11. 4% Paraformaldehyde solution (PFA)
12. Alcian Blue (Sigma cat. no. A5268)
13. Glacial acetic acid (Sigma cat. no. A9967)
14. Alcian blue staining solution:

Solution 1: 3ml glacial acetic acid + 97 mL Distilled water

Solution 2: 1g Alucian blue + 100 mL Solution 1

Mix Solution 2 well for 30 min. Then, filtrate it through filter paper. Store at 4℃ for 6 mo.

2.8. Reagents for hematopoietic cell differentiation from ES cell-derived mesoderm cells

1. αMEM
2. FBS pretested for in vitro ES cell differentiation. (see **Note 2**).
3. L-Glutamine (200mM 100X)
4. Penicillin-streptomycin (100X)
5. Cytokines: Recombinant human Erythropoietin (hEpo)(R&D systems cat. no. 287-TC) , Recombinant human Interleukin-3 (hIL-3) (R&D systems cat. no. 203-IL), Recombinant human Stem cell factor (SCF) (R&D systems cat. no. 255-SC).
6. 6-well culture plate (Becton Dickinson, Falcon 353046).
7. Hematopoietic cell differentiation medium: αMEM + 20%FBS + 2mM L-Glutamine + 1X P/S + 2 U/ml hEpo

2.9. Reagents for endothelial cell differentiation from ES cell-derived mesoderm cells (ES cell-derived endothelial colony assay)

1. αMEM
2. FBS pre-tested for in vitro ES cell differentiation. (see **Note 2**).
3. L-Glutamine (200mM 100X)
4. Penicillin-streptomycin (100X)
5. 24-well culture plate (Becton Dickinson, Falcon 353047)
6. Endothelial cell differentiation medium: αMEM + 20%FBS + 2mM L-Glutamine + 1X P/S

3. Methods

Feeder-independent ES cells such as CCE and E14tg2a are used for an *in vitro* ES cell differentiation because of the easy maintenance.

3.1.1. Gelatin coating of dishes

All dishes, flasks and plates should be gelatinized before use.

1. Add enough 0.1% gelatin solution to cover the plate surface.

6cm dish-3 ml, 10 cm dish-7ml, 1 well of 24-well plate-0.5 ml

2. Let the solution sit for at least 10 min at room temperature.
3. Aspirate the gelatin solution completely just before use.

3.1.2. Thawing of ES cells

1. ES cells are removed from liquid nitrogen storage or deep-freezer (-150℃) and quickly thawed in a 37℃ water both.

2. Transfer ES cells into a 15-ml centrifuge tube (BD, Falcon cat. no. 352095) containing 10ml of 37℃ prewarmed ES cell culture medium.

3. Spin down ES cells at low speed (190*g* for 5 min) at room temperature. Remove medium by suction and resuspend the cell pellet in 2ml of ES cell culture medium by gently repeated pipetting.

4. More than $2 x 10^6$ ES cells is transferred to a gelatin-coated 10 cm dish containing 8 ml of prewarmed ES cell culture medium and cultured in a tissue culture incubator (37℃, 5%CO_2).

5. Change entire medium daily until semi-confluent.

3.1.3. Passage of ES cells

1. Once the ES cells grow to 70% confluence, passage them to new tissues culture dishes treated by gelatin. They should be passaged every 2 to 3 days as described below.
2. Aspirate medium and wash cells once with 37℃ prewarmed D-PBS. Volume of D-PBS needed for: 6-cm dish-3ml, 10-cm dish-6ml.
3. To remove ES cells from dish, add 0.5ml of 37℃ prewarmed 0.25% trypsin-EDTA into the 10 cm dish. Incubate in tissue culture incubator at 37℃ for 5 min.
4. Add 5ml of 37℃ prewarmed ES cell culture medium and break up the cell aggregates by repeated pipetting 8-15 times (see **Note 4**).
5. Transfer ES cells into a 15-ml centrifuge tube and spin down them at 270*g* for 5 min at room temperature. Resuspend the cell pellets in 5 ml of 37℃ prewarmed ES cell culture medium. Count cell number and seed ES cells at :

Seeding Number	Days Needed for Confluence	Confluent cell number
$1x10^6$/10-cm dish	3d	2-$3x10^7$/dish
$2x10^6$/10-cm dish	2d	2-$3x10^7$/dish
$8x10^6$/10-cm dish	1d	2-$3x10^7$/dish

6. Daily complete medium change is required until confluent.

3.1.4. Cell freezing

1. Prepare 2X freezing solution: 20% dimethyl sulfoide (DMSO) + 80% FBS. Keep on ice. Make fresh every time.
2. Remove cells from dish as in **Subheading 3.1.3.**
3. Resuspend $4x10^6$ cells in 0.25 ml ice-cold FBS and keep on ice.
4. Add an equal amount of 2X freezing solution. Freeze the cells at -80℃ overnight. The next day, transfer vials to a liquid nitrogen tank or ultra deep-freezer (-150℃).

3.2. Maintenance of OP9 stromal cell line for in vitro ES cell differentiation.

For maintenance of OP9 stromal cell line, the over-confluent condition should be avoided because the cells that undergo an over-growth become to stop their growth. We recommend 90% confluence on passage (see **Note 5**).

3.2.1. Thawing of OP9 cells

1. Thaw frozen vial in a 37℃ water both.
2. Transfer OP9 cells into a 15-ml centrifuge tube containing 10ml of 37℃ prewarmed OP9 culture medium.
3. Spin down OP9 cells at 190*g* for 5 min at room temperature. Resuspend cells in 2ml of OP9 culture medium by gently repeated pipetting.
4. Seed $5x10^5$ OP9 cells/6-cm dish and cultured in a tissue culture incubator (37℃, 5%CO_2).

3.2.2. Passage of OP9

1. Aspirate medium and wash cells once with 37℃ prewarmed D-PBS.
   Volume of D-PBS needed for: 6-cm dish-3ml, 10-cm dish-6ml.
2. To remove OP9 cells from dish, add 0.5ml of 37℃ prewarmed 0.05% trypsin-EDTA. Incubate in tissue culture incubator at 37℃ for 5 min.
3. Add 5ml of 37℃ prewarmed OP9 culture medium and break up the cell aggregates by repeated pipetting 8-15 times.
4. Transfer OP9 cells into a 15-ml centrifuge tube and spin down them at 270*g* for 5 min at room temperature. Resuspend the cell pellets in 5 ml of 37℃ prewarmed ES cell culture medium. We usually obtain: $7\text{-}8x10^5$ cells/6-cm dish and $1.2\text{-}1.6x10^6$/10-cm dish.
5. Seed 2-4 x 10^5 cells/10-cm dish.
6. OP9 cells should not be cultured for longer than 1 mo after thawing. In addition, over twenty-passage OP9 cells should not be used for in vitro ES cell differentiation (see **Note 5**).

