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—Original Article—

Maintenance of mouse trophoblast stem cells in KSR-based medium allows conventional 3D culture

Shuai SUN[1)], Shota YANO[1)], Momo O NAKANISHI[1)], Michiko HIROSE[2)], Kazuhiko NAKABAYASHI[3)], Kenichiro HATA[3)], Atsuo OGURA[2, 4)] and Satoshi TANAKA[1)]

[1)]*Department of Animal Resource Sciences/Veterinary Medical Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan*
[2)]*RIKEN BRC, University of Tsukuba, Tsukuba, Japan*
[3)]*Department of Maternal-Fetal Biology, Research Institute, National Center for Child Health and Development, Tokyo 157-8535, Japan*
[4)]*Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Japan*

Abstract. Mouse trophoblast stem cells (TSCs) can differentiate into trophoblast cells, which constitute the placenta. Under conventional culture conditions, in a medium supplemented with 20% fetal bovine serum (FBS), fibroblast growth factor 4 (FGF4), and heparin and in the presence of mouse embryonic fibroblast cells (MEFs) as feeder cells, TSCs maintain their undifferentiated, proliferative status. MEFs can be replaced by a 70% MEF-conditioned medium (MEF-CM) or by TGF-ß/activin A. To find out if KnockOut™ Serum Replacement (KSR) can replace FBS for TSC maintenance, we cultured mouse TSCs in KSR-based, FBS-free medium and investigated their proliferation capacity, stemness, and differentiation potential. The results indicated that fibronectin, vitronectin, or laminin coating was necessary for adhesion of TSCs under KSR-based conditions but not for their survival or proliferation. While the presence of FGF4, heparin, and activin A was not sufficient to support the proliferation of TSCs, the addition of a pan-retinoic acid receptor inverse agonist and a ROCK-inhibitor yielded a proliferation rate comparable to that obtained under the conventional FBS-based conditions. TSCs cultured under the KSR-based conditions had a gene expression and DNA methylation profile characteristic of TSCs and exhibited a differentiation potential. Moreover, under KSR-based conditions, we could obtain a suspension culture of TSCs using extracellular matrix (ECM) coating-free dishes. Thus, we have established here, KSR-based culture conditions for the maintenance of TSCs, which should be useful for future studies.

Key words: Extracellular matrix, KnockOut™ Serum Replacement, Suspension culture, Trophoblast stem cell

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At the blastocyst stage, cells of mouse embryos divide into two distinctive populations: the inner cell mass and the trophectoderm (TE). Mouse trophoblast stem cells (TSCs) can be established from the TE and have the ability to differentiate into trophoblast cells, which constitute the placenta [1]. In conventional culture conditions, TSCs maintain their self-renewal capability and stemness in a medium supplemented with 20% fetal bovine serum (FBS), fibroblast growth factor 4 (FGF4), and heparin, when co-cultured with mouse embryonic fibroblast cells (MEFs) as feeder cells. MEFs can be replaced by a 70% MEF-conditioned medium (MEF-CM) [1, 2] or by TGF-β/activin A [3]. Removal of FGF4 or MEF/TGF-β/activin A induces TSC differentiation into all types of trophoblast cells, such as trophoblast giant cells (TGCs), spongiotrophoblast cells (SpTs), syncytiotrophoblast cells (SynTs), and glycogen trophoblast cells (GlyTs). Given their differentiation potential, TSCs have been used as an *in vitro* model to study the functions of genes in the development of the trophoblast lineage.

KnockOut™ Serum Replacement (KSR) is a serum-free formulation designed to replace FBS for culturing embryonic stem cells (ESCs) and has also been adopted for the culture of induced pluripotent stem cells (iPSCs) [4–6]. KSR is used not only in the monolayer/2D culture but also in the suspension/3D culture of pluripotent stem cells [7, 8]. Compared to FBS, KSR consists of more defined components, with less lot-to-lot variation, and shows advantages for stem cell culture. For example, KSR facilitates the generation of ESC lines in 2D culture conditions [9], prevents excess cell aggregation, and enhances the growth of iPSCs in 3D culture [10]. However, it is not yet known if KSR could also be adopted for TSC culture. To address this issue, in this study, we cultured mouse TSCs in a KSR-based, FBS-free medium and investigated their proliferation capacity, stemness, and differentiation potential. The results indicated stable maintenance of TSCs in the KSR-based medium, with a growth rate comparable to that in the conventional FBS-based conditions. Moreover, the KSR-based conditions enabled us to obtain a suspension culture of TSCs without the need for special dishes, such as those with ultra-low cell attachment surfaces, and should thus make 3D organoid studies of mouse TSCs easier.

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Correspondence: S Tanaka (e-mail: asatoshi@mail.ecc.u-tokyo.ac.jp)

Materials and Methods

Reagents

All reagents were purchased from Wako (Osaka, Japan), unless otherwise stated. All PCR primers were purchased from either Sigma-Aldrich (Tokyo, Japan) or Eurofins Genomics (Tokyo, Japan).

Cell lines

EGFP-$TS_{3.5}$ [1], GFPTS#1 [11], and B6TS2 [12] TSC lines were used in this study.

2D cell culture

In the conventional FBS-based conditions, TSCs were maintained in an undifferentiated state in TS medium supplemented with 70% MEF-CM, 25 ng/ml FGF4, and 1 μg/ml heparin (70CM+FH; Sigma-Aldrich) [2]. In the KSR-based conditions, 20% KSR (Gibco, Waltham, USA) was used instead of 20% FBS, and the antibiotics (penicillin and streptomycin) were removed (KSR-TS medium). To maintain stemness in the KSR-based conditions, 25 ng/ml FGF4, 1 μg/ml heparin, and 10 ng/ml activin A (R&D Systems, Minneapolis, USA) were added (KSR+FHA condition). We have also used 1 μM BMS493 (KSR+FHAB; Tocris Bioscience, Ellisville, USA) and 10 μM Y-27632 (KSR+FHABY). Dishes were coated with extracellular matrix (ECM) for KSR-based 2D culture. To induce differentiation, TSCs were cultured at a density of 2.0×10^5 cells/35-mm dish in a plain TS medium or KSR-TS medium.

