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Biochimica et Biophysica Acta 1719 (2005) 117 – 124
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Review

Involvement of gap junctions in placental functions and development

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Received 15 June 2005; received in revised form 23 September 2005; accepted 26 September 2005
Available online 18 October 2005

Abstract

Connexin (Cx) expression and gap junctional intercellular communication (GJIC) are involved in development and differentiation processes. Mediating exchanges between mother and fetus, the placenta is formed when fetal membranes are apposed or even fusing or destroying the uterine mucosa. Therefore, an extraordinary variability of placental structures is observed throughout the mammalian species. This variability affect mainly, the maternofetal blood flow interrelationships, the kind and number of tissue layers separating maternal and fetal bloods, the trophoblast invasiveness and the formation of a syncytium (syncytiotrophoblast). Here, the expression, the localisation and the possible role of Cx and GJIC in placental functions and development are discussed. In rodents, gene knock out in mice have vastly improved our understanding of the role of Cx genes in mouse placental development: Cx26 in transplacental uptake of glucose, Cx31 in the proliferative process of trophoblastic cells and Cx45 in placental vascularisation. In human, it appears that Cx43 allows a GJIC required for the fusion process of cytotrophoblastic cells leading to the formation of the syncytiotrophoblast, the site of the numerous placental functions. On other hands, Cx40 plays a critical role in the switch from a proliferative to an invasive phenotype of the trophoblastic cells invading the endometrium. Owing to the striking diversity of Cx expression in placental structures, we must be careful when extrapolating findings from one species to another.

Keywords: Gap junction; Placenta; Trophoblast; Connexin; Cell–cell communication; Differentiation process

Contents

1. Introduction

Gap junctions are clusters of transmembrane channels composed of connexin (Cx) dodecamers that mediate cell-to-cell communication in almost tissues. In mammals, gap junction channels are composed of two hemi-channels termed connexons, each provides by one of the two neighbouring cells and tightly associated in the intercellular space. In general,

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doi:10.1016/j.bbamem.2005.09.019

effects of Cx 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 (IP3) and Ca^{++}. Connexins represent a family of closely related membrane proteins, which are encoded by a multigene family that contains at least 20 members in human. All represent structurally conserved non-glycosyled transmembrane proteins, 25 to 62 kDa in size, that differ chiefly in the length of their C-terminal domain. Biophysical and functional properties of the 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. Although each connexin isoform exhibits a distinct tissue distribution, many cell types express more than one connexin [1]. The permeability of junctional channels is finely regulated: cAMP, Ca^{++} intracellular levels, pH, phosphorylation, transjunctional applied voltage [2]. In addition, the gap junction biosynthesis is a highly complex process and the delivery, assembly and removal of gap junctional channels appears to be an independent mechanism to control the GJIC [3]. Furthermore, secondary proteins interacting with connexins have been identified: chaperones, scaffolding proteins, kinases, phosphatases, cell signalling molecules [4]. These molecules will undoubtedly aid the multiple steps of gap junction channels.

Connexin expression varies during differentiation, proliferation and transformation processes and following treatment with biologically active substances such as growth factors and hormones [5–8]. The main effects of Cx expression have been attributed to GJIC [9] and to the interaction of connexin with the intracellular signal cascade via the carboxy-terminus [4].

In mammals, the blastocyst defines with the maternal organism by an implantation process a structure which allows the development of the embryo and the fetus: the placenta. This organ is formed when the fetal membranes surrounding the fetus are apposed or even fusing or destroying the uterine mucosa. The placenta is unique among other organs in that it possess the functional activities of other organs: gas and metabolic transfer, excretion, endocrine and immunological activities. To succeed these functions, an extraordinary variability of placental structures has been developed throughout the mammalian species. This variability affect mainly, the maternofetal blood flow interrelationships, the kind and number of tissue layers separating maternal and fetal bloods, the trophoblast invasiveness and the formation of a syncytium (syncytiotrophoblast) [10].

