



Expression of the fusogenic HERV-FRD Env glycoprotein (syncytin 2) in human placenta is restricted to villous cytotrophoblastic cells.

Malassiné A^{1,2}, Blaise S³, Handschuh K^{1,2}, Lalucque H³, Dupressoir A³, Evain-Brion D^{1,2*}, Heidmann T^{3*}.

1.INSERM, U767, 4 avenue de l'Observatoire 75006 Paris, France

2.Université Paris Descartes, Faculté des Sciences Pharmaceutiques et Biologiques, 4 avenue de l'Observatoire, 75006 Paris, France

3. Unité des Rétrovirus Endogènes et Eléments Rétroïdes des Eucaryotes Supérieurs UMR 8122 CNRS, Institut Gustave Roussy, 39 rue Camille Desmoulins, 94805 Villejuif, France

*Authors for correspondence (e-mail)

Danièle Evain-Brion: evain@pharmacie.univ-paris5.fr

Thierry Heidmann: heidmann@igr.fr

Key words: retrovirus, human trophoblast, cell fusion, syncytin.

Summary

Recently, the expression of a human endogenous retrovirus HERV-FRD able to encode a fusogenic envelope protein (syncytin 2), has been observed in human placenta. The aim of the present study was to localize the expression of syncytin 2 in first trimester placenta. In addition, we investigated the presence of HERV-FRD transcripts during the in vitro differentiation of isolated villous and extravillous trophoblastic cells from first trimester chorionic villi. Using a monoclonal antibody specifically raised against the HERV-FRD Env protein, syncytin 2 was immunolocalized only in the villous trophoblast of the chorionic villi, at the level of cytotrophoblastic cells. Interestingly, immunostaining was not observed in all cells but only in some of them, and was detected, more frequently, at the membrane level at the interface between the cytotrophoblastic cells and syncytiotrophoblast. Labeling was observed neither in the syncytiotrophoblast nor in the mesenchymal core of the villi nor in the extravillous trophoblast. In vitro detection of HERV-FRD transcripts was restricted to villous trophoblastic cells and decreased significantly with time in culture. These results suggest that syncytin 2 might play a role in human trophoblastic cell fusion.

INTRODUCTION

Human endogenous retroviruses (HERV) comprise approximately 8% of the human genome [1,2]. Most of the identified elements are defective due to mutations and/or deletions within their genes, but some elements have conserved intact open reading frames. A systematic search for non-defective endogenous retrovirus envelope genes has led to the identification of 16 genes [3]. Among them two can induce cell-cell fusion when expressed in different cells, and are highly and specifically expressed in the human placenta [4-6].

The products of these two genes are glycoproteins named HERV-W Env glycoprotein (syncytin 1) and HERV-FRD Env glycoprotein (syncytin 2). Syncytin 1 induces the formation of syncytia on interaction with the D type mammalian retrovirus receptor (RDR, also known to be the neutral amino acid transporter ATB0/ASCT2/SLC1A5) [5, 7]. In addition, Frendo et al. [8] showed that cell fusion and differentiation of villous trophoblastic cells in culture are markedly inhibited by specific HERV-W antisense oligonucleotides. Recently we demonstrated that syncytin 1 is expressed in all types of human trophoblastic cells [9]. Syncytin 1 and syncytin 2 originate from distinct retroviral elements, and disclose several differences. Syncytin 2 entered the primate genomes earlier than syncytin 1, namely before the split between New World and Old World Monkeys (i.e > 40 Myrs ago), whereas syncytin 1 entered about 25 Myrs ago, being not

found in Old World Monkeys. Yet, both genes remained functional in all the corresponding primate branches, thus strongly suggesting selection for a physiological role [2] Syncytin 1 and syncytin 2 also differ by their receptor (still not identified for syncytin 2), as demonstrated by *ex vivo* cell-cell fusion assays using different cell types [6].

