Fundamental differences in endoreplication in mammals and *Drosophila* revealed by analysis of endocycling and endomitotic cells

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Throughout the plant and animal kingdoms specific cell types become polyploid, increasing their DNA content to attain a large cell size. In mammals, megakaryocytes (MKs) become polyploid before fragmenting into platelets. The mammalian trophoblast giant cells (TGCs) exploit their size to form a barrier between the maternal and embryonic tissues. The mechanism of polyploidization has been investigated extensively in *Drosophila*, in which a modified cell cycle—the endocycle, consisting solely of alternating S and gap phases—produces polyploid tissues. During S phase in the *Drosophila* endocycle, heterochromatin and specific euchromatic regions are underreplicated and reduced in copy number. Here we investigate the properties of polyploidization in murine MKs and TGCs. We induced differentiation of primary MKs and directly microdissected TGCs from embryonic day 9.5 implantation sites. The copy number across the genome was analyzed by array-based comparative genome hybridization. In striking contrast to *Drosophila*, the genome was uniformly and integrally duplicated in both MKs and TGCs. This was true even for heterochromatic regions analyzed by quantitative PCR. Underreplication of specific regions in polyploid cells is proposed to be due to a slower S phase, resulting from low expression of S-phase genes, causing failure to duplicate late replicating genomic intervals. We defined the transcriptome of TGCs and found robust expression of S-phase genes. Similarly, S-phase gene expression is not repressed in MKs, providing an explanation for the distinct endoreplication parameters compared with *Drosophila*. Consistent with TGCs endocycling rather than undergoing endomitosis, they have low expression of M-phase genes.

DNA replication | polytene

Throughout the plant and animal kingdoms, cells in specific tissues increase their DNA content to become polyploid, making an understanding of the mechanism involved in polyploidization a fundamental question in biology (1, 2). The biological rationale for increasing genome content during differentiation appears most commonly to be to increase cell size, although it also may facilitate increased cell metabolism. Much remains to be discovered in terms of how genomic DNA content is increased during differentiation and how this affects gene expression.

The cell cycle alterations leading to polyploidy have been investigated in *Drosophila* and mammals, revealing diverse mechanisms. The endocycle is the modified cell cycle most variant from the canonical division cycle, consisting solely of alternating synthesis (S) and gap (G) phases. In *Drosophila*, nearly all differentiated tissues increase DNA content via the endocycle (3). The ploidy levels of differentiated *Drosophila* cells range from 16C (where C is the haploid genome content) to 1024C, depending on the cell type (4, 5). The structure of the chromosomes in most *Drosophila* cells is polytene, differing from polyploid cells in that all replicated copies of the sister chromatids are physically aligned, giving a consistent banding pattern. Some cell types, such as mammalian megakaryocytes (MKs), undergo endomitosis, a modified cell cycle with G1, S, and G2 phases but only a partial M phase in that nuclear division and cytokinesis do not occur (for review see ref. 6). Thus, the cells produced by endomitosis are mononucleated, like those from the endocycle, but the replicated sister chromatids are separated and polyploid rather than polytene. A third mechanism leading to polyploidy involves nuclear division but no cytokinesis to produce multinucleate cells, as in tetraploid hepatocytes (7).

In *Drosophila* endocycles, differential DNA replication occurs, rather than an integral doubling of the genome during S phase (8). This can lead to increased gene copy number, gene amplification, of specific genomic regions, as occurs at six sites in ovarian follicle cells (9). More commonly, underreplication leads to reduced gene copy number. All *Drosophila* polytene cells examined to date have a highly reduced copy number of heterochromatin as well as underreplicated euchromatic regions. These underreplicated regions outside of heterochromatin blocks can be either tissue specific or common to several tissues (10). Whereas gene amplification can be a strategy for robust expression of specific genes over a short developmental time, underreplication may conserve resources by avoiding replication of gene-poor regions of the genome.

Mammalian MKs achieve a ploidy of 128C, and the resulting increased cell size is necessary for sufficient platelet production (6). During MK endomitosis, anaphase A chromatid separation occurs, but the spindle does not elongate, and mitosis is aborted without nuclear division (11). Lagging chromosomes are observed during anaphase, possibly reflecting incomplete DNA replication, although this has not been investigated.