3.2.3. Storing of OP9

1. Prepare 2X freezing solution: 20% dimethyl sulfoide (DMSO) + 80% FBS. Keep on

ice. Make fresh every time.

2. Remove cells from dish as in **Subheading 3.2.2.**

3. Resuspend 6-8x10^5 cells in 0.25 ml ice-cold 100% FBS and keep on ice.

4. Add an equal amount of 2X freezing solution. Freeze the cells at -80℃ overnight. The next day, transfer vials to a liquid nitrogen tank.

3.3. In vitro ES cell differentiation

3.3.1. Induction of mesoderm cells without OP9 cells

Before induction, ES cells should be maintained for at least 1 wk after thawing. For the differentiation into hematopoietic and endothelial cells, we recommend the condition in the presence of OP9 cells.

1. Aspirate medium and wash cells **twice** with 37℃ prewarmed D-PBS. Volume of D-PBS needed for: 6-cm dish-3ml, 10-cm dish-6ml.

2. To remove ES cells from dish, add 0.5ml of 37℃ prewarmed 0.25% trypsin-EDTA into the 10 cm dish. Incubate in tissue culture incubator at 37℃ for 5 min.

3. Add 5ml of 37℃ prewarmed **differentiation culture medium** and break up the cell aggregates by repeated pipetting 8-15 times.

4. Transfer ES cells into a 15-ml centrifuge tube and spin down them at 270*g* for 5 min at room temperature. Resuspend the cell pellets in 5 ml of 37℃ prewarmed differentiation culture medium.

5. In the case of feeder-dependent ES cells, to remove feeder cells, ES cells harvested are incubated at 37℃ with 5% CO_2 for 30 min. Collect the floating cells by pipetting gently.

6. Add 8x10^4 undifferentiated ES cells into 10-cm collagenIV-coated dish containing **15 ml** of prewarmed differentiation culture medium.

7. Change a half of medium (~8ml) on day 3.

3.3.2. Induction of mesoderm cells with OP9 cells

1. Prepare 10-cm dish with 90% confluent OP9 cells. OP9 is splitted 3days before in vitro ES cell differentiation.

2. Aspirate medium and wash cells **twice** with 37℃ prewarmed D-PBS. Volume of D-PBS needed for: 6-cm dish-3ml, 10-cm dish-6ml.

3. To remove ES cells from dish, add 0.5ml of 37℃ prewarmed 0.25% trypsin-EDTA into the 10 cm dish. Incubate in tissue culture incubator at 37℃ for 5 min.

4. Add 5ml of 37℃ prewarmed **OP9 differentiation culture medium** and break up the cell aggregates by repeated pipetting 8-15 times.

5. Transfer ES cells into a 15-ml centrifuge tube and spin down them at 270*g* for 5 min at room temperature. Resuspend the cell pellets in 5 ml of 37℃ prewarmed OP9

differentiation culture medium.

6. In the case of feeder-dependent ES cells, to remove feeder cells, ES cells harvested are incubated at 37℃ with 5% CO_2 for 30 min. Collect the floating cells by pipetting gently.

7. Add $8x10^4$ undifferentiated ES cells into 10-cm dish containing both 90% confluent OP9 and 10 ml of prewarmed OP9 differentiation culture medium.

3.3.3. Purification of Mesoderm cells from the culture without OP9

On day4 culture day, the ES cells induced into mesoderm differentiaiotn are harvested and analyzed by FACS to examine the expression pattern of mesoderm markers (**Fig. 2A**) (see **Note 6**).

1. Aspirate medium and wash cells **twice** with 37℃ prewarmed D-PBS.

2. To remove the differentiated cells from dish, add 8 ml of 37℃ prewarmed cell-dissociation buffer into the 10 cm dish. Incubate in tissue culture incubator (37℃, 5%CO2) for 15 min.

3. Add 8ml of 37℃ prewarmed D-PBS with 10% FBS and break up the cell aggregates by repeated pipetting 10-15 times.

4. Transfer the cells into a 50-ml centrifuge tube (BD, Falcon 2070) and spin down them at 270*g* for 5 min at **4℃**. Resuspend the cell pellets in 8 ml of ice-cold D-PBS with 10% FBS. Count cell number. Transfer $1x10^7$ cells into 15-ml centrifuge tube and spin down at 270*g* for 5 min at 4℃.

5. Resuspend the cell pellet in 100μl of ice-cold NMS and incubate the single-cell suspensions for 20 min on ice.

6. Add an appropriate concentration of PE-labeled anti-VEGFR2 and biotin-labeled anti- PDGFRα mAbs to cell suspension in NMS and incubate for 20 min on ice.
Add 10 ml of ice-cold HBSS/BSA into cell solution and spin down it at 270*g* for 5 min at 4℃.

7. Resuspend cell pellet in 100μl of ice-cold HBSS/BSA and add an appropriate concentration of SA-APC. Incubate it for 20 min on ice.

8. Wash the cells **twice** with 10 ml ice-cold HBSS/BSA. Resuspend the cells in 1 ml of ice-cold HBSS/BSA/PI for dead cell exclusion.

9. Analyze and sort $VEGFR2^+PDGFR\alpha^+$, $VEGFR2^+PDGFR\alpha^-$ and/or $VEGFR2^-PDGFR\alpha^+$ according to your experiments (**Fig. 2A and B**) (see **Note 7**).

3.3.4. Purification of Mesoderm cells from the culture with OP9

On **day 5** culture day, the ES cells induced into mesoderm differentiaiotn are harvested and analyzed by FACS to examine the expression pattern of mesoderm markers. We recommend you to investigate VEGFR2 expression but not PDGFRα as

PDGFRα is also expressed in OP9 stromal cells.

1. Prepare the cells as in **Subheading 3.3.3 (from Step 1 to 5)**.

2. Add an appropriate concentration of PE-labeled or APC-labeled anti-VEGFR2 to cell suspension in NMS and incubate for 20 min on ice.

