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Original Article

Effect of a FOXO1 inhibitor on trophoblast differentiation from human pluripotent stem cells and ERV-associated gene expression

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ABSTRACT

Introduction: In human placental development, the trophectoderm (TE) appears in blastocysts on day 5 post-fertilization and develops after implantation into three types of trophoblast lineages: cytotrophoblast (CT), syncytiotrophoblast (ST), and extravillous trophoblast (EVT). CDX2/Cdx2 is expressed in the TE, and Cdx2 expression is upregulated by knockdown of Foxo1 in mouse ESCs. However, the significance of FOXO1 in trophoblast lineage differentiation during the early developmental period remains unclear. In this study, we examined the effect of FOXO1 inhibition on the differentiation of naive human induced pluripotent stem cells (iPSCs) into TE and trophoblast lineages.

Methods: We induced TE differentiation from naive iPSCs in the presence or absence of a FOXO1 inhibitor, and the resulting cells were subjected to trophoblast differentiation procedures without the FOXO1 inhibitor. The cells obtained in these processes were assessed for morphology, gene expression, and hCG secretion using phase-contrast microscopy, reverse transcription polymerase chain reaction (RT-PCR), quantitative RT-PCR (RT-qPCR), RNA-seq, immunochromatography, and a chemiluminescent enzyme immunoassay.

Results: In the induction of trophoblast differentiation from naive iPSCs, treatment with a FOXO1 inhibitor resulted in the enhanced expression of TE markers, CDX2 and HAND1, but conversely decreased the expression of ST markers, such as ERVW1 (Syncytin-1) and GCM1, and an EVT marker, HLA-G. The proportion of cells positive for an early TE marker TACSTD2 and negative for a late TE marker ENPEP was higher in FOXO1 inhibitor-treated cells than in non-treated cells. The expressions of ERVW1 (Syncytin-1), ERVFRD-1 (Syncytin-2), and other endogenous retrovirus (ERV)-associated genes that have been reported to be expressed in trophoblasts were suppressed in the cells obtained by differentiating the TE cells treated with FOXO1 inhibitor.

Conclusions: Treatment with a FOXO1 inhibitor during TE induction from naive iPSCs promotes early TE differentiation but hinders the progression of differentiation into ST and EVT. The suppression of ERV-associated genes may be involved in this process.

Abbreviations: iPSCs, induced pluripotent stem cells.; TE, trophectoderm; CT, cytotrophoblast; ST, syncytiotrophoblast; EVT, extravillous trophoblast; ERV, endogenous retrovirus; hCG, human chorionic gonadotropin.

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1. Introduction

The human trophectoderm appears in the blastocyst (day 5 after fertilization) and is the part that will become the placenta. After implantation, it develops into three types of trophoblasts: cytotrophoblasts (CT), syncytiotrophoblasts (ST), and extravillous trophoblasts (EVT) [1]. Placental dysfunction in early pregnancy causes fetal growth restriction, gestational hypertension, preeclampsia, miscarriage, and other pregnancy complications [2–5]. However, the mechanisms underlying these disorders remain unclear. This is, at least in part, because of the difficulty in obtaining human early trophoblast samples for study.

A promising strategy for obtaining sufficient material to understand the etiology of placental developmental disorders associated with trophoblast defects is to generate trophoblast cells from self-renewable human cell lines *in vitro*. Since 2018, several groups have generated human trophoblast stem cell (TSC) lines derived from human blastocysts and cytotrophoblasts [6–8]. Furthermore, human TSCs and trophoblast cell lines have recently been successfully generated *in vitro* from naive human pluripotent stem cell**s** (PSCs) [9,10]. This technology, using human PSCs, overcomes the need for human blastocysts or trophoblast cells in early pregnancy, allowing the generation of sufficient trophoblast-like cells without quantitative limitations.

In the long period of the first trimester (12 weeks after fertilization), the pre-implantation state of the trophectoderm (TE) in the first week after fertilization is poorly understood and should be studied to understand the healthy and pathological early developmental processes of trophoblasts by leveraging the advantages of human PSC technology. Recently, a model of implantation was reported using blastoids derived from naive ES cells [11]. However, only a few studies have focused on the differentiation of human PSCs into trophectoderm [12,13].

Upregulation of Cdx2, a transcription factor, has been reported to be important for TE formation in mouse blastocysts [14–16]. Although humans and mice do not have identical gene expression patterns during TE-inner cell mass (ICM) maturation [17,18], they are similar in that CDX2/Cdx2 is expressed in the TE but not in the ICM [19]. Notably, CDX2/Cdx2 is expressed only before implantation during placental development in both humans and mice [20]. In addition, during trophoblast lineage induction from human PSCs *in vitro*, CDX2 expression changes from negative to positive [21]. Thus, we employed CDX2 as an important indicator of trophoblast lineage specification initiation in our studies focusing on the “pre-implantation” period.

Cdx2 expression has been reported to be upregulated by knockdown of Foxo1 in mouse ESCs [22]. FOXO1, a Forkhead transcription factor of the FOXO subfamily, regulates various cellular functions, such as the cell differentiation, metabolism, proliferation, and survival [23]. However, the effects of FOXO1 on human trophoblast differentiation remain unclear.

In the present study, we investigated whether or not the addition of a FOXO1 inhibitor enhances the differentiation of naive human induced pluripotent stem cells (iPSCs) into TE cells and their derivative trophoblast cells by assessing the expression of placenta-associated markers and hCG secretion.

2. Materials and methods

2.1. Culture of human primed feeder-free human iPSCs

The validated human iPSC line 201B7 was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and transferred from on-feeder to feeder-free conditions in our laboratory. iPSCs were maintained in StemFit AK02 N medium (Ajinomoto, Tokyo, Japan) supplemented with penicillin (50 units/mL) and streptomycin (50 μg/mL) (Gibco, Carlsbad, CA, USA) at 37 °C and 5% CO_2. For passages (every 7 days), iPSCs were dissociated into single cells by treatment with 0.5X TrypLE Select (1X TrypLE Select [Thermo Fisher Scientific, Waltham, MA, USA] diluted 1:1 with 0.5 mM EDTA [Nacalai Tesque, Kyoto, Japan] in phosphate-buffered saline [PBS]) and seeded with StemFit medium with 10 μM Y-27632 (034–24024; Wako, Osaka, Japan) and iMatrix-511 silk (0.167 μg/cm^2; Nippi, Tokyo, Japan) in uncoated usage. The following day, the medium was replaced with AK02 N medium without Y-27632 and changed every other day.

2.2. Conversion from primed iPSCs to naive iPSCs

The conversion of primed feeder-free iPSCs to naive feeder-free iPSCs was performed using RSeT Feeder-Free medium (ST-05975, Stem Cell Technologies, Vancouver, Canada) according to the manufacturer's instructions. Primed iPSCs were passaged using Gentle Cell Dissociation Reagent (Stem Cell Technologies) and cultured in mTeSR1 (Stem Cell Technologies) on hESC-qualified Matrigel (#354277; Corning, New York, NY, USA)-coated culture plates. After incubating the cells at 37 °C with 20% O_2 and 5% CO_2 for 24–36 h, the mTeSR1 was replaced with RSeT Feeder-Free Medium. The cells were cultured under hypoxic conditions (37 °C with 5% O_2 and 5% CO_2), and the medium was changed every other day. After seeding for 4 or 5 days, the cells were passaged onto a non-Matrigel-coated 6-well plate with RSeT Feeder-Free Medium containing 5 μM Y-27632, using 1X TrypLE Select Enzyme (Stem Cell Technologies). The following day, the medium was replaced with RSeT Feeder-Free Medium without Y-27632 and changed every other day, and cells were passaged every 3–6 days.

