Development and function of trophoblast giant cells in the rodent placenta

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ABSTRACT  Trophoblast giant cells (TGCs) are the first cell type to terminally differentiate during embryogenesis and are of vital importance for implantation and modulation of post-implantation placentation. TGCs are mononuclear and polyploid but are heterogenous and dynamic. At least four different subtypes of TGCs are present within the mature placenta that have distinct cell lineage origins. The development of TGCs is complex and requires transition from the mitotic to the endoreduplication cell cycle and is regulated by a wide variety of factors. During early gestation, TGCs mediate blastocyst attachment and invasion into the uterine epithelium, regulate uterus decidualization, and anatomosis with maternal blood spaces to form the transient yolk sac placenta. During later gestation, TGCs secrete a wide array of hormones and paracrine factors, including steroid hormones and Prolactin-related cytokines, to target the maternal physiological systems for proper maternal adaptations to pregnancy and the fetal-maternal interface to ensure vasculature remodeling. The large number of mouse mutants with defects in TGC development and function are giving us significant new insights into the biology of these fascinating cells.

KEY WORDS: pregnancy, hormone, cell cycle, polyploid, endoreduplication

The enigmatic life of the trophoblast giant cell

Trophoblast giant cells (TGCs) are the first terminally differentiated cell type to form during embryogenesis in rodents and are of vital importance for embryo implantation and promoting maternal adaptations to pregnancy. They arise from the trophoderm layer in the blastocyst (Fig. 1), are endocrine in nature and characterized by their extremely large cytoplasm and polyploid nuclei that result from endoreduplication (Zybina and Zybina, 1996). TGCs are best studied in rodents and are usually mononucleated. In mice, there are several subtypes of TGCs with distinct functions that arise at different stages of gestation and in different locations within the placenta (Simmons et al., 2007; Simmons et al., 2008b). Mono-nucleated, bi-nucleated or occasionally multi-nucleated cells with polyploid nuclei have also been identified in the rabbit, vole, human, cow, water buffalo and alpaca placenta (Carvalho et al., 2006; Klisch et al., 2005; Klisch et al., 1999; Klisch et al., 2004; Zybina et al., 1975; Zybina and Zybina, 1985; Zybina et al., 1992; Zybina et al., 2004; Zybina et al., 2002; Zybina et al., 2001). In humans, the polyploid cells are the so-called extravillous cytotrophoblast cells that invade into the uterus (Zybina et al., 2002) and are associated with remodeling of the spiral arteries (Pijnenborg et al., 1980). In addition to polyploidy, many genes that are involved in TGC development and function in rodents are conserved between rodents and humans, such as transcription factors, proteases and cell adhesion molecules (Cross et al., 2003; Rawn and Cross, 2008). Therefore, studies of TGCs should give insights into human gestational diseases that are associated with extravillous cytotrophoblast cells such as preeclampsia and intrauterine growth restriction (Brosens et al., 1977; Brosens et al., 1972). We review here the development and functions of TGCs focusing on insights from mice, discussing the differences between TGC subtypes and implications of their diverse functions.

Characteristics, origins and regulation of TGC development

The mature placenta in rodents is composed of three broad zones including the maternal decidua on the outside, the junc-
The decidua is devoid of trophoblast cells until around mid-gestation when trophoblast cells invade both into the decidua and up the spiral arteries, replacing the endothelium and thereby promoting the transition from endothelial cell-lined to trophoblast cell-lined maternal blood spaces (hemo-chorial) in the placenta (Adamson et al., 2002). The junctional zone consists of spongiotrophoblast cells and a layer of TGCs that line the implantation site. The labyrinth is the region in which nutrient exchange occurs and the bulk of the trophoblast compartment is composed of two layers of multi-nucleated syncytiotrophoblast that arise from cell-cell fusion of post-mitotic cells (Hernandez-Verdun and Legrand, 1975) and not endomitosis or endoreduplication as with TGCs. Four TGC subtypes have been identified in the placenta (Simmons et al., 2007), and include parietal TGC (P-TGC) that line the implantation site and are in direct contact with decidual and immune cells in the uterus, spiral artery-associated TGCs (SpA-TGC), maternal blood canal-associated TGCs (C-TGC), and sinusoidal TGC (S-TGC) that are within the sinusoidal blood spaces of the labyrinth (Figs. 1 and 2). The four subtypes of TGCs are distinguished by their anatomical location and gene expression (Table 1) (Simmons et al., 2007).

**Characteristics of TGCs**

All TGC subtypes share the characteristics that they are large, have polyploid (usually single) nuclei, and are secretory in nature with their content of golgi and endoplasmic reticulum increasing during differentiation (Bevilaqua and Abrahamsohn, 1988). In contrast to proliferating cells, TGCs undergo rounds of DNA replication without intervening mitoses, a process called endoreduplication, and can accumulate DNA up to 1000C (Zybina and Zybina, 1996). The genome of P-TGCs is ‘polytene’, a state in which many sister chromatids are synapsed together resulting from multiple rounds of genome replication (Varmuza et al., 1988; Zybina and Zybina, 1996). While the other subtypes of TGCs have polyploid nuclei (Simmons et al., 2007), it is unclear whether their nuclei are polytene. Consistent with their large cytoplasm and extensive rough endoplasmic reticulum, TGCs secrete a variety of proteins including extracellular matrix, cell adhesion molecules, proteinases, cytokines and hormones (see below).