In this review, the expression, the localisation and the possible role of connexins and of GJIC in placental functions and development of the various placental types will be discussed (Table 1).

2. Epithelio and synepitheliochorial placentae

In the epitheliochorial placenta, the blastocyst did not invade the endometrium, subsequently, the fetal chorion

Table 1
Connexin expression in the various placental types

Placental type	Cell type	Connexin expression	Function	References
Epitheliochorial				
Horse	Stromal cells	Cx43		[11]
Pig	Stromal cells	Cx43		[11]
Synepitheliochorial				
Sheep	Stromal cells	Cx43		[12]
	Uterine epithelial cells	Cx26		[12]
Cow	Maternal and fetal mesenchyme	Cx43		[13]
	Uterine epithelial cells	Cx32		[13]
		Cx26		
	Trophoblastic giant cells	Cx26	Migration/fusion?	[13]
		Cx43		
Hemochorial				
Rat	Syncytiotrophoblastic Layer I and II	Cx26	Transfer	[20]
	Trophoblastic cells ectoplacental cone	Cx31		[19]
	Spongiotrophoblast	Cx43		[19]
	Trophoblastic giant cells	Cx43		[19]
Mouse	Syncytiotrophoblastic Layer I and II	Cx26		[21]
	Trophoblastic cells ectoplacental cone	Cx31	Cell proliferation	[25,27]
	Spongiotrophoblast	Cx43		
	Trophoblastic giant cells	Cx43		[23]
Human	*Villous trophoblast*			
	Cyto- and syncytiotrophoblast	Cx43	Cell fusion	[33,34,36,37]
	Fetal mesenchyme	Cx43		
	Extravillous trophoblast			
	Proximal proliferative cells of the column	Cx40	Cell proliferation	[34,44]
	Trophoblastic aggregated cells of the placental bed	Cx43	Cell fusion	[42]
		Cx32		

(cytotrophoblastic cells, chorionic mesenchyme and fetal endothelium) faces the intact uterine epithelium [10]. In these placentae (horse and pig), Cx26, Cx32 and Cx43 expression was not detected, neither in the maternal endometrium epithelium, nor in the trophoblast, these data were correlated to the absence of trophoblastic invasion in these placentae [11]. The synepitheliochorial placenta of the ruminants (sheep, cow) is similar to the epitheliochorial type, however some trophoblastic giant cells (TGC) or binucleate cells, migrate along tight junctions into the uterine epithelium were they fuse with the epithelial cells forming symplasma [10]. This process illustrates a restricted type of trophoblastic invasion. In the sheep placenta, connexin expression was only detected in the maternal endometrium: Cx26 in the epithelial cells and Cx43 in the stromal cells [12]. In the bovine placentome, Cx26 protein was detected at the feto-maternal interface and as a cytoplasmic staining in TGC. Transcripts expression of Cx26 was located in uterine epithelium and in TGC. The expression of Cx32 was localised in uterine epithelium cells while Cx43 protein and mRNA were present in TGC. Cx43 was also expressed in the chorionic and maternal mesenchymes [13]. According to the authors, this connexin expression pattern could be correlated with the trophoblastic migration and fusion processes observed in bovine placentome.

3. Endotheliochorial placentae

Higher invasion of the trophoblast results in the development of the endotheliochorial placenta. Following erosion of the uterine epithelium and maternal connective tissue, the trophoblast comes into intimate contact with the maternal epithelium. Furthermore, mononuclear cytotrophoblastic cells differentiate by fusion into a plurinuclear syncytiotrophoblast (carnivores, insectivores, bats, lower primates). Connexin expression was not investigated in these placentae, however the presence of gap junctions was ultrastructuraly demonstrated between cytotrophoblastic cells and syncytiotrophoblast, supporting a role for gap junctions in trophoblastic cell–cell fusion process [14].