2.3. Differentiation into TE

The culture plate for TE differentiation was precoated with iMatrix-511 silk (diluted to 0.5 μg/cm^2 in PBS) at 37 °C for 30 min. Naive iPSCs were dissociated using 1X TrypLE Select Enzyme and seeded on a pre-coated plate at a density of 4×10^4 cells/cm^2. Cells were cultured at 37 °C with 5% O_2 and 5% CO_2 in a medium composed of NDiff 227 supplemented with 2 μM A83-01 (SML0788; Sigma-Aldrich, St. Louis, MO, USA), 2 μM PD0325901 (4192; Tocris, Bristol, UK), and 10 ng/mL recombinant human BMP4 (314-BP-050/CF; R&D Systems, Minneapolis, MN, USA), as previously described [12]. On the following day (day 1), the medium was changed to a medium composed of Ndiff227 supplemented with 2 μM A83-01, 2 μM PD0325901, and 1 μg/mL JAK inhibitor I (420099; Merck KGaA, Darmstadt, Germany), as previously described [12]. The medium was changed the next day (day 2). The cells were analyzed on day 3 after TE differentiation.

2.4. Differentiation into CT

After performing *in vitro* differentiation of naive iPSCs into TE, we performed further differentiation into CT.

On day 3 of TE differentiation, the medium was changed to ACE medium, composed of NDiff 227 supplemented with 1 μM A83-01, 2 μM CHIR99021 (#4423; Tocris), and 50 ng/mL EGF (236-EG; R&D Systems), as previously described [12]. The CT-like cells were incubated at 37 °C with 5% O_2 and 5% CO_2. After the cells reached approximately 80% confluent, the cells were passaged at a density of 4×10^4 cells/cm^2 in ACE medium and 10 μM Y-27632 on the pre-coated plate (0.5 μg/cm^2 iMatrix-511 silk at 37 °C for 30 min) by

dissociation with Accutase for 30 min. The next day, the medium was removed and replaced with the ACE medium without Y-27632. The medium was then changed every other day and passaged every 3–6 days. CT-like cells at passages 1 or 2 (cultured for 4–7 days in ACE medium) were used for analysis.

2.5. Differentiation into ST

On the pre-coated plate (0.5 μg/cm^2 iMatrix-511 silk at 37 °C for 30 min), CT-like cells (at passage 1 or 2, cultured for 4–7 days in ACE medium) were seeded at a density of 4 × 10⁴ cells/cm^2 for differentiation into ST. ST-like cells were incubated at 37 °C with 20% O_2 and 5% CO_2. The cells cultured in ST medium composed of DMEM/Ham's F-12 (11320–033; Gibco) supplemented with 0.1 mM 2-Mercaptoethanol (21985–023; Gibco), 0.3% BSA (017–22231; Wako), 1% ITS-X supplement (51500056; Gibco), 4% KSR (10828028; Gibco), 2 μM forskolin (Abcam, Cambridge, UK), and 2.5 μM Y-27632, as previously described [6].

ST medium was replaced on day 3, and cells were cultured for an additional three days. The cells were analyzed on day 6 of the ST differentiation.

2.6. Differentiation into EVT

On the pre-coated plate (0.5 μg/cm^2 iMatrix-511 silk at 37 °C for 30 min), CT-like cells (at passage 1 or 2, cultured for 4–7 days in ACE medium) were seeded at a density of 4 × 10⁴ cells/cm^2 to for differentiation into EVT. The EVT-like cells were incubated at 37 °C with 20% O_2 and 5% CO_2. The cells cultured in EVT medium, which was composed of DMEM/Ham's F-12 supplemented with 0.1 mM 2-Mercaptoethanol, 0.3% BSA, 1% ITS-X supplement, 4% KSR, 7.5 μM A83-01, 100 ng/mL NRG1 (Cat. 5218SC; Cell Signaling), and 2.5 μM Y-27632, as previously described [6]. Geltrex (A1413302; Thermo Fisher Scientific, Waltham, MA, USA) was added to a final concentration of 2% shortly after the cells were suspended in the medium.

On day 3 of EVT differentiation, the medium was replaced with EVT medium without NRG1, and Geltrex was added to a final concentration of 0.5%. On day 6 of EVT differentiation, the medium was replaced with the EVT medium without NRG1 or KSR. Geltrex was added to a final concentration of 0.5% and cultured for 2 additional days. The cells were analyzed on day 8 of EVT differentiation.

2.7. AS1842856 treatment

Naive iPSCs were differentiated into TE with or without 100 nM FOXO1 inhibitor AS1842856 (344355; Sigma Aldrich) for the first 3 days (from day 0 to day 3). Subsequently, three trophoblast lineages (CT, ST, and EVT) were induced from each TE-like cell. For TE differentiation without AS1842856, DMSO was added as a negative control.

2.8. Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) or quantitative RT-PCR (RT-qPCR)

Total RNA was extracted using the TRIzol Reagent (Life Technologies, Grand Island, NY, USA). Treatment with ezDNase (Life Technologies) eliminated genomic DNA contamination. Total RNA was then reverse-transcribed to cDNA using the PrimeScript II 1st strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). For semi-quantitative RT-PCR, the resulting cDNA was subjected to PCR with the TaKaRa Ex Taq® PCR kit (Takara Bio). RT-qPCR was performed using a Quant Studio 1 Real-Time PCR System (Thermo Fisher Scientific) with Thunderbird™ SYBR® qPCR Mix (TOYOBO, Osaka, Japan). The PCR primers used are listed in Table S1.

Human iPSC-derived definitive endoderm (DE) cells were obtained using a previously reported method [24].

2.9. RNA sequencing

Total RNA was isolated as described above and sent to Macrogen (Seoul, South Korea). Library preparation using the SMARTer stranded total RNA-seq kit. Paired-end RNA sequencing datasets were created using an Illumina NovaSeq6000 (Illumina, San Diego, CA, USA). The Strand NGS software program (Strand Life Sciences, Bangalore, India) was used to align reads to the human transcriptome (hg38) reference sequences. RNA-seq data were deposited in GEO under the accession number GSE 267171. Human placenta (Thermo Fisher Scientific) was used as a positive control. Pathway analysis was performed using the Strand NGS.

2.10. Flow cytometry

Differentiated TE cells were dissociated into single cells using Accutase and adjusted to a concentration of 5 × 10^4 cells/mL in 10% human serum (H4522; Sigma-Aldrich)/PBS and incubated at room temperature for 15 min. Cells were incubated with anti-human TROP2 (TACSTD2)-FITC (clone77220 [MM0588-49D6]; R&D Systems, Minneapolis, MN, USA; dilution 1:25) and anti-human CD249 (ENPEP)-PE (clone2D3/APA BD Bioscience, San Jose, CA, USA; dilution 1:25) in 1% fetal bovine serum (FBS; S1650; Sigma Aldrich)/PBS on ice for 20 min. Flow cytometry was performed using a BD FACS Melody flow cytometer (BD Biosciences).

2.11. hCG measurement

During ST-like cell differentiation, the ST medium was replaced on day 3, and the supernatant was collected on day 6. The supernatant was poured onto the designated area of a pregnancy test by immunochromatography, GONASTICK W (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan), as a qualitative test of secreted human chorionic gonadotropin (hCG). The quantitative levels of hCG were determined by a chemiluminescent enzyme immunoassay (CLEIA; SRL, Tokyo, Japan). As a negative control, hCG levels in ST medium, which was not used for culture, were also measured in the same way.

2.12. Transposable elements quantification using TEtranscripts

BAM files were generated from FASTQ files of RNA-seq data (GSE 267171) using the Strand NGS software program. GTF files for gene annotations and transposable element annotations were downloaded from the TEtranscripts website (https://www.mghlab.org/software/tetranscripts, as of December 23, 2023). Placenta was used as a control sample, and a differential analysis was performed using DESeq2.

A total of 22926 genes and 1168 transposable elements that contained 579 LTR retrotransposons showed estimated log2FC values in at least 1 of the analyzed samples and were used for the analysis.

