

—Original Article—

## Cell proliferation potency is independent of FGF4 signaling in trophoblast stem cells derived from androgenetic embryos

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**Abstract.** We previously established trophoblast stem cells from mouse androgenetic embryos (AGTS cells). In this study, to further characterize AGTS cells, we compared cell proliferation activity between trophoblast stem (TS) cells and AGTS cells under fibroblast growth factor 4 (FGF4) signaling. TS cells continued to proliferate and maintained mitotic cell division in the presence of FGF4. After FGF4 deprivation, the cell proliferation stopped, the rate of M-phase cells decreased, and trophoblast giant cells formed. In contrast, some of AGTS cells continued to proliferate, and the rate of M-phase cells did not decrease after FGF4 deprivation, although the other cells differentiated into giant cells. RO3306, an ATP competitor that selectively inhibits CDK1, inhibited the cell proliferation of both TS and AGTS cells. Under RO3306 treatment, cell death was induced in AGTS cells but not in TS cells. These results indicate that RO3306 caused TS cells to shift mitotic cell division to endoreduplication but that some of AGTS cells did not shift to endoreduplication and induced cell death. In conclusion, the paternal genome facilitated the proliferation of trophoblast cells without FGF4 signaling.

**Key words:** Androgenetic embryo, Cell proliferation, FGF4 signaling, Parental genome, Trophoblast stem cell

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In mammals, one set of paternal and maternal genomes is necessary for embryogenesis. In mice, androgenetic (AG) embryos, which have two sets of paternal genomes, do not survive beyond embryonic day (E) 9.5. Although AG fetal development is quite suppressed, the size of the placenta is similar to that of the wild-type placenta [1, 2]. We observed cryosections of these placentae *in vivo* at E9.5 to compare the structure of the AG placenta to that of the fertilized placenta. The AG placenta *in vivo* at E9.5 did not show a functional structure because of numerous trophoblast giant cells and lack of spongiotrophoblast cells [3]. Therefore, both the parental genomes might be involved in placental development.

In mammals, the blastocysts have two types of trophoblast (TE): one is the polar TE that is attached to the inner cell mass (ICM), and the other is the mural TE that is away from the ICM. After implantation, ICM cells mainly differentiate into an embryo, and TE cells differentiate only into extraembryonic tissues. In murine TE cells, mural TE cells fail to proliferate, and they undergo endoreduplication to form giant cells. In contrast, murine polar TE cells continue to proliferate, and they differentiate into trophoblast subtypes to form the placenta [4].

In mice, trophoblast stem (TS) cells are derived from the polar TE cells of blastocysts at E3.5. These TS cells are diploid and self-renewing when they are cultured in an undifferentiated state

with fibroblast growth factor 4 (FGF4), heparin, and primary mouse embryonic fibroblast (MEF) or MEF-conditioned medium. TS cells express undifferentiated TS marker genes such as *Cdx2* and *Erbb*. When these factors are removed, cell proliferation stops, the expression of TS marker genes is repressed rapidly, and TS cells differentiate into all trophoblast cell subtypes including spongiotrophoblast, labyrinth, and trophoblast giant (TG) cells; they terminally differentiate into TG cells with a large amount of cytoplasm and high ploidy [5].

We established TS cells from AG embryos (AGTS cells) to clarify the role of parental genomes in trophoblast cells for differentiation *in vitro* [3]. Under undifferentiated culture conditions, AGTS cells show cell proliferation and express undifferentiated TS marker genes in a manner similar to TS cells. After FGF4 depletion, AGTS cells expressed a TG cell-specific gene, *Pl-1*, but they did not express the spongiotrophoblast cell-specific gene, *Tpbpa*, and the spongiotrophoblast cell- and labyrinth-specific gene, *Mash2*. These results indicated that AGTS cells differentiate preferentially into TG cells [3].

After FGF4 depletion, murine TS cells differentiate into giant cells via endoreduplication. Endoreduplication occurs when a cell undergoes multiple S-phases without entering mitosis and without undergoing cytokinesis [6], and it results in large polyploidy cells and giant cell formation [7]. Although endoreduplication is observed in protozoa, arthropods, mollusks, and plants, it is rare in mammals [6]. Endoreduplication is triggered by the suppression of cyclin-dependent kinase 1 (CDK1) activity by the CDK-specific inhibitor *P57/kip2* [8].

*P57/kip2* knockout TS cells express TS marker genes including *Cdx2*, *Erbb* and *Fgfr2* in the presence of FGF4. After FGF4 depletion, the expressions of *Pl-1* and *Pl-2* genes are increased [8]. However, FGF4-deprived *p57/kip2* knockout TS cells fail to undergo endoreduplication. Moreover, these TS cells form not giant cells but

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multinuclear cells. Therefore, *p57/kip2* knockout TS cells are not differentiated into TG cells via endoreduplication [8]. Interestingly, FGF4-deprived *p57/kip2* knockout TS cells continue to proliferate. As *p57/kip2* is a maternally expressed imprinted gene, the maternal genome might be necessary for stop the cell proliferation and shift to endoreduplication after FGF4 depletion.