3. To wash the cells, add 10 ml of ice-cold HBSS/BSA into the cell solution and spin down it at 270*g* for 5 min at 4℃.

4. Repeat above washing once and resuspend cell pellet in 100μl of ice-cold HBSS/BSA/PI.

5. Analyze and sort VEGFR2$^+$ mesoderm cells **(Fig. 2B)** (see **Note 7**).

3.4. Differentiation into descendants of the mesoderm cells

3.4.1.1. Induction of bone cells

1. Gelatinize the wells of 24-well plate as in **Subheading 3.1.1**

2. Seed 1-3x10^3 ES-derived mesoderm cells purified by FACS into one well of gelatinized 24-well plate with 1 ml of bone cell differentiation medium.

3. Change a half of medium (~0.5ml) every 3 days.

4. The calcium deposit can be observed around on day 28 **(Fig. 2C)** (see **Note 8**).

3.4.1.2. Alizarin red staining:

To confirm the bone cell formation, specific staining is needed.

1. To fix the cells, add 1ml of 4%PFA into the well. Keep at room temperature for 10 min.

2. Wash twice by D-PBS, 5 min, room temperature.

3. Add 1 ml of Alizarin red staining solution and keep for 5 min at room temperature.

4. Quickly wash 5-6 times by D-PBS

5. Observation: Calcium deposit is stained to red color.

3.4.2.1. Induction of cartilage cells

1. Resuspend ES cell-derived mesoderm cells in cartilage cell differentiation medium at 8x10^6/ml concentration and put 10 μl of this solution on a well of 24-well plate.

2. Incubate in tissue culture incubator at 37℃ for 30 min.

3. Add slowly 1ml of pre-warmed cartilage cell differentiation medium with 10 ng/ml **TGFβ3** into the well of plate.

4. One week later, change completely the medium by pre-warmed cartilage cell differentiation medium with 10 ng/ml **BMP2**. Do **not** add **TGFβ3**.

5. Change a half of cartilage cell differentiation medium with 10 ng/ml **BMP2** (~0.5ml) every 3 days.

6. Analyze the cartilage generation on day 21 **(Fig. 2C)** (see **Note 8**).

3.4.2.2. Alcian blue staining:

To confirm the cartilage cell formation, specific staining is needed.

1. To fix the cells, add 1ml of 4%PFA into the well. Keep at room temperature for 10 min.
2. Wash twice by D-PBS, 5 min, room temperature.
3. Add 1ml of 3% glacial acetic acid into the well and keep for 5 min at room temperature.
4. Discard the glacial acetic acid and add 1 ml of Alcian blue staining solution.
Keep for 30 min at room temperature.
5. Quickly wash 5-6 times by 3% glacial acetic acid.
6. Observation: Muco-glycoprotin is stained to blue color.

3.4.3. Induction of hematopoietic cells.

3.4.3.1. Generation of primitive erythrocytes

1. To prepare 6-well plate with confluent OP9 stromal cells, one confluent OP9 10-cm dish is spilt to four 6-well plates three days before the experiment. Prepare VEGFR2$^+$ ES cell-derived mesoderm cells as in **Subheading 3.3.**
2. Resuspend $1x10^4$ VEGFR2$^+$ ES cell-derived mesoderm cells in 2 ml of hematopoietic cell differentiation medium and seed to a well of 6-well plate with the 90% confluent OP9 stromal cell.
3. Analyze primitive erythrocytes that appeared after 3-4d.

3.4.3.2. Generation of definitive hematopoietic cells

1. Prepare 6-well plate with 90% OP9 stromal cells and VEGFR2$^+$ ES cell-derived mesoderm cells as in **Subheading 3.4.3.1. (Step1)**
2. Resuspend $1x10^4$ VEGFR2$^+$ ES cell-derived mesoderm cells in 2 ml of hematopoietic cell differentiation medium with 10 ng/ml hIL3 and 100 ng/ml SCF.
3. Seed it to a well of 6-well plate with the 90% confluent OP9 stromal cell.
4. Change the medium every 3-4d. In general, TER119$^+$ definitive erythroid cells initially appear in culture after 3d. Gr-1$^+$ mature myeloid cells appear after 5 to 7d. To confirm the definitive erythropoiesis, the expression of β-hemoglobin gene in the culture is examined (**Table 1** and **Fig. 2D**).

3.4.4. Endothelial cell colony assay

1. One confluent OP9 10-cm dish is spilt to four 24-well plates three days before the experiment. Prepare VEGFR2$^+$ ES cell-derived mesoderm cells as in **Subheading 3.3.**
2. Resuspend $5x10^2$-$1x10^3$ VEGFR2$^+$ ES cell-derived mesoderm cells in 2 ml of endothelial cell differentiation medium.
3. Seed it to a well of 24-well plate with the 90% confluent OP9 stromal cell.
4. Sheets of endothelial cells growing on OP9 stromal cells can be observed after 3d

(**Fig. 2E**) (see **Note 9**).

4. Notes

1. Test 10 different sera for ES and OP9 stromal cells. Select a serum lot that supports a good growth rate of ES and OP9 cells. Refer to Methods **subheading 3.1.3. and 3.2.2.** for the growth rates of ES and OP9 cells, respectively. Cell and colony morphologies are also the key factors to select a good serum.
2. FBS is a critical factor to induce a high rate of PDGFRα^+ and VEGFR2$^+$ cell induction. Lot no. checks of sera are highly recommended for finding appropriate serum lot. They are usually examined by the induction rate of ES cell-derived mesoderm cells. In general, using 20 different sera lots, the frequency of PDGFR2$^+$ and VEGFR2$^+$ cells generated after 4d under the condition without OP9 ranged from 30-60 %.
3. The medium for differentiation is used less than 4 wk as old medium affects the induction rate of mesoderm cells.
4. ES cells have to be plated as single cells, otherwise, ES cells will differentiate even in the presence of LIF. A long term culture (more than 2 wk) induces ES cells to accumulate genetic mutation including chromosomal abnormality. Therefore, thaw new ES cells every 2-3 wk.
5. The condition of OP9 stromal cells influences the generation rate of ES cell-derived mesoderm cells. OP9 cells should not be cultured for longer than 1 mo after thawing. OP9 cells may loose the ability to support ES cell differentiation as they easily differentiate into adipocytes by the long time culture. In addition, High passage (>20 passages) easily induce ES cells to be transformed and may loose their ability to support the in vitro ES cell differentiation.
6. In the case of CCE ES cells, 50-60% of d4 differentiated ES cells express PDGFRα and VEGFR2 (***16***).
7. To confirm the ES cell-derived mesoderm cells, the gene expression specific for paraxial and lateral mesoderm cells are examined by quantitative RT-PCR (qPCR). We use Tbx6 and Mesp2, and GATA2 and Tal1 for paraxial and lateral mesoderm markers, respectively (see **Table 1** and **Fig. 2B**) (***16***). GAPDH is used as a control (see **Table 1**).
8. The qPCR method is useful for examining the presence of bone and cartilage cells. Several markers such as Bglap 1 and 2, and Col2a1 and Col10a1 are suitable for defining the bone and cartilage cell lineages, respectively (see **Table 1** and **Fig. 2C**) (***16, 19***).