3. Results

3.1. Differentiation into trophoblast lineages via trophectoderm from naive iPSCs

Previous reports have shown TE differentiation from “naive human iPSCs on feeder cells” [9,10,12]. Therefore, we first investigated whether or not “feeder-free naive human iPSCs” could also differentiate into TE and trophoblast lineages (CT, ST, EVT) based on a previous differentiation protocol [12] (Fig. S1A). We established naive human iPSCs that formed dome-shaped colonies (Fig. S1B, right panel) from primed human iPSCs (Fig. S1B left panel) using

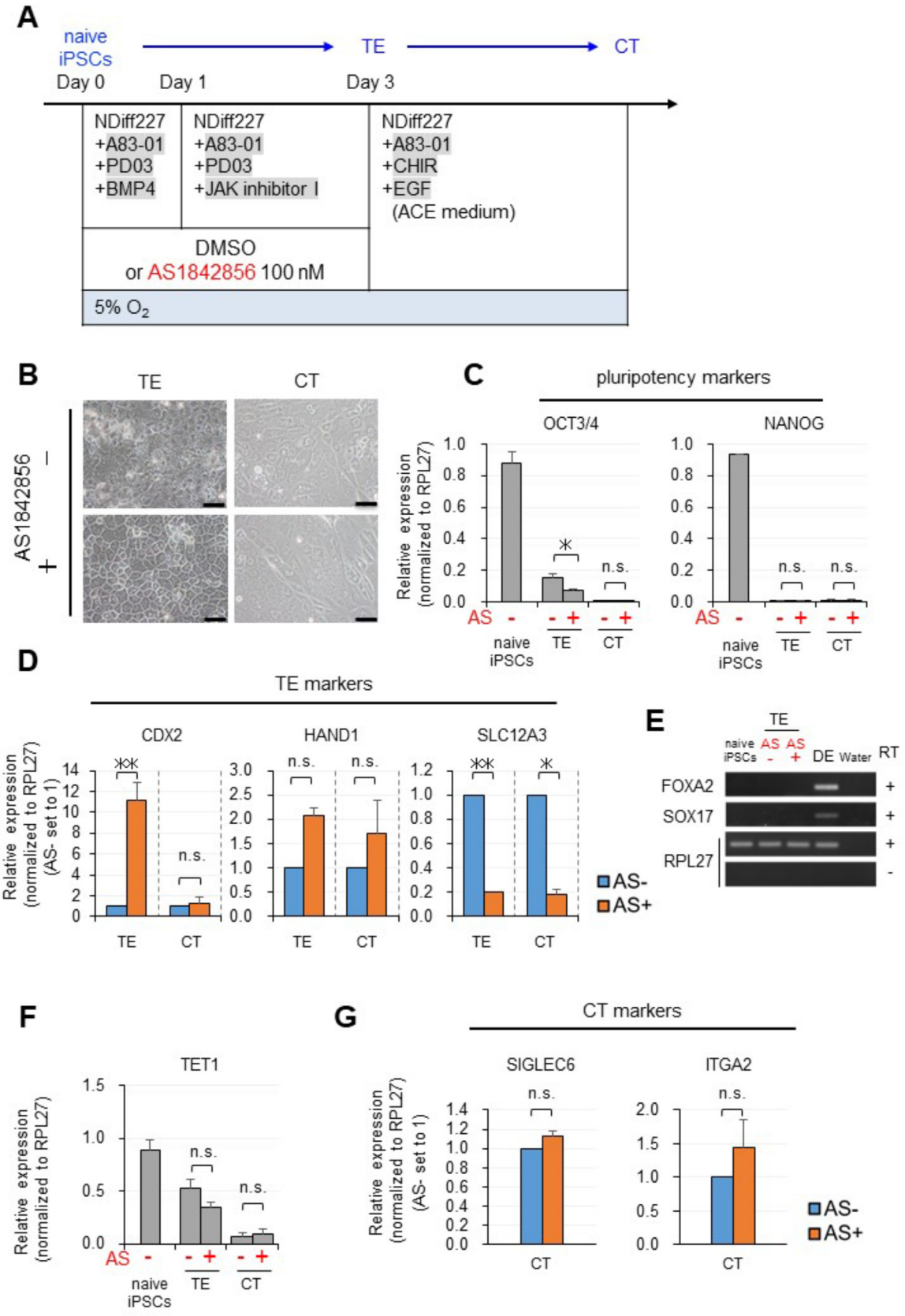

Fig. 1. Effect of a FOXO1 inhibitor on TE and CT differentiation. (A) A schematic diagram of the protocol for the induction of differentiation of naive iPSCs into TE-like cells in the absence or presence of AS1842856 from day 0 to day 3. (B) Representative phase-contrast images of TE-like cells (left panels) and CT-like cells (right panels) with or without the treatment of AS1842856 for the first three days of TE differentiation. Scale bars, 50 μm. (C) A qPCR analysis of pluripotency marker genes (OCT3/4 and NANOG) on day 3 after TE differentiation, with or without AS1842856 treatment. The mean value ± SD, paired *t*-test, n.s.: not significant, *p < 0.05 (n = 3). (D) A qPCR analysis of TE marker genes on day 3 after TE differentiation, with or without AS1842856 treatment. The mean value ± SD, paired t-test, *p<0.05, **p<0.01, n.s.: not significant (n = 4). AS- was set to 1. (E) A semi-

RSeT Feeder-Free medium (see *Materials and Methods* in detail). In naive hiPSCs, the expression of naive-related markers NANOG and KLF4 was increased and prime-related markers SOX1 and c-Myc were decreased relative to primed hiPSCs 25,26 (Fig. S1C). Then, we performed *in vitro* differentiation of "feeder-free naive human iPSCs" into TE and trophoblast lineages (Figs. S1A and D).

During TE differentiation for three days, the dome-shaped colonies became flat and composed of polygonal cells (Fig. S1D upper left panel). After TE differentiation for three days and subsequent culture, qPCR showed that the differentiated cells expressed CDX2, a TE marker gene (Fig. S1E). In addition, another TE marker gene, HAND1 [27], was also upregulated (Fig. S1E).

Next, we differentiated TE-like cells into CT, ST, and EVT and confirmed the expression of a CT marker SIGLEC6, a ST marker CGB7 and an EVT marker HLA-G. The results showed that the expression of each marker gene (SIGLEC6, CGB7, and HLA-G) increased 116-fold in CT-like cells, 15.4-fold in ST-like cells, and 2.2-fold in EVT-like cells compared to TE-like cells (Fig. S1E). Furthermore, hCG secretion was observed after six days of culture of CT-like cells in ST medium (Fig. S1F). Together, these findings indicate that "feeder-free naive iPSCs" could be differentiated into TE and trophoblast lineage cells, so we decided to use "feeder-free naive iPSCs" in this study. We confirmed the expression of FOXO1 at all stages from the naive iPSCs to ST/EVT by semi-quantitative PCR and qPCR (Fig. S1G).

3.2. Effect of a FOXO1 inhibitor treatment on TE differentiation

First, to reveal that AS1842856 inhibits FOXO1 function in naive hiPSCs, we compared the gene expression in hiPSCs treated with AS1842856 for 3 days and that in hiPSCs without AS1842856. AS1842856 inhibits the DNA-binding activity of FOXO1 [28]. FOXO1 binds directly to the regulatory regions of OCT4 and SOX2 pluripotency genes in human ES cells and activates the expression of these genes [22]. In naive hiPSCs, AS1842856 treatment tended to reduce the expression of OCT4, although not to a statistically significant extent, and significantly reduced the expression of SOX2 (Fig. S2), suggesting that AS1842856 suppresses the FOXO1 function in naive hiPSCs.

To clarify the role of a FOXO1 inhibitor in TE differentiation, TE induction was performed with or without 100 nM AS1842856 treatment for the first 3 days (Fig. 1A). Differentiated cells not treated with AS1842856 ("TE AS-") and differentiated cells treated with AS1842856 ("TE AS+") differed in morphology, with TE AS + having more distinct cell boundaries (Fig. 1B, left panels). OCT3/4 expression was significantly lower in TE AS + cells than in TE AS- cells, whereas NANOG expression was undetectable under both conditions (Fig. 1C). Furthermore, TE AS + cells showed a 10.3-fold higher expression of CDX2 than TE AS- cells, with statistical significance (Fig. 1D). On average, TE AS + cells tended to have a 1.7-fold higher expression of HAND1 than TE AS- cells, although the difference was not statistically significant (Fig. 1D). In contrast, TE AS + cells had 5.6-fold lower expression of SLC12A3, another TE marker gene, than TE AS- cells (Fig. 1D). In addition, we compared 3-day treatment with AS1842856 to 1-day treatment and found that 3-day treatment had a greater effect on the gene expression (data not shown). Therefore, we used 3-day AS1842856 treatment in the subsequent experiments.