The human trophoblast differentiates along two major pathways both critical for normal placental function [10]. In the villous trophoblast, the cytotrophoblastic cells of the floating villi proliferate and differentiate by fusion to form a syncytiotrophoblast that covers the entire surface of the villi. This syncytiotrophoblast is regenerated, by a continuous turnover process including proliferation of cytotrophoblast stem cells, fusion of differentiating cytotrophoblast into syncytiotrophoblast and progression towards apoptosis. The syncytiotrophoblast is multifunctional, but its primary functions are absorption, exchanges and specific hormonal functions. In the extravillous trophoblast, the cytotrophoblastic cells of the anchoring villi in contact with the uterus wall proliferate, detach from the basement membrane and aggregate into multilayered columns of non-polarized cells that invade the uterus wall. From there individual cells migrate into the decidua and the myometrium, remodeling the pregnant vasculature. Indeed, some of the extravillous cytotrophoblastic cells invade the uterine arterioles. Alternatively many extravillous interstitial cytotrophoblastic cells

are scattered through the deciduas and the myometrium and differentiate into multinucleated giant cells.

The aim of the present study was to localize in situ, in first trimester placenta, the expression of HERV-FRD Env glycoprotein in the villous and extravillous trophoblast. In addition, using our previously described in vitro model of villous and extravillous cytotrophoblastic cell isolation [11], we investigated the presence of HERV-FRD transcripts specific for syncytin 2 in these cells.

MATERIAL AND METHODS

Antibodies

A DNA fragment containing amino acids 16 to 166 of the HERV-FRD envelope ORF was amplified from pHCMV-EnvFRD [6], inserted into the pET28b (Novagen) procaryotic expression vector and expressed in BL21 bacteria. The recombinant protein was purified from bacteria lysates by nickel affinity chromatography. Mice immunization and generation of monoclonal antibodies producing hybridomas were performed in accordance with standard procedures [12]. The hybridoma supernatants were tested by ELISA assays using the recombinant protein, and by immunoblot analyses using lysates of cells transiently transfected with an HERV-FRD Env expression vector [6]. The hybridoma with the highest affinity was selected (6E1 hybridoma) and injected into the peritoneal

cavity of 5 mice to produce ascites. Monoclonal antibodies were then purified by using protein G sepharose 4 fast flow (Amersham, Orsay, France), according to the manufacturer instructions. The isotype of the anti-HERV-FRD Env monoclonal antibody, as determined by an ELISA assay, is IgG1. To test the specificity of the 6E1 monoclonal antibody, 293T human cells were transfected with expression vectors for HERV-FRD Env and HERV-W Env [6], by using lipofectamine (Invitrogen, 1-2 μg of DNA for 7.5×10^5 cells). Transfected cells were recovered 24 h after transfection in cold phosphate-buffered saline (PBS) supplemented with protease inhibitors, and centrifuged at $200 \times g$ at 4°C for 5 min. The cell pellets were then resuspended in Laemmli buffer and whole-cell lysates were separated by SDS-PAGE using gels containing 12% polyacrylamide. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell, Mantes La Ville, France) and incubated either with the HERV-FRD Env 6E1 mouse monoclonal antibody (1:500), or with HERV-W Env polyclonal antibodies (anti-SU rabbit antiserum ; gift from M. Mangeney, Villejuif, France) used as a control (1:500). A goat antibody to mouse or to rabbit IgG conjugated to horseradish peroxidase (Amersham, Orsay, France; dilution 1:10000) was used as the secondary antiserum.

