

## RESEARCH ARTICLE

## TECHNIQUES AND RESOURCES

# Visualizing developmentally programmed endoreplication in mammals using ubiquitin oscillators

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## ABSTRACT

The majority of mammalian somatic cells maintain a diploid genome. However, some mammalian cell types undergo multiple rounds of genome replication (endoreplication) as part of normal development and differentiation. For example, trophoblast giant cells (TGCs) in the placenta become polyploid through endoreduplication (bypassed mitosis), and megakaryocytes (MKCs) in the bone marrow become polyploid through endomitosis (abortive mitosis). During the normal mitotic cell cycle, geminin and Cdt1 are involved in 'licensing' of replication origins, which ensures that replication occurs only once in a cell cycle. Their protein accumulation is directly regulated by two E3 ubiquitin ligase activities, APC<sup>Cdh1</sup> and SCF<sup>Skp2</sup>, which oscillate reciprocally during the cell cycle. Although proteolysis-mediated, oscillatory accumulation of proteins has been documented in endoreplicating *Drosophila* cells, it is not known whether the ubiquitin oscillators that control normal cell cycle transitions also function during mammalian endoreplication. In this study, we used transgenic mice expressing Fucci fluorescent cell-cycle probes that report the activity of APC<sup>Cdh1</sup> and SCF<sup>Skp2</sup>. By performing long-term, high temporal-resolution Fucci imaging, we were able to visualize reciprocal activation of APC<sup>Cdh1</sup> and SCF<sup>Skp2</sup> in differentiating TGCs and MKCs grown in our custom-designed culture wells. We found that TGCs and MKCs both skip cytokinesis, but in different ways, and that the reciprocal activation of the ubiquitin oscillators in MKCs varies with the polyploidy level. We also obtained three-dimensional reconstructions of highly polyploid TGCs in whole, fixed mouse placentas. Thus, the Fucci technique is able to reveal the spatiotemporal regulation of the endoreplicative cell cycle during differentiation.

**KEY WORDS:** Fucci imaging, Endomitosis, Endoreduplication

## INTRODUCTION

Endoreplication is the process by which a cell undergoes successive rounds of DNA replication without intervening mitosis or cytokinesis, resulting in polyploidy (Zybina and Zybina, 1996; Edgar and Orr-Weaver, 2001; Lee et al., 2009; Ullah et al., 2009; Hu

and Cross, 2010; Davoli and de Lange, 2011). In lower eukaryotes, endoreplication is developmentally programmed and is a major phenomenon during embryogenesis and differentiation. For example, salivary gland and follicle cells in *Drosophila* and leaf epidermal cells in plants have polyploid nuclei (Schaeffer et al., 2004; Lammens et al., 2008; Narbonne-Reveau et al., 2008). In these cell types, anaphase-promoting complex/cyclosome (APC/C) and an activator (Cdh1, Fzr or ccs52a) are required to switch from the mitotic to the endoreplicative state. The cyclic activity of APC/C<sup>Cdh1/Fzr/ccs52a</sup> is also essential to sustain endoreplication.

Endoreplication is a relatively minor event in mammals and has been intensively studied in two cell types. Trophoblast giant cells (TGCs) and megakaryocytes (MKCs) undergo endoreduplication (bypassed mitosis) and endomitosis (abortive mitosis), respectively. TGCs are polyploid cells in the placenta that are essential for embryonic implantation in the uterus and for post-implantation placental development. TGCs avoid mitosis entirely, and this form of endoreplication is called endoreduplication. TGCs endoreduplicate as they differentiate from trophoblast stem cells (TSCs) in the trophectoderm layer of the blastocyst-stage embryo (Zybina and Zybina, 1996; Rossant and Cross, 2001; Martindill and Riley, 2008; Ullah et al., 2008; Ullah et al., 2009; Hu and Cross, 2010; Davoli and de Lange, 2011; Chen et al., 2012). Loss of fibroblast growth factor 4 (Fgf4) signaling or activation of the transcription factors Hand1 and Tead4 is necessary for TSCs to switch from the mitotic to the endoreduplicative cell cycle (Rossant and Cross, 2001; Martindill and Riley, 2008; Nishioka et al., 2008; Ullah et al., 2008; Chen et al., 2012). However, little is known about the molecular mechanisms responsible for progression of endoreduplication in TSCs.

MKCs in the bone marrow also show polyploidy. Hematopoietic stem cells (HSCs) differentiate first into megakaryoblasts (MKBs) and then into mature MKCs through endomitosis in the presence of the cytokine thrombopoietin (TPO; also known as THPO). Endomitosis is a form of endoreplication in which the cell undergoes early mitotic events such as chromosome condensation. Molecular candidates for the endomitotic switch include cyclin B1 and cyclin E (Carow et al., 2001; Bermejo et al., 2002; Eliades et al., 2010). In addition, live-imaging experiments were performed using the histone2B (H2B)-GFP or YFP-tubulin marker to monitor how mitosis aborted (Geddis et al., 2007; Papadantonakis et al., 2008).

Polyploidization or endoreplication is not only developmentally programmed, but is also an early step in the generation of aneuploid cancers. For instance, persistent DNA damage signals elicited by dysfunctional telomeres causes mitosis to be bypassed in p53-deficient cells (Davoli et al., 2010). Oscillations in ubiquitylation by the SCF<sup>Skp2</sup> and APC<sup>Cdh1</sup> complexes (Ang and Harper, 2004; Vodermaier, 2004; Nakayama and Nakayama, 2006) were observed during endoreplication in these cells using fluorescent ubiquitylation-based cell cycle indicator (Fucci) live-cell imaging.

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**Table 1. Summary of the Fucci transgenic mouse lines**

Mouse lines	Features	References
mAG-hGem(1/110)*		
FucciS/G2/M-#504	Constant expression in most organs except hematopoietic system	Sakaue-Sawano et al., 2008; Stuckey et al., 2011; Hama et al., 2011; Ge et al., 2012
FucciS/G2/M-#492	Constant expression in hematopoietic organ; used for lymphatic studies	Aiba et al., 2010; Maruya et al., 2011; Kawamoto et al., 2012
FucciS/G2/M-#474	Constant expression in hematopoietic organ; used for HSC studies	This article
mKO2-hCdt1(30/120)*		
FucciG1-#596	Constant expression in most organs except hematopoietic system	Sakaue-Sawano et al., 2008; Juuri et al., 2012
FucciG1-#639	Constant expression in hematopoietic organ; used for lymphatic studies	Aiba et al., 2010
FucciG1-#610	Constant expression in hematopoietic organ; used for HSC studies	This article

\*Under control of the CAGGS promoter.