To investigate the possibility that CDX2 expression indicates differentiation to definitive endoderm rather than to TE, we examined the expression of SOX17 and FOXA2, which are markers of definitive endoderm, in the resultant cells of our TE induction experiments and found no expression of these genes (Fig. 1E). Therefore, we considered that the expression of CDX2 in TE AS- and TE AS + cells indicated successful differentiation into TE.

Knockdown of TET1 in mouse ES cells upregulate CDX2 and HAND1, and knockdown of TET1 in preimplantation embryos leads to differentiation into TE [29]. We performed qPCR to determine whether or not TET1 expression was downregulated in TE-like cells treated with AS. The results showed that TET1 expression was similar between TE AS- and TE AS + cells (Fig. 1F).

3.3. Effect of FOXO1 inhibitor treatment on CT differentiation

We carried out a CT cell differentiation protocol based on a previous report [12] in naive iPSC-derived TE-like cells treated with AS1842856 and untreated for the first 3 days (Fig. 1A). The morphology of resultant cells derived from the TE-like cells with and without AS1842856 treatment ("CT AS+" and "CT AS-," respectively) was similar (Fig. 1B right panels). Neither CT AS + nor CT AS cells expressed pluripotency markers (Fig. 1C). Levels of CDX2 and HAND1, both TE markers, were not significantly different between CT AS+ and CT AS- cells, whereas the expression of SLC12A3 was significantly lower in CT AS + cells than in CT AS- cells (Fig. 1D). Furthermore, the expression of CT markers (SIGLEC6 and ITGA2) in CT AS- and CT AS + cells was not significantly different (Fig. 1G).

3.4. Effect of FOXO1 inhibitor treatment on ST and EVT differentiation

To examine the effect of FOXO1 inhibitor treatment during TE induction on ST and EVT differentiation, we differentiated CT-like cells (CT AS- and CT AS+) into ST or EVT lineages using previously reported protocols [6,12] (Fig. 2A). The morphology of resultant cells of the ST differentiation protocol derived from CT AS- and CT AS+ (abbreviated "ST AS-" and "ST AS+", respectively) was similar (Fig. 2B). Qualitative hCG tests showed that both cells secreted hCG into the culture supernatant (Fig. 2C). Quantitative tests showed that hCG levels in ST AS + cells were 10.2-fold lower than those in ST AS- cells (Fig. 2D). We examined the expression of ST marker genes (GCM1, ERVW-1[Syncytin-1], and CGB3/5/8[CGBtypeII]) in ST AS- and ST AS + cells using qPCR. Interestingly, ST AS + showed 6.5-fold, 5.8-fold, and 3.2-fold lower expression of GCM1, ERVW-1, and CGB3/5/8, respectively, compared to ST AS- with statistical significance (Fig. 2E).

The morphology of resultant cells of the EVT differentiation protocol derived from CT AS- and CT AS + cells ("EVT AS-" and "EVT AS+", respectively) did not show obvious differences (Fig. 2F). The expression of an EVT marker gene HLA-G was 1.9-fold lower in EVT AS + than in EVT AS- cells (Fig. 2G).

These results indicate that AS1842856 treatment during TE differentiation suppressed the induction of ST and EVT.

3.5. Effect of FOXO1 inhibitor treatment on developmental stage progression of TE from naive iPSCs

We investigated the stage of trophoblast differentiation at which the FOXO1 inhibitor affected differentiation. Since treatment with AS1842856 during TE induction increased the TE markers CDX2 and HAND1 but decreased the expression of SLC12A3, another TE marker, and suppressed differentiation into ST and EVT, we hypothesized that differentiation beyond trophectoderm specification was hindered by the FOXO1 inhibitor. Therefore, we

quantitative RT-PCR analysis of definitive endoderm (DE) marker genes (FOXA2 and SOX17). Human iPSC-derived DE cells were used as positive controls. RT: reverse transcriptase. (F) A qPCR analysis of TET1 expression on day 3 after TE differentiation with or without AS1842856 treatment. The mean value ± SD, paired *t*-test. n.s.: not significant (n = 4). (G) A qPCR analysis of CT marker genes (SIGLEC6 and ITGA2) in the absence and presence of AS1842856. The mean value ± SD, paired *t*-test, n.s.: not significant (n = 3). AS- was set to 1.

