placentation). This process involves the proliferation, the invasion and the differentiation of the cytotrophoblastic cells which are the stem cells from which the different trophoblast populations of the placenta are derived (Kaufmann and Castelluci, 1997). With the progression of placentation, two distinct pathways of differentiation lead to the formation of two distinct trophoblastic cell populations (Fig. 1).

In the extravillous phenotype, the cytotrophoblastic cells of the anchoring villi aggregate into cell columns to attach to the uterine wall. From there, individual cells migrate into the decidua and the myometrium remodelling the pregnant endometrium and its vasculature (Fig. 1). Indeed, some of the extravillous cytotrophoblastic cells (EVT) invade the uterine arterioles and destroy the media, thus creating low resistance, large diameter blood vessels. Alternatively, many of the EVT cells scattered through the decidua and the myometrium, differentiate into multinucleated placental giant cells. EVT cells share many characteristics with tumor cells, including similar mechanisms underlying their invasive ability. However,

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unlike tumor cells, proliferation, migration and invasiveness of EVT cells are exquisitely regulated *in situ*, both temporally and spatially, in order to maintain a healthy uteroplacental homeostasis. As they invade the uterine wall, EVT cells upregulate the expression of metalloproteinases (Polette et al., 1994; Bischof et al., 1995; Zhou et al., 1997; Lala et al., 1998) and HLA-G, a trophoblast-specific HLA class I molecule that is likely to be important in avoiding rejection of the conceptus by the maternal immune system (Kovats et al., 1990). The patterns of expression of proto-oncogenes, cell adhesion molecules and integrins have been well documented (Kaufmann and Castelluci, 1997; Vicovac and Aplin, 1996) and an "integrin switching" (Damsky et al., 1994) was demonstrated during trophoblast invasion. The villous cytotrophoblastic stem cells strongly express the $\alpha 6 \beta 4$ integrin (laminin receptor) and as they differentiate in the extravillous pathway, the cells downregulate $\alpha 6 \beta 4$ and sequentially upregulate expression of fibronectin and the $\alpha 5 \beta 1$ (fibronectin receptor) in cell columns and the $\alpha 1 \beta 1$ (laminin/collagen I receptor) in the uterine wall. Insufficient infiltration of the uterine wall is implicated in the pregnancy disorder pre-eclampsia in which the mother shows signs and symptoms such as high blood pressure, proteinuria and oedema and which is associated in some cases with fetal intrauterine growth retardation (Lim et al., 1997). Pre-eclampsia is a major cause of infant and maternal mortality worldwide and is resolved by childbirth.

On the other hand, uncontrolled trophoblast invasion is detrimental to feto-maternal health as occurs in invasive moles and choriocarcinomas (trophoblastic

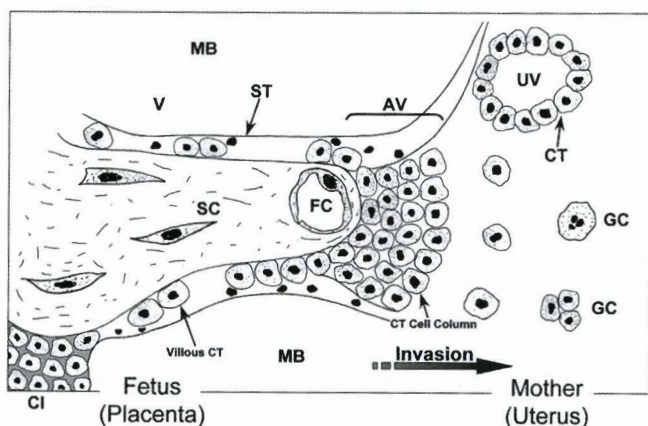


Fig. 1. Diagram of a longitudinal section of a villus at the fetal-maternal interface during gestation. The chorionic floating villus (V) is bathed with the maternal blood (MB). In the floating villus, cytotrophoblastic cells (CT) differentiate by fusion into syncytiotrophoblast (ST). The cell islands (CI) are an accumulation of cytotrophoblastic cells partly embedded in fibrinoid and attached to free floating tips of villi. The anchoring villus (AV) contains extravillous cytotrophoblastic cells (CT) which proliferate to form CT cell columns and then invade the uterine interstitium and the uterine maternal blood vessel (UV). The infiltrating cells eventually differentiate to become placental bed giant cells (GC). FC: fetal capillary; SC: stromal core.

cancer). Human choriocarcinomas cell lines are a frequently used model for studying the mechanisms of uncontrolled proliferation and invasion.

In the villous phenotype, cytotrophoblastic cells fuse to form the syncytiotrophoblast, a multinuclear syncytium primarily engaged in absorptive, exchange and specific endocrine functions. During gestation, the ability of villous trophoblast to differentiate by cytotrophoblastic fusion to the advanced stage of syncytiotrophoblastic phenotype is a major determinant for placental growth and development. This differentiation process is needed for syncytiotrophoblast building and also for regeneration of ageing syncytiotrophoblast. Thereby, a failure of cytotrophoblastic cell differentiation is associated with some major pregnancy disorders such as intra uterine growth retardation.