This technology harnesses the cell cycle-dependent proteolysis of two ubiquitin oscillators, human CDT1 and geminin, which are the direct substrates of SCF<sup>Skp2</sup> and APC<sup>Cdh1</sup> complexes, respectively (Sakaue-Sawano et al., 2008). The Fucci probe consists of two chimeric proteins: mKO2 (monomeric Kusabira-Orange 2) fused to the ubiquitylation domain of CDT1 [FucciG1=mKO2-hCdt1(30/120)], which labels G1 phase nuclei red, and mAG (monomeric Azami-Green) fused to the ubiquitylation domain of geminin [FucciS/G2/M=mAG-hGem(1/110)], which labels S/G2/M phase nuclei green. We also observed that DNA damage-induced endoreplication occurs when normal murine mammary gland (NMuMG) cells were treated with etoposide, an inhibitor of DNA topoisomerase II (Sakaue-Sawano et al., 2011). The nuclei of NMuMG cells stably expressing Fucci probes changed from green to red without cell division and failed to exhibit any features consistent with mitosis, including nuclear envelope breakdown (NEB). Thus, Fucci probes can report reciprocal E3 ligase activities in DNA damage-induced endoreplication.

Regulation of ubiquitin oscillators by SCF<sup>Skp2</sup> and APC<sup>Cdh1</sup> complexes is crucial for normal development. *Cdh1*<sup>-/-</sup> embryos die at embryonic day (E) 9.5–10.5 owing to defects in endoreduplication of TSCs (Garcia-Higuera et al., 2008; Li et al., 2008). Conversely, geminin<sup>-/-</sup> embryos fail to form an inner cell mass and exhibit premature endoreduplication at the eight-cell stage; as a result, all cells become committed to the trophoblast cell lineage (Gonzalez et al., 2006; Hara et al., 2006). In addition, early embryogenesis was examined using mice deficient in *Emi1*, which is known to be an APC/C inhibitor (Reimann et al., 2001). Although *Emi1*<sup>-/-</sup> embryos form polyploid TGCs, the inner cell mass does not develop properly, presumably because of the high degree of polyploidy and the abnormal cell-cycle progression (Lee et al., 2006). By contrast, *Skp2* appears to be less crucial than *Cdh1*. *Skp2*<sup>-/-</sup> embryos are viable, but cells in knockout mice contain markedly enlarged nuclei with polyploidy and multiple centrosomes, resulting in reduced growth and increased apoptosis (Nakayama et al., 2000). However, these studies do not provide direct evidence that SCF<sup>Skp2</sup> and APC<sup>Cdh1</sup> activities oscillate reciprocally during the endoreduplication cycle.

Here, we used Fucci technology to study endoreplication in differentiating mouse TGCs and MKCs. To do this, we screened transgenic mouse lines expressing Fucci probes and identified mice that mark differentiating trophoblast cells and hematopoietic cells. We also designed special culture wells to track the cell cycle progression of cells isolated from these mice as they differentiated into TGCs or MKCs. Finally, we used the Scale technique, which renders fixed biological samples optically transparent, but preserves fluorescent signals in the clarified structures (Hama et al., 2011). By this technique, we were able to perform three-dimensional (3D)

reconstruction of polyploid cells in whole placentas of transgenic pregnant mice. Using this combination of novel techniques, we show that TGCs and MKCs both skip cytokinesis, but using different mechanisms. In addition, we demonstrate that the transition from the SCF<sup>Skp2</sup>- to APC<sup>Cdh1</sup>-active state in differentiating MKCs varies with the polyploidy level.

## RESULTS

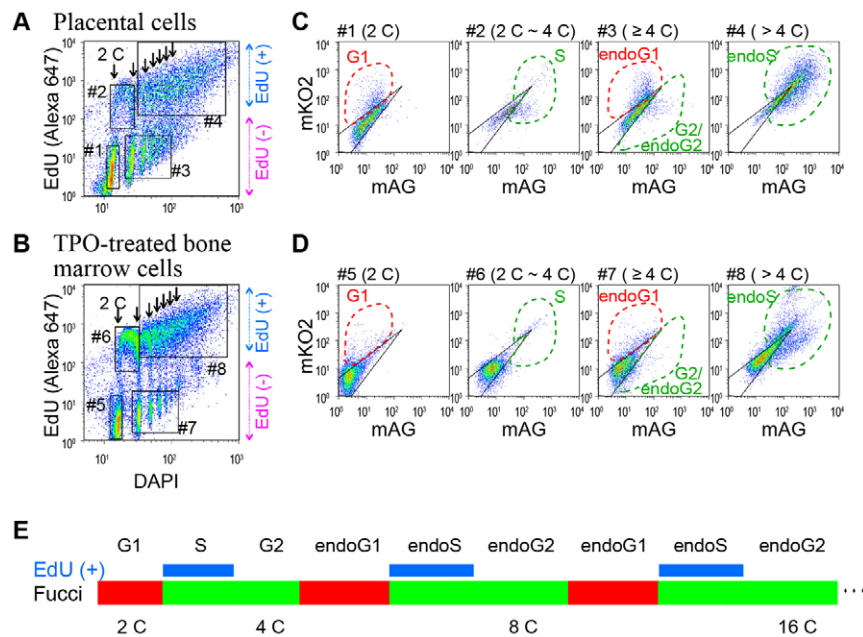
### Fucci transgenic mice

We previously generated eight transgenic mouse lines that constitutively express mAG-hGem(1/110) (FucciS/G2/M) and 16 lines constitutively expressing mKO2-hCdt1(30/120) (FucciG1) under the CAG promoter (Sakaue-Sawano et al., 2008). Our original work on the development of the Fucci technique used the FucciS/G2/M-#504 and FucciG1-#596 lines, which show nearly ubiquitous expression at early embryonic stages, with blastomere nuclei exhibiting green (FucciS/G2/M) or red (FucciG1) fluorescence (Table 1). Thus, to observe cell cycle changes during differentiation of trophoblast cells into TGCs, we cross-bred FucciS/G2/M-#504 and FucciG1-#596 mice and incubated blastocysts carrying both transgenes (#504/#596) in culture dishes.

For observation of MKC differentiation, these two lines were not suitable, as they did not show Fucci signals in the hematopoietic organs. We therefore performed extensive flow cytometry analysis using cell surface markers (Noda et al., 2008) to identify transgenic mouse lines expressing Fucci in the hematopoietic system. We found that FucciS/G2/M-#474 and FucciG1-#610 exhibited optimal signals in cells expressing a marker for cycling HSCs that have high repopulating activity (Table 1). We were therefore able to isolate HSCs from the bone marrow of the Fucci transgenic mice (#474/#610) and to observe cell cycle dynamics as they differentiated into MKCs.