Immunohistochemistry

Placental tissues (n= 4) from first trimester legal induced abortions (8-12 weeks of gestation) were obtained from the Department of Obstetrics and Gynecology at the Broussais, Saint Vincent de Paul and Cochin Hospitals and fixed in 4 % formalin

for 4 to 12 h at room temperature. In addition paraffin sections of an implantation site (placenta and myometrium) at 16 weeks of pregnancy was obtained from the Department of Pathology CHR La Citadelle, Liège. Hysterectomy was performed because of cervical carcinoma stage IIa diagnosed at 16 weeks of gestation. Samples were fixed in 4 % formalin for 4 to 12 h at room temperature. Use of these biological samples, were approved by local ethical committee. After fixation, samples were dehydrated and embedded in paraffin. Five μm sections were cut, deparaffined in xylene and rehydrated in ethanol/water. Antigens were retrieved by treatment for 45 min in 0.001M sodium citrate buffer (pH 6) in a water bath (95-99C), according to the Dako labs kit procedure (Trappes, France). Endogenous peroxydase activity was inhibited with 3% hydrogen peroxide for five minutes. Non specific binding was blocked by incubation for 30 min with 2% BSA in PBS, then, specimen were washed five times in PBS and incubated for 30 min at room temperature with antibody against HERV-FRD Env (1/400) or cytokeratin 07 (1/100). Then after five further washes in PBS specimens were immunostained by a streptavidin-biotin staining procedure, using the Peroxydase, Dako LSAB kit. Specimens were mounted in an aqueous medium (Aqueous Mounting Medium; Dako). All controls, performed with a mouse isotypic IgG1 at the same concentration as the primary antibody were negative.

Isolation and purification of cytotrophoblastic and fibroblastic cells from first

trimester chorionic villi.

Six different cell isolations were performed using chorionic villi from first trimester (7-12 weeks) legal induced abortion, as previously described [11]. In the first place dissected chorionic villi were incubated in Hank's solution containing 0.125% trypsin (Difco Laboratories, Detroit, Michigan), 4.2 mM MgSO₄, 25 mM Hepes, 50 U/ml Deoxyribonuclease type IV (Sigma, Saint-Quentin Fallavier, France) for 35 min at 37°C without agitation. Then the tissue was sedimented and the supernatant was taken and filtered (100 µm pores). Hank's solution was added six times to the tissue with subsequent sedimentations. The pooled supernatants were used for extravillous cytotrophoblastic cells (EVCT) isolation and trypsin digestion was stopped in these supernatants with 10% fetal calf serum (FCS). In the mean time the remaining villous tissue was further digested for villous cytotrophoblastic and fibroblastic cells isolations (see section below). Cells were centrifuged at 300 x g for 10 min, washed twice in Hank's solution supplemented with 10% FCS and filtered on a 50 µm pore nylon membrane. The cell suspension was carefully layered over a discontinuous Percoll gradient and centrifuged for 25 min at 1000 x g. The layer corresponding to 40-45% Percoll, was washed twice in Hank's solution supplemented with 10% FCS, diluted to a concentration of 1×10^5 cells/ ml in DMEM supplemented (10% FCS, 2 mM glutamine, 100 UI/ml penicillin and 100 µg/ml streptomycin) and plated on Matrigel-coated (5 mg/ml; Collaborative

Biomedical Products, Le Pont de Claix, France) 8- μ m pores Transwell insert (Costar, Cambridge, MA), as previously described [11] . After 2 h adhesion period in 5% CO₂ at 37°C, cells were carefully washed three times to eliminate non-adherent cells and cultured for 48 h in HAMF12/DMEM (10% FCS, 2 mM glutamine, 100 UI/ml penicillin and 100 μ g/ml streptomycin). Each preparation of purified EVCT cells were characterized using immunocytochemistry as previously described [11, 13 and table 1]. Briefly cells cultured for two days on Matrigel coated Transwell and crossing the 8- μ m diameter pores of the membrane were immunostained for vimentin, cytokeratin 07, human leukocyte antigen-G (HLA-G), human placental lactogen (hPL), the protooncogene c-erbB2 and alpha5 subunit of the alpha5-beta1-fibronectin receptor [13]. We observed that populations of isolated EVCT were 95 to 97% pure from one preparation to another, as analyzed by double labeling using antibodies against cytokeratin 07 and hPL. In addition these EVCT cells expressed c-erbB2 and HLA-G protein. These cell preparations were negative for vimentin.

