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Fusion of Cytotrophoblast with Syncytiotrophoblast in the Human Placenta: Factors Involved in **Syncytialization**

M. Gauster, B. Huppertz

Human placental villi are covered by a characteristic epithelial-like layer. It consists of mononucleated cytotrophoblasts and an overlying syncytiotrophoblast layer both in contact to the trophoblastic basement membrane. The syncytiotrophoblast mostly lacks DNA replication and seems to transcribe only barely mRNA. Therefore, the syncytiotrophoblast depends on cell compounds delivered by fusing cytotrophoblasts. Delivery of fresh cytoplasmic contents into the syncytiotrophoblast is achieved by continuous fusion with cytotrophoblasts throughout gestation. Fusion between cytotrophoblasts and the syncytiotrophoblast is driven by multiple factors, including environmental growth factors and cytokines, which turn on a specific cascade of fusogenic proteins in cytotrophoblasts destined for fusion. The cascade includes protein kinases and transcription factors, as well as induced expression of fusion-promoting proteins associated with the cell membrane. Additionally, specific proteases are activated, which cleave and remodel structural proteins to prepare the cell for fusion. However, not only fusogenic proteins, but also plasma membrane architecture and physicochemical factors such as calcium and oxygen affect intertrophoblastic fusion. Coordinated action of all factors involved is crucial for proper cytotrophoblast - syncytiotrophoblast fusion. Deregulation of a single factor might cause an inadequate fusion rate and could lead to pregnancy complications such as preeclampsia or even spontaneous abortion. J Reproduktionsmed Endokrinol 2008; 5 (2): 76-82.

Key words: cytotrophoblast, syncytiotrophoblast, syncytialization, intertrophoblastic fusion

Intercellular Fusion in the Human

In human, the phenomenon of cellcell fusion occurs in several different cell types. During fertilization, after penetration of the zona pellucida and entry of the sperm into the egg's perivitelline space, fusion of the egg and sperm cell membranes takes place [1]. Macrophages can differentiate and fuse to form multinucleated chondroclasts and osteoclasts [2], which are important for cartilage and bone development and remodelling. Macrophages are also able to fuse into giant cells important in immune defense. During embryonic development of skeletal muscle, mononucleated myoblasts fuse to form multinucleated myotubes. Even in the adult skeletal muscle satellite cells fuse with skeletal muscle fibers. In the human placenta, villous trophoblasts fuse to generate the characteristic multinucleated syncytial layer, the syncytiotrophoblast. Extravillous trophoblasts differentiate and fuse to generate the trophoblastic giant cells characteristic for the decidua basalis during pregnancy.

Human Trophoblast

Arising from the trophoblast of the blastocyst, two trophoblast populations differentiate early during gestation. Extravillous trophoblasts leave the basal membrane of anchoring villi and invade the tissues of the maternal decidua basalis to attach the placenta to the uterus and to remodel maternal spiral arteries.

The second population is the villous cytotrophoblast, which upon specific stimulation differentiate and fuse with the covering syncytiotrophoblast. The multinucleated syncytiotrophoblast in concert with the underlying mononucleated cytotrophoblasts are located on a basement membrane and constitute the villous trophoblast, the epithelial-like layer of the placental villous tree. Some of the cytotrophoblasts are trophoblast progenitor cells and divide in an asymmetric way. While one daughter cell retains its progenitor character, the other is destined for differentiation. The final differentiation stage of a cytotrophoblast is fusion with the syncytiotrophoblast. This way, cytotrophoblast derived nuclei and other organelles, proteins and RNA as well as cytoplasm and membranes are transferred into the syncytiotrophoblast.

Experiments using ³H-thymidine incorporation revealed that DNA synthesis does not occur in syncytiotrophoblast, implying that syncytial nuclei are unable to replicate [3]. In addition, ³H-uridine incorporation experiments showed that the syncytiotrophoblast is mostly lacking RNA synthesis [4]. Taking in consideration that the syncytiotrophoblast does not replicate and shows only little transcriptional activity, the mechanism of fusion with cytotrophoblasts becomes highly important. The integrity of the syncytiotrophoblast depends on continuous fusion and delivery of cytotrophoblast derived cytoplasmic contents throughout pregnancy.