The mechanisms controlling trophoblast invasiveness and differentiation are poorly understood so far. Previous *in vitro* studies have shown that the future fate of cytotrophoblastic cells probably only depends on the surrounding environment (Morrish et al., 1998). Contact to fibrin or other extracellular matrices could induce extravillous trophoblast phenotype; contact to the maternal blood could induce villous trophoblast phenotype. Recent results suggest that Inhibitor of DNA binding protein 2 (Id-2) and the basic helix-loop (bHLH) transcription factors play an important role in human EVT cell invasion (Janaptour et al., 2000). Villous syncytiotrophoblast formation is thought to involve cell aggregation, remodelling of the cortical cytoskeleton underlying the plasma membrane and fusion of the plasma membranes. Numerous specific cell surface interactions between adjacent cells are thought to be involved in regulating trophoblastic cells fusion. The process is preceded by cadherin and desmoplakin expression (Douglas and King, 1990; Coutifaris et al., 1991). The implication of endogenous retrovirus genome in the cytotrophoblastic cell fusion was suggested (Harris, 1998; Rote et al., 1998) and recently, the role of endo-genous retroviral envelope protein was demonstrated (Mi et al., 2000). Interestingly, soluble factors like hormones, growth factors and oxygen can modulate the cytotrophoblastic cell fusion process and the subsequent villous functional differentiation. Thus, epidermal growth factor (EGF), granulocyte-macrophage-colony-stimulating factor (GM-CSF), human chorionic gonadotropin (hCG) and glucocorticoids induce villous differentiation, whereas hypoxia, transforming growth factor $\beta 1$ (TGF $\beta 1$) and endothelins impair the phenomenon (for review, see Cronier et al., 1999c).

Gap junctional intercellular communication

Gap junction channel

Mammalian gap junctions consist of specialized cellular structures which permit direct communication between cytosols of adjacent cells and mediate the

exchange of low molecular weight hydrophilic molecules. This junctional complex consist of clusters of transmembraneous channels in closely apposed regions of membranes in contact.

The structure of gap junctions has been extensively reviewed in the last decade (Bennett et al., 1991; Severs, 1995; Yeager et al., 1998). Briefly, each aqueous junctional channel is formed by two hemi-channels named connexons tightly associated in the intercellular space. Each connexon consists of six transmembrane proteins termed connexins and symbolized by Cx followed by the predicted molecular mass in kDa (Beyer et al., 1987). Connexins represent closely-related proteins encoded by a multigene family. Biochemical studies have demonstrated that connexins share sequence homologies and a common molecular topology with highly conserved transmembraneous regions. The connexin diversity corresponds to variations in the cytoplasmic loop and C-terminus tail.

Connexin diversity

The carboxyl-terminal domain, which manifests the molecular weight and sequence differences between connexins, seems to be responsible for the regulation of the functional properties of the channels owing to the fact that post-translational modification sites are present (Paul, 1986; Saez et al., 1986; Laird and Revel, 1990). Moreover, biophysical and functional properties of intercellular channels depend on the type of connexins expressed. However, it is important to note that a specific function cannot be associated with one specific connexin. Indeed, the expression of most connexins is not restricted to one tissue or cell type and most if not all tissues express more than one connexin (Bruzzone et al., 1996). Moreover, the expression of multiple connexins in the same cell type lead to the possible formation of heterotypic (hemi-channels with different connexins) or heteromeric (mixture of connexins in one connexon) intercellular channels (Elfgang et al., 1995; Brink et al., 1996; Jiang and Goodenough, 1996).

Evaluation of GJIC functionality

Functional studies of GJIC were mainly performed by electrophysiological and dye coupling methods. The second way included microinjection of fluorescent dye (Stewart, 1978), scrape loading (El-Fouly et al., 1987) and gap-FRAP (Fluorescence Recovery After Photo-bleaching) method (Wade et al., 1986). Due to the fact that the gap-FRAP technique is not harmful to the cells, this technique is particularly adapted to the study of long term differentiation processes such as trophoblast differentiation. Briefly, this method is based on the analysis of the fluorescence recovery after photo-bleaching of one selected cell that is in contact with fluorescent adjacent cell(s). The fluorescence recovery due to permeable gap junctions between contiguous cells follows a characteristic exponential time course.

Moreover, because the diffusion of dye is neither prevented by the cell membrane nor rate limited by the presence of GJIC in case of cellular fusion, the discrimination between cellular fusion and intercellular communication is possible using gap-FRAP method by measuring the time constant of the fluorescence recovery.