The trophoblast giant cells (TGCs) provide a barrier between the maternal blood supply and the embryo proper, facilitated by the large size of the cells (12–14). In rodents, they become highly polyploid; reaching up to 512C in mouse (15). The murine TGCs differ from syncytiotrophoblasts found in both rodents and human in that they contain a single, highly polyploid nucleus, whereas the latter arise by cell fusion of G1 cells, yielding multinucleate cells with diploid nuclei (16, 17). In addition, TGCs are on an endocycle, and polytene structures have been visualized by in situ hybridization, although in contrast to *Drosophila* the polytene structures do not extend the length of each chromosome (18). The TGC chromosome morphology is dynamic during development, with the chromosomes appearing to fragment at day 10 of mouse embryogenesis (16). Mouse TGCs can be differentiated in culture from trophoblast stem cells by the absence of fibroblast growth factor 4, and a rat trophoblast cell culture line exists that becomes polyploid in culture, attaining up to 64C

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ploidy (19, 20). Analysis of these models revealed that the cyclin-dependent kinase (CDK) inhibitor p57 is necessary for the onset of the endocycle (21, 22). Differential DNA replication, however, was not examined in these cell culture models.

Here we investigate the nature of S phase in the modified cell cycles producing MKs and TGCs, using primary MKs differentiated from precursor cells and mouse TGCs isolated directly from implantation sites. Through array-based comparative genome hybridization (aCGH), we systematically analyzed the genome for regions subject to differential DNA replication, uncovering fundamental differences in endoreplication in mouse and *Drosophila*.

Results

Primary Polyploid Murine Megakaryocytes Undergo Uniform Genome Replication. Differential replication is a phenomenon common to all *Drosophila* polytene and polyploid tissues (8). Before this study, however, it was not known whether mammalian cells that become polyploid also change gene copy number in parts of the genome. aCGH permitted quantification of gene copy number across the euchromatic portion of the genome. We first investigated mouse bone marrow MKs, which polyploidize via endomitosis (Fig. 1*A*), the modified cell cycle least variant from the archetypal cell cycle. Recent studies suggested that low-ploidy (2C, 4C) murine MKs have uniform replication of the genome, given the relatively normal anaphase figures (23). In contrast, higher ploidy MKs could have differential replication that may contribute to the observed aberrant mitotic figures (23, 24).

We obtained polyploid MKs for aCGH by isolating bone marrow progenitor cells from C57BL/6J mice and culturing them in the presence of thrombopoietin (TPO) for 4 d to induce MK polyploidization. Following BSA gradient fractionation, we obtained populations of 2C and 16C–128C MKs (Fig. 1*B*). To validate the purity of our polyploid-enriched MK fraction, we analyzed DNA content by fluorescence-activated cell sorting (FACS) of total MKs after 4 d in TPO and of polyploid MKs from the 3% (wt/vol) BSA pellet (Fig. 1*B*). The 3% BSA pellet was highly enriched with MKs with higher than 8C ploidy. Genomic DNA from these cell populations was differentially labeled and cohybridized to a two-color, 1-million probe Agilent array spanning the entire C57BL/6J euchromatin. Copy number profiles revealed uniform copy number across all of the chromosomes in polyploid MKs relative to diploid MKs (Fig. 1*C* and Fig. S1). Thus, in contrast to *Drosophila*, no genomic intervals are resistant to replication and are underreplicated. In addition, genomic regions harboring some of the genes uniquely or selectively expressed abundantly in MKs, such as *glycoprotein 6* (*GPVI/Gp6*) (25), *c-myeloproliferative leukemia virus oncogene* (*c-Mpl*) (26, 27), *cyclin D3* (28), and *cyclin E* (29), do not have amplified copy number above the overall ploidy of the cells (Fig. S2).

Microdissection of a Pure Population of Embryonic Day 9.5 Murine Trophoblast Giant Cells. The TGCs are similar to *Drosophila* polytene cells in that they use the endocycle to become polytene and thus possibly undergo gene amplification or underreplication (16). Parietal TGCs (P-TGCs, termed TGCs for simplicity from here on) (30, 31) form a thin layer of extraembryonic cells separating the maternally derived decidua from the embryonically derived layers of the murine placenta (Fig. 2*A*), with placental morphology and TGC accessibility drastically changing throughout gestation (12, 13). We chose to isolate TGCs rather than analyze cell culture models to avoid potential differences in the endocycle that may occur in cell culture and also because the native TGCs attain a higher level of ploidy.