ECM coating

Fibronectin, vitronectin, laminin, collagen I, and collagen IV (Nitta Gelatin, Osaka, Japan) were diluted with PBS (–) to the working concentration. The ECM solution (1 ml/35-mm dish) was added to a culture dish and preserved at 4°C for more than 15 h. Then, the solution was removed, and the coated dishes were washed twice with PBS (–), air-dried, and stored at 4°C until use.

3D cell culture

TSCs were inoculated on a 35-mm non-coated Petri dish (Falcon, New York, USA) in KSR+FHABY at 2.0×10^5 cells per dish. Differentiation was induced by moving cell aggregates into the plain KSR-TS medium. For single-cell culture, cells were dissociated into single cells using trypsin. The cells were then diluted to approximately 30 cells/mL and seeded in a 60-well MicroWell™ (Sigma Aldrich) at 15 μl per well.

Statistical analyses

A two-tailed Student's *t*-test was performed for a comparison of gene expression levels between two samples (Figs. 3A–B, 4C, 5B, Supplementary Figs. 3A, 5A, and 5C: online only). Difference in the DNA methylation status (Fig. 2C) was detected using nonparametric two-tailed Mann-Whitney *U* test. Tukey-Kramer test was performed for a comparison among three or more samples (Supplementary Figs. 1A and 4: online only).

The methods for all biochemical experiments are described in detail in the "Supplementary Materials and Methods" section (online only).

Results

TSC survived in KSR-based medium

First, we replaced the FBS and MEF-CM of the conventional feeder-free TSC culture conditions (70CM+FH) [2] with KSR and 10 ng/ml activin A, respectively, and cultured TSCs (EGFP-$TS_{3.5}$ line) in this condition (KSR+FHA) in standard tissue culture dishes (Fig. 1A). Unless otherwise noted, we used the same cell line throughout the current study. While most of the cells adhered in 70CM+FH during 24 h of culture, most viable cells, as observed through Trypan blue staining, were found to be unattached in KSR+FHA (Fig. 1B). The unattached, floating cells in KSR+FHA were efficiently attached to culture dishes and grew when placed back into 70CM+FH (Fig. 1C). These results suggested that most TSCs survived in KSR+FHA for at least 24 h, but could not efficiently attach to culture dishes.

Further, we examined various ECMs to support the attachment of TSCs in KSR+FHA. Dishes coated with 2.5–5 μg/cm^2 fibronectin or vitronectin showed potent adhesive effect even in KSR+FHA, comparable to that in 70CM+FH (Supplementary Fig. 1A), confirming that fibronectin is as effective in KSR-based conditions as in the chemically defined condition [13]. Laminin, on the contrary, was less effective compared to fibronectin/vitronectin. Collagen I, collagen IV, and gelatin were even more ineffective (Supplementary Fig. 1A). Given these results, we chose 2.5 μg/cm^2 fibronectin coating for the experiments discussed further in this article.

In KSR+FHA, though most of the TSCs adhered efficiently on fibronectin-coated dishes, they ceased to proliferate after the second passage (Fig. 1D, Supplementary Fig. 1B). We searched for reagents that could retain the proliferative capacity of TSCs in KSR-based conditions and found that the Rho-associated protein kinase inhibitor, Y-27632 [14], was a potent cell-proliferation activator. In the presence of 10 μM Y-27632 along with FGF4, heparin, and activin A (KSR+FHAY), TSCs grew stably but slightly slower than in 70CM+FH (Fig. 1D). Another reagent, BMS493, a pan-RAR inverse agonist [15], stimulated the growth of TSCs [16], to a lesser extent compared to Y-27632, in KSR+FHA in a dose-dependent manner (Supplementary Fig. 1B). Thus, the addition of 10 μM Y-27632 and 1 μM BMS493 to KSR+FHA (KSR+FHABY) resulted in a cell proliferation rate comparable to that obtained using 70CM+FH (Fig. 1D, Supplementary Fig. 1C). The morphology of TSCs in KSR+FHABY was indistinguishable from that of TSCs in 70CM+FH (Fig. 1E).

Y-27632 suppresses the apoptosis of mouse TSCs, in chemically defined conditions [13], and dissociated human ESCs [17]. To assess if this was also the case in our study, we identified apoptotic cells by detecting activated caspases in the TSCs cultured without (KSR+FHAB) or with Y-27632 (KSR+FHABY). While only a minimal number of apoptotic cells were found in the early (day 2 after the passage) culture, apoptotic cells were identified on day 4 in both conditions. There were more apoptotic cells in KSR+FHABY than in KSR+FHAB (Supplementary Fig. 2: online only). This result indicated that apoptosis occurred when the colonies of TSCs expanded. It also revealed that apoptosis does not account for the growth retardation of mouse TSCs in KSR-based conditions without Y-27632.

