Trophoblast differentiation in vitro: establishment and characterisation of a serum-free culture model for murine secondary trophoblast giant cells

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Abstract

Differentiation of trophoblast giant cells is an early event during the process of murine embryo implantation. However, differentiation of secondary trophoblast giant cells in the rodent is still only partially understood, probably because of the lack of suitable in vitro models and cell markers. In order to advance our understanding of trophoblast differentiation, suitable in vitro models and markers are required to study their development. The objectives of this study were to establish and characterise a serum-free in vitro model for murine secondary trophoblast cells. Secondary trophoblast giant cells growing in vitro and paraffin sections of day 8.5 postcoitum mouse embryos were processed for immunostaining to establish the expression of potential markers using antibodies to blood group antigens, E-cadherin, α7 integrins and activator protein-γ, as well as placental lactogen-II. Within 3 days in serum-free culture, ectoplacental cone-derived secondary trophoblast cells underwent simultaneous induction of both morphological and functional differentiation. Secondary trophoblasts grew in vitro as a monolayer of cells with giant nuclei and expressed B and Le-b/Le-y blood group antigens, α7 integrins and placental lactogen-II, as well as activator protein-γ. Transcripts for activator protein-γ and placental lactogen-II were detected in cultures by RT-PCR and for placental lactogen-II by in situ hybridisation. At later time-points apoptosis increased. A fibronectin substrate significantly increased secondary trophoblast cell numbers and surface area of outgrowth. The increase in cells with giant nuclei coincided with induction of placental lactogen-II expression. A relationship was found between the nuclear area of secondary trophoblast cells and expression of placental lactogen-II.


Introduction

The outermost cells of the mammalian blastocyst, the epithelial trophectoderm, are composed of mural cells, away from the inner cell mass and polar cells overlying it. Trophoblast giant cells (TGCs) differentiate from both polar and mural trophectoderm but at different times during pregnancy in the mouse. Shortly after implantation (4.5 days postcoitum; p.c.), mural trophectoderm differentiates into primary TGCs, which later form an anastomosing network of blood sinuses at the periphery of the embryo. Acting as a part of the yolk sac placenta, TGC-derived blood sinuses facilitate diffusion of O2 and nutrients from the maternal circulation into the embryo.

Secondary TGCs arise from the outermost layer of the polar trophectoderm-derived ectoplacental cone (EPC), and later form the outermost layer of chorioallantoic placenta (Copp 1979, Cross et al. 1994, Cross 2000). The central cells of the EPC differentiate into the spongiotrophoblast that forms the intermediate layer of the placenta and secondary TGCs (Zybina & Zybina 1996, Cross 2000). Trophoblast differentiation and invasion have been modelled in vitro using embryos or explants cultured on substrates of extracellular matrix molecules. In vitro cultured blastocysts attached to fibronectin (FN), laminin or collagen substrates, forming primary TGCs after the trophectoderm–trophoblast transition (Arman et al. 1986a,b, Farach et al. 1987, Sutherland et al. 1988). In other studies, isolated day 7.5 EPC explants were cultured on a laminin (Romagnano & Babiarz 1990) or an FN (Sutherland et al. 1991, 1993) substrate and used as a model to study secondary TGC adhesion, migration and invasion into the uterus in the mouse. Paras et al. (2001) used a similar culture model to investigate the alterations of cytoskeleton organisation during the differentiation of secondary TGCs.
The process of trophoblast attachment and outgrowth in vitro is thought to be analogous to the adhesion and invasion stages of trophoblast development in utero (Sherman & Atienza-Samols 1978, Enders et al. 1981). During their growth in vitro, EPC explants first extend processes to attach to the culture substrate. Then they form secondary TGCs that spread as a monolayer of flattened cells (Romagnano & Babiarcz 1990). TGCs are identified as those cells that have characteristic large nuclei due to DNA endoreduplication (Zybina & Zybina 1996). Moreover, secondary TGCs can be distinguished from primary in vivo by their larger and irregular-shaped nuclei as well as by being multinucleated. Primary TGCs are mononucleated and have regular and smaller nuclei.

A number of cytoplasmic and cell surface molecules have been used as markers to identify TGCs in vitro and in vivo. Secondary TGCs express activator protein-2γ (AP-2γ) in vitro (Richardson et al. 2001) and in vivo (Sapin et al. 2000, Auman et al. 2002). In the mouse, AP-2γ regulates chorionic gonadotrophin-β (Johnston & Jameson 1999) and placental lactogens (Richardson et al. 2000), and has been shown to control trophoblast proliferation and differentiation in the mouse (Welting & Schorle 2002). Certain markers are selectively expressed on extra-embryonic cells of the peri-implantation embryo. For instance, blood group B antigen is expressed from the late morula stage as the outer cells of the embryo differentiate as trophoderm, on blastocyst trophectoderm and after implantation by primary and secondary TGCs and parietal endoderm (Brown et al. 1993), while Le-γ is expressed by the trophoderm at and after implantation, and to a very limited extent by ectoderm (Fenderson et al. 1986, Kimber 1990). These cell surface carbohydrate epitopes may play a role in cell interactions during early development and in the immune system (Kimber 1990, Springer 1990). Moreover, trophoderm and TGCs express α2 integrins (laminin receptors) in the early postimplantation mouse embryo which may facilitate their invasion into the uterine stroma (Sutherland et al. 1993, Klaffky et al. 2001). On the other hand, E-cadherin, a member of the cadherin family of adhesion molecules, is expressed by embryonic ectoderm, trophoderm and labyrinthine trophoblast cells, but is absent on TGCs in the rat (Reuss et al. 1996).

Differentiation of TGCs is characterised by exit from the cell cycle leading to DNA endoreduplication and formation of characteristic giant nuclei (Barlow & Sherman 1972, Snow & Ansell 1974, Cross 2000). Differentiated TGCs also produce metalloproteinases (Rinkenberger et al. 1997) and several members of the placental proline gene family, including placental lactogen (PL-I) (Faria et al. 1990, Peters et al. 2000), PL-II (Campbell et al. 1989, Linzer & Fisher 1999) and proliferin (Linzer & Nathans 1985).

Primary TGCs synthesise PL-I from early to mid pregnancy in the mouse, while PL-II expression serves as an endocrine marker for secondary TGCs in the mid- to late-gestation period (Lin & Linzer 1998). PL-1 and PL-II target the ovary and act to maintain the corpus luteum of pregnancy and stimulate luteal progesterone production (Glosy & Talamantes 1995, Linzer & Fisher 1999), as well as to promote mammary gland development and lactation (Colosi et al. 1988).

