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**BIOCHEMICAL CHARACTERIZATION AND MODULATION OF LH/CG -
RECEPTOR DURING HUMAN TROPHOBLAST DIFFERENTIATION.**

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Running title: LH/CG-R in human trophoblast differentiation.

Key words: hCG, hPL, placenta, cytotrophoblast, syncytiotrophoblast, cell fusion.

6 text figures and 1 table

ABSTRACT

1 Due to the key role of the human chorionic gonadotropin hormone (hCG) in placental
2 development, the aim of this study was to characterize the human trophoblastic luteinizing
3 hormone/chorionic gonadotropin receptor (LH/CG-R) and to investigate its expression using the *in*
4 *vitro* model of human cytotrophoblast differentiation into syncytiotrophoblast. We confirmed by *in*
5 *situ* immunocytochemistry and in cultured cells, that LH/CG-R is expressed in both villous
6 cytotrophoblasts and syncytiotrophoblasts. However, LH/CG-R expression decreased during
7 trophoblast fusion and differentiation, while the expression of hCG and hPL (specific markers of
8 syncytiotrophoblast formation) increased. A decrease in LH/CG-R mRNA during trophoblast
9 differentiation was observed by means of semi-quantitative RT-PCR with two sets of primers. A
10 corresponding decrease (~ 60%) in LH/CG-R protein content was shown by western-blot and
11 immunoprecipitation experiments. The amount of the mature form of LH/CG-R, detected as a 90-kDa
12 band specifically binding ¹²⁵I-hCG, was lower in syncytiotrophoblasts than in cytotrophoblasts. This
13 was confirmed by Scatchard analysis of binding data on cultured cells. Maximum binding at the cell
14 surface decreased from 3511 to about 929 molecules/seeded cells with a K_d of 0.4 - 0.5 nM.
15 Moreover, on stimulation by recombinant hCG, the syncytiotrophoblast produced less cyclic AMP
16 than cytotrophoblasts, indicating that LH/CG-R expression is regulated during human villous
17 trophoblast differentiation.

INTRODUCTION

Human chorionic gonadotropin (hCG) belongs to a family of glycoprotein hormones, which includes lutropin (LH), thyrotropin (TSH) and follitropin (FSH). These hormones composed of two non-covalently linked subunits, alpha (α) and beta (β), are active on bidentary form. The α -subunit is common to all glycoprotein hormones, whereas the β -subunits confer the hormonal specificity (Pierce and Parsons, 1981). Alpha hCG is encoded by a single gene and β hCG by six genes, one of which, *CG β 5*, is predominantly expressed in the placenta (Bo and Boime, 1992). HCG is essential for the initiation and maintenance of early pregnancy. After implantation, hCG is produced by the placenta and mainly by the trophoblast (Hoshina et al., 1985; Kliman et al., 1986; Muyan and Boime, 1997, Handschuh et al., 2006). It is used as a diagnostic marker of pregnancy.

The human placenta is characterized by extensive invasion of the trophoblast in the maternal uterus, creating direct trophoblast contact with maternal blood (haemochorial placentation). In early pregnancy, mononuclear cytotrophoblasts (CT) proliferate and invade the maternal endometrium to form the anchoring villi. (Aplin, 1991). Cytotrophoblasts also differentiate into a multinucleated continuous layer known as the syncytiotrophoblast (ST). This cell layer, which covers the chorionic villi, is bathed by maternal blood in the intervillous spaces from early gestation (Richard, 1961; Midgley et al., 1963; Boyd and Hamilton, 1970; Benirschke and Kaufmann, 2000). This syncytiotrophoblast is multifunctional, but its primary functions are exchange of oxygen, nutrients, removal of waste products and hormone production. The syncytiotrophoblast secretes hCG in large amounts, directly into the maternal blood bathing the chorionic villi in the intervillous space.

The mechanisms underlying villous trophoblast differentiation remain largely to be explored. Syncytiotrophoblast formation *in vivo* and *in vitro* arises from villous cytotrophoblast fusion and differentiation. Several factors modulate villous trophoblast differentiation, including EGF (epidermal growth factor) and EGF receptor expression (Morrish et al., 1987; Alsat et al., 1993), hypoxia (Alsat et al., 1996), cAMP-dependent protein kinase (PKA) (Keryer et al., 1998), granulocyte-macrophage stimulating factor (Garcia-Lloret et al., 1994), transforming growth factor β (TGF β) (Morrish et al.,

45 1991) and oxidative stress due to overexpression of copper zinc superoxide dismutase (Frendo et al.,
46 2000a, 2001). The molecular mechanisms underlying trophoblast membrane fusion are poorly
47 understood. Proteins involved in cell adhesion (cadherin 11) (Getsios and MacCalman, 2003) and cell-
48 cell communication (connexin 43) (Frendo et al., 2003a) are known to be directly involved. We
49 recently demonstrated the direct involvement of syncytin I, a human endogenous retroviral envelope
50 glycoprotein (Frendo et al., 2003b), and the presence of syncytin 2, restricted to some villous
51 cytotrophoblasts (Malassiné et al, 2006).

52 Several studies suggest that hCG stimulates villous trophoblast differentiation by acting on the
53 LH/CG-R (Shi et al., 1993; Cronier et al., 1994; Yang et al., 2003). This receptor, which has seven
54 transmembrane domains, belongs to the superfamily of G protein-coupled receptors (Pierce and
55 Parsons, 1981; Loosfelt et al., 1989; McFarland et al., 1989; Minegishi et al., 1990). The LH/CG
56 receptor gene has been cloned in pig, mouse, rat and human; in humans it is composed of 11 exons
57 and 10 introns, and its coding region is over 60 kb long (Segaloff and Ascoli, 1993). HCG binding to
58 its receptor activates adenylate cyclase, phospholipase C and ion channels, which in turn control
59 cellular cAMP, inositol phosphates, Ca²⁺ and other secondary messengers (Gudermann et al., 1992;
60 Hipkin et al., 1992).

