Transcription factor AP2 regulates human inhibin α subunit gene expression during in vitro trophoblast differentiation

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Submitted on November 25, 2010; resubmitted on May 18, 2011; accepted on May 30, 2011

ABSTRACT: During human trophoblast differentiation, inhibin α subunit mRNA expression and protein secretion are increased. To understand how inhibin α subunit gene was regulated during syncytialization, we firstly cloned the inhibin α promoter and found a region with transcriptional activity related to the differentiation state. In this paper, we identified this protein and defined its DNA-binding site. Protein purification and identification were done by DNA affinity chromatography followed by mass spectrometry and western blotting. In order to confirm the identity of the protein, characterize its DNA-binding properties and to measure its expression during in vitro trophoblast differentiation, gel retardation assays and real-time polymerase chain reaction were done. We found that the cytotrophoblastic protein interacting with the inhibin α promoter was the transcription factor activating protein 2 (AP2). Western blotting using specific antibodies against AP2α and AP2γ confirmed that AP2α was the main subtype present in trophoblast cells, while AP2γ was barely detectable. Supershift experiments indicated that these two factors were able to bind to the sequence 5′-GCCctcAGC-3′. We also observed an increase in AP2α mRNA and protein during in vitro trophoblast differentiation correlated with an increase in inhibin α subunit gene expression. Furthermore, AP2α and AP2γ overexpression in these cells was responsible for an increase in inhibin α subunit mRNA expression. We conclude that AP2 regulates the inhibin α subunit gene expression during trophoblast differentiation and may be a key regulator of syncytialization.

Key words: inhibin A / trophoblast differentiation / AP2 / syncytiotrophoblast / placenta

Introduction

Among transforming growth factor (TGF) β superfamily, inhibin and activin are important factors for successful implantation of the blastocyst into the endometrium and placental vascularization (Jones et al., 2006). These glycoproteins originally isolated from gonadal tissue are responsible for the regulation of the pituitary follicle-stimulating hormone secretion (Ying, 1988). Inhibin and activin are a combination of three related protein subunits (α, βA and βB) encoded by separate genes. Activin is a dimer of two β subunits, while inhibin is formed of one α subunit disulfide linked to one of the two β subunits.

During pregnancy, the placenta is the main source of inhibin A and activin A (Muttukrishna et al., 1997; Florio et al., 2001). Maternal serum concentrations increase rapidly during pregnancy up to 36 weeks’ gestation (Fowler et al., 1998). In pregnancies associated with Down’s syndrome or complicated by pre-eclampsia, inhibin A concentration in maternal serum is significantly increased (Dalgliesh et al., 2001). To the contrary, low inhibin A levels in early pregnancy are associated with subsequent spontaneous abortion risk (Muttukrishna et al., 2004). Thus, inhibin A secretion is related to pregnancy complications due to placental disease. The mechanisms leading to inhibin concentration changes in pathological situations are unclear and a better knowledge of inhibin α gene regulation could help us to understand these modifications and physiopathological process.

Placental development starts just after implantation of the blastocyst into the endometrium (Depoix and Taylor, 2010). Cytotrophoblastic cells proliferate extensively and invade the endometrium. They differentiate into hormone-secreting syncytiotrophoblastic cells which are polynucleated cells covering the villi. They are in direct contact with the maternal blood through the intervillous space. In vitro isolated cytотrophoblastic cells cultures differentiate spontaneously into syncytiotrophoblastic cells. During this fusion process, inhibin α subunit mRNA expression is correlated with increased inhibin A protein secretion into the cells supernatant (Debieve et al., 2000). For a better understanding of the regulation of inhibin A expression during syncytialization, our laboratory cloned the inhibin α subunit gene promoter. We found
by DNase protection assay that a sequence of the promoter was bound by a protein when cells were not yet undergoing differentiation (Debieve and Thomas, 2002). Retardation assays using nuclear extracts prepared from isolated trophoblastic cells before culture and after 3 days in culture incubated with a probe corresponding to the protected fragment of the inhibin α promoter showed that the protein ability to interact with DNA was dependent on the differentiation state of the cells. Transfection assays with a reporter gene under control of the inhibin α promoter confirmed the importance of the sequence in the regulation of the inhibin expression in trophoblastic cells. Nevertheless this transcription factor was not identified.

