Immortalization of equine trophoblast cell lines of chorionic girdle cell lineage by simian virus-40 large T antigen

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Abstract

Immortalized cell lines have many potential experimental applications including the analysis of molecular mechanisms underlying cell-specific gene expression. We have utilized a recombinant retrovirus encoding the simian virus-40 (SV-40) large T antigen to construct several immortalized cell lines of equine chorionic girdle cell lineage — the progenitor cells that differentiate into the equine chorionic gonadotropin (eCG) producing endometrial cups. Morphologically, the immortalized cell lines appear similar to normal chorionic girdle cells. Derivation of the immortalized cell lines from a chorionic girdle cell lineage was verified by immunological detection of cell-surface antigens specific to equine invasive trophoblasts. The cell lines differed, however, from mature chorionic girdle cells or endometrial cup cells in that they did not produce eCG and did express MHC class I molecules. Thus, these cell lines appear to have been arrested at a stage of development prior to final differentiation into endometrial cup cells. It was also determined that some of these cell lines as well as endometrial cups express the estrogen receptor-related receptor β gene, but not the glial cell missing gene (GCMa) both of which are expressed in the murine and human placenta. Among these cell lines, three (eCG 50.5, 100.6 and 500.1) express eCG α mRNA. Since regulation of eCG α subunit gene is largely unknown, we investigated the signal transduction pathways regulating the eCG α subunit gene. Both activators of protein kinase A (PKA) and protein kinase C (PKC) induced the expression of eCG α subunit expression 3·2 (P<0·05)- and 1·9 (P<0·05)-fold respectively, in the eCG 500.1 cell line. However, activation of these pathways failed to induce eCG β subunit expression. In conclusion, lines of equine trophoblast cells have been immortalized that display markers characteristic of those with the equine chorionic girdle and endometrial cup cell lineage. A subset of these cells expresses the eCG α subunit gene which is responsive to activators of the PKA and PKC signal transduction pathways.


Introduction

Primates and equids are the only species known to synthesize a placental gonadotropin (chorionic gonadotropin or CG) (Pierce & Parsons 1981). As a member of the glycoprotein hormone family, CG is a heterodimer composed of an α and β subunit (Pierce & Parsons 1981, Fiddes & Talmadge 1984). In both primates and equids, the α subunit is common to all glycoprotein hormones and is encoded by a single copy gene (Fiddes & Talmadge 1984, Fenstermaker et al. 1990). Thus, in primates and equids, the common α subunit gene must contain DNA regulatory elements that confer both pituitary- and placenta-specific expression. That placental expression of α is confined to primates and horses is intriguing, as horses are more closely related, phylogenetically, to other ungulates such as cattle and sheep that do not express their α subunit genes in placenta. This suggests that independent evolutionary events have twice given rise to placental expression of the α subunit gene. In fact, previous studies clearly indicate that different genetic mechanisms underlie placental expression of the α subunit gene in primates and horses (Fenstermaker et al. 1990).


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Acquisition of placental expression of the CG β subunit appears to differ in primates and horses. In primates, the CG β subunit is highly homologous to the LH (luteinizing hormone) β subunit but is encoded by different genes (Fiddes & Talmadge 1984). The LH β subunit gene exists as a single copy gene and is expressed solely in the anterior pituitary gland. In contrast, the genes encoding the CG β subunits are arranged in a gene cluster derived by a series of gene duplications of the single LH β gene (Fiddes & Talmadge 1984). The CG β genes are expressed solely in placenta. In horses, however, the CG and LH β subunit polypeptides are identical (Bousfield et al. 1987, Sugino et al. 1987) and are encoded by a single gene (Sherman et al. 1992). Thus, as is the case for the α subunit genes, the equine CG/LH β subunit gene must contain regulatory elements that confer both pituitary- and placenta-specific expression.

Due to the availability of human choriocarcinoma cell lines that produce CG, much has been learned about regulation of placenta-specific expression of the genes encoding the α and β subunits of human CG (hCG) (Nilson et al. 1991). However, the lack of homologous expression systems has hampered efforts to study expression of the equine CG (eCG) α and β subunit genes.