### Validation of Fucci performance during endoreplication

We first performed validation experiments using TGCs and MKCs isolated from #504/#596 and #474/#610 mice, respectively (supplementary material Fig. S1A–D), to demonstrate that the Fucci system can be used to study cell cycle regulation during endoreplication. Immediately after administration of 5-ethynyl-2'-deoxyuridine (EdU) to a pregnant mouse (#504/#596) for 20 minutes, E10.5 placental cells were isolated and subjected to the Click-iT reaction for labeling with Alexa 647 (supplementary material Fig. S1A). Bone marrow (BM) cells were isolated from a mouse (#474/#610), cultured in the presence of TPO for 7 days, treated with EdU for 20 minutes, and then subjected to the Click-iT reaction for labeling with Alexa 647 (supplementary material Fig. S1C). Prior to flow cytometric analysis, both samples were



**Fig. 1. Application of Fucci technology to analysis of endoreplicating cells.** (A,B) Flow cytometry analysis of EdU incorporation (DNA synthesis) versus DAPI staining (DNA content) of placental cells from a #504/#596 mouse (A) and TPO-treated bone marrow cells from a #474/#610 mouse (B). Four cell fractions from each (#1-#4 in A; #5-#8 in B), which were characterized further, are indicated by boxes.

(C,D) Flow cytometry analysis of Fucci signals (mKO2 versus mAG) of cells in fractions #1-#4 (C) and #5-#8 (D). Cells that are EdU(-), diploid (2 C) and mKO2(+) are assigned to cycling G1 (#1 and #5). Cells that are EdU(+), diploid ~tetraploid (2 C ~ 4 C) and mKO2(+)/mAG(+) or mAG(+) are assigned to S (#2 and #6). Cells that are EdU(-), polyploid ( $\geq 4$  C) and mKO2(+) are assigned to endoG1 (#3 and #7). Cells that are EdU(-), polyploid ( $\geq 4$  C) and mAG(+) are assigned to G2 or endoG2 (#3 and #7). Cells that are EdU(+), polyploid ( $> 4$  C) and mKO2(+)/mAG(+) or mAG(+) are assigned to endoS (#4 and #8). (E) The cell populations in terms of EdU incorporation, Fucci color and DNA content are schematized.

stained with 4',6-diamidino-2-phenylindole (DAPI) (supplementary material Fig. S1B,D). Thus, all of the cells that transgenically exhibited the two-color fluorescence signals of Fucci probes produced fluorescence signals for the analysis of DNA synthesis (Alexa 647) and DNA content (DAPI). We performed multi-color flow cytometric analysis using four laser lines (supplementary material Fig. S2) to characterize polyploid populations in the samples. As cells in the cerebral cortex of an adult mouse are non-dividing diploid cells resting in G0/G1, they were used as the reference G0/G1 population (supplementary material Fig. S1E,F). Substantial DNA synthesis was detected in the placental and BM cell samples. Furthermore, prominent polyploidy associated with DNA synthesis was identified in both samples. By contrast, neither DNA synthesis nor polyploidization was found in the brain cell sample. Next, we obtained four fractions according to the EdU and DAPI signals for placental (Fig. 1A) and TPO-treated BM (Fig. 1B) cell samples each, and characterized their cell cycle phases (Fig. 1C,D, respectively). Fractions #1 and #5 produced an mKO2 signal and were supposed to represent the G1 population, whereas fractions #2 and #6 should represent the S population. Remarkably, by contrast, fractions #3, #4, #7 and #8 were composed of cells with  $\geq 4$  C. As illustrated in Fig. 1E, fractions #3 and #7 were EdU(-) and thus should consist of cells in the endoG1 and endoG2 populations (Hu and Cross, 2010) as well as the G2 population, whereas fractions #4 and #8 were EdU(+) and should represent the endoS population (Hu and Cross, 2010). These cell population data demonstrated that polyploid cells ( $> 4$  C) prepared from the Fucci transgenic mice had green or red nuclei according to cell cycle progression, which indeed involved DNA synthesis.

We also performed similar validation at the single-cell level. Another placenta from an EdU-administered pregnant mouse (#504/#596) was optically cleared using ScaleA2 reagent (Hama et al., 2011) and then subjected to the Click-iT reaction and DAPI staining (Fig. 2). Multi-color imaging using a Zeiss LSM780 system with two-photon excitation and confocal configurations (supplementary material Fig. S2) allowed us to identify gigantic TGCs that carried EdU(-) red nuclei (endoG1), EdU(+) green nuclei (endoS) and EdU(-) green nuclei (endoG2).

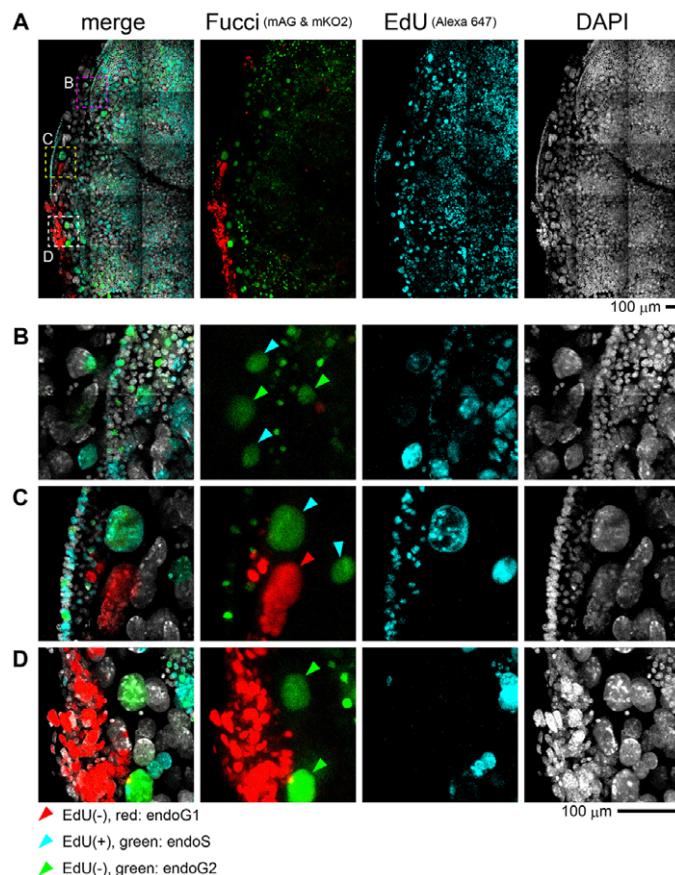
### Culture well for tracking all individual cells

During extended time-lapse imaging, target cells often migrate away from the field of view. To overcome this problem, we developed a culture well (FulTrac) with a poly(dimethylsiloxane) (PDMS) block stamped onto a glass coverslip (supplementary material Fig. S3). This allows us to fully track or trace individual cells as they progress through the cell cycle and differentiate. As the surface for cell growth is  $< 0.33$  mm in diameter, imaged cells remain inside the field of view of a  $20\times$  objective. Moreover, the FulTrac well has a stadium shape to maximize the numerical aperture (0.55) of the condenser for transmitted light. This enables high spatial resolution for differential interference contrast (DIC) imaging everywhere in the well, whereas conventional cylindrical wells give rise to shading in the periphery. For example, using an LCV100 microscope equipped with a FulTrac well, we were able to time-lapse image a morula-stage mouse embryo as it developed, all the way until it hatched from the zona pellucida (supplementary material Movie 1). Of particular importance is the design flexibility of FulTrac wells resulting from the ease of PDMS fabrication. For high-throughput analysis, we designed a PDMS block with four wells, performance of which can be maximized by a motorized microscope stage.