4. Hemochorial placentae

In some mammals, placenta is characterised by an extensive invasion of the trophoblast into the maternal uterus allowing direct contact of the trophoblast with the maternal blood (rodents, lagomorph, higher apes and human). In spite of similar interhemal structure, these placentae differ in their morphogenesis, maternal blood flow interactions and invasive ability. However, analogous cell types have been identified among the trophoblast: proliferative cells, invasive cells and cells differentiating into a syncytium. Unlike tumor cells, in the placenta proliferation, migration and invasiveness are exquisitely regulated both temporally and spatially.

Rodents placental development has been elegantly described in others review and transgenic mouse model has vastly improved our understanding of the genetic control of placental development in this species [15,16]. In the definitive chorioallantoic placenta, the maternal blood enters a labyrinth and bath the fetal blood (Fig. 1A). The trophoblast could be differentiated in one layer (caviomorph rodents), two layers (beaver, lagomorphs) or three layers (rat, mouse). Earlier ultrastructural studies have reported the presence of gap junctions between syncytiotrophoblastic layers of the labyrinth of rabbits, guinea-pig, rat and mice [17,18]. In rat, numerous gap junctions made of Cx26 connect the syncytiotrophoblastic layers I and II of the labyrinth [19]. These gap junctions in conjunction with glucose transporter 1 (Glut1) have been implicated as the structural basis for the transport of glucose across the rat placental barrier [20]. This was elegantly confirmed in the mouse by the fact that transplacental uptake of glucose was strongly decreased in embryonic lethal connexin26-deficient mice [21]. In rat, during the first stage of placentation (choriovittelline placenta), the invasive trophoblastic cells population of the ectoplacental cone express Cx31 which latter disappears with further trophoblast differentiation. Then, in the definitive chorioallantoic placenta, Cx26 appears in the labyrinthine trophoblast whereas Cx43 appears in the spongiotrophoblast and in the polyploid trophoblastic giant cells (TGC) of the junctional zone [19]. Furthermore, it must be pointed out that a normal rat trophoblastic cell line HRP1 expressed Cx43, whereas a rat choriocarcinoma cell line RCHO-1 expressed Cx31 [22].

During mouse placental development, a similar connexin pattern expression has been observed in the trophoblast [23]. However, according to Pauken and Lo, no Cx43 transcripts were found in the mouse placenta, but only in the maternally derived decidual cap covering the placenta [24]. Inactivation of the Cx31 gene results in a transient placental dismorphogenesis with an imbalance in the trophoblast cell lineage differentiation in favour of the polyploid cells (TGC) of the junctional zone [25]. Recently, as Cx31 and possibly Cx43 are coexpressed in the spongiotrophoblast, Cx31 and Cx43 double-deficient mice were investigated [26]. This study shows that Cx31/Cx43 double-deficient mice exhibit the known phenotypes of the single-knockout strains but not combined effects, each connexin having a unique function in placental development. Furthermore, it was demonstrated that undifferentiated mouse trophoblastic stem cells exclusively express Cx31 protein and Cx31 transcripts. Upon differentiation of trophoblastic cells, Cx26 and Cx43 are induced. Interestingly, Cx31 knockout trophoblastic cells revealed an accelerated differentiation process to the polyploid trophoblastic giant cells. All these results suggest that during mouse trophoblastic cell lineage differentiation, the Cx31 channels are required to maintain the proliferative diploid trophoblastic cells [27].

Inactivation of the Cx45 gene leads to defective vascular development in the mouse placenta: impaired formation of the vascular trees in the yolk sac, impaired allantoic mesenchymal ingrowth and fetal capillary formation in the labyrinth. Cx45-deficient embryos exhibited striking abnormalities in vascular development and died between days 9.5 and 10 [28].