At the same time acquisition of fresh cellular material requires deposition of aged cytosolic content to keep the biological balance of the syncytium. Apoptotic material is packed in

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Figure 1. Schematic representation of trophoblast differentiation within the villous trophoblast. Derived from trophoblast progenitor cells daughter cells start to differentiate and finally fuse with the overlying syncytiotrophoblast. Nuclei that have become an integrative part of the syncytiotrophoblast change their morphology and display chromatin condensation. Finally late apoptotic syncytial nuclei are packed into syncytial knots and are released into the maternal blood stream. The arrows follow the route of cell types and nuclear changes during trophoblast differentiation.

membrane sealed vesicles – the so called syncytial knots – and is released from the apical cytoplasmic membrane of the syncytiotrophoblast into the maternal circulation. Hence, the turnover of villous trophoblast in a placental villus is similar to the turnover in stratified epithelia and includes proliferation, differentiation, fusion and controlled release of apoptotic material (Fig. 1).

To study the fusion process between trophoblasts in greater detail in vitro the BeWo cell model has attracted a great deal of attention. The BeWo cell line is a choriocarcinoma cell line, which shows intercellular fusion when stimulated with forskolin [5]. Treatment of BeWo cells with this reagent leads to formation of multinucleated cells (syncytia) and an increase in the expression of human chorionic gonadotropin (hCG). Syncytialization can be visualized by staining these cells with membrane associated proteins such as desmoplakin or E-cadherin (Fig. 2).

Regulation of Trophoblast Differentiation

Since cytotrophoblasts can either acguire the extravillous (invasive pathway) or the villous (syncytial pathway) phenotype, it is apparent that differentiation has to be strictly regulated. Factors that are involved in governing these pathways are cytokines and growth factors derived from the maternal and fetal environment. The first growth factor described to induce syncytialization has been epidermal growth factor (EGF) leading to secretion of the hormones hCG and human placental lactogen (hPL) [6]. Colony stimulating factor (CSF) and granulocytemacrophage colony-stimulating fac-



Figure 2. A) Immunofluorescent staining of human first trimester placenta (week 8). Mononucleated cytotrophoblasts (arrow) show staining for E-cadherin in their apical and lateral cell membranes (red). The overlying multinucleated syncytiotrophoblast (arrowhead) lacks lateral cell walls and is negative for E-cadherin. The syncytiotrophoblast expresses hCG (green). B) and C) Immunofluorescent staining of forskolin and vehicle treated BeWo cells. Treatment of BeWo cells for 48h with forskolin (20 µM) induced formation of multinucleated syncytia, visualized by degradation of Ecadherin (stained in red). hCG expression (green) was predominantly observed in syncytia of forskolin treated BeWo cells, but was also rarely seen in mononucleated BeWo cells (treated and untreated).

tor (GM-CSF) have also been demonstrated to trigger syncytialization and hCG synthesis [7]. Moreover, transforming growth factor (TGF)- α and leukemia-inhibitory factor (LIF) have been suggested to promote syncytialization and production of hCG [8]. Interestingly, hCG itself, produced by the syncytiotrophoblast can act as inducer of cytotrophoblast differentiation [8, 9]. In contrast, transforming growth factor (TGF)-β inhibits syncytial formation as well as secretion of hCG and hPL [10]. Also tumor necrosis factor (TNF)-a impairs syncytium formation of primary term trophoblasts in vitro and represses hCG synthesis by downregulation of β-hCG subunit expression [11].

When environmental factors bind to their receptors on target trophoblasts, mitogen-activated protein kinases (MAPKs) may be activated which in turn regulate complex programs of cell differentiation. Two classical MAPKs, the extracellular signal-regulated kinase1/2 (ERK1/2) and p38, are implicated to play significant roles in initiation of trophoblast differentiation and fusion. Studies employing specific ERK1/2 and/ or p38 inhibitors to primary trophoblast cultures revealed impaired differentiation and syncytialization [12].

