snRNAs are reduced in the syncytiotrophoblast: a possible mechanism for regulation of human placental protein production

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ABSTRACT: The multinucleate syncytiotrophoblast of the human placenta is formed by fusion of the underlying cytotrophoblast progenitor cells. The large surface area of the syncytiotrophoblast is necessary for transport functions while it also serves as the site of synthesis of hormones and steroids. Studies of syncytiotrophoblast transcription are puzzling, demonstrating that many of the nuclei in the multinucleated syncytium are transcriptionally inactive. To further elucidate RNA activity in the syncytiotrophoblast, we investigated expression of snRNAs involved in RNA splicing. Using RNA in situ hybridization, we observed that snRNAs were markedly reduced in the syncytium throughout the course of pregnancy. Recaptulating these results in primary trophoblasts and in trophoblast cell lines in vitro, we found, using qRT-PCR and RNA in situ hybridization, that snRNA expression is reduced in trophoblasts cultured under fusion conditions. Our finding that snRNA is markedly reduced in the syncytiotrophoblast suggests that the placenta has evolved a balance between the large surface area essential for its transport function and the need to regulate protein production in the multinucleated syncytium.

Key words: cell fusion / placenta / snRNA / syncytiotrophoblast / transcriptional regulation

Introduction

In a process that continues throughout pregnancy, the syncytiotrophoblast of the growing placenta is formed by fusion of the underlying progenitor cells, the cytotrophoblasts. Development of the multinucleate syncytiotrophoblast creates a barrier between maternal blood filling the intervillous space between the chorionic villi and that of the fetal vessels within. This terminally differentiated and continuous epithelial syncytium provides a large surface area of almost 12 m² at term for an efficient discharge of the respiratory and transport functions of the placenta (Boyd, 1970; Ellery et al., 2009). Among its many tasks the syncytiotrophoblast functions as the site of protein synthesis. Yet, studies of syncytiotrophoblast transcription are intriguing, demonstrating that most of the nuclei in the multinucleated syncytium are transcriptionally inactive. To further elucidate RNA activity in the syncytiotrophoblast, we investigated expression of snRNAs involved in RNA splicing. Using RNA in situ hybridization, we observed that snRNAs were markedly reduced in the syncytium throughout the course of pregnancy. Recaptulating these results in primary trophoblasts and in trophoblast cell lines in vitro, we found, using qRT-PCR and RNA in situ hybridization, that snRNA expression is reduced in trophoblasts cultured under fusion conditions. Our finding that snRNA is markedly reduced in the syncytiotrophoblast suggests that the placenta has evolved a balance between the large surface area essential for its transport function and the need to regulate protein production in the multinucleated syncytium.
ribonucleoprotein (snRNP) complexes. The complexes then reenter the nucleus to engage in splicing of the pre-mRNA. The U6 snRNA is highly conserved in terms of size, structure and sequence in evolution from yeast to mammals (Brow and Guthrie, 1988). The U2/U6 snRNP complex initiates the first catalytic step in splicing (Sun and Manley, 1995). Using an RNA in situ hybridization procedure adapted from miRNA techniques, we observed that snRNAs are mostly absent from the syncytiotrophoblast. This finding was also recapitulated in cell culture in vitro fusion of primary trophoblasts and trophoblast cell lines and observed in both RNA in situ hybridization as well as quantitative real-time PCR. Term placenta produces 250 mg or more of progesterone per day and 1 g of human placental lactogen per day (Simpson and MacDonald, 1981). Our finding that snRNA is down-regulated in the syncytiotrophoblast adds another dimension to the enigma of a transcriptionally down-regulated but translationally active syncytiot. Since transport and protein production are two major physiological roles of the syncytiotrophoblast, a molecular process had to evolve to develop the large placental surface area while not overwhelming protein production. Our observation of down-regulation of splicing and hence transcription raises the possibility that this is a method that the placental syncytiotrophoblast employs to regulate levels of protein transcription during pregnancy while maintaining a very large surface area to fulfill the needs of the fetus through transport of nutrients, wastes and gases.