In the human, at 7 to 8 days post-conception, the blastocyst invades the uterus and the formation of the placenta is the result of a complex series of interaction between fetal trophoblast and

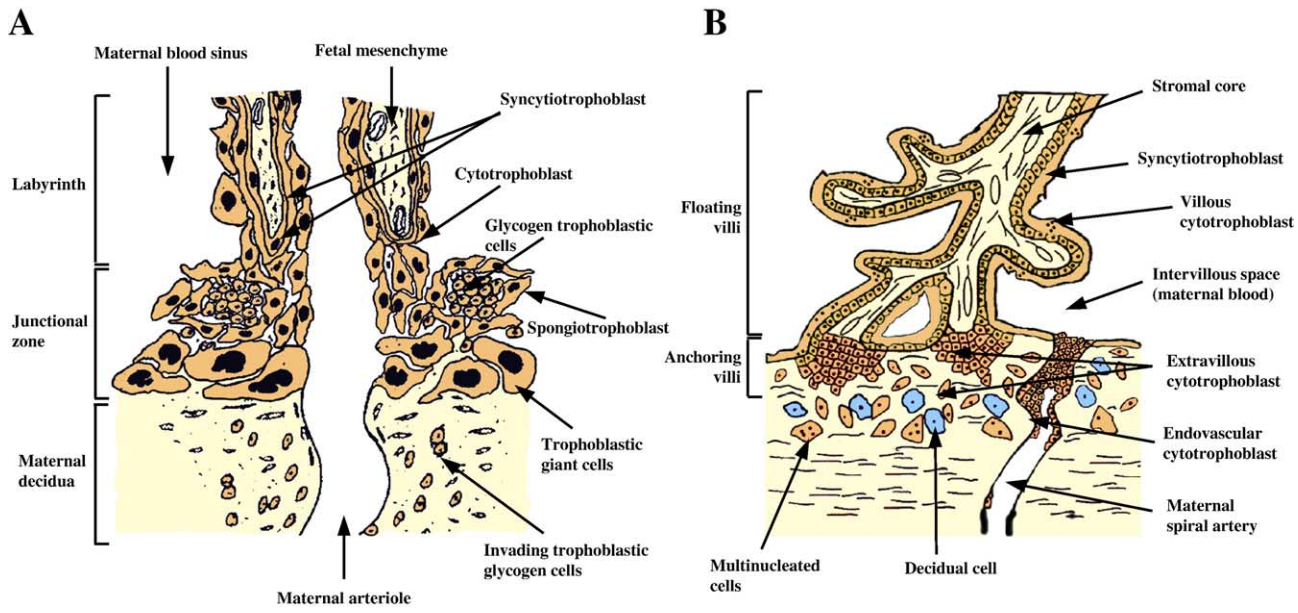

Fig. 1. The definitive structures of mouse (A) and human (B) placentae. In mouse, placentation changes from an initially choriovitelline pattern to a chorioallantoic pattern at 11.5 days (gestation: 19–20 days). In the definitive chorioallantoic placenta, the labyrinth is made up of trophoblastic septae containing a core of fetal mesenchyme. The trophoblast is differentiated into two syncytial layers in contact with the fetal endothelium and one cellular layer forming the wall of the maternal sinuses. The junctional zone is composed of vacuolated "glycogen cells", "spongiotrophoblastic cells" and "trophoblastic giant cells" (polyploid cells). By day 13 of gestation, interstitial invasion of the decidua basalis by trophoblastic glycogen cells occurs. In humans, the definitive chorioallantoic placenta appears by day 21 of pregnancy (gestation: 270 days). At around 10–12 weeks of pregnancy (diagram B), the chorionic floating villi are bathed by maternal blood in the intervillous space. In these villi, cytotrophoblastic cells differentiate by fusion to generate the syncytiotrophoblast. In the anchoring villi, the cytotrophoblastic cells proliferate and invade the decidua (interstitial invasion). The infiltrating cells eventually differentiate into multinucleated giant cells. The cytotrophoblastic cells also invade the lumen of maternal arteries (endovascular invasion) remodelling the spiral artery. Reproduced from Malassine et al. [58] with permission of the publisher OXFORD University Press.

maternal cells in the decidua of the uterus [29]. With progression of placentation, two pathways of differentiation lead to the formation of two distinct trophoblastic cell populations and by day 21 the definitive structural and functional units of the placenta are already present: the "floating villus" and the "anchoring villus" (Fig. 1B).