The PL-II is a non-glycosylated single-chain polypeptide with a molecular size of about 25 kDa. It has significant amino acid sequence homology with PL-I (Duckworth et al. 1986, Jackson et al. 1986). In the mouse, PL-II appears in the maternal circulation on day 9, increases until about day 14 and then either remains relatively constant in some strains of mice or continues increasing until the end of pregnancy in others (Soares & Talamantes 1983). Production of PL-II is controlled by several proteins such as epidermal growth factor (Yamaguchi et al. 1992), interleukin-1 and -6 (Yamaguchi et al. 1996) and decidua-derived calcyclin (Richard et al. 1998).

In this study, we have developed a serum-free model for secondary trophoblast outgrowth and differentiation in vitro based on those established by Romagnano & Babiarcz (1990) and Sutherland et al. (1991, 1993). We have used a panel of molecular markers to identify secondary TGCs in vitro and in vivo and track TGC differentiation and the relationship between secondary trophoblast nuclear area and PL-II expression. We have also used identification of apoptotic cells to monitor cell survival in vitro.

Materials and Methods

Animals

All embryos were produced by natural mating of MF1 female mice (7–12 weeks of age) with stud males of the same strain (Harlan Olac Ltd, Bicester, Oxon, UK). Females were caged with males for mating and examined the following morning (day 0.5 of pregnancy) for the presence of vaginal plugs to confirm that mating had occurred. Mice were kept under a standard 12 h light:12 h dark cycle with free access to water and food.

Culture of EPC explants

For culturing EPC explants, day 8.5 p.c. pregnant mice were killed by cervical dislocation. EPCs were dissected as described previously (Hogan et al. 1994). Briefly, decidual capsules were dissected from the uterus and placed in Dulbecco’s modified Eagles’ medium (DMEM) (Invitrogen, Paisley, Strathclyde, UK), with 10% (v/v) heat-inactivated foetal calf serum (Sigma, St Louis, MO, USA). The decidual capsules were split open to isolate the whole embryos from the surrounding decidual tissues. For separation from the whole embryos, EPCs were dissected at the junction with the extraembryonic ectoderm and separated from attached TGCs with fine sterilised needles.

Secondary TGC outgrowth in serum-free culture

Isolated EPC explants were incubated for 3 days either on bovine serum albumin (BSA)- or 100 μg/ml FN-coated
coverslips (Invitrogen) in a 24-well plastic tissue culture plate (Nunc, Roskilde, Denmark). Each well contained 0.5 ml culture medium. The culture medium consisted of DMEM supplemented with 20 mg/ml l-glutamine and penicillin–streptomycin (60 μg/ml and 50 μg/ml) (ICN Pharmaceutical, Basingstoke, Hampshire, UK), and 2% (v/v) Nutridoma-HU (a growth factor-free growth-supporting nutrient; Roche GmbH, Mannheim, Germany).

The time and percentage of EPC attachment to substrates were recorded at regular intervals after plating, as described for blastocyst outgrowth by Sherman (1988). Briefly, an EPC explant was considered to be attached when it remained attached to the coverslip when the culture plate was tapped gently or swirled in a circular motion. A giant cell was defined as one with a nucleus which had a diameter at least double that of nuclei (8–10 μm) from cells at plating. TGCs could also be recognised by the fact that they were nearly detached from the original cell explant but still shared at least 20% of their borders with other cells. Further identification of secondary TGCs was carried out by staining for PL-II and B blood group antigen antibodies (TGC and trophoderm marker respectively), and absence of staining for vimentin (marker for endoderm and mesoderm cells). The number of secondary TGCs was counted and the surface area of outgrowth was measured from the explant centre at regular time-intervals after attachment of the explant (every 6 h for 3 days). Fifty percent of the medium was replaced by fresh medium after 24 h of attachment. The progress of the outgrowth was recorded using a Leitz Dialux inverted phase microscope (Leitz, Wetzlar, Germany).

**Morphological and statistical analysis**

Outgrowth extension was measured as the distance between the farthest migrated, or pioneer, cells and the centre of the explant. A graticule was photographed at the same magnification and used to calibrate printed images. A cross was drawn through the centre of the outgrowth when it remained attached to the coverslip when the culture plate was tapped gently or swirled in a circular motion. A giant cell was defined as one with a nucleus which had a diameter at least double that of nuclei (8–10 μm) from cells at plating. TGCs could also be recognised by the fact that they were nearly detached from the original cell explant but still shared at least 20% of their borders with other cells. Further identification of secondary TGCs was carried out by staining for PL-II and B blood group antigen antibodies (TGC and trophoderm marker respectively), and absence of staining for vimentin (marker for endoderm and mesoderm cells). The number of secondary TGCs was counted and the surface area of outgrowth was measured from the explant centre at regular time-intervals after attachment of the explant (every 6 h for 3 days). Fifty percent of the medium was replaced by fresh medium after 24 h of attachment. The progress of the outgrowth was recorded using a Leitz Dialux inverted phase microscope (Leitz, Wetzlar, Germany).

**TUNEL assay**

The terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labelling (TUNEL) was carried out using a TUNEL kit (Roche) and following the company’s protocol. Briefly, EPC trophoblast cells were fixed in 4% (w/v) paraformaldehyde (PFA; Sigma) in phosphate-buffered saline (PBS; Oxoid Ltd, Basingstoke, Hampshire, UK), and washed in a PBS/PVP wash (PBS, pH 7.4 containing 3 mg/ml polyvinylpyrrolidone (PVP) (Sigma)). They were permeabilised in PBS containing 0.5% Triton X-100 (Sigma) for 60 min at room temperature, then washed in PBS/PVP. The terminal deoxynucleotide transferase (TdT) reaction mixture was prepared by mixing 10 μl TdT (enzyme solution) with 90 μl dUTP-fluorescein isothiocyanate (FITC) labelling solution. Outgrowths were incubated with the TdT reaction mixture for 60 min at 37°C in the dark before washing in PBS containing 0.5% Triton X-100, then in PBS/PVP. They were then mounted in Vectashield containing 4,6-diamidino-2-phenylindole hydrochloride (DAPI; Vector Laboratories, Burlingame, CA, USA). Controls were performed as above but without adding enzyme solution to the reaction mix. A positive control was also included by incubating outgrowths with 3 units/ml DNAse I (Roche). A trophoblast cell was considered apoptotic when showing fragmented nuclei (with blue channel for DAPI) which were also green-fluorescence labelled. The percentage of apoptotic cells was calculated from three sets of experiments performed on different occasions using three explant cultures in each set (excluding controls).