The aims of this study based on human placental cells cultured in vitro are (i) to identify the transcription factor interacting with the inhibin α gene promoter, (ii) to characterize the DNA sequence responsible for this interaction, (iii) to confirm the effect of this factor on endogenous inhibin α subunit gene expression and (iv) to investigate a possible relationship between the expression of this factor and syncytiotrophoblastic transformation.

Materials and Methods

Cytotrophoblastic cell isolation and purification

Isolated cytotrophoblastic cells were obtained from term placenta collected from uncomplicated pregnancies immediately after elective Cesarean section or vaginal delivery as previously described (Debieve et al., 2000). All tissues were collected with the approval of the ethical committee at Université Catholique de Louvain. Briefly, isolation and purification consisted of digestion of placentae tissues by dispase II followed by digestion of extracellular DNA by DNase I. After serial filtrations followed by density centrifugation through a 5–70% gradient Percoll (Sigma-Aldrich, Saint-Louis, MO, USA), the cellular fraction corresponding to the binding reactions and incubated for 15 min at room temperature. Protein–DNA–MACS™ beads were then loaded onto a 4-μm column placed in a strong magnet. Washing and elution were done following the manufacturer protocol. The beads were washed at least four times with a low stringency buffer then four times with a high stringency buffer. Proteins interacting with DNA were eluted with either 50 μl elution buffer containing 1 M NaCl, 10 mM Tris–HCl pH 7.5 and 0.5 mM EDTA and immediately sent for MS analysis or with 250 μl MACSTM native solution (high salt concentration) for further western blotting analysis. Proteins were precipitated for 1 h on ice with 10% trichloroacetic acid (TCA). After 1 h centrifugation at 15 000 g, the pellet was washed with 500 μl cold acetone overnight at −20°C. After 30 min centrifugation at 15 000 g, the pellet was air-dried and redissolved in 20 μl of 0.1 M NaOH.

Gel electrophoresis and Western blotting

Fifteen micrograms of nuclear extract and TCA-precipitated proteins from μMACS™ elution were loaded onto a 4–12% gradient acrylamide/bisacrylamide NuPage® Novex® Bis-Tris minigel (Invitrogen, Carlsbad, CA, USA) and separated in 4 × 12% NuPage® MES SDS running buffer for 35 min at 200 V. Proteins were then transferred onto a nitrocellulose membrane (Amersham Hybond™-ECL™, GE Healthcare UK limited, Buckinghamshire, UK) 1 h at 100 V. After complete transfer, the membrane was blocked for 1 h in 5% non-fat dry milk in 1 × PBS-T (50 mM Na/HPO4, 150 mM NaCl and 0.05% Tween-20) followed by overnight incubation at 4°C with a mouse anti-activating protein 2 (AP2) or anti-AP2γ monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted at 1/200 in 5% non-fat dry milk in 1 × PBS-T. The membrane was washed three times with 1 × PBS-T for 15 min each and then incubated for 1 h at room temperature with a 1/40 000 dilution of the anti-mouse Horseradish Peroxidase-conjugated antibody (Santa Cruz Biotechnology, Inc.) and bound antibody was detected using the ECL detection system (Amersham, GE Healthcare, Buckinghamshire, UK).