Regulation of GJIC

Using the methods mentioned above, a number of authors have firmly established that regulation of GJIC can take place at two main levels: number of gap junctional channels present at the junctional area and gating modifications of intercellular channels. Connexins differ notably from other membrane proteins in lacking glycosylation and being primarily localized in non-junctional regions of coupled cells (for review, see Bruzzone et al., 1996; Laird, 1996; Yeager et al., 1998). Moreover, the oligomerization seems to take place after the exit of the endoplasmic reticulum in the trans-Golgi network (Musil and Goodenough, 1991, 1995). Surprisingly, the half-life of gap junctions is short, thus the formation and the turnover of intercellular channels could largely influence the intercellular communication. Gap junctional intercellular communication appears to depend on both intrinsic and extrinsic factors. It is important to note that specific cell adhesion molecules such as cadherins are necessary for the establishment of cell-to-cell communication (Miner et al., 1995; Mege et al., 1988). The connexin expression varies during differentiation, proliferation, transformation processes or during long term treatment with biologically actives substances (Risek and Gilula, 1991; Meda et al., 1993; Cronier et al., 1994b; Munari-Silem et al., 1994). For example, the effects of cAMP can be exerted at the levels of transcription, mRNA stability and turnover (Kessler et al., 1985; Saez et al., 1989). Moreover, it is now well known that gating properties of intercellular channels vary under certain circumstances (pH_i , $[Ca^{2+}]_i$, voltage, phosphorylation by PKA, PKC, PKG, Tyrosine kinase and MAP kinase; Rose and Loewenstein, 1976; Turin and Warner, 1977; Atkinson et al., 1981; Giaume and Korn, 1983; Spray et al., 1993; Lau et al., 1996; Warn-Cramer et al., 1998).

A variety of lypophilic substances such as aliphatic alcohols (Johnston et al., 1980) can uncouple gap junction channels although their action appear non-specific. A compound isolated from licorice root, 18 β -glycyrrhetic acid (18 β -GA), has been reported to inhibit GJIC by gap junction disassembly and connexin dephosphorylation (Davidson and Baumgarten, 1988; Guan et al., 1996).

Functional role of gap junctions in development and differentiation processes

The rapid propagation of electrical activity between excitable cells is the most obvious function of the GJIC,

permitting nerve conduction at electrical synapses and coordinated contraction of cardiac and smooth muscle cells. Gap junction-diffusible molecules (PM < 1000 Da) also include metabolites and those involved in signal transduction like calcium ions, cAMP and IP₃ (Loewenstein, 1981; Bruzzone et al., 1996; Kumar and Gilula, 1996). Moreover, in developmental systems, differentiation and physiological processes are linked to dynamic spatiotemporal patterns of connexin expression (Saez et al., 1993). Many *in vitro* and *in vivo* studies have provided evidence for gap junctions being involved in the regulation of cell metabolism, growth and differentiation in different cell and tissue types (Lo, 1996; Bani-Yaghoub et al., 1999; Constantin and Cronier, 2000).

Different approaches are possible to study the involvement of GJIC in development and differentiation. Warner et al. (1984) have demonstrated the implication of GJIC in embryogenesis. Indeed, the injection of specific anti-connexin antibodies into *Xenopus* oocytes induced embryonic abnormalities. Long term treatments with uncouplers such as heptanol (Mège et al., 1994) or 18β-GA (Bani-Yaghoub et al., 1999) were also used to show the incidence of GJIC blockade on cellular differentiation. Finally, antisense oligonucleotide approach or connexin gene surexpression were used to further elucidate the link between regulation of growth/differentiation and gap junctional communication (Li et al., 1999).

Some critical functions have been demonstrated by the association of mutations on specific connexin genes with some pathologies (Donaldson et al., 1997). A critical role of connexins has been identified in one form of peripheral neuropathy, the X-linked Charcot Marie-Tooth disease (Bergoffen et al., 1993), developmental abnormalities of the cardiovascular system (Britz-Cunningham et al., 1995) and formation of lens cataract (Gong et al., 1997). Moreover, a number of arguments support the implication of GJIC dysfunction in heart disease (Bastide et al., 1993; Saffitz et al., 1994) and carcinogenesis (Krutovskikh and Yamasaki, 1997). Aberrant GJIC was demonstrated in tumor cells or between normal cells and tumorigenic cells (homologous and heterologous GJIC). Cancer-causing agents or oncogenes downregulate GJIC, whereas inhibitors of carcinogenesis or chemopreventive agents upregulate GJIC. Increase of connexin expression in tumor cells by transfection of Cx cDNA or by injection of Cx mRNA restores normal growth control (Mesnil et al., 1995). Connexin genes are considered as tumor suppressor genes (Lee et al., 1992).