### Visualizing endoreduplication of trophoblast cells

To visualize cell cycle dynamics in TGC development, we crossed a female FucciG1-#596 heterozygote to a male FucciS/G2/M-#504 homozygote and cultured individual blastocyst stage embryos in FulTrac wells. As was expected, half of the embryos carried both transgenes (#504/#596) and exhibited both green (FucciS/G2/M) and red (FucciG1) fluorescence. During incubation in KSOM-AA medium (Nishioka et al., 2008) for 1 day, most blastocysts completed hatching. Embryos were then subjected to long-term time-lapse imaging in RPMI1640 medium supplemented with 20% fetal bovine serum (FBS) (Fig. 3A). After a hatched blastocyst attached to the bottom surface of the well, cells in the trophectoderm layer migrated away from the inner cell mass (ICM) (Fig. 3B; supplementary material Movie 2). The nuclei of some cells switched between green and red (Fig. 3C), but no cellular features consistent with M phase, such as NEB, were ever observed during the color conversion. This is reminiscent of what was previously observed in

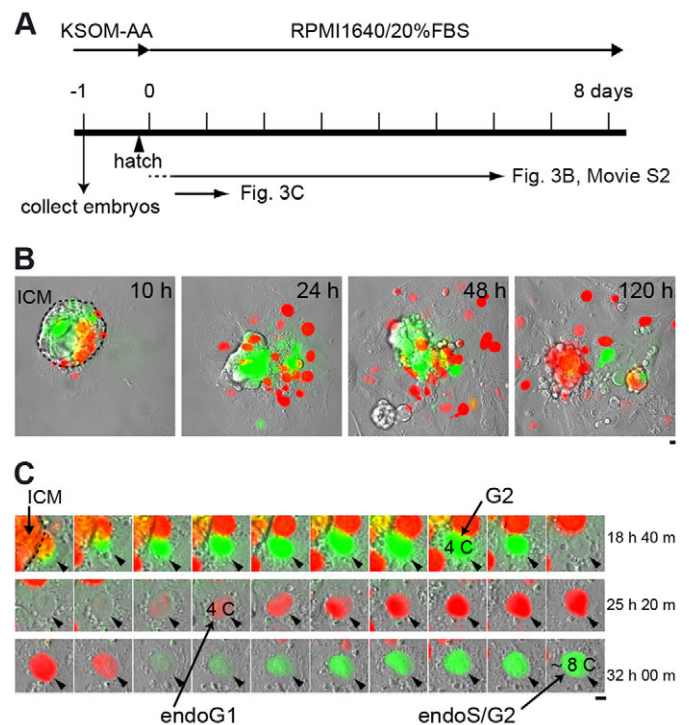




**Fig. 2. Visualization of endoreduplicating cells within a whole placenta.** Four-fluorophore imaging of endoreduplication in an optically cleared (ScaleA2-treated) whole placenta (E10.5), which was prepared from a Fucci transgenic mouse (#504/#596) and stained for DNA synthesis [EdU (Alexa 647)] and DNA content (DAPI). Images were acquired using confocal (Fucci and EdU) or two-photon excitation (DAPI) microscopy. (A) Multiple images were combined using the microscope system's 'tiling' software. (B–D) Three regions (indicated by dashed boxes in A) in the periphery are enlarged. TGCs in endoG1 [EdU(–), red], endoS [EdU(+), green] and endoG2 [EdU(–), green] are indicated by red, cyan and green arrowheads, respectively.

etoposide-treated NMuMG cells (supplementary material Fig. S4) (Sakaue-Sawano et al., 2011). These endoreduplicating cells eventually exhibited large, red (endoG1) or green (endoS/G2) nuclei and were morphologically identifiable as differentiated, polyploid TGCs (Fig. 3B, 120 hours). By reliably tracking individual cells on the ICM with 40-minute intervals (Fig. 3C), we were able to identify mitotic, diploid trophoblast cells (black arrowheads), which then differentiated into polyploid TGCs, and to assign chromatin (C) values to individual cells across time.

To gain a comprehensive 3D perspective of the endoreduplication cycle in TGCs, we examined Fucci signals in optically cleared placentas of #504/#596 embryos. Placentas with intact maternal decidua tissues at various gestation stages were treated with ScaleU2 (Hama et al., 2011) for >2 months. We imaged a whole E10.5 sample from the embryonic side (Fig. 4A). We used a confocal microscopy system (FV1000) equipped with a 40× silicone-oil objective to acquire high-resolution images of Fucci signals residing deep inside the sample and verified their nuclear localization by DAPI staining (Fig. 4B). Next, using a macro-confocal microscopy system (AZ-C1) equipped with a 2× objective, we obtained a 2D projection image of the entire placenta (Fig. 4C). Then we substituted a 4× objective for