**Immunofluorescence of outgrowths**

Trophoblast outgrowths were fixed in fresh 4% PFA for 10 min and washed in PBS. For staining with antibody to α2 integrins, outgrowths were fixed in 1% PFA for 10 min followed by another fixation in methanol for 8 min at −20°C. For cytoplasmic antigens, outgrowths were permeabilised in 0.01% Tween X-100 in PBS and blocked in normal goat serum (1:20 in PBS; Sigma). Outgrowths were incubated overnight at 4°C with a primary antibody as described in Table 1, or with irrelevant antibody of the same isotype as a negative control. The following day, outgrowths were washed and incubated with the appropriate FITC-conjugated secondary antibody for 1 h at room temperature. Outgrowths were washed, mounted in Vectashield containing DAPI and photographed using an Olympus Vannox microscope (Olympus Optical Ltd, Tokyo, Japan) with epifluorescence capacity.

**Immunofluorescence of sections**

Day 8.5 p.c. mated females were killed by cervical dislocation. Dissected uterine horns were fixed in fresh 4% PFA overnight at 4°C. Dissected decidua were dehydrated, cleared and then embedded in paraffin wax (BDH, Poole, Dorset, UK). Paraffin sections were cut at 5 μm thickness and mounted on cleaned slides. Sections were de-paraffinised in xylene (Genta Medical, York, Yorks, UK), hydrated through a series of ethanols and washed in distilled H2O before processing for antigen retrieval using microwave treatment. Sections were left to cool at room temperature, then treated with the following: 50 mM NH4Cl (BDH) in PBS for 30 min and 1%
(w/v) BSA (Sigma), 0.2% (w/v) gelatine (Sigma) and 0.05% (w/v) saponin (Sigma) in PBS three times for 10 min. Sections were then incubated overnight at 4°C with the appropriate primary antibody (Table 1) or irrelevant antibody. On the next day they were rinsed in 0.1% BSA, 0.2% gelatine and 0.05% saponin in PBS three times for 10 min before being incubated with the appropriate secondary antibody (1:125) diluted in 0.1%

Table 1 Antibodies used in this study.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specification</th>
<th>Dilution</th>
<th>Antigens</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-2γ</td>
<td>Rabbit IgG</td>
<td>1:250</td>
<td>AP-2γ</td>
<td>Active motif; Geneka, Rixensart, Belgium</td>
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<tr>
<td>α7 integrin</td>
<td>Rabbit IgG</td>
<td>1:200</td>
<td>α7 integrins</td>
<td>Dr U Mayer; University of Manchester, Manchester, UK</td>
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<tr>
<td>BOO6</td>
<td>Mouse IgM</td>
<td>1:40</td>
<td>B-histo-blood group antigens</td>
<td>BioCarb; Lund, Sweden</td>
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<tr>
<td>Decma-1</td>
<td>Rat IgG</td>
<td>1:50</td>
<td>E-cadherin</td>
<td>Sigma</td>
</tr>
<tr>
<td>HOO1</td>
<td>Mouse IgM</td>
<td>1:30</td>
<td>Le-b/Le-y-blood group antigens</td>
<td>MonoCarb; Lund, Sweden</td>
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<tr>
<td>PL-II</td>
<td>Rabbit IgG</td>
<td>1:250</td>
<td>PL-II</td>
<td>Chemicon, Harrow, UK</td>
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</tbody>
</table>

AP, activator protein.
BSA, 0.2% gelatine and 0.05% saponin for 1 h at room temperature. Sections were washed first in 0.1% BSA, 0.2% gelatine and 0.05% saponin three times for 10 min, then in PBS three times for 10 min and finally in distilled H₂O. Sections were mounted in a hydrophilic mounting media, Vectashield.

RNA extraction

Total RNA was extracted from secondary TGCs growing in culture using Qiagen RNeasy total RNA mini kit (Qiagen, Crawley, West Sussex, UK) following the manufacturer’s protocol. To ensure extraction of RNA from trophoblast outgrowths but not from the central explants, the latter were separated from the culture using sterile fine needles. Quantification of the RNA was carried out by measuring the absorbance at 260 nm in a u.v. spectrophotometer (GeneQuant DNA/RNA Calculator; Pharmacia Biotech, St Albans, Herts, UK). The purity of the RNA was assessed by measuring the ratio of the absorbance at 260:280 nm. RNA was considered pure when A_{260}/A_{280} ≥ 2.0.

RT-PCR

Relative changes in AP-2γ and PL-II mRNA were examined during 3 days of culture using RT-PCR. Changes in PCR products obtained for AP-2γ and PL-II were normalised by comparison with two housekeeping genes: glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) and S28.

First-strand cDNA synthesis

For annealing with the oligo-(dT) primer, 2 μg template RNA was incubated with 1 μg oligo-(dT)₁₂–₁₈ primer (0.5 μg/μl; Invitrogen) and sterile H₂O to make a total volume of 10 μl. The mixture was heated at 70°C for 10 min and chilled on ice. To allow cDNA synthesis, the following reagents (all from Invitrogen) were added to the mixture: 4 μl 5× strength first-strand buffer (250 mM Tris–HCl (pH 8.3), 375 mM KCl and 15 mM MgCl₂), 2 μl 0.1 M dithiothreitol, 1 μl dNTPs (10 mM each dNTP) and 1 μl moloney murine leukaemia virus reverse transcriptase (M-MLV RT; 200U). The reaction volume was made up to 20 μl by adding 2 μl sterile H₂O, then incubating at 37°C for 1 h. The RT reaction was inactivated by heating samples to 95°C for 5 min, cooling on ice and storage was at −20°C. A negative control omitting the M-MLV RT was run in parallel in all reactions. cDNA concentration was calculated by measuring the absorbance at 260 nm in a u.v. spectrophotometer.

PCR

An initial titration was carried out to determine that amplifications at the selected cycle number were within the linear range. All primer sets were designed to span at least one intron to ensure that cDNA, not genomic DNA, was being amplified. Prior to PCR, primers were optimised to adjust their annealing temperatures and times following Qiagen’s protocols. PCR was carried out with an Eppendorf mastercycler gradient (Hamburg, Germany) using primers and cycle numbers shown in Table 2. PCR amplification was performed using HotStarTaq DNA polymerase kit (Qiagen) following the manufacturer’s protocol. Briefly, 5 μl diluted cDNA (≤ 1 μg/reaction volume) was added to a mixture containing 5 μl 10× strength PCR buffer, 10 μl 5× strength Q solution (a PCR additive that facilitates template amplification by modifying DNA melting behaviour), 1 μl dNTP mix (200 μM of each dNTP), 0.25 μM appropriate primers (Table 2), 0.25 μl HotStarTaq DNA polymerase (2.5 units/reaction) and distilled H₂O to a total reaction volume of 50 μl. This mixture was incubated in the thermal cycler under the following conditions: denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and primer extension at 72°C for 1 min. PCR products were separated on 2% (w/v) agarose gels for size assessment. Gels were recorded using a Polaroid dual-intensity transilluminator camera (UVP Inc., San Gabriel, CA, USA).