61 The presence of LH/CG-R in human placenta was first described by Alsat (Alsat and Cedar,
62 1974) and has since been confirmed by other authors (Reshef et al., 1990; Lei and Rao, 1992).
63 Inhibition of LH/CG-R expression by specific antisense oligodeoxynucleotides during cytotrophoblast
64 culture results in time- and concentration-dependent inhibition of cytotrophoblast differentiation,
65 showing that hCG, *via* its receptor, is an autocrine and paracrine regulator of human placental
66 syncytiotrophoblast formation (Yang et al., 2003).

67 Most of the studies actually done, have used transfected cells with cDNA from LH/CG-
68 receptor in rat or mouse models. In human, the characterization and the modulation of LH/CG-R
69 expression during syncytiotrophoblast formation is poorly documented. Here we used the
70 physiological model of cultured primary human trophoblasts (Kliman et al., 1986; Frendo et al.,
71 2000b), in which isolated mononuclear cytotrophoblasts differentiate and fuse to form a
72 syncytiotrophoblast, which secretes large amounts of hCG and other pregnancy-related hormones. We

73 used various methodological approaches to characterize the hCG/LH receptor, and observed its down-
74 regulation during villous trophoblast differentiation. This was confirmed by *in situ* immunolocalisation
75 of the hCG receptor in sections of human placenta.

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77

78

MATERIALS AND METHODS

79

Placental tissue collection and trophoblast cell culture

81 These studies were performed in agreement with our local ethics committee and with written informed
82 consent of patients. Third trimester placentas were obtained immediately after iterative Caesarian
83 section from healthy mothers delivered at 35-39 weeks of amenorrhea. First trimester placentas (7-12
84 weeks of gestation) were collected following legal voluntary interruption of pregnancy from women
85 who gave their written informed consent. Cytotrophoblasts were isolated as previously described
86 (Alsat et al., 1993). After sequential trypsin (0.25%)/DNase I digestion followed by Percoll gradient
87 centrifugation (Frendo et al., 2003a), the cells were further purified by negative selection to obtain a
88 trophoblast preparation not contaminated by other cells, by using a monoclonal anti-human leukocytic
89 antigen A, B and C antibodies (W6-32HL, Sera Lab, Crawley Down, UK) according to a published
90 method (Schmon et al., 1991; Cronier et al., 2002). This antibody reacts with most cell types (e.g.
91 macrophages, fibroblasts, extravillous trophoblasts) but not with villous cyto- or syncytiotrophoblast.
92 Cytotrophoblasts were diluted to a final density of 2.7×10^6 cells in 3 ml of minimum essential medium
93 (MEM) containing 10% fetal calf serum (FCS). Cells were plated in 60-mm plastic dishes (TPP,
94 Trasadingen, Switzerland) and incubated at 37°C in 5% CO₂. Cytokeratin 7 immunocytochemistry
95 was performed to confirm the cytotrophoblastic nature of the attached cells: about 95-98% of the cells
96 were positively stained.

97

Hormone assay

99 The hCG concentration was determined in culture medium after 24 and 72 hours of culture by using
100 an enzyme-linked fluorescence assay (Vidas System, BioMerieux, Marcy l'Etoile, France) with a

101 detection limit of 2 mU/ml. The hPL concentration was determined in 4-fold-concentrated conditioned
102 medium by using a method (Amerlex IRMA, Amersham Pharmacia Biotech) with a detection limit of
103 0.5 µg/ml. All reported values are means ± SEM of triplicate determinations.

104

105 ***Immunohistochemistry***

106 Placental samples were obtained after first-trimester abortion. They were fixed by incubation in 4%
107 formalin for 4 to 12 h at room temperature and then embedded in paraffin, dewaxed in xylene and
108 rehydrated in ethanol/water. Immunostaining was performed with a universal streptavidin-peroxidase
109 immunostaining kit (Peroxidase, Dako LSAB®+Kit, DAKO®, Glostrup, Denmark). Non-specific
110 binding was blocked by incubation for 5 min in a blocking reagent containing 3% H₂O₂ and then in
111 3% serum albumin in PBS for 30 min. The sections were incubated with the primary antibody for 30
112 min at room temperature. The primary antibodies (table 1) were polyclonal anti-human LH/CG-R
113 (LHR-K15, Santa Cruz Biotechnology Inc, CA, USA, at 2 µg/ml), monoclonal anti-cytokeratin 7
114 (M7018, DAKO®, Glostrup, Denmark, at 1 µg/ml), and polyclonal anti-hCG (A0231 against the beta
115 subunit of hCG, DAKO®, Glostrup, Denmark, at 2 µg/ml). Sections were washed in PBS and
116 incubated with a biotinylated secondary antibody for 15 min. They were then washed three times in
117 PBS and incubated with streptavidin conjugated to horseradish peroxidase for 15 min. The sections
118 were washed in PBS and staining was detected by incubation for 30 seconds with the DAB (3,3'-
119 diaminobenzidine) chromogen. Controls were performed by incubating the sections with nonspecific
120 IgG at the same concentration as the primary antibody. Successive pre-adsorptions of LH/CG-R
121 antibody with trophoblastic cells in culture abrogate LH/CG-R immunodetection.

