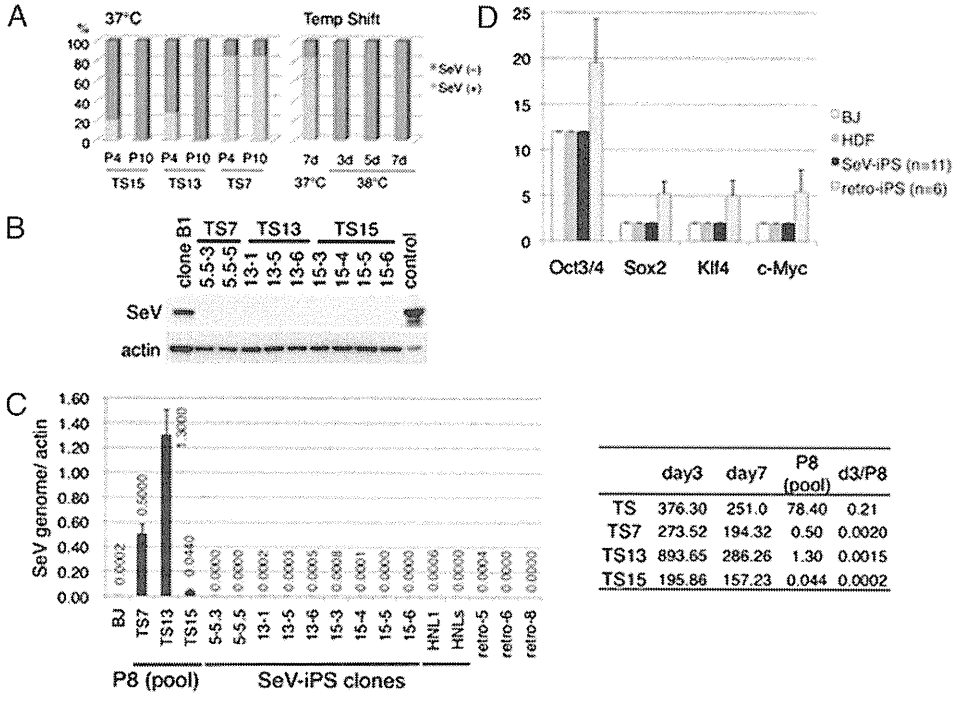

	day3	day7	P8 (pool)	d3/P8
TS	376.30	251.0	78.40	0.21
TS7	273.52	194.32	0.50	0.0020
TS13	893.65	286.26	1.30	0.0015
TS15	195.86	157.23	0.044	0.0002

Fig. 3. (*A*) Ratio of SeV-positive colonies. P, passage number. (*Left*) Randomly chosen colonies were expanded independently, and the existence of SeV vectors was evaluated by qRT-PCR at P4 and P10. The number of positive and negative colonies was counted and is expressed for each as a ratio of all colonies chosen (n = 12 per each TS vector). (*Right*) Temperature shift to a nonpermissive temperature of 38 °C effectively removed SeV vectors from the iPSC colonies generated using TS7 vectors. The culture dishes at P4 were split and transferred to culture at 37 or 38 °C for the number of days indicated. The ratio was calculated as left panel. (*B*) SeV proteins were not detected in iPSC colonies by Western blot analysis with anti-SeV antibodies. Clone B1, positive control for iPSCs in which the SeV persisted (17); control, LLC-MK2 cells transfected with plasmids encoding SeV vectors. 5.5-3 and 5.5-5 were generated with MYC/TS7; 13-1, 13-5, and 13-6 were generated with MYC/TS13; and 15-3, 15-4, 15-5, and 15-6 were generated with MYC/TS15. (*C*) qRT-PCR of viral genome in SeV-generated iPSCs (viral genome/actin). BJ, parental fibroblasts; day 3 and day 7, BJ cells infected with SeV vectors after 3 and 7 d; HNL1 and HNLs, iPSCs established using conventional SeV (17). P8, pooled colonies at passage 8. Values <0.001 were backgrounds under the calculation curve as detected in parental cells. (*D*) Copy numbers of *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* in parental cells [BJ and human dermal fibroblast (HDF) cells] and iPSCs generated by SeV (SeV-iPS; n = 11) or retrovirus (retro-iPS; n = 6), as determined by qRT-PCR of genomic DNA. The passage numbers of tested clones are listed in Table S3.

