Up-regulation of lymphocyte antigen 6 complex expression in side-population cells derived from a human trophoblast cell line HTR-8/SVneo

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Running title characterization of HTR-8/SVneo SP cells

Abstract

The continual proliferation and differentiation of trophoblasts are critical for the maintenance of pregnancy. It is well known that the tissue stem cells are associated with the development of tissues and pathologies. It has been demonstrated that side-population (SP) cells identified by fluorescence-activated cell sorting (FACS) are enriched with stem cells. The SP cells in HTR-8/SVneo cells derived from human primary trophoblast cells were isolated by FACS. HTR-8/SVneo-SP cell cultures generated both SP and non-SP (NSP) subpopulations. In contrast, NSP cell cultures produced NSP cells and failed to produce SP cells. These SP cells showed self-renewal capability by serial colony-forming assay. Microarray expression analysis using a set of HTR-8/SVneo-SP and -NSP cells revealed that SP cells overexpressed several stemness genes including *caudal type homeobox2*(*CDX2*) and *bone morphogenic proteins*(*BMP*s), and *lymphocyte antigen 6 complex locus D (LY6D) gene* was the most highly up-regulated in HTR-8/SVneo-SP cells. *LY6D* gene reduced its expression in the course of seven days' cultivation in differentiation medium. SP cells tended to reduce its fraction by treatment of *LY6D* siRNA indicating that *LY6D* had potential to maintain cell proliferation of HTR-8/SVneo-SP cells. On ontology analysis, epithelial mesenchymal transition (EMT) pathway was involved in the up-regulated genes on

microarray analysis. HTR-SVneo-SP cells showed enhanced migration. This is the first report that LY6D was important for the maintenance of HTR-8/SVneo-SP cells. EMT was associated with the phenotype of these SP cells.

key word

Trophoblast, Epithelial-mesenchymal transition, HTR-8/SVneo, LY6D, side population cell

Acknowledgement

We thank Laboratory of Molecular and Biochemical Research, Research Support Center, Juntendo University Graduate School of Medicine, Tokyo, Japan, for technical assistance.

This work was in part supported by MEXT KAKENHI Grant Number 22130008 (T.T.).

Conflict of interest

There is no conflict of interest with regard to this paper.

<abbreviation> vSTB: syncytiotrophoblast, vCTB: Cytotrophoblast, EVT: extravillous cytotrophoblast, EMT: epithelial-mesenchymal transition, TSCs: trophoblast stem cells, hESCs: human embryonic stem cells, SP: side-population, NSP: non-side population, BMP: bone morphogenic protein, OCT4: octamer-binding transcription factor4, CDX2: caudal type homeobox2, FBS: fetal bovine serum, HBM: HTR-8/SVneo basal medium, HSM: HTR-8/SVneo-SP medium, SOX2: sex determining region Y-box 2, NANOG: Nanog homeobox, POU5F1: Pou class 5 homeobox 1, LY6D: lymphocyte antigen 6 complex locus D, LY6K: lymphocyte

antigen 6 complex locus K

Introduction

The placenta plays important roles in nutrition, fetal circulation and hormonal secretion maintenance of pregnancy and fetal growth [1]. The continual proliferation and differentiation of trophoblasts are critical for the maintenance of pregnancy. Placental trophoblasts consist of villous cytotrophoblast, syncytiotrophoblast (vSTB), and extravillous cytotrophoblast (EVT). Improper formation of the placenta leads to fetal growth restriction and reduced trophoblast invasion into the uterus causes pregnancy-induced hypertension [2-4]. It is believed that adherent and migratory processes of the EVTs are important to the success of the early phases of pregnancy as well as the continual development of a normal placenta.

It is well known that the tissue stem cells are associated with the development of tissues and pathologies. It is supposed that trophoblast stem cells (TSCs) exist in the placenta and that their continual proliferation and differentiation are crucial for the maintenance of pregnancy. In the mouse, TSCs have been isolated from pre- and post-implantation embryos, and they differentiated along the trophoblast lineage. TSCs of mice have been cultured and maintained in the presence of FGF4 and feeder cells [5]. However, the derivation of human TSC has not been achieved under conditions similar to those used for mouse TSC lines.

Experiments have demonstrated that side-population (SP) cells identified by cells' ability to remove the intracellular fluorescent dye Hoechst 33342 are enriched with stem cell [6]. We have previously reported the existence of SP cells in a HTR-8/SVneo cell line obtained from human primary trophoblast cells in the first trimester and determined the condition medium for maintenance of these SP cells [7]. However, it remains uncertain which pathways or genes are associated with the maintenance of these SP cells. Therefore, we have investigated the up-regulated genes by microarray expression analysis using the most recent chips and performed the pathway analysis.

In the present study, we showed that SP cells overexpressed several stemness genes including *caudal type homeobox2(CDX2)* and *bone morphogenic proteins*(*BMPs*) compared with non-SP cells, and the most up-regulated gene in SP cells was *lymphocyte antigen 6 complex locus D (Ly6D)*. On ontology analysis, the epithelial-mesenchymal transition (EMT) pathway was suggested to involve the up-regulated genes in microarray analysis. We also demonstrated that migration activity was enhanced in SP cells compared with NSP cells.

Material and Methods

Cell lines and culture

The HTR-8/SVneo cell line obtained from human trophoblast cells in the first trimester was established previously [8], and is distributed by Kyushu University. HTR-8/SVneo parental cells, SP cells and non-SP (NSP) cells were cultured in the medium described by Takao et al. [7]. To maintain the SP fraction, SP cells were cultured in 50% RPMI1640 containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (HTR-8/SVneo basal medium[HBM]), and 50% mouse embryonic fibroblasts (MEFs)-conditioned medium containing one μg heparin/mL and 25 ng /mL FGF2 (HTR-8/SVneo SP medium [HSM]). Alternatively, parental and NSP cells were cultured in HBM providing differentiation conditions.

Isolation of SP and NSP cells

To identify and isolate HTR-8/SVneo-SP and -NSP cells, cultured cells were dislodged from dishes with trypsin and EDTA, washed, and suspended at a concentration of 1x 10^6 cells/mL in RPMI1640 containing 2% FBS, one mM Hepes, and five mM EDTA. The cells were labeled in the same medium at 37℃ for 90 minutes with 2.5 μg/mL Hoechst 33342 dye (Thermo Fisher Scientific Inc., MA, USA), either alone or in combination with 50 μmol/L verapamil (SIGMA-Aldrich, Missouri, USA). Finally, the cells were counterstained with one μg/mL propidium iodide (PI) to label dead cells. The cells were then analyzed by FACS Vantage or Aria II fluorescence-activated cell sorter (BD Bioscience, California, USA) using dual wavelength analysis (blue 424-444 nm and red 675- nm for

Vantage; 395-415 nm and 670- nm for Aria II) after excitation with UV light (350 nm for Vantage; 355 nm for Aria II) [9].PI-positive dead cells were excluded from the analysis.

SP cells and NSP cells were separated by flow cytometry and both fractions were seeded in HSM or HBM on collagen-coated 24-well plates (two cm^2) (Iwaki, Funabashi, Japan). The cells were cultured for one week, and then transferred to collagen-coated 60 mm dishes (Iwaki, Funabashi, Japan).

Colony-forming unit assay

The cells were seeded at a density of 100 per well in 24-well tissue culture plates (BD, New Jersey, USA). Two weeks later, cells were trypsinized, washed with PBS, and re-suspended in cell culture medium at a density of 10^3 per well in 60mm culture dishes, and colony formation was evaluated. Colonies were stained with 1% crystal violet, 95% ethanol and distilled water, and then rinsed with distilled water several times to remove excess stain. Dishes were dried for 24 hours. Three independent experiments were conducted.

RNA purification

RNA was isolated with Qiagen RNeasy Plus Mini kit (Qiagen, Hilden, Germany) as directed by the manufacturer. 5 μ g of total RNA was reverse-transcribed to cDNA using oligo dT primer and Superscript Ⅱ reverse-transcriptase (Invitrogen, Carlsbad ,USA).

Real-time PCR

Real-time PCR was carried out using a 7300 Real-Time PCR System with SDS RQ Study software (Applied Biosystems, California, USA). The cDNA templates were combined with SYBER Green premix (Takara Bio, Otsu, Japan) to perform quantitative-PCR reactions. Primers used in this study are shown in Supplementary Table 1. Reactions were carried out as follows: for one cycle at 94° C for five minutes; 40 cycles at 94° C for 30 seconds , 59° C for 30 seconds, and 72° C for 30 seconds; one cycle at 94° C for one minutes; and one cycle at 55° C for 30 seconds. The transcript level of each specific gene was normalized to *hypoxanthine phosphoribosyltransferase (HPRT)* amplification. Three independent experiments were conducted.