Materials and Methods

Placental tissue

For isolation of first-trimester trophoblasts we used (Institutional Review Board approved) de-identified, pooled and waste placenta materials from first-trimester elective terminations of pregnancy (8–12 weeks gestation) with the explicit condition that DNA not be isolated from the samples. The archived paraffin placenta samples are de-identified. Ten placentas from normal pregnancies were examined: four from the first-trimester (gestational range 8–12 weeks) and six from the third-trimester (38–41 weeks of gestation, four vaginal deliveries and two Cesarean deliveries). A minimum of five slides were examined from each block.

snRNA in situ hybridization

Using a technique modified for use from miRNA in situ hybridization, U6 and U2 snRNA were detected using a double DIG labeled antisen probe, (U6 5′CACTGAATTGGCTGTCATCCTT, U2 5′ATATTTGCTCCTGAGTAA GAGGG) and as negative control a scrambled (5′GTGTAACACGCTTA TACGCCCC) LNA™ probe (Exiqon, Denmark) according to the manufacturer’s instructions with minor modifications. Following deparaffinization, slides were incubated with proteinase K (10 μg/ml, Sigma-Aldrich) for 20 min at 37°C. After RNA in situ hybridization and washings, slides were treated with peroxidase and incubated overnight at 4°C with Anti-Digoxigenin-POD (poly), FAB fragments, (Roche Diagnostics, Germany), 1:50 in a humidified chamber. The following morning the slides were washed (phosphate-buffered saline, PBS) and incubated at RT for 10 min in the dark with TSA™ Plus Cyanine 3 (Cy3) system (Tyramide Signal Amplification with Cyanine 3 fluorescence producing a red fluorescent signal, catalogue number NEL74401KT, PerkinElmer, Massachusetts). Slides were washed and mounted with DAPI mounting (UltraCruz™ Mounting Medium, sc-24941, Santa Cruz Biotechnology, CA).

Fluorescent microscopy was performed using an Olympus BX41, U-RFL-T microscope. Confocal microscopy was performed at the Bioimaging facility of Hebrew University. Imaging was performed using the FV-1000 confocal microscope (Olympus, Japan), equipped with an IX81 inverted microscope. A 60 x /1.4 oil immersion objective was used.

snRNA in situ co-detection with E-cadherin

E-cadherin co-detection with the snRNA was performed using a modified frozen section procedure. Briefly, slides were washed (in PBS) and fixed with 4% paraformaldehyde on ice for ten minutes. Slides were washed and treated with triton X-100, washed and de-acetylated. Probe incubation and detection were performed as for FFPE sections. After TSA-cy3 amplification the slides were blocked with CAS buffer (a universal blocking reagent, Zymed laboratories, CA) for 15 min and incubated for 3 h at room temperature with anti-human E-cadherin antibody, NorthernLights™ 493 conjugated goat polyclonal antibody, 1:20 (R&D systems, Minneapolis, MN). Slides were washed and mounted with DAPI mounting medium.

Tissue culture

Single-cell cytotrophoblast cultures were established from pooled first-trimester human placentas (8–12 weeks of gestation). First-trimester placentals cells from elective pregnancy terminations were studied in accordance with the protocol for the study of human subjects approved by our institutional board. Cytotrophoblasts were isolated and purified as described (Kliman et al., 1998) with some modifications of our laboratory (Shimonovitz et al., 1994; Hanna et al., 2006). Placental tissue was placed in ice-cold saline and processed within the hour. Briefly, the placental tissue was rinsed with PBS and the soft villous material was cut away from connective tissue and bloody tissue fragments. The villous tissue was incubated for 30 min at 37°C in Hank’s balanced salt solution (HBSS) containing 0.125% trypsin type XII-S (Sigma, St. Louis, MO), 3 mM EDTA and 0.2 mg/ml deoxyribonuclease I (Sigma, St. Louis, MO, USA). The dissociated cells were isolated by centrifugation, resuspended in medium and layered over a discontinuous Percoll (Pharmacia, Sweden) gradient (15–70%) prepared in HBSS. The middle bands of the gradient (35–55%), containing the cytotrophoblasts were collected and washed several times with culture media (DMEM:F12(HAM), 1:1) and 15% heat-inactivated fetal calf serum (FCS). The remaining leukocytes were removed as previously described by our laboratory (Shimonovitz et al., 1994). All tissue culture supplies were purchased from Biological Industries Inc. (Beit Haemek, Israel) unless otherwise specified. Using this method we isolate trophoblasts with cell purities of over 95% (Hanna et al., 2006). Our methods for validating purity of the isolated trophoblasts have been described (Hanna et al., 2006). All cell culture experiments were performed in a humidified incubator (Heraeus, Thermo Electron Corporation, Germany) at 37°C, 5% CO₂ in air.