an MOI of only 2 was sufficient to generate iPSCs from the CB cells described below, possibly because of the highly efficient gene transfer into human CB stem cells (19). We infected freshly isolated mononuclear cells with the GFP construct at an MOI of 2 and found that GFP expression was limited to the $CD34^+$ fraction (43% of the $CD34^+$ cells were GFP^+ cells), whereas no GFP^+ cells were found in the $CD34^-$ fraction at 2 d after SeV infection (19). The rate of SeV infection determined by GFP expression in $CD34^+$ cells was increased up to 100% if we used SeV vectors at an MOI of 20. This fraction corresponds to hematopoietic stem cells or progenitors, as reported elsewhere (28). Then freshly isolated purified $CD34^+$ CB cells (purity of $CD34^+$, 96–99%) or the frozen $CD34^+$ CB cells (RIKEN BioResource Center; purity of $CD34^+$, 97–99%), thawed 1 d before infection, were used for SeV infection. These cells were treated with a SeV vector mixture consisting of SeV TS7-*OCT3/4*, *-SOX2*, *-KLF4*, and *-c-MYC*. Infected cells were incubated under hematopoietic cell culture conditions, followed by cocultivation on mitomycin C (MMC)-treated SNL cells on day 4 or day 10 (Fig. 4*C*). hESC-like colonies expressing SSEA-4 appeared after 14 d of cocultivation with SNL (18 d after SeV infection), whereas no colonies were obtained after transfer onto SNL on day 10 (Table S2). The efficiency with which iPSC colonies emerged on SNL feeder cells using SeV TS7 vectors is also shown in Table S2. To eliminate remaining SeV virus constructs, ESC-like colonies were subjected to heat treatment at 38 °C for 3 d and recloned. SeV-positive cells constituted 60–80% of total cells within hESC-like colonies before heat treatment (clones SeV iPSC 4, 15, and 17; passage 2). This was reduced to 0% after recloning (clones SeV iPSC 4A, 15A, and 17A; passage 4), followed by the heat treatment. The percentages of SeV-positive cells were determined by the proportion of sum of the area positively detected with anti-SeV HN (envelope) antibody against total colony area. In the agreement with this staining result, SeV constructs were not detected by qRT-PCR at passage 4 after heat treatment (Fig. 4*E*). The virus-negative ESC-like clones showed the expression of hESC markers (Fig. S5 *A* and *B*), global gene expression profile similar to that of hESCs (Fig. S5 *C* and *E*), and normal karyotype (Fig. S5*D*). Seven established cell clones were tested for embryoid body–mediated in vitro differentiation potential, and three out of the seven clones were found to have in vivo differentiation potential in a teratoma formation assay with SCID mice. All three of these clones were able to give rise to cells of all three germ layers as detected by immunocytochemistry and cell morphology studies (Fig. S5*F*). These three lines formed the teratomas with a cystic mass containing differentiated tissues morphologically corresponding to all three germ layers (Fig. S5*F*). The characterization of established clones from fibroblasts and CB cells is summarized in Table S3.