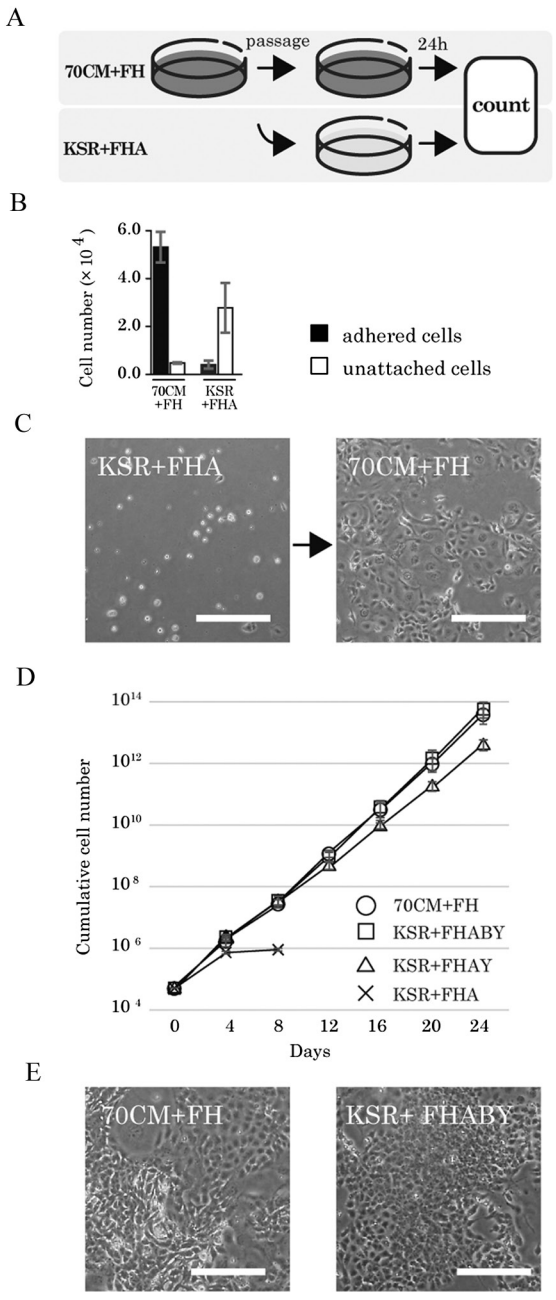

Fig. 1. TSC culture in KSR-based medium. (A) Schematic representation of experimental protocol. (B) Number of adhered and unattached viable cells, as observed by Trypan blue staining, 24 h after inoculation (5×10^4 cells/35-mm dish) in the 70CM+FH or KSR+FHA condition. Mean ± SD is shown (n = 3). (C) Unattached TSCs in KSR+FHA 24 h after the inoculation (left) were collected, re-seeded, and cultured for further 4 days in 70CM+FH (right). Scale bars = 200 µm. (D) Growth in different conditions in fibronectin-coated dishes. Cells were counted and passaged every 4 days (n = 3 for each time point). (E) Appearance of TSCs in 70CM+FH (left) and KSR+FHABY (right) 4 days after the passage. Scale bars = 200 µm.

TSCs in KSR+FHABY maintained a unique transcriptome and methylation status

To characterize TSCs cultured in KSR+FHABY, we performed RNA-seq and compared the transcriptome of these TSCs with that of TSCs cultured in 70CM+FH. Clustering analysis based on Pearson correlation coefficients of RNA-seq data did not separate cells depending on the culture conditions, demonstrating high similarity among the analyzed TSC samples (correlation coefficients > 0.96) (Fig. 2A and Supplementary Table 1: online only). There were 270 and 325 upregulated (fold change ≥ 2) and downregulated (fold change ≤ 0.5) genes, respectively, in KSR+FHABY compared to 70CM+FH (Fig. 2B and Supplementary Tables 2 and 3: online only). Gene ontology (GO) term enrichment analysis using these differentially expressed genes did not identify any enriched GO term, implying that no particular biological process was significantly affected by KSR+FHABY. In addition, TSC marker genes, such as *Cdx2*, *Eomes*, and *Elf5* [18–21], did not show a significant difference between KSR+FHABY and 70CM+FH (log2(fold change) ≥ 1, $P < 0.01$) in the RNA-seq assay (Fig. 2B).

We had previously identified some genomic regions that were differentially methylated between trophoblast and embryonic cell lineages and named them T-E T-DMRs [22]. We then analyzed the DNA methylation status of these regions in TSCs in KSR+FHABY by bisulfite sequencing and compared them to that of these regions in TSCs in 70CM+FH. As shown in Fig. 2C, TSCs in KSR+FHABY and 70CM+FH showed a similar CpG methylation profile of T-E T-DMRs, with only a modest difference, indicating that the DNA methylation status of T-E T-DMRs was not significantly affected in KSR+FHABY.

These results suggest that TSCs maintained their unique gene expression and DNA methylation profile in KSR+FHABY.

TSCs in KSR+FHABY retained stemness and differentiation potential

We further analyzed the expression of TSC marker genes by qPCR. Although the expression levels of none of the marker genes examined disappeared in KSR+FHABY, suggesting maintenance of stemness, those of some showed statistically significant differences from those in 70CM+FH in this assay; *Cdx2*, *Fgfr2*, and *Eomes* showed lower expression and *Elf5* showed higher expression in KSR+FHABY than in 70CM+FH (Fig. 3A).

In 70CM+FH, the removal of FGF4, heparin, and CM induces the differentiation of TSCs [1]. To verify the differentiation potential of TSCs in KSR+FHABY, TSCs maintained in KSR+FHABY for nine passages were seeded in KSR-based basal medium without FGF4, heparin, activin A, BMS493, and Y27632 (KSR-TS medium). The expression of marker genes for differentiated trophoblast subtypes [23, 24] was examined by qPCR on days 2, 4, 6, and 8 of differentiation (Fig. 3B). Compared to TSCs in FBS-based condition (‘TS medium’ in Fig. 3B), cells in the KSR-TS medium exhibited increased levels of markers for TGCs (*Prl3d1* and *Prl3b1*) and SpTs (*Tpbpa* and *Ascl2*); levels of *Prl3b1* in both conditions were almost equal at day 8. In contrast, markers for SynTs (*Gcm1, Syna,* and *Synb*) generally showed lower expression in KSR-based conditions, with several exceptional cases (Fig. 3B). Overall, TSCs appeared to retain differentiation potential in KSR+FHABY; this was confirmed