However, the complexity to study GJIC implication in physiological processes is important and well illustrated by analysis of the connexin-deficient transgenic mice (Reaume et al., 1995; Nicholson and Bruzzone, 1997; Lo, 1999). These experiments have yielded only a limited insight due to multiple connexin isotypes expression in one cell type and to the possible compensatory mechanisms between connexins

(Steinberg, 1998; Minkoff et al., 1999). As mentioned by Lo (1999), in considering the role of gap junctions in growth, development and differentiation, the determination of connexin mRNA or protein levels or of assembly in plasma membrane are not sufficient and the critical importance is functional analysis of GJIC. Thus, a number of recent studies focus on the functional role of connexins and gap junctional communication during development and differentiation processes including the placenta (Bani-Yaghoub et al., 1999; Constantin et Cronier, 2000; Dowling-Warriner and Trosko, 2000).

Gap junctions and trophoblast

Little is known about cell-cell communication properties in trophoblast during placental development. Earlier ultrastructural studies have reported the presence of gap junctions between syncytiotrophoblastic layers of hemochorial placentae of rabbits, guinea-pig, rats and mice (Firth et al., 1980; Metz and Weihe, 1980) and between cytotrophoblastic cells and the syncytiotrophoblast of the endotheliochorial placenta of the mole (Malassiné and Leiser, 1984). Furthermore, gap junctions are present during cytotrophoblastic cell fusion in the guinea-pig placenta (Firth et al., 1980). In the human hemomonochorial placenta, gap junctions were not observed by Metz and Weihe (1980) between trophoblastic cells in full term placenta, while they were described by De Virgiliis et al. (1982) between cytotrophoblastic cells and syncytiotrophoblast in first trimester placenta. In non primate placentas, immunolocalization studies indicated that connexins are expressed by trophoblastic cells in mice (Pauken and Lo, 1995) and rats (Risek and Gilula, 1991; Grummer et al., 1996; Reuss et al., 1996; Winterhager et al., 1996). In rat embryos, the invasive ectoplacental cone trophoblastic cells express Cx31, which later disappears with further trophoblast differentiation. Then Cx26 appears in the labyrinthine trophoblast whereas Cx43 appears in the spongiotrophoblast and trophoblast giant cell layer. This switch in connexin gene expression is directly correlated to functional switch during rat trophoblast development. A normal rat trophoblastic cell line HRP1 expressed Cx43, whereas a rat choriocarcinoma cell line RCHO-1 expressed Cx31 (Grummer et al., 1996; Winterhager et al., 1996).

Gap junctions and villous trophoblast: a role in trophoblastic cells fusion

In the human placenta, the chorionic villi, in direct contact with the maternal blood consist of the trophoblast surrounding a core of connective tissue including fetal vessels, fibroblasts and macrophage (Fig. 1). Thus, investigations including morphologically and physiologically well defined placental components are necessary for fine biochemical studies. Villous trophoblastic cell cultures are now currently used to study certain aspects of the dynamic processes occurring

during villous differentiation (Kliman et al., 1986). Isolated mononuclear cytotrophoblastic cells aggregate and fuse together to form a non proliferative multinucleated syncytiotrophoblast which expresses specific hormonal productions (human Chorionic Somatomammotropin (hCS), oestrogens and leptine). During this *in vitro* villous differentiation, Cx26, Cx32 and Cx40 could not be detected, whereas Cx43 was expressed at low levels (Cronier et al., 1994c) (Fig. 2). RT-PCR analysis of highly purified cytotrophoblastic cells and of syncytiotrophoblast in culture demonstrated the presence of Cx43 mRNA. Furthermore, Cx26, Cx32, Cx33 and Cx40 containing gap junctions were not detected *in vitro* by immunofluorescence study while punctuate staining for Cx43 was observed at intercellular boundaries of contiguous trophoblast (Fig. 3) and in some perinuclear zone presumed to be associated with the Golgi. After cellular fusion, Cx43 staining was only observed within the cytoplasm of the trophoblastic cells (Cronier et al., 1999a). The presence of Cx43 (mRNA

and protein) in isolated villous trophoblastic cells was recently confirmed (Winterhager et al., 1999).

From all connexins tested *in situ* by immunofluorescence and confocal microscopy only Cx43 was detected between trophoblastic cells in first trimester placenta. *In situ* hybridization with [³⁵S]-labelled Cx43 sense and antisense, detected Cx43 mRNA in the cytotrophoblastic layer and in the mesenchymal compartment of the first trimester villus (Fig. 4).