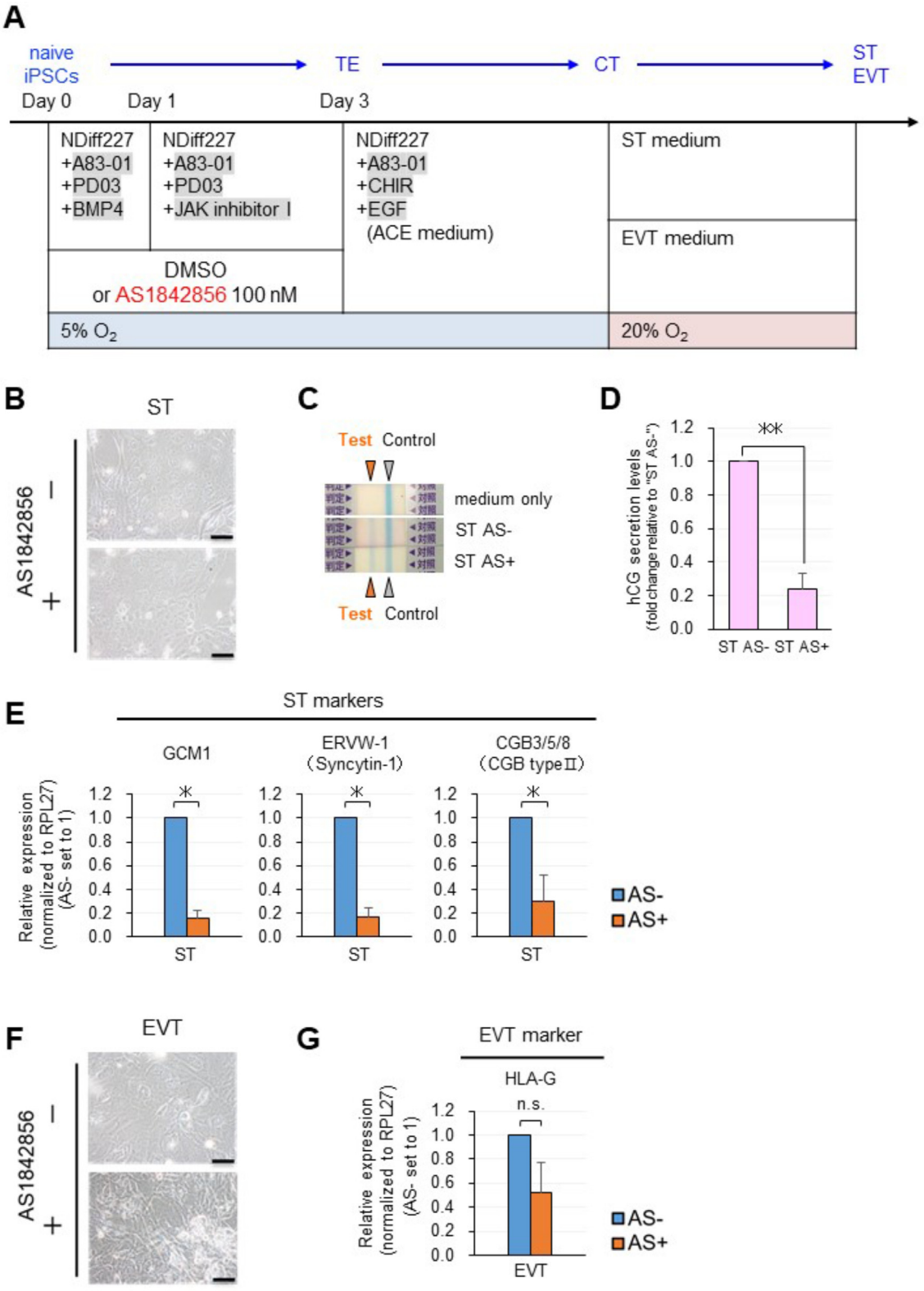

Fig. 2. Effect of a FOXO1 inhibitor on ST and EVT. (A) A schematic diagram of the protocol for the differentiation of CT-like cells into ST-like and EVT-like cells with or without the treatment of AS1842856 for three days during TE differentiation. (B) Representative phase-contrast images of ST-like cells generated from CT-like cells with or without the treatment of AS1842856 for three days during TE differentiation. Scale bars, 50 μm. (C) Pregnancy test results using supernatants of ST-like cells generated from CT-like cells with or without the treatment of AS1842856 for three days during TE differentiation. The grey arrowhead indicates the control line, and the orange arrowhead indicates the test line. (D) Levels of hCG secreted by ST-like cells generated from CT-like cells with or without the treatment of AS1842856 for three days during TE differentiation. The mean value ± SD, paired *t*-test, **p < 0.01 (n = 3). ST AS- was set to 1. (E) A qPCR analysis of ST marker genes (GCM1, Syncytin-1 and CGB typeII) in ST-like cells generated from CT-like cells with or without the treatment of AS1842856 for 3 days during TE differentiation. Mean ± SD, paired *t*-test, *p < 0.05 (n = 4). ST AS- was set to 1. (F) Representative phase-contrast images of EVT-like cells generated from CT-like cells with or without the treatment of AS1842856 for three days during TE differentiation. Scale bars, 50 μm. (G) A qPCR analysis of an EVT marker gene HLA-G in EVT-like cells generated from CT-like cells with or without the treatment of AS1842856 for three days during TE differentiation. The mean value ± SD, paired *t*-test, n.s.: not significant (n = 3). EVT AS- was set to 1.

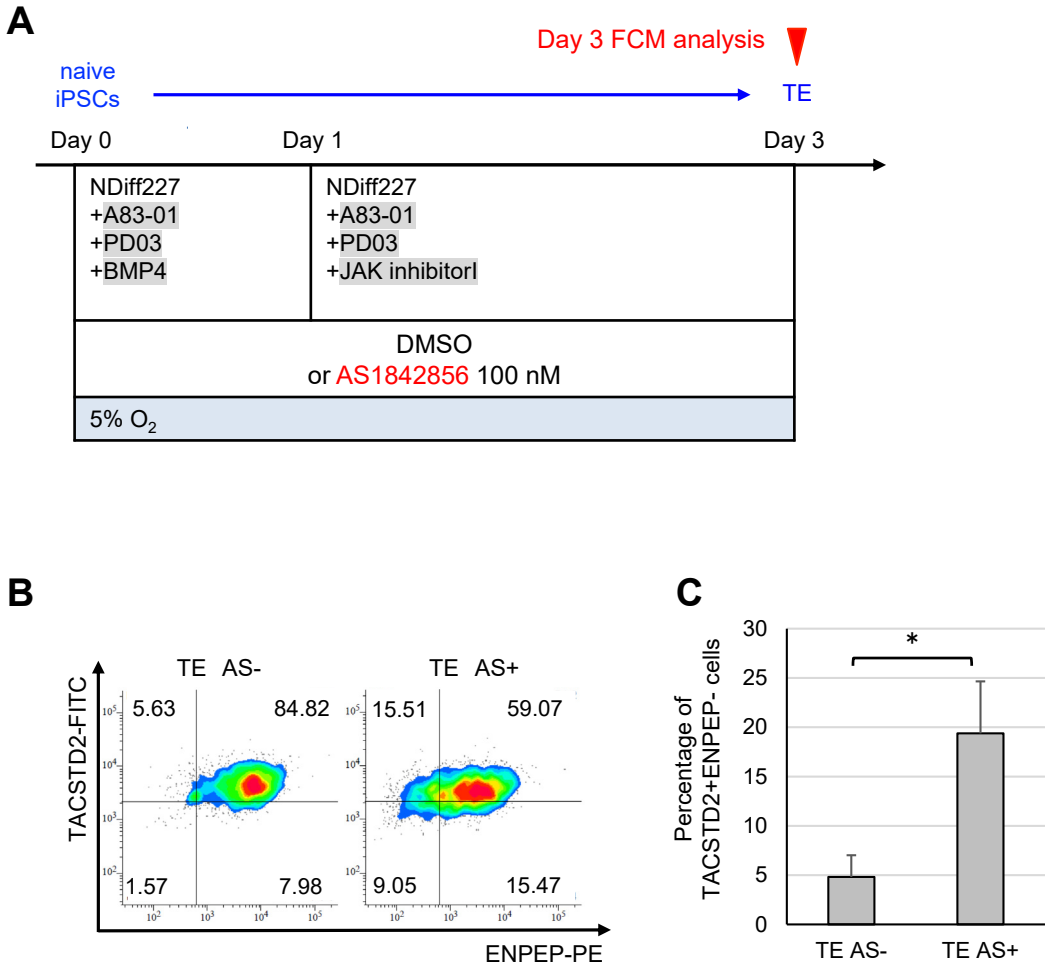

Fig. 3. Effect of FOXO1 treatment on different stages of TE. (A) A schematic diagram of the protocol for the differentiation of naive iPSCs into TE-like cells in the absence or presence of AS1842856 from day 0 to day 3. A flow cytometry analysis was performed on day 3. (B) The expression of trophectoderm cell surface marker genes (TACSTD2 and ENPEP) was analyzed using flow cytometry on day 3 in the absence and presence of AS1842856. A representative density plot is shown. (C) The percentage of TACSTD2+ ENPEP- cells is shown. The mean value ± SD, paired *t*-test, *p < 0.05 (n = 3).

decided to distinguish between early and late TE to assess TE AS- and TE AS + cells. TACSTD2 is reportedly expressed in early (E5) and late blastocyst (E7) TE, whereas ENPEP expression increases in late blastocyst (E7) TE [12]. Consistently, it has been reported that during the *in vitro* TE induction from naive iPSCs, TACSTD2+ENPEP- cells appeared first, followed by TACSTD2+ENPEP + cells [12].

In our data, TE AS + cells had a higher percentage of TACSTD2+ENPEP- cells than TE AS- cells in the flow cytometry analysis (Fig. 3A and Fig. 3B). Three independent experiments showed 4.0-fold significant differences (Fig. 3C), suggesting that AS1842856 treatment hinders progression from early-to late-stage TE.

3.6. RNA-seq of differentiation into each trophoblast lineage from naive iPSCs with or without FOXO1 inhibitor treatment

To reveal the effect of FOXO1 inhibitor treatment during TE differentiation on the global gene expression of induced three trophoblast lineages via TE from naive iPSCs, we performed RNA-seq.

A principal component analysis (PCA) revealed that among TE, CT, ST, and EVT, differences in gene expression patterns between AS- and AS + cells were most apparent in ST (Fig. 4A).

First, we focused on TE, the first step in differentiation induction, and compared the gene expression profiles in TE with and without AS treatment. In the scatterplot, the expression of 169 genes was found to be more than 5-fold higher in TE AS(+) than in TE AS(−), while 72 genes showed lower expression levels (Fig. 4B, Table S2). A pathway analysis was performed for each of these gene groups, and no particularly distinctive pathways were found for the genes for which the expression was more than 5-fold lower in TE AS(+) than in TE AS(−) ($p < 10^{-3}$). On the other hand, 169 highly expressed genes were significantly enriched in multiple pathways, including "Cancer pathways," "lncRNA in canonical Wnt signaling and colorectal cancer," and "Wnt signaling pathway and pluripotency" (Fig. 4C). By acting on some pathway related to WNT signaling, AS treatment may affect later differentiation stages, CT and ST/EVT differentiation.