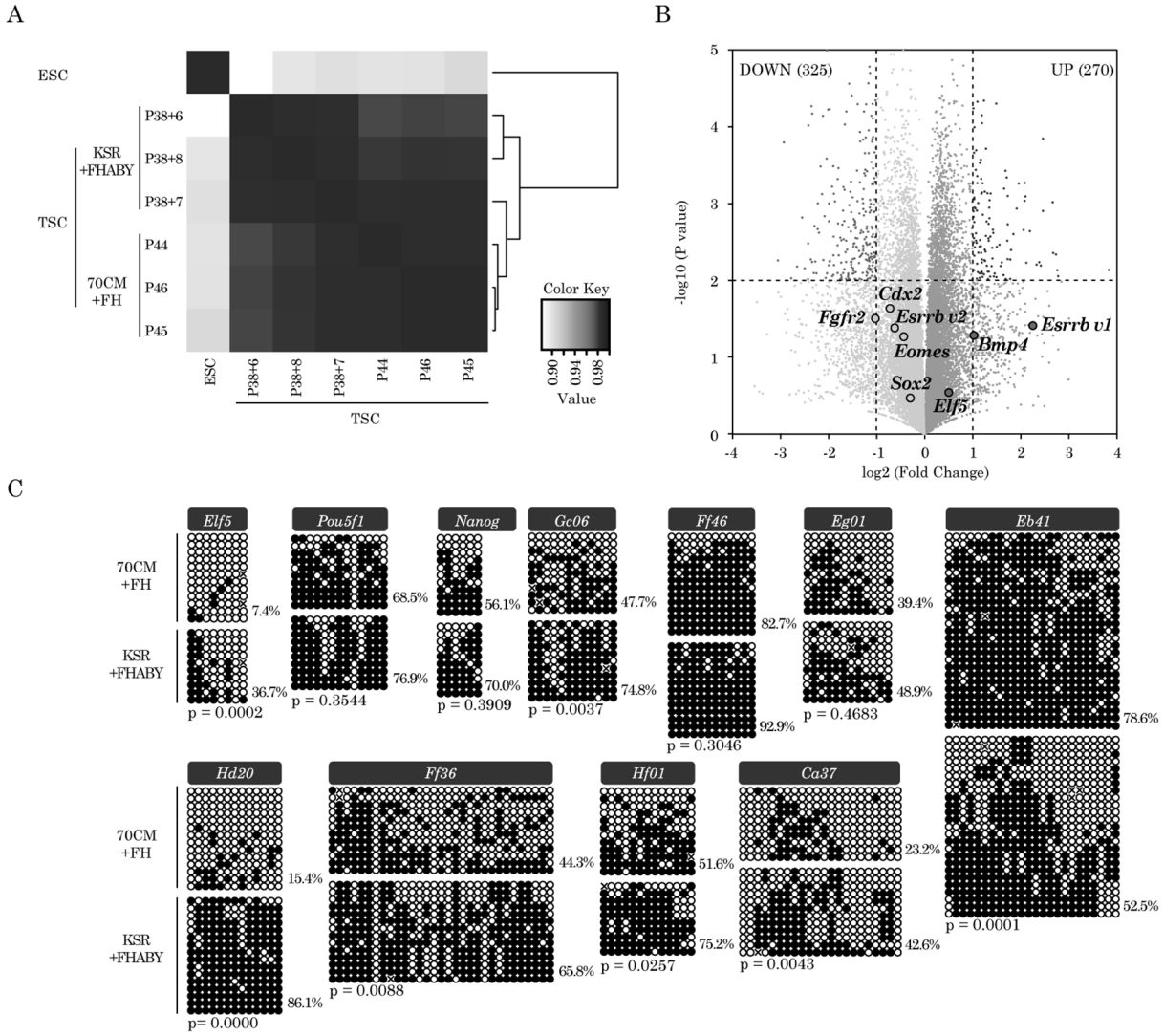

Fig. 2. Transcriptome and methylation status of TSCs in KSR+FHABY. (A) Similarity between TSCs in KSR+FHABY and 70CM+FH. A heatmap was drawn using the values of Pearson correlation coefficients obtained using the RNA-seq datasets. TSCs maintained in 70CM+FH were divided into two aliquots at passage 38 (P38). One of them was kept in the same conditions to up to P46, and total RNA was prepared at the indicated three sequential passages. The other was switched to KSR+FHABY and maintained for more 6–8 passages (P38+6, P38+7, P38+8) to match the total passage numbers with 70CM+FH samples. RNA-seq data of ESCs (J1) (GEO Accession: GSM2064389) was used as a comparison. (B) Volcano plot shows differential gene expression between TSCs in KSR+FHABY and 70CM+FH. There were 270 and 325 upregulated and downregulated (fold change ≥ 2 or ≤ 0.5, $P < 0.01$) genes, respectively. The average RNA-seq value from samples of three sequential passages for each condition were used. TSC marker genes are highlighted. (C) DNA methylation status of the T-E T-DMRs (Nakanishi *et al*., 2012). Open and filled circles represent unmethylated and methylated cytosines, respectively. The overall methylation percentage (the number of methylated CpGs per number of total CpGs) is shown at the right side of each part. The *p* values were calculated using the nonparametric two-tailed Mann-Whitney test.

using another TSC line (GFPTS#1) (Supplementary Fig. 3A). The differentiation potential of TSCs, maintained in KSR+FHABY for 13 passages, was also demonstrated by the contribution of the derivatives of GFP-positive GFPTS#1 TSCs in all layers of E10.5 chimeric placentas (Supplementary Figure 3B).

In FBS-based conditions, the ECM composition has been reported to change the differentiation fate of TSCs through regulation of HIF stabilization [25]. To find out if defined ECM in the KSR-based conditions affects the differentiation fate of TSCs, we employed four types of ECMs. First, TSCs were cultured in dishes coated with 2.5 μg/cm^2 fibronectin, 1 μg/cm^2 laminin, 1.5 μg/cm^2 collagen I, or 5 μg/cm^2 collagen IV in KSR+FHABY for three passages before the induction of differentiation. The adhesion induced by collagens was low (Supplementary Fig. 1A); hence, we used a mixture of 1 μg/cm^2 fibronectin with collagen I and collagen IV. Then, TSCs were induced to differentiate on each type of ECM for up to 8 days, and the expression dynamics of marker genes for differentiated trophoblasts were analyzed by qPCR. The results showed no significant difference