DNA sequencing

To confirm identity, amplified DNAs were subcloned into a plasmid TA cloning vector (pCRII; Invitrogen) and sequenced using an automated ABI DNA sequencer (ABI Analytical, Ramsey, NJ, USA). The sequencing results were verified by a BLAST search.

Table 2 Primers used in this study.

<table>
<thead>
<tr>
<th>Primer⁴</th>
<th>Primer sequence 5’ → 3’ (base number of primer)</th>
<th>Product size (bp)</th>
<th>No. of cycles</th>
<th>Gene Bank no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-2γ</td>
<td>(f) GAGATGGCTCACCCCATAGA</td>
<td>181</td>
<td>28</td>
<td>BC003778</td>
</tr>
<tr>
<td></td>
<td>(r) CAGGGACTGAGCAGAAGGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PL-II</td>
<td>(f) CACCAGACACATCCGAGA</td>
<td>243</td>
<td>28</td>
<td>M14647</td>
</tr>
<tr>
<td></td>
<td>(r) TGACCATGCGACCCACAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>(f) TCTGAGGGCCACTGAAG</td>
<td>200</td>
<td>28</td>
<td>NM008084</td>
</tr>
<tr>
<td></td>
<td>(r) AGGGTTTCTTACTCCCTGAGG</td>
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<td></td>
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</tr>
<tr>
<td>S28</td>
<td>(f) TAAACTGTCTCAGCAGGCTCCT</td>
<td>180</td>
<td>28</td>
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</tr>
<tr>
<td></td>
<td>(r) CAGGGATAAACTGGCTTGGG</td>
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</tr>
</tbody>
</table>

⁴ (f), forward primer; (r), reverse primer.

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In situ hybridisation

Preparation of the probes

Mouse cDNA for PL-II was generated and amplified as described above. The identity of the oligonucleotide sequence for PL-II cDNA was determined by DNA sequencing and BLAST search, which showed 98% homology with the target gene. Further identification of the PL-II cDNA was carried out by restriction enzyme digestion using appropriate enzymes, which resulted in two fragments of the expected size. Purity of PL-II cDNA was assessed using the u.v. spectrophotometer by measuring the ratio of the absorbance at 260:280 nm. PL-II cDNA was considered pure when \( A_{260}/A_{280} = 1.8–2.0 \). To further confirm its purity, PL-II cDNA was run on a 2% agarose gel giving a single distinct band. Before subcloning into the appropriate plasmid, PL-II cDNAs were purified by Gene-clean kit (Q.Biogene, Nottingham, Notts, UK), following the manufacturer’s protocol in order to clean PL-II cDNA from non-specific products.

PL-II cDNA was subcloned into pGEM-T plasmid (Promega, Southampton, Hants, UK), and transformed into the E.coli host strain, XL-1 blue (Stratagene, San Diego, CA, USA) as instructed by the manufacturer. PL-II-ligated plasmids were purified using a Qiagen plasmid miniprep purification kit and analysed for their identity by DNA sequencing and BLAST search, which showed 98% homology with the target gene. Further identification of the PL-II cDNA was carried out by restriction enzyme digestion using appropriate enzymes, which resulted in two fragments of the expected size. Purity of PL-II cDNA was assessed using the u.v. spectrophotometer by measuring the ratio of the absorbance at 260:280 nm. PL-II cDNA was considered pure when \( A_{260}/A_{280} = 1.8–2.0 \). To further confirm its purity, PL-II cDNA was run on a 2% agarose gel giving a single distinct band. Before subcloning into the appropriate plasmid, PL-II cDNAs were purified by Gene-clean kit (Q.Biogene, Nottingham, Notts, UK), following the manufacturer’s protocol in order to clean PL-II cDNA from non-specific products.

Paraffin-embedded tissues

Uterine horns were fixed in 4% PFA and embedded in paraffin wax (Sidhu & Kimber 1999). After dehydration, decidual tissue was transferred to chloroform:ethanol (1:1) and 100% chloroform for 30 min and 1 h respectively and then vacuum embedded. Sections of 7 \( \mu \)m thickness were floated out on diethyl pyrocarbonate (DEPC; Sigma)-treated, warmed H\(_2\)O to remove wrinkles before mounting on 2% (v/v, acetone) aminopropyltriethoxysilane (Sigma)-coated slides.

Pre-hybridisation

Paraffin sections were dewaxed, rehydrated and refixed in 4% PFA in PBS for 20 min. After washing in PBS, sections were treated with proteinase K (20 \( \mu \)g/ml; Sigma) in Tris–EDTA (BDH) buffer for 10 min, washed again in PBS, and then incubated in 0.1 M triethanolamine HCl (pH 8.0; Sigma) containing acetic anhydride (0.25% v/v; Sigma). They were then washed in PBS and dehydrated through ethanol before air-drying.

In situ hybridisation (ISH)