Chorionic gonadotropin production in the horse is confined to the invasive trophoblast. In this regard, the equine invasive trophoblast displays at least 2 distinct phases of development that culminate in the histogenesis of endometrial cups (Allen & Moor 1972, Allen et al. 1973, Ginther 1992). These fetally derived structures found at the base of the gravid uterine horn represent the primary site of eCG production during equine pregnancy. The initial phase of development occurs between days 25 and 32 post-conception and is characterized by differentiation of the chorionic girdle. This morphologically distinct band of cells is formed at the junction of the regressing yolk sac and enlarging allantois and completely circumscribes the developing conceptus. At days 36–38 post-conception, the girdle cells attach to the uterine wall and begin infiltrating the endometrium, characterizing the second stage of development. Between days 40 and 50, the chorionic girdle cells aggregate, hypertrophy, and differentiate into morphologically discrete structures known as endometrial cups. Peak production of eCG occurs between days 50 and 70 of gestation. Eventually, the cups are destroyed by a mechanism that is poorly understood, and eCG production ceases by approximately day 100.

While eCG production in pregnant mares peaks at about day 60, eCG is in fact produced as early as day 33 by the most differentiated cells of the chorionic girdle (Allen & Moor 1972, Allen et al. 1973). This is important, because the embryo is only loosely associated with the endometrium during this period. Thus, the embryo and all of the extra-embryonic membranes can be collected from the uterus non-surgically by uterine lavage (McDowell et al. 1990). Isolated chorionic girdle cells are easily adapted to primary culture and have been maintained for up to 180 days, during which time they continue to secrete eCG and develop a binucleate phenotype characteristic of mature endometrial cup cells (Allen & Moor 1972). Although primary cultures of chorionic girdle cells are an important biological resource, horses are seasonal breeders and generally have a single conceptus per pregnancy. Hence one can only recover a single chorionic girdle from each pregnancy and it takes at least 1 month post mating before the material can be obtained. This precludes routine use of primary cultures for studies that demand frequent availability since establishment of the cultures requires access to pregnant mares. At least one solution to this practical limitation is to immortalize chorionic girdle cells. Toward this end, we have explored the use of a recombinant retrovirus to immortalize equine chorionic girdle cells. Using this approach, we have isolated 12 different G418 resistant clones. As determined immunologically, at least nine of these clones are of chorionic girdle cell origin. Herein we will provide evidence indicating that these clones represent cells arrested in different developmental stages that precede maximal expression of the genes encoding the α and β subunits of eCG. As such, they provide new models for investigating the molecular mechanisms responsible for temporal and spatial patterns of expression of eCG α mRNA.

**Materials and Methods**

**Isolation of chorionic girdle cells**

Two equine conceptuses were obtained by non-surgical uterine lavage at 33 days of gestation (McDowell et al. 1990). An extended Foley catheter equipped with a balloon cuff was introduced trans-cervically into the uterine body. The cuff was inflated to prevent reflux of uterine contents through the cervix. Approximately 1 liter of PBS was introduced into the uterus via the catheter. The ruptured conceptus was flushed out of the uterus through the catheter and the embryo and all of the extra-embryonic membranes were collected in a sterile 1 liter cylinder (McDowell et al. 1990). After several washes in PBS supplemented with antibiotics, the chorionic girdle was identified with the aid of a dissecting microscope and dissected free of the surrounding chorion with fine forceps and iris scissors and rinsed several times in Hank’s balanced salt solution (HBSS) (McDowell et al. 1990). The dissected chorionic girdles from both mares were combined and placed in 10 ml HBSS with 0-25% trypsin and incubated for 20 min at 37 °C with shaking. After 20 min, cells were dispersed by aspiration with a small-bore Pasteur pipette and then centrifuged at 500 g for 10 min. Cells were resuspended in HBSS+0-25% trypsin and incubated for an additional 20 min at 37 °C. Cells were harvested by centrifugation and resuspended in 10 ml HAM’s F12
containing 10% fetal bovine serum (FBS), 5% horse serum (HS), 100 U/ml penicillin, 100 mg/ml streptomycin and plated in 100 mm tissue culture plates.