Then 2 × 10⁵ isolated primary trophoblasts were plated on permenox Lab Tek chamber slides (Naige Nunc International, IL); cells and were maintained up to Day 4 in culture in DMEM:F12 + 15% FCS. In addition, trophoblast identity was ascertained using a monoclonal mouse anti-human antibody to cytokeratin 7 (mouse anti-human Cytokeratin 7, mouse IgG1 clone OV-TL 12/30 Dako, Denmark) on the plated cells.

JEG-3 cells (purchased from ATCC) were maintained in RPMI 1640 with 10% FCS. Cells were plated confluential for the fusion experiments at 5 × 10⁵ cells per well.

BeWo cells (purchased from ATCC) were cultured in F-12 (Hams) with 10% FCS. Cells were plated subconfluent for the fusion experiments at 1 × 10⁶ cells per well (Nunc, Naperville, IL, permenox four well, catalogue number 177445).

Cell culture reagents were purchased from Biological Industries (Beit Haemek). For fusion experiments, 24 h after cell plating the media was changed and forskolin (prepared in DMSO) with final concentration...
20 μM μg/ml (Sigma-Aldrich), was added, with only the DMSO carrier added to control wells.

For real-time PCR experiments, optimal cell seeding was found to be \(2.5 \times 10^5\) cells/well for BeWo cells and \(1.75 \times 10^5\) cells/well for JEG-3 cells, in a 12-well plate. All cell culture experiments were performed in duplicate wells for both forskolin treated and controls (DMSO only). Medium was removed for determination of βhCG concentration and RNA was isolated at time 0, 24, 48 and 72 h after the addition of forskolin. Cells were photographed at 72 h. The entire experiment was repeated three times. SD error bars are included. Fusion was evaluated with the formation of nuclear aggregates and the immunohistochemical observation of the disappearance of E-cadherin expression in fused cells.

βhCG was examined by immunoassay (Roche) on the Cobas E601 machine.

### Quantitative RT–PCR

RNA was isolated from the BeWo and JEG-3 forskolin and control time course cell culture experiments (all performed in duplicate wells) using the miRNeasy mini kit according to the manufacturer’s instructions (Quiaegen, Germany). RNA concentration was determined using the NanoDrop 2000 (Thermo-Scientific, Wilmington, DE). RNA (20 ng) was reversed transcribed using the mirCURY LNA Universal RT microRNA/PCR kit (Exiqon, Denmark) according to the manufacturer’s instructions with the PTC-200 machine (MJ Research). Real-time PCR was performed using SYBR Green detection with the Light Cycler (Roche-Applied Sciences, Germany) and U6 snRNA and 5S RNA primers (catalogue numbers 203907 and 203906, respectively, Exiqon, Denmark). Reactions were performed in duplicate with no-template control reactions. snRNA U6 expression was normalized to 5S.