Whole mount ISH on EPC culture

The procedure for non-radioactive whole mount in situ hybridisation was adapted from Conlon & Rossant (1992) and Carney et al. (1993). Briefly, EPC outgrowths were rehydrated in a methanol:PBS (containing 0.1% Tween-20 (PBT)) series of 75%/25%, 50%/50% and 25%/75% (v/v) before washing in PBT. Cultures were then treated with proteinase K (50 \( \mu \)g/ml), washed in PBT containing freshly added glycine (2 mg/ml), then PBT before re-fixing in 0.2% (v/v) glutaraldehyde (Sigma)/4.0% PFA. They were treated with 0.1% (v/v) NaBH\(_4\) before pre-hybridisation by incubating cultures for 4 h in the following buffer (PE): 50% deionised formamide (Sigma), 0.75 M NaCl (BDH), 100 \( \mu \)g/ml tRNA (Roche), 5 mg/ml sodium heparin (Sigma), 1 mg/ml fatty acid free-BSA (Roche) and 1% (w/v) SDS (Sigma) in 10 mM PIPES (Sigma)/1 mM EDTA (BDH), pH 6.8. For hybridisation with the riboprobe, fresh buffer containing DIG-labelled PL-II or \( \beta \)-actin riboprobe (1 \( \mu \)g/ml) was added. Cultures were incubated with the riboprobes overnight at 50°C in humid chambers. The following day, three fresh hybridisation washes, A, B and C were prepared as follows: wash A (= 0.3 M NaCl, 1% (v/v) SDS in PE), wash B (= 0.05 M NaCl, 0.1% SDS in PE) and wash C (= 0.5 M NaCl, 0.1% (v/v) Tween-20 in PE). Cultures were incubated in 75%/25%, 50%/50% and 25%/75% (v/v) hybridisation buffer/wash for 3 min each. They were then incubated twice for 30 min at 50°C in wash A, then in wash B, equilibrated by incubation in RNAse buffer (= 0.5 M NaCl, 10 mM PIPES, pH 7.2, 0.1% (v/v) Tween-20) for 10 min, and then incubated with 0.1 mg/ml RNAse A in RNAse buffer for 30 min at 37°C. Further washings were carried out in wash A and wash B at 50°C for 30 min each, followed by wash C for 20 min at 70°C. Immunological detection of DIG was carried out by incubating EPC cultures with anti-DIG antibody conjugated to alkaline phosphatase (Roche) overnight at 4°C, followed by colour development with nitro-blue-tetrazolium-chloride (Sigma) and 5-bromo-4-chloro-3-indolyl-phosphate (Roche). The reaction was stopped by rinsing in DEPC-treated distilled water. Cultures were then dried and mounted in gelvatol (Fisher Scientific, Loughborough,
Leics, UK). Cells were examined under bright field (Olympus Vannox; Olympus, Tokyo, Japan).

**Paraffin-embedded tissues** In situ hybridisation on paraffin-embedded sections was carried out following the methods of Wilkinson & Nieto (1993) and Sidhu & Kimber (1999). Briefly, sections were incubated overnight at 65°C with either PL-II or β-actin riboprobes at 1 μg/ml in hybridisation buffer containing 50% (v/v) formamide (Sigma), 1% (v/v) SDS, 50 μg/ml tRNA (Roche), 5× strength SSC (pH 4.5) and 50 μg/ml heparin (Sigma). Hybridisation was performed in sealed humidified chambers to prevent sections from drying. The following day, sections were given a series of stringent washes to reduce background. Immunological detection was carried out as described for EPC cultures.

**Image analysis**

To examine the relationship between nuclear size and PL-II expression, the nuclear area of PL-II expressing and non-expressing cells were measured with the assistance of Image-Pro Plus analysis software (Media Cybernetics, Silver Spring, MD, USA). Nuclear area distributions were determined by measuring the nuclear area of cells in the top right quadrant of outgrowths on each day of culture. Within each field the nuclear area of PL-II-positive cells and unstained cells were measured. For each culture day, 12 outgrowths were analysed in each experiment over six sets of experiments performed on different occasions.

**Results**

Cells of the growing EPC undergo final cell-fate decisions around day 7.5 p.c. in the mouse in vivo (Yan et al. 2001). Therefore, day 8.5 p.c. was chosen for excision of EPC explants to be used as progenitors for secondary TGCs.

**Establishment of an in vitro serum-free culture model for secondary TGC outgrowth**

Our first goal was to determine the substrate requirements for secondary TGCs in order to establish an in vitro model to study their development. When EPC explants were plated on a low concentration FN substrate (10 μg/ml) in the presence of Nutridoma-HU (1%) in the medium, initial attachment was observed after an average time of 17 h (Fig. 1a). A small trophoblast outgrowth was formed only after 2 days of attachment (Fig. 2b). At 1% Nutridoma-HU, increasing the FN concentration to 100 μg/ml did not increase trophoblast outgrowth (Fig. 2c), or significantly reduce the time of initial attachment of the explants (Fig. 1a), suggesting that increasing the FN concentration is not enough to produce further trophoblast outgrowth.

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**Figure 2** Phase contrast micrographs of secondary TGCs on (a) BSA (control)-, (b) 10 μg/ml FN- and (c) 100 μg/ml FN-coated coverslips with 1% Nutridoma-HU in the medium after 2 days of attachment. (d–h) Outgrowths on 100 μg/ml FN substrate were with 2% Nutridoma-HU in the medium: (e) after 12 h of attachment, (d and f) after 30 h of attachment and (g and h) after 2 days of attachment. Arrowheads refer to (f) small rounded cells growing in clusters, (g) elongated/spindle-shape TGCs and (h) multinucleated cells and giant nucleus insert is higher magnification (×400) of (h). Scale bars = (a, b, c and d) 250 μm, (e and f) 50 μm and (g and h) 30 μm.
However, using FN substrate (100 μg/ml) and increasing Nutridoma-HU to 2% in the medium significantly reduced the average time taken for initial attachment of EPC explants to occur, compared with attachment on BSA (control) (Fig. 1a). The majority of EPC explants attached within 26 h; 70% initiated attachment in the period 10.5–26 h after plating (Fig. 1b). In addition, at FN (100 μg/ml) and Nutridoma-HU (2%), a greater number of secondary TGCs as well as an increased surface area of outgrowth (Figs 1c and e and 2d) was observed, compared with outgrowth on BSA-coated coverslips (Figs 1c and e and 2a).

After 30 h of attachment, the rate of trophoblast outgrowth was greatly reduced, probably due to consumption of essential nutrients from the medium (data not shown). Replacing 50% of the culture medium after 24 h of culture maintained a continuous increase of trophoblast outgrowth (Fig. 1c–e).

**Morphology of secondary TGCs in culture**

EPC outgrowths exhibited different cell morphologies and patterns of growth during their development in culture. After about 12 h of attachment, EPC explants extended long processes on the surface of the culture substrate (FN; 100 μg/ml) (Fig. 2e). Outgrowing cells formed a radial outgrowth (Fig. 2d), which appeared as a monolayer sheet of cohesive cells after approximately 30 h of attachment (Fig. 2f). Regions on the periphery of the EPC explant did not appear to produce outgrowing cells equally at the start of outgrowth (Fig. 2e). However, trophoblast outgrowth appeared to be formed from all around the EPC explant by approximately 30 h of attachment (Fig. 2d and f).

The morphology of secondary TGC-like cells was diverse during their development in culture. By 24 h of EPC attachment, cells were small, rounded and growing in clusters (data not shown). At 30 h of attachment, cells became flattened with a pavement-like morphology (Fig. 2f). Some cells also grew in clusters at the periphery of the outgrowth (Fig. 2f). Fibroblast-like trophoblast cells were shown in culture after 2 days of attachment (Fig. 2g). Many outgrowing cells showed large cell size and characteristic, easily distinguished giant nuclei (Fig. 2h), a characteristic of TGCs. Some cells were also multinucleated (Fig. 2g and h) and aggregated together to form cell islands (Fig. 2h), suggesting that these cells were TGCs.