In the present study, to obtain further insights into the feature of AGTS cells, we addressed a question concerning whether or not AGTS cells that lack maternally expressed imprinted genes have the ability to stop cell proliferation and shift into endoreduplication after FGF4 depletion and to differentiate into TG cells.

## Materials and Methods

### Production of AG embryos

B6D2F1 (C57BL/6 X DBA2) mice were used. AG embryos were produced as described previously [3]. Female mice were superovulated with 5 IU equine chorionic gonadotropin (eCG), followed by an injection of 5 IU human chorionic gonadotropin (hCG) 48 h later. Freshly ovulated metaphase II (MII) oocytes were collected at 13–16 h post-hCG injection, and the cumulus cells were removed by using 300 U/ml hyaluronidase in M2 medium [9]. The AG embryos were produced by *in vitro* fertilization using enucleated oocytes [10]. A pronuclear transfer was performed to produce diploid AG embryos as needed. The diploid AG embryos were cultured for 3.5 days to yield expanded blastocysts. To obtain conceptuses, expanded blastocysts from these embryos were transferred into the uterine horns of CD-1 female mice at day 2.5 of pseudopregnancy. At E9.5, the uteri containing the conceptuses were fixed in 4% paraformaldehyde. Samples were separated into each conceptus containing a portion of the uterus, and soaked in 10%, 15% and 20% sucrose in phosphate-buffered saline (PBS). They were frozen in an embedding OCT compound (Sakura Finetechnical, Tokyo, Japan) at –80 C until cryosectioning. The fertilized embryos obtained by mating B6D2F1 male and female mice were used as wild-type embryos.

### Cell culture

TS cells from AG and wild-type blastocysts were established from expanded blastocysts as described previously [3, 5]. In this study, two lines of AGTS cells were used. TS cells were cultured in TS medium: RPMI 1640 (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 20% fetal bovine serum (FBS), 1 mM sodium pyruvate (Gibco), 100 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 2 mM L-glutamine (Gibco) and 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco). Mitomycin-treated mouse embryonic fibroblast-conditioned medium (MEF-CM) was prepared as described previously [3].

TS cell lines were maintained in an undifferentiated condition by using 70cond F4H medium, which consist of 70% MEF-CM and 30% TS medium, containing 25 ng/ml FGF4 (PeproTech EC, London, UK) and 1 mg/ml heparin (Sigma-Aldrich). Differentiation of TS cells was induced by culturing in TS medium without FGF4, heparin and MEF-CM.

NIH3T3 cells were cultured in DMEM medium (Gibco) containing 10% FBS (DMEM + FBS). RO3306 (Merck, Darmstadt, Germany), an ATP competitor that selectively inhibits CDK1 activity [11, 12],

was used at a final concentration of 10  $\mu$ M.

### Cell proliferation assay

TS and AGTS cells plated at  $2 \times 10^4$  cells/35-mm dish were cultured for 48 h in 70cond F4H medium, and then the culture medium was changed as follows. TS cells were cultured in 70cond F4H medium with or without 10  $\mu$ M RO3306 or in TS medium. AGTS cells were cultured in 70cond F4H medium or in TS medium with or without 10  $\mu$ M RO3306. At 0, 1, 2 and 3 days after changing the culture medium, cells were incubated in 0.05% Trypsin-EDTA (Gibco Invitrogen) at 37 C for 4.5 min, and then the cells were dissociated by pipetting and checked microscopically to ensure that all of them were floating. These floating cells were collected and stained with trypan blue to qualify viable nucleated cells, and we calculated the number of cells by using a hemocytometer. Daily cell numbers were expressed relative to the number on day 0. Three independent experiments were performed.

### Viability assay

The viability of the cells treated with RO3306 was determined using a Live/Dead Cell Staining Kit II (PromoKine, Heidelberg, Germany) according to the manufacturer's protocol. Live and dead cells were determined by calcein-AM (green fluorescence) and ethidium homodimer III (red fluorescence) staining, respectively, and were detected by green and red signals under a fluorescence microscope. In this assay, NIH3T3 cells were used as a positive control that blocked the proliferation by RO3306.

### In situ hybridization (ISH)

Mouse *Tpbpa* and *Pl-1* cDNA fragments cloned in pGEM1 and pGEM2 (Promega, Madison, WI, USA), respectively, were kindly provided by Dr. Janet Rossant (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada). A 257-bp fragment of *Plpa* was amplified by polymerase chain reaction (PCR) using cDNA from a placenta at E12.5 as the template with the following primers: *Plpa*-F, 5'-gatcccagcccttcacacat-3', and *Plpa*-R, 5'-ggcaatccagttatcccaga-3'. The PCR products were ligated into pGEM-T Easy Vector (Promega), cloned and sequenced using an ABI DNA sequencer (Applied Biosystems, Foster City, CA, USA). The plasmid DNAs were linearized, and sense and antisense digoxigenin (DIG)-labeled riboprobes were generated using a DIG RNA Labeling Kit (Sp6/T7) (Roche, Mannheim, Germany).