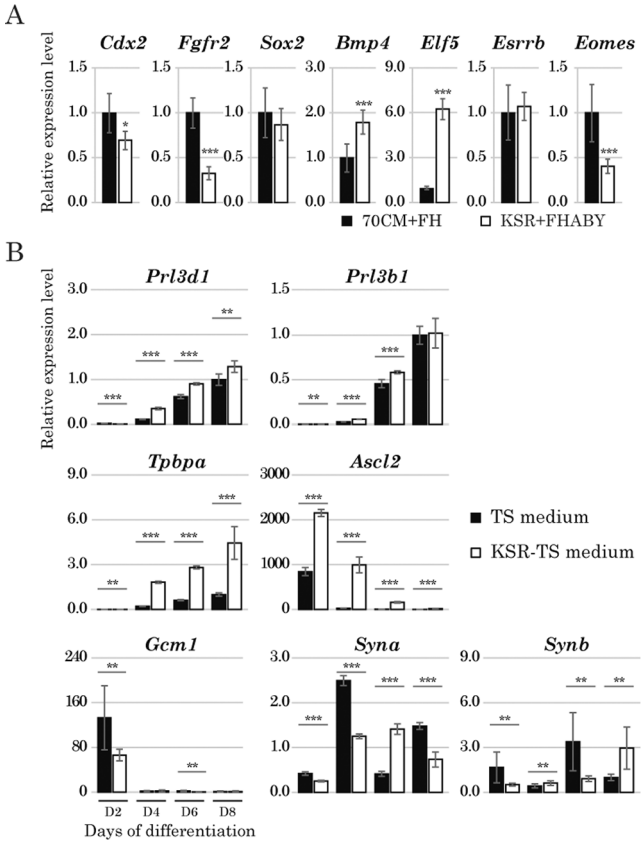

Fig. 3. Expression analysis of marker genes. (A) Expression of marker genes in undifferentiated TSCs. The same set of TSC RNA samples used for RNA-seq in Fig. 2A was subjected to qPCR. Mean ± SD of three samples for each condition is shown as a relative value (70CM+FH = 1). (B) Expression dynamics of marker genes for TGCs (*Prl3d1* and *Prl3b1*), SpTs (*Tpbpa* and *Ascl2*), and SynTs (*Gcm1*, *Syna*, and *Synb*). TSCs were induced to differentiate and collected for RNA preparation on days 2, 4, 6, and 8 (D2-D8) of differentiation. Mean ± SD (technical triplicates each for biological duplicate) was normalized to the expression of *Actb* and is shown as a relative value (D8 of "TS medium" = 1). The statistically significant differences are represented as follows: * $P < 0.05$; ** $P < 0.01$; and ***, $P < 0.001$ (Student's *t*-test).

on day 8 among different ECMs, indicating that the types of ECM examined did not affect the differentiation of TSCs in KSR+FHABY (Supplementary Fig. 4).

TSCs in KSR+FHABY could be maintained in a suspension culture

Because most TSCs survived without adhesion for at least 24 h and could be maintained for multiple passages as adhered culture in KSR+FHABY, we tested whether TSCs can be maintained in ECM coating-free Petri dishes as a suspension culture. In this suspension condition, TSCs formed cell aggregates, which were trypsinized into single cells and passaged every 3–4 days (Fig. 4A); the cells grew stably for at least four passages, with a comparable proliferation rate to that in adhered cultures in 70CM+FH and KSR+FHABY (Fig. 4B). When we extended the culture period to up to 8 days, the aggregates increased in diameter and cell number (Fig. 4D). The expression analysis of TSC marker genes on day 3 of incubation, after five and seven passages in the suspension culture, revealed no significant difference in the expression levels of marker genes between the suspension and the adhered cultures in KSR+FHABY, except for a higher expression of *Eomes* in the suspension culture (Fig. 4C). In the day 8 aggregates, although the levels tended to be lower compared to those in the day 3 aggregates, the expression of these TSC markers was retained (Supplementary Fig. 5A). Moreover, using immunostaining, we detected Cdx2 protein expression inside the aggregates on days 3 and 8, with relatively low-Cdx2 expressing cells in the cores of day 8 aggregates (Fig. 4D). These results indicate that the ECM coating for cell attachment is dispensable for the growth of TSCs in KSR+FHABY.

In an earlier study, Motomura *et al*. showed that dissociated TSCs move actively and aggregate on the culture dish surface, forming small cell masses that eventually give rise to TSC colonies when cultured on feeder cells in the conventional FBS-based adhered culture. The authors also mentioned that TSCs never formed colonies from single cells in low-density culture [26]. To find out if a single TSC can divide and form a clump of cells in the KSR+FHABY suspension culture, we separately seeded dissociated TSCs into 60-well flat-bottomed plates and continuously observed them for 4 days. As a result, 8.2% of single TSCs formed compact aggregates on day 4 (type A), 6.6% attached even to the non-coated surface (type B), 8.2% formed loosely assembled cluster(s) of relatively large cells (type C), and the rest (77.0%) degraded during the culture period (Fig. 4E and Supplementary Table 4: online only). Type A aggregates could be further expanded and maintained over two passages in suspension culture (data not shown).

TSCs in the KSR+FHABY suspension culture retained differentiation potential

In the past decade, suspension cell culture systems have gained popularity in stem cell research. In such studies, cell spheroids were typically grown to up to 200–500 μm in diameter before being induced to differentiate [7, 8, 27]. Additionally, it was shown that the necrotic core frequently appears in tumor cell spheroids with diameters over 500 μm [28]. Therefore, to find out if TSCs retain their differentiation potential in the suspension culture and if TSC aggregates in the suspension culture differentiate differently from those in the conventional adhered culture conditions, we grew TSC aggregates to a diameter of up to 500 μm and put them in the differentiation condition.

It took approximately 8 days for almost all aggregates to grow to ~500 μm in diameter in our proposed conditions. After 8 days of KSR+FHABY suspension culture, TSC aggregates were transferred to Petri dishes containing KSR-TS medium, that is, without FHABY, to induce differentiation (Fig. 5A). Around day 4 of differentiation, TSC aggregates attached to the surface, and some cells with mesenchymal appearance migrated out of the aggregates, even on the non-coated Petri dishes (Supplementary Fig. 5B). In contrast, the main body of aggregates remained as a three-dimensional cell clump (Supplementary Fig. 5B). The expression analysis of marker genes for differentiated trophoblasts by qPCR showed upregulation of all marker genes examined during differentiation (Fig. 5B and