To further elucidate the role of gap junctions during villous trophoblast differentiation, gap junctional intercellular communication was investigated by means of fluorescence recovery after photobleaching method (gap-FRAP). In presence of fetal calf serum, GJIC was observed as well between aggregated cytotrophoblastic cells, as between cytotrophoblastic cells and syncytiotrophoblast and as between contiguous syncytiotrophoblasts. This intercellular communication precedes the cellular fusion (Cronier et al., 1994b, 1997b) and parallels the morphological and biochemical

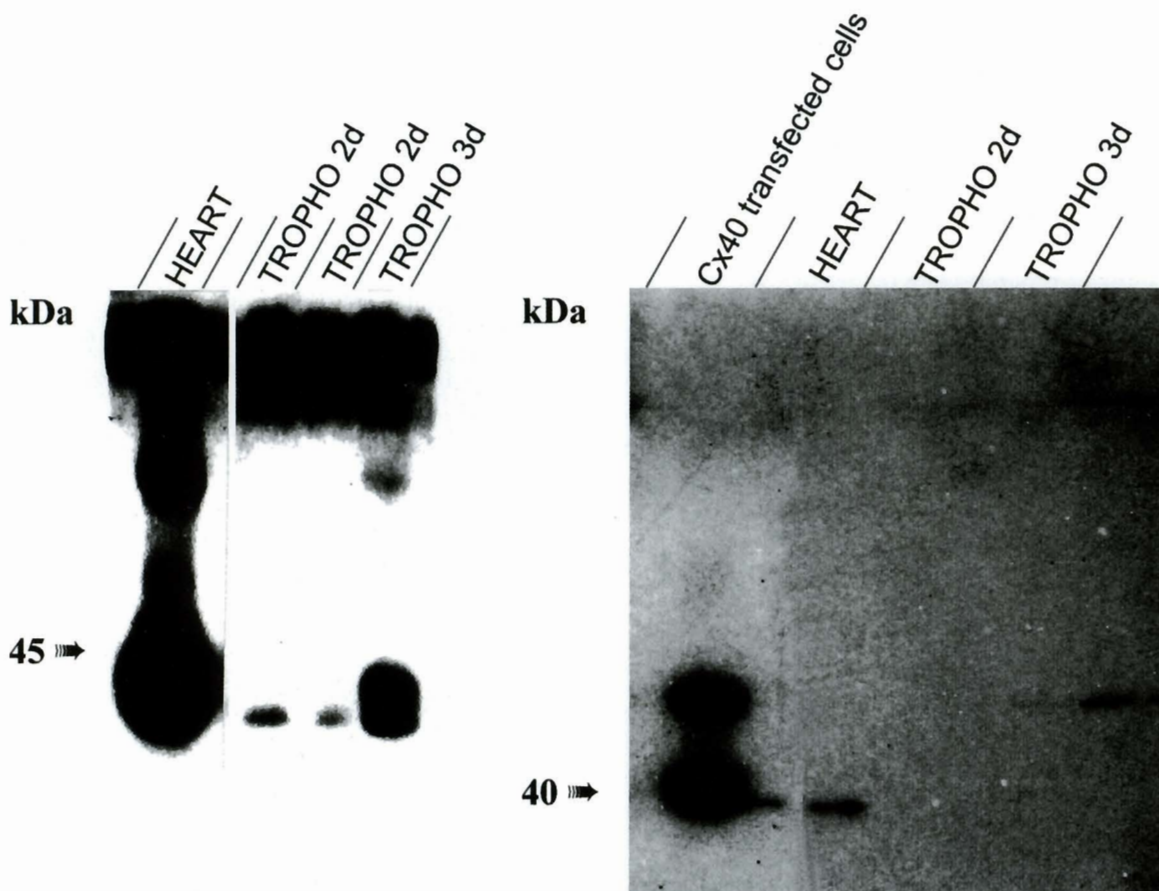


Fig. 2. Immunoblot analyses of connexin expression in villous trophoblastic cells. After 2 or 3 days of culture (2d or 3d), five micrograms of total proteins per lane were electrophoresed. Rat heart homogenates or Cx40-transfected cells were used as control for Cx43 (left panel) and Cx40 (right panel) expression. Blots were probed by means of affinity-purified antibodies raised to residues 360-382 of rat connexin 43 (1/500) or to residues 337-358 of mouse connexin 40 (1/500). Only Cx43 was detected in trophoblastic cells during villous differentiation with an increase of expression between 2 and 3 days of culture.

differentiation (Malassiné et al., 1990; Fig. 5). One way to investigate the role of gap junctions in differentiation is by chemically inhibiting GJIC by a junctional uncoupler. The presence of 1.5 to 3 mM of heptanol in the culture medium during 2 days dramatically decreased the number of coupled cells, the syncytiotrophoblast formation, the hCG secretion and the hCS expression. Viability was not affected by this uncoupling treatment and the level of Cx43 was maintained during the treatment. This effect was reversible since heptanol removal restored the GJIC, the syncytium formation and the specific hormonal secretion. These results demonstrated the requirement of GJIC for trophoblastic cells fusion and subsequent differentiation (Cronier et al., 1994a). Furthermore, the action of soluble factors acting in both autocrine and paracrine fashion and the signalling mechanisms implicated in GJIC and Cx43 expression were investigated. The earliest hormonal production of the human trophoblast is the human chorionic gonadotropin (hCG) and hCG/LH receptor is expressed by the trophoblast. Villous trophoblastic GJIC and the expression of Cx43 are specifically stimulated by hCG (Fig. 5); the phenomenon involves the cAMP-Protein Kinase A pathway. Furthermore, inhibition of Protein Kinase C strongly stimulated trophoblastic GJIC (Cronier et al., 1997b). Glucocorticoids (Cronier et al., 1998) and Oestradiol (Cronier et al., 1999c) specifically stimulated GJIC, Cx43 expression and trophoblast differentiation, whereas TGF β 1 (Cronier et al., 1997a)