Cryosections (20  $\mu$ m thick) were placed on slides, and ISH was performed as described previously [13]. After hybridization with DIG-labeled probes, the slides were counterstained with eosin, dehydrated, cleared in xylene and mounted in Canada balsam.

### Immunostaining for M-phase cell detection

TS and AGTS cells were fixed by 4% paraformaldehyde for 20 min at room temperature (RT) and then permeabilized in 100% methanol for 10 min at –20 C. Samples were blocked in PBS containing 2.5% bovine serum albumin (BSA; Sigma-Aldrich) for 1 h at RT and incubated with the antibody to anti-phospho Histone H3 (pHH3) (1:300; Millipore, Temecula, CA, USA), a mitotic-specific marker [14], overnight at 4 C. After washing three times, samples were incubated with Alexa 488 donkey anti-rabbit antibody (1:300;

Invitrogen) for 1 h at RT. DNA staining and mounting on slides were conducted using VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA, USA). Image acquisition was performed using a fluorescence microscope. Three independent experiments were performed, and M-phase cells were counted in four fields in each experiment.

*Multinuclear cells detection*

TS and AGTS cells were cultured in TS medium for 10 days and then were fixed by 4% paraformaldehyde for 20 min at RT. DNA staining and mounting on slides were conducted using VECTASHIELD with DAPI. Image acquisition was performed using a fluorescence microscope. Ten fields of cells were scored at  $\times 200$  magnification.

*Western blotting*

TS and AGTS cells were washed once with PBS and lysed on ice in 10 ml of buffer comprised of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2.5%  $\beta$ -mercaptoethanol and 1.25% bromophenol blue. The cells were then boiled at 95 C for 5 min.

Electrophoresis was carried out on a 10% polyacrylamide gel, and separated proteins were transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare, Piscataway, NJ, USA). The membrane was blocked in 5% skim milk in washing buffer (25 mM Tris-HCl, 150 mM NaCl and 0.05% Tween-20) for 1 h and incubated with primary antibodies (anti-rabbit P57/KIP2 polyclonal antibody [1:1000; Abcam, Cambridge, UK], anti-CHK1 mouse monoclonal antibody [Santa Cruz] and anti-human G3PDH antibody [Trevigen, Gaithersburg, MD, USA]) overnight at 4 C, followed by anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:1000; GE Healthcare). Signals were detected with an ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized by a luminescent image analyzer (LAS-1000plus; Fujifilm, Tokyo, Japan). Protein samples were from  $1 \times 10^4$  cells for P57/KIP2 detection and 20  $\mu$ g proteins for CHK1 detection.

*Statistical analysis*

We used the Student's *t*-test to analyze the significance of differences in cell proliferation, percentages of M-phase cells and percentages of dead cells compared with TS or AGTS cells cultured in 70cond F4H, TS cells at each day and NIH3T3, TS or AGTS cells at day 1, respectively. Differences with  $P < 0.05$  were considered significant.