9. Immuno-cytostaining is the easiest method for clarifying the presence of endothelial colonies in the culture. We routinely examine the expression of either VE-cadherin or PCAM-1(CD31) as the markers for endothelial cells (***16***, ***18***).

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Figure legends

Fig. 1 Differentiation pathway of mesoderm in *in vitro* ES cell culture.

The analyses of differentiated ES cells reveal the three types of mesoderm cells, PDGFRα^+VEGFR2$^+$ population (PDGFRα and VEGFR2 double positive population, DP), PDGFRα^+VEGFR2$^-$ population (PDGFRα single positive population, PSP) and PDGFRα^-VEGFR2$^+$ population (VEGFR2 single positive population, VSP). The DP is the most immature and can give rise to both the PSP and the VSP. Both the VSP and the PSP exhibit the specific properties of paraxial and lateral mesoderm respectively,

Fig. 2 Fate of the ES cell-derived mesoderm cells

(A) Day4 ES cell differentiation. CCE ES cells are cultured on type IV collagen-coated dishes with the differentiation medium in the absence of LIF. Four days after the induction, differentiated ES cells are harvested and the expression of PDGFRα and VEGFR2 are examined by FACS. Four populations (PDGFRα^+VEGFR2$^+$,DP; PDGFRα^+VEGFR2$^-$, PSP; PDGFRα^-VEGFR2$^+$, VSP; PDGFRα^-VEGFR2$^-$, DN) are observed in day 4 differentiated ES cells. **(B)** Marker expression by quantitative RT-PCR (qPCR). PSP expresses the markers specific for paraxial mesoderm such as Tbx6 and Mesp2. In contrast, VSP expresses the markers specific for lateral mesoderm such as GATA2 and Tal1, suggesting that it represents lateral mesoderm in actual embryo. Black and white squares indicate the expression in

PSP and VSP, respectively. **(C)** Marker expression of bone and cartilage cells. The ES cell-derived mesoderm populations are cultured under distinct conditions that allow the differentiation of osteocytes or chondrocytes. After differentiation, RNA is purified and the expression levels of individual specific markers are measured by qPCR. Culture cells derived from the PSP expresses osteogenesis-(Bglap1 and Bglap2, **left panel**) and chondrogenesis-(col2a1 and col10a1, **right panel**) related genes at higher level than that of the VSP. Black and white squares indicate the expression in PSP and VSP, respectively. **(D)** The expression level of βHI in the cultures of PSP and VSP. The expression level of βHI is measured by qPCR and normalized by GAPDH expression level. Culture of VSP exhibits the higher expression of βHI than that of PSP. This suggests that VSP generates hematopoietic cells more efficiently than PSP. **(E)** The number of endothelial colonies derived from different mesoderm populations. 500 sorted cells are cultured on confluent OP9 cell layer for three days. Endothelial colonies are visualized by VE-cadherin immunostaining. The number of VE-cadherin$^+$ colonies is counted in each well of 24-well plates. (Error bars = SD). The frequency of endothelial progenitors in the PSP is a quarter of those of the VSP.

Table 1 Primers for quantitative RT-PCR

Gene		Sequence
GAPDH	sense	5'-GGAGCGAGACCCCACTAACA-3'
	antisense	5'-GCCTTCTCCATGGTGGTGAA-3'
Tbx6	sense	5'-CCCAACTATGCAGCCAACACT-3'
	antisense	5'-CTGTGTGATCCTAGGGTTCTGGTA-3'
Mesp2	sense	5'-CTGAAAACCTTGGGAACAGGAT-3'
	antisense	5'-GGCTCTTTCTAGGGACTGGTGTAA-3'
GATA2	sense	5'-CGGCCTCTTCTTCTGCAGG-3'
	antisense	5'-TGGTACTTGACGCCATCCTTG-3'
Tal1	sense	5'-CCCACCAGACAAGAAACTAAGCA-3'
	antisense	5'-GGCCAGGAAATTGATGTACTTCA-3'
Bglap1	sense	5'-GAGGACCATCTTTCTGCTCACTCT-3'
	antisense	5'-GACATGAAGGCTTTGTCAGACTCA-3'
Bglap2	sense	5'-GCGCTACCTTGGAGCTTCAG-3'
	antisense	5'-CATACTGGTTTGATAGCTCGTCACA-3'
Col2a1	sense	5'-CCTTGGACGCCATGAAAGTT-3'
	antisense	5'-CTTGCTGCTCCACCAGTTTTT-3'
Col10a1	sense	5'-CCTGGTTCATGGGATGTTTTATG-3'
	antisense	5'-TGGCGTATGGGATGAAGTATTG-3'
βH1	sense	5'-TGTTTACCCATGGACTCAGAGATTC-3'
	antisense	5'-CTTTCTTGCCATGGGCTCTAA-3'