Microarray

Total RNA was extracted using RNeasy Mini Kits according to the manufacturer's instruction (QIAGEN, Hilden, Germany).

Microarray analysis was performed by CERI customer service (Saitama, Japan) in accordance with the instruction of One-Color Microarray-Based Gene Expression Analysis, ver 6.0. The integrity of the RNA was checked using an RNA 6000 Nano kit and 2100 Bioanalyzer (Agilent Technologies). Hybridization and washing were performed in accordance with the instructions of the manufacturer. Microarray expression analysis was performed for screening of up-regulated genes in a set of HTR-8/SVneo SP and NSP cells using Whole human Genome Array (4X44X) ver 2.0 (G4845A)(Agilent Technologies).

Western blotting

To measure protein expression, subconfluent cells were lysed with ice-cold lysis buffer (CelLytic M Cell Lysis Reagent; Sigma, MO, USA) containing freshly added protease inhibitors (Protease inhibitor Cocktail; Sigma, MO, USA). After centrifugation at 13,000 X g for ten minutes to remove debris, 10 μg of the proteins were subjected to SDS-PAGE and transferred onto a nitrocellulose membrane in a semi-dry transfer cell (Bio Rad Laboratories, Hercules, USA). The blots were incubated with diluted primary antibodies specific for fibronectin (Abcam plc, Cambridge UK) and GAPDH (Santa Cruz, TX, USA) overnight at 4°C. After incubation with each primary antibody (1:1000 dilution), the blots were incubated with horseradish peroxidase-linked anti-rabbit antibodies and analyzed with an ECL system (Amersham Bioscience, Buckinghamshire, UK).

Immunocytochemical staining

Cultured cells were incubated on glass chamber slides (LAB-TEK; Nalge Nunc International Corp. Naperville, USA) and fixed by treatment with 10% formalin. Slides were washed in PBS solution, treated with H_2O_2 in methanol at room temperature for ten minutes, incubated overnight at 4℃ with different primary antibodies (Supplementary Table 2), rinsed with PBS, and incubated with anti-mouse secondary antibody (1:200 dilution; Invitrogen) at room temperature for one hour. Visualization of the immunoreaction was carried out by incubation with 3,3′-diaminobenzidine (DAB) for two minutes. Finally, sections were counterstained with hematoxylin, covered, and viewed under a light microscope. PBS buffer was used as a substitute for primary antibodies in the negative control. Three independent experiments were conducted.

Treatment with siRNA

The design and synthesis of *LY6D* siRNAs were done at Invitrogen, USA. The nucleotide sequences of sense and antisense siRNAs are described in Supplementary Table 3. Negative control siRNA, which does not target any known cellular mRNA, was provided by Invitrogen.

Transfection was performed with Amaxa® Cell Line Nucleofector® Kit (Lonza, Basel, Switzerland) according to the manufacturer's protocol. For electroporation with Amaxa® Cell Line Nucleofector® Kit L (Lonza, Basel,Switzerland), cells were washed with PBS and detached with trypsin (Invitrogen , California,USA). Approximately 1 x10^6 cells were transferred to a new tube, centrifuged at 200 x g for ten min at room temperature and the pellet was suspended in 100 μL of transfection solution. The cells were transfected using either 30 nM siRNA or two μg GFP plasmid. In the electroporation cuvette, the cells were nucleofected with program T-020. The sample was transferred to prepared six-well plates after the program was finished. Cells were incubated at 37^{O}C for 48 hours and flow cytometric analysis was performed. Five independent experiments were conducted.

In vitro scratch assay

SP and NSP cells were cultured on 24-well plates in HBM. Upon reaching confluence, the medium was replaced with RPMI1640 serum free medium, and the cell layer was wounded with 200 μL tips. After 24 hours of incubation, the cells were photographed with a BZ-8100 microscope (Keyence, Osaka, Japan). The area filled with cells after 24 hours was measured. Three independent experiments were conducted.

Invasion assay

Cell invasiveness was assessed with a BioCoat Matrigel Invasion Chamber kit according to the protocol of the manufacturer (Becton Dickinson Company, New Jersey, USA). After the hydration of chambers with the culture medium, 5 x 10^5 cells (HTR-8/SVneo Parent, SP and NSP) in 500 μL HBM were transferred into the chamber. The cells that invaded into the lower side of the filter were viewed under a Nikon phase-contrast microscope and counted in ten fields of view at ×200 magnification. The number of cells on the lower side of the filter was normalized to cells using the control chamber without Matrigel. The assay was done in triplicate.

Pathway analysis

Genetic pathways were evaluated using the MetaCore Analytical Suite (Gene Go Inc.). Enrichment analysis consisted of mapping gene IDs of the dataset onto IDs in entities of built-in functional ontologies represented in MetaCore by pathway maps and networks.

Statistical analysis

Data are represented as the means ± SEM and were analyzed with Student's t-test. For all analyses, two-sided tests of significance were used, with $p < 0.05$ considered significant.

Results

Isolation of SP cells from HTR-8/SVneo cells

SP cells were isolated from HTR-8/SVneo by FACS and were cultured with the medium as described in Materials and Methods. Verapamil blocked the dye efflux,

increased staining, and rendered the SP cells undetectable by FACS (Fig.1A-a, b). Next, both SP cells and NSP cells were cultured for two weeks, stained with Hoechst 33342, and then reanalyzed by FACS. SP cell cultures generated both SP and NSP subpopulations (Fig.1A-c). In contrast, NSP cell cultures produced NSP cells, but failed to produce SP cells (data not shown). SP cells cultured in HBM for 7 days couldn't retain SP cells (Fig.1A-d). When SP cells or NSP cells were plated in 60 mm collagen-coated dishes (50 cells/cm^2), they proliferated and SP cells formed more colonies than NSP cells (Fig.1B-a). To test the self-renewal capability of SP cells within each colony, we dissociated the primary colonies into a single cell, and then cultured these cells in 60 mm collagen-coated dishes (50 cells/cm^2). A single SP cell formed secondary or tertiary colonies. This indicates that the colony-forming cells isolated from existing colonies retain the same colony-forming potential and self-renewal capability of the primary SP cells. NSP cells produced a few secondary colonies and no tertiary colonies (Fig. 1B-b).

***LY6D* was the most highly up-regulated in HTR-SP cells.**

We performed microarray expression analysis to screen for up-regulated genes in HTR-SP cells compared to NSP cells using the most recent chips. We identified 4149 genes that were up-regulated more than two-fold in HTR-SP cells compared with those in HTR-NSP cells. *Lymphocyte antigen 6 complex locus D (LY6D)* was the most highly

up-regulated in SP cells compared with NSP cells. Another *LY6* family, *lymphocyte antigen 6 complex locus K* (*LY6K)* was also highly up-regulated. In addition, the genes related to the trophoblast stemness among them were picked up. In the present study, *CDX2* (a TSC marker in mice), *BMP3, BMP4,* and *BMP7* (that support the conversion of hESC to trophoblasts) [10-12], *sex determining region Y-box 2* (*SOX2)*, *Nanog homeobox* (*NANOG),* and *Pou class 5 homeobox 1* (*POU5F1)* (highly expressed in hESC) [13-15] were overexpressed in HTR-SP cells compared with NSP cells. Representative genes were shown in Table 1. Overexpression of these mRNA was confirmed by real-time PCR (Fig.2A). Immunocytochemical staining confirmed the elevated expression of LY6D, LY6K, BMP7, and BMP3 proteins in SP cells compared to NSP cells (Fig. 2B). We pursued the genes' expression levels after SP cells had been cultured in the differentiation medium (HBM). *LY6D*, *BMP7, BMP3, CDX2,* and *SOX2* gradually reduced their expression in the course of seven days' cultivation, but *LY6K* showed no change of expression in this experiment (Fig. 3).

***LY6D* has potential to maintain cell proliferation of HTR-8/SVneo-SP cells.**

Next, we investigated the effect of *LY6D* expression, which was the most up-regulated gene, on the percentage of SP cells.

LY6D was down-regulated in HTR-8/SVneo SP cells by the introduction of *LY6D* siRNA by electroporation (Fig.4A). The proportion of SP cells in the presence or absence of *LY6D* siRNA was analyzed by flow cytometry. By treatment of *LY6D* siRNA, cell growth was suppressed ($1x10^6$ SP cells reduced the number to 643,333±10,969 cells in 48 hours by treatment of LY6D siRNA), and total cell number cultured for 48 hours

reduced. The SP fraction was significantly reduced by treatment with *LY6D* siRNA compared with control siRNA or no treatment (Fig.4B-a,b). These results demonstrated that *LY6D* has potential to maintain cell proliferation of HTR-8/SVneo-SP cells.