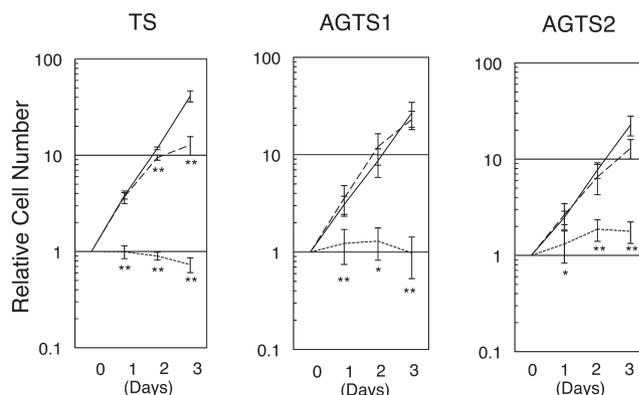
**Results**

*Cell proliferation assay*

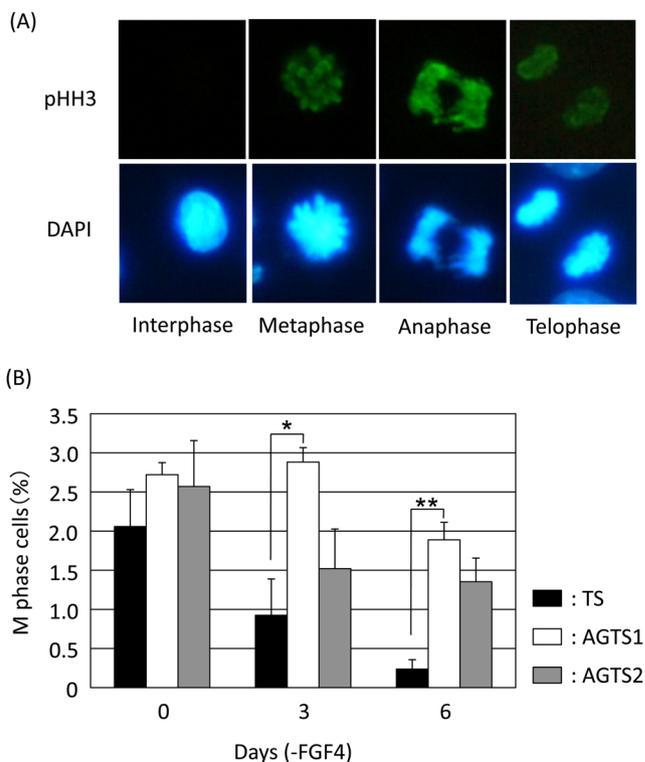
In both TS and AGTS cells, cell proliferation continued in the presence of FGF4 (Fig. 1). After FGF4 deprivation, TS cells abruptly stopped proliferating. In contrast, the AGTS cells continued to proliferate in the absence of FGF4. When cells were treated with RO3306 to block the cell cycle at the G2/M phase, both TS cells (with FGF4) and AGTS cells (without FGF4) showed markedly inhibited cell proliferation.

*The number of M-phase cells after FGF4 depletion*

M-phase cells were detected by anti- pHH3 antibody (Fig. 2) [15]. Metaphase, anaphase and telophase cells were counted as M-phase



**Fig. 1.** Growth curves of TS and AGTS cells. TS cells were cultured in 70cond F4H medium with and without RO3306 (dotted and solid lines respectively) and TS medium (broken line). AGTS cells were cultured in 70cond F4H medium (solid line) and TS medium with or without RO3306 (dotted and broken lines, respectively). Cells were harvested and counted in triplicate each day. Daily cell numbers are expressed relative to the number at day 0. Values represent means  $\pm$  SEM for three to four experiments. Asterisks indicate statistical significance (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ) compared with TS or AGTS cells cultured in 70cond F4H medium by Student's *t*-test.



**Fig. 2.** The M-phase TS and AGTS cells at days 0, 3 and 6 after FGF4 depletion. (A) Immunofluorescence images using pHH3 antibody. (B) The percentage of M-phase cells at days 0, 3 and 6 after FGF4 depletion. Three independent experiments were performed. Error bars represent the SEM. Asterisks indicate statistical significance (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ) by Student's *t*-test.

cells (Fig. 2A). In both TS cells and AGTS cells, the percentages of M-phase cells were 2%–2.5% in the presence of FGF4. After FGF4 depletion, the numbers of M-phase TS cells gradually decreased, and they could hardly be detected (0.25%) at day 6 after FGF4 depletion. In AGTS cells, in contrast, 1.5%–2.0% of the cells were M-phase cells at day 6 after FGF4 depletion (Fig. 2B). These results indicate that cell proliferation of AGTS cells is also maintained without FGF4.

#### Viability assay

At day 3 after FGF4 deprivation, almost all TS cells showed a giant cell-specific morphology with a large amount of cytoplasm and a large nucleus (Fig. 3). In contrast, although AGTS cells also formed giant cells after FGF4 deprivation, the number of giant cells was fewer than in the case of TS cells, and some cells remained diploid cells in morphology. After 3 days of RO3306 treatment, both TS and AGTS cells showed giant cells that had cell morphologies similar to differentiated cells.

The effect of RO3306 treatment on cell viability in NIH3T3, TS and AGTS cells is shown in Fig. 4. In NIH3T3 cells, the percentage of dead cells was less than 1%. Following treatment with RO3306 for 1, 2 and 3 days, the percentages of dead cells in NIH3T3 cells increased and were 7%, 12% and 30%, respectively. Less than 1% of TS and AGTS cells cultured without RO3306 were dead. Following treatment with RO3306, 5% of TS cells were dead, and the percentages of dead cells in AGTS cells treated with RO3306 for 1, 2 and 3 days were 5%, 10% and 18%, respectively. These results suggest that RO3306 might induce cell death in NIH3T3 and AGTS cells but not in TS cells.

#### Multinuclear cells formation

The nuclei of TS and AGTS cells were observed at day 10 after FGF4 deprivation (Fig. 5). The rates of multinuclear cells were 3.0%, 6.2% and 4.1% in TS, AGTS1 and AGTS2 cells respectively. These rates were quite lower than those of *P57/kip2* knockout TS cells (80% at day 8 after FGF4 deprivation) [8].