After the first 35min digestion period the remaining villous tissue was subjected to sequential trypsin DNase digestion (3-4) (Hank's solution containing 0.0625 % trypsin, 4.2 mM MgSO₄, 25 mM Hepes, 50 U/ml DNase type IV) for villous cytotrophoblastic cells isolation as previously described [11-14]. Cells suspensions were carefully layered over a discontinuous Percoll Gradient (70% to 5%, in 5%

steps) and centrifuged. The middle layer (30-45%) was removed and washed in DMEM. Cells were diluted to a final concentration of 1.25×10^5 cells/ml DMEM supplemented with 10% FCS, 2 mM glutamine, 100 UI/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin and then plated in 35 mm plastic dishes (TPP, Switzerland) and incubated at 37°C in humidified 5 % CO_2 , 95%. After one night incubation non adherent cells and syncytial fragments were removed by three washes with Hank's solution. These cells were positively stained for cytokeratin 07 at 95%. These villous trophoblastic cells were cultured for 24 and 72 h and the culture medium was changed daily. For fibroblast isolation, several (3-5) trypsin-DNase (Hank's solution containing 0.0625 % trypsin, 4.2 mM MgSO_4 , 25 mM HEPES, 50 U/ml DNase type IV) digestions were further performed on the remaining mesenchymal core of the chorionic villi. After discarding cells from the first digestion, cells were pooled, diluted to a final concentration of 2×10^5 cells/ml in DMEM supplemented (10% FCS, 2 mM glutamine, 100 UI/ml penicillin and 100 $\mu\text{g/ml}$ streptomycin). Cells (1.25×10^5 cells/ml) were plated on 35 mm plastic dishes (TPP, Switzerland) and cultured for 48 h and the culture medium was changed daily. These cultured fibroblasts were characterized using immunocytochemistry. 98% of the cells were vimentin positive and also positive to a monoclonal antibody against specific fibroblast antigen (clone ASO2, Dianova, Hambourg, Germany).

Quantification of specific transcripts by real-time RT-PCR.

Total RNA was extracted from EVCT cells cultured for 48 h (10^6 cells), from villous trophoblastic cells (6×10^6 cells) cultured for 24 and 72 h and from fibroblasts (6×10^6 cells) cultured for 48 h using QIAGEN Rneasy mini kit (Courtabeuf, France). cDNA synthesis and PCR amplification were performed as described previously [15]. All PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Corp., Applied Biosystems, Foster City, CA) and the SYBER Green PCR Core Reagents kit (Perkin-Elmer Corp. Applied Biosystems). We used the following primers FRDf (+) GCCTGCAAATAGTCTTCTTT and FRDr (-) ATAGGGGCTATTCCCATTAG [3]; PPIA (+) CTGAAGGCCTGCATGGATTC and PPIA (-) CTGCTGTCTTTGGGACCTTGT [15]. The gene PPIA coding for cyclophilin A was used as internal control and each sample was normalized on the basis of its PPIA content.

RESULTS

HERV-FRD Env glycoprotein immunolocalization in human placenta

To detect the HERV-FRD Env (syncytin 2) expression, we raised a monoclonal antibody directed against part of the HERV-FRD Env surface (SU) subunit (6E1

antibody; Material and Methods). The specificity of the 6E1 monoclonal antibody was shown by western blot experiments (Fig 1). Human 293T cells were transiently transfected with plasmids expressing the HERV-FRD Env protein (pCMV-EnvFRD), or the HERV-W Env protein (pCMV-EnvW) as a control. Immunoblotting of the lysates with the 6E1 monoclonal antibody revealed, only in cells transfected with HERV-FRD Env, a major band with an apparent molecular mass of approximately 75 kDa, consistent with the size of the Env precursor, and a fainter band of approximately 60 kDa, most probably corresponding to the cleaved Env SU subunit. No band was observed with the 6E1 antibody in lysates of cells transfected with the HERV-W Env (syncytin 1) expression vector. Expression of the HERV-W Env protein was controlled by immunoblotting the same lysates with a polyclonal antibody raised against HERV-W Env SU protein. These results demonstrate that the 6E1 monoclonal antibody is specific for HERV-FRD Env (syncytin 2), and does not cross react with the syncytin 1 protein.