The importance of endoreduplication and polyploidy for TGC function is a matter of speculation. Polyploid nuclei have been identified in many plant and animal cells with secretory or nutritive function such as salivary gland and follicle cells in *Drosophila melanogaster*, and leaf cells in plants (Edgar and Orr-Weaver, 2001). It has been suggested that polyploidy may increase their capacity for protein synthesis. Alternatively, since TGCs have a relatively short lifespan, it may be that endoreduplication and the associated cell hypertrophy allow tissue growth with less time and energy expenditure. An extension of this hypothesis is that because TGCs are post-mitotic, they function without risk of forming

**TABLE 1**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Location</th>
<th>Temporal appearance</th>
<th>Gene expression</th>
<th>Suggested function</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpA-TGC</td>
<td>Lining maternal spiral arteries bringing blood into placenta</td>
<td>E10.5</td>
<td>PIF</td>
<td>Regulate maternal spiral artery remodeling and blood flow into the placenta</td>
</tr>
<tr>
<td>P-TGC</td>
<td>Lining implantation site and outer layer of parietal yolk sac</td>
<td>E7.5</td>
<td>PIF, P2, P4</td>
<td>Facilitate implantation and initial maternal vascular connections, regulate decidual cell differentiation, and maternal physiology</td>
</tr>
<tr>
<td>C-TGC</td>
<td>Lining canals that bring maternal blood to base of labyrinth</td>
<td>E10.5</td>
<td>PIF, P2</td>
<td>Regulate maternal vasculature remodeling and maternal physiology</td>
</tr>
<tr>
<td>S-TGC</td>
<td>Within maternal blood sinusoids of the labyrinth layer</td>
<td>E10.5</td>
<td>Ctsq, P2</td>
<td>Modulation of hormone and growth factor activity before they enter fetal and/or maternal circulation, regulate maternal physiology</td>
</tr>
</tbody>
</table>
tumours (Hemberger, 2008). This is important since TGCs have the ability to invade and promote local angiogenesis (see below). Interestingly though, TGC nuclei in voles become fragmented during late gestation with sex chromosomes in each sub-domain, and this suggests that there is some structural order to the polyploid nuclei (Zybina et al., 2005). The formal test of whether or not polyploidy is critical for function is to analyze mutants in which it does not occur.

Endoreduplication is compromised in cyclin E1/E2-deficient mice but markers of TGCs are still induced, indicating that endoreduplication and differentiation are not linked (Geng et al., 2003; Parisi et al., 2003).

Transformation of the mitotic cell cycle to the endocycle in TGCs
Mechanisms concerning the initiation and maintenance of endoreduplication have been well studied in flies and plants (Edgar and Orr-Weaver, 2001; Larkins et al., 2001), though there are some data from rodent TGCs as well (Fig. 3) (Hattori et al., 2000; MacAuley et al., 1998; Nakayama et al., 1998). The most obvious change in the cell cycle is that S phase is dissociated from M phase. This is not trivial since checkpoint controls normally prevent initiation of DNA replication until completion of mitosis and entry into mitosis is prevented until completion of S phase (Elledge, 1996). TGCs show other changes in checkpoint controls such that they continue through DNA replication even after sustaining DNA damage (MacAuley et al., 1998).

The G2 decision point: mitosis or endoreduplication?
In a mitotic cell cycle, cyclin B/Cdk1 promotes entry into mitosis (Sherr and Roberts, 2004). The Rcho-1 trophoblast cell line has been used to study TGC differentiation as precursor cells that have committed to leave the mitotic cell cycle can be selected as a result in a change in cell adhesiveness even before they have begun to endoreduplicate (MacAuley et al., 1998). The transition occurs in the G2 phase but cyclin B/Cdk1 complex is not activated due to reduced association of cyclin B and Cdk1 (MacAuley et al., 1998). This is likely due to the effect of the Cdk inhibitor p57Kip2 which has recently been shown to inhibit Cdk1 activity during TGC differentiation (Ullah et al., 2008). In the subsequent endocycle, cyclin B expression is suppressed (MacAuley et al., 1998; Palazon et al., 1998). The zinc finger transcription factor Snail regulates the ‘G2 decision point’ of whether trophoblast cells go through mitosis or enter the endocycle (Fig. 3) (Nakayama et al., 1998). Its precise mechanism is unknown but over-expression of Snail increases expression of the mitotic cyclins A and B (Nakayama et al., 1998).

Resetting the periodic S phases during endoreduplication
During the mitotic cell cycle, biochemical events coincident with mitosis lead to the re-setting of the origins of replication (Elledge, 1996). This includes degradation of the protein Geminin which otherwise suppresses the firing of origins of replication (McGarry and Kirschner, 1998). Geminin mutant embryos die during pre-implantation development and show ectopic endoreduplication in blastomeres (Gonzalez et al., 2006). Another important mechanism to maintain periodicity during the endocycle involves cyclic expression of p57Kip2, a G1/S Cdk inhibitor (Hattori et al., 2000). When TGC differentiation is initiated, p57Kip2 mRNA expression appears during the transition endocycle (Fig. 3) (Hattori et al., 2000). During subsequent endocycles, p57Kip2 protein levels fluctuate and this defines two phases: a
**TABLE 2**