Cell culture and transfections

Isolated cytotrophoblastic cells from three different placentae were cultured in 35 mm Primaria tissue culture dish (Falcon, Beckton Dickinson Labware, Franklin Lakes, NJ, USA) in duplicate at a density of 4.10⁶ viable

<table>
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<th>Oligo</th>
<th>5′ modification</th>
<th>Sequence (5′–3′)</th>
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<tr>
<td>S</td>
<td>dR-Biotine</td>
<td>ATATCAAGATCTGGCCAAAGGATTTCGCCTCAAGCGACCTGACTCACACATG pGL4b 1–16 INH</td>
</tr>
<tr>
<td>NS</td>
<td>dR-Biotine</td>
<td>ATATCAAGATCTGGCCGCGCCGAAGCTTGGCAATCCGGTACTGTTGGT pGL4b 1–51</td>
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cells. After 12 h, the medium was changed and cells cultured for 4 days. The medium was changed every day. Each day, two dishes were used for total RNA extraction and purification.

For transfection experiments, isolated cytotrophoblastic cells were cultured on 6-well plates at a density of 4.10^6 viable cells per well. Immediately after plating, cells in suspension were transfected with 500 ng per well of the following expression vectors: pCDNA3.1(+) (Invitrogen, Carlsbad, CA, USA), AP2α/pCDNA3.1(+) or AP2γ/pCDNA3.1(+) using FuGENE®6 (Roche Life Sciences, Mannheim, Germany) at a 1:3 ratio. AP2 expression vectors were kindly provided by Dr Ronald Weigel, University of Iowa, Iowa City, IA, USA. Cells were allowed to attach for 12 h and the medium was replaced. Attached cells were then transfected again with the same conditions. Forty-eight hours after the second transfection, cells were harvested for total RNA extraction and purification. All transfections were done in duplicate.

**Electrophoretic mobility shift assay**

Five micromolars of nuclear proteins was incubated in 10 μl containing 10 mM Tris–HCl pH 7.5, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl and 4% glycerol for 10 min on ice. For competition experiments, 100-fold excess unlabeled probe was added to the binding reaction. For supershift experiments, 0.4 μg anti-AP2 antibodies were added to the reaction mixture. After incubation at 4°C for 1 h, 25 fmol biotinylated probes were added and the reactions were kept at 4°C for another 1 h (see Table II for list of oligos used).

One microliter of 10× loading buffer (250 mM Tris–HCl pH 7.5, 0.2% bromophenol blue, 40% glycerol) was added before loading. The DNA–protein complexes were then separated at 4°C in a 4% acrylamide/ bisacrylamide (BioRad, Hercules, CA, USA) and 0.5× TBE (50 mM NaCl, 45 mM borate and 0.5 mM EDTA pH 8.3) running buffer.

Free DNA and DNA–protein complexes were transferred onto a Byodine® B 0.45 μm modified nylon membrane (ThermoScientific-Pierce, Rockford, IL, USA) in 0.5× TBE buffer at 350 V for 40 min and then UV cross linked. Complexes were visualized by chemiluminescence (Chemiluminescent Nucleic Acid Detection Module, Pierce).

**RNA extraction**

Total RNA was extracted from trophoblasts using the PureLink Micro-to-Midi total RNA Purification System (Invitrogen, Carlsbad, CA, USA) and quantified by absorbance at 260 nm in a NanoDrop™ 1000 spectrophotometer (Thermo-Fisher Scientific, Wilmington, DE, USA).

**Reverse transcription and real-time polymerase chain reaction**

Total RNA (0.5 μg) was used as template for reverse transcription in 20 μl reaction volume, using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA, USA) then diluted five times with RNase-free water.

Two microliters of cDNA and 0.6 μM specific primers for human inhibin α subunit (forward: CAA-GTA-TGA-GAC-AGT-GCC-C; reverse: GCC-ATC-TAT-TTC-CCA-ACT-CTG), AP2α (forward: TGT-ACC-CTG-CTC-ACA-CTA-G; reverse: TCT-TGT-CAC-TTG-CTC-ATT-GG), AP2γ (forward: CGG-GAG-AAG-ATT-GC; reverse: ATT-CGG-CTT-CAC-AGA-CAT-AGG) and RNA polymerase II (RPII) (forward: GCA-CCA-CGT-CTT-AAT-ACA-T; reverse: GTG-CCG-CTT-CAC-CTA) were added to SYBR Green Master Mix (Applied Biosystem). The PCR amplification was performed on a StepOne™ Realtime PCR system (Applied Biosystem) using the following thermal protocol: incubation at 95°C for 10 min followed by 40 cycles of a denaturation step at 95°C for 30 s, a hybridization step at 61°C for 60 s and signal reading.