### Figure 1

SnRNA in situ hybridization of normal placental sections. First-trimester (A–D) and third-trimester (E) placental sections. In the nuclei (DAPI, blue) snRNA expression (Cy3, red) is detected in cells other than syncytiotrophoblasts (open arrows). snRNA expression is indicated in the progenitor cytotrophoblasts (closed arrows). Trophoblast cell column (CC), sporadic clusters of snRNA expression in syncytial nuclei (+) and an area with blood (B) are indicated. Original magnification 200×, scale bar 100 μm (A, B, C and E); 400×, scale bar 50 μm (D). RNA in situ probes for U6 snRNA (A–C) and U2 snRNA (D and E).
The real-time PCR was performed twice, in two independent experiments. Each one included cDNA synthesized from two sets of cell cultures, for all time points analyzed, including BeWo, JEG-3 cells with forskolin and control.

Results

snRNA expression is reduced in syncytiotrophoblast of normal placenta

First-trimester, formalin-fixed paraffin-embedded (FFPE) placental sections (from four different placentas) were examined for snRNA expression (U2 and U6). In the chorionic villi, we observed snRNA expression in the nuclei of: stromal cells, cells of the fetal vasculature, cytotrophoblasts and in extravillous trophoblasts forming cell columns but not in the majority of the syncytiotrophoblast nuclei (Fig. 1A–E). We observed the same pattern of expression using U2 and U6 snRNA probes in situ and representative sections are shown for both probes (Fig. 1). Occasionally, we were able to observe several positive nuclei in the syncytium (Fig. 1B). Using the RNA in situ technique, we are unable to discern whether these positive cells represented recently fused nuclei that continue to retain the snRNAs or if they represented a subset of syncytiotroblasts where transcription persists post fusion. This latter possibility raises questions of how lateral control of transcriptional regulation of the nuclei within the syncytiotrophoblast occurs. In third-trimester placental sections (from six different placentas), similarly to first-trimester sections, the snRNAs were down-regulated in the syncytiotrophoblast (Fig. 1E) but were readily observed in the stromal cells of the chorionic villi and also in cytotrophoblasts. Furthermore, using confocal microscopy to view the villous structures more clearly in DIC, snRNA in situ hybridization was not observed in the syncytiotrophoblast of third-trimester placental sections (Fig. 1A) but was readily observed in villous stromal cells. A scrambled probe was included in each experiment (Fig. 2A) as a negative control.

snRNA is down-regulated in fusion of trophoblasts in cell culture

The cytotrophoblast progenitor cells fuse throughout pregnancy to form the syncytiotrophoblast. Hence we next performed cell fusion experiments in tissue culture. In an effort to recapitulate in vitro the observed down-regulation of snRNA expression in syncytiotrophoblast sections we characterized snRNA expression in trophoblast cell lines (choriocarcinoma and primary trophoblasts). JEG-3 cells do not fuse in vitro but BeWo cells will fuse upon treatment with forskolin (an activator of adenylate cyclase; Al-Nasiry et al., 2006; Fradet et al., 2012). Nuclear aggregation and dissociation of E-cadherin are used as indicators of cell fusion (Coutifaris et al., 1991; Al-Nasiry et al., 2006). In situ hybridization of JEG-3 cells with a U6 probe revealed expression of U6 snRNA in all nuclei examined with co-detection of the cell membrane using an E-cadherin antibody (Fig. 3A). In situ hybridization revealed distribution of snRNA with nucleolar sparing (Fig. 3A). Of particular interest is that the snRNA appears in a speckled pattern reminiscent of that previously described in the immunofluorescence of proteins of the snRNP complex (Fig. 3A) (Spector and Lamond, 2011). This speckled snRNA pattern of the interchromatin region of the nucleoplasm, the interchromatin granule clusters, are storage sites of splicing factors. The distribution of the snRNA in structures of mitotic nuclei was also observed (Fig. 3B), with a striking appearance as the snRNA appears excluded from the chromatin. Furthermore, we observed snRNA in the focal plane of the cytoplasm, which is most likely indicative of the snRNA in the process of either exiting or entering the cytoplasm and forming the snRNP complex for transport to the nucleus (Fig. 3C).