122

123 ***Immunocytochemistry***

124 To detect desmoplakin, LH/CG-R, hCG, cytokeratin 7 and hPL, cultured cells were rinsed with PBS,
125 fixed and permeabilized in methanol at -20°C for 8 min. Alternatively, cultured cells were fixed with
126 4% paraformaldehyde at 4°C for 20 min. After washing once with PBS, the remaining free aldehyde
127 groups were blocked by adding 50mM NH₄Cl for 10 min. A polyclonal anti-desmoplakin (AHP320,
128 Serotec, Oxford, UK at 2.5 µg/ml), two polyclonal anti-LH/CG-R (LHR-K15 and LHR-H50, Santa

129 Cruz Biotechnology Inc, CA, USA, at 2 µg/ml), two polyclonal anti-hCG (A0231, DAKO[®], Glostrup,
130 Denmark at 2 µg/ml and SC-7821, Santa Cruz Biotechnology Inc, CA, USA at 2µg/µl), a monoclonal
131 anti-cytokeratin 7 (M7018, DAKO[®], Glostrup, Denmark, at 2.6 µg/ml), or a polyclonal anti-hPL
132 (A0137, DAKO[®], Glostrup, Denmark, at 1.6 µg/ml) was then applied (table 1), followed by
133 fluorescein isothiocyanate-labeled goat anti-mouse IgG, or fluorescein isothiocyanate-labeled goat
134 anti-rabbit IgG (Jackson Immuno Research, Baltimore, USA at 1:150), or Alexa 488-labeled donkey
135 anti rabbit (Molecular probes Inc, OR, USA at 1:400), or Texas red labeled donkey anti goat (Jackson
136 Immuno Research, Baltimore, USA at 1:400), or Cy^{TM3} goat anti-rabbit IgG, as previously described
137 (Frendo et al., 2001). The controls, which consisted of omitting the primary antibody or applying the
138 non specific IgG of the same isotype, were all negative.

139

140 ***Immunoblotting***

141 Cell extracts were prepared as previously described (Alsat et al., 1996). Protein (70 µg) was
142 solubilized in RIPA (radioimmunoprecipitation) buffer (50 mM Tris, 150 mM NaCl, 1% Triton X100,
143 1% deoxycholate, 0.1% SDS, pH: 8), and stained markers were submitted to 7.5% SDS-PAGE and
144 transferred to nitrocellulose sheets. Membranes were immunoblotted with two polyclonal antibodies
145 against LH/CG-R, LHR-K15 (goat anti human, Santa Cruz Biotechnology Inc, CA, USA) and LHR-
146 H50 (rabbit anti human, Santa Cruz Biotechnology Inc, CA, USA) at 2 µg/ml each, and the specific
147 band was revealed by chemiluminescence (West Pico Chemiluminescent, Pierce, Rockford, IL, USA)
148 after incubation with an anti-goat or anti-rabbit peroxidase-coupled antibody (Jackson Immuno
149 Research, Baltimore, USA). To detect actin, cytokeratin 7, hCG and hPL, we proceeded as described
150 above, except that proteins were immunoblotted with rabbit polyclonal antibody at 0.7 µg/ml for actin
151 (Sigma-Aldrich, MO, USA), rabbit polyclonal antibody at 0.4 µg/ml for hCG and 0.32 µg/ml for hPL
152 (DAKO[®], Glostrup, Denmark) and mouse monoclonal antibody at 0.5 µg/ml for cytokeratin 7
153 (DAKO[®], Glostrup, Denmark). Successive pre-adsorptions of LH/CG-R antibody with trophoblastic
154 cells in culture abrogate LH/CG-R immunodetection in western-blot analysis.

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156

157 ***Immunoprecipitation and ligand blotting***

158 Protein G Plus-Agarose (Immuno precipitation Reagent, Santa Cruz Biotechnology Inc, CA, USA)
159 was pre-mixed with a polyclonal antibody against human LHCG-R (K15, Santa Cruz Biotechnology
160 Inc, CA, USA), or without antibody. Cells (1.0×10^6 /well) were seeded in six-well plates and cultured
161 as previously described. After 24 hours of culture, cells were washed with PBS and scraped free in
162 ice-cold RIPA buffer. After sonication, the cellular lysate and debris were separated by centrifugation
163 at 10000 g for 10 min at 4°C. The supernatant was transferred to the protein G-anti-human LHCG-R
164 immunocomplex and incubated overnight at 4°C on a rocker platform, followed by four washes in
165 RIPA buffer. Protein was eluted by heating at 60°C for 10 min in 1X electrophoresis sample buffer
166 (Bio-Rad laboratories, CA, USA). Aliquots were submitted to 7.5% SDS-PAGE and transferred to
167 nitrocellulose membranes. Membranes were exposed to antibody as previously described, or the blots
168 were incubated with ^{125}I -hCG at 10^{-11} M (PerkinElmer Life and Analytical Sciences Inc. MA, USA)
169 for 16 h at 4°C in the absence or presence of excess unlabeled hCG at 10^{-6} M (Organon SA, Puteaux,
170 France). The blots were washed with PBS containing 0.1% Tween 20, then dried. Bound ^{125}I -hCG was
171 visualized by autoradiography and analyzed by Cyclone (Storage phosphorImaging System, Hewlett
172 Packard, France).

173

174 ***RNA extraction***

175 Total RNA was extracted from trophoblastic cells after 24 or 72 hours of culture by using the Trizol
176 reagent (Invitrogen Life Technologies, CA, USA) and was stored at -80°C or at -20°C in 75% ethanol
177 until use. The total RNA concentration was determined at 260 nm and RNA integrity was checked in
178 1% agarose gel. The relative LH/CG-R mRNA levels were determined by semi-quantitative reverse
179 transcriptase-polymerase chain reaction (RT-PCR). The transcript level was normalized to the actin
180 mRNA level (endogenous control).

181

182 ***RT-polymerase chain reaction***

183 RNA samples were pretreated with DNase I using the RQ1 RNase-Free DNase kit (Promega Inc, WI,
184 USA). Briefly, we used 5 units of RQ1 RNase-free DNase per 5 micrograms of RNA, we then added
185 RQ1 RNase-free 10x reaction buffer and TE buffer. Mixture was incubated at 37°C for 30 min and the
186 digestion was terminated by the RQ1 DNase stop solution. DNase was then inactivated by heating at
187 65°C for 10 min.