**GENES REGULATING TGC DEVELOPMENT**

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene product</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eomes</em></td>
<td>T-box transcription factor</td>
<td>Mutants die at peri-implantation stage, primary TGCs absent</td>
<td>(Russ et al., 2000)</td>
</tr>
<tr>
<td><em>Ets</em></td>
<td>Ets transcription factor</td>
<td>Mutants die by E8.5 with extraembryonic ectoderm absent, ectoplacental cone present</td>
<td>(Donnison et al., 2005)</td>
</tr>
<tr>
<td><em>End2/Entl</em></td>
<td>Orphan nuclear receptor</td>
<td>Mutants die by E9.5 with chorion absent, TGC number increased</td>
<td>(Luo et al., 1997, Tremblay et al., 2001a)</td>
</tr>
<tr>
<td><em>Foxd3</em></td>
<td>Forkhead transcription factor</td>
<td>Mutants die by E6.5 with TGC number increased</td>
<td>(Tompers et al., 2005)</td>
</tr>
<tr>
<td><em>AP-2γ</em></td>
<td>AP-2 family of transcription factors</td>
<td>Mutants die by E8.5 with trophoblast stem cells and ectoplacental cone reduced, Primary TGCs reduced</td>
<td>(Auman et al., 2002, Werling and Schorle, 2002)</td>
</tr>
<tr>
<td><em>Erf</em></td>
<td>Ets domain transcriptional repressor</td>
<td>Mutants die by 10.5 with persistence of ectoplacental cavity, failure in chorioallantoic attachment, expanded TGCs layer</td>
<td>(Papadaki et al., 2007)</td>
</tr>
<tr>
<td><em>Ets2</em></td>
<td>Ets transcription factor</td>
<td>Mutants die by E8.5 with chorion absent and extraembryonic ectoderm reduction and defective trophoblast stem cells self renewal</td>
<td>(Kohn et al., 2003)</td>
</tr>
<tr>
<td><em>Dp1</em></td>
<td>DP family transcription factor</td>
<td>Mutants die by E12.5 with reduced chorion, fewer TGCs and TGCs with minimal nuclear enlargement</td>
<td>(Yamamoto et al., 1998, Wen et al., 2007)</td>
</tr>
<tr>
<td><em>FGF4</em></td>
<td>Fibroblast growth factor</td>
<td>Mutants die shortly after implantation with failure to maintain trophoblast stem cells, premature TGC formation</td>
<td>(Feldman et al., 1995, Tanaka et al., 1998)</td>
</tr>
<tr>
<td><em>FGFR2</em></td>
<td>Fibroblast growth factor receptor 2</td>
<td>Mutants die shortly after implantation with failure to maintain trophoblast stem cells, premature TGC formation</td>
<td>(Arman et al., 1998)</td>
</tr>
<tr>
<td><em>Erk2</em></td>
<td>Extracellular signal-regulated kinase 2</td>
<td>Mutants die shortly after implantation, extraembryonic ectoderm and ectoplacental cone not formed</td>
<td>(Hatano et al., 2003, Saba-El-Leil et al., 2003)</td>
</tr>
<tr>
<td><em>Shp2</em></td>
<td>Non-receptor protein-tyrosine phosphatase</td>
<td>Mutants die at peri-implantation stage, trophoblast stem cells not generated</td>
<td>(Yang et al., 2006)</td>
</tr>
<tr>
<td><em>Nodal</em></td>
<td>Transforming growth factor β1 superfamily member</td>
<td>Mutants die by E9.5 with spongiotrophoblast layer reduced and TGC number increased</td>
<td>(Guzman-Ayala et al., 2004, Ma et al., 2001)</td>
</tr>
<tr>
<td><em>Activin</em></td>
<td>Transforming growth factor β1 superfamily member</td>
<td>Promotes maintenance of cultured trophoblast stem cells</td>
<td>(Erlebacher et al., 2004)</td>
</tr>
<tr>
<td><em>Tgfβ</em></td>
<td>Transforming growth factor β1 superfamily member</td>
<td>Promotes maintenance of cultured trophoblast stem cells</td>
<td>(Erlebacher et al., 2004)</td>
</tr>
<tr>
<td><em>BMP2</em></td>
<td>Transforming growth factor β1 superfamily member</td>
<td>Mutants die by around E8.5 with amnion/chorion defects caused by an open proamniotic canal</td>
<td>(Zhang and Bradley, 1996)</td>
</tr>
<tr>
<td><em>Acvr1b</em></td>
<td>Activin/Nodal receptor 1B</td>
<td>Mutants die by E9.5 with disorganized extraembryonic ectoderm</td>
<td>(Gu et al., 1998)</td>
</tr>
<tr>
<td><em>Acvr2b</em></td>
<td>Activin/Nodal receptor 2 and 2B</td>
<td>Compound homozygous mutants die by E8.5</td>
<td>(Song et al., 1999)</td>
</tr>
<tr>
<td><em>Smad1</em></td>
<td>Intracellular transducer of TGF-β signals</td>
<td>Mutants die by E10.5 with chorion erratically folded and allantois growth defects</td>
<td>(Arnold et al., 2006, Lechleider et al., 2001, Tremblay et al., 2001b)</td>
</tr>
<tr>
<td><em>mTOR</em></td>
<td>Mammalian TOR (target of rapamycin)</td>
<td>Mutants die shortly after implantation. Mutant trophoblast fails to proliferate in vitro.</td>
<td>(Murakami et al., 2004)</td>
</tr>
</tbody>
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**Ectoplacental cone and spongiotrophoblast maintenance**

