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Syncytins expression in cultured trophoblast cells according to differentiation status.

Research Article

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Abstract: Background: Data of syncytin 1 and 2 *env* gene expression in human placenta and participation in the syncytialisation phenomena has been reported. However, there are not many studies on simultaneous changes in expression of both syncytins in culture. We sought evidence on the relative expression of syncytins and syncytin 1 receptors in trophoblast cell culture treated with a differentiation inducing factor (forskolin). Methodology: Total RNA was isolated from normal karyotype specimens after chorionic villous sampling of a mean gestational week of 12^{+5} after 10 days of culture with or without the addition of forskolin. Real time quantitative PCR was used to measure the expression of syncytins and syncytin 1 receptors. Results: In cultures of trophoblast cells treated with the inducing factor we noted an inverse expression pattern of syncytins. When forskolin was added, syncytin 1 levels increased, while syncytin 2 levels decreased. Expression levels of syncytin 1 receptors remained unchanged. Conclusions: Our data provide evidence for the expression of syncytins in normal trophoblastic tissue from early placentas and advance the understanding of the physiological changes of syncytins upon differentiation towards syncytium.

Keywords: *ASCT1 • ASCT2 • Forskolin • Human placenta • Syncytin 1 • Syncytin 2 • Trophoblastic cultures*

1. Introduction

Endogenous retroviruses (ERVs) are the proviral form of exogenous retroviruses that have become integrated into the germ line of the host, most probably through infection mechanisms, and subsequently transmitted in a Mendelian manner. Human endogenous retroviruses (HERVs) entered the germ line of primates 25 to 40 million years ago [1] and comprise 8% of the human genome [2].

Among the various HERV families, two *env* genes, from the HERV- W and the HERV- FRD families, produce glycoproteins named syncytin 1 and 2, respectively, that possess fusiogenic capabilities when expressed in cells having the corresponding receptors [3].

Syncytin 1 is thought to be expressed mainly in the syncytiotrophoblast layer of villous trophoblasts [4-6] while others report that syncytin 1 is also expressed in the extravillous trophoblast, all along its differentiation pathway: cytotrophoblastic cells of the column, interstitial extravillous trophoblast (EVCT), multinucleated giant cells, and endovascular trophoblast [7]. On the other hand, syncytin 2 expression was found in villous cytotrophoblasts, but not in extravillous trophoblast. Moreover, the authors provide evidence of syncytin 2 localization not only in the membrane level, but also in the cytoplasm of cytotrophoblasts in proximity with the syncytiotrophoblast border [8].

Syncytin 1 is thought to interact with the D type mammalian retrovirus receptor (RDR, also known as neutral amino acid transporter ASCT2/*Slc1a5*, ATB0) [1,9] and also with the related ASCT1 (*Slc1a4*), while scarce data exist about the recently proposed syncytin 2 receptor [10]. ASCT2 has been shown to be expressed in the basal membrane of human placental syncytiotrophoblast [11] and hence co-localize with syncytin 1. This co-localization may facilitate fusion of cytotrophoblasts to syncytiotrophoblast. Similarly,

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studies about ASCT1 localization report its presence at the basal membrane of the syncytiotrophoblast layer of human placenta [12,13].

Most of these studies were performed on very short term cultures of trophoblast tissue obtained from term placentas, not abundant in cytotrophoblasts. To our knowledge, there are limited data from studies using mid-term cultures or trophoblast tissue from early placentae. Additionally, no data exist so far for any correlation between syncytin 1 and syncytin 2 expression.

In this study, syncytin 1 and syncytin 2 expression as well as the receptors ASCT1 and ASCT2 levels were investigated in cultured trophoblast tissue obtained from early placentas and seek for any changes in their expression after the addition of a differentiation-inducing substance, forskolin, in trophoblast cultures. Our results show interesting functional relationships between investigated genes with a possible use *in vivo*. Identification of the normal pattern of expression in trophoblast tissue and any changes upon differentiation from cytotrophoblasts to syncytiotrophoblasts could prove useful if sought in conditions affecting placental implantation and function, such as preeclampsia and intrauterine growth restriction (IUGR).