Using the 6E1 monoclonal antibody, specific expression of syncytin 2 was analyzed by immunostaining of earlyplacenta sections. In the chorionic villi, syncytin 2 was detected at the level of the cytotrophoblast (Figure 2). Interestingly, immunostaining was not observed in all cytotrophoblastic cells but in only some of them (Figure 3 and 4). In these cells, immunostaining occurred sometimes in the cytoplasm (Figure 3 and 4) and, interestingly also at the membrane level, at the

interface between cytotrophoblastic cells and the syncytiotrophoblast (Figure 3). Immunostaining was never observed in the syncytiotrophoblast (Figure 2, 3 and 4) and in the mesenchymal core of the villi (Figure 2 and 4).

As shown in figure 5, syncytin 2 immunostaining was not observed in the extravillous cytotrophoblastic cells: proliferative cells of the column (panel A), interstitial extravillous cytotrophoblastic cells (panel A), multinucleated giant cells (panel C) and peri and endovascular trophoblastic cells (panel C). The trophoblastic identity of the investigated cells was characterized by cytokeratin 07 immunostaining (panels B and D).

In control experiments, replacing the primary antibody by a non specific IgG1 antibody, no immunoreactivity was detected (Figure 3: insert, Figure 5: panels E and F).

HERV-FRD transcript levels in cultured villous and extravillous cytotrophoblastic cells.

Cytotrophoblastic cells obtained after 35 minutes enzymatic digestion of first trimester chorionic villi and further purified (see Materials and Methods) were cultured for two days on Matrigel-coated Transwell (see Materials and Methods). After crossing the membranes, cells visible on the bottom side of the porous

membrane were shown to express the specific markers of human invasive EVCT, as previously described [9, 11, 13]. We determined, by real-time quantitative RT-PCR, the expression of HERV-FRD mRNA in EVCT cells cultured for two days. HERV-FRD transcripts were not detected in these cells and in human fibroblasts isolated from the same villi (Figure 6), in agreement with the absence of HERV-FRD Env glycoprotein immunodetection in EVCT and fibroblasts.

In contrast HERV-FRD transcripts were detected in cultured villous trophoblastic cells isolated from the same villi, with variations from one placenta to another (Figure 6). In culture, cytotrophoblastic cells isolated from first trimester chorionic villi after sequential enzymatic digestion adhere to plastic dishes, aggregate and fuse together to form a syncytiotrophoblast within 48-72 h (8, 11, 14,15) . In these cells, HERV-FRD transcript levels decreased significantly between 24 hours and 72 hours of culture (Figure 6).

DISCUSSION

In this study, using a monoclonal antibody specifically raised against the HERV-FRD glycoprotein, we demonstrate for the first time that this protein, also called syncytin 2, is expressed in human trophoblast. HERV-FRD Env is immunodetected in the villous trophoblast at the cytotrophoblastic cell level and not in the

extravillous trophoblast. Interestingly, the expression of this fusogenic retroviral envelope glycoprotein is not uniformly distributed in the villous cytotrophoblastic cell layer. Villous cytotrophoblastic cells are immunostained sometimes in the cytoplasm but more frequently at the cell membrane, the membranous staining occurring at the interface between cytotrophoblastic cells and the syncytiotrophoblast. Localization at this interface is precisely that expected for a protein directly involved in the fusion of the mononuclear cytotrophoblastic cells into the syncytiotrophoblast. Moreover, the scattered localization of syncytin 2 may be linked to the dynamic process of trophoblast fusion. These observations argue for a role of syncytin 2 in syncytiotrophoblast formation and regeneration.