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene product</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mash2</em></td>
<td>Basic helix-loop-helix transcription factor</td>
<td>Mutants die by E10 with smaller ectoplacental cone and lack of spongiotrophoblast, TGC number increased, labyrinth layer reduced</td>
<td>(Guillenot et al., 1994, Scott et al., 2000, Tanaka et al., 1997)</td>
</tr>
<tr>
<td><em>Sp1,3</em></td>
<td>Zinc finger transcription factors</td>
<td>Sp1/3−/− die by E10.5, Sp3/3−/− die postnatally, spongiotrophoblast layer decreased in Sp1/Sp3 compound heterozygous and Sp3/−; mutants</td>
<td>(Kruger et al., 2007)</td>
</tr>
<tr>
<td><em>PPARγ</em></td>
<td>nuclear receptor peroxisome proliferator-activated receptor β/δ; lipid-activated transcription factors</td>
<td>Mutants die by E10.5 with reduced spongiotrophoblast and TGC</td>
<td>(Nadra et al., 2006, Wang et al., 2007)</td>
</tr>
<tr>
<td><em>HiFα</em></td>
<td>bHLH/PAS transcription factors composed of HiFα and HiFβ/β/Amh subunits</td>
<td>Amf−/− and HiFα−/− HiFβ−/− die by E10.5 with TGC number increased, smaller ectoplacental cone and reduced spongiotrophoblast</td>
<td>(Abbott and Buckalew, 2000, Adelman et al., 2000, Cowden Dahl et al., 2005)</td>
</tr>
<tr>
<td><em>Cited 1</em></td>
<td>CBP/p300-interacting transactivator</td>
<td>Mutants die shortly before birth, spongiotrophoblast layer irregular in shape and enlarged</td>
<td>(Rodriguez et al., 2004)</td>
</tr>
<tr>
<td><em>Cited 2</em></td>
<td>CBP/p300-interacting transactivator</td>
<td>Mutants die by E14.5 with reduced spongiotrophoblast, glycoen trophoblast cells and TGCs</td>
<td>(Withington et al., 2006)</td>
</tr>
<tr>
<td><em>Dmdm3L</em></td>
<td>DNA methyltransferase 3-like protein</td>
<td>Mutants die by E10.5 with TGCs number increased, spongiotrophoblast and labyrinth reduced</td>
<td>(Arna et al., 2006)</td>
</tr>
<tr>
<td><em>Keratin 8, 18, 19</em></td>
<td>Cytokeratin-intermediate filaments</td>
<td>Kβ−/− die by E12.5, Kβ−/−/K19−/− die by E10.5 and K18−/−/K19−/− die by E9.5, all with altered TGCs</td>
<td>(Hesse et al., 2000, Jaquemart et al., 2003, Tamai et al., 2000)</td>
</tr>
<tr>
<td><em>Connexin 31</em></td>
<td>Connexin; Gap junction protein</td>
<td>60% mutants die between E10.5 and 13.5 TGC number increased, spongiotrophoblast and labyrinth decreased</td>
<td>(Kibschiull et al., 2004, Plum et al., 2001)</td>
</tr>
<tr>
<td><em>Connexin 31.1</em></td>
<td>Connexin; Gap junction protein</td>
<td>Mutants die by E14.5, compact spongiotrophoblast with increased thickness</td>
<td>(Zheng-Fischhofer et al., 2007)</td>
</tr>
<tr>
<td><em>Bruce</em></td>
<td>BIR repeat-containing ubiquitin-conjugating enzyme</td>
<td>Mutants in C57BL/6 background die perinatally with spongiotrophoblast reduced</td>
<td>(Hitz et al., 2005, Lotz et al., 2004)</td>
</tr>
<tr>
<td><em>HOP/NECC1</em></td>
<td>Homeodomain-only protein/not expressed in choriocarcinoma clone 1 (HOP/NECC1)</td>
<td>Mutants have TGCs number increased, spongiotrophoblast reduced</td>
<td>(Asanoma et al., 2007)</td>
</tr>
<tr>
<td><em>Talin</em></td>
<td>Cytoplasmic protein associated with integrin-containing cellular junctions</td>
<td>Mutants die by E9.5 with disorganized extraembryonic tissues and the ectoplacental and extraembryonic cavities are not formed</td>
<td>(Monkley et al., 2000)</td>
</tr>
</tbody>
</table>

**TGC terminal differentiation**

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene product</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hand1</em></td>
<td>Basic helix-loop-helix transcription factor</td>
<td>Mutants die by E8.5 with smaller ectoplacental cone, TGC number reduced and nuclear size reduced</td>
<td>(Furili et al., 1998, Riley et al., 1998, Scott et al., 2000)</td>
</tr>
<tr>
<td><em>Stra13</em></td>
<td>Basic helix-loop-helix transcription factor</td>
<td>Over-expression stimulates TGC differentiation</td>
<td>(Hughes et al., 2004)</td>
</tr>
</tbody>
</table>
both cyclin A and E are expressed during endo-S phase (MacAuley et al., 2000). The p53 tumour suppressor protein helps ensure that chromosomes are intact before replication. At the G1 checkpoint, the Rb tumor suppressor protein is phosphorylated in vitro, which in turn drives the cell cycle into S phase (Sherr, 2000). During the transition from the mitotic cell cycle to the endocycle in trophoblast cells, cyclin D isoform expression switches from D3 to D1 (MacAuley et al., 2000). Expression of p53 and Rb declines during TGC differentiation whereas their forced over-expression inhibits differentiation (Soloveva and Linzer, 2004), and p53 tumour suppressor protein, transcriptional factor, promotes secondary TGC differentiation. Historically, it has been hypothesized that periodic expression of p57Kip2 protein promotes alternating S and gap phases during the endocycle.

Altered G1 to S checkpoint

A G1 to S checkpoint is present during the mitotic cell cycle to ensure that chromosomes are intact before replication. At the G1 checkpoint, the Rb tumor suppressor protein is phosphorylated by cyclin D/Cdk allowing the E2F transcription factor to be liberated, which in turn drives the cell cycle into S phase (Sherr and Roberts, 2004). The p53 tumour suppressor protein helps cells to survey genotoxic damage and cooperates with Rb to regulate G1 arrest (Sherr, 2000). During the transition from the mitotic cell cycle to the endocycle in trophoblast cells, cyclin D isoform expression switches from D3 to D1 (MacAuley et al., 1998). Expression of p53 and Rb declines during TGC differentiation whereas their forced over-expression inhibits differentiation (Soloveva and Linzer, 2004). This altered G1 checkpoint control might allow cells to go through repeated S phases without intervening mitoses.