Discussion

The present study has demonstrated that the established TS SeV vectors TS15, TS13, and TS7 are highly effective tools with which we were able to obtain transgene-free human iPSCs. These iPSCs were generated efficiently by robust gene replication, with a subsequent rapid decrease in the level of factor-carrying SeV vectors

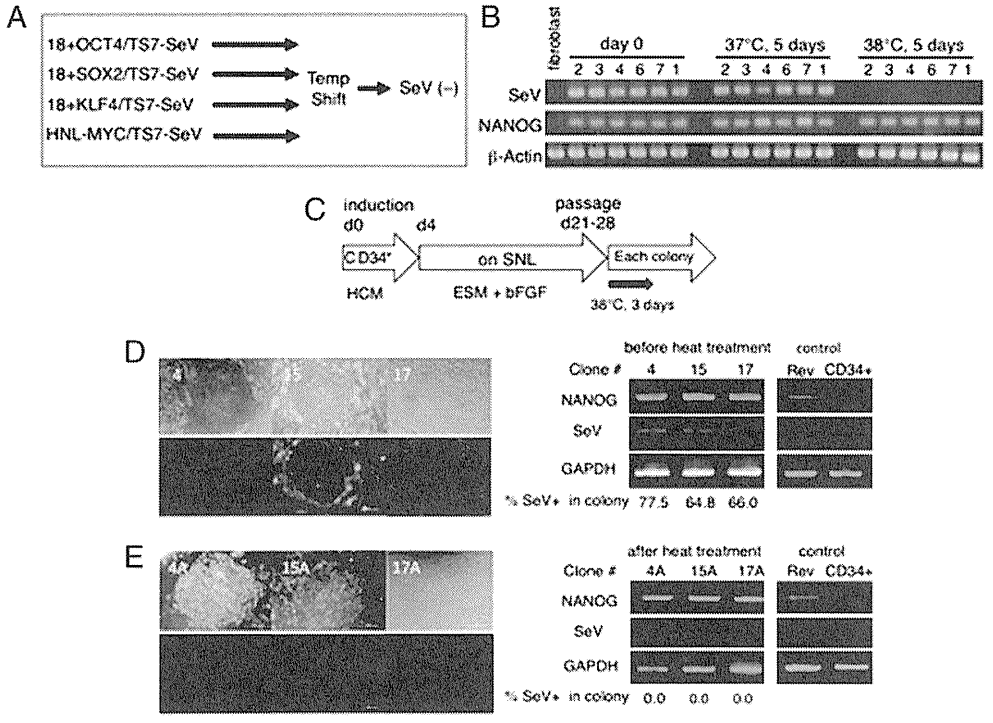

Fig. 4. Our second strategy with temperature-shift treatment. (*A*) Schemes to remove viral genome. (*B*) qRT-PCR of the SeV genome and hESC markers before and after temperature-shift treatment. iPSCs generated from HDF cells using TS7 vectors were treated with a temperature shift to 38 °C for 5 d. The SeV genome was not detected after this temperature-shift treatment, whereas expression of *NANOG* was not affected. (*C*) Schemes for generation of virus vector-free iPSCs from $CD34^+$ CB cells with SeV vectors. The ESC-like colonies 4, 15, and 17 that emerged were subcloned after heat treatment for 3 d at 38 °C. (*D* and *E*) The remaining SeV construct in SeV iPSC 4, SeV iPSC 15, and SeV iPSC 17 (*D*; passage 2, before heat treatment) and heat-treated subclone SeV iPSC 4A (from 4), SeV iPSC 15A (from 15), and SeV iPSC 17A (from 17) (*E*; passage 4) were determined by immunostaining with anti-HN antibody (*Left*) and by qRT-PCR against endogenous *NANOG* transcript and SeV RNA construct (*Right*). Retrovirally generated iPSCs from CB cells (Rev) and $CD34^+$ CB cells (CD34) were used as controls. The percentage of SeV-positive cells in the respective colony was determined using a two-value recognition function and is given below the qRT-PCR image.

during cell expansion (Fig. 2*C*). Almost all of the colonies picked up were virus-negative at late passages, and even if vectors were present, they could be easily removed using the temperature-shift protocol (Figs. 3*A* and 4). Indeed, the iPSCs generated using TS SeV vectors were free of any integrated viral factors, in contrast to retroviral-generated iPSCs, which express variable copy numbers of *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* (Fig. 3*D*). We also demonstrated that using TS SeV vectors, virus-negative iPSCs were efficiently generated from $CD34^+$ human CB cells (Fig. 4).