Fig. 3. Immunofluorescent localization of Cx43 protein in first trimester trophoblastic cells cultured for 24h on plastic dishes. Strong immunostainings are observed at the borders of adjacent cells (Cx43 polyclonal Ab/TRITC anti-rabbit). x 500

and endothelin (Cronier et al., 1999b) inhibited the phenomenon. In conclusion, the ability of villous cytotrophoblastic cells to express Cx43 and to develop a transient GJIC is a prerequisite for the formation of the syncytiotrophoblast. *In vitro*, villous differentiation occurs earlier when cell-to-cell communication is enhanced (hCG, cAMP analogs, PKC inhibitors, oestradiol, glucocorticoid). On the contrary, differentiation process is delayed when this communication is decreased by TGF β or endothelins.

Connexin switch in the extravillous trophoblast phenotype

Extravillous trophoblastic cells of the human placenta proliferate, migrate and invade the decidua and its vasculature. Since a large number of studies indicate that a reduction or a loss of GJIC is a common event during the transition of a normal tissue to a malignant tissue, connexin expression and GJIC were examined in malignant trophoblastic cells. Human choriocarcinoma cell lines (Jeg 3, BeWo and Jar) express variable amounts of Cx40 with Jeg3 cells revealing the lowest

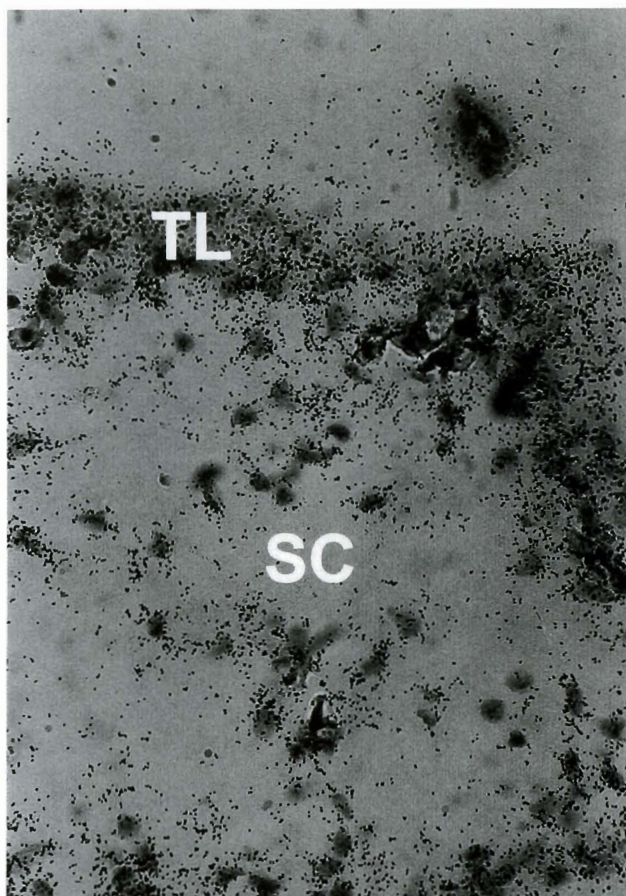


Fig. 4. Localization of Cx43 mRNA in a first trimester chorionic floating villus section hybridized with [35 S]-labeled antisense Cx43cDNA probe. The hybridization signal is strong within the trophoblastic layer (TL) and is also present in the stromal core (SC). x 900

Gap junctions and human trophoblast

levels of Cx40 transcripts leading to an extremely low cell-cell communication (Hellmann et al., 1996). Interestingly the transfection of Jeg3 cells with Cx26, Cx40 and Cx43 genes, restored cell coupling and reduced the *in vitro* growth of all clones. In contrast to Cx40, the Cx26 channel was more potent in reducing proliferation and induce differentiation indicated by BhCG secretion (Hellmann et al., 1999). Recently, in an *in vitro* propagated first trimester human EVT cell population, only Cx43 (protein and mRNA) was detectable, while Cx26, Cx32, and Cx40 were not detected (Khoo et al., 1998). When these cells were immortalized by SV40 Tag transfection, premalignant derivatives were produced with hyperproliferative and hyperinvasive phenotype. Cx43 and GJIC were reduced in long lived cell line and undetectable in the immortal cell line, confirming that down regulation of connexin is an early event in tumor progression. Cx40 was detected in the cell islands of first trimester placenta, a highly proliferative EVT cell population partly embedded infibrinoid and attached to floating villi (Hellmann et al., 1996). Recently, Cx40 was also immunolocalized *in situ* in the columnar extravillous trophoblast (Cronier et al., 1999a; Winterhager et al., 1999) and in non-proliferating deeply invasive extravillous cells (Winterhager et al., 1999). Furthermore, immunolabelling for Cx32 and Cx43 was also detected in the placental bed aggregated