Figure 1 Era

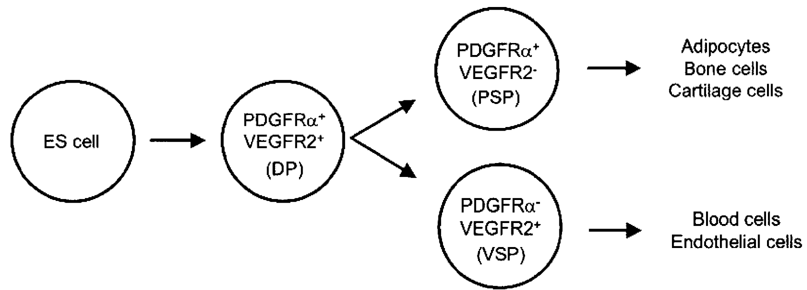
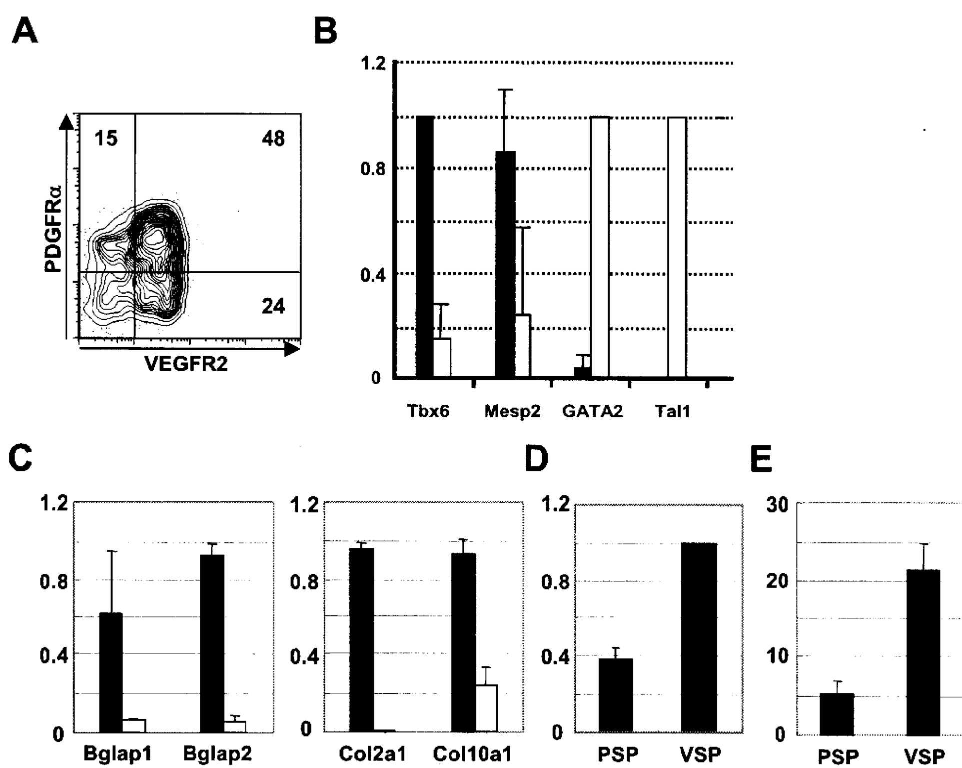


Figure 2 Era



Cell Stem Cell
Brief Report

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Generation of Induced Pluripotent Stem Cells from Human Terminally Differentiated Circulating T Cells

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The direct reprogramming of somatic cells to produce induced pluripotent stem cells (iPSCs) is a prominent recent advance in stem cell biology (Takahashi and Yamanaka, 2006). Generation of iPSCs without genomic integration of extrinsic genes is highly desirable. Initially, human dermal fibroblasts were used to derive human iPSCs (hiPSCs) (Takahashi et al., 2007; Yu et al., 2007). However, recent studies have shown that other human somatic stem cells can be used (Aasen et al., 2008; Eminli et al., 2009; Kim et al., 2009; Ye et al., 2009). It is difficult to obtain human somatic stem cells, but human terminally differentiated circulating T cells (hTDCTCs) are readily available from peripheral blood. Here, we show that a combination of activated T cell cultivation and a temperature-sensitive mutated Sendai virus (SeV) that encodes human OCT3/4, SOX2, KLF4, and c-MYC allows the generation of hiPSCs easily, efficiently, and safely within a 1 month time frame.

Sampling of peripheral blood is one of the least invasive procedures performed routinely in clinics, and surplus peripheral blood samples are often left unused after clinical examinations. Among peripheral blood mononuclear cells (PBMCs), T cells can be readily cultured in vitro by means of a plate-bound anti-CD3 monoclonal antibody and recombinant (r)IL-2 (Desai-Mehta et al., 1996), and we used such an approach to expand hTDCTCs from peripheral blood samples. From 1 ml of whole blood, PBMCs were separated on a Ficoll gradient and then cultured with plate-bound anti-CD3 monoclonal antibody and rIL-2 (Figure 1A). Although PBMC fractions contain lymphocytes and monocytes, T cells are selectively cultured under these conditions. In culture, the number of activated T cells increased gradually but consistently. Five days after blood sampling, the cultured cells were morphologically identical to pure CD3-positive T cells collected by fluorescence-activated cell sorting (FACS) (Figure 1B). We used a whole-PBMC culture method because it is technically simpler than FACS, in which the sorted cells are frequently damaged by laser emission and the process of single-cell sorting.

To avoid transgene integration during iPSC generation, we used an SeV vector, which is a minus-strand RNA virus that is not integrated into the host genome and is not pathogenic for humans (Li et al., 2000). We used a temperature-sensitive mutated SeV vector in these experiments to reduce transgene expression and SeV residue in generated lines. This form of SeV vector generates weaker transgene expression and cannot proliferate at standard culture temperatures (data not shown). SeV can be efficiently transduced into human T cells and can express exogenous genes (Okano et al., 2003). We first introduced green fluorescent protein (GFP) into human T cells by SeV in a dose-dependent manner; toxicity for the infected cells was minimal at the virus dosages used (Figure 1C). To generate iPSCs from hTDCTCs, we used SeV to deliver multiple transgenes that encoded stem cell-specific transcription factors, such as *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*, into cells on day 6 of culture. Two days after gene introduction, the cells were replated onto feeder layers of SNL cells. On day 9, the cells were transferred to human ES cell (ESC) medium that contained 4 ng/ml bFGF. Within 3 weeks of infection, we identified a colony that resembled human ESCs (hESCs) among the T cell derivatives. On day 25, colonies that were larger and morphologically similar to hESC-like colonies were picked (Figure 1D). Of these initial colonies, which were identified by crystal violet staining, most were positive for alkaline phosphatase (ALP), which is a characteristic marker of stem cells (Figure 1E). T cells that had been transfected with SeV vectors carrying *OCT3/4, SOX2, KLF4,* and *c-MYC* were plated onto mitomycin C-treated SNL feeder cells at 5×10^4 cells per 10 cm dish. Around day 25 after blood sampling, the number of ALP-positive hESC-like colonies was counted and approximately 50 colonies were observed at MOI 20 (Figure 1E) (an efficiency of 0.1%). Moreover, the efficiency of iPSC colony generation was dependent upon the dosage of virus used for gene introduction (Figure 1F). We named these established T cell-derived iPSCs as "TiPS cells (TiPSCs)." After expansion, the cloned TiPSCs displayed typical hESC/iPSC morphology and had a normal karyotype (Figures S1A and S1B available online).