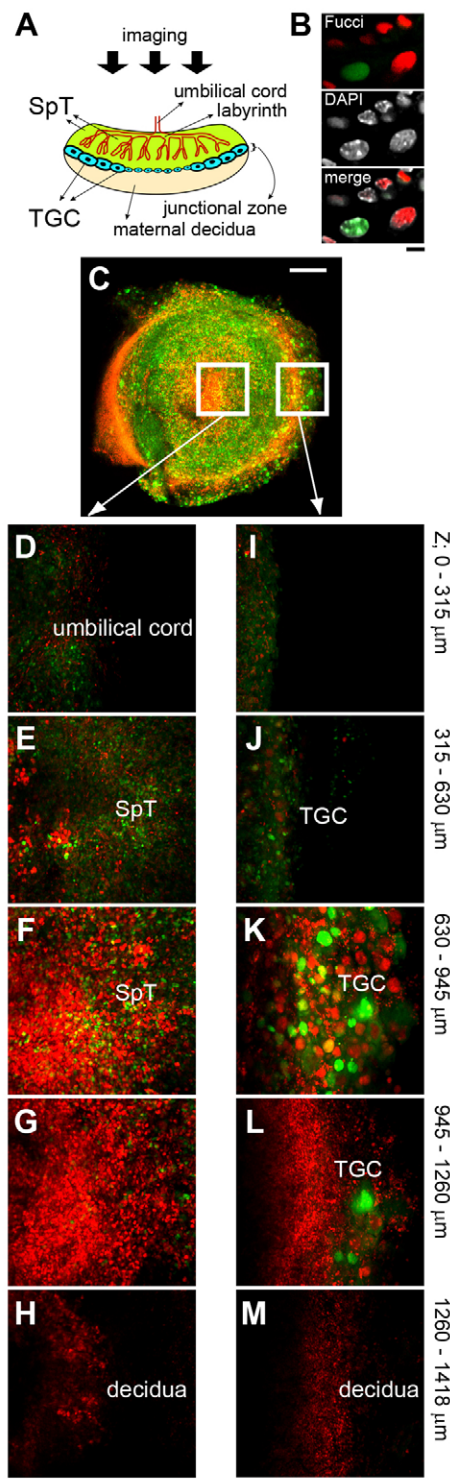


**Fig. 3. Visualization of trophoblast endoreduplication in FulTrac wells by Fucci live-cell imaging.** (A) A timetable of the preparation of blastocysts and time-lapse imaging. The endoreduplicative processes that were visualized in this study are indicated by solid lines. (B) Snapshots of trophoctoderm outgrowth from the ICM. (C) Endoreduplication of a mouse trophoblast giant cell with increasing chromatin ('C') values from 4 C to 8 C. C values denote DNA content as a multiple of the normal haploid genome. The Fucci fluorescence and DIC images were merged. Arrowheads indicate an endoreduplicating trophoblast cell. Scale bars: 10 μm.

higher magnification views in central (Fig. 4D–H) and peripheral (Fig. 4I–M) regions of the placenta. Highly polyploid TGCs with giant nuclei (>100 μm in diameter) were located peripherally at a depth of 0.6–1.0 mm, in the junctional zone (Fig. 4J–L). At this stage (E10.5), the green (endoS/G2) and red (endoG1) giant nuclei were observed in equal proportion. As their distributions appeared to be random, it is likely that no intercellular or spatial regulation governs progression of the endoreduplication cycle. By contrast, the central region was abundant with green and red nuclei of normal size (~10 μm in diameter), which might belong to diploid spongiotrophoblast (SpT) cells or less differentiated TGCs. The lack of highly polyploid TGCs in the central region was confirmed by 3D reconstruction experiments using two other E10.5 samples. These results suggest a centripetal pattern of differentiation of TGCs in the placenta. Highly polyploid TGCs with nuclear size >150,000 μm<sup>3</sup> were automatically identified using commercial software (Volocity) in the peripheral regions of transparent placentas prepared at E8.5, E9.5, E10.5, E11.5, E12.5 and E13.5 (supplementary material Fig. S5). Their number increased drastically between E9.5 and E10.5, and then the ratio of the green (endoS/G2) to red (endoG1) nuclei decreased. By E13.5, >99% of the gigantic cells had red (endoG1) nuclei. Thus, endoreduplicative cycling appears to be almost complete by E13.5 and produces terminally differentiated TGCs.

#### Visualizing endomitosis of MKBs and MKCs

To monitor endomitosis in HSCs as they differentiate into MKCs, we used two transgenic lines that mark hematopoietic cell nuclei



**Fig. 4. Three-dimensional reconstructions of a Fucci-expressing mouse placenta.** (A) Schematic of an E10.5 mouse conceptus. Tissue was fixed with 4% PFA and treated with ScaleU2 for 2 months. (B) High-resolution confocal images of giant nuclei labeled with Fucci and DAPI inside the placenta (#504/#596). Unsectioned tissue was imaged using a confocal microscope. (C) A projection image of Fucci-labeled nuclei in the placenta (#504/#596) and the maternal decidua (#596). Unsectioned tissue was imaged using a macro-zoom confocal microscope. (D-H) Five consecutive projection images of the center region (left white box in C). Each projection image consisted of 100 confocal images and spanned 315  $\mu\text{m}$  along the z-axis. (I-M) Five consecutive projection images of the peripheral region (right white box in C). Scale bars: 10  $\mu\text{m}$  (B); 1 mm (C); 100  $\mu\text{m}$  (D-M).

with green (FucciS/G2/M-#474) and red (FucciG1-#610) fluorescence (Table 1; Fig. 5). CD34<sup>+</sup>/c-Kit<sup>+</sup>/Sca-1<sup>+</sup>/Lin<sup>-</sup> cells (HSCs) were isolated from the bone marrow of adult #474/#610 mice and were used for further studies (supplementary material Fig. S6A). First, we analyzed the fluorescence intensities of mKO2 (red) and mAG (green) of these HSC, multipotent progenitor (MPP), mature B and immature B cell fractions by flow cytometry (supplementary material Fig. S6B). In each fraction, we identified two cell populations with high and low intensities of mKO2 signals for CDT1 accumulation, which we hypothesize correspond to quiescent G0 and cycling G1 cells, respectively. Because the HSCs exhibited a high intensity of mKO2 (red) fluorescence (supplementary material Fig. S6B, HSC), the #474/#610-derived HSC fraction was assumed to be enriched with quiescent G0 cells. We transferred the HSCs into the FulTrac well with a medium containing TPO (Fig. 5A). Nearly all of the cell nuclei were red (G0) in the beginning, but turned green (S/G2/M) after ~2 days, as revealed by the time-lapse imaging (supplementary material Fig. S7 and Movie 3). This slow proliferation switch in HSCs is corroborated by previous bromodeoxyuridine (BrdU) incorporation analyses (Passequé et al., 2005). After re-entering the cell cycle, some cells switch to an endomitotic cell cycle. At DIV (days *in vitro*) 8, giant cells with green (endoS/G2) or red (endoG1) nuclei appeared (Fig. 5B, HSC, DIV 8), which we assumed to be highly polyploid megakaryoblasts (supplementary material Fig. S8). These cell nuclei alternated between green and red multiple times as they grew and differentiated. The cells finally became presumptive MKCs with red nuclei.