Using confocal microscopy of U6 snRNA in situ hybridization in first-trimester placental sections, we observed a positive signal in the cytotrophoblasts in a punctate pattern with down-regulation of snRNA in the syncytiotrophoblast. However, the resolution is less clear than in the cell culture, most likely because of the thickness of the sections (Fig. 4).

Figure 2 Confocal microscopy of third-trimester placental section snRNA and scrambled probes. Confocal microscopy of snRNA expression in a third-trimester placental section (A). U6 snRNA expression (Cy3, red) is observed in villous stromal cells but not in the syncytiotrophoblasts. Negative results are shown with a scrambled probe (B). Scale bar 50 μm. Arrowheads syncytial nuclei negative for snRNA expression.
Figure 3  Co-detection of snRNA and E-Cadherin in JEG-3 cells. Confocal microscopy of snRNA and E-cadherin co-detection in JEG-3 cells. In row A snRNA is detected in the nucleus with nucleolar sparing (open arrowhead). In row B mitotic cells (carat) are visible in another microscopic field. In row C, snRNA is observed as the confocal approaches the cytoplasmic focal plane (closed arrow). Scale bar 10 μm.

Figure 4  High magnification and confocal microscopy distribution of snRNA in first-trimester placental section. First-trimester placental section RNA in situ hybridization with a U6 snRNA probe (A) original magnification 200 x. An area of a chorionic villus with both cytotrophoblasts and syncytiotrophoblasts is boxed in white. (B) Confocal microscopy of the boxed area in (A) (original magnification 600 x) with cytotrophoblasts positive for snRNA expression (white arrow) and negative or dimly visible for snRNA in the syncytiotrophoblast (hollow arrow). Nuclei are stained blue with DAPI and snRNA appears red (cy3) and in the merged image the snRNA co-localizing with the DAPI stain appears pink.
Using the co-detection technique (snRNA and E-cadherin) for BeWo cells grown in cell culture, we observed, as in JEG-3 cells, snRNA expression in all nuclei (Fig. 5A). However, under fusion conditions, snRNA nuclear staining was no longer uniform (Fig. 5B): some nuclei were brightly and others dimly stained. The fused cell nuclei appeared to aggregate, and, as expected, the E-cadherin staining pattern disappeared or became indistinct, cloudy and disorganized (Fig. 5B), while the snRNA signal was dim or below our level of detection. Within the same microscopic field (Fig. 5B) unfused cells were also observed and these have maintained snRNA and E-cadherin expression. Expanding these experiments to primary culture of cytotrophoblasts, which spontaneously fuse when cultured in vitro, we found

**Figure 5** snRNA down-regulation under fusion conditions in vitro. Fluorescent microscopy of BeWo cells (A and B) and primary trophoblasts (C) taken at 72 h in culture. (A) BeWo cells cultured without forskolin; snRNA is observed in all nuclei and E-cadherin membranes are clearly visible. Down-regulation of snRNA, dissolution of E-cadherin and aggregation of nuclei (open arrow) are observed in fused BeWo and primary trophoblast cells (B and C). In (B), an area of positive snRNA expression in unfused cells with distinct E-cadherin staining of cell membrane (closed arrow) is also observed. βhCG levels in BeWo (D) and JEG-3 cultured (E) with and without forskolin (DMSO carrier only). The conditioned media was collected at 0, 24, 48 and 72 h. This experiment was repeated three times. (F) Real-time PCR of U6 snRNA normalized to 5S RNA of BeWo and JEG-3 cells grown in culture and harvested 0, 24 or 48 h after the addition of forskolin when RNA was extracted. The real-time PCR was performed twice, in two independent experiments. Each one included cDNA synthesized from two sets of cell cultures, for all time points analyzed, including BeWo, JEG-3 cells with forskolin and control.
that the snRNA was also dimly visible or below our level of detection in the fused cells (Fig. 5C).