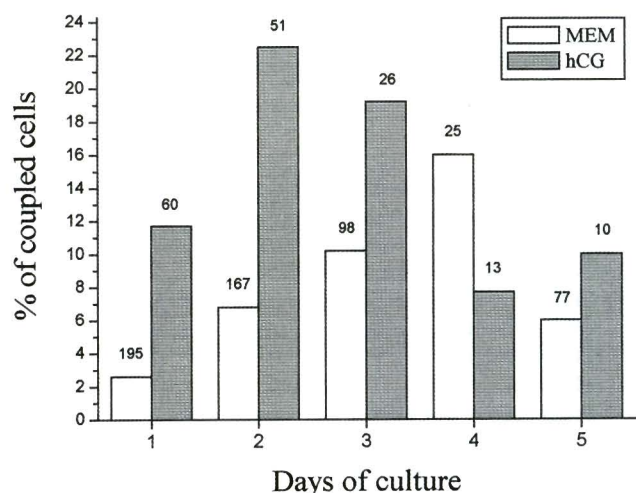


Fig. 5. Evolution of gap junctional intercellular communication vs time in control conditions and under exogenous hCG (human Chorionic Gonadotropin) stimulation. Percentage of coupled cells vs time of culture in MEM (Minimum Essential Medium) and in MEM supplemented with 500 mIU/ml hCG. N values (number of experiments) are indicated in the middle of bars. The fluorescence recovery after photobleaching technique was used to estimate the transfer of 6-carboxyfluorescein from contiguous trophoblastic elements (cytotrophoblastic cells, syncytiotrophoblasts) into photobleach cells. Fluorescence recovery follows a slow exponential time course when the cell-to-cell process is rate limited by the presence of gap junctional channels between contiguous cells, contrasting with a much faster step-like course in the case of fusion of plasma membrane. (Reproduced from Cronier et al., 1994b, with permission of the publisher The Endocrine Society).

giant cells (Fig. 6) (Lamki et al., 1999; Malassiné: personal observation). Interestingly, when first trimester cytotrophoblastic cells were cultured on Matrigel®, $\alpha 5\beta 1$ integrin immunoreactivity was observed in cells, concomitant with an abundant Cx40 expression between cells. Furthermore, no evidence of GJIC was detected in this extravillous-induced phenotype (Cronier et al., 1999a). In conclusion, these results suggest a switch in connexin gene expression correlated to the functional switches during human trophoblast development.

Concluding remarks

We have seen over the course of this review that the pattern of different connexins depends on the trophoblastic phenotype (Fig. 7). In the villous trophoblast, Cx43 is the only connexin expressed *in vitro* and *in situ*. On the contrary, the extravillous phenotype is characterized by a switch in connexin expression like in rodents where the connexin expression is spatially and temporally regulated during placental development. The functional significance of the differential expression of connexin is important because of the specific properties of permeability and regulation of homo or heterotypic

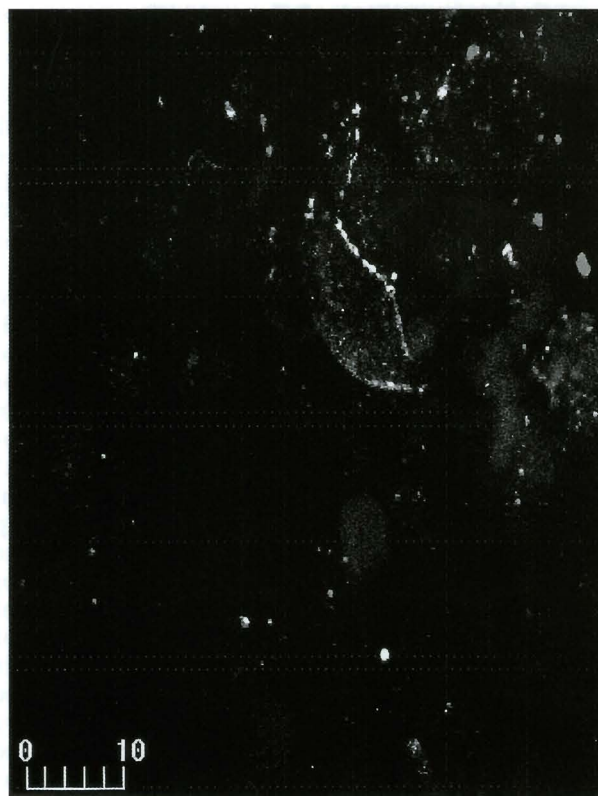


Fig. 6. Immunofluorescence image of placental bed section with Cx32 monoclonal antibody and Fluorescein-isothiocyanate (FITC). Punctate intercellular staining is observed in contiguous giant cells. Cell type was identified by positive staining with anti hPL (human Placental Lactogen). (Bar: 20 μ m).