In the *villous phenotype*, the cytotrophoblastic cells of the floating villi remain attached to the villous basement membrane, forming a monolayer of epithelial cells that proliferate and differentiate by fusion to form a syncytiotrophoblast covering the entire surface of the villus. This trophoblastic epithelium surrounds a core of connective tissue including fetal vessels, fibroblasts and macrophages. The syncytiotrophoblast is engaged in absorptive, exchange and specific endocrine functions of the placenta.

In the *extravillous phenotype*, cytotrophoblastic cells of the anchoring villi proliferate, detach from the basement membrane, and 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. Indeed, some of the extravillous cytotrophoblastic cells invade the uterine arterioles, destroy the media and replace the endothelial cells, thus creating low-resistance, large-diameter blood vessels. Alternatively, many extravillous cytotrophoblastic cells scattered through the decidua and the myometrium and then differentiate into multinucleated cells. This trophoblastic invasion is precisely regulated, confined spatially to the endometrium, the first third of the myometrium, and the associated spiral arterioles and temporally to early pregnancy. On the other hand, uncontrolled trophoblast invasion is detrimental to feto-maternal health as occurs in invasive moles and choriocarcinomas (trophoblastic cancers). In vitro studies have shown that the future of cytotrophoblastic cells into one or the other differentiation pathways depends on the surrounding environment and several types of regulators have been investigated: cytokines, hormones, growth factors, extracellular matrix (ECM), and oxygen tension [30–33]. Furthermore, gap junctional intercellular communication also appears to be implicated in each pathway.

In the *villous phenotype*, Cx43 was immunodetected in situ in the trophoblast of the floating villi, whereas Cx40, Cx33, Cx 32 and Cx 26 could not be detected [34] (Fig. 2). Cx43 protein and Cx43 mRNA are present between cytotrophoblastic cells and between cytotrophoblastic cells and syncytiotrophoblast. The Cx43 protein was also present in the mesenchymal core of the villi. In vitro, isolated mononucleated cytotrophoblastic cells, cultured on plastic dishes in presence of fetal calf serum, aggregate and fuse forming a non proliferative multinucleated syncytiotrophoblast that produces pregnancy specific hormones [35]. In this model, Cx43 was immunodetected at the intercellular boundaries between aggregated cells and after cellular fusion the signal disappeared from the cell membrane [8]. Furthermore, using the gap-fluorescence recovery after photobleaching (gap-FRAP) method, a transient gap junctional inter-trophoblastic communication was demonstrated before trophoblast fusion suggesting a role for GJIC in villous