2. Experimental Procedures

Trophoblastic tissue was obtained from women undergoing chorionic villous sampling (CVS) for diagnosis of chromosomal abnormalities antenatally. Study was conducted with the approval of the Ethics Committee of the University Hospital of Thessaly. Informed consent was taken from every human subject that participated. Samples used for the experiments were those of a normal karyotype.

2.1 Trophoblast tissue culture

Trophoblastic tissue was thoroughly and repeatedly washed with PBS solution to remove any blood clots present. The washed pellet was incubated for 40 minutes in enzymatic dissociation solution containing 1 mg/ml collagenase (Type I, GIBCO) and 1 mg/ml trypsin (Trypsin⁄EDTA Solution, GIBCO). Subsequently, enzymatic solution was neutralized adding an excess volume of culture medium and cells were mechanically dissociated to a further extent. Afterwards, the cells were centrifuged at 1700 rpm for 7 minutes, twice. Supernatant was discarded and pellet was resuspended to Chang-D medium (T105, Irvine Scientific, USA) which is specially optimized for culturing chorionic villi samples (CVS) during prenatal care. Each sample was divided in half. Verification of a similar number of cells in each sub culture was performed. Afterwards, cells were transferred to 25 cm^2 flasks (Sarstedt, Germany) and cultured at 37°C in a humidified incubator with 5% CO_2 air atmosphere. Culture medium was changed at culture day 5. Cells for RNA extraction and flow cytometry were used after 10 days culture. By that day, the cells had reached confluence. Forskolin (a cAMP analogue commonly used to induce differentiation of cytotrophoblasts to syncytiotrophoblasts) at a final concentration of 20 μM [14] was added in randomly selected samples at culture day 6. A variety of forskolin concentrations has been used elsewhere, ranging from 10 to 100 μM [15-18] added at the beginning of culture but in our cultures optimum results were noted when using 20 μM, added at day 6, a concentration used in previous studies [19].

2.2 Biochemical assays - flow cytometry

In order to determine cell type population, an anti-cytokeratine antibody was used. Briefly, 50-100 μl of cells were incubated for 15 min with cytokeratin clone LP34 (Beckman Coulter, Miami USA) and CD45 was used as a reference marker [20-22]. For estimation of cytokeratin levels, DNA PrepReagent Kit (Bechman Coulter) and cytomics FC 500 Flow cytometer (Beckman Coulter) were used.

Additionally, β-hCG secretion was measured in the supernatant of the culture medium of several samples on different culture days. Briefly, 1 ml of the supernatant was removed from cultures and β-hCG levels were measured using the appropriate ELISA-kit (Elecsys and COBAS E analyzers).

2.3 Qualitative RT-PCR assessment of syncytin 1, 2 ASCT1 and ASCT2 mRNA expression

Total cellular RNA isolation, cDNA preparation (after DNAse treatment), and RT-PCR amplification of cDNA sequences corresponding to the syncytin 1, syncytin 2, ASCT1 and ASCT2 mRNA transcripts were performed as previously described [23]. Qualitative assessment of isolated RNA was carried out, both spectrophotometrically and *via* 2% agarose gel electrophoresis.

2.4 Complementary DNA (cDNA) synthesis

For quantitative purposes, volume equivalent to 1 μg of total RNA was diluted with random primers to a final volume of 13 μl and incubated at 65°C for 10 minutes. Cooling on ice for 5 minutes followed. Afterwards, samples were diluted with reagents mix (final volume: 20 μl) and amplification followed (PCR conditions: 25°C for 10 min, 42°C for 60 min and 99°C for 5 min). Products were stored at 4°C. Retinoic acid receptor alpha sequences (RARα) were used as a control in cDNA synthesis.