Another potential key player in regulating intercellular trophoblast fusion is protein kinase A (PKA). Transfection of the catalytic subunit of PKA into BeWo cells demonstrated that PKA activity is sufficient to increase BeWo cell fusion [13]. Activation of the PKA pathway led to a diminution of the desmosomal protein desmoplakin and an increase of nuclei within syncytia. Stimulation of PKA with either forskolin or cAMP (both induce fusion) upregulated transcription of the transcription factor glial cell missing a (GCMa) [13], which belongs to the family of zinccontaining transcription factors [14].

Initial overexpression experiments indicated a functional role of human GCM in nervous system development and gliogenesis in *Drosophila* and mammals [15, 16]. However, in the human nervous system GCMa was not detected in significant amounts, but was rather present in mammalian placenta [17, 18]. In human placenta, expression of GCMa is restricted to a subset of villous cytotrophoblasts and is implicated to be associated with trophoblast differentiation [19]. The involvement of GCMa in trophoblast fusion is substantiated by knockdown experiments, revealing that specific GCMa targeting led to diminished intercellular fusion in BeWo cells. GCMa knockout mice show defective placental labyrinth formation and embryonic lethality, implying an integral role of this transcription factor in placental development [20]. So far, two placental target genes are described for GCMa. One is human aromatase [21] and the second is human syncytin 1 [22], which is supposed to trigger membrane fusion.

Membrane Proteins Involved in Trophoblast Fusion

Though syncytialization is an event of membrane fusion, it is obvious that membrane proteins are involved in triggering the interaction of the two different plasma membranes. One membrane protein playing a key role in trophoblast fusion seems to be syncytin 1. Syncytin 1 is a transmembrane protein encoded by an envelope gene of an endogenous retrovirus of the HERV-W family [23]. When functional, it consists of a transmembrane unit and an extracellular domain exposed on the surface of the cell. Initial experiments revealed that overexpression of syncytin 1 in COS cells resulted in the formation of multinucleated syncytia [23]. In primary cytotrophoblasts, expression of syncytin 1 was upregulated when cells were stimulated with a cAMP analogue, which is known to induce fusion [24]. In contrast, silencing of syncytin 1 in primary trophoblasts inhibited syncytialization, substantiating an instrumental role of syncytin 1 in trophoblast fusion [24].

Interestingly, screening of retroviral sequences in the human genome for potential other viral envelope encoding genes revealed a second fusogenic gene product termed syncytin 2. Syncytin 2 is encoded by an endogenous retroviral envelope gene, which belongs to the HERV-FRD family [25]. So far no fusogenic properties have been allocated to syncytin 2. The question how morphology and physiology of the human placenta would have proceeded without integration of retroviruses into the human genome is rather speculative and remains unsolved. However, the fact that syncytin 1 is abundantly expressed in placental trophoblast, but is suppressed by CpG methylation in non placental cells [26] emphasizes the important role of this protein in placental development. The combined action of the two syncytins may well have been important for the evolution of the human placenta with syncytin 1 being crucial for trophoblast fusion and syncytin 2 displaying immunosuppressive activities [27].

Though syncytin 1 and 2 are factors involved in placental syncytialization and immunosuppression, rare and rather controversial information is available on their localization in the human placenta. In situ hybridization detected syncytin 1 mRNA in the syncytiotrophoblast [23]. In contrast, the syncytin 1 protein was localized in either in the syncytiotrophoblast alone [23], in the syncytiotrophoblast and some villous cytotrophoblasts [28], or in villous cytotrophoblasts and extravillous trophoblasts [29]. Varying antibody clones, staining protocols and tissue handling may have given rise to these discrepancies.

So far, two receptors have been described for syncytin 1, the amino acid transporters ASCT1 and ASCT2 [30]. They are sodium dependent transporters of neutral amino acids such as alanine, cysteine and serine [31]. Both mRNAs were detected in placenta, but ASCT1 expression was faint [32]. The ASCT2 protein, also designated as type-D retrovirus receptor (RDR) or amino acid transporter B0 (ATB⁰), was localized exclusively in the cytotrophoblast [33].