188 Complementary DNA was synthesized from 5 µg of total RNA. The reaction mixture had a final
189 volume of 20 µl and contained 375 mM KCl, 250 mM Tris-HCl (pH 8.3), 15 mM MgCl₂, 0.1 M DTT,
190 40 U of RNAsin®, 200 U of reverse transcriptase Superscript II (Invitrogen Life Technologies, CA,
191 USA), 10 mM each dNTP and 200 ng of random primers (Invitrogen Life Technologies, CA, USA).
192 Mixture of total RNA, DTT and random primers was heat at 65°C for 5 min. Annealing was run for 10
193 min at 25°C and primer extension for 50 min at 42°C. An aliquot of the reaction mixture (5 µl) was
194 then made up to 45 µl with Taq polymerase buffer containing 1 unit of Taq polymerase Platinum
195 (Invitrogen Life Technologies, CA, USA). Before heating to 94°C (hot-start), 50 pmol of each specific
196 primer was added. Amplification was run for 40 cycles for LH/CG-R and for 20 cycles for actin,
197 consisting of 1 min at 94°C (denaturation), 1 min at 55°C (annealing) and 1 min at 72°C (extension).
198 Oligonucleotide primers specific for the coding sequence of LH/CG-R (NM_000233) were used (Fig.
199 3A): P1 (+): 5'-CAAGCTTTCAGAGGACTTAATGAGGTC-3'; P1 (-): 5'-AAAGCACAGCAGTGG
200 CTGGGGTA-3'; P2 (+): 5'-TCGACTATCACTTGCCTACC-3'; P2 (-): 5'-GGAGAAGACCTTCGTA
201 ACAT-3'; Actin (NM_001101) (+): 5'-GTGGGGCGCCCCAGGCACCA-3'; Actin (-): 5'-CTCCTTA
202 ATGTCACGCACGATTTC-3'. Amplified products were analyzed by electrophoresis on 1.8% agarose
203 gels and visualized by ethidium bromide staining.

204

205 ***Cloning and DNA sequencing of LH/CG-R from trophoblastic cells***

206 PCR products were eluted from agarose gel by using the Macherey Nagel kit (NucleoSpin Extract II,
207 MN, Hoerd, France) and purified DNA fragments were cloned into the pCRII-TOPO vector by using
208 the TOPO-TA Cloning kit (Invitrogen Life Technologies, CA, USA). Positive clones were selected by
209 PCR and were sequenced by Genome Express (Meylan, France). Both strands of DNA fragments were
210 sequenced, using M13 reverse and M13 forward primers.

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Intracellular cAMP determination

Cells (1.0×10^6 /well) were seeded in six-well plates and cultured as described above. After 24 h or 72 h, cells were preincubated with 10mM IBMX (3-isobutyl-1-methylxanthine) for 1 hour to prevent cAMP degradation and were stimulated for 20 min with 10^{-8} M hCG (C6322, Sigma-Aldrich, MO, USA). Cells were frozen on dry ice and cAMP was extracted with ice-cold 65% ethanol. The extracts were dried and kept at -20°C until use. Cyclic AMP was assayed after acetylation by using a method (Amersham Biosciences, NJ, USA) based on the competition between unlabelled cAMP and a fixed quantity of ^{125}I -labelled cAMP for binding to a cAMP-specific antibody. Bound antibody was separated from free fraction by magnetic separation with a second antibody AmerlexTM-M preparation that is bound to magnetizable polymer particles. Separation of the antibody bound fraction is effected either by magnetic separation of the AmerlexTM-M suspension or decantation of the supernatant. The concentration of unlabelled cAMP in the sample was then determined by interpolation from a standard curve.

Binding assay and Scatchard analyses

Trophoblastic cells (1.0×10^6 /well) were seeded in six-well plates and cultured as described above. After 24 h or 72 h of culture the cells were washed five times and cultured in DMEM, 0.1% BSA for 2 hours to dissociate any bound endogenous hCG. The cells were then washed and placed in 1 ml of DMEM containing 0.1% BSA and 1 mM HEPES, pH 7.3. Cells were incubated for 30 min at room temperature with 0.5 nM ^{125}I -hCG and an increasing concentration of unlabelled hCG (from 10^{-12} M to 10^{-8} M, C6322, Sigma-Aldrich, MO, USA) on a shaker platform at 50 cycles/min. At the end of the incubation period the cells were washed and scraped free, and bound radioactivity was counted. Each assay was performed in triplicate. Data were analyzed by using the LIGAND fitting program (version 4.97) (Munson and Rodbard, 1980). For Scatchard analysis, the results showing the number of labeled molecules associated with the cellular membrane were expressed in a number of molecule associated per seeded cells. For comparison between CT and ST experiments, nuclei were counted at 24h and 72h

239 of culture after staining with DAPI, as previously described in the immunocytochemistry section. We
240 did not observe difference between the number of nuclei at 24h and 72h of culture (CT are non
241 proliferative cells and apoptosis or cellular loss account for about 4% (data not shown).
242 ¹²⁵I-labeled hCG was prepared using chloramine T as oxidant (Hunter and Greenwood, 1962). In a
243 final volume of 20 µl, hCG (5 µg, 4.4 µM) was added to 0.5 mCi of Na¹²⁵I (Perkin-Elmer Life and
244 Analytical Sciences, MA, USA; 17.4 Ci/mg, 11.5 µM) neutralized with 0.1 M Mops and poly(ethylene
245 glycol) 1000 (1%). The reaction in 25 mM Mops buffer pH 7.2 was started by adding 100 µM
246 chloramine-T for 3 min at room temperature and was stopped by adding 120 µM sodium bisulfite for 3
247 min and 2 mM NaI for 1 min. The volume was then adjusted to 0.5 ml with Mops-buffered saline (20
248 mM Mops, 130 mM NaCl, pH 7.2) containing 1 mg/ml BSA. Iodinated-hCG was desalted on a PD10
249 Sephadex G25-M column in the same buffer. Specific activity of ¹²⁵I-hCG was 2.1-2.4 Ci/µmole
250 corresponding to about 1 atom of iodine per molecule hCG.