**Trophoblast cell death in culture**

To examine the differentiation and survival of trophoblast cells after a longer time in culture, we grew EPC outgrowth for 6 days after attachment. Trophoblast cells continued outgrowing during this period but with no significant change in total cell number (data not shown). In addition, the morphology of the cells changed dramatically during days 5 and 6 after attachment. At day 6, EPC outgrowth appeared as a flattened sheet of trophoblast cells which were mainly in close cell–cell contact with an epithelial appearance (i.e. epithelial pavement-like morphology) (Fig. 3a). These cells have large nuclei (Fig. 3a). However, the number of detached, probably apoptotic cells, greatly increased during days 4–6 post attachment.

EPC cultures grown for 1–3 days showed very few TUNEL-positive cells. After 1 day of attachment, the percentage of TUNEL-positive cells was 2% (Fig. 3b) while at 2–3 days, the percentage of apoptotic cells had increased to 6% (Fig. 3b). After 3 days, however, TUNEL-positive cells increased markedly reaching 10%, 12.2% and 15.3% of the total cell population at days 4, 5 and 6 after attachment respectively (Fig. 3b). Consequently, we used day 1–3 EPC cultures in this study.

**Characterisation of secondary TGCs in vitro**

TGCs were morphologically identified by their giant nuclear size. To further identify secondary TGC-like cells, we established a panel of molecular markers to the EPC outgrowth in vitro. We examined the expression of B and Cdk4 genes to identify TGCs. These genes were selected because they are known to be expressed in TGCs. The results showed that both genes were expressed in TGCs, indicating that these cells were TGCs.

**Figure 3** (a) Phase contrast micrograph of secondary TGCs after 6 days of attachment. Note the pavement-like morphology of the cells. Scale bar = 30 μm. (b) Histogram showing the percentage of TUNEL-labelled cells at different days after attachment. Each bar represents the means±S.E.M. of three independent experiments, each using three explants (i.e. a total of nine explants for each day).
Le-b/Le-y blood group antigens, α7 integrins (laminin receptors) and E-cadherin (Figs 4 and 5). mRNA and protein expression of AP-2γ and secondary TGC-specific protein PL-II were also examined (Figs 5, 6 and 7).

To ensure that no other types of cell were growing in the EPC culture, EPC explants were dissected and isolated from attached tissues with care. The absence of staining for vimentin confirmed the lack of mesoderm or endoderm cells in the culture. EPC culture cells stained positively for Le-b/Le-y blood group antigens (Fig. 4a), but were negative for E-cadherin (Fig. 4c). Intense homogenous staining for Le-b/Le-y blood group antigens was mainly on the peripheral cell surface between spreading cells (Fig. 4a). α7 integrin was expressed strongly, in a punctate pattern, at the periphery of cells containing giant nuclei (Fig. 4b). The pattern of expression of B blood group antigens on the trophoblast cell surface changed with time in culture (Fig. 5). In early EPC outgrowths (12 h after attachment), B blood group antigens were expressed at cell surface peripheries between crowded cells in contact (Fig. 5a). After 2 days of attachment, B blood group antigens appeared as punctate staining on the cell surface (Fig. 5b). With increasing cell spreading, staining appeared to have increased and was homogenous at the cell surface periphery with punctate/patch-like staining abundant on the rest of cell surface of more developmentally advanced outgrowths (Fig. 5e). These results suggested that changes in the pattern of distribution of B blood group antigens may be correlated with the reduction of cell–cell adhesion and increasing cell spreading. Control immunocytochemical reactions (irrelevant primary) showed the absence of non-specific staining (Fig. 5f).

To further identify secondary TGCs in culture, we examined gene and protein expression of AP-2γ and PL-II. Transcripts for AP-2γ, a gene acting to regulate TGC proliferation and differentiation, were expressed by secondary TGCs on all 3 days of culture (Fig. 6a). Trophoblast-like cells with giant nuclei showed positive staining for AP-2γ protein (Fig. 6b). AP-2γ stain was localised mainly to the nuclei, with a diffuse reticulated pattern. However, less intense cytoplasmic staining was also observed (Fig. 6b). Control outgrowths showed the absence of non-specific staining (Fig. 6c). A population of trophoblast-like cells with giant nuclei expressed PL-II protein in vitro (Fig. 7). At day 1 of attachment, trophoblast cells spread as cell islands and expressed PL-II mainly at the periphery of the cytoplasm (Fig. 7a). By day 3 of attachment, cytoplasmic staining of PL-II had increased; however, the highest staining intensity was still observed at the cell periphery (Fig. 7b). Cells with small, non-giant nuclei showed no staining for PL-II (Fig. 7a and b). Outgrowths incubated with irrelevant antibody of the same species/isotype showed the absence of non-specific staining (Fig. 7c).
Expression of PL-II transcripts

Changes in the expression of transcripts for PL-II from day 1 to day 3 in culture were examined by semi-quantitative RT-PCR. The expression of PL-II RNA appeared relatively similar at all 3 days of culture as compared with GAPDH and S28 mRNA (Fig. 8a). Expression of PL-II RNA by secondary TGCs was further analysed by in situ hybridisation both in vitro (Fig. 8) and in vivo (Fig. 9). Using a riboprobe specific for PL-II RNA transcripts, PL-II were localised exclusively to secondary TGCs in vitro (Fig. 8) and in vivo (Fig. 9). The sense probe for PL-II showed no detectable hybridisation (Figs 8c and 9a and d). Secondary TGC-like cells spreading as cell clusters/islands exhibited a heterogeneous pattern of staining for PL-II, with signals concentrated in the cytoplasm (Fig. 8b). In contrast, all cells were hybridised with the riboprobe for β-actin (Fig. 8d). PL-II message was detected in secondary TGCs at days 7.5 and 8.5 of pregnancy (Fig. 9b, c, e and f). PL-II signals were more abundant in peripheral trophoblast cells than those growing near the EPC (Fig. 9b and f).
c), suggesting that PL-II expression increases as trophoblast cells are displaced peripherally. A strong hybridisation signal was also observed in secondary TGC islands/clusters (Fig. 9e and f).

**Relationship between nuclear size and PL-II expression**

In preliminary experiments, we observed that the increase in the number of cells expressing PL-II, a marker of functional differentiation of secondary TGCs, coincided with the elevation of the number of cells with giant nuclei, a marker for morphological differentiation of secondary TGCs. Consequently, we assessed the relationship between PL-II expression and giant cell nuclear size during secondary TGC development in culture (Fig. 10).