**Feeder layers and infections**

Details of the retroviral infection protocol have been published previously (Agarwal & Eckert 1990). Briefly, a 3T3 packaging cell line constructed to package and secrete a recombinant retrovirus (Jat et al. 1986, Agarwal & Eckert 1990) encoding the simian virus-40 (SV-40) large T antigen as well as the gene conferring neomycin resistance was irradiated with 6000 rads ($^{60}$Co). The viral feeder cells were then seeded into 100 mm tissue culture dishes. After 72 h in culture, chorionic girdle cells were harvested and added to the 3T3 feeder cells at approximately $2 \times 10^6$ cells per 100 mm plate. Chorionic girdle cells were allowed to grow to confluence and then passaged onto additional feeder cells for 2 weeks.

**Selection of G418 resistant clones**

At the end of 2 weeks, three confluent plates of girdle cells were passaged and plated onto 12, 100 mm tissue culture dishes (approximately 20% confluent). Duplicate dishes of cells then received 10 ml complete medium containing 0, 25, 50, 100, 300, or 500 µg/ml G418. The media were changed 3 days later and every fourth day thereafter. For each dose of G418, individual clones were isolated using cloning cylinders and trypsin. Any remaining colonies were pooled and designated as mixed cultures. Analyses reported herein are confined to individual G418 resistant clones.

**Monoclonal antibodies**

The rat monoclonal antibodies used to characterize the clonal cell lines have been described previously (Antczak et al. 1987, Donaldson et al. 1990, 1992). Antibody F67.1 is specific for eCG, whereas F71.1, F71.8, and 102.1 recognize cell surface antigens found on both invasive (chorionic girdle) and non-invasive trophoblast (allanto-chorion) as well as some maternal tissues. F71.3 reacts with a surface antigen expressed on invasive trophoblast and endometrial cup cells, but not on non-invasive trophoblast of the allantochorion. F71.7 recognizes an antigen on non-invasive trophoblast that is not detected on the invasive trophoblast. WS54 and WS48 are monoclonal antibodies specific for either MHC class I or MHC class II molecules respectively (Kydd et al. 1991). Peroxidase- or fluorescein-conjugated goat anti-mouse IgG and goat anti-rat IgG were obtained from Jackson Immunoresearch (Avondale, PA, USA). Either rat or mouse anti–canine parvovirus were used as negative controls for immunohistochemistry (Donaldson et al. 1990).

**Immunohistochemistry**

Clonal cell lines were grown to confluence in 100 mm tissue culture dishes in complete medium. The adherent cells were harvested by trypsinization for 2–4 min at 37 ºC using 0.25% trypsin, 1 mM EDTA in HBSS. The cell pellet was resuspended at a concentration of 2.5 x 10^5 cells/ml in DMEM containing 20% FBS. Twenty-five thousand cells were placed onto APTS- or poly-l-lysine coated slides using a cytopsin centrifuge. Cytospin slides were washed briefly in PBS, fixed in cold acetone for 10 min and stored at −20 ºC until analyzed by indirect immunoperoxidase labeling.

**Indirect immunoperoxidase labeling**

Cells were incubated for 30 min at room temperature in 70 µl undiluted hybridoma culture supernatant containing the specific monoclonal antibodies (first stage reagent). A mouse or rat monoclonal antibody to canine parvovirus was used to assess non-specific staining. After three washes in Tris-buffered saline (TBS), non-specific binding of the peroxidase-conjugated second stage antibody was blocked by incubation of the cells with 10% normal goat serum. The viral feeder cells were irradiated with 6000 rads ($^{60}$Co). The viral feeder cells were washed briefly in PBS, fixed in cold acetone for 10 min and stored at −20 ºC until analyzed by indirect immunoperoxidase labeling.

**Hematoxylin–eosin staining**

Cells were plated (25 000 cells per chamber) on Lab-Tek II (Nalge Nunc International Corp., Naperville, IL, USA) chamber slides and cultured in growth media (DMEM containing 10% FBS and 5% HS). At 80–85% confluence, cells were fixed using 4% paraformaldehyde solution at 4 ºC for 15 min. Fixation was stopped by adding PBS and washing two additional times with PBS. The slides were subsequently dehydrated and stained with hematoxylin for 30–90 s. Coverslips were permanently mounted using Glycergel and examined with a Nikon Diaphot-TMD inverted microscope.