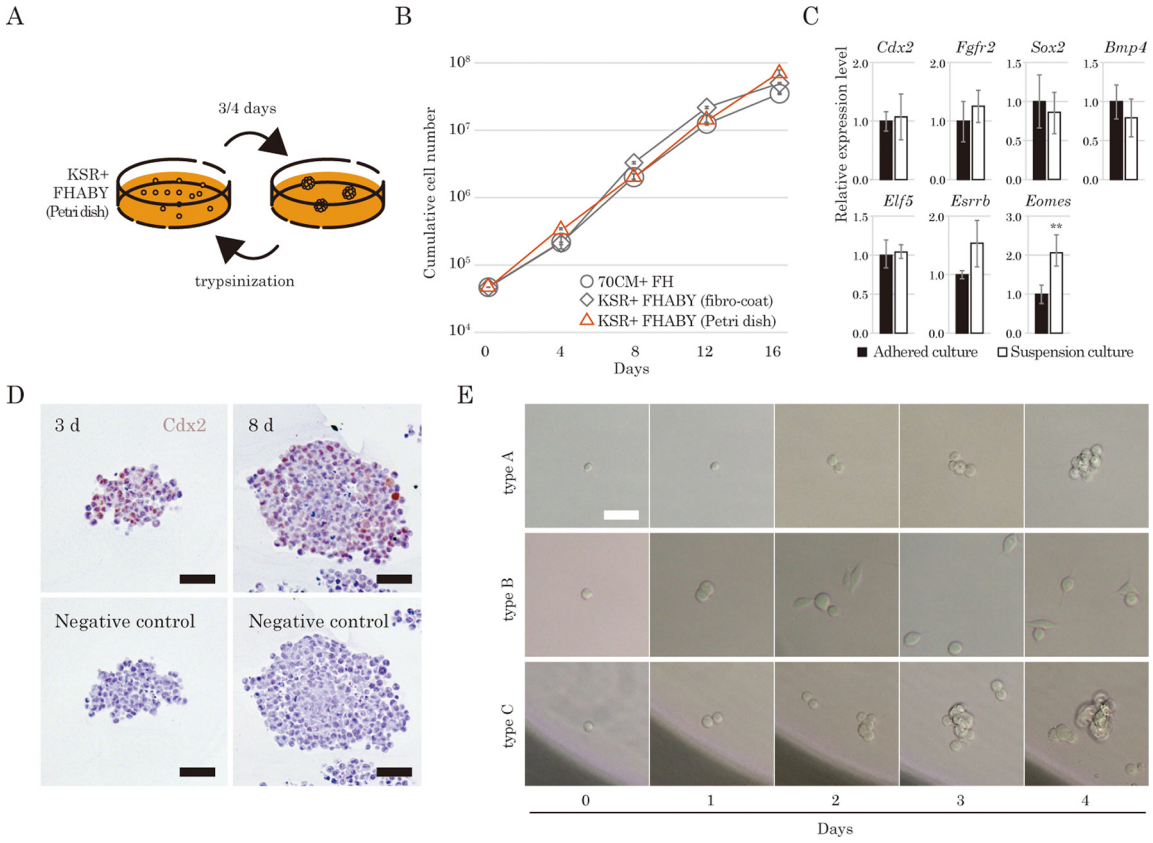

Fig. 4. TSCs in suspension culture. (A) Schematic representation of the TSC suspension culture. (B) Comparison of TSC growth in different culture conditions. Aliquots (5×10^4 cells/35-mm dish) of TSCs maintained in 70CM+FH were placed in the indicated three different conditions on day 0, then passaged every 3 or 4 days at 5×10^4 cells/35-mm dish. KSR+FHABY (fibro-coat), KSR+FHABY on the fibronectin-coated dish. Mean ± SD is shown (n = 3). (C) Expression analysis of TSC marker genes by qPCR. Adhered culture, TSCs maintained in KSR+FHABY in the fibronectin-coated dish for 7 and 8 passages, after 38 passages in 70CM+FH (3 days after the last passage); Suspension culture, TSCs maintained in KSR+FHABY in non-coated Petri dish for 5 and 7 passages after 22 passages in 70CM+FH (3 days after the last passage). Mean ± SD (technical triplicate of two samples per culture condition) was normalized to the expression of *Actb* and is shown as a relative value (Adhered culture = 1). ** $P < 0.01$ (Student's t-test). (D) Cdx2 immunostaining on days 3 (3 d) and 8 (8 d) TSC aggregates in the suspension culture. Scale bars = 100 µm. (E) TSCs in the single-cell suspension culture. Scale bar = 50 µm.

Supplementary Fig. 6: online only).

As for the expression of TGC markers (*Prl3d1*, *Prl3b1*, *Prl2c2*, and *Ctsq*), there was no significant increase on day 0 of differentiation (D0) even after 8 days of suspension culture, except for *Ctsq*, which showed a slight increase in expression, with a statistical significance, in the aggregates compared with that in the adhered undifferentiated TSCs (Fig. 5B). After the induction of differentiation, the expression of *Prl3d1* increased earlier in the suspension culture than in the adhered culture, but it decreased by day 8 of differentiation (D8), becoming lower than that in the adhered culture. On the contrary, the expression of *Prl3b1*, *Prl2c2*, and *Ctsq* was higher in the suspension condition throughout the culture period, with a drastic increase on day 6 of differentiation (D6). An SpT marker, *Tpbpa*, also showed expression dynamics similar to those of *Ctsq*. There was no difference between the suspension and adhered cultures at the level of *Ascl2* expression. Meanwhile, the GlyT marker *Pcdh12* [29] was downregulated on D6 and D8. The expression of SynT markers (*Gcm1*, *Syna*, and *Synb*) fluctuated to some extent. *Gcm1* was higher in the suspension on D0 and D2. Although there was a statistically significant difference between the suspension and adhered cultures, only residual *Gcm1* expression was detected on D8. *Synb* and *Syna* were higher in the suspension than in the adhered cultures on D2, D6, and D8 and D2 and D4, respectively. Differentiation analysis using another TSC line (B6TS2) also revealed a higher expression of most marker genes in the suspension culture than in the adhered culture (Supplementary Fig. 5C).

These results indicated that the TSC aggregates formed in the 8 days of KSR+FHABY suspension culture retained their differentiation potential.