A computer software-based measurement of nuclear area was used to determine whether nuclear enlargement, the main morphological character of differentiated TGC, was related to the expression of PL-II. The two-dimensional area covered by nuclei was therefore examined in relation to expression of PL-II (Fig. 10). Expression of PL-II was restricted to cultured cells containing large/giant nuclei (Fig. 10). At all culture days, there was a highly significant difference ($P \leq 0.0001$) in nuclear area between PL-II-expressing and non-expressing cells. The observed area covered by nuclei in PL-II-expressing cells was 306–440 $\mu m^2$, while nuclei in non-expressing cells covered a smaller area (30–304 $\mu m^2$) (Fig. 10).

Moreover, examination of EPC cultures by image analysis indicated an obvious shift in the nuclear area of cells over the 3 days of culture (Fig. 10). This shift in nuclear area was associated with the increase of PL-II-positively stained cells with time in culture (Fig. 10).

**Expression of TGC markers in vivo**

The expression of TGC markers, B, Le-b/Le-y blood group antigens, $\alpha_7$ integrins and AP-2$\gamma$ as well as PL-II in the mouse uterus was examined to see whether these proteins were expressed by specific cells at day 8.5 of pregnancy (Fig. 11). Multicellular islands of secondary TGCs showed intense staining for all these markers (Fig. 11), but no immunoreactivity was observed in the uterine cells (Fig. 11). No staining was observed with irrelevant primary controls (data not shown).

**Discussion**

In the present study, we have investigated the development of secondary TGCs in culture using EPC explants isolated from day 8.5 p.c. mouse embryos as a source of secondary TGCs. The current in vitro culture model, like similar secondary TGC culture systems (Romagnano & Babiarz 1990, Sutherland et al. 1991, 1993), showed that EPC explants could attach and form secondary TGC outgrowths on an FN substrate. Outgrowth occurred in the absence of serum or medium conditioned by other cells.
High concentration of FN stimulates secondary TGC outgrowth

Trophoblasts growing on an extracellular matrix (ECM) substrate such as FN in culture can mimic the progression of secondary TGCs along the endometrial basement membrane in vivo (Sherman & Atienza-Samols 1978, Enders et al. 1981, Armant et al. 1986a). FN has also been shown to effectively promote blastocyst attachment and outgrowth of primary (Armant et al. 1986a) and secondary TGCs in vitro (Romagnano & Babiarz 1990). Secondary TGC outgrowth was therefore assessed on FN substrates produced using two different concentrations of FN, and two different concentrations of the serum substitute, Nutridoma-HU, were also tested.

Our study established that the highest concentrations of FN (100 μg/ml) and Nutridoma-HU (2%) stimulate rapid attachment of a high percentage (86%) of EPC explants in vitro. The number of secondary TGCs and degree of trophoblast spreading was also high under these conditions. FN is present in the serum and the basement membranes of the endometrial epithelium and interstitial matrix of uterine stroma (Wartiovaara et al. 1979, Grinnell et al. 1982). Attachment and outgrowth on an FN substrate in vivo and in vitro required trophoblast cells to differentiate and express FN receptors (Armant et al. 1986a). Trophoblast outgrowth on FN substrate in vitro has been suggested to represent a model for trophoblast invasion in vivo where trophoblast cells attach and degrade matrix FN in order to penetrate the stroma (Armant et al. 1986a).

As a cell attachment molecule (Yamada 1983), FN promotes the migration of many cell types, including parietal endoderm (Behrendtsen et al. 1995), neural crest (Bronner-Fraser 1986), primordial germ cells (Ffrench-Constant et al. 1991) and avian precardiac mesoderm (Linsker & Lash 1988). Inhibiting FN interaction with these cells using FN-inhibitory antibodies, RGD-containing peptides or antibodies to FN receptors results in loss of cell migration and reduced outgrowth (Bronner-Fraser 1986, Linsker & Lash 1988, Ffrench-Constant et al. 1991).
Survival of trophoblast cells in culture

Very few cells were lost from the EPC culture system during the first 72 h after attachment. The percentage of apoptotic cells was low and these were located close to the EPC explants. They were absent in the peripheral (pioneer) trophoblast cells that have previously been identified as differentiated TGCs (Guillemot et al. 1994, Cross 2000, Scott et al. 2000). A possible explanation may be the production of anti-apoptotic factor(s) such as parathyroid hormone-related protein (PTHrP) which has been reported to be produced by differentiated secondary TGCs (Karperiien et al. 1996, A K H El-Hashash S J Kimber, unpublished observations), or the presence of other apoptosis-inhibitory factor such as transforming growth factor-α (Brison & Shultz 1998), while EPC outgrowth central cells lack both these factors. Furthermore, cell death may be triggered if cells fail to enter either the mitotic cell cycle or the programme of endoreduplication (Goncalves et al. 2003).

Characteristic morphology of secondary TGCs growing in vitro

In the present study, secondary TGCs grew in vitro as a monolayer sheet or meshwork of flattened cells, in agreement with Romagnano & Babiarz (1990). Multinucleated cells with large and irregular-shaped nuclei were observed as previously reported for secondary TGCs (Zybina & Zybina 1996). The characteristic increase in the nuclear size was reported by Barlow & Sherman (1972) in the mouse to be due to DNA endoreduplication.

Characterisation of the secondary trophoblast outgrowth model

In this study, we have described the expression of a number of antigens that have previously been reported to be expressed by primary and/or secondary trophoblast cells at postimplantation development in the mouse. There are limited specific markers for secondary TGCs. Only PL-II has been used extensively as a specific molecular marker for secondary TGCs. Therefore, we used known general markers for trophoblast cells, e.g. blood group antigens, α7 integrins and AP-2γ, after ensuring that cultures were free from other embryonic cell types. Generating a culture model for secondary TGCs free of other cell types was achieved by careful isolation of EPC explants from attached embryonic and extraembryonic tissues, and confirmed by lack of staining of EPC culture cells for vimentin and negligible E-cadherin.

The distribution patterns of B and Le-b/Le-y blood group antigens and α7 integrins were fairly similar. Blood group antigens and α7 integrins were localised mainly to cell peripheries with some punctate staining on cell surfaces.
Le-b/Le-y blood group antigens have been shown to be expressed on the surface of TGCs at pre- and postimplantation stages, while B blood group antigens are expressed at day 6–7 p.c. by both primary and secondary TGCs as well as parietal endoderm (Kimber 1990, Brown et al. 1993). A change in the distribution of B blood group antigens was observed from mainly restricted to the cell margins to covering the entire cell surface. Whether this relates to the reduction of cell–cell adhesion and increase in cell spreading remains to be determined.