To obtain DNA and RNA with minimal contamination from the surrounding layers, we microdissected TGCs from embryonic day 9.5 (E9.5) implantation sites from C57BL/J6 mice (32), as cells at this stage are still in a layer amenable to microdissection. Furthermore, we manually removed the portion of the TGCs in direct contact with the spongiotrophoblast layer and the labyrinth layer to avoid collecting any polyploid cells from the former or multinucleated syncytiotrophoblast cells from the latter (12). We then analyzed the ploidy of the isolated TGC population, measuring DAPI intensity as described (33) and normalizing to diploid embryonic DNA content. TGCs had ~80-fold (median = 82) more DNA than embryonic cells (Fig. 2*B*), indicating that the majority of the cells had undergone roughly six ($\log_2$ 128C) endocycles. It has been estimated that the maximal ploidy level at day 9 is 128–256C (34, 35), which is in agreement with our day 9.5 DAPI quantifications. RNA-Sequencing (RNA-Seq) identification of the transcriptome from these isolated TGCs further supported the purity of the cells (see below and Fig. S4).

Trophoblast Giant Cells Do Not Undergo Significant Differential DNA Replication. Genomic DNA was isolated from the purified TGCs to perform aCGH to search for over- or underreplicated

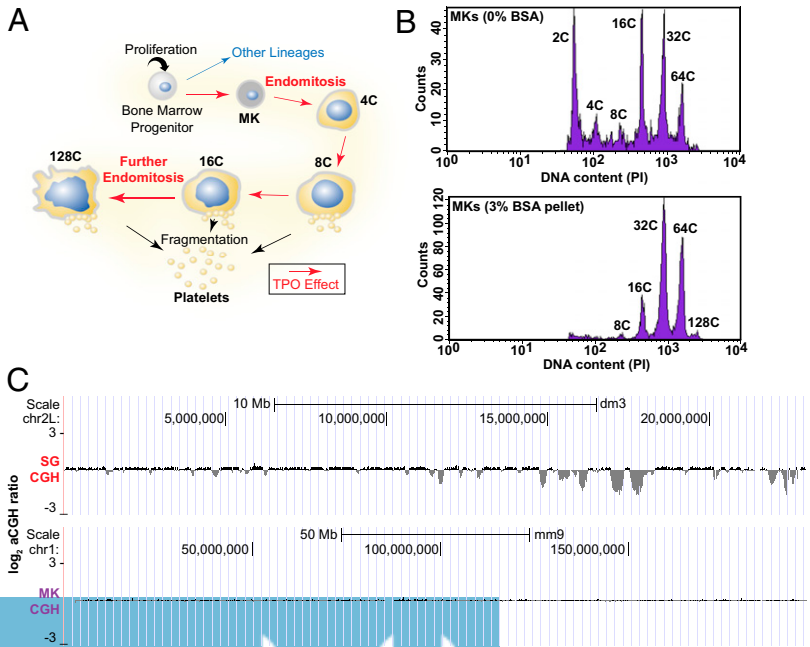

Fig. 1. MKs do not exhibit over- or underreplicated genomic regions. (*A*) Schematic diagram illustrating thrombopoietin (TPO) induction of megakaryocyte (MK) differentiation and polyploidization via endomitosis, adapted from ref. 6. (*B*, *Upper*) Flow cytometry analysis of CD41-positive MKs after 4 d in culture with TPO. (*B*, *Lower*) Flow cytometry analysis of CD41-positive MKs from 3% BSA pellet, illustrating the purity of our polyploid-enriched fraction. (*C*, *Upper*) Array-based comparative genome hybridization (aCGH) array for *Drosophila* chromosome arm *2L* showing copy number in polytene salivary glands relative to diploid embryonic DNA (data from ref. 64). Domains of 100–300 kb have up to a 10-fold reduction in copy number. (*C*, *Lower*) aCGH array of mouse chromosome 1 comparing copy number of polyploid (>16C) MKs relative to diploid (2C) MKs. Copy number is uniform across the chromosome, without gene amplification or underreplication. This figure, Fig. 2*C*, and Figs. S1–S4 were created using the University of California Santa Cruz genome browser (build dm3 and mm9) and are plotted on a $\log_2$ scale (70, 71).