Next, focusing on ST, we selected 268 genes with more than 5-fold higher expression in ST AS- cells than in ST AS + cells in scatter plots (Fig. 4D). These genes included GCM1, CGA, ERVW-1, CGB3, CGB5, and CGB8, which are known to be important markers of ST (Fig. 4D) [6,30–33].

A pathway analysis of the 268 genes was performed. "Steroidogenic pathway", "Metabolism of steroid hormones" pathway, and "Interleukin signaling pathways" were strongly involved (Fig. 4E, Table S3). In addition, from the non-hierarchical clustering for the 268 genes and 4 conditions (naive iPSCs, ST AS- cells, ST AS + cells, and placenta) (Fig. 4F), the genes appeared to be divided into several groups: (i) group 1, lower expression only in ST AS + cells; (ii) group 2, lower expression in naive iPSCs and ST AS + cells and higher expression in ST AS- cells and placenta; (iii) group 3, higher expression only in ST AS- cells; (iv) group 4, lower in naive and ST AS + cells,

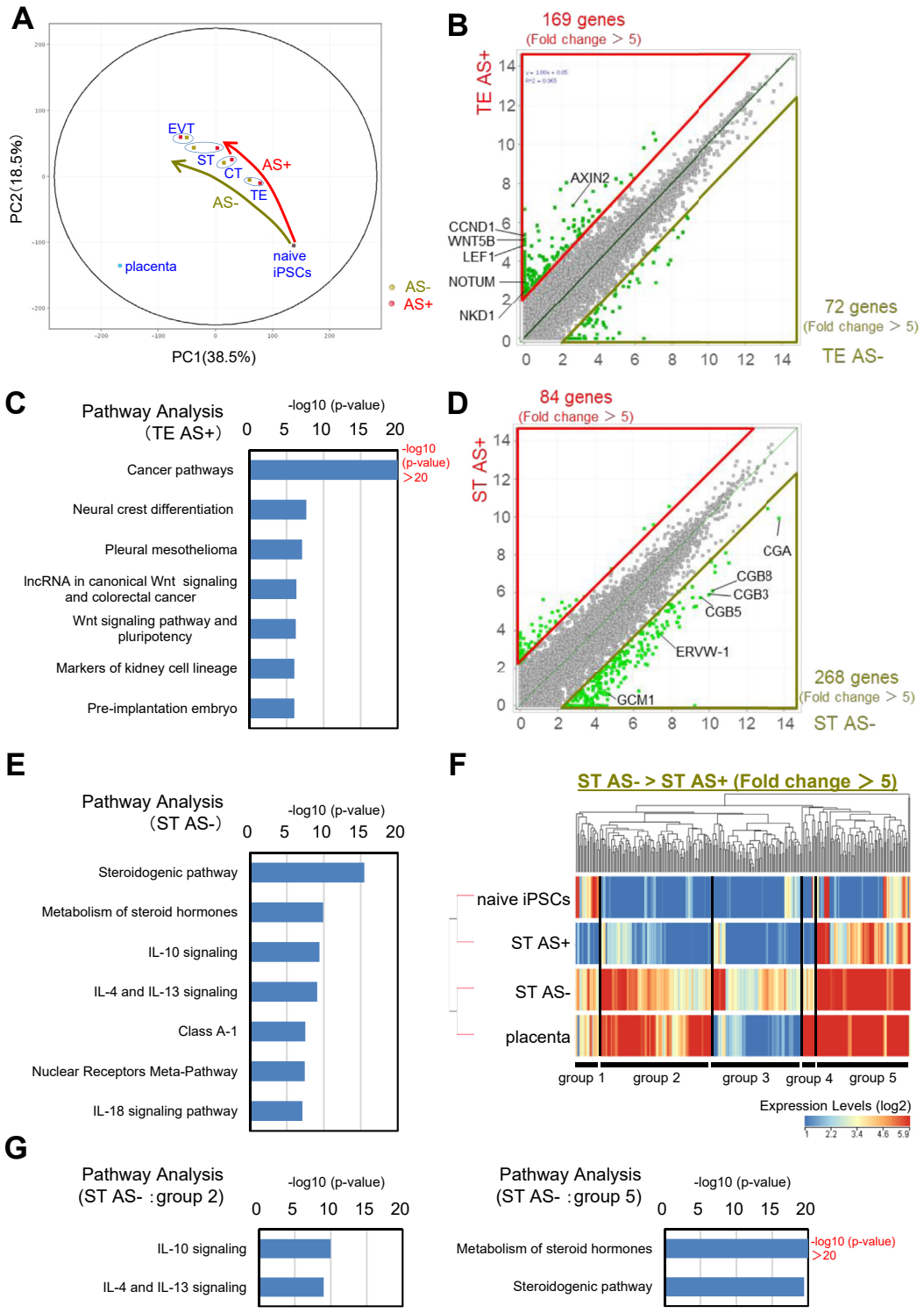

Fig. 4. A global gene expression analysis of differentiated cells in each trophoblast lineage from naive iPSCs with and without a FOXO1 inhibitor treatment. (A) A principal component analysis (PCA) of naive iPSCs, TE, CT, ST, EVT, and placenta without AS1842856 treatment (AS-) and with AS1842856 treatment (AS+). TE: trophectoderm-like cells from naive iPSCs, CT: cytotrophoblast-like cells from TE, ST: syncytiotrophoblast-like cells from CT, EVT: extravillous trophoblast-like cells from CT. (B) Scatterplot of the expression of all genes in TE AS- and TE AS+. Relative to TE AS+, the expression of 72 genes was increased more than 5-fold in TE AS-. A more than 5-fold decrease in the expression of 169 genes was observed in TE AS- relative to TE AS+. (C) A pathway analysis was performed for 169 genes with higher expression levels in TE AS + than in TE AS+. Pathways with a -log10 (p-value) >6 are listed. (D) Scatterplot of all genes between ST AS- and ST AS+. Compared to ST AS+, the expression of 268 genes was more than 5-fold increased in ST AS-. A more than 5-fold decrease in the expression of 84 genes was observed in ST AS- compared to ST AS+. (E) A pathway analysis was performed for 268 genes that showed a higher expression in ST AS- than in ST AS+. Pathways with a -log10 (p-value) >7 are listed. (F) Non-hierarchical clustering (Euclidean, average) was performed for 268 genes and 4 conditions (naive iPSCs, ST AS-, ST AS+, and placenta). These 268 genes were divided into 5 groups. (G) Results of the pathway analysis for genes in groups 2 and 5 are shown. Pathways with a -log10 (p-value) >7 are listed. There were no pathways with a -log10 (p-value) >7 in groups 1, 3, or 4.