PCR reactions were performed in duplicate for each cDNA, averaged and normalized to RPII reference transcripts. The specificity of the amplification was determined by doing a melting curve (60–95°C, read every 0.3°C and hold 10 s) which generated single-peak product for each transcript.

RNA was quantified using the ΔΔCt method of relative quantification using a Microsoft® Excel® Macro spreadsheet designed for gene expression analysis for iCycler iQ® Real-Time PCR Detection System (Bio-Rad).

**Statistical analysis**

Each experiment was done in triplicates, with the results normalized to control conditions for statistical analyses. The data are expressed as mean ± standard error and were compared using Kruskall–Wallis test (GraphPad Prism® version 5.03 for Windows, GraphPad Software, San Diego, CA, USA). Two-tailed probabilities <0.05 were considered statistically significant.

**Results**

In order to identify the transcription factor interacting with the inhibin α gene promoter, we used the DNA-binding properties of this transcription factor in electromobility shift assay (EMSA)-binding conditions applied to DNA affinity chromatography with

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**Table II List of oligonucleotide probes tested in electrophoretic mobility shift assay (underlined: transcription factor binding sites; lower case: mutated nucleotides).**

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<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
<th>References</th>
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<tr>
<td>INH</td>
<td>AAGGATTTCGCCTCAAGCGACCTGACTCAAGCTACAGAGT</td>
<td>Williams et al.</td>
</tr>
<tr>
<td>mLINH</td>
<td>GATTTCATTCAAGGACCCT</td>
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<tr>
<td>csAP2</td>
<td>GATCGAAGTGCACCGGCACCGGCCGCT</td>
<td>Remacle et al.</td>
</tr>
<tr>
<td>mAP2</td>
<td>GATCGAAGTGCACCGGCACCGGCCGCT</td>
<td></td>
</tr>
<tr>
<td>E-cad</td>
<td>TGGCCGGCGAGGTGAAGACCTGACGGCGATCGGCGCGCT</td>
<td></td>
</tr>
<tr>
<td>Ecad1</td>
<td>TGGCCGGCGAGGTGAAGACCTGACGGCGATCGGCGCGCT</td>
<td></td>
</tr>
<tr>
<td>Ecad2</td>
<td>TGGCCGGCGAGGTGAAGACCTGACGGCGATCGGCGCGCT</td>
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streptavidin-coated magnetic μMACS® beads. This procedure was scaled up to allow purification of enough proteins to be analyzed by MS. Nuclear protein extracts from isolated cytотrophoblastic cells were incubated with biotinylated double-stranded oligonucleotides corresponding to either the −219 bp to −185 inhibit α-promoter fragment (S) or to a non-specific unrelated oligonucleotide (NS) corresponding to the multiple cloning site of pGL4 basic vector (Promega) (Table I). Streptavidin-coated magnetic μMACS® beads were added to the binding reaction and the mixtures were loaded onto a μcolumn placed into a strong magnetic field. Unbound proteins were removed by sequential washes with increasing ionic strength. Finally, bound proteins were eluted in their native state with a high salt solution. The eluted fraction was then analyzed by MS. Fine analysis of the spectra showed that there were two minor peaks corresponding to peptides related to the human transcription factor AP2, isoform alpha (data not shown). Sequences of the identified peptides responding to peptides related to the human transcription factor AP2, sis of the spectra showed that there were two minor peaks corresponding to peptides related to the human transcription factor AP2. We confirmed by western blotting using monoclonal antibodies raised against AP2α and AP2γ that these factors were interacting with the inhibit α promoter fragment. The factor purification was done according to the previously described protocol. Eluted proteins from each binding condition were TCA concentrated and loaded onto a denaturing polyacrylamide gel followed by western blotting (Fig. 1). Cytotrophoblastic nuclear proteins were loaded onto lane 1 (NE) to confirm the presence of the factors in these cells (Fig. 1). Lane 2 corresponds to the non-specific interaction (NS) and lane 3 corresponds to purification using the specific inhibit α promoter oligonucleotide (S). A band was detected at ~52 kDa corresponding to the expected AP2 molecular weight. Cytotrophoblastic cells essentially expressed AP2α and very little AP2γ. No AP2 factor was detected in the non-specific eluate. In the specific lane (S), only purified AP2α was detected. This confirms the MS results and the ability of this factor to interact in solution with the inhibit α promoter.