In vitro, several factors have been demonstrated to be directly involved in trophoblastic cell-cell fusion, including the phosphatidylserine flip [16], connexin 43 [17, 18], cadherin 11 [19], CD98 [20], caspase 8 [21] and syncytin 1 [8].

Syncytin 1 expression is observed in all trophoblastic cell types (villous and extravillous) [4, 22, 23, 8, 24, 25, 26, 9]. Such a profile is clearly different from that observed in the present study for syncytin 2, thus suggesting that the activities of these two proteins of retroviral origin are non-redundant. Yet, this does not exclude their possible cooperation in the fusion process, as hypothesized for the other above-mentioned proteins

The fusion process of villous cytotrophoblast into syncytiotrophoblast requires the

interaction of the retroviral envelope proteins with membranous receptors. The D type mammalian retrovirus receptor RDR, functional receptor for syncytin 1, is present in both the villous and extravillous trophoblast [9]. The nature of the HERV-FRD Env receptor mediating cell fusion is unknown and needs to be investigated in the near future.

In conclusion, we have demonstrated for the first time that the HERV-FRD Env glycoprotein (syncytin 2) is only expressed in villous cytotrophoblastic cells, suggesting a direct role in human syncytiotrophoblast formation. Cell fusion is an essential step in human trophoblast differentiation and studies are still required to improve our understanding of the various factors directly involved in this process. Pathological models, such as cytotrophoblasts isolated from Trisomy 21-affected placentas [27] and in which cell fusion and syncytiotrophoblast formation are defective [15], should help to further our understanding of the cell-cell fusion process.

ACKNOWLEDGEMENTS

The authors wish to thank Pr Foidart (Université de Liège, Belgium) and the staff of Cochin Obstetrics Department (Paris, France) for assistance with specimen collection, M. Huesca (Institut André Lwoff) for the production of the monoclonal antibodies, B. Ducos for a series of antibody assays, P Gerbaud for placental

fibroblast cell culture and Ingrid Laurendeau for expertise in RT-PCR analysis. We thank C. Laviolle for critical reading of the manuscript

REFERENCES

- [1] Landers ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, *et al.* Initial sequencing and analysis of the human genome. *Nature* 2001;409:860-921.
- [2] de Parseval N, Heidmann T. Human endogenous retroviruses: from infectious elements to human genes. *Cytogenet Genome Res* 2005;110:318-32.
- [3] de Parseval N, Lazar V, Casella JF, Benit L, Heidmann T. Survey of human genes of retroviral origin: identification and transcriptome of the genes with coding capacity for complete envelope proteins. *J Virol* 2003; 77: 10414-22.
- [4] Mi S, Lee X, Li X, Veldman GM, Finnerty H, Racie L *et al.* Syncytin is a captive retroviral envelope protein involved in human placental morphogenesis. *Nature* 2000;403:785-789.
- [5] Blond JL, Lavillette V, Cheynet V, Bouton G, Oriol S, *et al.* An envelope glycoprotein of the human endogenous retrovirus HERV-W is expressed in the human placenta and fuses cells expressing the type D mammalian retrovirus receptor. *J Virol* 2000;74:3321-3329.