Gap junctions and human trophoblast

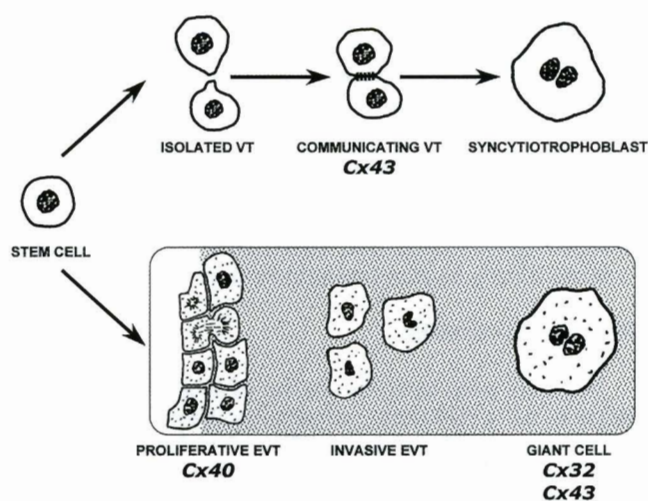


Fig. 7. Schematic representation of the two major pathways of cytotrophoblastic differentiation and the associated expression of different connexins. A trophoblastic stem cell gives rise to two distinct pathways: 1) Villous trophoblast (VT) differentiation leading to the formation of syncytiotrophoblast after adhesion, intercellular communication and fusion. 2) Extravillous trophoblast (EVT) differentiation leading to the formation of giant cells after proliferation and invasion of the uterine wall (gray). The different trophoblastic lineage shares distinct connexin expressions with Cx43 during VT differentiation, whereas proliferative EVT and giant cells are respectively characterized by Cx40 and Cx32/Cx43.

gap junction channels. Thus, Cx40 protein which characterises the malignant trophoblast and the proliferative and invasive phenotypes forms channels where dye transfer is very low. Furthermore, Cx40 transfectant does not pair functionally with most of the other connexins (Elfgang et al., 1995).

The ability of the cytotrophoblastic cells to differentiate by aggregation, expression of Cx43, development of a transient GJIC and cellular fusion to form the villous syncytiotrophoblast is a major determinant of structure and function of the human placenta. Thus, the expression of specific syncytiotrophoblastic genes (hCS, P₄₅₀ aromatase, leptine) depends on the formation of the syncytium. In rodent placenta, it has been demonstrated that Cx26 gap junctional channels, connecting different layers of the trophoblast could act as a transplacental pathway for glucose (Gabriel et al., 1998). Thus, the existence of cell-to-cell channels would permit the exchange of second messengers between trophoblastic cells and it is reasonable to postulate that this exchange can regulate the fusion process. The nature of the messengers that are involved in the intertrophoblastic GJIC is certainly one of the crucial questions to be addressed in the near future. It is conceivable to assume that the second messengers such as Ca²⁺, IP₃ and cAMP could be exchanged during the fusion period controlling various cellular effectors involved in the cell fusion and in the transcription of syncytiotrophoblast-specific genes.

These intercellular messengers may also cross talk with GJIC, since gap junction channels are known to be regulated by cAMP and Ca²⁺. The process of fusion could be correlated with a concomitant increase in cellular levels of cAMP (Roulier et al., 1994) and with a decrease in basal Ca²⁺ activity (Cronier et al., 1999b). Trophoblastic cells are not the only cell type to communicate through GJIC prior to fusion, with other tissues such as myoblast displaying the same phenomenon. This apparent similarity raises the possibility that the presence of GJIC may be a functional criterion in all fusion processes in different types of tissues that depend on the formation of syncytia. Questions still remain to be answered concerning the involvement of GJIC in human placental development. Data concerning this problem have been obtained with the cultured trophoblastic models and by *in situ* hybridization and immunostaining. The role of Cx26 protein has been analysed *in vivo* in Cx26-deficient transgenic mice but human placental structure and development are different. Finally, the modulation by extracellular matrix of EVT cells connexin expression should be explored using invasiveness test on Matrigel®, a well known model used for integrin switch studies.

In conclusion, trophoblast offers a physiological tool for cell biologists to study the molecular mechanisms involved in the control of proliferation, invasion and differentiation in human at the cellular level.

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