Maintenance of S phase cyclin/Cdk activities

Cyclin E promotes the G1 to S phase transition whereas cyclin A promotes S phase progression (Sherr and Roberts, 2004), and both cyclin A and E are expressed during endo-S phase (MacAuley et al., 1998). Cyclin E1/E2-deficient mice show a reduced endoreduplication in TGCs, indicating that cyclin E is essential for endoreduplication (Geng et al., 2003; Parisi et al., 2003). Conversely, cyclin E levels are elevated in P-TGCs that are present by mid-gestation, and all of the other subtypes, arise at different times during development (Fig. 2) (Simmons et al., 2007). Some of the P-TGCs arise directly from the ~60 mural trophectoderm cells in the blastocoel in a process called primary TGC differentiation. However, most of the several hundred P-TGCs that are present by mid-gestation, and all of the other subtypes, arise from the polar trophectoderm through so-called secondary TGC differentiation. Historically, it has been hypothesized that secondary TGCs are derived from progenitor cells within the ectoplacental cone and the spongiosotrophoblast layers that are Mash2 and Tpbpa/4311 positive (Simmons et al., 2007). Some of the P-TGCs arise from the S-TGCs that are present by mid-gestation, and all of the other subtypes, arise from the polar trophectoderm through so-called secondary TGC differentiation. Historically, it has been hypothesized that secondary TGCs are derived from progenitor cells within the ectoplacental cone and the spongiosotrophoblast layers that are Mash2 and Tpbpa/4311 positive (Simmons et al., 2007). However, lineage-tracing studies have shown that only some TGC subtypes arise from the Tpbpa-positive precursor cells (Fig. 2) (Simmons et al., 2007). Both P-TGCs and C-TGCs have mixed developmental origins. In contrast, all of the SpA-TGCs originate from the Tpbpa-positive cells, whereas all of the S-TGCs arise from the Tpbpa-negative precursors (Fig. 2). While the Tpbpa-positive precursors are located in the outer ectoplacental cone
starting at ~E8.5 and later in the spongiotrophoblast, the source of the Tpbpap-negative precursors is unknown but could be the extraembryonic ectoderm/chorion trophoblast cells, inner ectoplacental cone cells, or both. The chorion has distinct layers of cells by E8.5 that are thought to give rise to the three different trophoblast cell layers in the labyrinth including two multinucleated syncytiotrophoblast layers and S-TGCs (Fig. 1 and 2) (Simmons et al., 2008a).

**Regulators of TGC development**

A variety of factors regulate TGC development (Table 2). After implantation, the trophoblast lineage is maintained by proliferation of trophoblast stem cells that reside in the polar trophoderm and that produce the bulk of the trophoblast lineage save for ~60% of the P-TGCs that derive from mural trophoderm (Fig. 2). The trophoblast stem cell pool is maintained by FGF4/FGFR2 (Arman et al., 1998; Tanaka et al., 1998) and Nodal (Guzman-Ayala et al., 2004) signaling and the AP2α (Auman et al., 2002), Eomes (Russ et al., 2000), Er2 (Luo et al., 1997; Tremblay et al., 2001a), Foxd3 (Tompers et al., 2005) and Elf5 (Donnison et al., 2005) transcription factors. Mice that are deficient for these factors, in general, show premature TGC differentiation as a consequence of the failure to maintain trophoblast stem cells (Table 2).

**Genes involved in maintenance of the ectoplacental cone and/or spongiotrophoblast**

There are many genes involved in maintaining the ectoplacental cone and/or spongiotrophoblast (Table 2) and, in general, loss-of-function mutations result in an increase in the number of TGCs. The precise mechanisms of action are established for only a few of these factors and only these will be discussed in detail. The basic helix-loop-helix (bHLH) transcription factor Mash2 plays an essential role in maintenance of the ectoplacental cone and spongiotrophoblast and negatively affects TGC differentiation (Guillemot et al., 1994; Scott et al., 2000; Tanaka et al., 1997). Mash2 is expressed in the in the chorion, ectoplacental cone and later spongiotrophoblast (Scott et al., 2000). Mash2-deficient mice die by E10 due to placenta defects, which include the absence of spongiotrophoblast, an increase of TGCs and a failure of labyrinth formation (Guillemot et al., 1994; Tanaka et al., 1997). A similar phenotype is observed in mutants for the DNA methyltransferase 3-like gene Dnmt3L (Arima et al., 2006). Dnmt3L is required for the establishment of maternal methylation imprints, and Dnmt3L heterozygous mutants die by E10.5 due to an imprinting defect and expression of Mash2 is diminished (Arima et al., 2006).

Oxygen levels can regulate trophoblast lineage cell fate in mice both in vitro and in vivo (Adelman et al., 2000; Cowden Dahl et al., 2005). Hypoxia promotes in vitro differentiation of trophoblast stem cells into spongiotrophoblast cells as opposed to TGCS (Adelman et al., 2000; Takeda et al., 2006). Hypoxia inducible factors (HIFs) are heterodimeric basic helix-loop-helix/Per-Arnt-Sim (bHLH/PAS) transcription factors composed of HIF1β/Arnt and HIF1α or HIF2α, that are activated by hypoxia (Semenza, 2007). In HIF1α/HIF2α double mutants, there are fewer spongiotrophoblast and syncytiotrophoblast cells, and more TGCS (Cowden Dahl et al., 2005). Arnt mutant placentas are similar to HIF1α/HIF2α mutants (Adelman et al., 2000; Cowden Dahl et al., 2005).