Although the use of synthetic modified mRNA to generate iPSCs has been reported, this is highly dependent on the gene delivery system, because it requires repetitive transfection for 16 d (13). Therefore, it might not be applicable to difficult-to-transfect cells, such as primary peripheral blood cells. The addition of decoy receptor to prevent IFN production by host cells is also needed. In contrast, SeV vectors require no transfection reagents or decoy receptors. We suggest that this is a considerable advantage to using nonintegrating SeV; the iPSCs thus generated have a homogeneous genetic background. Several studies on the generation of iPSCs from blood cells and CB cells with integration-free episomal vectors have been published recently (29–31). The efficiency of generating iPSCs is lower with episomal vectors than with SeV (>0.1% at an MOI of 2) and varies depending on the construction of the vectors and the nature of the transfection medium used. The major advantage of using nonintegrated SeV vectors is a potent and robust protein-expressing property that does not require optimization of the transfection medium. The volume of collected CB is 80–120 mL and may contain 2–4 × 10^5 $CD34^+$ cells on average. Our results show that only 1 × 10^4 $CD34^+$ cells, corresponding to ~5 mL of CB, are needed to obtain 10 independent iPSC clones with SeV. The rest of the CB can be sorted and used for the regular bone marrow transplantation therapy. In addition, there have been no reports of pathogenicity associated with SeV in primates, and the safety of the SeV vector is further enhanced by the F-deficiency that makes the vector nontransmissible (16).

In the present study, we have confirmed that the SeV vectors were not reactivated or detected in iPSCs at late passages or after temperature-shift treatment (Figs. 1*C* and 3). It has been suggested that TS mutations in *P* and *L* affect polymerase activity and promote degradation of the virus vector after treatment at nonpermissive temperatures (23). Based on our findings, we believe the TS SeV vector system that allows the elimination of remaining SeV construct from reprogrammed cells by temperature-shift treatment could accelerate future clinical application of iPSCs generated from cells obtained by less invasive or even noninvasive methods.

Materials and Methods

The induction of human iPSCs was done as reported previously (1, 17). Unless indicated otherwise, SeV vectors (*OCT3/4, SOX2, KLF4,* and *c-MYC*) were used at an MOI of 3 or 30 for 1 × 10^6 fibroblasts. For retroviral induction, fibroblasts were infected with mCAT containing simian immunodeficiency virus (SIV) vector at an MOI of 50, and then expanded by induction with retrovirus vectors containing reprogramming factors. Infected cells were transferred onto MMC-treated mouse embryonic fibroblast (MEF) feeder cells on day 6 after induction. Details of the experimental procedures are provided in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank Satomi Nishikawa and K. Kobayashi for useful discussions; T. Aoi for RNA from hESCs; A. Iida for a critical reading of

the manuscript; T. Kitamura for pMX vector; RIKEN BioResource Center for CD34$^+$ CB cells; S. Seino, T. Kanaya, K. Washizawa, T. Fujikawa, E. Suzuki, and T. Yamamoto for technical assistance; H. Iwasaki for help with the histological studies; and T. Ofuji and M. Miyako for help with manuscript editing. This work was supported in part by the Japan Science and Technology Agency's Precursory Research for Embryonic Science and Technology and S-Innovation Programs, Regional Consortium Project Grant 19K5510 from the Ministry of Industry and Economy (2007–2009), Grant-in-Aid 2210853 from the Japan Society for the Promotion of Science Fellows, and Japan Science and Technology Agency "Tests for Safety Issue of Pluripotent Stem Cell" (2010-14).