251

252 *Statistical analysis*

253 We used the StatView F-4.5 software package (Abacus Concepts, Inc., CA, USA). Values are reported
254 as means ± SEM. Significant differences (p<0.05) were identified by analysis of variance (ANOVA).

255

256

257

RESULTS

258

259 *Human villous trophoblast differentiation in vitro*

260 We used the primary cell culture model of villous cytotrophoblasts isolated from term placenta
261 (Kliman et al., 1986; Alsat et al., 1991). Figure 1 shows purified cytotrophoblasts cultured on plastic
262 dishes for 24 and 72 hours. Mononuclear cytotrophoblasts fused and formed multinucleated
263 syncytiotrophoblasts, 72 hours after plating (Kliman et al., 1986). Syncytiotrophoblast formation was
264 associated with a significant increase in hCG and hPL levels in the culture medium (Fig. 1 I).
265 Concomitantly, immunostaining for hCG (Fig. 1 A and B) and hPL (Fig. 1 F and H) showed an
266 increase in intensity during *in vitro* syncytiotrophoblast formation. HPL, expressed mainly by the

267 syncytiotrophoblast (Handwerger, 1991), was detected by immunostaining at 72 h (Fig. 1 D and H)
268 but not at 24 h (Fig. 1 C and F). Immunostaining of cytokeratin 7, expressed by trophoblastic cells
269 (Blaschitz et al., 2000), was positive at 24 h (Fig. 1 C and E) and 72 h (Fig. 1 D and G).

270 These results showed that differentiation of isolated cytotrophoblasts into a syncytiotrophoblast is
271 associated with an increase in the expression and secretion of hCG and hPL, hormones mainly
272 synthesized by the syncytiotrophoblast.

273

274 ***Decrease in LH/CG-R protein levels during in vitro trophoblast differentiation***

275 As shown in figures 2 A and B, LH/CG-R was expressed by cultured cytotrophoblasts. The LH/CG-R
276 immunostaining shown in this figure was obtained with the polyclonal antibody LHR-K15. Another
277 antibody (LHR-H50) gave the same results (data not shown). LH/CG-R was expressed in both
278 cytotrophoblasts (24 h) and syncytiotrophoblasts (72 h), with punctuate immunolabeling. LH/CG-R
279 immunostaining appeared stronger in cytotrophoblasts than in syncytiotrophoblasts. Double
280 immunostaining for LH/CG-R (LHR-50) and hCG (C-20) of trophoblasts cultured for 48 hours (Fig. 2
281 C and D respectively, merge Fig. 2 E) illustrated the dynamics of the process. A mononucleated
282 cytotrophoblast (Fig. 2 C arrow head) expressed LH/CG-R, whereas aggregated trophoblasts showed
283 and heterogenous immunostaining of both LH/CG-R and hCG (Fig. 2 E). To validate this observation,
284 western-blot analysis was performed on extracts of cytotrophoblasts (24 h) and syncytiotrophoblasts
285 (72 h) (Fig. 3 A). At 24 h and 72 h of culture, two major bands with molecular masses (estimated from
286 SDS gels) of 65-75 kDa and 85-95 kDa were observed, as described in other cellular models and in
287 mammalian cells transfected with LH/CG-R cDNA. In the literature, the 85-95 kDa band corresponds
288 to the mature LH/CG-R present at the cell surface, and the 65-75 kDa band is the precursor of the cell-
289 surface receptor (for review see Ascoli et al., 2002).

290 Our results show that the expression of the mature LH/CG-R and its precursor (respectively designated
291 m and p in Fig. 3 A) decreases during cytotrophoblast differentiation. At the same time, actin
292 expression remains constant. Normalization of mature LH/CG-R protein expression to actin
293 expression showed a significant decrease ($58.6 \pm 6.7\%$; $p < 0.0001$) in cell-surface receptor expression.
294 We obtained similar results with the two antibodies used (LHR-K15 and LHR-H50).

295 Interestingly, in the same cellular extracts, the decrease in precursor and mature LH/CG-R expression
296 coincided with an increase in hCG and hPL expression (Fig. 3 A).

297 To further characterize LH/CG-R expression during trophoblast differentiation, we performed
298 immunoprecipitation (IP) with anti-human LH/CG-R antibody (K15). Cellular extracts were purified
299 by immobilized anti-receptor antibody (IP) and eluates were analyzed by SDS-PAGE and
300 immunoblotting using the receptor-specific antibody (K15). A 90 kDa band corresponded to the
301 mature form of LH/CG-R (m), and a major band of 75 kDa corresponded to the precursor (p).

302 To determine which molecular form of the receptor bound the hormone, we used ^{125}I -hCG in ligand-
303 blot experiments (Fig. 3 B). Incubation of the IP blot with ^{125}I -hCG (10^{-11}M) revealed a major band of
304 90 kDa. This band was absent when the blot was incubated with an excess of unlabeled hCG (10^{-6}M),
305 showing that the 90-kDa LH/CG-R specifically binds the hormone. In these conditions, ^{125}I - hCG
306 binding to the mature form of the receptor (90 kDa) was lower in the syncytiotrophoblast than in
307 cytotrophoblasts.

308

309 ***Decrease in LH/CG-R mRNA expression during in vitro trophoblast differentiation***

310 We conducted semi-quantitative RT-PCR experiments with two different sets of primers (P1 and P2)
311 (for primer positions see Fig. 4 A). To avoid contamination by genomic DNA, each primer was
312 located on a separate exon and RNA extracts were pretreated with DNase I.