Discussion

In this study, we attempted to culture TSCs in serum-free conditions using KSR. We found that the addition of BMS493 and Y-27632 to KSR-based conditions, along with exogenous factors essential for the maintenance of TSC stemness, supports the proliferation of

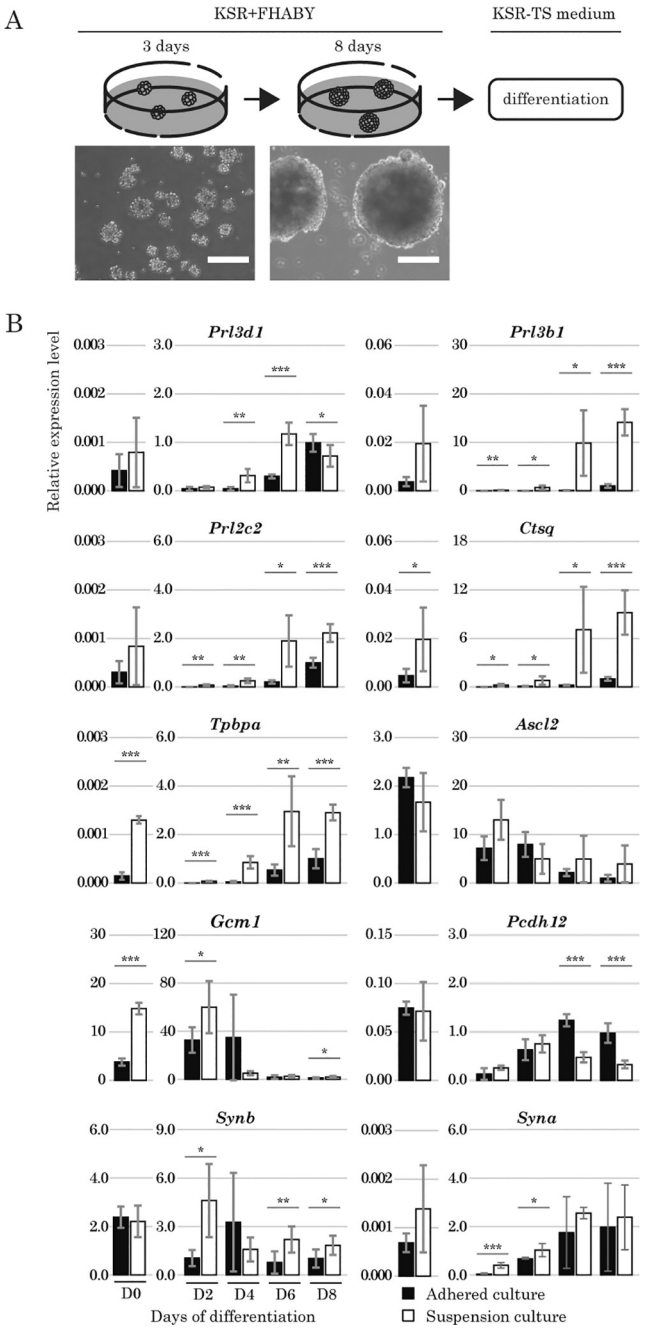

Fig. 5. Differentiation of TSCs in the suspension culture. (A) Schematic representation of the differentiation-in-suspension experiment. TSC aggregates on days 3 (3 days) and 8 (8 days) are shown. Scale bars = 200 μm. (B). Expression dynamics of marker genes for TGCs (*Prl3d1*, *Prl3b1*, *Prl2c2*, and *Ctsq*), SpTs (*Tpbpa* and *Ascl2*), GlyTs (*Pcdh12*), and SynTs (*Gcm1*, *Syna*, and *Synb*) in the suspension (white bars) and the adherent (black bars) conditions. TSCs were induced to differentiate and collected for RNA preparation on days 0, 2, 4, 6, and 8 (D0-D8) of differentiation. Mean ± SD (technical triplicates each for biological duplicate) was normalized to the expression of *Actb*, and then shown as a relative value (D8 of "Adhered culture" = 1). Note that the *y*-axis is magnified for D0 because of the very weak expression of the marker genes. Statistically significant differences are represented as follows: * $P < 0.05$; ** $P < 0.01$; and *** $P < 0.001$ (Student's *t*-test).

TSCs on fibronectin-coated dishes at a rate comparable to that in the conventional FBS-based conditions. In addition, we maintained TSCs in suspension in non-coated Petri dishes. In KSR-based 2D and 3D conditions, TSCs retained their differentiation potential. In the suspension culture condition, TSCs formed cell aggregates, which showed an enhancement of differentiation marker gene expression upon induction of differentiation compared with that in the 2D monolayer culture.

In our KSR-based conditions, Y-27632 significantly accelerated the growth of TSCs. Y-27632 inhibits ROCK kinases by competing with ATP for binding to kinases [30]. It suppresses the dissociation-induced apoptosis of human ESCs caused by RhoA/ROCK overactivation [31]. The anti-apoptotic effect of Y-27632 has also been observed in some other cell culture systems [32–34], including mouse TSCs under the chemically defined conditions [13]. In contrast, Y-27632 has also been reported to induce apoptosis of the attached growing human ESCs [35]. However, in the present study, we barely detected apoptotic TSCs 2 days after passage, regardless of Y-27632 (Supplementary Fig. 2), indicating that Y-27632 neither suppressed nor induced the apoptosis of TSCs in the KSR-based conditions. The presence of a substantial number of viable unattached TSCs in KSR+FHA without fibronectin-coating 24 h after inoculation (Fig. 1B and C) also implies that the anti-apoptotic role of Y-27632 is unlikely. Apoptotic TSCs appeared 4 days after the passage, and Y-27632 increased their number (Supplementary Fig. 2). We suppose that this result likely reflects an increase in cell density through the enhancement of cell growth rather than a direct induction of apoptosis by Y-27632, because a comparable number of apoptotic cells was also detected 4 days after the passage in the conventional FBS-based conditions (data not shown). Y-27632 can also promote cell proliferation and cell cycle progression, for example, by activating the ERK signaling pathway [36]. Based on these observations and reports, we hypothesize that a cell cycle delay occurred in TSCs under the KSR+FHAB conditions and that Y-27632 may have relieved it. Although Y-27632 appeared to be essential for the attachment of human TSCs [37], it was not the case in this study. It should be noted that Y-27632 also accelerated the growth of macaque TSCs in FBS-based conditions [38]. Thus, it may be worthwhile to study the underlying mechanisms of the effect of Y-27632 in TSCs of different species for further understanding TSC maintenance and proliferation.