1. Takahashi K, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872.
2. Yu J, et al. (2007) Induced pluripotent stem cell lines derived from human somatic cells. *Science* 318:1917–1920.
3. Okita K, Nakagawa M, Hyenjong H, Ichisaka T, Yamanaka S (2008) Generation of mouse-induced pluripotent stem cells without viral vectors. *Science* 322:949–953.
4. Yu J, et al. (2009) Human induced pluripotent stem cells free of vector and transgene sequences. *Science* 324:797–801.
5. Soldner F, et al. (2009) Parkinson's disease patient–derived induced pluripotent stem cells free of viral reprogramming factors. *Cell* 136:964–977.
6. Sommer CA, et al. (2009) Induced pluripotent stem cell generation using a single lentiviral stem cell cassette. *Stem Cells* 27:543–549.
7. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K (2008) Induced pluripotent stem cells generated without viral integration. *Science* 322:945–949.
8. Woltjen K, et al. (2009) piggyBac transposition reprograms fibroblasts to induced pluripotent stem cells. *Nature* 458:766–770.
9. Kaji K, et al. (2009) Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 458:771–775.
10. Jia F, et al. (2010) A nonviral minicircle vector for deriving human iPS cells. *Nat Methods* 7:197–199.
11. Zhou H, et al. (2009) Generation of induced pluripotent stem cells using recombinant proteins. *Cell Stem Cell* 4:381–384.
12. Kim D, et al. (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. *Cell Stem Cell* 4:472–476.
13. Warren L, et al. (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. *Cell Stem Cell* 7: 618–630.
14. Lamb RA, Kolakofsky D (1996) Paramyxoviridae: The viruses and their replication. Fields Virology, eds Fields BN, Knipe DM, Howley PM (Lippincott-Raven, Philadelphia), 3rd Ed, pp 1177–1204.
15. Li HO, et al. (2000) A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. *J Virol* 74:6564–6569.
16. Nagai Y, Takakura A, Irie T, Yonemitsu M, Gotoh B (2011) Evolution of Sendai virus: The journey from mouse pathogen to a state-of-the-art tool in virus research and biotechnology. The Paramyxoviruses, ed Samal SK (Horizon Scientific Press, Norwich, UK).
17. Fusaki N, Ban H, Nishiyama A, Saeki K, Hasegawa M (2009) Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. *Proc Jpn Acad, Ser B, Phys Biol Sci* 85:348–362.
18. Seki T, et al. (2010) Generation of induced pluripotent stem cells from human terminally differentiated circulating T cells. *Cell Stem Cell* 7:11–14.
19. Jin CH, et al. (2003) Recombinant Sendai virus provides a highly efficient gene transfer into human cord blood–derived hematopoietic stem cells. *Gene Ther* 10:272–277.
20. Giorgetti A, et al. (2009) Generation of induced pluripotent stem cells from human cord blood using OCT4 and SOX2. *Cell Stem Cell* 5:353–357.
21. Takenaka C, Nishishita N, Takada N, Jakt LM, Kawamata S (2010) Effective generation of iPS cells from CD34$^+$ cord blood cells by inhibition of p53. *Exp Hematol* 38:154–162.
22. Bowman MC, Smallwood S, Moyer SA (1999) Dissection of individual functions of the Sendai virus phosphoprotein in transcription. *J Virol* 73:6474–6483.
23. Feller JA, Smallwood S, Skiadopoulos MH, Murphy BR, Moyer SA (2000) Comparison of identical temperature-sensitive mutations in the L polymerase proteins of Sendai and parainfluenza3 viruses. *Virology* 276:190–201.
24. Inoue M, et al. (2003) Nontransmissible virus-like particle formation by F-deficient Sendai virus is temperature-sensitive and reduced by mutations in M and HN proteins. *J Virol* 77:3238–3246.
25. Guo XM, et al. (2006) Creation of engineered cardiac tissue in vitro from mouse embryonic stem cells. *Circulation* 113:2229–2237.
26. D'Amour KA, et al. (2005) Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nat Biotechnol* 23:1534–1541.
27. Kawasaki H, et al. (2000) Induction of midbrain dopaminergic neurons from ES cells by stromal cell–derived inducing activity. *Neuron* 28:31–40.
28. Majeti R, Park CY, Weissman IL (2007) Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. *Cell Stem Cell* 1:635–645.
29. Chou BK, et al. (2011) Efficient human iPS cell derivation by a non-integrating plasmid from blood cells with unique epigenetic and gene expression signatures. *Cell Res* 21: 518–529.
30. Hu K, et al. (2011) Efficient generation of transgene-free induced pluripotent stem cells from normal and neoplastic bone marrow and cord blood mononuclear cells. *Blood* 117:e109–e119.
31. Yu J, Chau KF, Vodyanik MA, Jiang J, Jiang Y (2011) Efficient feeder-free episomal reprogramming with small molecules. *PLoS ONE* 6:e17557.