The Cx31 and Cx31.1 genes encode for connexin gap junction proteins and are involved in maintaining TGC progenitor cells within the ectoplacental cone and spongiotrophoblast (Kibschull et al., 2005; Kibschull et al., 2004; Plum et al., 2001; Zheng-Fischhofer et al., 2007). Cx31 is expressed at pre-implantation stages, but is restricted to the ectoplacental cone and extraembryonic ectoderm after implantation and then it persists in spongiotrophoblast (Plum et al., 2001). Cx31.1 is co-expressed with Cx31 at post-implantation stages, except that its expression is suppressed in spongiotrophoblast after E11.5 and persists in glycogen trophoblast cells (Zheng-Fischhofer et al., 2007). Cx31 and Cx31.1 deficient mice show similar placental defects including excessive TGCs (Plum et al., 2001; Zheng-Fischhofer et al., 2007).

**Genes involved in TGC terminal differentiation**

A large number of factors are implicated in promoting the terminal differentiation of TGCs though most of the information to date is limited to insights into P-TGCs (Table 2). Only some of these factors will be discussed in detail in which the molecular and cellular function is understood.

**Transcription factors.** It is well established that bHLH factors play key roles in TGC differentiation. Hand1 plays an essential role in promoting TGC differentiation (Firulli et al., 1998; Riley et al., 1998; Scott et al., 2000). It is expressed in the upper layer of the chorion, ectoplacental cone and all TGC subtypes. In Hand1 mutants, the ectoplacental cone is smaller, the number of cells lining the implantation site is reduced and they are strikingly smaller than normal P-TGCs. Hand1 mutants die by ~E8.5 but, in studying Hand1-deficient trophoblast stem cells in culture, it appears that Hand1 is essential for differentiation of all four TGC subtypes (Simmons et al., 2007). Mash2, the bHLH protein that maintains the diploid TGC progenitors, antagonizes Hand1 action (Scott et al., 2000). Stra13, another bHLH factor that is induced by retinoic acid (Sapin et al., 2000), can induce TGC differentiation in vitro (Hughes et al., 2004). I-mfa, a bHLH factor interacting protein, promotes TGC differentiation by inhibiting Mash2 (Kraut et al., 1998).

Gata transcription factors are also implicated in TGC development. Gata2 and Gata3 regulate transcription of TGC-specific hormone genes (Ma et al., 1997; Ng et al., 1993; Ng et al., 1994). In addition, Gata2 has been implicated in restricting expression of the Plpa gene to P-TGCs that surround the ectoplacental cone (Ma and Linzer, 2000). The results are interesting because they may imply distinct regulatory mechanisms for TGCs that are derived from Tpbpap-positive versus Tpbpap-negative precursors.

**Intercellular signaling pathways.** Several signaling pathways are implicated in TGC development and highlight the importance of paracrine interactions. Leukemia inhibitory factor (LIF) is a member of the interleukin-6 cytokine family and has several biological functions (Metcalf, 2003). LIF binds to a low-affinity receptor (LIFR), which in turn forms a high-affinity complex with the gp130 receptor protein. The LIFR-gp130 heterodimer complex transduces the LIF signal through activation of JAK kinase and STAT transcription factors (Metcalf, 2003). Suppressor of cytokine signaling (SOCS) proteins are important negative regulators of JAK-STAT signaling that form a negative-feedback loop—(Metcalf,
Functions of TGCs

TGCs have diverse functions that are crucial for implantation and subsequent placental function (Fig. 4). The mural trophectoderm-derived TGCs mediate attachment of blastocyst to the uterine epithelium, induce uterine decidualization, invade into the uterine stroma, and anastomose to form the yolk sac placenta for early exchange of nutrients and endocrine signals between mother and fetus. After implantation, TGCs produce hormones and cytokines for maintenance of the feto-maternal interface and regulation of maternal adaptations to pregnancy.

Functions of TGCs in establishment of fetal-maternal interface

Adhesion to the uterine epithelium

At the time of implantation, mural trophectoderm cells increase their adhesiveness and become competent to attach to the uterus (Arman, 2005). Meanwhile, uterine epithelial cells are primed by progesterone and estrogen from the ovary and become capable of attaching to the blastocyst (Dey et al., 2004). TGCs also produce progesterone (Yamamoto et al., 1994) that may contribute to regulation of uterine changes. During implantation, trophoblast cells attach to extracellular matrix (ECM) in the receptive uterus, which is composed of fibronectin, laminin, vitronectin and collagen (Sutherland, 2003; Wang and Arman, 2002). As the blastocyst and uterine epithelium attach, the now differentiating P-TGCs express several integrins such as αβ3 (Metcalfe, 2003; Schultz and Arman, 1995), αβ1 (Klaffky et al., 2001), α4β1 (Basak et al., 2002), and αβ3 (Rout et al., 2004) and αβ3 (Rout et al., 2004).

TGCs affect decidualization of the uterine stromal cells

Upon attachment of the blastocyst to the uterine epithelium, the uterine stromal cells at sites of blastocyst apposition undergo proliferation and differentiation into decidual cells, a process called decidualization. The uterine stroma can undergo decidualization in response to even an artificial stimulus such as scratching of the epithelium or intraluminal injection of lectin-coated beads or oil, as long as the uterus has been exposed to appropriate priming by estrogen and progesterone (Dey et al., 2004). TGCs are thought to be indispensable for decidualization because of their production either of progesterone or other signals that affect decidual cell differentiation (Bany and Cross, 2006). These latter signals are inferred from the fact that there are differences in gene expression between the decidua surrounding a normally implanted embryo compared to an artificially induced one, or surrounding mutant mouse embryos that have TGC
defects (Bany and Cross, 2006). At least one of the TGC signals is a type I interferon (Bany and Cross, 2006).