313 As shown in figure 4 B, amplification of the 647-bp and 282-bp fragments, obtained with primers P1
314 and P2 respectively, indicated that LH/CG-R mRNA was significantly less abundant in the
315 syncytiotrophoblast (72 h) than in cytotrophoblasts (24 h). No significant difference was noted in the
316 actin mRNA level. We obtained similar results with the two sets of primers. The amplification
317 products were then purified from the agarose gel and cloned into the pCRII-TOPO vector. Sequencing
318 confirmed that both the 647-bp and 282-bp fragments were part of the human LH/CG receptor.
319 Normalization of LH/CG-R mRNA to actin mRNA after RT-PCR with primer sets P1 and P2 showed
320 a significant decrease in LH/CG-R mRNA levels during differentiation (Fig. 4 C). With the P1
321 primers, LH/CG-R mRNA levels fell from 0.33 ± 0.01 at 24 h to 0.13 ± 0.01 at 72 h ($p < 0.0001$). A
322 similar decrease was observed with the P2 primers (from 0.82 ± 0.02 at 24 h to 0.36 ± 0.01 at 72 h; $p <$

323 0.0001). Although the amplification product obtained with primers P2 appeared to be at least twice as
324 abundant as that obtained with primers P1 (probably because the P2 amplicon is about half the length
325 of the P1 amplicon), the size of the decrease in LH/CG-R levels at 72 h was similar with the two
326 primer sets (respectively 2.5- and 2.3-fold).

327

328 ***Decrease in ¹²⁵I-hCG binding to cell-surface LH/CG-R during in vitro trophoblast differentiation***

329 To confirm the decrease in LH/CG-R mRNA and protein levels, we performed binding saturation
330 experiments with iodinated hCG at 24 h and 72 h of culture (Fig. 5). Scatchard analysis of binding
331 data showed that the number of molecules bound per seeded cell at 24 h of culture (cytotrophoblasts)
332 was 3511 ± 693 . After differentiation, at 72 h of culture, this number fell significantly ($p=0.02$) to
333 929 ± 583 . No significant difference in K_d values was observed between 24 h (0.5 ± 0.1 nM) and 72 h
334 (0.4 ± 0.1 nM).

335

336 ***LH/CG-R stimulation during in vitro trophoblast differentiation***

337 In order to confirm the reduction in functional mature hCG receptor expression at the
338 syncytiotrophoblast surface compared to the cytotrophoblast surface, we determined cAMP production
339 in response to an effective hCG concentration for 20 min (Fig. 6). As cAMP is a second messenger for
340 hCG signaling in trophoblastic cells, the decrease in LH/CG-R transcript and protein levels ought to be
341 associated with a decrease in cAMP production. Determination of the most effective hCG
342 concentration was carried out by stimulating trophoblasts with 10^{-12} M to 10^{-6} M hCG; 10^{-8} M hCG was
343 the most effective concentration (data not shown). As shown in figure 6, hCG-stimulated cAMP
344 production by trophoblasts was higher at 24 h than at 72 h of culture ($p= 0.0021$). Trophoblast
345 stimulation by hCG (10^{-8} M) at 24 h of culture induced at least a 2-fold increase in cAMP production
346 compared to the basal level ($p= 0.0016$), but did not induce detectable cAMP production at 72 h of
347 culture ($p= 0.7644$). In contrast, epinephrine (which stimulates camp production and is used as a
348 positive control) induced similar cAMP production at 24 h and 72 h of culture, indicating that the cells
349 were functional and that the decrease in cAMP production observed at 72 h was not due to a defective
350 cAMP pathway.

351 ***Immunolocalization of LH/CG-R in villous sections***

352 These *in vitro* findings were confirmed by examining placental LH/CG-R expression *in situ*, on villous
353 sections. First-trimester placenta was chosen because cytotrophoblasts are more abundant than at other
354 stages of pregnancy and form a continuous layer.

355 LH/CG-R was detected in villous cytotrophoblasts and syncytiotrophoblasts. Use of a polyclonal
356 antibody raised against the extracellular domain of human LH/CG-R showed that LH/CG-R is mainly
357 expressed by the cytotrophoblast layer (Fig. 7 A). Weaker staining was observed in the
358 syncytiotrophoblast (ST). LH/CG-R was also expressed by perivascular cells (VC) of the villous core.
359 We obtained similar results with two other monoclonal antibodies (LHR 29 and LHR 1055) which
360 recognize two different epitopes of the extracellular domain of LH/CG-R (Vuhai et al., 1990; Méduri
361 et al. 1997) (data not shown). No staining was detected in negative control sections (Fig. 7 D).
362 Interestingly, strong hCG immunostaining was observed in the syncytiotrophoblast (Fig. 7 B) while
363 cytokeratin 7 was mainly located in the cytotrophoblast layer (Fig. 7 C).

364

365 Taken together, these results strongly suggest that the expression of a functional cell-surface
366 LH/CG-R decreases during cytotrophoblast differentiation into a syncytiotrophoblast.

367

368

369

DISCUSSION

370

371 By using several complementary methods and a well-characterized *in vitro* model of human
372 villous trophoblast differentiation, we clearly observed that LH/CG-R mRNA and protein expression
373 is lower in syncytiotrophoblasts than in cytotrophoblasts and that this down-regulation is associated
374 with an apparent decrease of receptor activation by its specific hormone. These results differ from
375 those of two previous studies published by CV. Rao, who described stronger expression of LH/CG-R
376 in syncytiotrophoblasts than in cytotrophoblasts (Reshef et al., 1990; Lei and Rao, 1992). This
377 divergence may come from the use of different tools. Anti-human LH/CG-R antibodies were not
378 available in the early 1990s, and most immunohistochemical and western-blotting studies used

379 antibodies raised against the N-terminal part of the rat LH/CG receptor. The amino acid sequence
380 identity between the rat and human receptors is 85%, with the strongest similitude in the
381 transmembrane portion of the molecule and not in the N-terminal region (Segaloff and Ascoli, 1993).
382 Moreover, experiments involving radiolabeled probes, such as northern blotting and *in situ*
383 hybridization, used porcine cDNA with 88% sequence identity to the human sequence. In this study,
384 we cloned PCR fragments of the human hCG/LH receptor from villous cytotrophoblasts and used
385 antibodies specific for the human receptor.