New Technology

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。

Noemi Fusaki, Ph.D
ディナベック株式会社 技術開発室 細胞工学グループ
Cell Engineering Group, Department of Technology Development, DNAVEC Corporation

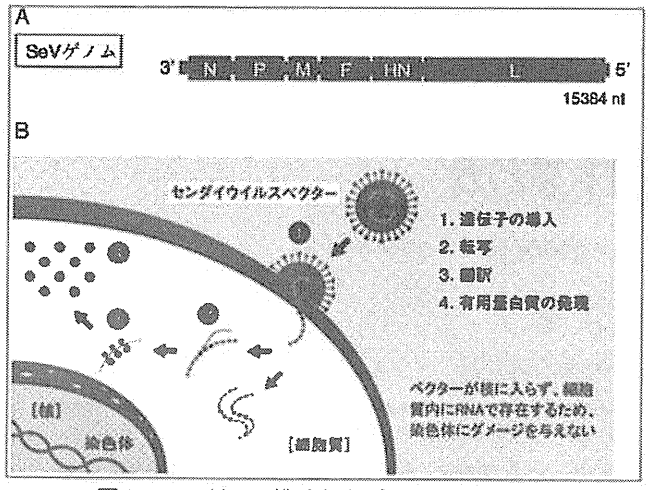

図1　SeVゲノム構造およびSeVベクター生活環

表　iPS細胞誘導法一覧

	ベクター/物質		備考
組込み型	レトロウイルスベクター	RNA&DNA	ランダム挿入
	レンチウイルスベクター		ランダム挿入
切り出し型	レンチウイルスベクター (Cre/LoxP)		一部残存
	トランスポゾン (piggyBac)	DNA	ヒト細胞では切り出し難い
非組込み型	アデノウイルスベクター		低効率/組込可能性あり
	プラスミド		低効率/組込可能性あり
	エピゾーマルベクター		
	バキュロウイルス		
	SeVベクター	RNA	高効率・1回の感染
	人工染色体		
	タンパク質		低効率・繰り返し導入
	改変RNA		高効率・繰り返し導入
その他	miRNA		
	化合物		

■iPS細胞作製への応用

体細胞に初期化因子（OCT3/4, SOX2, KLF4, c-MYC，あるいはNANOG, LIN28など）を導入することにより，数多くの組織に分化可能な，人工多能性幹細胞（iPS細胞）が作製できることは，2006年の京都大学・山中伸弥教授グループによるマウスiPS細胞および2007年のヒトiPS細胞樹立発表[2]以来，周知の通りである。この発明により，従来胚を破壊しなければ得られなかったヒトES細胞の倫理面の問題の克服や，個別化医療への応用の可能性など数多くの再生医療の可能性が現実のものとなってきた。さらに，望むヒト分化細胞を比較的容易に実験室で調製ないし使用できるようになったことも重要である。初期のiPS細胞誘導では初期化因子の導入に，レトロウイルスベクターが主流であったが，宿主ゲノムへの外来遺伝子のランダムな組み込みが，がん化のリスクを高めることが明らかになっており，それに替わる安全な誘導法が求められてきた。現在，レトロ/レンチウイルスベクター以外のウイルスベクターやプラスミド，トランスポゾン，核初期化タンパク質導入，最近では改変RNAや核初期化遺伝子に替わるものとしてのmiRNAや化学物質を用いた方法などが考案されている。また補助的に効率を上げるための薬剤なども頻繁に使われている。現在までのiPS細胞作製法については表にまとめた通りである。