2.5 Quantitative, real-time RT-PCR assessment of syncytin 1, 2 ASCT1 and ASCT2 mRNA expression.

For quantitative PCR purposes, we developed and evaluated real-time fluorescence PCR assays for the Roche LightCycler (LC; Roche Diagnostics). Primers and probes for genes *syncytin 1*, *syncytin 2, ASCT1* and *ASCT2* were amplified with primers designed to span exons matching the corresponding gene. Primers and probes were synthesized by TIB Molbiol (Berlin, Germany). The adjacent ends of the hybridization probes were labelled with fluorophores. The 5' end of the first probe was labelled with the acceptor fluorophore LC Red 640 and the 3' end of the second probe with the donor fluorescein (FITC, 3FL). The 5'-labeled probes were 3'-phosphorylated to block polymerase extension during PCR. Sequences: for syncytin 1, forward primer sequence CCA ATG GAT GCC CTG and reverse CCA GGG GTC TGC GGT A. FL sequence TCC AGA ATC GAA GCT GTA AAA CTA CAA ATG and LC sequence GCC CAA GAT GCA GTC CAA GAC TAA GA. For syncytin 2, forward CAC TCT TCC AAG TAC AGT CTG TAA TG and reverse GAT TTA TCT AGC AAA GTT CCC TGA G. FL sequence AAC CAA CAG ACT TAC CAA ACA TAC ACC C and LC sequence CAA CCA ATT CCG CCA TCA ACC A. For ASCT1, forward primer sequence TGT TTG CTC TGG TGT TAG GAG T and reverse TTC CAA CAA GGA ACA TGA TGC were used. FL sequence CCG AAG GAG AAG ACC TCA TCC GT and LC sequence TCT TCA ATT CCC TCA ACG AGG CGA. ASCT2 forward sequence CCG CTT CTT CAA CTC CTT CAA T and reverse CCA GCA GGC AGC ACA GAA T. FL sequence TCA TGT TCC TGG TGG CTG GCA A and LC sequence ATC GTG GAG ATG GAG GAT GTG GGT TT. Real time PCR was performed using the *Light Cycler FastStart DNA Master Hybridization Probes* kit and Light Cycler from Roche. SYBR green detection method was used. Each sample was analyzed for both the gene probed and PBGD (Porphobilinogen Deaminase) as reference gene. Negative samples were used as standards in each panel of samples. For each experiment, a standard curve of the reference gene was also estimated using PBGD samples with constant number of replicates. The amplification mixture consisted of 2 µL of 10x reaction mix (LC-FastStart master hybridization probes; Roche Diagnostics), a 0.5 µM concentration of each oligonucleotide primer, a 0.15 µM concentration of each oligonucleotide probe, and 2 µL of template cDNA in a final volume of 20 µL. In a separate PCR reaction, the same cDNA was evaluated for expression of the PBGD gene. Samples were amplified as follows: an initial denaturation step at 95°C for 10 min to activate the FastStart Taq DNA polymerase, 45 cycles of denaturation at 95°C for 10 sec, annealing at 56°C for 10 sec, and extension at 72°C for 5 sec. Cooling step was set at 40°C for 30 sec. The temperature transition rate was 20°C/sec.

Results were calculated as the ratio of the number of each sample target- gene transcripts to the number of reference gene (PBGD) transcripts of the same sample.

2.6 Sequence analysis

Primers used were designed to span exons in order to avoid contamination with intronic DNA. Sequences were aligned against exons of the genes probed using Basic Local Alignment Tool (BLAST, http://blast.ncbi.nlm.nih.gov). Additionally, all PCR products sequences were verified using the Sanger method in an automated analyzer [Applied Biosystems ABI 3730 sequencer, version 1 "Big Dye" dye-terminator chemistry (ABI)] and proved to be the anticipated ones.