**Real-time PCR detection of snRNA down-regulation**

In an effort to quantify the down-regulation of snRNA in the cell culture experiments, BeWo and JEG-3 cells were grown under fusion and non-fusion conditions. βhCG levels in the conditioned media of both JEG-3 and BeWo cells cultured with forskolin rise precipitously over a 3-day period, with levels in the JEG-3 cells almost 4-fold higher than those observed in the BeWo cells (Fig. 5D and E). βhCG levels were similar at 48 h and 72 h in the forskolin-treated BeWo cells but continued to rise in the forskolin-treated JEG-3 cells (Fig. 5D and E). As reported by others, JEG-3 cells produce βhCG under forskolin conditions although they do not fuse. This has led researchers to conclude that not all morphological changes (fusion in the choriocarcinoma cells) go hand in hand with biochemical changes in protein expression, in this case βhCG (Al-Nasiry et al., 2006; Orendi et al., 2010). Real-time PCR was performed to quantify U6 expression in cells grown under fusion conditions in culture. In JEG-3 cells treated with forskolin, U6 snRNA expression remained fairly steady over a 48-h period, whereas in BeWo cells cultured under fusion conditions, U6 snRNA decreased by ~3-fold (Fig. 5F). Thus, while continuing to produce βhCG, the BeWo cells down-regulated U6 expression.

**Discussion**

Using RNA in situ hybridization, we observed that snRNAs (U2 and U6) are markedly reduced in the syncytiotrophoblast of the human placenta. Recapitulating these results in primary trophoblasts and in trophoblast cell lines in vitro using qRT-PCR and RNA in situ hybridization, we found that snRNA expression is reduced in trophoblasts cultured under fusion conditions. We interpret these results as suggesting that, while the placenta has evolved a large surface area to provide nutrients, gas exchange, and waste removal and to act as a partial barrier to nurture and protect the developing fetus, a mechanism evolved to control protein production in the multinucleate syncytiotrophoblast. Our snRNA data, supported by previous reports of transcriptional down-regulation, suggest that one such mechanism of protein regulation is transcriptional down-regulation in the syncytiotrophoblast (Huppertz et al., 1998; Ellery et al., 2009; Huppertz, 2010). The extent of the reported syncytiotrophoblast with transcriptional reduction is most likely a reflection of the techniques employed. When immunohistochemistry and immunofluorescence combined with the disector were used, active pol II was observed in only 20–40% of total nuclei (Fogarty et al., 2011). Overall we observed a greater transcriptional reduction in syncytial nuclei than Fogarty et al. However, Fogarty et al. used immunohistochemistry and immunofluorescence (for protein detection), while we used RNA in situ hybridization (for detection of splicing snRNAs). It is very likely that the half-lives (protein or snRNA) may differ. Furthermore, down-regulation may be staggered, with splicing down-regulated before active transcription. Neither we nor the Fogarty group measured transcription directly as we both analyzed placental sections. In addition, we performed our experiments on individual sections that do not reflect the dramatic gestational increase in placental volume.

In some ways the difference between our findings reflects a difference in interpretation. Fogarty et al. interpreted the finding of transcription factors in nuclei as evidence for transcriptional activity in the syncytiotrophoblast and as a basis for explaining the fact that the syncytiotrophoblast is actively producing proteins. Conversely, we view the biologically unusual finding of lack of transcription in many syncytial nuclei as a clue to an idiosyncratic evolutionary balance between the need for large surface area versus down-regulated protein production. The biological mechanism for this down-regulation is an area ripe for further research, particularly surrounding the questions of lateral control and the positional effect of transcriptional activity in the multinucleate syncytiotrophoblast.

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**Authors’ roles**

D.G.-W., C.G. and G.S. performed the experiments. D.G.-W., C.G., S.Y. and I.A. interpreted the results. I.A. analyzed the results for appropriate pathology. D.G.-W., C.G., R.H.-K. and S.Y. designed the experiments and contributed substantially to conception of the experiments. TI directed the real-time PCR experiment and I.E.-L. performed the analysis of those results. D.G.-W. wrote the manuscript and all authors approved the final version of the manuscript. None of the authors report a conflict of interest.

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Conflict of interest

All authors declare no conflict of interest.

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