The EMT-associated pathway were enhanced in HTR-SVneo-SP cells

Next, we performed the pathway analysis. Microarray data from HTR-8/SVneo SP and NSP were evaluated using the MetaCore system, an approach to evaluating ontology. As seen in GO process, most of the up-regulated genes were associated with development (Supplementary Fig. 1A).

We used the MetaCore package to identify the Gene GO pathway maps involved in up-regulated genes in HTR-8/SVneo cells. Two Gene GO pathways of EMT were involved in the top ten scoring pathways (Supplementary Fig. 1B).

HTR-8/SVneo-SP cells showed enhanced motility.

It is well known that epithelial-mesenchymal transition (EMT) is associated with cell migration and invasion. At first, we investigated the cell growth rate of HTR-8/SVneo SP or NSP cells in the course of 6 days' cultivation in the Supplementary Fig. 2. There is no significant difference of the growth rate between SP cells and NSP cells in the course of 6 days' cultivation. Therefore, we evaluated the migration capacity, which is one of phenotype of EMT, using the *in vitro* scratch assay in 24 hours. Migration

capacity was enhanced in SP cells compared with NSP cells (Fig. 5A,B).

We also examined cell invasiveness using the Boyden chamber assay with a filter coated with Matrigel. Migration ability was also evaluated by the number of cells that went through the control chamber without Matrigel. The invasion/migration ratio was used for the comparison of cells' ability for invasion. Although the difference was not significant, SP cells' capacity for invasion tended to be higher than that of NSP or parental cells (Fig.5C).

Finally we evaluated the protein level of fibronectin, which is one of EMT markers, in HTR-8/SVneo SP by the Western blotting. Fibronectin expression was enhanced in SP cells compare with that in NSP cells (Fig.6).

Discussion

We isolated SP cells from the human trophoblast cell line HTR-8/SVneo and analyzed their characteristics. In the present study, we showed that SP cells overexpressed several stemness genes including the pluripotency factors associated with ESC (Oct4, NANOG, SOX2) and the TSC maintenance factors (CDX2, GATA3 and EOMES) and BMPs (BMP 7, 3 and 4) compared with non-SP cells. The most up-regulated gene in SP cells was *LY6D*. In addition, *LY6D* gradually reduced its expression in SP cells during differentiation, suggesting that *LY6D* is an undifferentiated marker of trophoblast cells. SP cells tended to reduce its fraction by treatment of *LY6D* siRNA indicating that *LY6D* has potential to maintain cell proliferation of HTR-8/SVneo-SP cells.

LY-6 molecules were initially identified in mice as lymphocyte differentiation antigens [16] and as membrane bound proteins with GPI-anchors [17,18].

LY6D protein expression marks the earliest stage of B-cell specification and identifies the branch point between B-cell and T-cell development [19]. Although previous studies have shown that LY6D protein is expressed exclusively on normal squamous epithelia and transitional epithelium and their malignant counterparts [20,21], there is no report of its expression in the placenta. The function of LY6D protein in the trophoblast remains unclear. After knockdown of *LY6D* gene expression by siRNA, the proportion of SP cells tended to be reduced. These results demonstrate that *LY6D* can play a role in the maintenance of HTR-8/SVneo -SP cells.

To supply the fetus with oxygen and other key molecules, the EVTs invade the uterine decidua and myometrium. These adherent and migratory processes are important to the success of the early phases of pregnancy as well as the continual development of a healthy placenta. It is well known that EMT is associated with cell migration and invasion. Kokkinon et al. proposed the role for EMT during placentation, and its importance to proliferation, migration, and invasion by EVT [22].

We used the MetaCore package to identify the Gene GO pathway maps involved in the up-regulated genes in HTR-SP cells. We have recently shown that the pathways associated with EMT were important characteristics of endometrial cancer stem cells

(CSCs) [23]. In agreement with the previous study, two pathways of EMT were involved in the scoring pathways (Supplementary Fig. 1B). Migration assays revealed that SP cells showed enhanced migration activity compared with that of NSP cells. These characteristics of HTR-8/SVneo-SP cells might be associated with the process of uterine invasion of trophoblast cells.

In this study, we identified the up-regulated genes "*LY6D*" which had potential to maintain cell proliferation and demonstrated induction of EMT in trophoblastic SP cells. It is suggested that the abnormal placental formation causes fetal growth restriction and pregnancy-induced hypertension can be induced. Further study for the placentation system from the point of interaction between trophoblast stem cells and endometrial cells should be necessary for analysis of the cause of the clinical problem.

Microarray data

The data associated with this paper is registered in GEO (study No GSE61547) at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE61547.

Study No GSE61547

References

1 Roberts, R. M. and Fisher, S. J. Trophoblast stem cells. *Biol Reprod* .2001;**84**, 412-421.

2 Khong, T. Y., Staples, A., Chan, A. S., Keane, R. J. and Wilkinson, C. S. Pregnancies complicated by retained placenta: sex ratio and relation to pre-eclampsia. *Placenta* .1998;**19**, 577-580.

3 McFadyen, I. R., Greenhouse, P., Price, A. B. and Geirsson, R. T. The relation between plasma urate and placental bed vascular adaptation to pregnancy. *Br J Obstet Gynaecol* .1986;**93**, 482-487.

4 Pijnenborg, R., Anthony, J., Davey, D. A., et al. Placental bed spiral arteries in the hypertensive disorders of pregnancy. *Br J Obstet Gynaecol* .1991;**98**, 648-655.

5 Tanaka, S., Kunath, T., Hadjantonakis, A. K., Nagy, A. and Rossant, J. Promotion of trophoblast stem cell proliferation by FGF4. *Science* .1998;**282**, 2072-2075.

6 Zhou, S., Schuetz, J. D., Bunting, K. D., et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med.*2001; **7**, 1028-1034.

7 Takao, T., Asanoma, K., Kato, K., Fukushima, K.,et al. Isolation and characterization of human trophoblast side-population (SP) cells in primary villous cytotrophoblasts and HTR-8/SVneo cell line. *PLoS One* .2011;**6**, e21990.

8 Graham C., Hawley T., Hawley R.,et al. Establishment and characterization of first trimester human trophoblast cells withexpanded lifespan. Exp. Cell Res.1993; 206, 204-211.

9 **Kondo T., Setoguchi T., and Taga T.** Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci U S A*. 2004;101, 781-786.

10 Xu, R. H., Chen, X., Li, D. S., et al. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nat Biotechnol* .2002;**20**, 1261-1264.

11 Amita, M., Adachi, K., Alexenko, A. P., et al. Complete and unidirectional conversion of human embryonic stem cells to trophoblast by BMP4. *Proc Natl Acad Sci U S A* .2013;**110**, E1212-1221.

12 Giakoumopoulos, M. and Golos, T. G. Embryonic stem cell-derived trophoblast differentiation: a comparative review of the biology, function, and signaling mechanisms. *J Endocrinol* .2013;**216**, R33-45.

13 Niwa, H., Toyooka, Y., Shimosato, D.,et al. Interaction between Oct3/4 and Cdx2 determines trophectoderm differentiation. *Cell* .2005;**123**, 917-929.

14 Keramari, M., Razavi, J., Ingman, K. A., ei al. Sox2 is essential for formation of trophectoderm in the preimplantation embryo. *PLoS One* .2010;**5**, e13952.

15 Desmarais, J. A., Demers, S. P., Suzuki, J., et al. Trophoblast stem cell marker gene expression in inner cell mass-derived cells from parthenogenetic equine embryos. *Reproduction* .2011;**141**, 321-332.

16 McKenzie, I. F., Gardiner, J., Cherry, M. and Snell, G. D. Lymphocyte antigens: Ly-4, Ly-6, and Ly-7. *Transplant Proc* .1977;**9**, 667-669.

17 Gumley, T. P., McKenzie, I. F. and Sandrin, M. S. Tissue expression, structure and function of the murine Ly-6 family of molecules. *Immunol Cell Biol* .1995;**73**, 277-296.

18 Brakenhoff, R. H., Gerretsen, M., Knippels, E. M.,et al. The human E48 antigen, highly homologous to the murine Ly-6 antigen ThB, is a GPI-anchored molecule apparently involved in keratinocyte cell-cell adhesion. *J Cell Biol* .1995;**129**, 1677-1689.

19 Inlay, M. A., Bhattacharya, D., Sahoo, D., et al. Ly6d marks the earliest stage of B-cell specification and identifies the branchpoint between B-cell and T-cell development. *Genes Dev* .2009;**23**, 2376-2381.

20 Quak, J. J., Balm, A. J., van Dongen, G. A.,et al. A 22-kd surface antigen detected by monoclonal antibody E 48 is exclusively expressed in stratified squamous and transitional epithelia. *Am J Pathol* .1990;**136**, 191-197.