#### Expression of *CHK1* and *P57/KIP2* in TS and AGTS cells

The expression patterns of *CHK1* in TS and AGTS cells at days 0, 2, 4 and 6 after FGF4 depletion are shown in Fig. 6. At day 0 after FGF4 depletion, the protein expression of *CHK1* was detected in both TS and AGTS cells. At days 2, 4 and 6 after FGF4 depletion, *CHK1* was not detected in TS cells, whereas its expression was detected in AGTS cells.

The expression patterns of *P57/KIP2* in TS and AGTS cells at days 0, 2, 4 and 6 after FGF4 depletion are shown in Fig. 7. At day 0 after FGF4 depletion, the protein expression of *P57/KIP2* was not detected. At days 2, 4 and 6 after FGF4 depletion, the protein expression of *P57/KIP2* was detected in both TS and AGTS cells.

#### *Pl-1*, *Plpa* and *Tpbpa* mRNA expression in conceptuses at E9.5

To clarify the differential ability of trophoblast cells from AG embryos during placentation, we analyzed the localization of *Pl-1*, *Plpa* and *Tpbpa* mRNA at E9.5 conceptuses by ISH (Fig. 8). In the wild-type conceptuses, *Pl-1* was localized in primary and secondary TG cells, *Plpa* was detected only in secondary TG cells, and *Tpbpa*

was detected in the ectoplacental cone. In the AG conceptuses, *Pl-1* was localized in primary and secondary TG cells, and *Plpa* was detected only in secondary TG cells, but *Tpbpa* was not detected. These results confirmed that AG embryos preferentially differentiated into TG cells and were inhibited to induce the spongiotrophoblast cell formation.