To validate our hypothesis, we visualized the protein–DNA interaction by EMSA. Cytotrophoblastic nuclear protein extracts were incubated with the inhibit α-promoter oligonucleotide (Table II). A very strong protein–DNA complex was formed and 100-fold excess of wild-type competitor (WT INH) completely removed the binding confirming the specificity of the interaction (Fig. 2). Competition with an oligonucleotide containing a consensus sequence for AP2 (csAP2) strongly diminished the binding. To confirm that the protein recognized the AP2-binding site, we mutated the AP2 consensus sequence (mAP2). This oligonucleotide lost its competition ability, confirming that the protein interacting with the inhibit α promoter fragment was AP2. To define which AP2 protein interacted with the inhibit α promoter fragment, we added either anti-AP2α or anti-AP2γ antibodies to the binding reactions. A supershift was formed with both antibodies indicating that the two subtypes interacted and bound in solution to our oligonucleotide. In respect of the previous western blotting results, we got a stronger supershift with the anti-AP2α antibody than with the anti-AP2γ antibody.

All members of the AP2 family recognize and bind to the same DNA sequence with the same affinity as homo- or heterodimer. The consensus AP2-binding site is 5’-GCGN3GCC-3’ with N corresponding to any nucleotide (Williams and Tjian, 1991). Using this palindromic core sequence, we analyzed the inhibit α promoter fragment but could not find any homology. A study on the comparison of AP2-binding site specificity for AP2α and AP2γ determined that the
consensus sequence for both factors was 5′-GCCCGaAGC/CTCCGT/ C−3′ (McPherson and Weigel, 1999). Using this consensus sequence as a template, we found a putative AP2-binding site 5′-GCTCCAAGC-3′ from −194 to −202. To verify that this sequence was effectively an AP2-binding site, we mutated the first three nucleotides of the core sequence and used this oligonucleotide in competitive gel shift assay (Fig. 3). This mutant oligonucleotide completely lost its ability to compete.

Among the other possible transcription factors investigated by Debieve and Thomas in 2002, there was δEF1, a zinc finger protein known to bind to the ACCT sequence (Remacle et al., 1999). While a competitor oligonucleotide derived from E-cadherin promoter containing a δEF1-binding site proved to be effective in suppressing the protein–DNA complex formation, the use of another δEF1-binding competitor derived from α4-integrin promoter did not compete meaning that this δEF1 factor was not binding to the ACCT sequence of the inhibin α promoter. E-cadherin is a transmembrane glycoprotein involved in cell–cell adhesion. During differentiation and fusion of the cytotrophoblasts, E-cadherin disappears (Coutifaris et al., 1999). It is possible that E-cadherin may also be regulated by AP2 during syncytialization. We decided to define which part of the E-cadherin promoter fragment (Ecad) was able to compete for a transcriptional activity as shown by luciferase reporter gene assay in transfection experiments (Debieve and Thomas, 2002). This transcriptional activity is located in the protected region of the promoter and we demonstrated here that the factor responsible for this regulation is AP2. We measured AP2α and AP2γ mRNA expression by reverse transcription followed by quantitative real-time polymerase chain reaction (RT-qPCR) in cytotrophoblastic cells during in vitro syncytialization from Day 0 (D0) to Day 4 (D4). In undifferentiated cytotrophoblasts (D0), both AP2α and γ mRNAs were expressed at the same level. After 4 days in culture, AP2α mRNA expression started to increase to reach a maximum of 1.6 fold at D3 (Fig. 4A). This increase in messenger RNA was correlated with a trend in an increase in nuclear protein (Fig. 4B), but nevertheless not statistically significant in densitometry analysis. To the contrary, AP2γ mRNA expression was characterized by a 40% decrease at Day 1. From Day 2 to Day 4, mRNA levels increased again to reach the D0 level. These fluctuations in AP2γ mRNA expression were not correlated with protein production.