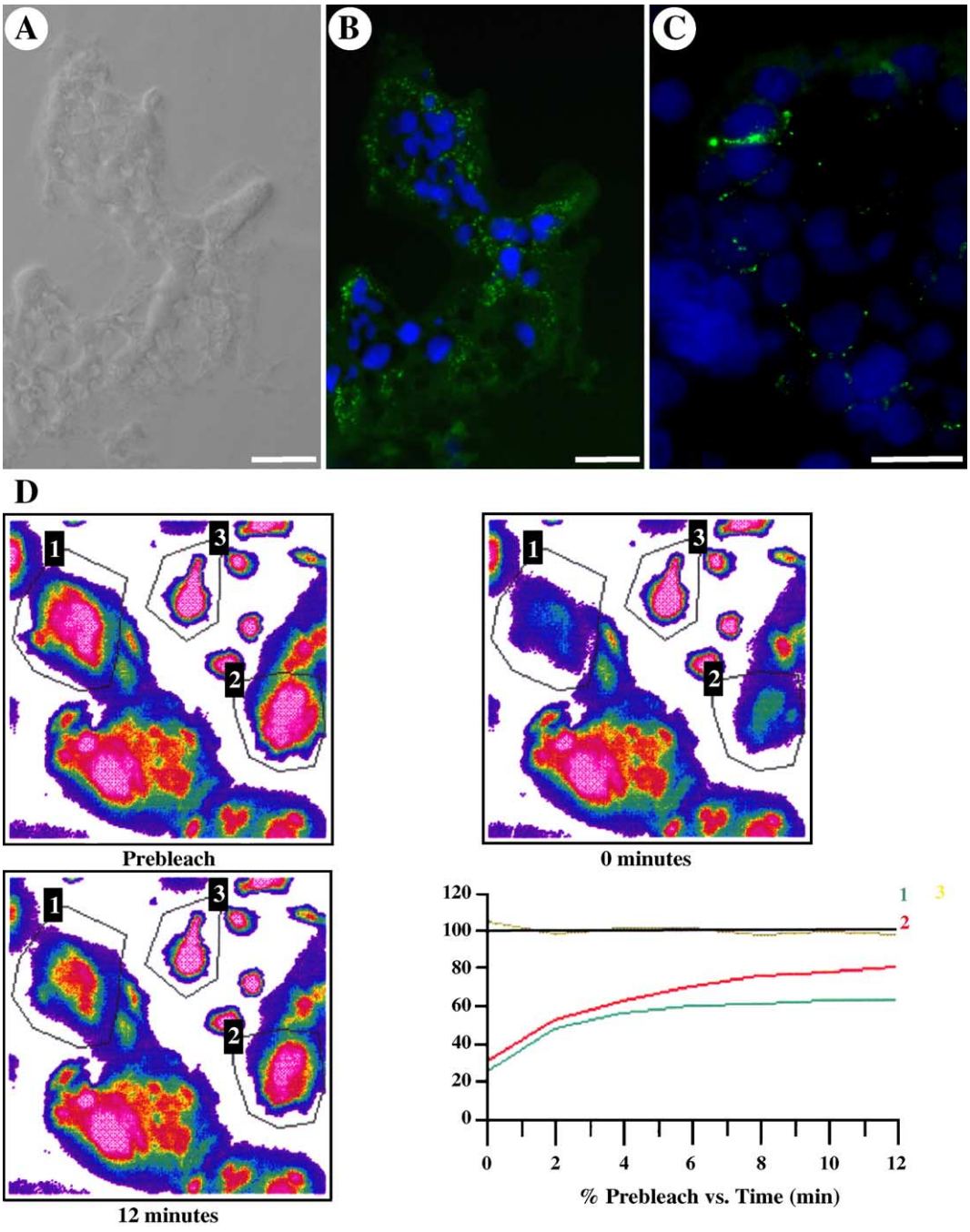

Fig. 2. (A) Brightfield image of first trimester floating villous section immunostained for Cx43 and DAPI in Fig. 1B. (B) Cx43 immunostaining is observed in the trophoblastic layer between contacting cells and within the cytoplasm. (C) Transversal section of another floating villous at higher magnification immunostained for Cx43. Cx43 punctuate IF is observed between 2 contiguous nuclei presumably of cytotrophoblastic cells and between other contacting nuclei (presumably cyto- and syncytiotrophoblast). Scale bars=20 μm. (D) Gap-FRAP experimentation. Typical computer-generated images of fluorescence distribution in villous trophoblastic cells cultured for 3 days measured during gap-FRAP experiment. After a prebleach scan, the fluorescent dye was photobleach in some selected cells (polygons 1 and 2) by means of a strong laser illumination. Isolated cell (polygon 3) kept unbleached served as control for the spontaneous fading of fluorescent emission. Then, the evolution of fluorescence intensities was measured just after photobleaching until 12 min with a scanning period of 2 min. Corresponding curves of fluorescence evolution in selected cells: fluorescence recoveries in cells 1 and 2 follow a closely exponential time course, reflecting the presence of open gap junctional channels. Note the low decrease of fluorescence intensity in control unbleached cell (3) due to repeated scanning. Reproduced from Cronier et al. [34] with permission of the publisher OXFORD University Press.