21 Nieuwenhuis, E. J., Jaspars, L. H., Castelijns, J. A.,et al. Quantitative molecular detection of minimal residual head and neck cancer in lymph node aspirates. *Clin Cancer Res* .2003;**9**, 755-761.

22 Kokkinos, M. I., Murthi, P., Wafai, R., Thompson, E. W. and Newgreen, D. F. Cadherins in the human placenta--epithelial-mesenchymal transition (EMT) and placental development. *Placenta* .2010;**31**, 747-755.

23 Kusunoki, S., Kato, K., Tabu, K., et al. The inhibitory effect of salinomycin on the proliferation, migration and invasion of human endometrial cancer stem-like cells. *Gynecol Oncol* .2013;**129**, 598-605.

Figure legend

Fig.1 Isolation of trophoblast SP cells

A: Representative data of one experiment is shown in Fig.1 A.

a) HTR-8/SV neo parental cells were analyzed by flow cytometry .

SP fraction of HTR-8/SV neo parent cells was 1.16% of total cells.

b) Verapamil blocked Hoechst33342 efflux by ABC transporter, increased staining, and makes SP cells undetectable. SP fraction disappeared by adding verapamil.

c) SP cell re-culture generated both SP and NSP subpopulations. SP fraction is well retained. (20.3% of total cells)

d) SP cells cultured for 7 days in the differentiation medium couldn't retain SP fraction. SP cells reduced its fraction from 20.3% to 0.17%.

B: Dissociated SP cells or NSP cells were plated in 60 mm collagen-coated dishes (50 cells/cm^2).

a) SP cells formed more colonies than NSP cells.

b) SP cells increased the colonies by times of passage.

NSP cells produced a few secondary colonies and no tertiary colonies.

Fig. 2 Up-regulation genes in SP cells

A: Microarray analysis demonstrated that *CDX2*, *SOX2*, *BMP3* and *BMP7* were

overexpressed in HTR-8/SV neo SP cells compared with NSP cells (Table 1).

Lymphocyte antigens, *LY6D* and *LY6K* were highly up-regulated in SP cells. Overexpression of mRNA of each gene was confirmed by real-time PCR.

Relative ratio : the ratio of each gene expression in SP cells to that in NSP cells

B: IHC proved the high expression of LY6D, LY6K, BMP3 and BMP7 in HTR-8/SVneo SP cells than NSP cells

Fig. 3 Levels of the SP cells' up-regulated genes in differentiation medium culture

The up-regulated genes' expression levels of SP cells cultured in the differentiation medium were measured by real time PCR. *LY6D*, S*OX2*, *CDX2*, *BMP3* and *BMP7* gradually reduced their expressions in the course of seven days' cultivation, but *LY6K* showed no change of expression.

Relative ratio : the ratio of each gene expression to that in NSP cell on day 0

Fig. 4 *LY6D* has potential to maintain cell proliferation of HTR-8/SVneo-SP cells.

No treatment cells are those without electroporation and control cells were those electroporated with control siRNA.

A: *LY6D* was down regulated in HTR-8/SVneo SP cells by electroporation of *LY6D* siRNA.

Relative ratio: the ratio of each *LY6D* expression to that in No treatment SP cell

B: a,) SP cells reduced their fraction by treatment with *LY6D* siRNA (1.67±0.39%) compared with control siRNA (3.51±0.66%) or no treatment (4.45±0.53%), and the total number of SP cells reduced. Representative data were shown.

b) Data of cell numbers were represented as the mean ± SEM from three independent experiments. No treatment 29,433±4,166, Control siRNA 21,166±2,723 , LY6D siRNA 11,900±1,344

Fig. 5 HTR-8/SVneo SP cells showed enhanced migration compared with NSP cells.

A,B: Cell migration activity was investigated with the *in vitro* scratch assay.

The area filled with cells within 24hours was measured. Migration capacity was enhanced in SP cells compared with NSP cells

C: Cell invasion activity was examined with the Boyden chamber assay with a filter coated with Matrigel. The ratio of cells those went through invasion chamber and control chamber is shown in the figure.

Fig. 6

Fibronectin expression was enhanced in SP cells compared with that in NSP cells.

The protein level of fibronectin, which is one of EMT markers, in HTR-8/SVneo-SP and -NSP cells was measured by the Western blotting.

Supplementary Fig. 1 MetaCore analysis between HTR-8/SVneo SP and NSP cells

We used the MetaCore package to identify the Gene GO pathway MAPs involved in upregulated genes in the microarray data on a set of HTR-8/SVneo SP cells and -NSP cells.

A) Most of the upregulated genes were associated with development.

B) Two Gene GO pathways of EMT were involved in the top ten scoring pathways.

Supplementary Fig. 2 Cell growth of HTR-8/SVneo-SP and -NSP cells

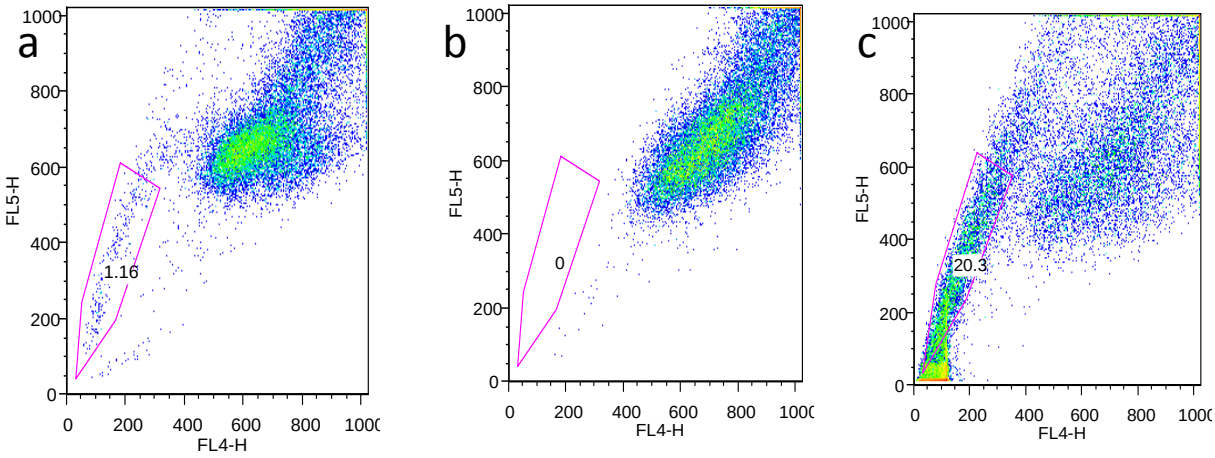
2×10^4 SP and NSP cells were cultured with HSM medium and cell growth rate was assessed.