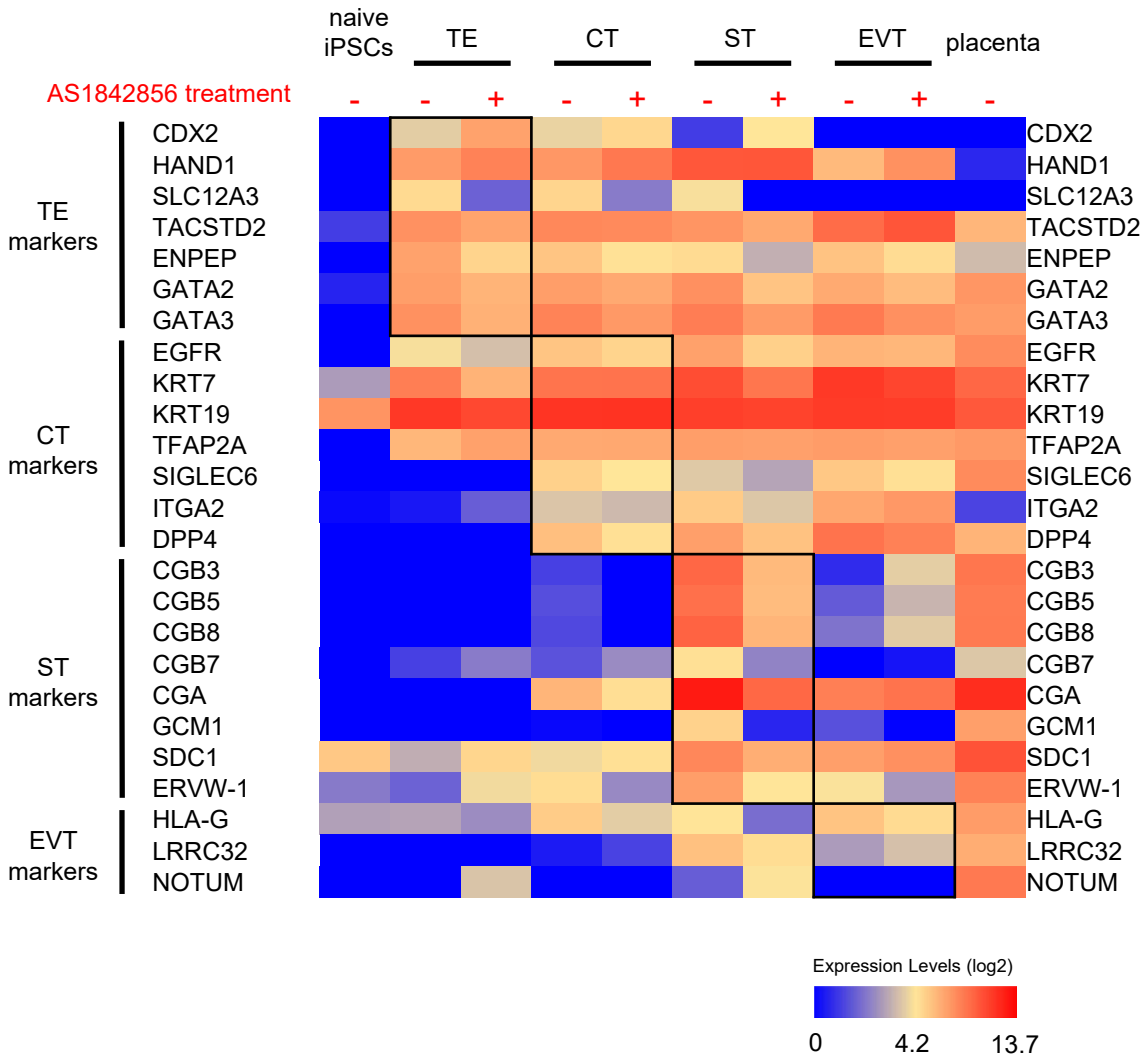

Fig. 5. An evaluation of gene expression at each developmental stage of trophoblast induction cells from naive iPSCs with and without FOXO1 inhibitor treatment.Heatmaps of trophectoderm and trophoblasts in differentiated cells at various stages. A heatmap was generated based on transcript per million (TPM) values.

middle in ST AS- cells, and higher in placenta; and (v) group 5, lower in naive iPSCs, middle in ST AS + cells, and higher in ST AS- cells and placenta (Fig. 4F, Table S4). A pathway analysis of genes from each group showed that the "Interleukin-10 signaling" pathway was strongly involved in group 2, and the "Metabolism of steroid hormones" and "Steroidogenic" pathways were strongly involved in group 5 (Fig. 4G). In contrast, there were 84 genes whose expression decreased by more than 5-fold in scatter plots with ST AS- compared to ST AS+ (Fig. 4D, Table S4). A pathway analysis of 84 genes was performed. Seven pathways with a p-value $<10^{-7}$ were identified among the 268 genes downregulated in ST AS- cells (Fig. 4E), while no pathways were identified with p-values $<10^{-7}$ among the 84 genes. Although nine pathways showed a p-value $<10^{-3}$, the significance of any pathway in placental development was unclear (Fig. S3).

3.7. Suppression of endogenous retroviral envelope-derived genes in FOXO1 inhibitor-treated cells

Next, we compared the gene expression patterns of stage-specific marker genes between AS- and AS+ in TE, CT, ST, and EVT-like cells, as shown in Fig. 5. Naive iPSCs and placenta were used as controls. Among the ST markers whose expression is suppressed by AS1842856 treatment, ERVW-1 encodes Syncytin-1, which is a protein encoded by the env gene of endogenous retrovirus (ERV), and GCM1 is known to be a transcription factor that controls the expression of ERVW-1 (Syncytin-1) and ERVFRD-1 (Syncytin-2) [34]. Since at least 8 retroviral envelope-derived genes (ERVW1, ERVFRD-1, ERVV-1, ERVV-2, ERVH48-1, ERVMER34-1, ERV3-1, and ERVK13-1) have been reported to be expressed in human trophoblast lineages [35], we investigated whether or not the expression of other retroviral envelope-derived genes is affected by treatment with AS1842856 during TE differentiation. As shown in Fig. 6A and B, the expression of 6 retroviral envelope-derived genes other than ERV3-1 and ERVK13-1 tended to decrease under AS + conditions in CT, ST, and EVT lineages. Therefore, AS1842856 treatment during TE differentiation inhibited the expression of retroviral envelope-derived genes in not only ST-like cells but also CT-like and EVT-like cells, although the mechanism is unknown. Furthermore, referring to The University of California Santa Cruz (UCSC) Genome Browser Database, of TE markers, SLC12A3 and ENPEP, which were suppressed by AS1342856 treatment, had a long terminal repeat (LTR) around the transcription start sites, whereas CDX2 and HAND1, a gene upregulated by AS1842856, and TACSTD2, a gene apparently not downregulated by AS1842856, did not have an LTR (Fig. 6C).