differentiation [32] (Fig. 2). The requirement of a GJIC and the involvement of Cx43 expression in trophoblastic cells fusion were then demonstrated using gap junctional uncouplers and an antisense strategy in primary culture of trophoblastic cells. Treatment of cells with heptanol (a non specific junctional uncoupler blocking all connexin channels) inhibits GJIC leading to a decrease in syncytiotrophoblast formation and in trophoblastic-specific gene expression. This blocking action was reversible and the inhibition of trophoblast differentiation did not affect Cx43 transcripts and protein expression

[36]. Cytotrophoblastic cells treated with Cx43 antisense fused poorly and trophoblastic gene expression was largely reduced. Furthermore, the mRNA of HERV-W env, encoding an endogenous retroviral envelope glycoprotein (syncytin-I) involved in trophoblastic cell fusion, is significantly decreased. Treatment with Cx43 antisense also dramatically reduced the percentage of coupled cells [37]. Taken together, these data suggest that the molecular exchanges through Cx43 channels preceding fusion are essential for villous trophoblast differentiation.

The *extravillous trophoblast* differentiation occurs during the first weeks of pregnancy, therefore its in situ study is difficult for evident ethical reasons. In the first trimester anchoring villi, Cx40 is expressed in the proximal proliferative part of the cell column [38,39,34]. This localisation is substantiated by the ultrastructural demonstration of gap junctions in the proximal cytotrophoblastic cells of the columns [40]. When cytotrophoblastic cells were cultured for 2 days on Matrigel®, they never fuse and expressed an extravillous phenotype concomitant with the presence of Cx40 mRNA and of Cx40 protein at the border of aggregated cells [34]. In these extravillous trophoblastic cells, only the Cx40 transcript 1B is expressed as in malignant trophoblastic cells [41]. Furthermore, no evidence of GJIC was detected in the cells when investigated by gap-FRAP method. Despite the fact that electrophysiological studies support the hypothesis of an ionic coupling in cell lines transfected by Cx40 and Cx43, it is currently admitted that Cx40 cannot form functional heterotypic channels with most others connexins. Thus, Cronier et al. [34] have hypothesised that the cells of the proximal part of the column (expressing Cx40) cannot communicate with the villous trophoblastic cells (expressing Cx43), thus preventing them to follow a villous differentiation process. According to Winterhager et al. [38], when the cytotrophoblastic cells are leaving the column (in the distal part) the Cx40 immunostaining disappeared and was re-expressed in the trophoblastic cells aggregates within the decidua. Furthermore, Cx32 and Cx43 immunostainings were detected in these trophoblastic cells aggregates [42,8].

Another in vitro model for studying the extravillous phenotype is the culture of first trimester villous explants [43]. When small villous explants are cultured on Matrigel® in low oxygen (3%), the cytotrophoblastic cells proliferate forming compact and organised outgrowths similar to cell column and expressing Cx40 mRNA and protein. When measured by a microinjection method, a GJIC was detected between cells. The GJIC-blockade by carbenoxolone or heptanol (non specific gap junctional uncouplers) induced the presence of scattered group of rounded individual trophoblastic cells reminiscent of an early invasive phenotype. Furthermore, Cx40 antisense treatment also resulted in the abolishment of outgrowth extravillous cells proliferation. These results suggested to Nishimura et al. [44] that Cx40 channels are required for the proliferation of extravillous trophoblastic cells in cell columns and that a loss of GJIC contributes to differentiation to the invasive extravillous trophoblastic cell phenotype.