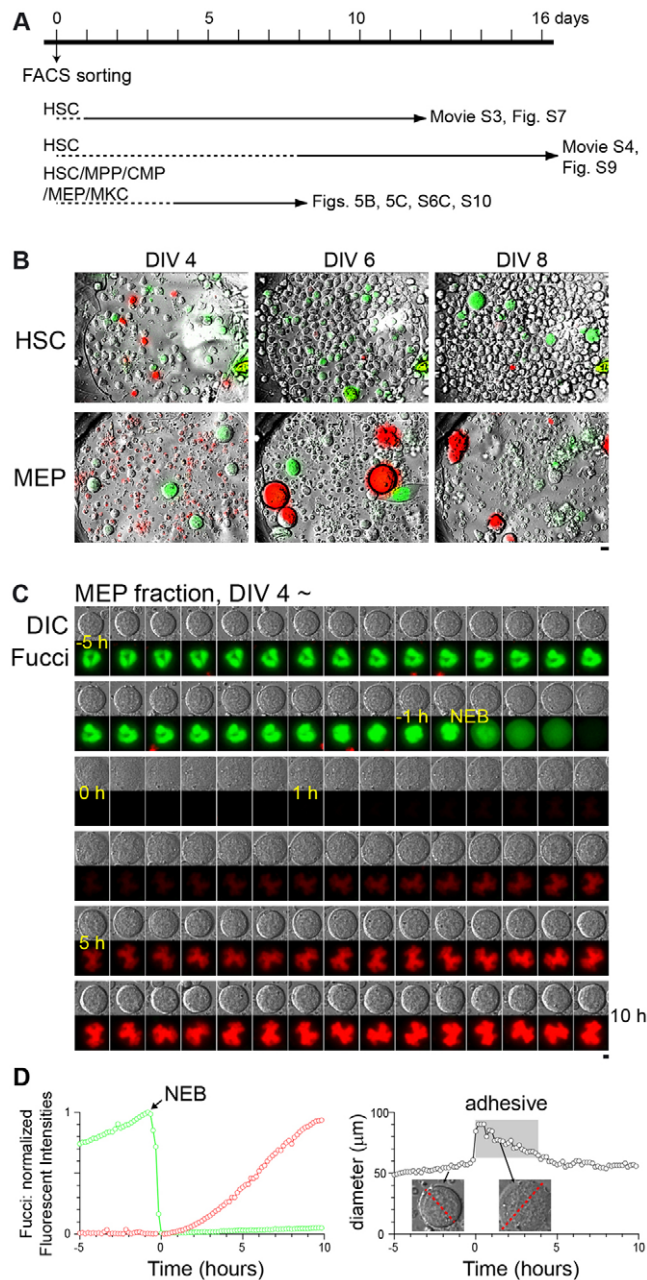
Using combinations of cell surface markers, we isolated MPPs, common myeloid progenitors (CMPs), megakaryocyte-erythroid progenitors (MEPs) and MKCs (supplementary material Fig. S6A). They were kept in FulTrac wells for time-lapse imaging (Fig. 5B; supplementary material Fig. S6C). We observed endomitosing megakaryoblasts in MPP, CMP and MEP cultures. In the MKC culture, we observed mature MKCs with red nuclei exhibiting hallmark features of proplatelets (supplementary material Fig. S6C, arrowhead) (Patel et al., 2005).

We also examined nuclear structures during endomitosis in MKCs (Fig. 5C). The green-to-red conversion (the endoG2/endoG1 transition) was accompanied by breakdown of the nuclear envelope, consistent with the fact that polyploidy in MKCs is achieved via endomitosis. Interestingly, upon the disappearance of the green fluorescence, cells rapidly adhered to the coverslip and then gradually returned to spheres. This transient morphological change was easily quantified by measuring the cell diameter (Fig. 5D). We were also able to determine the C values of individual differentiating cells and observed that nuclei spent a longer time in the green (endoS/G2) or red (endoG1) state as polyploidy increased (supplementary material Fig. S9 and Movie 4). During the green-to-red conversion, in particular, the latency time for the emergence of the red signal (endoG1 entry) was longer in highly polyploid cells ( $\geq 16\text{C}$ ) (supplementary material Fig. S10).

#### Comparison of endoreplication in TGCs and MKCs

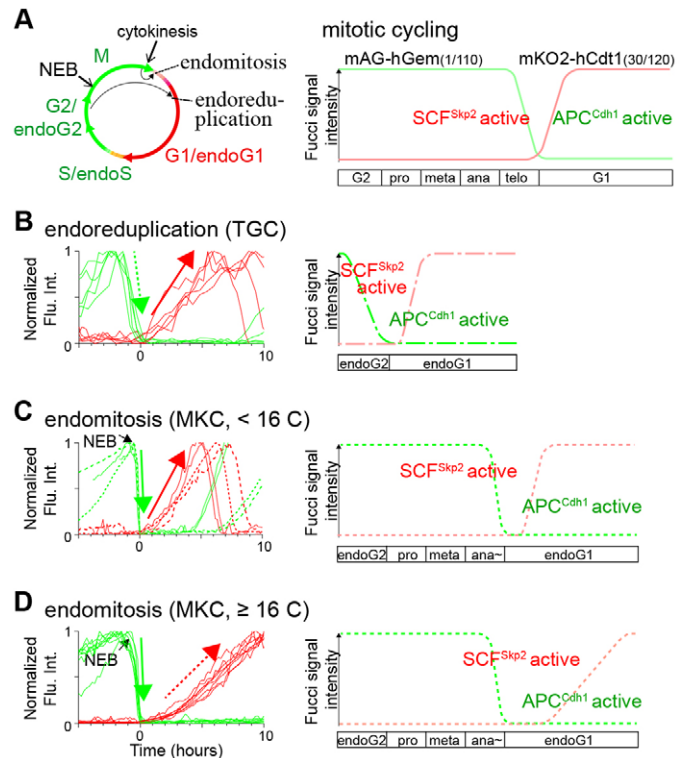
Although APC<sup>Cdh1</sup> is activated at the metaphase/anaphase transition in the mitotic cell cycle, it also functions at the endoG2/endoG1 transition in TGC endoreduplication and MKC endomitosis. However, cellular features consistent with M phase were observed, including NEB, during the endoG2/endoG1 transition of MKCs but not TGCs (Fig. 6A). We detailed the temporal profiles of the transition in multiple cells and attempted to superimpose them (Fig. 6B-D). In TGCs, the rate at which the green fluorescence





**Fig. 5. Visualization of MKB endomitosis in FulTrac wells by Fucci live-cell imaging.** (A) A timetable of the preparation of cell fractions and time-lapse imaging. The endomitotic processes that were visualized in this study are indicated by solid lines. (B) Typical fluorescence and DIC images of Fucci-expressing cells at DIV 4, 6 and 8 originating in HSC and MEP fractions. (C) Endomitosis tracking of a cell derived from the MEP fraction during a green-to-red conversion. A series of DIC (top) and Fucci fluorescence (bottom) images later than DIV 4. (D) Temporal profiles of fluorescence intensities of mAG-hGem(1/110) (green line) and mKO2-hCdt1(30/120) (red line) (left) and morphology (right) tracked from images shown in C images. NEB was identified by the abrupt spread of Fucci green fluorescence over the entire cell (Leung et al., 2004). Scale bars: 10 μm.

disappeared and the red fluorescence appeared was constant, irrespective of polyploidy level (Fig. 6B). By contrast, the polyploidy of MKCs determined the speed of green-to-red conversion (endoG2/endoG1 transition). In low polyploidy MKCs (<16 C), the emergence and subsequent extinction of red