To confirm that the TiPSCs had the characteristics of typical ESC/iPSCs, we

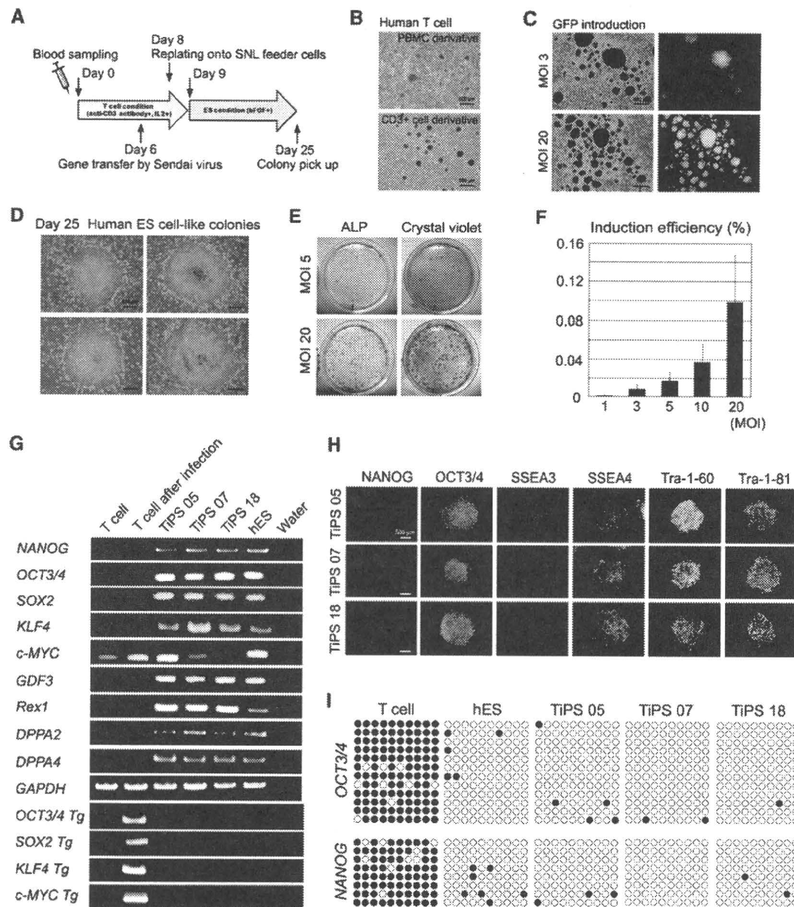

Figure 1. hTDCTCs-Derived iPSC Colonies

(A) Strategy used in the present study for reprogramming T cells.

(B) Morphologies of T cells derived from whole PBMCs or FACS-sorted T cells grown in the presence of CD3 antibody and rIL2.

(C) Efficient GFP introduction by SeV in T cells transfected at an MOI of 3 or MOI of 20.

(D) Typical ESC-like iPSC colonies on day 25 after blood sampling.

(E) Examples of 10 cm dishes stained for ALP on day 25, showing numerous ALP-positive colonies of T cells that were transfected at an MOI of 5 or MOI of 20.

(F) Numbers of ALP-positive colonies in relation to multiplicity of infection.

(G) RT-PCR analyses for the hESC marker genes *NANOG*, *OCT3/4*, *SOX2*, *KLF4*, *c-MYC*, *GDF3*, *REX1*, *DPPA2*, and *DPPA4* and the transgenes *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*.

(H) Immunofluorescence staining for pluripotency and surface markers (NANOG, OCT3/4, SSEA3, SSEA4, TRA-1-60, and TRA-1-81) in TiPS 05, 07, and 18. Scale bars represent 500 μm.

(I) Bisulfite sequencing analysis of the *NANOG* and *OCT3/4* promoter regions in peripheral T cells, hESCs, and hTiPSCs 05, 07, and 18. Each row of circles for a given amplicon represents the methylation status of the CpG dinucleotides in one bacterial clone for that region. Open circles represent unmethylated CpGs; closed circles represent methylated CpGs.

See also Figure S1 and Table S1.

examined stem cell marker expression. Reverse-transcription PCR (RT-PCR) analyses revealed that the TiPS 05, 07, and 18 clones expressed ESC marker transcripts for *NANOG*, *OCT3/4*, *SOX2*, *KLF4*, *c-MYC*, *GDF3*, *REX1*, *DPPA2*, and *DPPA4*. The original T cells also expressed *c-MYC* at a basal level, as previously reported (Douglas et al., 2001). In the TiPSCs, the *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* transgenes were lost after several passages (Figure 1G; Figure S1C). Immunostaining revealed that the TiPSCs expressed the Nanog, Oct3/4, SSEA3, SSEA4, Tra-1-60, and Tra-1-81 proteins (Figure 1H). High telomerase activity is also an important characteristic of iPSCs, and, appropriately, TiPSCs showed high levels of telomerase activity (Figure S1D). Another signature of iPSCs is epigenetic remodeling. We used bisulfite sequencing to examine the methylation status of the *NANOG* and *OCT3/4* promoters. T cells, which do not express *NANOG* or *OCT3/4*, showed mostly methylated CpGs in those promoters. hESCs, which do express *NANOG* and *OCT3/4*, showed unmethylated CpGs in those promoters. As in hESCs, the CpGs in these promoter regions were predominantly unmethylated in the TiPSCs (Figure 1I). These results suggest that SeV-mediated gene transfer successfully reprograms hTDCTCs.