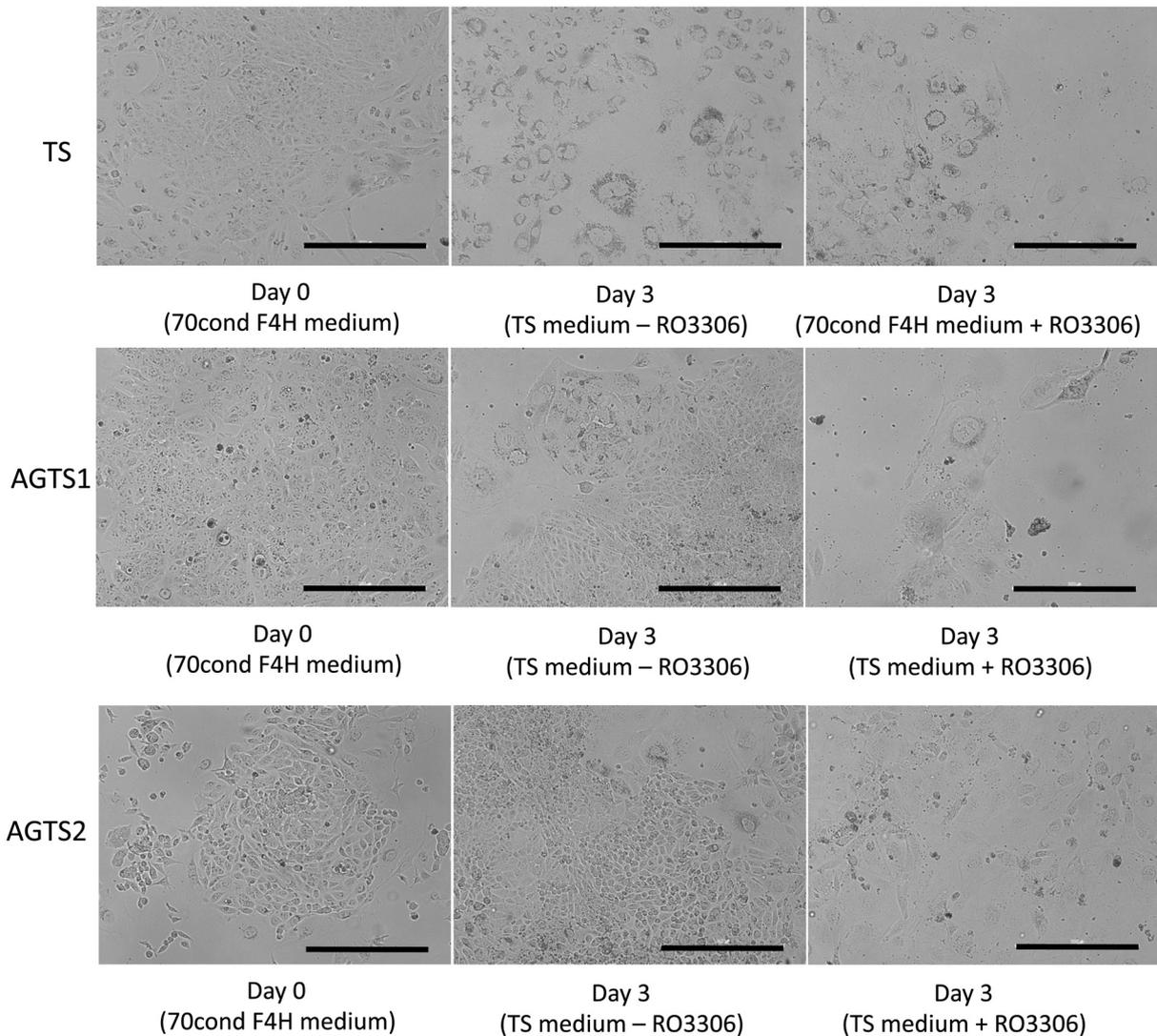
## Discussion

We previously reported that AGTS cells expressed the TG cell-specific gene *Pl-1* but did not express the spongiotrophoblast cell-specific gene *Tpbpa* and the labyrinth-specific gene *Mash2* [3]. These results indicate that AGTS cells differentiate preferentially into TG cells [3]. In the present study, AG conceptuses expressed *Pl-1* but not *Tpbpa*, so the AG embryos were able to differentiate into TG cells *in vivo*. Thus, we speculate that the differentiation ability of AGTS cells is similar to that of AG trophoblast cells *in vivo*.

AGTS cells express paternally expressed imprinted genes (*Peg1*, *Peg3*, *Peg10*, *Snrpn*, *U2af1-rs1* and *Igf2*) but not maternally expressed imprinted genes (*H19*, *Igf2r* and *Tssc3*) [3]. Moreover, a genome-wide methylation analysis in AGTS cells detected three differentially methylated regions (DMRs), which are paternally methylated regions, localized in the *Gpr1-Zbdf2* region on chromosome 1. We also detected a novel paternally expressed imprinted gene named *Gpr1* [16]. Therefore, the AGTS cells used in the present study might be useful to understand the role of the parental genome controlled by epigenetic modification in trophoblast cell differentiation.

Previous reports have indicated that paternally expressed genes promote fetal growth and that maternally expressed genes suppress growth [17]. More than 100 imprinted genes have been identified in mice. In addition to this, many more tissue-specific human-imprinted loci have been discovered [17]. The majority of imprinted genes expressed in the fetus are also expressed and imprinted in the placenta. Several reports of phenotypes associated with the targeted deletion of imprinted genes confirm the function of imprinting in placental development. These results clearly indicate that the loss of maternally expressed genes (*p57kip2*, *Grb10*, *H19*, *Igf2r* and *Tssc3*) results in placentomegaly, and the loss of paternally expressed genes (*Dlk1*, *Igf2*, *Kcnq1ot1*, *Peg1*, *Peg3* and *Plagl1/Zac1*) results in placental growth deficit [18]. Moreover, two maternally expressed genes, *Mash2* and *p57/kip2*, control differentiation into spongiotrophoblast and TGC cells, respectively [6, 8, 19, 20]. These findings indicate that the parental genomes affect the proliferation and the differentiation of murine placenta tissues.

In humans, hydatidiform moles are gestational trophoblastic tumors that result from abnormal fertilization [21]. Hydatidiform moles can be classified into complete hydatidiform moles and partial hydatidiform moles [22]. A hydatidiform mole forms as a result of fertilization of an oocyte without a nucleus by a single sperm, followed by duplication of the haploid genome [23, 24], or alternatively, fertilization of an enucleated oocyte by two sperm cells [24, 25]. Consequently, these oocytes have only paternal genomes. Hydatidiform moles are a human disease characterized by abnormally proliferating trophoblastic tissues [26]. Therefore, the proliferation of human trophoblast cells might be controlled by the paternal genome.



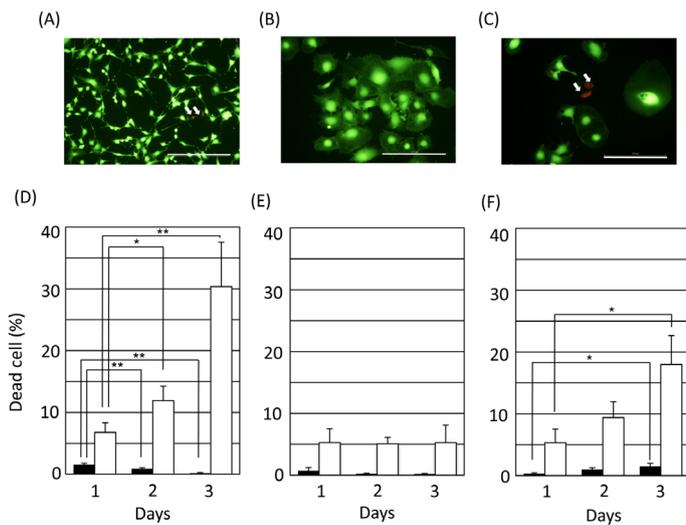
**Fig. 3.** Morphology of TS and AGTS cells cultured with DMSO or RO3306 for 0 and 3 days after FGF4 depletion. Scale bars, 300  $\mu$ m.