Yet another putative amino acid transporter, the CD98 antigen was demonstrated to be important in trophoblast fusion [34]. CD98, also known as FRP-1 and 4F2, is a heterodimeric integral membrane protein, consisting of a heavy chain and a covalently attached light chain. The role of CD98 in membrane fusion is substantiated by the fact that its expression is required for virus induced cell fusion, as well as osteoclast formation [35]. In human trophoblast derived BeWo cells, knockdown of CD98 expression by antisense and siRNA techniques suppressed cell fusion [34, 36]. Interestingly, treatment of BeWo cells with the fusion inducing reagent forskolin led to an increased expression of CD98 [37]. In the human placenta, CD98 was detected in cytotrophoblast and the syncytiotrophoblast [38]. The proposed ligand of CD98 is galectin 3, which is expressed in epithelial and immune cells [39]. Co-immunoprecipitation in BeWo lysates clearly demonstrates binding of galectin 3 to CD98 in vitro [37]. Galectin 3 is suggested to bind to glycosylated sites of CD98 by its carbohydrate recognition domain. Experiments with lactose, which binds with high affinity to lectins, revealed an inhibition of galectin 3 binding to CD98 and decreased fusion in BeWo cells [37]. Like CD98, galectin 3 was detected in both cytotrophoblasts and syncytiotrophoblast of the human placenta [40].

Gap junctional communication between cytotrophoblasts and the syncytiotrophoblast was shown to be important for syncytialization [41]. Gap junctions are composed of clusters of connexin hexamers, which act as transmembrane channels. In the human placenta, connexin 43 (Cx43) was detected between cytotrophoblasts and between cytotrophoblasts and the syncytiotrophoblast [42]. Studies with heptanol, a non specific junctional uncoupler blocking all connexin channels, displayed inhibited intertrophoblastic communication and a decrease in syncytiotrophoblast formation [43]. Antisense oligonucleotides targeted against Cx43 mRNA resulted in poor cytotrophoblast fusion and decreased hCG secretion [44]. Hence, Cx43 is directly involved in intertrophoblastic communication, differentiation and fusion.

Proteases

Remodelling of membranes and cytoskeletal proteins are crucial steps in the process of intercellular fusion. Proteins of the sub-membranous cytoskeleton are degraded by proteases to prepare defined areas of the plasma membrane for fusion. Some of the proteases involved in tro-

phoblast fusion are family members of the caspase family. The majority of caspases is basically known to drive apoptosis, but recent studies revealed key functions of such caspases in cell differentiation [45-47]. The term caspase (cysteine aspartase) arises from the proteolytic behaviour of this protease family. Caspases are cysteine proteases (use cysteine as the nucleophilic group for substrate cleavage) as well as aspases (cleave the peptide bond C-terminal to aspartic acid residues). In the human placenta, caspase 8 is implicated to play a key role in differentiation of cytotrophoblasts and subsequent fusion with the syncytiotrophoblast [4, 48]. When specific peptide inhibitors or antisense oligonucleotides interfering with caspase 8 activity or transcription, respectively, were added to first trimester villous explant cultures, syncytialization was blocked [48]. Active caspases 8 and 10, which are activated by cleavage of their proenzyme states, were detected in a subset of differentiated cytotrophoblasts [4]. Recently, caspase 14 was suggested to participate in trophoblast fusion. Forskolin stimulated BeWo cells showed increased expression of caspase 14, while treatment with staurosporine and induction of apoptosis had no effect on caspase 14 expression [49].

Other candidate proteins facilitating intercellular fusion are members of the ADAM (a disintegrin and a metalloproteinase domain) family. ADAM proteins contain disintegrin and metalloproteinase domains and many of them also comprise putative hydrophobic fusion peptides, which mediate cell-cell fusion [50, 51]. Two members of this family, fertilin α and β (ADAM 1 and 2), were shown to be involved in murine sperm-egg fusion [52]. In human, fertilins are not functional in fertilization [53]. However, other members of the ADAM family, the meltrins, were suggested to trigger fusion, as shown for meltrin α (ADAM12) in myoblast fusion [54] and osteoclast formation [55]. In the human placenta two splice variants of ADAM12 were detected by northern blots [56]. One long transcription variant, which includes a transmembrane domain, was designated as ADAM12-L. The alternative short splice variant, ADAM12-S, lacks the membrane anchor and is the secreted isoform.

ADAM12 is located in cytotrophoblasts and apical areas of the syncytiotrophoblast in first trimester placenta. At term, immunohistochemistry revealed positive staining of cytotrophoblasts [57]. However, immunohistochemistry alone is not sufficient to demonstrate a direct fusogenic potential of ADAM12. Functional fusion assays are pending to elucidate this issue.