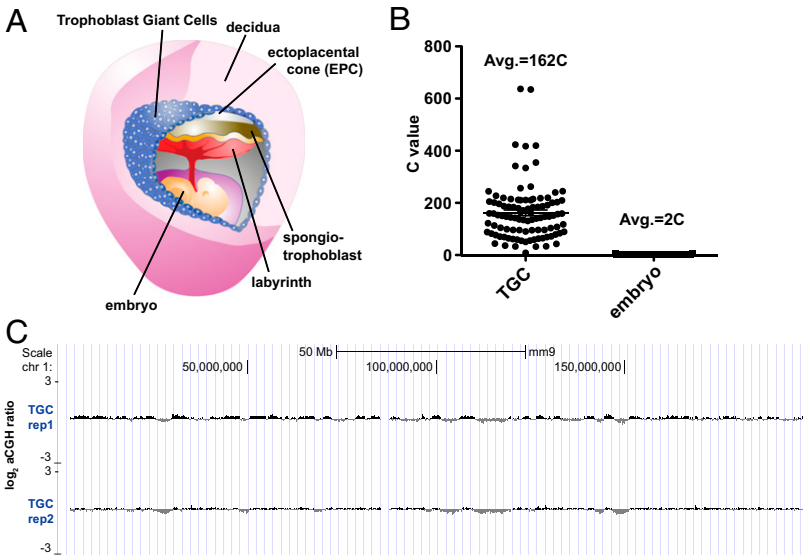

Fig. 2. Trophoblast giant cells are devoid of differential replication. (*A*) Illustration of day 9.5 mouse implantation site. In blue is shown the trophoblast giant cell layer that was microdissected for this study. (*B*) Scatterplot showing ploidy C values from DAPI microdensitometry of day E9.5 trophoblast giant cells (TGCs) and E9.5 embryonic cells (n = 98 for TGCs and n = 68 for embryos). Polytene TGCs contain 80-fold more DNA content than embryonic diploid cells; the average C value for TGCs is 162C compared with the average embryonic value set at 2C. (*C*) Two biological replicates of aCGH arrays on chromosome 1 of the mouse genome, comparing DNA levels from the TGCs at day E9.5 relative to diploid embryonic DNA levels.

genomic regions. DNA copy number measurements of E9.5 TGCs relative to E9.5 embryos from two biological replicate aCGH experiments showed no significant (>1.3-fold relative to overall ploidy) differential replication in TGCs (Fig. 2C and Fig. S3), indicating that no euchromatic regions were over- or underreplicated for even one round of endoreplication at this stage.

Heterochromatin Is Fully Replicated in Polyploid TGCs and MKs. In *Drosophila*, underreplication is prevalent in both euchromatic (10) and heterochromatic regions (36, 37). The blocks of heterochromatin surrounding the centromeres and accounting for 20–30% of the length of each chromosome arm are not visible in *Drosophila* polytene chromosomes. Cytological studies on the chromosomes of TGCs have yielded conflicting conclusions about whether and to what extent heterochromatin is underreplicated (15, 18). The bridges observed in anaphase in MKs could be due to underreplication of the heterochromatin. Because the microarrays do not contain probes for heterochromatic sequences, we measured copy number changes in heterochromatin in isolated TGCs and MKs by quantitative real-time PCR (qPCR). Using primer sequences derived from bacterial artificial chromosome (BAC) clones, we performed qPCR on three different heterochromatic regions including centromeric and telomeric sequences (Fig. 3). Copy number changes were obtained relative to two single-copy genes (*β-actin* and *γ-tubulin*) in two biological replicates. Strikingly, our results demonstrate that both TGCs and MKs undergo full replication of heterochromatin.

Transcriptome of Trophoblast Giant Cells. To identify gene expression signatures associated with TGC differentiation and endoreplication, we determined the transcriptome and microRNA (miRNA) expression profile from the purified trophoblasts. The transcriptome analysis confirmed a high level of purity for our hand-dissected samples, with high expression of known TGC markers, and negligible expression of genes known to be expressed in nearby tissues (Fig. S4). As expected, among the most abundant protein-coding RNA molecules of the TGCs (top 30 expressed protein-coding genes shown in Table S1) were many hormone genes (such as the prolactin family) and genes involved in steroid biosynthesis [*cytochrome P450, family 11, subfamily A, polypeptide 1* (*Cyp11a1*), *steriodogenic acute regulatory protein* (*STAR*), and *hydroxy-delta-5-steriod dehydrogenase 3 beta-and steroid delta-isomerase 6* (*Hsd3b6*)). The *heart and neural crest derivatives expressed transcript 1* (*Hand1*) gene, an important regulator of TGC differentiation, also was highly expressed (19). Other highly expressed genes included *scavenger receptor class B, member 1* (*Scarb 1*), required for the phagocytic activity of TGCs (38, 39), and the *Cdk1 inhibitor p57* (*Cdkn1c*), required for TGC endoreplication (22).