TS cells need FGF4 for maintenance of the undifferentiated state and for cell proliferation. After FGF4 depletion, TS cells stopped proliferating [5] and decreased the number of cells in M-phase. On the other hand, the number of AGTS cells increased after FGF4 depletion. These FGF4-deprived AGTS cells were delayed decreasing the number of cells in M-phase. Moreover, AGTS cells expressed CHK1 after FGF4 depletion. CHK1 is an essential protein kinase that is required for mammalian cell proliferation and for preventing cells from entering mitosis with DNA damage or stalled replication forks [27, 28]. In humans, Ckh1 is expressed specifically at the S to M phase of the cell cycle at both the RNA and protein levels and that is localized to the nucleus at that time. Chk1 activity can be readily detected at the S to M phase of the cell cycle [29]. Therefore, CHK1 was detected in asynchronous proliferating cells. CHK1 was also detected in TS cells in the presence of FGF4. In contrast, FGF4-depleted TS cells suppressed CHK1 expression because

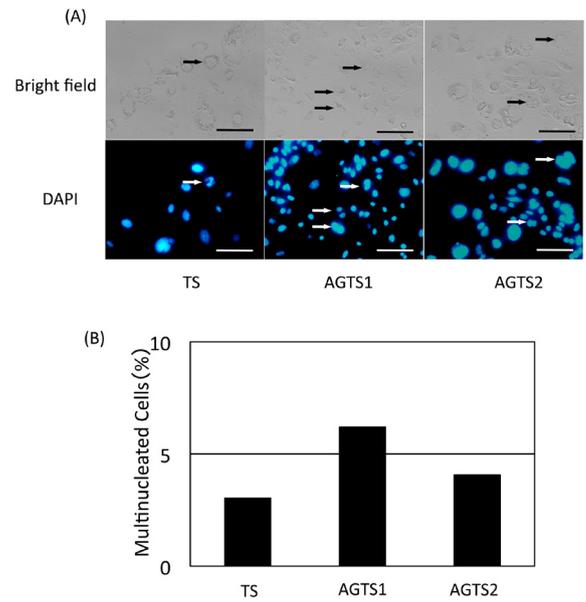
the cell cycle shifted from mitotic division to endoreduplication. These results indicated that AGTS cells are able to proliferate by FGF4-independent signaling.

In the present study, RO3306 stopped the cell proliferation of both TS and AGTS cells. In TS cells, RO3306 induced TG cell formation and did not affect cell death. RO3306 induced apoptosis in tumor cells [11], embryonic stem (ES) cells [8] and cell lines including NIH3T3 (shown in the present study) because of G2/M-phase arrest. On the other hand, in the AGTS cells, although RO3306 induced TG cell formation, cell death was also facilitated. The results of this study indicate that some of the AGTS cells in which cell death was induced by RO3306 were able to proliferate and not shift to endoreduplication. When AGTS cells were cultured with RO3306, only TG cells were observed, indicating that endoreduplication was induced in some AGTS cells by RO3306.

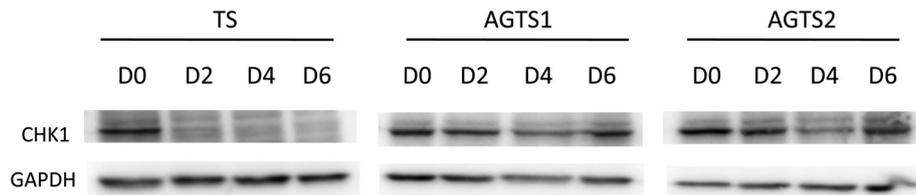
A previous study showed that endoreduplication is triggered by the



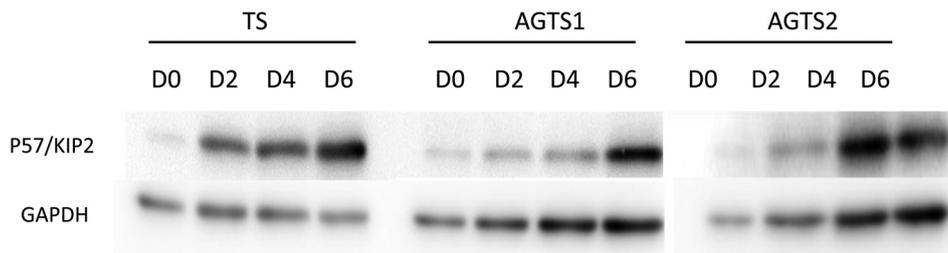
**Fig. 4.** The effects of RO3306 on cell death in TS and AGTS cells. Live and dead cells were detected by fluorescent staining of green and red cells, respectively, in (A) NIH3T3 cells, (B) TS cells and (C) AGTS1 cells. Arrows indicate the dead cells. Scale bars, 300  $\mu$ m. The percentages of dead cells in (D) NIH3T3 cells, (E) TS cells and (F) AGTS1 cells. Black bars: RO3306-untreated cells. White bars: RO3306-treated cells. Asterisks indicate statistical significance (\*  $P < 0.05$ ; \*\*  $P < 0.01$ ) by Student's *t*-test.



