Short Communication

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Human endogenous retrovirus-W envelope (syncytin) is expressed in both villous and extravillous trophoblast populations

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The placenta is unique amongst normal tissues in transcribing numerous different human endogenous retroviruses at high levels. In this study, RT-PCR and immunohistochemistry were used to investigate the expression of syncytin in human trophoblast. Syncytin transcripts were found in first-trimester trophoblast cells with both villous and extravillous phenotypes and also in the JAR and JEG-3 choriocarcinoma cell lines. Syncytin protein was detected in villous trophoblast and in all extravillous trophoblast subpopulations of first- and second-trimester placental tissues. It was also present in ectopic trophoblast from tubal implantations. This study confirms that syncytin is expressed widely by a variety of normal human trophoblast populations, as well as choriocarcinoma cell lines.

Approximately 5% of the human genome is made up of retrovirus-like sequences (International Human Genome Sequencing Consortium, 2001). These human endogenous retroviruses (HERVs) arose from ancient germ-cell infections by exogenous retroviruses. During the course of evolution, most HERVs have accumulated mutations that have disrupted their protein-encoding potential. However, a small number of HERV genes have retained open reading frames (ORFs) and particular interest has been focused on the expression and functions of the corresponding products. From studies of the tissue distribution of various HERV transcripts, it has become apparent that the placenta is a preferential site of expression. It is the only normal tissue to have such a wide range and high level of HERV mRNAs (Taruscio & Mantovani, 1998; Muir *et al.*, 2004).

The human placenta is made up of finger-like chorionic villi that are covered by a double layer of fetally derived trophoblast cells: the outer fused syncytiotrophoblast is formed from the underlying mononuclear cytotrophoblast cells. These two layers together comprise the villous trophoblast. Some of the cytotrophoblast cells grow through the syncytial layer to form columns of extravillous trophoblast cells. These extend towards the uterine wall, which they ultimately contact and invade, when several different subpopulations may be distinguished: endovascular cells lie within the uterine arteries and interstitial cells migrate through the decidua (pregnancy-transformed endometrium).

These finally halt and form multinucleated, placental-bed giant cells (Loke & King, 1995; Moffett-King, 2002).

Placental expression of HERV-W *env* has attracted particular attention. HERV-W is a multi-copy family with an estimated 70 *gag*, 100 *pro* and 30 *env* genes in the genome (Voisset *et al.*, 2000). Although most of these sequences are defective, an ORF has been reported for an *env* gene, now known as syncytin, on chromosome 7 and for single *gag* and *pro* sequences on chromosomes 3 and 6, respectively (Blond *et al.*, 1999; Mi *et al.*, 2000; Voisset *et al.*, 2000). The putative Env product was predicted to have features typical of a retroviral envelope protein with surface and transmembrane portions, the latter containing regions with potential fusogenic and immunosuppressive activities (Blond *et al.*, 1999; de Parseval *et al.*, 2003). Interestingly, this gene appears to be expressed at high levels only in the placenta. Multiple-tissue Northern analysis detected substantial transcription only in the placenta, with weaker expression in the testis (Mi *et al.*, 2000). A later study using quantitative PCR confirmed the uniquely high placental expression and revealed lower levels of transcription in several normal tissues (de Parseval *et al.*, 2003). HERV-W *env* RNA was localized to the syncytiotrophoblast of term villi by *in situ* hybridization (Mi *et al.*, 2000). However, *in vitro*, isolated primary cytotrophoblasts from both early and term placentae expressed the transcripts (Frendo *et al.*, 2003). A recent study also demonstrated syncytin expression throughout gestation (Okahara *et al.*, 2004). The translation of a corresponding protein has now been confirmed. HERV-W Env was detected in a placental extract and in differentiating primary cytotrophoblast cells

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by Western blot (Voisset *et al.*, 2000; Frendo *et al.*, 2003). Immunohistochemical staining of villi with a range of gestational ages from first trimester to term has consistently shown preferential expression in the syncytiotrophoblast compared with the underlying cytotrophoblast (Blond *et al.*, 2000; Lee *et al.*, 2001; Frendo *et al.*, 2003; Smallwood *et al.*, 2003). However, the extravillous trophoblast populations have been little studied. There had been only a single report of syncytin expression in the cytotrophoblast cell columns (Smallwood *et al.*, 2003), but, since the submission of our manuscript, another report has appeared in the literature (Malassiné *et al.*, 2005). Our present study using RT-PCR and immunohistochemical techniques confirms the expression of syncytin in extravillous trophoblast.