We analyzed the E9.5 TGC mRNA-Seq profile with the GOrilla gene ontology (GO) tool (http://www.ncbi.nim.nih.gov/pubmed/19192299), which uses an algorithm that precludes the choice of an arbitrary P value cutoff. Consistent with TGCs being highly metabolic steroidogenic cells, strongly expressed genes are enriched in GO terms for intracellular transport, Golgi vesicle transport, and ATP and steroid biosynthesis (Table S2). Among the enriched GO process categories (Table S2) were metabolism, translation, intracellular transport, Golgi vesicle transport, negative regulation of cell death, ATP biosynthetic process, steroid biosynthetic process, angiogenesis, and cell differentiation involved in embryonic placenta development. The enriched expression of genes involved in ATP biosynthesis and the robust expression of mitochondrial genes are consistent with a recently proposed role of mitochondria in polyploidization of maternal decidual cells (40). GO terms unique to the mitotic cell cycle and apoptosis were less common, in agreement with recent studies showing polyploid cells to have antiapoptotic properties, important in avoiding cell cycle checkpoints (22, 41).

Expression Levels of Replication Factors Vary Among Different Polyploid Cell Types. The *Drosophila* larval polytene salivary gland (SG), fat body, and midgut tissues undergo a dramatic

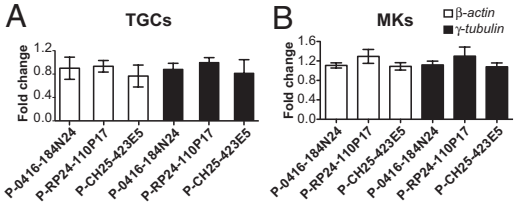

Fig. 3. Heterochromatin in TGCs and MKs is fully replicated. qPCR measurements of DNA copy number at three independent heterochromatic regions relative to two single-copy genes (*β-actin* and *γ-tubulin*). (*A*) Fold changes in heterochromatin copy number were similar (ratio of 1) between polytene TGCs and diploid embryonic cells, and between polyploid and diploid MKs (*B*), indicating no underreplication in these regions. BAC sequence primers used for this analysis covered both centromeric (P-0416-184N24 and P-RP24-110P17) and telomeric (P-CH25-423E5) regions. Error bars, SEM of two independent biological replicates.

reduction in the expression of genes involved in DNA replication (10, 42). It has been proposed that reduced expression of genes required for DNA replication leads to a slowed S phase and failure to replicate genomic regions normally replicated late in S phase (43), possibly leading to underreplication in these tissues.

We analyzed the expression status of S-phase genes in mammalian TGCs and MKs. S-phase gene transcript levels were quantile normalized (*Materials and Methods*) (Dataset S1), and compared between polytene TGC or polyploid MK cells and diploid control cells. In these analyses we used our RNA-Seq data from TGCs compared with published RNA-Seq data from mouse embryonic brains and published microarray data sets for polyploid versus diploid human MKs (Fig. 4). We compared expression levels of orthologous genes in the DNA replication pathway and cell cycle genes controlling the G1–S transition and S phase. Strikingly, we found that components of the DNA replication machinery including the *origin recognition complex* (*ORC*), *minichromosome maintenance* (*MCM*), and *proliferating cell nuclear antigen* (*PCNA*) genes were strongly expressed in the TGC lineage (Fig. 4). In contrast to TGCs, polyploid MKs exhibited a reduction in the expression of these genes, but the normalized expression levels were higher than in the *Drosophila* salivary gland (Fig. 4).

In *Drosophila*, the onset of endoreplication strongly correlates with the transcriptional and translational down-regulation of key M-phase promoting genes (1). To investigate the expression patterns of these mitotic genes in mammalian polytene and polyploid cells, we compared TGCs and MKs (Fig. S5 and Dataset S1). Similar to *Drosophila* polytene cells, TGCs show a reduction in the expression of mitotic genes compared with diploid control embryonic cells (Fig. S5), which tightly correlates with the nature of the endocycle and is consistent with previously described findings by MacAuley et al. (20) and Hattori et al. (21). Consistent with their ability to enter mitosis, polyploid MKs showed a different expression pattern, with increased expression of M-phase factors relative to TGCs or *Drosophila* SGs (Fig. S5).