**Invasion into the decidua and anastomosis with the maternal vasculature**

After attachment of the blastocyst to the uterine luminal epithelium, P-TGCs invade the uterus by remodeling of the ECM, phagocytosis and cell motility (Cross et al., 1994). They form a transient structure called the parietal yolk sac (Fig. 1) that is the site of exchange for nutrients and gases between the mother and fetus in the early post-implantation conceptus (Cross et al., 1994). It is composed of P-TGCs, parietal endoderm cells and an extensive basement membrane (Reichert’s membrane) between them (Welsh and Enders, 1987). The formation of the yolk sac placenta is highly dependent on the ability of the TGCs to penetrate the uterine epithelium and anastomose with maternal blood spaces surrounding the implantation site. During anastomosis, the P-TGCs are highly protrusive, with long cytoplasmic lamina extending to envelope a diffuse network of maternal blood sinuses (Bevilacqua and Abrahamsohn, 1989; McRae and Church, 1990).

The mechanisms of trophoblast invasion are best studied in P-TGCs but SpA-TGCs and glycogen trophoblast cells also invade into the uterus (Adamson et al., 2002; Pijnenborg et al., 1981). TGCs secrete a variety of proteins that are thought to digest the ECM as well as phagocytosed maternal cells and matrix materials. They include matrix metalloproteinases (MMP-2, -3, -9, -13) and inhibitors of metalloproteinases (TIMP-1, -2, -3, -4) (Alexander et al., 1996; Das et al., 1997; Harvey et al., 1995; Teesalu et al., 1999; Zhang et al., 2003) urokinase plasminogen activators (Teesalu et al., 1998; Teesalu et al., 1999) and cathepsins (Afonso et al., 1999; Deussing et al., 2002; Hemberger et al., 2000; Iida et al., 2004).

**Functions of TGCs after implantation**

After implantation, TGCs produce many paracrine and endocrine factors that target various maternal physiological systems to maintain maternal adaptations to pregnancy.

**Production of hormones that regulate various maternal physiological systems**

TGCs produce a broad range of hormones that regulate several maternal adaptations to pregnancy. In particular, the prolactin/placental lactogen (PL)/prolactin-like protein (PLP) gene family is highly evolved in rodents. There are 23 members in mice and all except for the pituitary prolactin gene are exclusively expressed in the placenta and in TGCs in particular (Simmons et al., 2008b, Wiemers et al., 2003). The expression patterns indicate that the 22 placenta-specific genes have diverse functions (Simmons et al., 2008b).

The PL were first identified in rodents as prolactin-related hormones that stimulate the mammary gland similar to prolactin and indeed they work through the prolactin receptor (Linzer and Fisher, 1999), though it is clear that PL has a variety of other target tissues. TGCs produce PL-I starting soon after implantation until mid-gestation and subsequently PL-II from mid-gestation until term (Talamantes, 1990). In mice in which the pituitary gland is removed as the source of prolactin, secretion of both PL-I (Lopez et al., 1991) and PL-II (Kishi et al., 1988) is elevated and some milk production occurs indicating that the placental lactogens are partially sufficient to promote mammary development (Thordarson et al., 1989). PL-I and PL-II also have luteotrophic effects on the ovary and support progesterone production (Galosy and Talamantes, 1995; Thordarson et al., 1997). PL-I and PL-II can also increase insulin secretion (Brejle et al., 1993; Fleenor et al., 2000; Nielsen et al., 1999; Sorensen and Brejle, 1997) and stimulate an increase in the number of insulin producing β cells in pancreatic islets. By contrast, progesterone inhibits insulin secretion and β cell division (Sorensen et al., 1993). PL-I affects the liver and induces expression of Na+/taurocholate-co-transporting polypeptide (NTCP) (Cao et al., 2001), which is critical for bile salt transport. Finally, prolactin modulates the response of the immune system to stress (Dorshkind and Horseman, 2001; Dugan et al., 2007; Dugan et al., 2002). It is not yet clear if PL-I and/or PL-II have similar effects.

TGCs secrete several PLPs that regulate hematopoiesis. PLP-E and PLP-F can stimulate megakaryocytepoiesis and erythropoiesis (Bhattacharyya et al., 2002; Lefebvre et al., 2001; Lin and Linzer, 1999; Zhou et al., 2005). PLP-E is expressed at the first half of pregnancy by P-TGCs, whereas PLP-F is secreted later in pregnancy by the spongiotrophoblast layer (Simmons et al., 2008b), suggesting that they function in a sequential manner. PLP-E has been shown to stimulate human and mouse erythroid progenitor cell proliferation and differentiation through activation of the JAK/STAT pathway (Bittorf et al., 2000). Proliferin 2 (PLF2) stimulates an increase in the fraction of long-term culture-initiating cells (LTC-IC) in cultured bone marrow cells (Choong et al., 2003).

**Production of paracrine factors that regulate the feto-maternal interface**

The vascular bed and repertoire of immune cells in the uterus changes dramatically during gestation. TGCs secrete the PL cytokines proliferin (PLF) and proliferin-related protein (PRP) that stimulate and inhibit endothelial cell migration, respectively (Jackson et al., 1994). PLF is expressed in the early half of gestation and in all TGC subtypes except for S-TGCs (Simmons et al., 2007). PRP is subsequently expressed in the latter half of gestation and in all four subtypes of TGCs. TGCs also express vascular endothelial growth factor (VEGF) (Voss et al., 2000) and placental-like growth factor (PLGF) (Tayade et al., 2007) in early gestation. Antagonists of VEGF/PLGF are also expressed in the placenta. Flt-1 is a VEGF receptor that can undergo alternative splicing to result in a secreted Flt-1 protein (sFlt-1) that blocks VEGF action. sFlt-1 transcripts are detected in the spongiotrophoblast that lies beneath the P-TGCs (Cross et al., 2002; He et al., 1999), implying a mechanism by which maternal blood vessels are prevented from growing into the junctional zone.