**RNA isolation and analysis**

Cells were cultured in 100 mm plates until they reached 80–85% confluency as stated above and treated with various concentrations of forskolin or phorbol 12–mymristate 13-acetate (PMA) overnight. Total RNA was isolated using Trizol Reagent (Life Technologies, Inc., Gaithersburg, MD, USA). Total RNA (10–15 µg) was separated by electrophoresis through a 1-0%
formaldehyde–agarose gel and subsequently transferred to a Nytran nylon membrane (Schleicher & Schuell, Keene, NH, USA) using downward capillary action. The transferred RNA was then UV cross-linked and stained with methylene blue to assure equivalent loading of samples. Membranes were pre-hybridized at 42 °C for 12–16 h in 50% formamide/Denhardt’s buffer (5 × Denhardt’s, 6 × SSC, 0.5% SDS, 10% dextran sulfate, 10 mM EDTA and 100 µg/ml denatured ssDNA) and hybridized to random primer-generated 32P-labeled cDNA probes under the same conditions. Non-specific binding of probe was removed by washing with 2 × SSC, 0.1% SDS at room temperature followed by 1 × SSC, 0.1% SDS at 50 °C and subsequently exposed to X-ray film. Following autoradiography, the blots were stripped and re-hybridized with a radiolabeled cDNA probe for equine ribosomal protein L7 as an internal control. Specific signals were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA) and these values were adjusted by taking the ratio of specific signals (such as eα1) to that of the control L7.

Analysis of tumorigenicity in nude mice

Tumorigenicity was tested by subcutaneous injection of 10^5–10^6 cells in 100 µl PBS into nude mice (HSD athymic mice). Mice were examined for tumor formation at weekly intervals for 8–12 weeks following injection.

Statistical analysis

Northern analysis experiments were performed a minimum of three times. Statistics were performed where noted using one-way ANOVA followed by post hoc comparisons of means using Duncan’s multiple range test and/or t-test. P<0.05 was considered as significant.

Results

Establishment of immortalized cell lines

A recombinant retrovirus encoding large T antigen and conferring neomycin resistance (Jat et al. 1986) was used to infect chorionic girdle cells derived from day 33 equine conceptuses. After 2 weeks of growth on 3T3 viral feeder layers, girdle cells were harvested and selection for stable transformants using G418 was initiated. After 3 weeks of selection in G418, independent colonies were apparent at each dose of G418; however, the total number of clones diminished with increasing doses of G418. Selected, well-defined colonies were isolated using cloning cylinders and individually propagated. Isolated clones displaying a phenotype most similar to chorionic girdle cells were analyzed further.

During growth, the cells flatten out on the surface of the plastic dish (Fig. 1). A small population of large binucleate...
or multinucleate cells are apparent. *In vivo*, eCG production is typically associated with endometrial cup cells that display a large, polygonal, binucleate morphology (Allen & Moor 1972). At confluence, the majority of cells are tightly packed, displaying a cuboidal or polygonal, epithelial type morphology similar to primary cultures of chorionic girdle cells.

Immortalization by SV-40 requires the expression of large T antigen (Monier 1986), therefore RNA was prepared from selected clonal cell lines and tested for the presence of large T antigen transcripts by Northern blot analysis. All of the clonal cell lines tested, with the exception of 50.5, were found to express large T antigen mRNA (data not shown). Expression of large T antigen was not evident in either BeWo, NIH 3T3, or CHO cell lines but was evident for αT3 cells, consistent with immortalization of this cell line by large T antigen (Windle *et al.* 1990).

**Figure 2** Representative indirect immunoperoxidase staining of the 50.1 cell line. Approximately 2.5 × 10⁵ cells were incubated with the indicated primary antibody. Following incubation with peroxidase-conjugated goat anti-mouse IgG, slides were developed by immersion in aminoethyl-carbamizol and hydrogen peroxide. (A) Trophoblast-specific, 102.1, (B) endometrial cup-specific, 71.3, (C) allantochorion-specific, 71.7, (D) MHC class I-specific, WS54, (E) MHC class II-specific, 130.8, (F) chorionic gonadotropin-specific, 67.1. Scale bars=35 μm.
One hallmark of acute transformation is the ability to induce tumor formation in nude mice (Monier 1986). Thus, the 50.4 cell line was tested for tumorigenicity. Despite expression of large T antigen, no tumors were detected in four mice injected with 50.4 cells. In contrast, both MCF-7 cells, a human breast carcinoma cell line (Horwitz & McGuire 1980), and Rcho-1 cells, a mouse mammary carcinoma cells (Rcho-1) are cultured in 10% FBS, and co-workers (Peters et al. 1998). When rat choriocarcinoma cells (Rcho-1) are cultured in 10–20% HS, proliferative stem cells differentiate into giant cells and express markers for giant cells. We chose four cell lines (eCG 50.1, 50.8, 100.6, and 500.1) to determine their ability to differentiate towards a binucleate phenotype and express the eCG subunit genes. Interestingly, eCG 100.6 and 500.1 cells cultured in 20% HS had increased expression of eCG α subunit mRNA, but again failed to induce β subunit expression (data not shown).