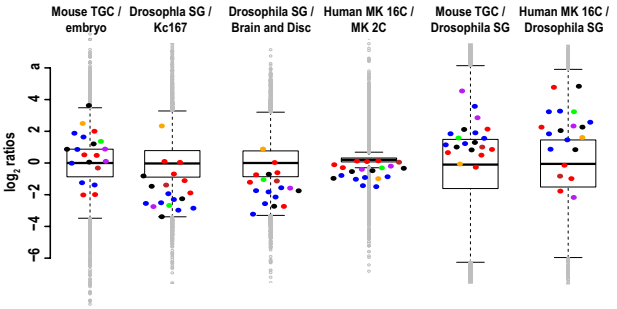

Fig. 4. DNA replication factors are increased in expression in TGC and MK cells relative to *Drosophila* salivary gland cells. Illumina RNA Sequencing or microarray gene expression levels from murine TGCs (this study), human MKs (69), polytene *Drosophila* salivary glands (42, 64), mouse E14.5 fetal heads (67), Drosophila brains and imaginal discs (42), and Kc167 cells (68). Shown are boxplots of $\log_2$-transformed quantile normalized expression ratios of S-phase genes between diploid and polyploid/polytene cells of the same species or between orthologous pairs of mammalian and *Drosophila* genes. Genes involved in DNA replication are color coded [red, *ORC* complex; orange, *cell division cycle 6* (*cdc6*); green, *double parked* (*dup*)/*Cdt1*; blue, *MCM* complex; purple, *cyclin E1* and *E2f/Cce1–2*; black, *DNA polymerase/primase* (*DNApol/PRIM*), *geminin*, *PCNA*]. Statistical analysis shows that the fold increase in expression of this set of genes is significant (one-way ANOVA with a Tukey post hoc test: $P = 5.33E\text{-}06$ (TGC/embryo vs. SG/Kc167); $P = 2.93E\text{-}05$ (TGC/embryo vs. SG/brain and discs); $P = 0.335122$ (MK16C/2C vs. SG/Kc167); $P = 0.550709$ (MK16C/2C vs. SG/brain and discs); $P = 4.47E\text{-}10$ (TGC/SG vs. SG/Kc167); $P = 3.57E\text{-}09$ (TGC/SG vs. SG/brain and discs); $P = 2.15E\text{-}10$ (MK16C/SG vs. SG/Kc167); $P = 1.81E\text{-}09$ (MK16C/SG vs. SG/brain and discs), indicating that as a group, the replication genes are more strongly expressed in murine TGCs and MKs than in *Drosophila* salivary glands.

miRNA Profile of Trophoblast Giant Cells. One of the most abundant noncoding RNA transcripts found in our TGC RNA-Seq data set was *H19* [Fragments Per Kilobase of transcript per Million reads mapped (FPKM) = 24,368], a tumor-suppresor noncoding RNA gene (44–47). As *H19* is not only the first imprinted noncoding transcript discovered, but also is the known precursor for *miR-675* (48, 49), we analyzed the miRNA composition of the TGCs by deep sequencing. Although we did see strong expression of *miR-675* (Table S3), it was far from the most abundant miRNA. Therefore, it is likely that the high expression of *H19* in TGCs functions to maintain high levels of the long noncoding RNA for its tumor-suppressor properties rather than primarily to give rise to *miR-675*. Importantly, *H19* is expressed in human placental extravillous cytotrophoblast cells (50). These are polyploid cells that invade the uterus in humans and share the expression of many transcription factors, proteases, and cell adhesion molecules with TGCs (30). It is attractive to speculate that *H19* plays a role in controlling the extent of invasiveness of the TGC cell population.

The two most highly expressed miRNAs in TGCs were *miR-322* and *miR-503* (Table S3), recently shown to down-regulate *cell division cycle 25A* (*cdc25A*) (51), whose expression in TGCs is indeed extremely low according to our mRNA-Seq data (FPKM= 20). Many of the *let-7* family members, implicated in promoting terminal differentiation, tumor suppression, and in being proangiogenic (52–54), are highly expressed in TGCs. *miR-182*, an antiapoptotic miRNA that is abundantly expressed in TGCs, was recently shown to decrease in human placentae of patients with preeclampsia, a disease associated with extravillous cytotrophoblast cells that are analogous in many respects to the TGCs of the rodent placenta (55). Little is known about the function of other highly expressed miRNAs (e.g., *miR-451*), making them interesting candidates for future studies. This catalog of mRNAs and miRNAs expressed in E9.5 TGCs will be a resource for further study of trophoblast differentiation and function.