**Fig. 5.** Multinucleated cell formation in TS and AGTS cells. (A) Images of TS and AGTS cells at day 10 after FGF4 deprivation. Arrows indicate the multinucleated cells. Scale bars, 100  $\mu$ m. (B) The percentage of multinucleated cells in TS and AGTS cells at day 10 after FGF4 deprivation. Ten fields of cells were scored at  $\times 200$  magnification.



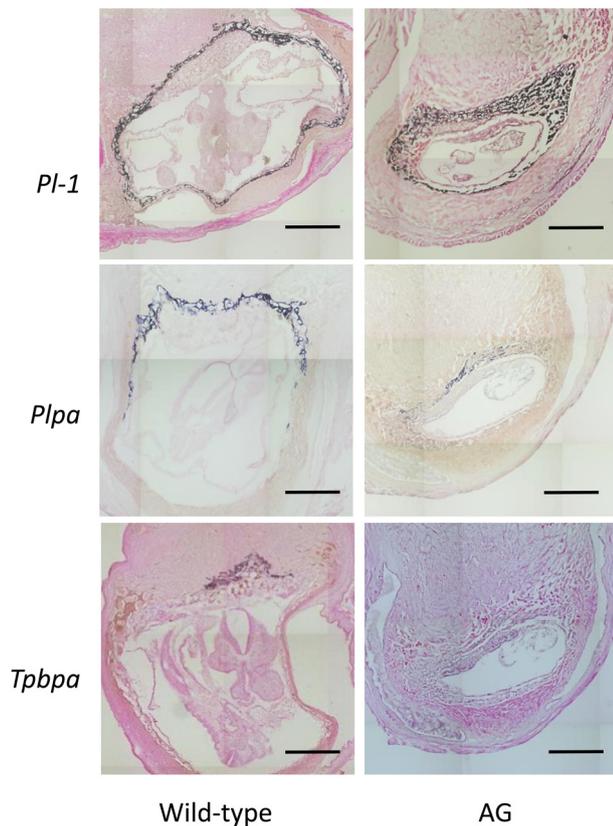
**Fig. 6.** Western blot analysis of CHK1 protein in TS and AGTS cells at days 0, 2, 4, and 6 after FGF4 deprivation. GAPDH was examined as a loading control.



**Fig. 7.** Western blot analysis of P57/KIP2 protein in TS and AGTS cells at days 0, 2, 4, and 6 after FGF4 deprivation. GAPDH was examined as a loading control.

suppression of CDK1 activity by the CDK-specific inhibitor *p57/kip2* [8]. *p57/kip2* knockout TS cells did not undergo endoreduplication and formed multinucleated cells, although these cells expressed TG cell-specific genes [8]. RO3306 induced endoreduplication and TG formation in *p57/kip2* knockout TS cells, indicating that *p57/kip2*

triggered the endoreduplication. *p57/kip2* is a maternally expressed imprinted gene [30], and thus it is presumed that *p57/kip2* expression is at a negligible level in AGTS cells. However, contrary to our expectations, AG trophoblast cells formed giant cells and hardly formed multinucleated cells both *in vivo* and *in vitro* because of



**Fig. 8.** Localization of *Pl-1*, *Plpa* and *Tpbpa* mRNA in extraembryonic tissues of conceptuses from fertilized and AG embryos at E9.5. In the extraembryonic tissues from wild-type embryos, *Pl-1* was detected in both primary and secondary TG cells, and *Plpa* was detected only in secondary TG cells. *Tpbpa* was detected in the ectoplacental cone. In AG extraembryonic tissues, *Pl-1* was detected in both primary and secondary TG cells, *Plpa* was detected only in secondary TG cells, and *Tpbpa* was not detected. Bars: 500  $\mu$ m.

*p57/kip2* expression. In uniparental fetuses, the expression of some imprinted genes deviated from the presumed levels, suggesting that trans-acting mechanisms between paternal and maternal alleles are involved in the appropriate expression of imprinted genes [31].

It has been reported that FGF4-deprived *P57/kip2* knockout TS cells continued to proliferate until they began to produce multinucleated giant cells around day 3 [8]. Interestingly, FGF4-deprived AGTS cells continued to proliferate up to day 6 (data not shown), although *p57/kip2* expression was detected, indicating that repression of *p57/kip2* is not sufficient for the trophoblast cell proliferation caused by FGF4-independent signaling. Therefore, the genes expressed from the paternal genome might strongly control trophoblast cell proliferation.

In conclusion, this study indicated that AGTS cells have the ability to proliferate in an FGF4-independent manner. The lack of maternally expressed imprinted genes might be involved in this feature of AGTS cells.

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