Figure 1

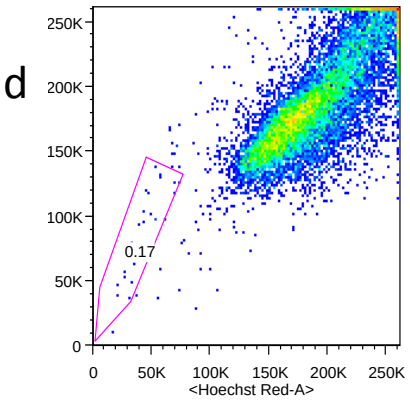
HTR-8/SVneo

Sorted SP, after 2 weeks' cultivation

A



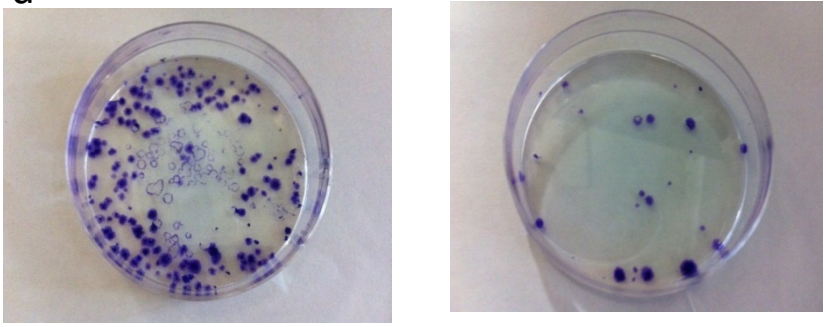
Verapamil — +



SP cells after seven days' cultivation in the differentiation medium

B

a



HTR-8/SVneo SP

HTR-8/SVneo NSP

b

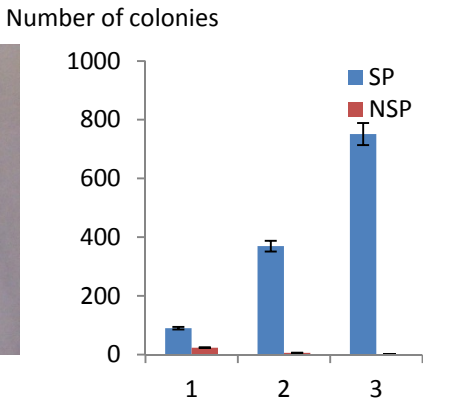
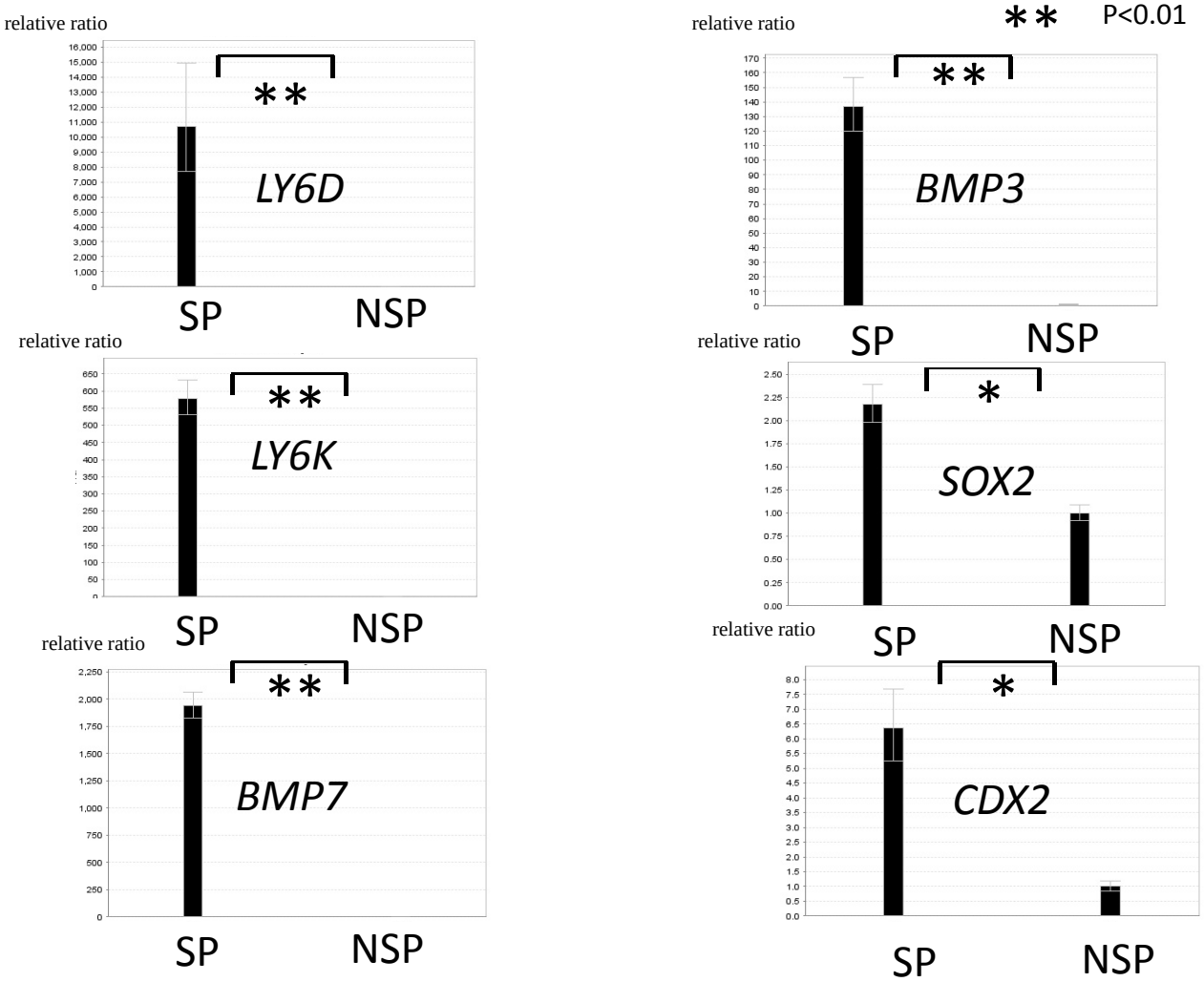