We isolated first-trimester trophoblast cells from routine vaginal terminations of pregnancy as described previously (Loke *et al.*, 1997). Freshly purified cells consist primarily of villous trophoblast, defined phenotypically as HLA-G-negative (Chumbley *et al.*, 1994). After plating on 20 µg human plasma fibronectin ml⁻¹ (Strattech) for 72 h in Ham's F12 medium/20 % fetal calf serum, the cells have the phenotype of extravillous trophoblast: 80–90 % express the extravillous markers HLA-G, BC-1 and c-erbB2. Multi-nucleated, HLA-G-positive cells, which resemble placental-bed giant cells, also arise (Loke & Burland, 1988; Chumbley *et al.*, 1991; Loke *et al.*, 1992, 1997; Burrows *et al.*, 1993). In addition to isolated trophoblast cells, we also obtained decidual tissues from decidua parietalis that did not contain trophoblast cells. Endometrial tissues from the follicular and secretory phases of the menstrual cycle were obtained during gynaecological laparoscopy. Term placental tissues were taken after delivery of normal pregnancies. Peripheral blood leukocytes were derived from buffy-coat blood samples. RT-PCR was performed on the above tissues. PCR used *Taq* DNA polymerase (Qiagen). Reactions (50 µl) contained 2 µl template (water, RT-negative control or cDNA), 1 µM each primer (Sigma-Genosys) and 0.2 mM each dNTP (Roche). Novel HERV-W *env*-specific primers were designed to detect a 1253 bp segment of the ORF: HERV-W *env* F, 5'-TGATGGGGGTGGAGTTCAAG-3'; HERV-W *env* R, 5'-TTGGGGCGTAGTAGAGTTG-3'. The housekeeping gene *β-actin* was amplified in parallel to generate a 520 bp product, which both verified the success of cDNA synthesis and allowed comparison of relative expression levels between tissues. Fig. 1 shows the results of HERV-W *env* RT-PCR performed on a number of different cell and tissue samples, including cells with both villous and extravillous phenotypes. One representative lane is shown for each triplicate set tested, but results were consistent for all three samples. Term placental tissue was positive for the 1253 bp HERV-W *env* mRNA (lane 6b). In addition, pooled first-trimester trophoblast cells, both of villous (lane 3b) and extravillous (lane 4b) phenotype, contained syncytin transcripts. The choriocarcinoma cell lines JAR (lane 1b) and JEG-3 (lane 2b), which are used as models of villous and extravillous cells, respectively, were also positive. The trophoblast specificity of expression was confirmed by the

Fig. 1. RT-PCR products obtained by using primers specific for HERV-W *env* [lanes (a) and (b), 1253 bp] and *β-actin* [lanes (c), 520 bp]. For each cell or tissue type, there are three lanes: lane (a) shows the results of amplifying the RT-negative control template and lanes (b) and (c) contain products from the corresponding cDNA. The tissue and cell types were: (1) JAR choriocarcinoma cells; (2) JEG-3 choriocarcinoma cells; (3) freshly isolated first-trimester trophoblast (villous phenotype); (4) first-trimester trophoblast plated for 72 h on fibronectin (extravillous phenotype); (5) first-trimester decidua parietalis; (6) term placenta; (7) endometrium; (8) peripheral blood leukocytes.

absence of transcripts in other uterine tissues, both decidua parietalis (lane 5b) and non-pregnant endometrium (lane 7b). Peripheral blood leukocytes (lane 8b) provided a further negative control.

The localization of syncytin protein expression in the various trophoblast subpopulations was studied by the Vectastain ABC immunohistochemical technique (Vector Laboratories). Fig. 2 shows staining with a rabbit polyclonal antibody towards syncytin and corresponding negative-control sections. Syncytin expression was observed in first-trimester villous trophoblast as early as 6 weeks gestation (Fig. 2a). Villi from second-trimester samples also stained with the antiserum, but staining intensity became weak at term (not shown). The syncytial layer stained diffusely with increased intensity at the apical membrane. However, in all first- and second-trimester tissues examined, syncytin was not confined to the syncytiotrophoblast, but was also detected strongly in the underlying cytotrophoblast cells. In each case, expression was maintained in the implanting columns (Fig. 2a) and in all populations of extravillous cells within the decidua: the invading interstitial trophoblast cells, endovascular trophoblast (Fig. 2c) and placental-bed giant cells (Fig. 2e). Interestingly, ectopic trophoblast in tubal pregnancies also expressed syncytin in both villous and extravillous cells (Fig. 2g). In ectopic syncytiotrophoblast, the same diffuse cytoplasmic staining and apical enhancement were observed as were found in normal trophoblast.

Fig. 2. Immunohistochemistry of formalin-fixed tissues using a rabbit polyclonal anti-syncytin antiserum (left-hand column) and corresponding controls (right-hand column). The top three panels show first (a, b, c, d)- and second (e, f)-trimester tissues stained with the anti-syncytin antibody (a, c, e) and corresponding control sections stained with rabbit IgG (b, d) or anti-von Willebrand factor, an endothelial cell (EC) marker (f). Villous syncytiotrophoblast (ST) and cytotrophoblast (CT) were stained. The extravillous trophoblast populations were also labelled: cytotrophoblast columns (COL), interstitial trophoblast (IT), endovascular trophoblast (VT) and placental-bed giant cells (GC). The lowest panel depicts sections from an ectopic pregnancy within a Fallopian tube stained with the anti-syncytin antibody (g) and anti-von Willebrand factor (h). Bars, 200 μ m (a, b); 30 μ m (c, d); 100 μ m (e–h).