Somatic recombination of T cell receptor (TCR) genes generates a diverse T cell repertoire that allows adaptation for antigen responses (Krangel, 2009). To confirm that the TiPSCs were derived from hTDCTCs, we analyzed TCR rearrangements. A hallmark of the TCR-β locus is developmentally ordered recombination, with Dβ-to-Jβ recombination preceding Vβ-to-DβJβ recombination. We performed capillary electrophoresis of the PCR products for the genomic DNA of the TCR-β regions. As a positive control, we used monoclonal T cells, which are derived from patients with lymphocyte malignancies and show a specific peak, because these T cells have only a single genetic variation in their TCR regions (Figure S2A). Peripheral T cells from people without lymphocytic diseases are polyclonal, with diverse genetic variations in their TCR rearrangements, and show a broad and low band without a specific peak. ESCs do not have TCR rearrangements and do not show a specific positive peak. The TiPS 05, 07, and 18 cell lines showed specific peaks for Dβ/Jβ recombination. TiPS 05 showed Vβ/Jβ2 recombination. TiPS 07 and TiPS 18 showed Vβ/Jβ1,2 recombination, albeit with different bands (Figure 2A). TCR rearrangement is specific for T cell development, so these results confirm that TiPSCs are derived from T cells. They also indicate that the TiPS 05, 07, and 18 lines originated from different T cells. We analyzed the rearrangement pattern of 10 independent TiPSCs and confirmed that every TiPSCs showed different rearrangement pattern (Figure S2A).

We also performed global gene expression analyses with DNA chips. Scatter plot analyses revealed global gene

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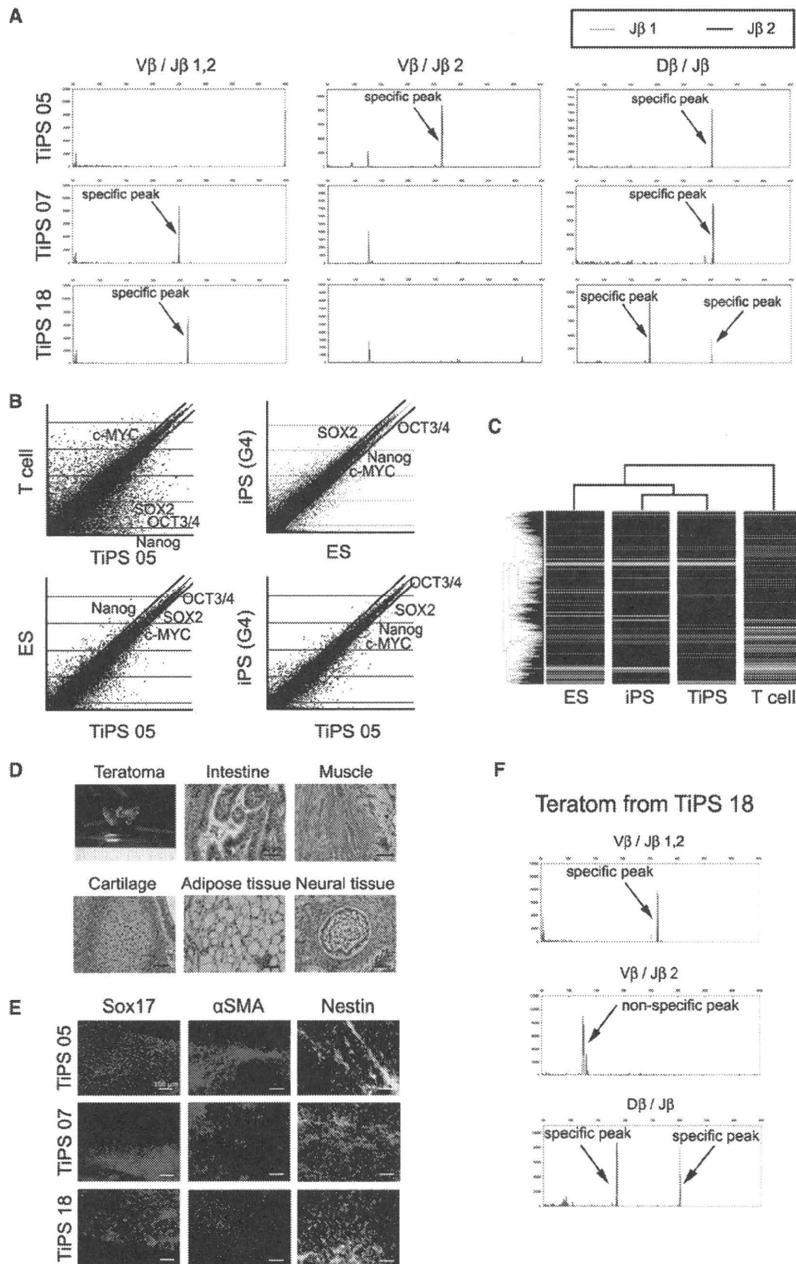

Figure 2. Detail Characterizations of TiPSCs

(A) Characterization of the TCR-β rearrangement by capillary electrophoresis. The green line is derived from the band for the Jβ1 gene, and blue line is derived from the band for the Jβ2 gene. TiPS 05 shows rearrangements of Vβ/Jβ2 and Dβ/Jβ. TiPS 07 shows rearrangements of Vβ/Jβ1,2 and Dβ/Jβ. TiPS 18 shows rearrangements of Vβ/Jβ1,2 and Dβ/Jβ.

(B) Scatter plots comparing the global gene expression profiles of T cells and TiPS cells, dermal fibroblast-derived iPSCs (G4) and ESCs, ESCs and TiPSCs, and dermal fibroblast-derived iPSCs (G4) and TiPSCs. The black lines indicate 2-fold differences in gene expression levels between the paired cell populations. The transcript expression levels are shown on a $\log_2$ scale. The expression levels of *NANOG*, *OCT3/4*, *SOX2*, and *c-MYC* are shown.

(C) Heat map analyses of hESCs, dermal fibroblast-derived iPSCs, TiPSCs, and the parental human T cells.

(D) Gross morphology, hematoxylin and eosin-stained representative teratomas derived from TiPS 05.

(E) Immunofluorescence staining for Sox17 (endodermal marker), αSMA (mesodermal marker), and Nestin (ectodermal marker) in each TiPSC-derived differentiated cell.

(F) Characterization of the TCR-β rearrangement for teratoma from TiPS 18.