Discussion

In this study, we compared mechanisms producing polyploid cells by analyzing gene expression and genome copy number in two polyploid mammalian cell types, the TGCs and MKs. By directly isolating TGCs from mouse placenta, we were able to obtain cells of high ploidy in their native state to analyze the parameters of DNA replication by quantification of genome copy number. The advantage of this approach over the use of cell culture models was the ability to analyze higher levels of ploidy and to avoid potential S phase alterations arising in vitro. Similarly, by examining primary MKs differentiated for only 4 d from bone marrow progenitor cells, possible genome aberrations from cell culture lines were eliminated. The transcriptome of the endocycling TGCs described here, compared with published data on endomitotic MKs, supports the notion that repression of the mitotic machinery is an integral component of endocycling cells both in *Drosophila* and mammals, in contrast to its presence in cells undergoing endomitosis.

Our aCGH studies and qPCR copy number measurements revealed integral genome doublings in mouse TGCs and MKs, and an absence of differential (>1.3-fold) DNA replication. Thus, in these cells, robust gene expression is not accompanied by gene amplification. It is notable that several extensive domains in TGCs appear reproducibly reduced in copy number and are called peaks by the Ringo and MA2C programs if thresholds are set to less than 1.3-fold. The slight copy number reduction may be a consequence of these domains replicating slowly, thus requiring a longer period of S phase to be duplicated. Therefore, in a high percentage of S-phase cells these regions may not yet be replicated.

The genome-wide aCGH analysis provided a quantitative and direct examination of copy number. Previous cytological studies in MKs raised the possibility of underreplication, given the chromosome bridges observed during aberrant anaphases (11). Because TGCs use an endocycle and all examined endocycling cells in *Drosophila* exhibit underreplication, we hypothesized that at the very least TGCs heterochromatin would be underreplicated. It was

not possible from previous cytological studies to ascertain gene copy number with certainty or to survey the entire euchromatic genome, and results yielded inconsistent conclusions about differential replication (56–58). TGC chromosomes showed detectable heterochromatin staining and the presence of Barr bodies, but there was no increase in the number and size of heterochromatin blocks with increasing ploidy (15). Attempts to quantify DNA content by cytophotometry were consistent with integral genome doublings in some cells, but showed others with DNA values lower than multiples of 2C (15). A restriction landmark genomic scanning study of CpG islands in late gestation rat placental junctional zone tissue, which contains TGCs, indicated that nearly all of the ~1,000 loci tested showed no differential replication (59). In situ hybridization signals with satellite DNA probes indicated replication of the heterochromatin in TGCs (18).

Given the evolutionary conservation of the endocycle and its universal use in the plant and animal kingdoms, it was surprising to observe the absence of differential replication in TGCs compared with its presence in all examined *Drosophila* tissues. Analysis of the transcriptome of the purified TGCs revealed a striking elevation in the normalized level of expression of genes required for S phase relative to the transcriptome of the *Drosophila* salivary gland. S-phase genes are also more highly expressed in MKs compared with *Drosophila* endocycling cells. These observations suggest that there are fundamental differences in the parameters of the endocycle between different organisms.

The differences in expression of replication genes support the model (42, 43) that limiting expression of proteins needed for initiation of DNA replication leads to a slower S phase and prevents the duplication of genome regions that are late replicating. This provides an advantage of not replicating gene-poor regions such as heterochromatin, but at the cost of slow endocycles. The endocycles indeed proceed much faster in the mouse TGCs than in *Drosophila*. The 256C ploidy at day 9.5 is achieved in 4–5 d of embryogenesis (15), in contrast to the *Drosophila* salivary gland that takes 7 d to achieve a ploidy of 1024C for a genome only 5% the size of the mouse genome (4). We speculate that the developmental window during which TGCs must attain the proper ploidy is short enough that a faster endocycle is required, necessitating high expression of S-phase genes and forcing replication of heterochromatic regions.

A second potential explanation for why TGCs and MKs integrally replicate their genomes in contrast to *Drosophila* tissues may lie in differences in genomic organization of the euchromatin and heterochromatin in the two species. In *Drosophila*, nearly all of the heterochromatin is present in large blocks surrounding each centromere, accounting for 20%–30% of the length of each chromosome arm (60). In contrast, in mouse, although there are satellite DNAs present in each centromere and telomere, heterochromatin is dispersed throughout the genome (61–63). We have shown that underreplication in *Drosophila* results both from an absence of origins across a region and from the presence of flanking chromatin that blocks progression of replication forks that initiate outside of an underreplicated region (64). The presence of heterochromatin in large domains may facilitate its underreplication, as such a domain necessitates only one region per side to impede fork progression. Interspersed murine heterochromatin would require many such blocking regions per chromosome.