2.7 Statistical analysis

Statistical analysis was performed using SPSS v.15 statistical package for Windows. Kolmogorov-Smirnov normality test was used to study gestational ages distribution. To compare expression patterns between cultured samples T-test was used whenever normality test was passed, otherwise Mann-Whitney was the test of choice. Confidence interval was set at 95% (P=0.05)

3. Results

3.1 Samples statistical analysis

CVS procedure was performed at an average gestational age of 12^{+5} gw (gestational week) (n=77, median=12^{+4}, min=11^{+0} gw, max=14^{+1} gw, stdev=0.89, percentiles 25%=12^{+1} gw, 50%=12^{+4} gw, 75%=13^{+3} gw). Kolmogorov-Smirnov test was applied on samples gestational age and normality could not be excluded (Z=0.999, P>0.05).

3.2 Beta chorionic gonadotropin (β-hCG) production, cytokeratin and CD 45 expression.

As proved by serial measurements in the supernatant of the culture at different days, β-hCG declined rapidly during the first days of culture but then remained relatively steady for the rest of the culture period (Figure 1).

On samples examined for the presence of cytokeratin, fresh (untreated) samples proved to have an average percentage score of approximately 17%. Later during culture, cytokeratin levels increased and remained steady reaching 50% at the end of the 10 days

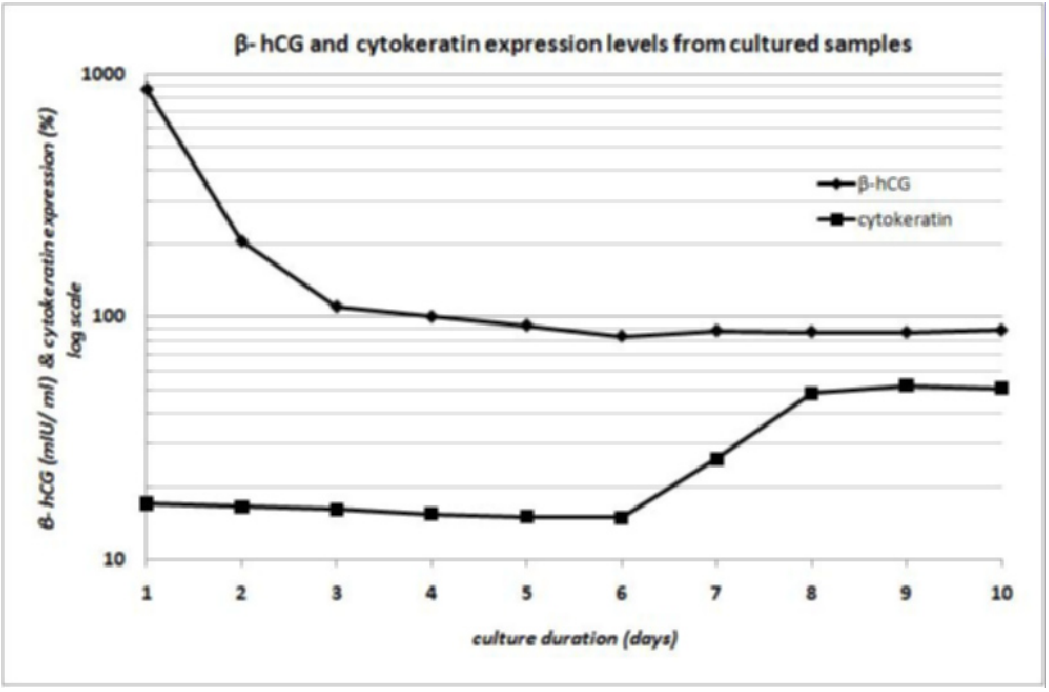

Figure 1. Mean values of β-hCG secretion and cytokeratin expression from trophoblast tissue cultures on different culture days. Both markers were used as evidence of cells differentiation in cultures were no forskolin was added to induce syncytialization. Y-axis in log scale and X-axis in days.

long culture (Figure 1). All samples were negative for CD 45 expression.

3.3 Quantitative PCR

PCR products were run on 3% agarose gels and the respective single bands matched the anticipated weight of molecules probed, thus verifying our results (data not shown). Statistical process was used to determine possible significant differences among the expression patterns of the biomolecules probed in cultured samples.