Figure 2

* P<0.05

** P<0.01

A



B

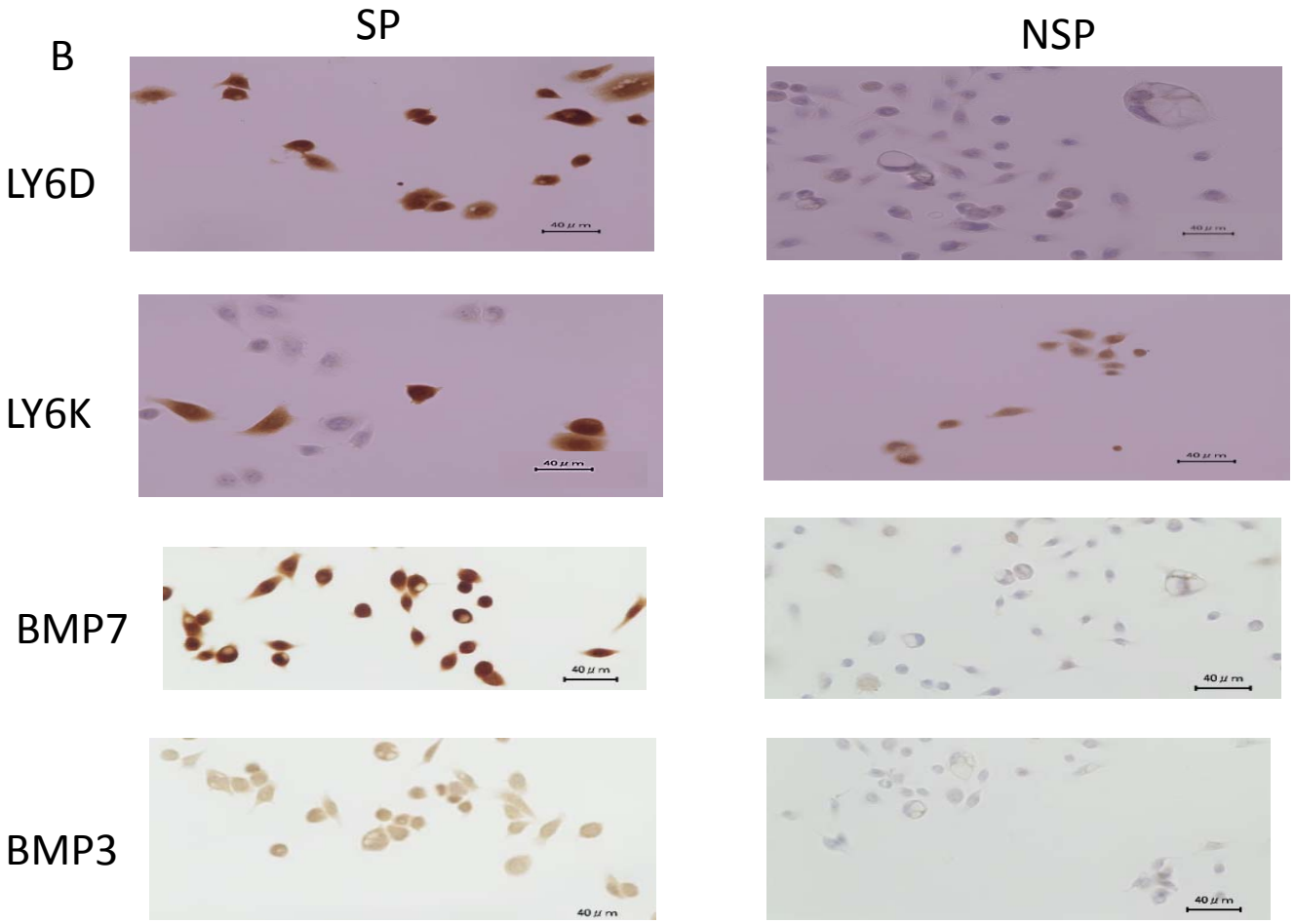


Figure 3

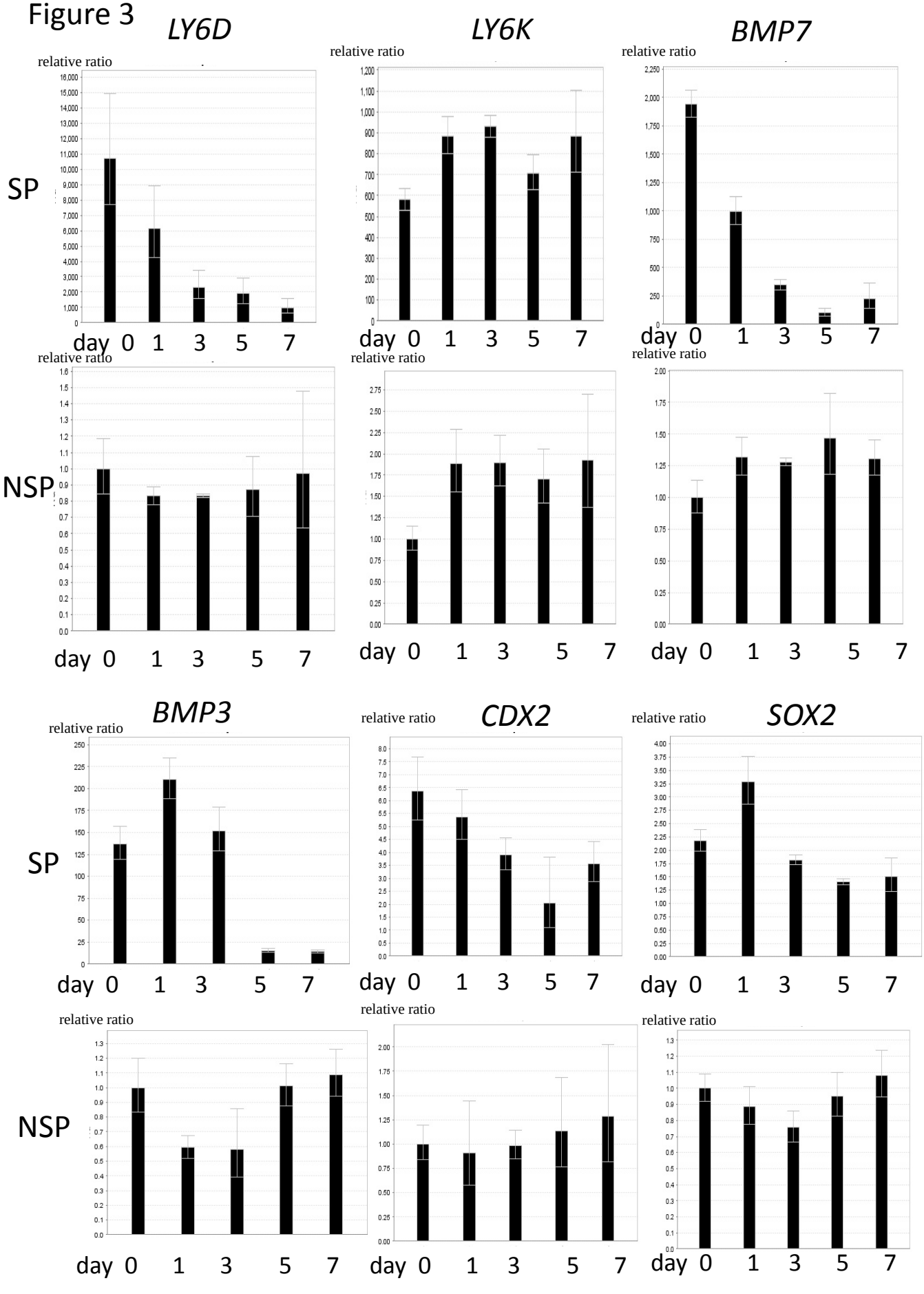
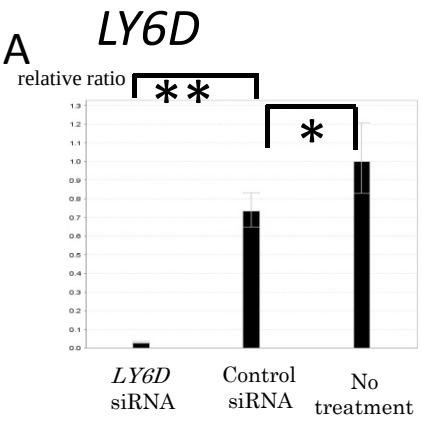