To verify that the anti-syncytin antibody used for immunohistochemical staining did indeed detect this protein, Western analysis was performed to compare protein lysates from freshly isolated pooled first-trimester trophoblast, 293T cells, which do not ordinarily transcribe syncytin, and 293T cells transiently transfected with a syncytin-expressing construct, P-I-S. Fig. 3 shows an immunoblot of untransfected 293T cells, 293T cells transiently transfected with the P-I-S construct and pooled, freshly isolated first-trimester trophoblast cells. An 80 kDa protein was detected in the P-I-S transfectants (Fig. 3b) and trophoblast cells (Fig. 3c). This was consistent with the size of the glycosylated HERV-W Env proteins detected by others in placental extracts, transfected cell lines and following *in vitro* transcription-translation (Blond *et al.*, 1999, 2000; Voisset *et al.*, 2000; Smallwood *et al.*, 2003). This shows one representative blot; a band of the same size was detected in three further trophoblast samples analysed.

Our observations confirm previous studies showing that syncytin is transcribed and translated specifically in trophoblast throughout gestation. Our trophoblast sample from a 6-week conceptus is the youngest studied to date. Our study also provides the first report of syncytin expression in ectopic trophoblast. Initially, syncytin expression was thought to be confined to the syncytiotrophoblast layer, but our findings, as well as those of others, have now shown that all of the other villous and extravillous trophoblast populations, including the placental-bed giant cells, also express this protein (Frendo *et al.*, 2003; Smallwood *et al.*, 2003; Malassiné *et al.*, 2005). This raises the question of the possible function of syncytin in these cells. There is much evidence to suggest that syncytin contributes to the cell-fusion events involved in generating syncytiotrophoblast (Blond *et al.*, 2000; Mi *et al.*, 2000; Frendo *et al.*, 2003; Pötgens *et al.*, 2004). The possibility that placental-bed giant cells might also arise by fusion is interesting, because

Fig. 3. Western blot of protein extracts from (a) untransfected 293T cells, (b) 293T cells transiently transfected with a syncytin-encoding vector and (c) first-trimester pooled freshly isolated trophoblast cells, stained with the anti-syncytin polyclonal antibody.

there is still debate about whether the mechanism involves fusion or endoreduplication (Al-Lamki *et al.*, 1999). Besides HERV-W *env* (syncytin), two other complete coding envelope genes are transcribed at high levels in the placenta: ERV3 *env* and HERV-FRD *env* (de Parseval *et al.*, 2003; Muir *et al.*, 2004). Interestingly, HERV-FRD *env* also has fusogenic properties, so it appears that there might be a second potential HERV mediator of fusion within the placenta (Blaise *et al.*, 2003).

There have been reports of spatially abnormal expression of syncytin in diseases of pregnancy such as pre-eclampsia, but it is unclear whether these observations reflect a cause or effect of the placental abnormalities (Lee *et al.*, 2001; Knerr *et al.*, 2002). This is further complicated by the fact that there is still much confusion regarding the subcellular distribution of syncytin in the layer of syncytiotrophoblast in normal pregnancy. Observations have varied between studies, even when using the same antibody. We have found diffuse cytoplasmic labelling of syncytiotrophoblast with enhancement at the apical membrane. This is in accord with the observation of Frendo *et al.* (2003), but contrasts with the findings of Lee *et al.* (2001), who showed syncytin to be located predominantly on the basal aspect of syncytiotrophoblast in normal pregnancies, but apically in pre-eclamptic placentae.

As our laboratory has a special focus on pregnancy immunology, we are particularly interested in a possible immunological function for syncytin in the uterus. The invading trophoblast cells are in close proximity to large numbers of maternal leukocytes at the implantation site, primarily uterine natural killer (NK) cells (Moffett-King, 2002). The nature and functional importance of trophoblast leukocyte interactions are poorly understood, but syncytin may have a

modulatory role via a region of amino acids that is highly conserved between the envelope proteins of many retroviruses, both endogenous and exogenous (Bénit *et al.*, 2001). CKS-17, a synthetic peptide derived from this sequence, has numerous immunological effects *in vitro*, for example influencing the cytokine production, proliferation, chemotaxis and killing activity of various immune-cell types (Cianciolo *et al.*, 1985; Nakagawa & Harrison, 1996). Interestingly, CKS-17 inhibited killing by peripheral-blood NK cells (Harris *et al.*, 1987).

The available data are presently insufficient to designate a definitive function for syncytin in the placenta. Whether the expression of this protein is vital to the success of pregnancy is also unknown. The fact that some mammals lack a syncytin homologue, yet undergo normal pregnancies, implies redundancy (Stoye & Coffin, 2000). However, our findings do suggest that, in addition to its probable contribution to cell fusion within the placenta, syncytin may have other important effects in the extravillous cells.

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