Kiyozumi *et al*. recently reported that laminin is the *in vivo* ECM niche for mouse TSCs [39]. The authors demonstrated that TSC proliferation was significantly suppressed in FBS-based conditions when TSCs were seeded into the TC dishes coated with a recombinant mutant laminin protein, which is inactive in integrin binding. They also showed that the size of CDX2-positive TSC populations in genetically modified mouse embryos with the integrin-binding–inert mutant allele of the laminin gene was significantly smaller than that of such populations in control embryos. However, laminin-integrin signaling could not replace FGF4-signaling for the maintenance of TSCs *in vitro*, indicating that laminin/integrin-mediated cell adhesion is a prerequisite for the propagation of TSCs [39]. Our results from this study do not contradict this report. Although it appeared less effective than fibronectin, laminin also facilitated cell attachment in the KSR-based conditions (Supplementary Fig. 1A). TSCs express fibronectin-binding integrins [39]. Our results thus indicated that

fibronectin could substitute laminin for cell attachment, at least *in vitro*. However, the successful maintenance of TSCs for multiple passages in suspension culture made the relevance of ECM/integrin-signaling for the expansion of TSCs obscure. One possibility may be that TSCs secrete ECM to secure prerequisite ECM/integrin signaling.

While the global gene expression profile was not drastically disturbed (Fig. 2A), and TSCs retained differentiation potential (Fig. 3B), there was a statistically significant difference in the expression levels of stem marker genes between TSCs in KSR+FHABY and 70CM+FH. Of these, *Elf5* was the most upregulated gene in KSR+FHABY (Fig. 3A). *Elf5* is critical for maintaining the stem cell state of TSCs [40]. It has also been reported that overexpression of *Elf5* induces trophoblast differentiation [41]. However, under KSR+FHABY condition, even with the elevated *Elf5* expression, the global gene expression profile was not drastically disturbed (Fig. 2A). The TSCs retained their proliferation and differentiation potential (Fig. 1D and 3B). Given that the TSCs are a heterogeneous cell population with various levels of *Elf5* expression [26], we assume that the ratio of the "*Elf5*-high" populations was increased under the KSR+FHABY conditions, rather than that the *Elf5* expression was augmented in each cell. Thus, the augmented expression of *Elf5* in KSR+FHABY might indicate that it is better for maintaining the undifferentiated state of TSCs than conventional FBS-based conditions.

Upon induction of TSC differentiation by switching conditions from KSR+FHABY to KSR-TS, marker gene expression increased, with similar expression dynamics to those in FBS-based conditions (Fig. 3B). Choi *et al.* have reported a significantly stimulated SynT differentiation at the expense of TGC differentiation in the serum-based conditions on the xeno-free defined ECM substrate CELLstart. Differentiating TSCs failed to stabilize HIF proteins on CELLstart under normoxic (21% O_2) conditions, which was accounted for the impaired TGC differentiation [25]. From proteomic analysis, fibronectin was found to be the most abundant ECM component of CELLstart [42], implying that TGC differentiation may require ECM components other than fibronectin. Therefore, we hypothesized that TGC differentiation could also be hindered in KSR+FHABY on fibronectin. However, expression levels of TGC markers were even higher in KSR-based conditions than FBS-based conditions, albeit only slightly. The expression of SpT marker genes was also higher in KSR-based conditions on fibronectin than in FBS-based conditions, while that of SynT marker genes was not enhanced. These results indicated that fibronectin had no inhibitory effect on TGC differentiation under our KSR-based conditions. Our results also indicated that TGC differentiation does not require any other exogenous ECM or that differentiating TSCs may produce such ECM components by themselves. Further studies are required to investigate how ECM, integrin, and HIF mRNA/protein expression are regulated in our KSR-based conditions.

When cultured as a suspension in KSR+FHABY, TSCs formed cell aggregates on Petri or non-coated TC dishes. While the expression level of *Cdx2* mRNA was retained in 8 d aggregates (Supplementary Fig. 5A), staining for Cdx2 protein appeared weaker in the core of aggregates (Fig. 4D). Other stem marker expression levels were generally lower in 8 d aggregates than in 3 d aggregates, with (*Elf5*, *Essrb*, and *Eomes*) or without (*Fgfr2*, *Sox2*, and *Bmp4*) statistically significant differences, suggesting that the cells inside 8 d aggregates tended to lose stemness. The availability of stemness-maintaining factors, such as FGF4, activin A, and oxygen, may be responsible. However, the expression of differentiation markers was not dramatically induced even in 8 d aggregates (Fig. 5B), although some of the marker genes showed a statistically significant increase, indicating that most of the cells remained in an undifferentiated state. 3D spheroid or organoid culture systems can mimic organ development *in vivo* [43]. It has also been effectively utilized for human trophoblast research [44, 45]. As for the mouse TSCs, Rai and Cross have adopted a 3D culture system using ultra-low attachment dishes and hanging-drop culture systems in FBS-based conditions [46]. Our KSR-based 3D culture conditions can be achieved more easily, providing another option for trophoblast research.

Albeit low frequency (8.2%), a single TSC could divide and form a cell clump, which could be passaged (type A in Fig. 4E). In preliminary observations, TSCs of types A, B, and C could re-form cell clusters after returning to a single cell state by trypsinization. This observation suggests that these three types of TSCs are still in an undifferentiated proliferative state. Meanwhile, TSCs of type A clusters gave rise to types B and C, whereas TSCs from types B and C did not form type A clusters. From these preliminary results, we assume that the type A TSCs are in a more "undifferentiated state" than types B and C. Further careful experiments are required to determine whether this assumption is correct. It would be interesting to investigate how these cells are different from other types of TSCs that functioned differently in single-cell culture experiments, to further understand the heterogeneity of TSCs suggested by Motomura *et al.* (2016). This single-cell culture system is also practically useful for obtaining a genuine clone of genetically modified TSCs, which has never been strictly obtained. We have already obtained several gene knock-out TSC lines by utilizing this single cloning system (data not shown).

In conclusion, we demonstrated here, that mouse TSCs could be maintained in KSR-based conditions while maintaining their differentiation potential. Our KSR-based conditions were also applicable to 3D culture systems, which may provide ways to classify the status of the stemness of TSCs and to analyze mechanisms underlying cell fate decisions regulated by extracellular stimuli.

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