In order to evaluate the effect of forskolin (a factor known to promote syncytia formation) in trophoblast cultures, this substance was added in samples being cultured. Under these conditions, changes in the mRNA expression levels of the biomolecules probed (in relation to the level of the reference gene mRNA, PBGD) were noted. Specifically, syncytin 1 levels increased, while syncytin 2 levels decreased. For *syncytin* 1 there was a 2.6-fold increase (mean expression in samples without forskolin: 0.068, mean expression in samples with forskolin: 0.257, U=22, P=0.015). For *syncytin 2* there was a 4.7 fold decrease (mean expression in samples without forskolin: 0.173, mean expression in samples with forskolin: 0.037, U=34, P<0.05) (Figure 2). Statistical analysis failed to show any significant change in both *ASCT1* and *ASCT2* receptors following the addition of forskolin in the culture medium. For *ASCT1,*

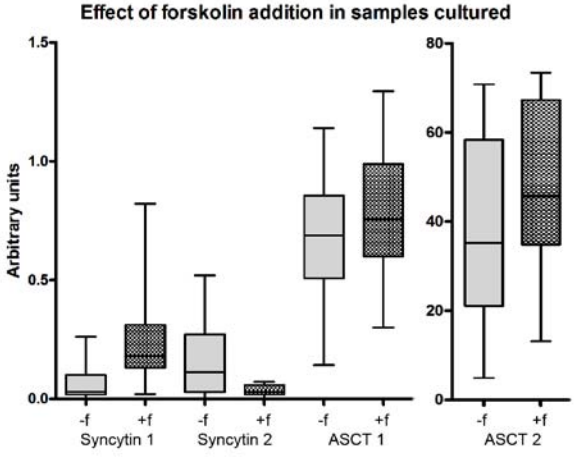

Figure 2. Expression of *syncytin 1, syncytin 2, ASCT1* and *ASCT2* mRNA at the end of 10 days long cultures with (+f) or without (-f) the addition of forskolin measured in arbitrary units. *Syncytin* 1 without forskolin: 0.068, with forskolin: 0.257, P<0.05. *Syncytin* 2 without forskolin: 0.173, with forskolin: 0.037, P<0.05). ASCT1 without forskolin: 0.066, with forskolin: 0.783, P>0.05. ASCT2 without forskolin: 37.42, with forskolin: 47.61, P>0.05.

mean expression in samples without forskolin was 0.066 while the mean expression in samples with forskolin was 0.783 (U=40, P>0.05). For *ASCT2,* mean expression in samples without forskolin was 37.42, while the mean

expression in samples with forskolin was 47.61 (U=28, P>0.05) (Figure 2).

4. Discussion

Most of the papers published so far about the topic studied in our research come either from cell lines or from very short term cultures of trophoblast tissue obtained from term placentas that are not abundant in cytotrophoblast cells. To our knowledge, no data from trophoblast cultures matching the gestational age nor the duration of our study have been published. Our specimens came from late first to early second trimester normal gestations using CVS samples. Similar studies in early pregnancies as well as the use of trophoblast tissue from CVS are few in the literature. Although Kliman's Percoll gradient protocol of purification is routinely used in trophoblast cell cultures, the amount of tissue collected using CVS was not enough for this protocol. Nevertheless, results obtained from our culture model are still comparable since cells population in each sub group was similar and directly compared to its counterpart. Additionally, data after simultaneous study of both the syncytin 1 and syncytin 2 biomolecules are very scarce. Similarly, there are no extensive data on the relative expression of those molecules and their changes under the effect of factors promoting cytotrophoblasts to syncytiotrophoblasts differentiation *in vitro* [24].