Plasma Membrane Architecture

Plasma membranes of human cells are bilayers of asymmetrically distributed phospholipids. While neutral cholinephospholipids (e.g. phosphatidylcholine, PC) are predominantly located in the outer leaflet, negatively charged aminophospholipids (phosphatidylethanolamine, PE and phosphatidylserine, PS) are located in the inner leaflet [58]. The membrane asymmetry is actively maintained by an aminophospholipid translocase, which handles misdirected PE and PS from the outer leaflet back to the inner leaflet [58, 59]. These translocases (flippases) are inactivated by caspases [60] and consequently the asymmetry can no longer be maintained. Pronounced PS externalization to the outer leaflet was observed prior to fusion events

Table 1. Factors that promote (+) or impair (-) fusion of trophoblasts. Evidence for involvement of listed fusion factors were provided either in BeWo cells (B), villous explants (VE) or isolated primary trophoblast cells (T). (?) indicates that functional fusion assays, demonstrating a role of this factor in fusion, are pending.

Growth factors	, hormones and cytokines			
EGF	growth factor	+	Т	[6]
CSF	growth factor	+	Т	[7]
GM-CSF	growth factor	+	Т	[7]
TGF-α	growth factor	+	Т	[8]
LIF	cytokine	+	Т	[8]
hCG	peptide hormone	+	Т	[8, 9]
TGF-β	growth factor	-	Т	[10]
TNF-α	cytokine	-	Т	[11]
Protein kinases	and transcription factors			
ERK1/2	mitogen activated protein kinase (MAPK)	+	Т	[12]
p38	mitogen activated protein kinase (MAPK)	+	Т	[12]
PKA	protein kinase	+	В	[13]
GCMa	transcription factor	+	В	[22]
Mash-2	transcription factor	-	Т	[74]
Membrane prot	teins			
Syncytin 1	endogenous retroviral envelope protein	+	Т, В	[23, 24]
Syncytin 2	endogenous retroviral envelope protein	?		[25]
ASCT1	amino acid transporter	?		[30-32]
ASCT2	amino acid transporter	+	В	[30-32]
CD98	amino acid transporter	+	В	[34, 36]
Galectin 3	lectin	+	В	[37]
Connexin 43	gap junction protein	+	Т	[44]
Proteases				
Caspase 8	protease	+	VE	[4, 48]
Caspase 10	protease	?		[4]
Caspase 14	protease	?		[49]
ADAM12	protease	?		[57]
Membrane arch	itecture			
PS flip	externalization of PS to outer leaflet of		D	1(2) (5)
ABCC2	membrane bilayer	+ 2	В	[63-65]
		i		[07]
Physicochemica	al factors			
hypoxia	low oxygen tension	-	Т, В	[71–73]
calcium		+	В	[78]

such as myotube formation [61], membrane fusion of sperm and egg [62] and trophoblast syncytialization [63]. The assumption that PS externalization, the so-called PS-flip, is a prerequisite of intertrophoblastic fusion was substantiated by in vitro experiments using monoclonal antiphosphatidylserine antibodies. Fusion efficiency of stimulated choriocarcinoma cells was decreased when these antibodies were additionally applied [64, 65]. However, these antibodies do not recognize phospholipids alone, but usually require a co-factor (e.g. beta-2-glycoprotein I) for sufficient binding [66]. Therefore, it is likely that proteinlipid complexes trigger syncytial fusion rather than PS externalization alone.

Recently, the xenobiotic/lipid transporter ABCG2, a member of the ATP binding cassette (ABC) family, was suggested to play a role in counterbalancing the increased PS externalization during trophoblast fusion [67]. Indeed, ABC transporters have been implicated to regulate phospholipid asymmetry by trafficking structural lipids within plasma membranes [68, 69]. ABCG2 was shown to be up-regulated during trophoblast fusion [70]. Knockdown of ABCG2 with siRNA increased PS externalization and resulted in higher rates of apoptosis in forskolin induced BeWo differentiation [67]. Thus, ABCG2 was hypothesized to protect cells as survival factor during the period of asymmetry and reorganization of the plasma membrane during trophoblast syncytialization.