Sutherland et al. (1993) and Klaffky et al. (2001) previously reported that α7β1 integrin receptors are expressed on trophectoderm cells at late blastocyst and on both primary and secondary TGCs until day 8.5 p.c. They are essential for trophoblast–laminin interactions that facilitate trophoblast invasion into the uterine stroma during implantation. In agreement with these authors, we found secondary TGCs expressed intense α7 integrins both in vivo and in our cultures. Our study demonstrated that E-cadherin protein expression was extremely low or undetectable in secondary trophoblast outgrowth in vitro. Similar results were shown in other mammalian embryos where downregulation of E-cadherin was coincident with the differentiation of TGCs in the rat (Reuss et al. 1996), and with the differentiation of non-invasive cytotrophoblast into invasive trophoblast in the human (Coutifaris et al. 1991, Floridon et al. 2000).

Secondary TGCs cultured from day 8.5 p.c. EPC explants expressed PL-II in their cytoplasm in keeping with previous in vivo and in vitro studies. PL-II has been used as a specific marker for secondary TGCs (Linzer & Nathans 1985, Soares et al. 1991). PL-II produced by secondary TGCs has a lactogenic effect and promotes mammary gland growth (Robertson et al. 1982). It enhances the expression of both oestrogen receptors α and β (Tellier et al. 1998), and stimulates luteal progesterone production (Linzer & Fisher 1999). We also demonstrated the expression of AP-2γ in the nuclei of secondary TGCs in vitro. AP-2γ protein has been shown to be present in murine secondary TGCs in vivo (Sapin et al. 2000, Auman et al. 2002), and detected at the RNA level in human cytotrophoblast cells in vitro (Richardson et al. 2001). As an upstream regulator of several trophoblast-specific genes, AP-2γ has been suggested to regulate the genetic programmes controlling murine trophoblast proliferation and differentiation (Werling & Schorle 2002). In other studies,

![Figure 9](image_url)
AP-2γ has also been shown to control murine adenosine deaminase gene expression (Shi et al. 1997, Shi & Kellems 1998), and human placental lactogen (Richardson et al. 2000), as well as chorionic gonadotrophin-β (John-son & Jameson 1999).

**Morphological and functional differentiation of secondary TGC in vitro**

EPC-derived secondary TGCs differentiate in a serum-free medium in vitro and the degree of their differentiation increased with time after attachment. This was shown morphologically by increasing numbers of cells with giant nuclei, and functionally by increasing numbers of PL-II-expressing cells. Our results relating cell morphology and nuclear area to PL-II expression confirm in vitro differentiation of secondary TGCs. If secondary TGCs differentiate normally in culture, one can expect PL-II to be expressed only by cells with giant nuclear size, but not by small nuclei cells. This expectation was investigated quantitatively using image analysis to measure the nuclear area of EPC trophoblast cells. PL-II-expressing cells were found to have significantly ($P < 0.0001$) larger nuclear areas than non-expressing cells. These results were in keeping with those reported in similar studies by Carney et al. (1993) on cultured isolated EPC explants, and by Faria & Soares (1991) on Rcho1 cells. Although our data showed a distinct relationship between nuclear area and PL-II expression by secondary TGCs, they did not demonstrate cause and effect. Increasing cell DNA content of TGCs reported here and by others in vitro (Goncalves et al. 2003) and in vivo (Barlow & Sherman 1972) will result in...
not only in nuclear enlargement but changing nuclear/cytoplasmic volume and surface ratio (Ilgren 1980, Kuhn et al. 1991). These changes have been suggested to alter the availability or effective concentrations of differentiation-regulatory molecules within the trophoblast cell (Carney et al. 1993).

Several reports identified the cells of trophoblast outgrowth growing close to the EPC explant as proliferative stem cells and the peripheral cells as differentiated secondary TGCs as evidenced by their expression of specific transcription factors (Guillemot et al. 1994, Cross 2000, Scott et al. 2000). In this study, we found that PL-II RNA expression was more abundant in peripheral trophoblast cells than those growing near the EPC in day 7.5 embryos in vivo. These results support a previous suggestion that EPC trophoblast cells differentiate into secondary TGC as they are displaced peripherally (Hoffman & Wooding 1993, Cross 2000). We have also detected a change in the expression of cyclin B1 and cyclin D1 correlating with an increase in the nuclear size as trophoblast cells spread away from the original EPC explant in vitro (A H K El-Hashash & S J Kimber, unpublished observations).

The mechanisms responsible for TGC differentiation are starting to be understood, particularly for primary TGCs. However, factors that trigger secondary TGC differentiation are still poorly defined. Morphological differentiation of TGCs is accompanied by arrest of cell proliferation but continued DNA synthesis (Snow & Ansell 1974, Varmuza et al. 1988, Hoffman & Wooding 1993, Goncalves et al. 2003). DNA endoreduplication and formation of secondary TGCs with giant nuclei are associated with their functional differentiation, i.e. production of PL-II (Soares & Glasser 1987) and progesterone (Glasser et al. 1987). The arrest of cell proliferation may be the first step of trophoblast cell differentiation (Faria & Soares 1991). In the present work, the in vitro induction of secondary TGC differentiation may be due to autocrine factors produced by the trophoblast cells themselves. One potential factor that may induce in vitro differentiation of secondary TGC is PTHrP. PTHrP is produced by secondary TGCs, acts to increase DNA synthesis in vitro (Centrella et al. 1989), and stimulates primary TGC outgrowth in vitro (Nowak et al. 1999). In parallel work, we are studying the role of PTHrP and other factors in regulating secondary trophoblast outgrowth and differentiation using the model system described here (A H K El-Hashash & S J Kimber, unpublished observations).

In conclusion, we have established a serum-free model in which secondary TGCs express differentiated phenotypes with a characteristic time-course on a FN substrate. The differentiated phenotypes include both morphological and functional (i.e. PL-II expression) parameters. In this model, PL-II, AP-2γ and α7 integrins proved to be characteristic markers for secondary TGCs.

Figure 11 Expression of different secondary TGCs markers in day 8.5 mouse embryos in vivo. (a) Haematoxylin and eosin staining. Paraffin sections were stained for (b) B blood group antigens, (c) Le-b/Le-y blood group antigens, (d) α7 integrins, (e) PL-II and (f) AP-2γ. Arrowheads refer to intense staining of secondary TGC islands. Am, amnion. Scale bars = 10 μm.

in vitro, and PL-II protein expression was related to cell nuclear area. Apoptosis was shown to increase in parallel with the differentiation of cells. This culture model can be used to study the factors and mechanisms regulating secondary TGC differentiation.

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