TGCs also produce several factors that can regulate blood flow. First, they secrete PLP-A that in vitro can inhibit the ability of NK cells to produce interferon-γ (IFNγ) (Muller et al., 1999). Uterine NK cells are important for spiral artery dilatation through their production of interferon-γ (Ashkar et al., 2000). TGCs also produce interferon-γ during mid-gestation (Platt and Hunt, 1998), and could affect NK cell function directly. Despite the predictions from the expression patterns and in vitro activity of PLP-A, Plpa-deficient mice have normal pregnancies unless the pregnant female mice are exposed to hypoxia (Ain et al., 2004). Second,
TGCs express adrenomedullin (Montuenga et al., 1997; Yotsumoto et al., 1998), a vasodilator, and endothelial nitric oxide synthetase (eNOS/Nos3) (Hemberger et al., 2003), an enzyme that produces the vasodilator nitric oxide (NO) and that is implicated in vasorelaxation during pregnancy (Gagioti et al., 2000). However, since spiral arteries at the feto-maternal interface lack smooth muscle, the targets of these vasodilators are unclear (Cross et al., 2002). Finally, trophoblast cells, like endothelial cells, suppress coagulation of blood whereas blood normally rapidly clots when hemorrhage occurs. P-TGCs, ectoplacental cone and spongiotrophoblast cells express thrombomodulin, a protein that has anti-coagulant effects on maternal blood within the parietal yolk sac and placenta (Isermann et al., 2003; Weiler-Guettler et al., 1996).

The activity of the local immune function is also altered during pregnancy in rodents to prevent the maternal immune system from killing the allogeneic conceptus. The precise mechanisms are unknown. Progesterone produced by TGCs may have some effect since it can stimulate activities of type 2 T helper cells (Th2) (Szekeres-Bartho and Wegmann, 1996) that secrete cytokines (e.g., IL-10) that have feto-protective effects. There are significant changes in the distribution of NK cells during pregnancy associated with the presence of a normal conceptus (Herington and Bany, 2007). This implies that factors from the conceptus, likely from TGCs, regulate NK cell homing, proliferation and/or survival.

**Distinct or overlapping functions of the four different TGC subtypes?**

Based on their distinct locations in the placenta and different gene expression patterns (Simmons et al., 2007; Simmons et al., 2008b), we speculate that the four subtypes of TGCs have distinct functions (Table 1). P-TGCs express the greatest variety of PLPs among all subtypes of TGCs (Simmons et al., 2008b). PL-I and PL-II can act on many maternal physiological systems such as corpus luteum, mammary gland, brain, and pancreas (Soares et al., 2007). The angiogenesis and hematopoiesis related hormones (PLF, PRP, PLP-A, PLP-E, PLP-F) are also expressed by P-TGCs (Simmons et al., 2008b), and may function early in establishing the parietal yolk sac before the circulation into the mature placenta is established. P-TGCs also express progesterone and interferon-γ (Yamamoto et al., 1994; Platt and Hunt, 1998) which are important for decidualization and NK cell function. Thus, the functions of P-TGCs are very broad. In general, SpA-TGCs express factors that regulate cardiovascular functions including formation of blood vessels (PLF and PRP) and blood cells (PLF2, PLF-E, PLF-F), and dilation of spiral arteries (PLP-A by affecting NK cells) (Simmons et al., 2008b). SpA-TGCs also express placenta-specific cathepsins, Cts7 and Cts8, and Cts8 in particular is capable of mediating smooth muscle degradation and blood vessel disintegration to facilitate formation of trophoblast-lined blood sinuses (Screen et al., 2008). These would facilitate the maternal blood supply to the conceptus. The C-TGCs line the canals, but other than this structural role, it is difficult to imagine their function. S-TGCs produce the least number of PLP hormones (PL-II, PRP, PLP-K) (Simmons et al., 2008b). Their expression of PRP, but not PLF, suggests that growth of endothelial cells into the labyrinth may be inhibited which would be critical for maintaining the hemo-chorial blood space. S-TGCs also secrete cathepsin Q (Simmons et al., 2007), a cysteine protease with related family members implicated in trophoblast invasion, as described above, and hormone regulation. Some cathepsins can cleave prolactin into peptides that have alternative functions (Clapp et al., 2006; Hilfiker-Kleiner et al., 2007; Piwnica et al., 2006). The location of S-TGCs on the maternal side of the feto-maternal interface implies that they could cleave prolactin-like hormones before they leave the placenta and enter the maternal circulation.

**Summary**

Recent research has made striking progress in understanding the development and function of TGCs. There are at least four different subtypes of TGCs within the mature placenta, each arising at different times and locations in the placenta, and likely having distinct functions. While we now have a fairly good understanding of the regulation of TGC development, the functions of the different TGC subtypes remain very active areas of investigation and there are key open questions that should guide future studies. First, it will be intriguing to understand more details about the different functions of the TGC subtypes and how they are differentially regulated. Second, TGCs express a wide repertoire of hormones, but the biological function of most of these is unknown. Third, since it appears that TGCs regulate homeostatic physiological systems in the mother, it will be intriguing to see if and what type of physiological changes in the mother can alter the development and/or function of TGCs. There are insights from a variety of experimental animals that alteration of diet can affect placental development. Hypoxia during pregnancy can also alter expression of prolactin-like protein genes and supports the notion that these hormones may mediate responses to pregnancy stressors. These emerging themes will be important to pursue in order to gain better insights into the dialogue between the mother and fetus that occurs during pregnancy.

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