It should be noted that the maximal ploidy levels of murine TGCs has been estimated to be 512C–850C at embryonic day 11 (16, 34). Thus, the TGCs undergo one or two more rounds of endoreplication beyond the stage at which our aCGH experiments were performed. It is conceivable that underreplication or gene amplification do not begin until these last one or two S phases. It is not possible to test this experimentally for TGCs because after embryonic day 9.5 the cells are embedded and cannot be isolated by microdissection. Underreplication for only the last two endocycles, however, would at most lead to a fourfold decrease in copy number. In the *Drosophila* larval tissues, underreplication begins at the first endocycle in embryogenesis (3, 64), although the *Drosophila* ovarian nurse cells undergo five endocycles with full replication followed by five with underreplication (65). Consequently, we cannot exclude the possibility that a later developmental change in the endocycle in the mouse TGCs could produce differential DNA replication. In addition, only the parietal TGC subtype was analyzed; it is still possible that other TGC subtypes (30) do exhibit differential replication.

Increased DNA content is used as a developmental strategy throughout the plant and animal kingdoms. Despite this evolutionary conservation, we found striking differences between *Drosophila* and mouse in the use of differential DNA replication within the context of endoreduplication. These differences suggest distinct parameters of the endocycle and possible effects of the genomic organization of heterochromatin and euchromatin on underreplication. It will be informative to examine endocycling cells in plants and other animal tissues to determine the association between differential DNA replication and heterochromatin organization and the link with levels of expression of S-phase genes.

Materials and Methods

Isolation of Mouse MKs and FACS. Isolation of mouse bone marrow cells, culture, purification of polyploid MKs, and FACS are detailed in *SI Materials and Methods*. These procedures were approved by the Institutional Animal Care and Use Committee at Boston University Medical Center.

Isolation of TGC Cells. Day E9.5 trophoblast giant cells were microdissected from C57B6/J mice as previously described (32) from two biological replicates. TGCs were isolated from 29 implantation sites from three pregnant mice (replicate one), and from 14 implantation sites from one pregnant mouse (replicate two). Diploid embryonic controls were E9.5 embryos from the dissected implantation sites. These procedures were approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

aCGH. DNA was prepared from diploid (2C) and polyploid (>16C) MK cell fractions or embryonic cells and purified TGCs, labeled, and hybridized to microarrays as described in *SI Materials and Methods*.

mRNA-Seq. Day E9.5 TGCs were microdissected from nine implantation sites from one pregnant mouse, immediately transferred into TRIzol (Invitrogen), and RNA extracted according to the manufacturer's protocol. Ten micrograms of RNA were processed with an Illumina mRNA Sample Preparation kit. Sequencing was done as described in *SI Materials and Methods*.

miRNA-Seq. TGCs miRNA sequencing samples were processed using an Illumina miRNA kit as in ref. 66. After adapter trimming, Illumina reads were aligned to mature miRNAs (miRBase Release 16) using Novoalign, and reads per miRNA were calculated.

Comparison of Expression Profiles. Expression data (microarrays and short reads) were downloaded, analyzed separately, and replicates were mean summarized. RNA-Seq profiles were generated using mouse TGCs (this study; 36-nt Illumina reads; $n = 1$), mouse E14.5 fetal heads (67) [National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) GEO series (GSE)33017; 36-nt Illumina reads; $n = 3$], *Drosophila* salivary glands (64) (NCBI GEO GSE33017; 36-nt Illumina reads; $n = 2$), and Kc167 cells [modENCODE 68; modENCODE_2593; 37-nt Illumina reads; $n = 2$] datasets. Microarray expression profiles were generated using published *Drosophila* salivary gland and brain-disk complexes (42) (NCBI GEO GSE19029; Genepix two-channel microarrays; $n = 1$) and human 2C and 16C MKs (69) (EBI ArrayExpress E-TABM-133; Agilent two-channel microarrays; $n = 2$). Mouse–fly ortholog pairs were obtained from Ensembl v67 and linked to RNA-Seq and expression microarray identifiers to obtain gene sets for further analysis.

Real-Time qPCR. Real-time qPCR was performed in two biological replicates with primers against BAC sequences for centromeric [BAC-RP24-184N24 (Y) and BAC-RP24-110P17 (Y)] and telomeric [BAC-CH25-423E5 (X)] heterochromatic regions in E9.5 TGCs (polytene sample) and E9.5 embryos (diploid sample), using Perfecta SYBR Green FastMix (Quanta BioSciences) with an Applied Biosystems 7300 Real-Time PCR machine according to the manufacturer's instructions. Primers are listed in *SI Materials and Methods*. PCR values were normalized to levels of two single-copy genes (β-*actin* and γ-*tubulin*).

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