Table 1 Summary of antibody staining of chorionic girdle cell lines

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>50.1</th>
<th>50.4</th>
<th>50.5</th>
<th>50.7</th>
<th>50.8</th>
<th>50.9</th>
<th>100.2</th>
<th>100.6</th>
<th>500.1</th>
</tr>
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<tbody>
<tr>
<td>W554</td>
<td>Class I MHC</td>
<td>95</td>
<td>80</td>
<td>95</td>
<td>70</td>
<td>var.</td>
<td>80</td>
<td>75</td>
<td>weak</td>
<td>80</td>
</tr>
<tr>
<td>130.8</td>
<td>Class II MHC</td>
<td>20</td>
<td>&lt;1</td>
<td>5</td>
<td>10</td>
<td>mod.</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>&lt;10</td>
</tr>
<tr>
<td>102.1</td>
<td>Trophoblast</td>
<td>55</td>
<td>70</td>
<td>35</td>
<td>35</td>
<td>var.</td>
<td>80</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>71.3</td>
<td>Invasive trophoblast</td>
<td>95</td>
<td>99</td>
<td>95</td>
<td>99</td>
<td>99</td>
<td>&gt;95</td>
<td>100</td>
<td>99</td>
<td>80</td>
</tr>
<tr>
<td>71.7</td>
<td>Allantochorion</td>
<td>0</td>
<td>&lt;1</td>
<td>&lt;10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>&lt;10</td>
<td></td>
</tr>
<tr>
<td>71.1</td>
<td>Trophoblast+</td>
<td>50</td>
<td>50</td>
<td>75</td>
<td>60</td>
<td>&lt;10</td>
<td>10</td>
<td>90</td>
<td>70</td>
<td>80</td>
</tr>
<tr>
<td>71.8</td>
<td>Trophoblast+</td>
<td>70</td>
<td>95</td>
<td>75</td>
<td>35</td>
<td>var.</td>
<td>90</td>
<td>40</td>
<td>90</td>
<td>70</td>
</tr>
<tr>
<td>67.1</td>
<td>eCG</td>
<td>&lt;10</td>
<td>weak</td>
<td>&lt;10</td>
<td>weak</td>
<td>0</td>
<td>&lt;1</td>
<td>weak</td>
<td>20</td>
<td>0</td>
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<tr>
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<td>Non-specific</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
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</tr>
<tr>
<td>R anti-CP</td>
<td>Non-specific</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>0</td>
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</table>

var., variable; mod., moderate.

Analysis of eCG α and β subunit gene expression

Equine chorionic girdle cells are the progenitor cells of the peripheral blood, the site of eCG production during pregnancy. Although mature endometrial cups are not morphologically distinct until about day 40, some differentiated chorionic girdle cells begin to synthesize and secrete small amounts of eCG by day 33 of gestation. Therefore, we examined selected clonal cell lines for eCG production using both immunological methods and by Northern blot analysis.

Immunostaining with antibody 67.1 (anti-eCG antibody) revealed extremely low to non-detectable levels of eCG expression (Fig. 3; Table 1). Northern blot analysis of RNA derived from the same cell lines confirmed these results. As a rule, expression of eCG β mRNA was undetectable for any of the cell lines tested (data not shown) whereas mRNA for eCG α subunit were readily detectable in three of the cell lines (Fig. 3). Cell lines 50.5, 100.6 and 500.1 exhibited high levels of eCG α subunit gene expression. From the results stated above, these cell lines appeared to be arrested at an early stage of differentiation prior to β subunit expression. We attempted to manipulate the culture system toward differentiation by using a method similar to that used by Peters and co-workers (Peters et al. 1998). When rat choriocarcinoma cells (Rcho-1) are cultured in 10–20% HS, proliferative stem cells differentiate into giant cells and express markers for giant cells. We chose four cell lines (eCG 50.1, 50.8, 100.6, and 500.1) to determine their ability to differentiate towards a binucleate phenotype and express the eCG subunit genes. Interestingly, eCG 100.6 and 500.1 cells cultured in 20% HS had increased expression of eCG α subunit mRNA, but again failed to induce β subunit expression (data not shown). The eCG 50.1 and
50.8 lines were not induced to express either subunit gene (data not shown). These data indicate that unlike Rcho-1 cells, these culture conditions do not stimulate differentiation of the equine cell lines.