**Fig. 6. Endoreduplication and endomitosis bypass aspects of mitosis to different extents.** (A) The generic mitotic cell cycle is indicated by the round circle. The cell cycle phases bypassed in TGCs and MKBs are indicated by black arrows. (B–D) Observed Fucci signals during green-to-red conversions (transitions from endoG2 to endoG1) in TGCs (B), and low ploidy (<16 C) (C) and high ploidy (≥16 C) (D) differentiating MKBs. Temporal profiles of fluorescence intensities of mAG-hGem(1/110) and mKO2-hCdt1(30/120) are indicated by green and red lines, respectively. The ubiquitin oscillators composed of SCF<sup>Skip2</sup> and APC<sup>Cdh1</sup> maintain the bistability at the direct transition from endoG2 to endoG1 in endoreduplication or at the endoG2/endoG1 transition through the abortive M phase in endomitosis.

fluorescence (endoG1 entry and exit) was variable but rapid (Fig. 6C), whereas the appearance of red fluorescence (endoG1 entry) in high polyploidy MKCs (≥16 C) was consistently slow (Fig. 6D; supplementary material Fig. S10). To summarize, green-to-red conversions of Fucci signals during mitotic cycling, endoreduplication and endomitosis are illustrated with respect to the normal cell cycle phases, from G2 to G1 to M (Fig. 6).

## DISCUSSION

Visualizing the cell cycle behavior of individual cells within complex tissues has in the past been extremely difficult. For instance, it has not been clear whether the ubiquitin oscillators that control normal mitotic cell cycle transitions (Ang and Harper, 2004; Vodermaier, 2004; Nakayama and Nakayama, 2006) function during endoreplication. Previous work showed that the protein level of the cyclin-dependent kinase inhibitor p57 (also known as Cdkn1c), which is targeted by SCF<sup>Skip2</sup> for degradation, oscillates in endoreduplicating mammalian cells (TGCs) in culture (Hattori et al., 2000; Ullah et al., 2008). Because the promoter activity of p57 is constant in TGCs, it was surmised that the enzymatic activity of SCF<sup>Skip2</sup> must oscillate. In endoreplicating *Drosophila* cells, oscillation in APC<sup>Fzr/Cdh1</sup> activity was verified by measuring the protein level of its substrate, Origin recognition complex 1 (Orc1) (Asano and Wharton, 1999; Narbonne-Reveau et al., 2008). Although these findings support the function of

ubiquitin oscillators in endoreplicative cycling, most of the data are snapshots correlating substrate expression levels and S phase markers, such as BrdU incorporation. Importantly, in addition, none of these studies examined both APC<sup>Cdh1</sup> and SCF<sup>Skp2</sup> activities at the same time. Thus, little was known about the precise timing of the transitions between the APC<sup>Cdh1</sup>- and SCF<sup>Skp2</sup>-active states during endoreplicative cycles. A recent computational study simulated the oscillatory dynamics of the APC<sup>Fzr/Cdh1</sup> and CRL4<sup>CDT2</sup> ubiquitin ligases in endoreplicating *Drosophila* cells (Zielke et al., 2011).

In the present study, we overcame previous limitations by performing live imaging of TGCs and MKCs using Fucci probes. This allowed us to monitor the anti-phase oscillating activities of the two E3 ligases, with high contrast and high temporal resolution. In particular, our *ex vivo* imaging using custom FulTrac wells permitted us to observe individual cells over a long time period. This work lays the foundation for future studies of endoreduplication and endomitosis in a multicellular context; for example, in living intact placenta and bone marrow. This is important because the endoreplicative cell cycle is regulated not only by intracellular signals but also by extracellular signals, although a recent integrative model for the cyclin/Cdk network in mammalian endoreplicative cells suggests that it functions as a self-sustained oscillator (Gérard and Goldbeter, 2009).

The temporal profiles of the green-to-red conversion of Fucci signals (Fig. 6) might yield clues as to what activates APC<sup>Cdh1</sup> during endoG2 phase or abortive mitosis. It is thus interesting to investigate temporal patterns of the activation of APC<sup>Cdh1</sup> regulators, such as Emi1. Furthermore, the reciprocal activation of SCF<sup>Skp2</sup> and APC<sup>Cdh1</sup> is modified during differentiation into MKCs. The relatively slow conversion seen in high-polyploidy ( $\geq 16$  C) MKCs suggests the existence of a state in which APC<sup>Cdh1</sup> is fully activated whereas SCF<sup>Skp2</sup> is not yet fully inactivated. Although the APC<sup>Cdh1</sup> complex directly targets the SCF<sup>Skp2</sup> complex for degradation and vice versa, there might be other mechanisms that regulate the anti-phase oscillation between the two E3 ligase activities. Notably, similar ploidy-level dependence was observed for the differential downregulation of two guanine nucleotide exchange factors [GEF-H1 (also known as Arhgef2) and ECT2] in endomitosing MKCs (Gao et al., 2012).

The Fucci transgenic mouse lines we have generated will be very useful for a variety of future studies. Crossing Fucci lines to the many mutant lines isolated by forward genetic screens will allow identification of the molecules responsible for the initiation, progression and termination of endoreplication. This information will refine computational models, which attempt to account for mammalian endoreplication. Also, crossing Fucci mice to the numerous transgenic lines expressing fluorescent markers under the control of cell type- or stage-specific promoters will be fruitful. Finally, Fucci transgenic mice will help researchers to re-evaluate polyploidy in cell types other than TGCs and MKCs. For example, the presence of polyploid neurons in vertebrates has been debated for the last half century; tetraploidy has been described in retinal ganglion cells (Morillo et al., 2010), cerebellar Purkinje neurons (Das, 1977) and cortical neurons of Alzheimer's disease patients (Mosch et al., 2007). In addition, an intriguing attempt would be the three-dimensional reconstructions of Fucci-labeled hepatocytes in the developing or regenerating liver, because hepatocytes show unique polyploidization (Duncan et al., 2010).

## MATERIALS AND METHODS

### Fucci transgenic mice

Fucci transgenic mice were generated by expressing mAG-hGem(1/110) [DDBJ/EMBL/GenBank, AB370333] (FucciS/G2/M) or mKO2-

hCdt1(30/120) [DDBJ/EMBL/GenBank, AB370332] (FucciG1) under the control of the CAGGS promoter (Sakae-Sawano et al., 2008). Transgenic mouse lines FucciS/G2/M-#474, -#492 or -#504, and FucciG1-#596, -#610 or -#639 along with genotyping protocols can be obtained from the RIKEN Bio Resource Center (BRC) website <http://www.brc.riken.jp/lab/animal/en/>. The experimental procedures and housing conditions for the animals were approved by the Animal Experimental Committees at the Institute of Physical and Chemical Research (RIKEN) – Brain Science Institute (BSI) and Bio Resource Center (BRC) – and all animals were cared for and treated humanely in accordance with the Institutional Guidelines for Experiments using Animals.

### FulTrac

PDMS-based devices (FulTrac) were constructed as described previously (Hirano et al., 2010).