To measure the effect of AP2 factors on the expression of endogenous inhibin α subunit gene, isolated cytotrophoblastic cells were transfected with AP2α and AP2γ expression vectors (McPherson and Weigel, 1999). A control experiment consisted of cytotrophoblastic cells transfected with the empty vector. Forty-eight hours after transfection, total RNA was extracted and purified. To verify the transfection efficiency, AP2α and AP2γ relative expressions were quantified by RT-qPCR. AP2α and AP2γ mRNA expressions were increased by 8.6- and 7.3-fold, respectively (Fig. 5A). Transfection efficiency was also checked at the protein level with the expression of AP2 factors in empty and AP2α- and AP2γ overexpressing cells (Fig. 5C). We then quantified the inhibin α subunit mRNA expression. In AP2α-overexpressing cells, inhibin α subunit mRNA expression was 1.2-fold increased over control and in AP2γ-overexpressing cells, the effect was even stronger with a 1.34-fold increase over control (Fig. 5B). This increase was statistically significant.

**Discussion**

During in vitro cytotrophoblast differentiation into syncytiotrophoblast, activin A is secreted during cellular aggregation while inhibin A is secreted during syncytialization (Debieve et al., 2000). Inhibin α subunit is the limiting factor for inhibin A production and secretion, and could be also considered as a marker of syncytialization. Our laboratory cloned previously a 692 bp fragment of the human inhibin α promoter. DNAse I footprinting experiments using nuclear extracts from isolated cytotrophoblastic cells showed that a region corresponding to nucleotides −221 to −185 was protected. EMSA experiments with a probe corresponding to this protected region confirmed the interaction between the inhibin α promoter fragment and a cytotrophoblastic nuclear protein. This interaction appeared to be dependent on the differentiation state of the cells. The nuclear protein preferentially bound to the promoter when cells were

![Figure 3](image_url)
undifferentiated. Moreover, transfection experiments with the inhibin α promoter fragment cloned into a luciferase reporter vector revealed that the protein presented activating transcriptional activity since deletion of the DNA-binding site abolished the luciferase activity (Debieve and Thomas, 2002).

In this study, we purified and identified the factor(s) interacting with the inhibin α promoter by DNA affinity chromatography coupled to MS and western blotting. We found that the previously unidentified protein was a member of the AP2 family. In human and mouse, the family consists of five subtypes α, β, γ, ε, and δ (Eckert et al., 2005). The AP2α, β and γ factors were found to be important for embryonic development (Schorle et al., 1996; Zhang et al., 1996). Gene suppression experiments in mouse revealed that AP2α and β were involved in fetal development, while AP2γ was important for placental formation (Auman et al., 2002).

Human cytotrophoblastic cells isolated from term placenta differentially express AP2α and AP2γ mRNA during in vitro syncytialization (Richardson et al., 2001). Our work indicates that these cells essentially produce AP2α protein and have very little AP2γ. Nevertheless, even if there are very little AP2γ factors in these cells, they still participate to the protein–DNA complex formation in solution as seen by supershift experiments with specific antibodies.

AP2 factors bind indistinctly to the same DNA consensus core sequence 5′-GCCN3GGC-3′ as homo- or heterodimer but endogenous binding sites from AP2-regulated genes may differ considerably (Hilger-Eversheim et al., 2000). Based on the consensus sequence defined by McPherson and Weigel, we identified a putative AP2-binding site 5′-GCCtcaAGC-3′. Mutation of the three nucleotides GCC of the 5′ half site of the palindromic core sequence completely suppressed the ability of the oligonucleotides to compete for the protein–DNA complex formation in gelshift experiments.