Beta chorionic gonadotropin (β-hCG) production is widely used as a marker of differentiation in trophoblast cells cultures and is thought to correlate positively with the differentiated syncytiotrophoblast presence [4,25-27]. Syncytiotrophoblast is considered to be the main site of β-hCG production and higher levels of this molecule are compatible with increased levels of syncytia in the cell population [4]. Levels of β-hCG declined during the first days of culture but remained relatively steady afterwards. Cytokeratin levels increased and remained relatively high throughout the culture period. Cytokeratin levels also correlated with syncytiotrophoblast levels with higher levels shown on cell populations rich with syncytia. The augmentation of cytokeratin levels further verified that trophoblast tissue was present. Additionally, cultures were inspected under microscope under direct vision or crystal violet staining and evidence of syncytialisation were noted and recorded (data not shown). Therefore, it is quite safe to speculate that trophoblast cells in the dish plate were viable, thus leading to a cell population comprised of a high cytotrophoblast/syncytiotrophoblast fraction. This is in agreement with published data from others showing that syncytiotrophoblast cells lack adherence to the flask well and thus are readily flushed away during flask washing or culture medium renewing while cytotrophoblasts can adhere to the dish plate surface [21].

When forskolin was added and cytotrophoblasts differentiated to syncytiotrophoblasts, *syncytin 1* levels increased and this finding is in complete accordance with studies in trophoblastic cells from term placentas being treated with forskolin [4]. Upon treatment with forskolin, a decline in levels of *syncytin 2* expression was noted, a pattern not yet, to our knowledge, being studied and published.

Known syncytin 1 receptors probed in our work have not been studied before. Syncytin 1 receptors followed a distinct expression pattern. In the presence of forskolin, the expression of syncytin 1 receptors did not show a statistically significant change (Figure 2). Similar data are available only for ASCT2 in BeWo cell line studies. In a single report, forskolin addition led to a decrease in their expression levels [28,15].

In an elegant review written by Potgens *et al.*, [29] the authors propose various models for syncytiotrophoblast replenishment from the underlying cytotrophoblast pool. In the most probable one, a theory was proposed where the relative equilibrium of syncytin 1 and its receptors plays a key role in the fusion process. Hypothetically, non-fusiogenic status is switched to fusiogenic when either of the signal (syncytin 1) or the receptor (mainly ASCT2; no conclusive data exist for ASCT1) is in excess. The factor in excess causes the counterpart to fuse. Thus, the authors [29] are introducing a feedback mechanism between cytotrophoblast and syncytiotrophoblast without either one holding the lead role in this process.

In this study, we showed, for the first time, that the presence of forskolin causes reverse changes in the expression levels of *syncytin 1* and syncytin 2 levels when studied together. This is compatible with evidence from authors who describe the presence of syncytin 2 solely to the cytotrophoblastic counterpart in close proximity to the syncytiotrophoblast layer [8]. It could be the result of depletion of the cytotrophoblast cells pool and the relative excess of syncytiotrophoblasts. Quantitative and functional analysis of syncytin 2 receptor could be the continuity of the work since no abundant or conclusive data exist upon its function [10] and protein specific antibodies are now commercially available. Finally, studies of the relative expression of the syncytins in abnormal placentation and myometrial invasion conditions and comparison to the normal expression pattern of these biomolecules could provide with useful information regarding the pathophysiology of placental dysfunction. Evidence of lower syncytins expression exist

in preeclampsia [30,31], HELLP [32] or IUGR [27] with no apparent effect on ASCT2 expression [33] but no studies have been conducted upon syncytin 2 receptor.

5. Conclusions

In conclusion, it is hard to fully elucidate the regulatory mechanisms behind the *in vivo* trophoblastic fusion process and replenishment of syncytiotrophoblasts from the underlying cytotrophoblastic pool. In this study, we provide evidence regarding the expression changes of syncytins when a fusiogenic agent is present *in vitro*. The exact role of each counterpart (syncytin 1, 2 and ASCT1, 2 receptors as well as the newly found syncytin 2 receptor) remains to be further investigated. Implications of syncytins expression in placenta dysfunction exist and are suggestive of a role in HELLP, preeclampsia and IUGR.

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