- [6] Blaise S, de Parseval N, Benit L, Heidmann T. Genomewide screening for fusogenic human endogenous retrovirus envelopes identifies syncytin 2, a gene conserved on primate evolution. *Proc Natl Acad Sci U S A*. 2003; 100:13013-8
- [7] Lavillette D, Marin M, Ruggieri A, Mallet F, Cosset FL, Kabat D. The envelope glycoprotein of human endogenous retrovirus type W uses a divergent family of amino acid transporters/cell surface receptors. *J Virol* 2002;76:6442-52.
- [8] Frendo JL, Olivier D, Cheynet V, Blond JL, Bouton O, Vidaud M et al. Direct involvement of HERV-W Env glycoprotein in human trophoblast cell fusion and differentiation. *Mol Cell Biol* 2003;23:3566-3574.
- [9] Malassine A, Handschuh K, Tsatsaris V, Gerbaud P, Cheynet V, Oriol G, et al. Expression of HERV-W Env glycoprotein (syncytin) in the extravillous trophoblast of first trimester human placenta. *Placenta*. 2005; 7:556-62.
- [10] Aplin J. Implantation, trophoblast differentiation and hemochorial placentation: mechanistic evidence in vivo and in vitro. *J Cell Sci* 1991;99:681-692.
- [11] Tarrade A, Lai Kuen R, Pavan L, Malassiné A, Tricottet V, Blain P et al. Characterization of human villous and extravillous trophoblasts isolated from first trimester placenta. *Lab Invest* 2001;81:1199-1211.
- [12] Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975; 256: 495-7.
- [13] Pavan L, Tarrade A, Hermouet A, Delouis C, Titeux M, Vidaud M, et al. Human invasive trophoblasts transformed with simian virus 40 provide a new tool

to study the role of PPAR in cell invasion process. *Carcinogenesis* 2003;24:1325-1336.

[14] Kliman H, Nestler J, Sermasi E, Sanger J, Strauss J III Purification, characterization and in vitro differentiation of cytotrophoblasts from the human term placenta. *Endocrinology* 1986;118:1567-1582.

[15] Frendo JL, Vidaud M, Guibourdenche J, Luton D, Muller F, Bellet D, et al. Defect of villous cytotrophoblast differentiation into syncytiotrophoblast in Down's syndrome. *J Clin Endocrinol Metab* 2000;85:3700-3707.

[16] Adler RR, Ng AK, Rote NS. Monoclonal antiphosphatidylserine antibody inhibits intercellular fusion of the choriocarcinoma line, JAR. *Biol Reprod.* 1995; 53:905-10.

[17] Frendo JL, Cronier L, Bertin G, Guibourdenche J, Vidaud M, Evain-Brion D et al. Involvement of connexin43 in human trophoblast cell fusion and differentiation. *J Cell Sci* 2003;116:3413-3421.

[18] Cronier L, Defamie N, Dupays L , Théveniau-Ruissy M , Goffin F , Pointis G et al. Connexin expression and gap junctional communication in human first trimester trophoblast . *Mol Hum Reprod* 2002;8:1005-1013.

[19] Getsios S, MacCalman CD. Cadherin-11 modulates the terminal differentiation and fusion of human trophoblastic cells in vitro. *Dev Biol.* 2003; 257: 41-54.

- [20] Kudo Y, Boyd CA. RNA interference-induced reduction in CD98 expression suppresses cell fusion during syncytialization of human placental BeWo cells. *FEBS Lett.* 2004; 577: 473-7.
- [21] Black S, Kadyrov M, Kaufmann P, Ugele B, Emans N, Huppertz B. Syncytial fusion of human trophoblast depends on caspase 8. *Cell Death Differ.* 2004; 11: 90-8.
- [22] Lee X, Keith JC Jr, Stumm N, Moutsatsos I, McCoy JM, Crum CP, et al. Downregulation of placental syncytin expression and abnormal protein localization in pre-eclampsia. *Placenta* 2001; 22:808-12.
- [23] Muir A, Lever A, Moffet A Expression and Functions of human endogenous retroviruses in the placenta : an update. *Placenta* 2004;25 SupplementA:S16-S25.
- [24] Smallwood A, Papageorghiou A, Nocolaides K , Alley MK , Alice J , Nargund G. Temporal regulation of the expression of syncytin (HERV-W), Maternally imprinted PEG10, and SGCE in human placenta. *Biol Reprod* 2003;69:286-293.
- [25] Yu C, Shen K, Lin M, Chen P, Lin C, Chang GD, et al. GCMa regulates the syncytin-mediated trophoblastic fusion. *J Biol Chem* 2002 ; 20: 50062-8.
- [26] Potgens AJ, Drewlo S, Kokozidou M, Kaufmann P. Syncytin: the major regulator of trophoblast fusion? Recent developments and hypotheses on its action. *Hum Reprod Update* 2004; 10: 487-96.