Cytotrophoblastic genes coding for human prolactin, human chorionic gonadotropin α and γ among others, are known to be increased during trophoblast fusion, and to be regulated by AP2 factors. Therefore, these transcription factors may play an essential role in regulating key genes of trophoblast differentiation. Overexpression of a dominant-negative mutant of AP2 in isolated cytotrophoblasts undergoing syncytialization revealed that inhibin α subunit was probably up-regulated by AP2 (Cheng et al., 2004). Here, we measured AP2α and AP2γ mRNA and protein expression during trophoblast differentiation. Interestingly, AP2α and AP2γ mRNA expressions were partially different from those previously published (Richardson et al., 2001; Johnson et al., 1997). AP2α is effectively increased during trophoblast differentiation at the mRNA level, but at the protein level, we only observed a trend without statistical significance. On the other hand, AP2γ mRNA is decreased the first day of culture, which corresponds to cell aggregation. Then AP2γ mRNA is increased again to reach the level measured in undifferentiated cytotrophoblasts. These changes in AP2γ mRNA are not correlated with protein production, which stayed nearly undetected during the 4-day in vitro culture. These changes in AP2 factor expression are small but could play a role in transcriptional regulation of their target genes. Indeed,
AP2α and γ overexpressions in trophoblasts lead to 1.2- and 1.34-fold increase, respectively, in inhibin α subunit mRNA expression over mock-transfected control trophoblasts. This increase is statistically significant only for AP2γ. The weak effect of AP2α and AP2γ overexpression may be due to the fact that these cells are already expressing endogenous AP2α and AP2γ. There could be also regulatory mechanisms involved since the cells are already undergoing syncytialization at the time of transfection. Primary trophoblasts purified and isolated from term placenta are not proliferating. They already express AP2α factors and their expression may be already at a maximum level, which would explain why the effect on inhibin α subunit expression is not more important. Nevertheless, our observations strongly support that AP2α regulates inhibin α subunit expression and production during the in vitro syncytialization.

Trophoblast fusion and syncytium formation are still unknown mechanisms. Studies on membrane glycoproteins expression during in vitro trophoblast differentiation indicated that E-cadherin, membrane retroviral envelop syncytin-1 and -2 and gap junctional proteins are essential. E-cadherin is not expressed in undifferentiated cytotrophoblasts (Coutifaris et al., 1991). Once in culture, E-cadherin protein starts to appear reaching a peak after 24 h, when cells form aggregates. After 24 h, E-cadherin protein expression diminishes and is not detected anymore when cells are completely differentiated into syncytiotrophoblasts. This E-cadherin expression is correlated with the increase in AP2α mRNA expression and protein synthesis and with the decrease in AP2γ mRNA expression seen at Day 1 in our 4-day cytotrophoblast cultures. Our competitive gel shift assay experiments also indicated that AP2 probably binds to an E-cadherin promoter fragment. We may speculate that AP2 factors are regulating key genes of syncytiotrophoblast formation. A defect in expression at the right time during trophoblast cellular fusion could lead to placental dysfunction as seen in pre-eclampsia (Brown et al., 2005; Langbein et al., 2008) or in trisomy 21-affected placenta (Massin et al., 2001).

**Authors’ roles**

F.D. designed the experiments and co-wrote the paper; C.D. performed the experiments and co-wrote the paper; C.H. supervised the research and corrected the paper.

**Acknowledgements**

Mass spectrophotometry results and analysis were kindly provided by D. Vertommen from the platform for proteomic and protein analysis by mass spectrometry (MASSPROT), de Duve Institute, Belgium.

**Funding**

This work was fully supported by ‘Fetus for live Foundation’ and by a grant from the ‘Fondation Saint-Luc’ to C.D.

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