Physicochemical Factors

Several in vitro studies demonstrated an inhibitory effect of low oxygen tension on trophoblast fusion and differentiation. An oxygen concentration of approximately 9% impaired fusion of isolated cytotrophoblasts and led to decreased hCG and hPL expression [71]. Forskolin stimulated BeWo cells, cultured under low oxygen tensions (2 % oxygen) showed suppressed intercellular fusion [72], which was associated with relatively lower expression of syncytin and its receptor compared to control conditions (20 % oxygen) [73]. Additionally, hypoxia was demonstrated to affect expression and activity of transcription factors. Studies with isolated primary trophoblasts revealed an oxygen dependent effect on the basic helix-loop-helix transcription factor Mash-2 (mammalian achaete/scute homologue 2) [74]. While transcription of Mash-2 was down-regulated in primary trophoblasts cultured at 20 % oxygen, its expression was increased at 2 % oxygen. The fact that overexpression of Mash-2 markedly inhibited trophoblast fusion [74] substantiated the inhibitory effect of low oxygen via Mash-2 on intercellular fusion.

Environmental calcium ions are required for fusion of artificial as well as biological membranes [75]. Various cell fusion experiments using cells overexpressing viral envelope glycoproteins emphasized the reguirement of an appropriate extracellular calcium ion concentration [76, 77]. Cells expressing the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (gp120gp41) and its receptor CD4 only fused when calcium (millimolar concentration range) was supplemented to the culture medium. In contrast, the addition of calcium chelators (EDTA, EGTA) inhibited syncytium formation in a concentration dependent way [76]. Considering the fact that trophoblast fusion is mediated by syncytin 1, which is an endogenously expressed retroviral envelope protein, it is tempting to speculate that trophoblastic syncytialization requires extracellular calcium. BeWo cells, known for their fusogenic potential, also require calcium for spontaneous, as well as forskolin induced intercellular fusion [78]. Primary cytotrophoblasts isolated from first trimester placenta remain in a mononuclear state when cultured in culture medium containing low calcium concentrations [79].

Conclusions

Fusion of cytotrophoblasts with the overlying syncytiotrophoblast is crucial for expansion, maintenance and functionality of the placental epithelium-like layer, the villous trophoblast. An increasing number of factors, involved in intertrophoblastic fusion, emerged in the recent past (Tab. 1). They comprise proteins with diverse cellular functions, but also include "non-protein" factors such as calcium concentration, oxygen tension



Figure 3. Illustration of factors involved in trophoblast fusion. Environmentally derived growth factors, hormones and cytokines bind to their cognate receptors at the plasma membrane of cytotrophoblasts. Activation of either protein kinase A (PKA) or MAP kinases ERK1/2 and p38 leads to increased protein expression of the transcription factor GCMa, which in turn drives transcription of fusogenic genes. Several structural and membrane proteins were suggested to promote trophoblast fusion, including syncytin 1 and its receptor ASCT2 (1), CD98 and its receptor galectin 3 (2) as well as connexin 43 (3). Beside activation of PKA or MAP kinase pathways, cytokines induce conversion of pro-caspase 8 into active caspase 8. Once activated, caspase 8 can mediate inactivation (PS flip) (4). Additionally, caspase 8 triggers remodelling of the sub-membraneus cytoskeleton by degrading structural proteins such as α -fodrin (5).

and architecture of the phospholipid bilayer. The studies reviewed herein suggest that fusion of trophoblasts is administrated by a panel of factors and rather exclude a hypothesis of a single fusing factor. Involved proteins such as growth factors and cytokines, protein kinases, transcription factors, proteases and membrane associated proteins imply activation of a specific fusogenic cascade in cytotrophoblasts destined for fusion (Fig. 3).

However, if one factor is dysregulated or non functional, the consequences on trophoblast fusion and placental development might be devastating. Downregulation of one of the best investigated fusion factors, syncytin 1, was associated with preeclampsia and intrauterine growth restriction [80, 81]. This can be explained by the fact that the transcription factor GCMa, which regulates syncytin 1 expression, was also downregulated in preeclamptic placentas [82]. Hence, regulation and interaction of all involved factors must be coordinated precisely.

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