LY6D
LY6K
BMP7
relative ratio
SP
NSP
day 0 1 3 5 7
BMP3
CDX2
SOX2

Figure 4

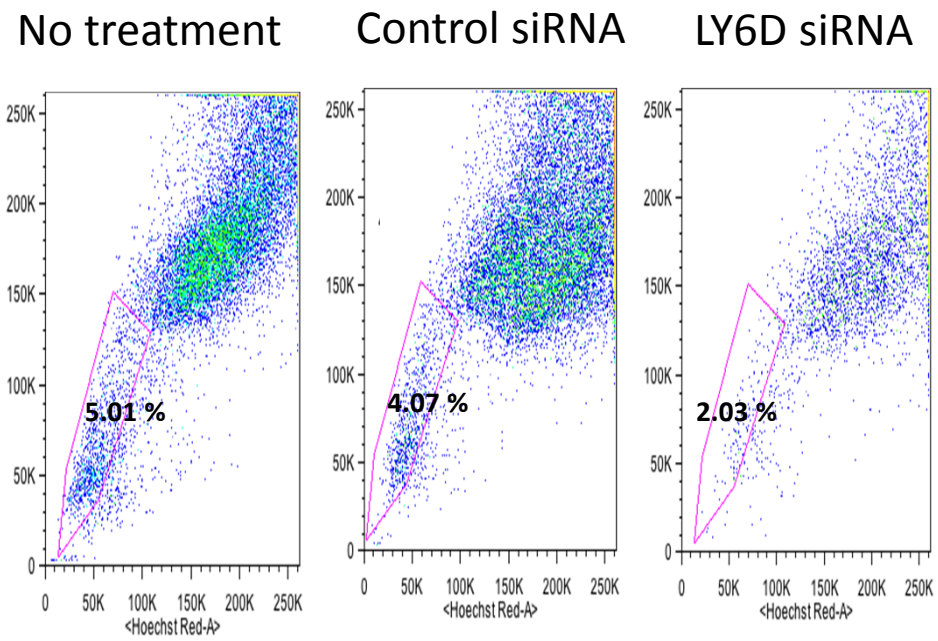
A



* NS

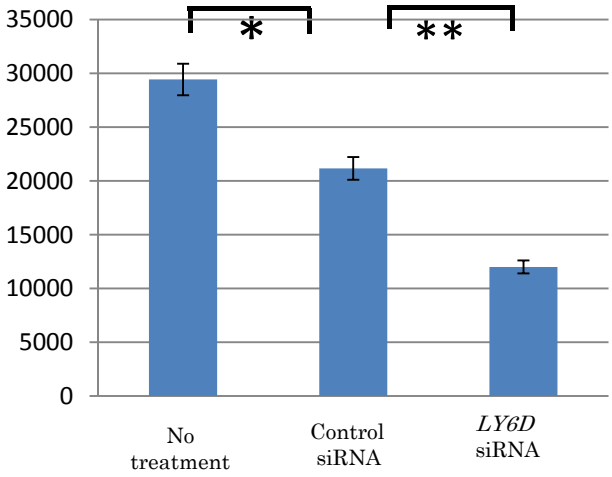
** P＜0.01

B a



b

SP cell number per 1x10^6 cells



* P=0.125

** P=0.027

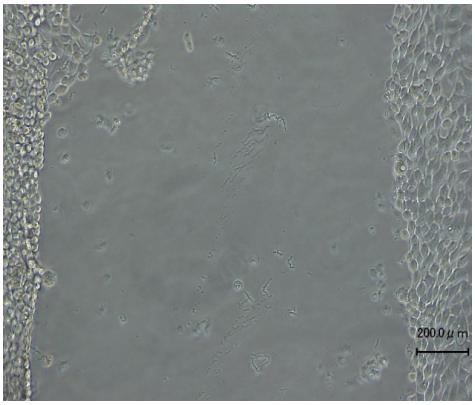
Figure 5

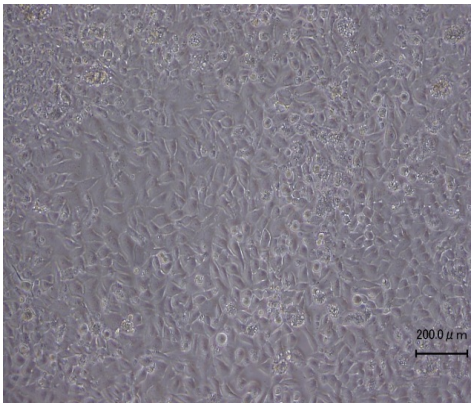
A

0hr

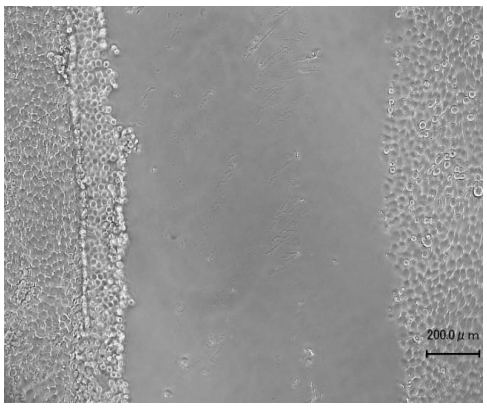
24hr

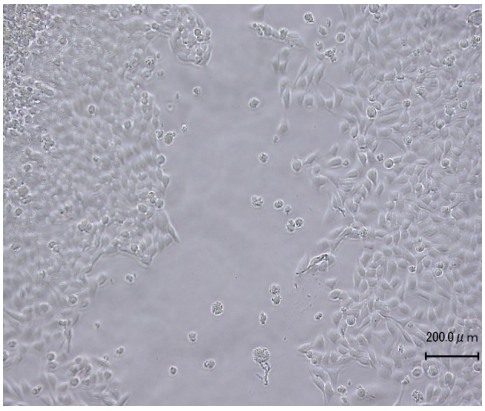
HTR8/Svneo
SP cells



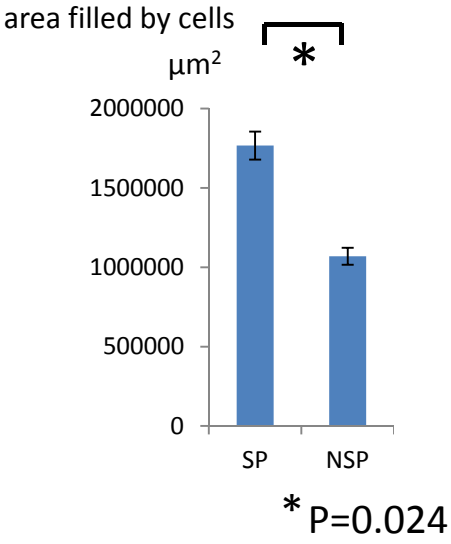


HTR8/Svneo
NSP cells





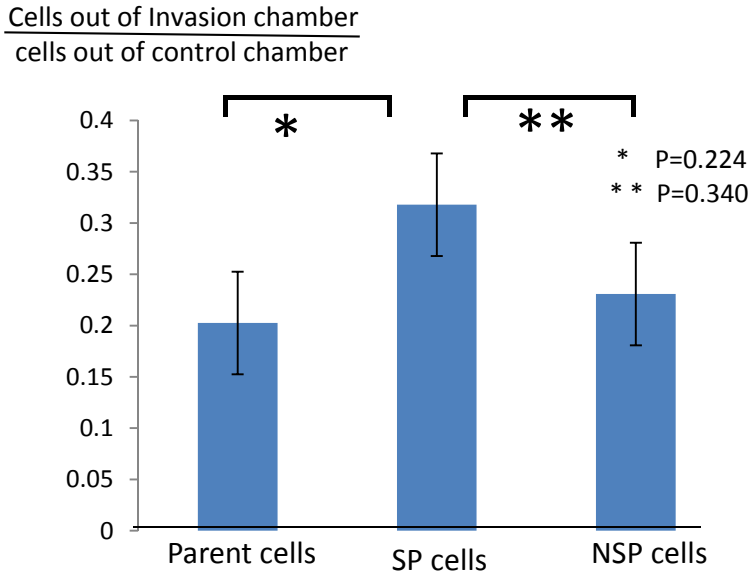
B



* P=0.024

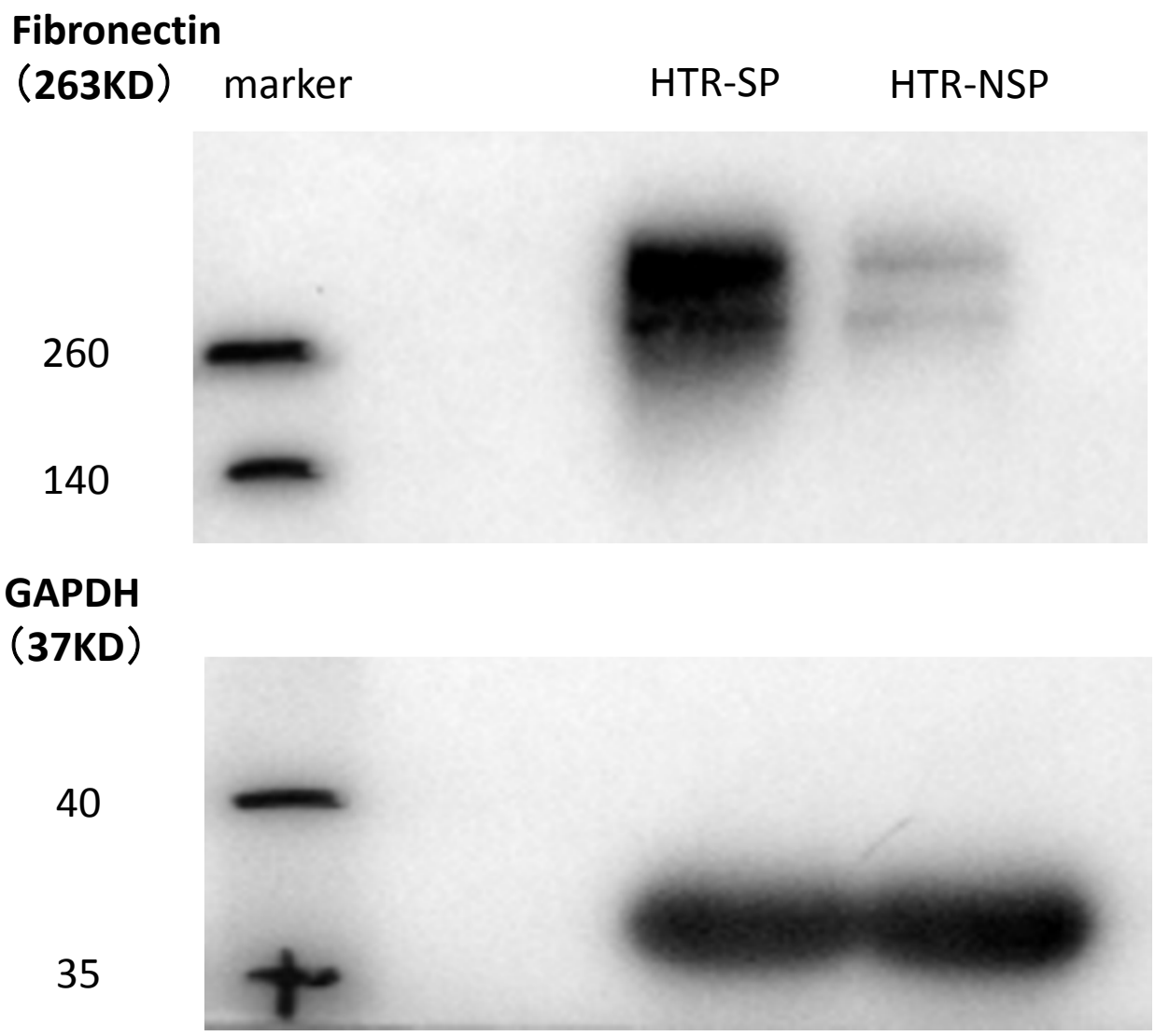
Areas filled within
24 hours after scratch

C



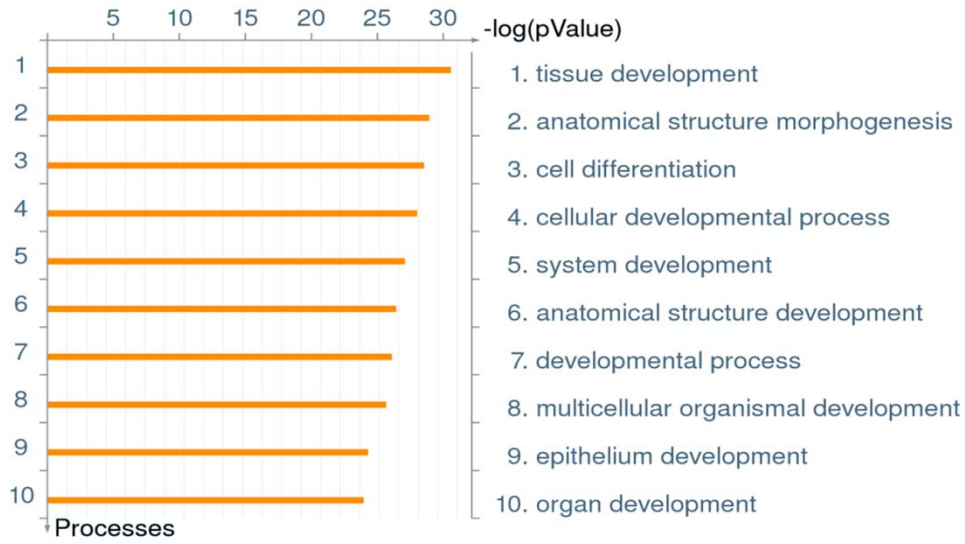
The ratio of cells those went through
Invasion chamber and control chamber