386 HCG, which is produced in large amounts by the syncytiotrophoblast, plays an important role
387 in cytotrophoblast differentiation into syncytiotrophoblast. An increasing number of studies have
388 investigated the central role of hCG and its receptor in the trophoblastic differentiation process. Many
389 authors have described down-regulation of LH/CG-R expression by increasing concentrations of hCG.
390 Indeed, exposure of ovarian or testicular cells expressing the endogenous LH/CG-R to a high
391 concentration of hCG down-regulates cell-surface receptor expression. This coincides with a decrease
392 in the abundance of LH/CG-R transcripts (Segaloff et al., 1990; Peegel et al., 1994; Hoffman et al.,
393 1991; LaPolt et al., 1990; Hu et al., 1990). It is noteworthy in this respect that hCG is secreted in large
394 amounts during syncytiotrophoblast formation. The decrease in cell-surface receptor expression was
395 confirmed in our study by the clear decrease in cAMP production by the syncytiotrophoblast after
396 stimulation by recombinant hCG. Interestingly, the decrease in cAMP production by the
397 syncytiotrophoblast was not due to a loss of affinity or to weak binding between the receptor and its
398 hormone, as we found no difference in LH/CG-R K_d values between 24 h and 72 h of culture.
399 Moreover, Scatchard plots clearly showed that the maximum number of hCG molecules bound per
400 seeded cell was significantly lower at 72 h of culture than at 24 h (~74%). This result confirms the
401 decrease of LH/CG-R (~60%) observed by western-blot analysis. The difference in LH/CG-R decrease
402 (60% *versus* 74%) may be due to the technical approaches used for the purpose. By western-blot
403 analysis, we quantified the mature form of the LH/CG-R in proteins from total cellular extracts. In
404 binding experiments, we used living cells, meaning that only the mature form of the LH/CG-R present
405 at the cell surface was quantified. Some mature forms internalized or present in the endosome might
406 not be accessible to ¹²⁵I-hCG.

407 LH/CG-R desensitization has been described in rat ovary and is accompanied by a transient
408 loss of responsiveness to LH, the receptor being temporarily uncoupled from its Gs protein (Segaloff
409 et al., 1990). We observed here that syncytiotrophoblast stimulation by recombinant hCG (10^{-8} M) did
410 not induce detectable cAMP production although the cAMP pathway was functional as shown by
411 epinephrine stimulation. This loss of responsiveness to recombinant hCG may thus be due in part to
412 cell-surface receptor desensitization.

413 Western blotting showed that two major species of LH/CG-R with molecular masses of 65-75
414 kDa and 85-95 kDa were expressed by cytotrophoblasts and by syncytiotrophoblasts.
415 Immunoprecipitation experiments and ligand blot analysis confirmed that the 65-75 kDa band was the
416 intracellular precursor of the cell-surface receptor and that the 85-95 kDa band corresponded to mature
417 LH/CG-R present at the cell surface, as shown by its ability to bind specifically labeled 125 I-hCG. Our
418 results show that the expression of the mature LH/CG-R and its intracellular precursor decreased
419 during cytotrophoblast differentiation. Furthermore, the precursor form seemed to be more strongly
420 expressed than the mature form in trophoblastic cells. Most studies of these two forms of LH/CG-R
421 have used mammalian cells transfected with the cDNA for the porcine, rat or human receptor (for
422 review see Ascoli and al., 2002), but as shown here, primary cultured human trophoblasts may be an
423 excellent model for studying the maturation of the intracellular precursor into the mature cell-surface
424 protein. Recently, Pietila et al using transfection models have shown that regulation of the immature
425 form into the mature form might considered be important in LH/CG-R expression (Pietila et al., 2005).

426 In this study, we characterized for the first time, in a human physiological model, the
427 expression and regulation of LH/CG-receptor. We demonstrate, both *in situ* and *in vitro*, that LH/CG-
428 R is expressed by human cytotrophoblasts and, albeit to a lesser extent, by the syncytiotrophoblast.
429 LH/CG-R expression thus seems to be regulated during villous trophoblast differentiation, and this
430 regulation may involve down-regulation of the receptor by its ligand. Abnormal regulation of this
431 process might be involved in trisomy 21-associated pregnancies, in which we recently observed an
432 abnormal glycosylated form of hCG associated with defective syncytiotrophoblast formation (Frendo
433 et al., 2000b, 2004). Abnormal syncytiotrophoblast formation might lead to complications such as
434 preeclampsia and intrauterine growth retardation.

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584

FOOTNOTES

585

586 [§] These two authors contributed equally to the work.

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592

593

FIGURE LEGENDS

594

595 Fig. 1 ***In vitro* human villous trophoblast differentiation.** (A) and (B): hCG immunodetection after
596 24 and 72 hours of culture of villous cytotrophoblasts isolated from term placentas. At 24 hours the
597 cells are sparse or aggregated (A). At 72 hours, they have fused to form the syncytiotrophoblast,
598 characterized by multiple nuclei and a strong positive immunofluorescent staining for hCG (B). Nuclei
599 were labeled with DAPI (blue fluorescence). (C) and (D): co-immunolocalization of cytokeratin 7 (in
600 green) and hPL (in red) at 24 hours (C) and 72 hours of culture (D). Nuclei are stained blue with
601 DAPI. HPL, known to be expressed mainly by the syncytiotrophoblast, was detected by
602 immunostaining at 72 h (H) but not at 24 h of culture (F). Cytokeratin 7 immunostaining, was positive
603 at 24 h (E) and 72 h (G). (I): levels of hCG and hPL (expressed respectively in milli-international
604 units per milliliter and micrograms per milliliter of medium) secreted into the culture medium at the
605 indicated times. Since cells were plated in triplicate (see Experimental procedures), hCG and hPL
606 levels were determined for each plate. ND: non detectable. Results are means \pm SEM of the three
607 culture dishes. This figure illustrates one experiment representative of three. Scale for pictures A-D: 1
608 cm = 30 μ m. Scale for pictures E-H: 0.5 cm = 30 μ m.