### Cell cycle analysis of endoreplicating cells

Pregnant mice were injected intravenously with EdU solution (Life Technologies) (50 mg/kg body weight). After 20 minutes, placentas at E10.5 were removed, minced, and dissociated with 500 U/ml collagenase I (Worthington) at 37°C for 30 minutes and then with 6.25 U/ml DNase I (Worthington) for 30 minutes. After being washed and passed through a 100- $\mu$ m nylon mesh, the placental cells were fixed with 4% paraformaldehyde (PFA) for 10 minutes on ice. The fixed cells were treated with azide-conjugated Alexa 647 by the Click-iT reaction (Life Technologies) with slight modification. As copper ion used for the conjugation between EdU and Alexa dyes in the Click-iT reaction tended to quench fluorescent proteins, we attempted to lower the copper ion concentration. We found that one-third of the copper ion concentration recommended by the manufacturer was sufficient for the reaction while effectively avoiding the quenching reaction. Finally, the cell samples were stained with 3  $\mu$ M DAPI and analyzed using a FACSaria II (BD Biosciences). DAPI was excited by a 355-nm laser line and its emission collected through 450/50BP; mAG was excited by a 488-nm laser line and its emission collected through 530/30BP; mKO2 was excited by a 561-nm laser line and its emission collected through 610/20BP; and Alexa 647 was excited by a 640-nm laser line and its emission collected through 710/50BP. The data were analyzed using FlowJo software (Tree Star).

Mouse bone marrow cells were cultured for 7 days with DMEM/F12 supplemented with 5% FBS, 10 ng/ml mouse stem cell factor (Peprotech), 100 ng/ml human thrombopoietin (TPO) (Peprotech), 10 ng/ml mouse Fgf1 (Invitrogen), and 20 ng/ml mouse insulin-like growth factor 2 (Igf2) (R&D Systems). The cells were treated with 100  $\mu$ M EdU for 20 minutes and the Click-iT reaction was subsequently performed as for placental cells.

### LCV100 microscopy

Cells were subjected to long-term, time-lapse imaging using a computer-assisted fluorescence microscope (Olympus, LCV100) equipped with an objective lens (Olympus, UAPO 40 $\times$ /340 N.A.=0.90), a 0.5 $\times$ zoom lens, a halogen lamp, a red light emitting diode (LED) (620 nm), a charge coupled device camera (Olympus, DP30), DIC optical components, and interference filters. The halogen lamp was used with a 470DF35 excitation filter, a 505DRLP dichroic mirror and a 510WB40 emission filter to observe the mAG fluorescence, and a BP520-540HQ excitation filter, a DM545HQ dichroic mirror and a BP555-600HQ emission filter to observe the mKO2 fluorescence. For DIC imaging, the red LED was used with a filter cube containing an analyzer. Image acquisition and analysis were performed using MetaMorph 6.37 and 7.6.0.0 software (Universal Imaging Corporation), respectively.

### Time-lapse imaging of trophoblast cell endoreduplication

FucciG1-#596 heterozygous eggs and FucciS/G2/M-#504 homozygous sperm were used for *in vitro* fertilization. Fertilized eggs were cultured to the two-cell stage and then were frozen as stocks. After thawing, two-cell stage embryos were transplanted into pseudopregnant female mice. After 3 days, blastocysts were collected from the uterus, and were individually cultured in FulTrac wells in KSOM-AA medium for 24 hours in a CO<sub>2</sub>



incubator. Then, the medium was changed to RPMI1640 containing 20% fetal calf serum. Trophoectoderm outgrowth from the inner cell mass was time-lapse imaged using an LCV100 microscope.

### Whole-placenta imaging

FucciS/G2/M-#504 and FucciG1-#596 mice were cross-bred and placentas were excised at E8.5, E9.5, E10.5, E11.5, E12.5 and E13.5 and fixed with 4% PFA. Tissue was cryoprotected in PBS containing 20% sucrose, and optically cleared with Sca/eU2 solution (4 M urea, 30% glycerol and 0.1% Triton X-100) (Hama et al., 2011). Cleared placentas were embedded in a Sca/eU2-soaked gel. Whole-placenta imaging was performed using an AZ-C1 macro-zoom confocal microscope (Nikon) equipped with 488 nm and 561 nm laser lines, an AZ-Plan Fluor 2×objective lens (N.A.=0.2, W.D.=45 mm), or an AZ-Plan Apo 4×objective lens (N.A.=0.4, W.D.=20 mm). Confocal images were taken every 315 μm along the z-axis to create z stacks. Also, a fixed #504/#596 placenta at E10.5, which had been labeled with EdU (Alexa 647) and DAPI, was imaged using an Olympus FV1000 confocal microscope equipped with a UPLSAPO 40×silicone-oil objective lens (N.A.=1.25, W.D.=0.3 mm) or a Zeiss LSM 780 confocal/multiphoton microscope equipped with a W Plan-Apochromat 20×objective lens (N.A.=1.0, W.D.=1.9 mm). The DAPI dye was excited by a 405-nm laser line (FV1000) or two-photon excitation at 780 nm (LSM 780).

### Fractionation of blood cells

Cell fractionation was carried out as described previously (Noda et al., 2008). Briefly, blood cells isolated from Fucci transgenic mice (#474/#610) (6–7 months old) were stained with cell surface markers and sorted using a FACS Vantage SE (BD Biosciences). Fractionated cells were cultured in FulTrac wells containing DMEM/F12 supplemented with 10 ng/ml mouse stem cell factor (Peprotech), 100 ng/ml human thrombopoietin (TPO) (Peprotech), 10 ng/ml mouse Fgf1 (Invitrogen) and 20 ng/ml mouse insulin-like growth factor 2 (Igf2) (R&D Systems).

### Time-lapse imaging of megakaryocyte endomitosis

Bone marrow cells were isolated from an adult Fucci transgenic mouse (#474/#610), and hematopoietic stem cells [CD34(–)/c-kit(+)/Sca1(+)/lineage(–)] were purified using flow cytometry (Noda et al., 2008). The cells were cultured in the FulTrac wells under conditions that promote differentiation into megakaryocytes, and were time-lapse imaged using an LCV100 microscope.

### Estimation of ploidy level

In this study, we determined the ploidy (the C value) for some TGCs and MKCs cultured in FulTrac wells, which enabled full tracking of individual cells over an extended period. We traced TGCs and MKCs back to cells inside the ICM and cells before exposure to TPO, respectively. These original cells had not entered the endoreplicative cell cycle yet and were supposed to be in the normal mitotic cell cycle (2 C ~4 C).

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

A.S.-S., E.T., H.M. and A.M. conceived and designed the study. A.S.-S., T.H., M.Y., R.T., K.O., T.A. and S.N. carried out the experiments. A.S.-S., M.Y. and S.N. analyzed the data. A.S.-S. and A.M. prepared the manuscript. A.M. supervised the project.

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### Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.099226/-/DC1>

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