See also Figure S2 and Table S2.

expression differences between peripheral T cells and TiPSCs. Comparison of hESCs and human dermal fibroblast-derived iPSCs, hESCs, and human TiPSCs (hTiPSCs), and dermal fibroblast-derived iPSCs and TiPSCs showed high levels of similarity (Figure 2B). Heat map analysis showed that the global gene expression profiles were overall similar in ESCs, dermal fibroblast-derived iPSCs, and TiPSCs, and different from T cells (Figure 2C). To further demonstrate the pluripotency of hTiPSCs, they were transplanted into the subcutaneous tissue of severe combined immunodeficient (SCID) mice. Six to eight weeks after injection, each TiPSC line tested gave rise to teratomas that contained derivatives of all three germ layers (Figure 2D; Figure S2B). We also examined the in vitro differentiation potential of TiPSCs. Each TiPSC line tested generated embryoid bodies that contained derivatives of all three germ layers (Figure 2E). These results indicate that hTiPSCs are pluripotent stem cells. Although it was reported that Trp53 null murine T cells could be reprogrammed into iPSCs (Hong et al., 2009), we have successfully reprogrammed wild-type human T cells. In our hands, the efficiency of conventional retrovirus-mediated gene transfer into wild-type human T cells was very low compared to SeV (data not shown). In our view, the efficiency of gene transfer is a major determining factor in successful iPSC generation.

With current technology, if iPSC-derived mature cells are transplanted into diseased patients, there is no good procedure for following their progeny, which could eventually form malignant or benign tumors. In animal models, several marker genes can be used to chart the progression and consequences of iPSC-derived mature cell transplantation, such as GFP and luciferase. However, it is not desirable to insert exogenous marker genes into the genomes of hiPSCs for clinical use. TiPSCs, however, already have a traceable genetic signature through TCR locus rearrangement. Consistent with this idea, teratomas derived from TiPSCs had

same signature as undifferentiated TiPSCs (Figure 2F; Figure S2C). Therefore, the descendents of TiPSCs can be identified by analyzing their TCR rearrangement patterns.

In conclusion, we have developed a minimally invasive method for hiPSC generation without genomic integration that uses low numbers of hTDCTCs from peripheral blood. This method has advantages for research into stem cell reprogramming, TCR rearrangement, immunologic disorders, and the development of genetic markers for future applications of regenerative medicine. TiPSCs may well be relatively easy to use in a clinical setting.

ACCESSION NUMBERS

The microarray data have been deposited in GEO and given the series accession number GSE22088.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and two tables and can be found with this article online at doi:10.1016/j.stem.2010.06.003.

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Note Added in Proof

A manuscript has appeared online demonstrating isolation of iPSCs from peripheral blood, including a single line that showed evidence for both TCR-β and TCR-γ rearrangement by PCR (Kunisato, A., Wakatsuki, M., Shinba, H., Ota, T., Ishida, I., and Nagao, K. [2010]. Direct generation of induced pluripotent stem cells from human non-mobilized blood. Stem Cells Dev., in press. Published online May 24, 2010. 10.1089/scd.2010.0063).

センダイウイルスベクターによるiPS細胞作製法

Generation of iPS cells using non-integrating Sendai virus vectors

房木 ノエミ

Key Words：iPS細胞，センダイウイルスベクター，安全性

Abstract

ディナベック株式会社は「染色体を傷つけない高効率な新しいiPS細胞作製法」のツールとして，当社独自の細胞質増殖型センダイウイルスベクターに，iPSアカデミアジャパンからライセンスを受けた山中初期化4因子(OCT3/4, KLF4, SOX2, c-MYC)を搭載したキットCytoTune™-iPSを本年6月より株式会社医学生物学研究所(MBL)を通じ，国内販売を開始している。本製品は，効率よくヒト線維芽細胞からiPS細胞を樹立できるだけでなく，染色体に外来遺伝子が組み込まれず，容易に外来遺伝子とベクターが除去出来ることを特徴としている。このため，従来のような組み込まれた遺伝子のコピー数や再活性化による影響に左右されずに，細胞の解析や分化実験が出来ることが大きなメリットである。

■はじめに

センダイウイルス（SeV）は1952年に東北大学にて当時の実験用マウスから単離された内在性ウイルスであり，地名にちなんでこの名前で呼ばれている。日本での正式名称は，日本で同定された血球凝集能をもつウイルスということで，HVJ (Hemagglutinating virus of Japan)である。SeVは非分節型の(-)鎖RNAをゲノムとして持つパラミクソウイルス亜科に分類される。なお，近縁種ではムンプスウイルスや麻疹ウイルス，ニューカッスル病ウイルスなどがあり，ヒトや家畜に対する病原体が含まれるが，SeVは野生型ではマウス，ラット等げっ歯類を宿主として呼吸器へ感染するウイルスであり，ヒトやサルといった霊長類には病原性の報告はない。全長15,384塩基のSeVゲノムRNAには図１Aに示すように，3'側から順にRNAゲノムと結合して鋳型活性を安定化させるヌクレオカプシドタンパク質(N)，RNAポリメラーゼの小サブユニットであるリン酸化タンパク質(P)，ウイルス粒子構造を内側から維持するマトリックスタンパク質(M)，そして宿主細胞への侵入に関わる膜融合タンパク質（F）と細胞への結合に関わる赤血球凝集素/ノイラミニダーゼ（HN），最後に巨大（ラージ）タンパク質（L）をコードする遺伝子が並ぶ。さらに，P遺伝子からは異なる読み枠を利用して非構造タンパク質CとVが作られる。SeVはウイルス粒子上のFやHN蛋白によって，哺乳類および鳥類などの殆どの細胞種に容易に感染することが可能であることから，歴史的には膜融合といったツールとして幅広く使われてきた[1]。

我々のグループは，十数年前にSeVをウイルスベクターとして開発することに成功し[1]，現在このベクターの，①受容体がシアル酸タンパク質であるため，幅広い動物種に対する高い感染能，②感染後も細胞質で複製するため非常に高い外来遺伝子発現量を誇る，③生活環においてすべてがRNA相であるため宿主ゲノムに組み込まれる恐れがない（図１B），といった優れた特徴を活かし，遺伝子治療・遺伝子ワクチン製剤の開発，抗体発現ツール，分子生物学的な研究ツールとしての提供などを行っている。また，用途に合わせて安全性を高めるための欠失型や，遺伝子発現量をコントロールできる数多くのベクターバックボーンを用意している。なお，センダイウイルスベクターに関する基本特許は，日本を含め世界の主要国で成立しており，ディナベック株式会社がその権利を独占的に保有している。

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