5. Human choriocarcinoma cell lines

Various trophoblastic cell lines (Jeg-3, BeWo, Jar) have been used as model for studies of certain aspects of gene expression and regulation in cells derived from the trophoblast. However, these highly proliferative cell lines do not necessary reflect the complex phenotype of villous or extravillous trophoblast [45,46]. Since a reduction or a loss of GJIC is a common event during the transition of a normal tissue to a malignant tissue [47], connexin expression and GJIC were examined in malignant trophoblastic cells. Human choriocarcinoma cell lines express variable amounts of Cx40 with Jeg-3 revealing the lowest levels of Cx40 transcripts leading to an extremely low cell–cell communication [48]. Interestingly, the transfection of Jeg-3 cells with Cx26, Cx40 and Cx43 genes, restored cell coupling and reduced the in vitro growth of all clones [48,49]. In contrast to Cx40, the Cx26 channel was more potent in reducing proliferation and inducing differentiation [49]. In an in vitro propagated first trimester human extravillous trophoblastic cell population, only Cx43 was detectable while Cx26, Cx32 and Cx40 were not detected [50]. Recently, it was demonstrated in choriocarcinoma cells that Cx43 interacts with NOV (nephroblastoma overexpressed) a member of the CCN family (Cyr61/connective tissue growth factor/nephroblastoma overexpressed), a factor involved in numerous cellular processes including cell proliferation, differentiation and development [51]. In Jeg-3 cells transfected either with Cx40, Cx43 or C-terminal truncated Cx43 (trCx43), only Cx43 displayed a reduced cell growth of the cells in vitro and in vivo within nude mice, suggesting a role of the C-terminus of Cx43 in growth regulation. NOV was found to be up-regulated only in the Cx43 transfected cells. The induction of Cx43 have lead to a switch in localisation of NOV from the nucleus to the cell membrane where it is colocalised with Cx43. As NOV itself acts as a growth regulator when transfected to Jeg-3, it is suggested that Cx43 is able to regulate cell growth via: an up-regulation of NOV transcription, a change in the localisation of the NOV protein and a binding of NOV to the C-terminus of Cx43 [52].

6. Concluding remarks

Experimental data regarding biological involvement of Cx expression and of GJIC in placental development and functions were mainly obtained in vivo in rodents (transgenic mice) and in vitro in human. It appears in human placenta, like in osteoclasts [53] and in myoblasts [54] that Cx43 is required for the fusion process leading to the villous syncytiotrophoblast. The main effects of Cx expression have been attributed to GJIC, therefore, the nature of the messengers involved in the inter-trophoblastic gap junctional communication needs to be addressed in the future. It is conceivable that Ca^{++}, IP3 and cAMP are exchanged, thus controlling various effectors involved in the transcription of other genes implicated in the fusion. These intercellular messengers may also cross talk with GJIC, as gap junction channels are regulated by cAMP and Ca^{++}. Thus, fusion may be correlated with a concomitant

increase in cellular levels of cAMP [55] and with a decrease in basal Ca^{++} activity [56].

On the other hand, Cx40 plays a critical role in the switch from a proliferative to an invasive extravillous trophoblast phenotype in the cell column. The implication of decidual growth factors of the extracellular matrix and of proteins interacting with Cx40 needs to be addressed in the future.

Recently, the principles of rodents hemochorial placental development have been explained by gene knockout approaches in mice [57]. Owing to striking diversity in placental structures and endocrine functions [58], we must be careful when extrapolating results obtained from one species to another. In mice, Cx26 and Cx31 cause placental alterations whereas in the human placenta, Cx26 and Cx31 are not expressed.

Acknowledgements

We would like to thank Dr. A. Cantereau for her expert assistance and J. Habrioux for his technical assistance. Studies carried out in the authors' laboratories were partly supported by grants from the Langlois foundation and from the Ligue Nationale contre le cancer.

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