Expression of trophoblast genes

A number of transcription factors have been identified as playing a role in trophoblast development. A member of the orphan nuclear hormone receptor family, estrogen receptor-related receptor β (ERRβ), is first expressed in extra-embryonic ectoderm at 5–6 days post-coitum (d.p.c.) in mice (Luo et al. 1997). Although ERRα and ERRβ are closely related to the estrogen receptor at the amino acid level, they function quite differently by binding to a half-site of an estrogen response element (AGGTCA) (Heard et al. 2000). Targeted disruption of ERRβ resulted in abnormal chorion development and embryonic death at 10.5 d.p.c. (Luo et al. 1997). Gland cell missing gene (GCMa), a mammalian homolog of the Drosophila gland cell missing gene, is required for gland cell differentiation and is also expressed in embryonic trophoblast. GCMa-deficient mice lack a functional labyrinth layer of the placenta and die at 9–5 d.p.c. In addition, GCMa has been shown to be involved in placenta-specific expression of the human aromatase gene (Yamada et al. 1999). Therefore, we investigated whether these two trophoblast genes were also expressed in equine trophoblast. Expression was evaluated in two of the lines that expressed the α subunit gene (100.6 and 500.1) and a non-expressing line (eCG 50.8) as well as day 38 endometrial cups. All three cell lines expressed ERRβ, and had levels similar to that seen in RNA isolated from endometrial cups (Table 2). In contrast, we were unable to detect the GCMa transcript in any of these clonal cell lines or endometrial cups.

Regulation of eCG α subunit gene expression

To date, the signal transduction pathways regulating expression of the eCG α and β subunit genes remain obscure primarily due to the lack of availability of equine placental cell lines. Since a subset of the clonal cell lines expressed the eCG α mRNA, we investigated the involvement of the protein kinase A (PKA) and protein kinase C (PKC) signal transduction pathways in regulating eCG α subunit gene expression. Treatment with 50 μM forskolin, activator of the PKA pathway, stimulated expression of the eCG α subunit mRNA 3-2-fold ± 0.696 (P<0.05) in the eCG 500.1 (Fig. 4A) and 100.6 cell lines (data not shown). In addition to the PKA pathway, treatment with 10 nM PMA, a classical PKC activator, induced eCG α subunit gene expression 1-9-fold ± 0.131 (P<0.05) in the 500.1 cell line (Fig. 4B). A similar increase was observed in the 100.6 cell line (data not shown). These data suggest that the PKA pathway plays a more prominent role in activation of eCG α subunit gene than does the PKC pathway.

Discussion

The transformation of mammalian cells using cellular oncogenes has proven to be a useful tool for the study of cell function, lineage development and gene expression. In fact, much of our knowledge of gene regulation in endocrine cells is due to the availability of permanent, hormone-producing cell lines. Toward this end we have used a recombinant retrovirus encoding the SV-40 large T antigen to construct several cell lines of equine chorionic girdle cell origin.

Table 2 Expression of trophoblast genes

<table>
<thead>
<tr>
<th>Genes</th>
<th>eCG 50.8</th>
<th>eCG 100.6 or 500.1</th>
<th>Endometrial cup</th>
<th>JEG-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>−</td>
<td>++</td>
<td>+++</td>
<td>++</td>
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<tr>
<td>β</td>
<td>−</td>
<td>−</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>ERRβ</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GCMa</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