Figure 6

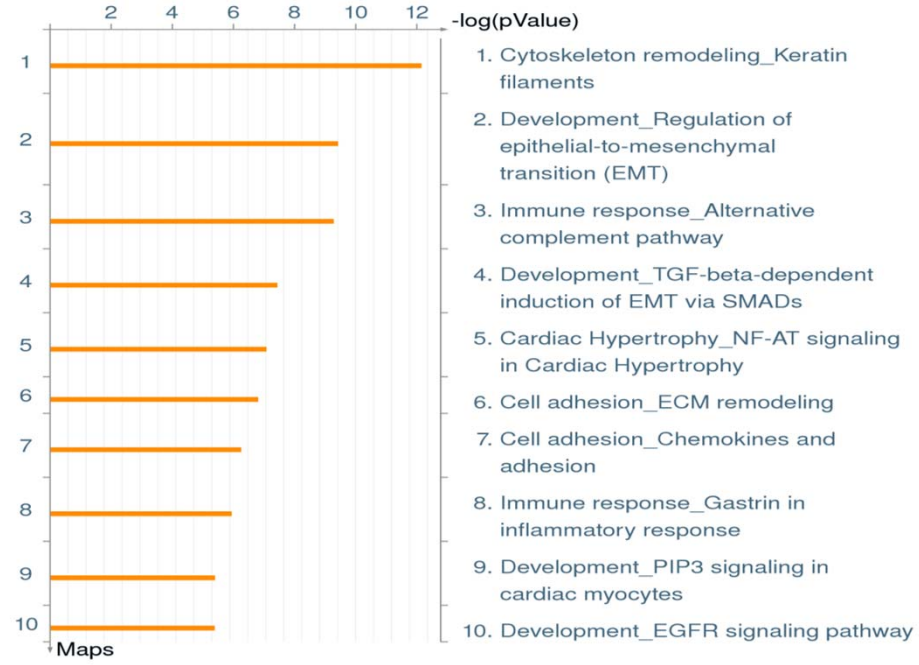

Fibronectin
（263KD）
marker
HTR-SP
HTR-NSP
260
140
GAPDH
（37KD）
40
35

Supplementary Figure 1

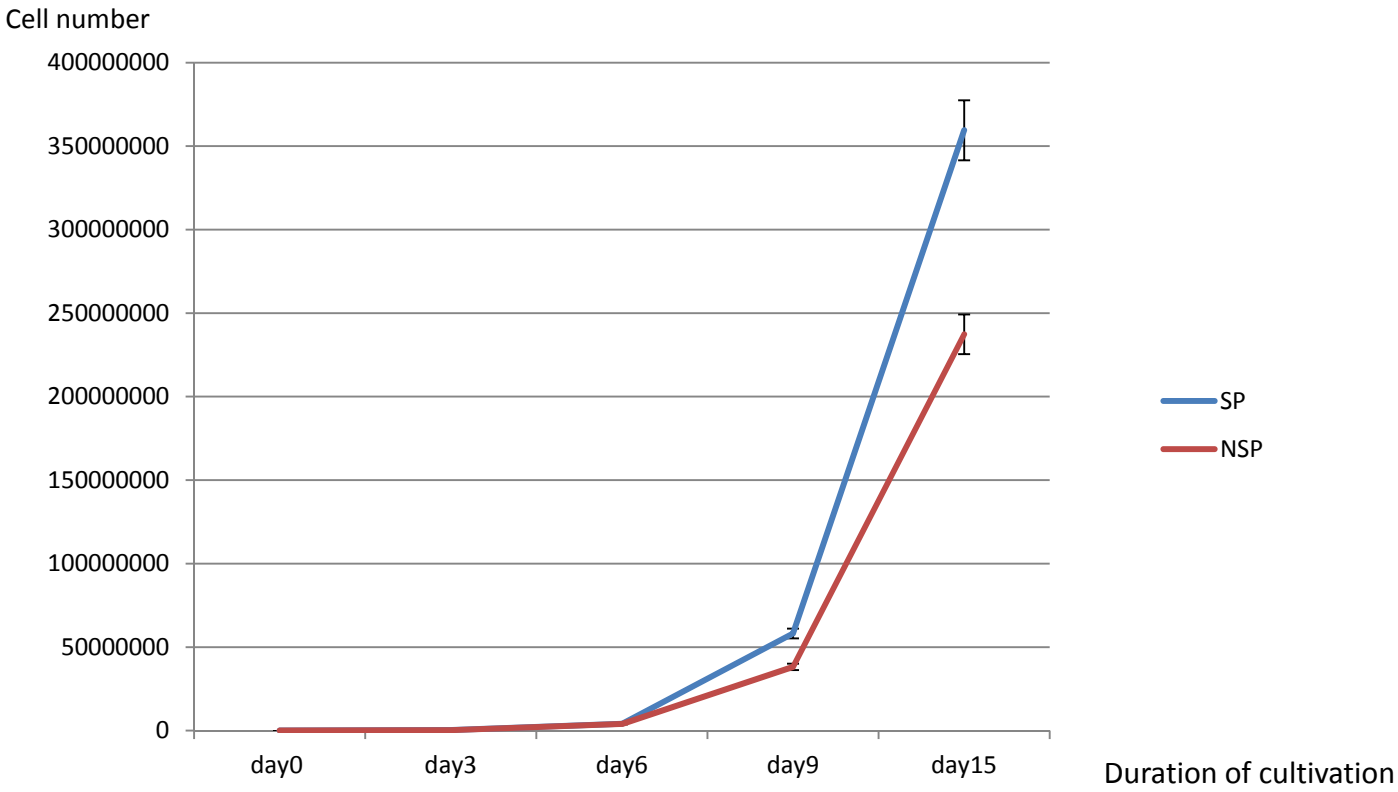
A



B



Supplementary Figure 2



Cell growth of HTR-8/SVneo SP or NSP cells

Table 1 Overexpressed genes in HTR-8/SVneo SP cells compared with NSP cells

Gene symbol	Fold change (SP/NSP)	Gene name
LY6D	7626.280077	**lymphocyte antigen 6 complex, locus D**
LY6K	1009.68076	**lymphocyte antigen 6 complex, locus K**
BMP7	2200.765632	**bone morphogenetic protein 7**
BMP3	100.5674554	**bone morphogenetic protein 3**
SOX2	260.5518449	**sex determining region Y-box 2**
GATA3	90.93055794	**GATA binding protein 3**
NANOG	14.54076231	**Nanog homeobox**
POU5F1	12.76913152	**POU class 5 homeobox 1**
ETS2	6.712241856	**v-ets erythroblastosis virus E26 oncogene homolog 2**
BMP4	6.21421174	**bone morphogenetic protein 4**
CDX2	2.964041814	**caudal type homeobox 2**
EOMES	2.074054781	**eomesodermin**

スライド 1

I1 add the title

Inagaki, 2015/05/27

Supplementary Table 1

Gene name	primer
HPRT	F GGCAGTATAATCCAAAGATGGTCAA R GTCAAGGGCATATCCTACAACAAAC
LY6D	F ACATCAGAGATGAGGACAGCATT R AGAATGCTTGCAGTTGCTGGAG
LY6K	F ACTGCGAGACAACGAGATCCA R CATGACACCACACTCTATTGTCACC
CDX2	F GGAACCTGTGCGAGTGGATG R CGGATGGTGATGTAGCGACTGTA
SOX2	F GTGAGCGCCCTGCAGTACA R GCGAGTAGGACATGCTGTAGGTG
BMP3	F CTTGCAGATATTGGCTGGAGTGA R CTGGATGGTAGCATGATTTGATGG
BMP7	F GGTGCAGGATGTGCTGGACTTA R CTCCAAATCGGGACACTTGGTTA

Supplementary Table 2

antibody	animal	company	country
LY6D	rabbit	SIGMA	USA
LY6K	rabbit	Santa Cruz	USA
BMP3	mouse	Abcam plc	UK
BMP7	rabbit	Abcam plc	UK
GAPDH	rabbit	Santa Cruz	USA
fibronectin	rabbit	Abcam plc	UK

Supplementary Table 3

RNAi	lot	Description Sequence	Quantity	company
LY6D	10620318 205728E05	GCCAGCUCUCGCU UCUGCAAGACCA	stealth	Invitrogen USA
LY6D	106203192 05962 F01	UGGUCUUGCAGAA GCGAGAGCUGGC	stealth	Invitrogen USA