[27] Frenzo JL, Therond P, Bird T, Massin N, Muller F, Guibourdenche J et al. Overexpression of copper zinc superoxide dismutase impairs human trophoblast cell fusion and differentiation. *Endocrinology* 2001; 142: 3638-48.

LEGENDS

Figure 1: Characterization of the 6E1 monoclonal antibody raised against the HERV-FRD Env. Whole-cell extracts of 293T cells, transiently transfected with HERV-FRD Env (pCMV-EnvFRD) or HERV-W Env (pCMV-EnvW) expression vectors, were separated by SDS-12% PAGE. The blots were probed with the 6E1 anti-EnvFRD mouse monoclonal antibody or with an anti-EnvW rabbit polyclonal antibody as a control.

Figure 2 Immunohistochemical analysis of syncytin 2 (HERV-FRD Env) in the villous trophoblast of placenta floating villi (16 weeks of gestation). Immunostaining with anti-syncytin 2 monoclonal antibody shows positive reactivity in cytotrophoblastic cells (black arrows), not uniformly distributed along the cytotrophoblastic cell layer. No labeling can be detected at the level of the syncytiotrophoblast or the mesenchymal core of the villi. Scale bar= 50µm.

Figure 3. Immunohistochemical analysis of syncytin 2 (HERV-FRD Env) in the villous trophoblast (12 weeks). Immunostaining was observed in the cytoplasm of cytotrophoblastic cells (black arrow) and at the membrane level, at the interface

between cytotrophoblastic cells and the syncytiotrophoblast (red arrow). Some cytotrophoblastic cells were not immunostained (green arrow). No reactivity was observed in control sections incubated with a non specific IgG1 antibody (insert). Scale bar= 40 μ m.

Figure 4. Immunohistochemical analysis of syncytin 2 (HERV-FRD Env) in the villous trophoblast of first trimester placenta floating villi (8 weeks of gestation). This large magnification allows to clearly establish the cytoplasmic localization in the cytotrophoblastic cells (CT). No immunostaining was observed in the syncytiotrophoblast (ST) and in the mesenchymal core (MC). Scale bar= 10 μ m.

Figure 5. Immunohistochemical analysis of syncytin 2 (HERV-FRD Env) in the implantation site of 16-week gestation placenta. Sections were stained for cytokeratin 07 to identify trophoblastic cells. The anti-syncytin 2 monoclonal antibody shows no reactivity in the EVCT of the column (A, bold arrow), in the interstitial extravillous cytotrophoblastic cells (C, arrow head) and in the multinucleated giant cells (C, asterix) identified by positive cytokeratin 07 immunostaining (B and D). No reactivity is observed in control sections incubated with a non specific IgG1 antibody (E and F). These results are representative of all the tissues studied. Scale bar = 40 μ m.

Figure 6. Syncytin 2 (HERV-FRD Env) transcript levels in villous and extravillous trophoblastic cells isolated from the same first trimester chorionic villi. Extravillous trophoblastic cells (EVCT) were cultured for 48h on Matrigel, villous cytotrophoblastic cells (VCT) for 24h and 72h on plastic dishes and fibroblasts for 48h on plastic dishes, as described in methods. Data are expressed as levels of syncytin 2 mRNA normalized by PPIA mRNA levels. Values are the mean \pm SEM of six different experiments carried out with cell cultures from six different first trimester placentas. $*=p<0.05$ using the Wilcoxon range test.