−, none; ++, weak; ++++, moderate; ++++, high expression.
The cells of the equine chorionic girdle are the progenitor cells of the endometrial cups. The histogenesis of these cells involves differentiation of non-invasive trophoblasts into invasive trophoblasts, active invasion of the endometrium, and terminal differentiation into mature, typically binucleate, endometrial cup cells. Several lines of evidence support the derivation of the clonal cell lines described herein as being of chorionic girdle cell origin. First, the immortalized cell lines resemble chorionic girdle cells in that they display a cuboidal or polygonal epithelial type morphology. Secondly, each cell line stained intensely with antibody 71.3, which reacts with the invasive trophoblast of the chorionic girdle and endometrial cups, but not with the non-invasive trophoblast cells of the allanto-chorion. Finally, no staining was evident with antibody 71.7, which identifies an antigen expressed by non-invasive trophoblast cells of the allanto-chorion but does not react with invasive trophoblasts. The inverse pattern of antibody reactivity observed with antibodies 71.3 and 71.7 as well as the presence of eCG α subunit mRNA confirms the identity of the cell lines as invasive trophoblast.

Each cell line expressed the horse trophoblast-specific molecule detected by antibody 102.1; however, differences in the intensity of staining suggests variable levels of expression of the antigen. Surface staining by antibody 102.1 has been shown to vary with the developmental stage of horse invasive trophoblast cells; it strongly labels chorionic girdle cells but the staining intensity fades on newly differentiated endometrial cup cells (Oriol et al., 1989). Thus, the variable staining intensities observed for the different cell lines may reflect slight differences in the stage at which girdle cell development was arrested by immortalization.

Similarly, expression of the molecules detected by antibodies 71.1 and 71.8 was highly variable. For some cell lines, there seemed to be an inverse relationship between expression of the molecules detected by these antibodies. For example, antibody 71.1 stains cell lines 50.5 and 50.9 intensely, while labeling by antibody 71.8 was much weaker. The inverse was true for cell lines 500.1, 50.7, and 100.6. As stated above, these results suggest different stages of developmental arrest and that expression of the molecules recognized by antibodies 71.1 and 71.8 may be developmentally regulated on the invasive trophoblast of the horse.

Expression of MHC class I molecules was consistently observed for each of the clonal cell lines examined. Studies of chorionic girdle cells in vivo or in vitro have revealed a significant reduction in class I antigen expression by developing invasive trophoblast cells of the endometrial cups. In general, chorionic girdle cells that label strongly for eCG expression, the phenotype of mature endometrial cup cells, have greatly decreased expression of MHC class I antigens. It has been suggested previously that chorionic girdle cells committed to eCG secretion are also committed to reduction of MHC class I expression to low or undetectable levels. The data presented herein support this hypothesis. That is, the concomitant absence of eCG

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**Figure 4** Induction of eCG α subunit expression in eCG 500.1 cell line following activation of the PKA (A) and PKC (B) pathways. The PKA (A) and PKC (B) pathways were activated following treatment (overnight) with increasing concentrations of forskolin (1, 10, 25, and 50 μM) or PMA (0, 1, 10, and 100 nM) (Sigma Chemical Co., St Louis, MO, USA) respectively. Forskolin induced eCGα mRNA levels in a dose-dependent manner, while only 10 nM PMA induced eCGα mRNA levels significantly. Fold induction is expressed as the mean ± s.e. of each treatment. (*P<0.05).
production by any of the clonal cell lines and generally robust expression of MHC class I molecules suggest developmental arrest of chorionic girdle cells prior to final commitment or maturation to eCG-producing endometrial cup cells.

Since three of the cell lines expressed eCG α subunit mRNA they represent a model to study the mechanisms regulating expression of this gene in trophoblasts as well as the importance of other transcription factors that have been implicated in trophoblast development and function. GCMa has been shown to regulate the expression of the aromatase gene in placenta (Yamada et al. 1999). In addition, a previous study has shown that this protein can bind to the trophoblast-specific element (TSE) within the human α subunit promoter (Yamada et al. 1999). However, the ability of GCMa to transactivate the human α subunit promoter has not been determined. Our data indicate that GCMa is not expressed in the equine clonal cell lines or endometrial cups and, therefore, presumably does not play a role in initiating expression of the eCG α subunit gene. The 5′ flanking region of eCG α subunit promoter harbors a consensus nuclear receptor half-site (AGGTCA) (Farmerie et al. 1997), and so far, two orphan nuclear receptors (ERRβ and γ) have been shown to be expressed in the placenta and are essential for placentation development (Luo et al. 1997, Heard et al. 2000). ERRβ expression is restricted to the chorion during embryogenesis and to a limited number of adult tissues (Pettersson et al. 1996). Therefore, we determined whether the clonal cell lines expressed ERRβ. Our data indicated that ERRβ is expressed fairly abundantly in JEG-3 cells and modestly in the equine clonal cell lines. To date, the role of ERRβ in regulating expression of the human CG subunit genes has not been examined. Because ERRβ is expressed in the clonal cell lines that express the α subunit (100.6 and 500.1) as well as in a non-expressing cell line (50.8), it is unlikely that the ERRβ alone plays a major role in regulating eCG α subunit gene expression. Additional studies are required to determine whether ERRβ might play a role in regulating expression of the α subunit gene in conjunction with other transcription factors and/or co-activators.