609

610 Fig. 2 **LH/CG-R immunodetection during *in vitro* trophoblast differentiation.** (A) and (B):
611 immunostaining for LH/CG-R by using the polyclonal antibody LHR-K15 raised against the human
612 LH/CG receptor. LH/CG-R was expressed in both cyto- (A; 24h) and syncytiotrophoblasts (B; 72h),
613 albeit more strongly in cytotrophoblasts. (E): co-immunodetection of LH/CG-R and hCG by using the
614 polyclonal antibodies LHR-H50 (C; in green) and hCG-C20 (D; in red) respectively at 48 hours of
615 culture. Single trophoblast (arrowed) was stained for LH/CG-R and aggregated trophoblasts were
616 stained for both LH/CG-R and hCG. Nuclei were labeled with DAPI (blue fluorescence). Scale for
617 pictures A and B: 0.5 cm = 30 μ m; scale for pictures C-E: 1 cm = 15 μ m.

618

619 Fig. 3 **LH/CG-R protein expression during *in vitro* trophoblast differentiation.** (A) and (B):
620 Western-blot analyses (A) were performed using the same antibody on extracts from cytotrophoblasts

621 (24 h) and syncytiotrophoblasts (72 h). At 24 h and 72 h of culture, two major bands with molecular
622 masses of 65-75 kDa, corresponding to the precursor (p) of the cell-surface receptor and 85-95 kDa,
623 corresponding to the mature LH/CG-R (m) present at the cell surface, were observed. The histogram
624 presents the normalization of mature LH/CG-R protein expression (m) by actin expression (43kDa)
625 (***: $p < 0.0001$). Results are expressed as the mean \pm SEM of three culture dishes. In the same
626 cellular extracts, decrease in precursor and mature LH/CG-R expression was concomitant with an
627 increase in hCG (38kDa) and hPL (22 kDa) expression. **(B):** immuno-precipitation and ligand-blot
628 analysis. Cellular extracts were purified by immobilized anti-receptor antibody. Eluates were analyzed
629 by SDS-PAGE and immunoblotting using the receptor-specific antibody. A 90 kDa band
630 corresponding to the mature form of LH/CG-R (m) and a major band with a molecular mass of 75 kDa
631 corresponding to the precursor (p) were observed. Incubation of the IP blot with labeled ^{125}I -hCG (10^{-6}
632 ^{11}M) revealed a major radioactive band at a molecular weight of 90 kDa, which was not detected when
633 the blot was incubated with an excess of unlabeled hCG (10^{-6} M). Figures A and B illustrate one
634 experiment representative of five.

635

636 **Fig. 4 LH/CG-R mRNA expression during *in vitro* trophoblast differentiation. (A):** diagram
637 showing the seven transmembrane domains of the LH/CG-receptor and the location of the primers sets
638 used in this study. The two sets of primers (P1 and P2) are located on the extracellular domain. P1
639 amplifies a fragment of 647-bp in the exons 2-9 and P2 amplifies a fragment of 282-bp in the exons 1-
640 5. **(B):** Ethidium bromide-staining gel of one representative of five independent experiments. Semi-
641 quantitative RT-PCR experiments with both the primers P1 and P2 shows respectively a 647-bp and a
642 282-bp amplified fragment. RT-PCR products were separated on 1.8% agarose gel and analysed by
643 densitometry. Sequencing confirmed that both the 647-bp and the 282-bp fragments are part of the
644 LH/CG receptor. **(C):** histograms represent the normalization of LH/CG-R mRNA by actin mRNA
645 after RT-PCR with primers sets P1 (upper histogram) and P2 (lower histogram). Data are expressed as
646 mean \pm SEM of five independent experiments similar to the one shown in B. bp: base pairs; ***: $p <$
647 0.0001.

648

649 Fig. 5 **Scatchard analyses of ^{125}I -hCG binding to trophoblasts during *in vitro* differentiation.**
650 Binding was performed for 30 minutes at room temperature, on cells at 24 hours (\square) or 72 hours (\bullet)
651 of culture. The apparent dissociation constants (Kd) and the maximum number of molecules bound per
652 mg of protein at 24 hours and 72 hours of culture were calculated by the LIGAND program (lower
653 table). Results are expressed as the mean \pm SEM of three experiments.

654

655 Fig. 6 **Intracellular cAMP production after LH/CG-R stimulation during *in vitro* trophoblasts**
656 **differentiation.** Stimulation of cells at 24 hours and 72 hours of culture was performed with 10^{-8} M of
657 hCG or with epinephrine (used as a positive control) for 20 min and compared to non stimulated cells
658 (0). **: $p < 0.005$ and ***: $p < 0.0001$

659

660 Fig. 7 **Immunolocalization of LH/CG-R, hCG and cytokeratin 7 in villous sections. (A):**
661 immunohistochemical staining of LH/CG-R, using the polyclonal antibody (H50) raised against the
662 extracellular domain. Villous cytotrophoblasts (CT), syncytiotrophoblast (ST) and perivascular cells
663 (VC) of the villous core were positively stained. **(B):** a strong immunostaining of hCG was observed
664 in the syncytiotrophoblast. **(C):** immunostaining of cytokeratin 7 was mainly located in
665 cytotrophoblasts layer. **(D):** No staining was observed in control sections treated with non specific
666 isotypic immunoglobulins.