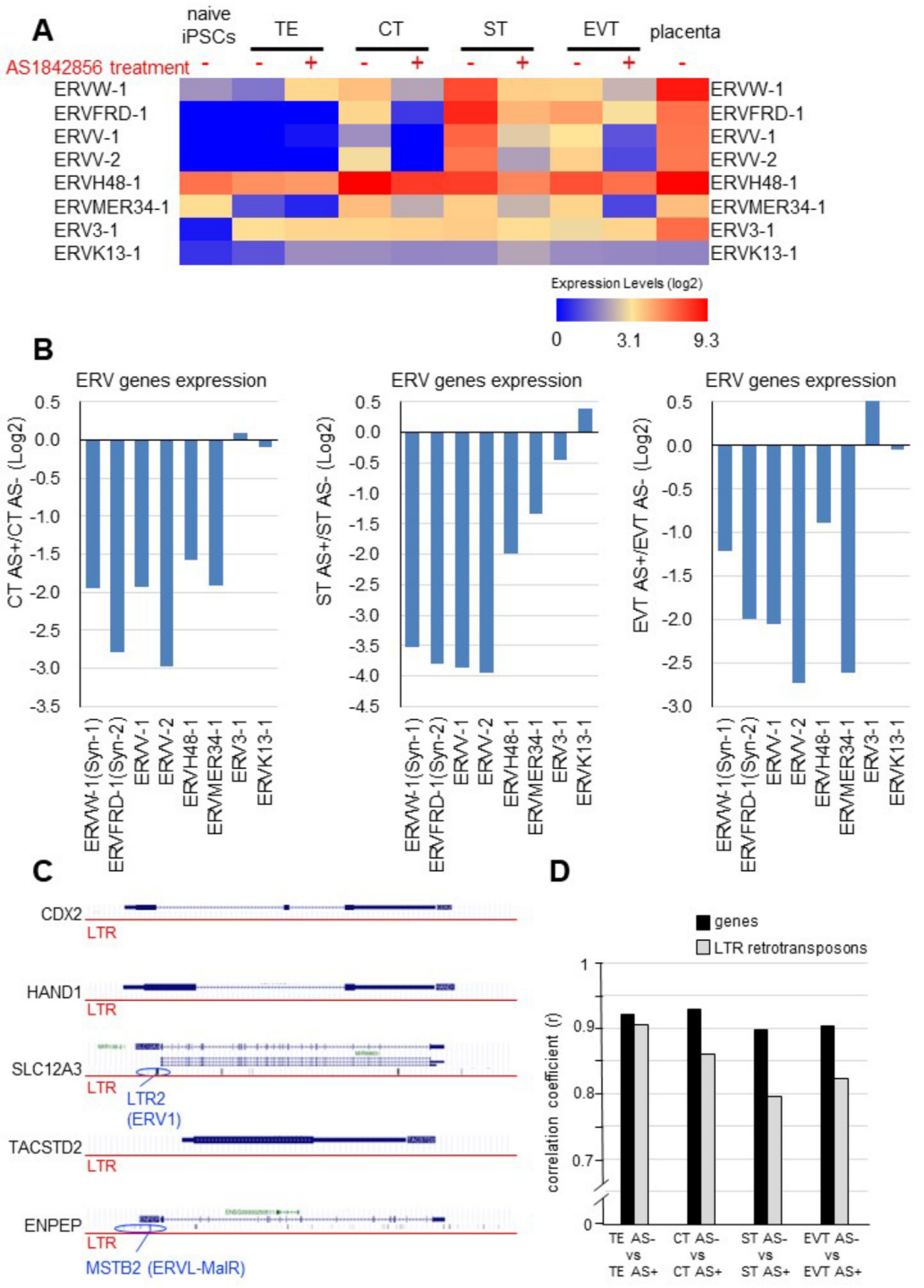

Fig. 6. Relationship between the addition of a FOXO1 inhibitor and endogenous retroviral suppression. (A) Heatmap of endogenous retrovirus (ERV)-related gene expression in syncytiotrophoblast lineages. (B) The difference in the expression of endogenous retroviral envelope-derived genes between AS+ and AS− in CT, ST and EVT was calculated by subtracting the log2 TPM value of AS− from that of AS+. (C) The positions of long terminal repeat (LTR) elements by RepeatMasker near the loci of five TE marker genes (CDX2, HAND1, SLC12A3, TACSTD2, and ENPEP) are shown. These diagrams were extracted from UCSC genome browser on human (GRCh38/hg38). (D) Correlation coefficient between AS (−) and AS (+) in TE, CT, ST and EVT samples for genes (black bars) and LTR retrotransposons (grey bars).

We used TEtranscripts, a widely used transposable element quantification method [36], to examine the expression of LTR retrotransposons, including those other than retroviral envelope-derived genes. We compared the estimated log2FC values of these genes (Fig. S4A), and LTR retrotransposon transcripts (Fig. S4B) of the TE, CT, ST, and EVT samples with (y-axis) and without AS1842856 treatment (x-axis). Consistent with Fig. 6B, which is the result of an analysis using the Strand NGS software program, 6 of 8 retroviral envelope-derived genes (shown in magenta) showed lower expression levels with AS treatment in CT, ST, and EVT samples in the analysis using TEtranscripts (Fig. S4A). The correlation coefficients for genes and LTR retrotransposons between AS (+) and AS (−) in TE, CT, ST, and EVT are summarized in Fig. 6D. AS1842856 treatment affected the expression of many (but not all) ERV genes (Fig. S4B), and the effect of AS1842856 treatment on the expression of LTR retrotransposons was greater than that on the expression of genes in CT, ST, and EVT (Fig. 6D).

4. Discussion

In this study, we succeeded in differentiating naive human iPSCs into trophoblasts via the trophectoderm *in vitro* without feeder cells. Several methods for differentiation of naive human PSCs to trophoblast lineages have been reported [9,10,12]; however, to our knowledge, all of the previous protocols employed “on feeder” culture systems. Our feeder-free system has the advantage of eliminating cost, time, and effort to prepare feeder cells and avoiding potential problems of variability in feeder cell quality attributes affecting trophoblast differentiation, leading to a more convenient and robust procedure for inducing trophectoderm and trophoblast lineages from iPSCs.

We demonstrated that treatment with a FOXO1 inhibitor during TE induction from naive iPSCs resulted in a decrease in ST markers, such as ERVW-1 (Syncytin-1) expression and hCG secretion, during ST differentiation, and a decrease in HLA-G during EVT differentiation, suggesting that FOXO1 inhibitor treatment during TE induction suppressed trophoblast differentiation. Foxo1-null mouse embryos reportedly manifested a profoundly swollen/hydropic allantois, which failed to fuse with the chorion and resulted in fetal lethality at embryonic day 10.5 [37]. In humans, it has been reported that placentas in preeclampsia and fetal growth restriction have a low number of chorionic trophoblast nuclei expressing FOXO1 [38]. Our present data are consistent with these previous reports suggesting crucial roles of FOXO1 in normal placental development. In addition, while it was unclear from previous reports at which stage of development FOXO1 is important, our findings add to the knowledge that FOXO1 is important in the early period up to TE formation in normal placenta development.

We found that even in a group of genes known as TE markers, the addition of a FOXO1 inhibitor resulted in a decrease in the expression of ENPEP, a late TE marker, whereas the expression of TACSTD2, an early TE marker, remained high. At what point the developmental process of TE is disrupted, causing miscarriage, is unclear. We hypothesized that the association between the TE quality among blastocysts prior to implantation in assisted reproduction and pregnancy rates may be informative. It has been reported that 47% of human blastocysts in intracytoplasmic sperm injection (ICSI) result in miscarriage, even with the highest TE grade in the blastocyst grading system introduced by Gardner and Schoolcraft [39]. This suggests that failure of TE development may occur after day 5 post-fertilization, when the TE quality was assessed in ICSI, leading to miscarriage. FOXO1 signaling may, at least in part, be involved in the failure of late TE development.

In the present study, treatment with a FOXO1 inhibitor during differentiation from naive iPSCs into TE reduced the expression of 6 of 8 retroviral envelope-derived genes reported to be expressed in trophoblasts, including ERVW-1 (Syncytin-1), a known ST marker, in the resulting cells in subsequent trophoblast induction. We also found a decrease in GCM1, which has been reported to act upstream of ERVW-1 (Syncytin-1) and ERVFRD-1 (Syncytin-2) [34]. A previous report argued that human placentas isolated and cultured from preeclampsia and placental dysfunction showed a lower ERVW-1 (Syncytin-1) expression, higher apoptosis rate, and lower hCG secretion than those from normal placenta [40]. Whether or not GCM1 regulates ERV-related genes other than ERVW-1 (Syncytin-1) and ERVFRD-1 (Syncytin-2) is unclear. TE was affected by a FOXO1 inhibitor despite a low GCM1 expression, suggesting the existence of a regulatory mechanism for the ERV-related genes that is not mediated by GCM1. Therefore, a FOXO1 signaling defect may downregulate ERV-related genes, either through decreased expression of GCM1 or not, leading to placental dysfunction during early pregnancy.

Several limitations associated with the present study warrant mention. First, only one iPSC line, 201B7, was used, raising the possibility that the findings of this study were specific to this line. We plan to perform similar experiments using other iPSC lines from other cell lines. Second, regarding the assessment of differentiation from naive iPSCs to trophoblasts, we focused on TE/trophoblast-marker gene expression but have not yet evaluated the functions of the resultant cells, except for hCG secretion. In future studies, we will investigate the effect of FOXO1 inhibition during the early period on the ability of CT to proliferate and whether or not CT can be cultured for a long time. In addition, whether or not the derivatives of FOXO-signaling-defective TE cells can undergo cell fusion as ST and have invasive potential as EVT needs to be clarified. Third, since only one FOXO1 inhibitor, AS1842856, was tested, we cannot rule out the possibility of any off-target effect rather than the effect of FOXO1 inhibition, and have not clarified whether other methods of suppressing the FOXO1 function can produce similar results. We plan to study other FOXO1 inhibitors and conduct FOXO1-knockdown experiments. Finally, the significance of the suppression of ERV-related genes other than ERVW-1 (Syncytin-1) and ERVFRD-1 (Syncytin-2) in the placental development and function has not been clarified.

5. Conclusions

Treatment with a FOXO1 inhibitor during TE induction from naive human iPSCs promotes early TE differentiation but hinders the progression of differentiation into ST and EVT. These findings suggest that an impaired FOXO1 pathway during TE formation leads to placental dysfunction in early pregnancy. The suppression of some ERV-associated genes may be involved in this process.

Declaration of competing interest

The authors have no competing financial interests to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.08.020.

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