The signal transduction mechanisms regulating expression of the human CG subunit genes have been extensively studied and this has been facilitated by the availability of human choriocarcinoma cell lines. In contrast, very little is known about the modulators and mechanisms regulating expression of the eCG α subunit gene. Our studies have identified two signal transduction pathways that induce eCG α subunit gene expression. Furthermore, these data suggest that similar signal transduction pathways may activate the human and equine α subunit genes. The PKA and PKC signal transduction pathways have been shown to regulate the expression of both human CG subunits (Jameson et al. 1987, Milsted et al. 1987, Andersen et al. 1988, Nilson et al. 1989, Albanese et al. 1991). Two previous studies have examined PKA regulation of the eCG α subunit promoter in human placental cell lines (Steger et al. 1991, Farmerie et al. 1997). Steger and co-workers reported that an α-activating element located within proximal 5′ flanking region of eCG α subunit promoter was responsible for PKA induction when evaluated in the JEG-3 human choriocarcinoma cell line (Steger et al. 1991). In contrast, Farmerie and co-workers reported that activation of the PKA pathway did not stimulate activity of the eCG α subunit promoter in the BeWo human choriocarcinoma cell line (Farmerie et al. 1997). Nonetheless, our data strongly suggest that the PKA pathway regulates expression of the endogenous eCG α subunit gene in our clonal cell lines. The ligand–receptor system that activates this PKA pathway remains to be identified. In contrast, the PKC pathway can be activated by various ligand–receptor systems. One possible candidate is the epidermal growth factor receptor (EGFR) system. A potential role for EGFR in placental development and implantation has been previously suggested (Adamson & Meek 1984, Lim et al. 1998). Stewart and co-workers have shown that expression of EGF increases in the uterine endometrium of the pregnant mare at 35 days of gestation (Stewart et al. 1994), which coincides with onset of implantation, placentation and CG secretion. Expression of EGF remains elevated in the epithelium of the endometrial glands until 250 days of gestation (term=320–340 days) (Lennard et al. 1998). Therefore, the possibility exists that the EGF/PKC pathway may function in vivo to regulate eCG α subunit gene expression in the horse. There is also a possibility that basal expression of α subunit may be regulated either in an autocrine manner or by growth factors present in FBS and/or HS, since bisindolylmaleimide-I, a specific PKC inhibitor, reduced basal expression of the eCG α subunit gene by 50% (T Thway & M Wolfe, unpublished data).

In conclusion, we have used a recombinant retrovirus encoding the SV-40 large T antigen to construct several clonal cell lines of equine chorionic girdle cell lineage. Differences in expression of cell-surface antigens suggest these cell lines have been arrested at slightly different stages of development prior to their final differentiation into mature endometrial cup cells, i.e. prior to secretion of eCG. In this regard, while none of the cell lines express the eCG β subunit gene, three of the cell lines express the common α subunit gene. Interestingly, the entrapment of equine chorionic girdle cells prior to final differentiation described in this study is reminiscent of the αT3 gonadotrope cell line established by targeted oncogenesis in transgenic mice (Windle et al. 1990). The αT3 cell line expresses the α subunit gene but neither the LH or FSH β subunit genes. Nevertheless, the αT3 cell line has proven extremely valuable for studying the molecular mechanisms underlying pituitary expression of the α subunit gene. Similarly, the equine chorionic girdle cells described herein should provide a useful model system for analyzing
the differential requirements for placental expression of the equine α subunit gene by multiple signal transduction pathways and possibly other trophoblast-specific genes.

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