これらの手法の中で，我々が昨年発表したセンダイウイルスベクターによるヒトiPS細胞作製法は，非常に高い外来遺伝子発現効率に起因する高誘導効率と，宿主ゲノムに非組み込み型であること，ベクター除去の容易性・簡便性という点で，より優れた方法として評価を受けている[3]。そのiPS細胞誘導効率はヒト線維芽細胞をソースとした場合，最大で1～4%程度，また従来難しいと言われていたヒトT細胞からも，効率よくiPS細胞を誘導することが可能となっている。末梢血をソースとした場合，レンチウイルスベクターやレトロウイルスベクターの手法に比較し，非常に少量の血液（1 ml）からiPS細胞樹立が可能であることを今年度発表している[4]。このことは，例えば疾患患者由来iPS細胞作製の際の検体の取得時，患者の負担を軽減することに貢献できる。また線維芽細胞の初代培養の時間やレトロウイルスベクターの受容体強制発現のステップも省け，iPS細胞の樹立期間も短縮出来ている。

■ベクターの構成および樹立方法

CytoTune™-iPSキットは，山中初期化4因子（ヒトOCT3/4, SOX2, KLF4, c-MYC遺伝子）をそれぞれ搭載した4つのベクターから構成されている。それぞれのベクターは精製され，力価測定された凍結溶液として提供される。保存はマイナス80℃で安定である。F欠失型ベクターを用いているため，感染後に細胞から感染性のウイルス粒子が再放出される恐れもなく，取り扱い条件はP2である。

ベクターカクテルには，温度高感受性ベクターが組み込まれ，従来のベクターより容易に除去可能となっている。iPS細胞樹立後，37℃で培養を続

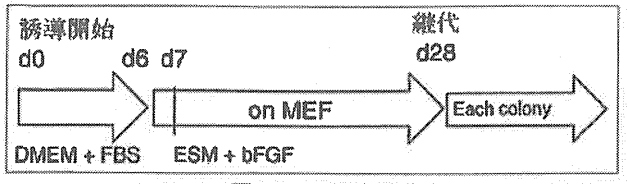

図2　iPS細胞誘導法

けることにより，特別な操作不要で自然に消えるような組み合わせとなっている[4]（論文投稿中）。もちろん，サブクローニングとRT-PCRおよび抗SeV抗体による免疫染色確認を経て，更に早くSeV陰性コロニーを得ることも可能である。

キットには，ヒト新生児包皮由来線維芽細胞，BJ細胞をソースとしたiPS細胞誘導法の例についてマニュアルを添付しているが，特にtransfection試薬もウイルス受容体の導入も必要なく，ただベクターカクテルを混ぜた培地を1回感染させるだけで，容易に線維芽細胞由来iPS細胞を得ることが出来る（図2）。T細胞をソースとする場合は，CD3でT細胞を活性化する必要がある[4]。得られたES細胞様コロニーは容易に継代可能で，ヒトES細胞のマーカーを発現し，かつ無限増殖能と三胚葉への多分化能を獲得しており，iPS細胞の特徴を持っている（図3）。ベクターフリーになった後は，得られたiPS細胞と，それから得られた分化細胞・組織は外来遺伝子をもはや保持していない。レトロウイルスベクターによるiPS細胞誘導のように，非常に数多くのiPS細胞コロニーから，低い挿入因子コピー数，外来遺伝子の発現抑制（サイレンシング），分化後の外来遺伝子の再活性化のリスクを考慮した大規模スクリーニングをする必要がなく，均質なiPS細胞が得られるため，その解析および省力化が可能である。この点で，我々の方法は疾患細胞由来iPS細胞の樹立等に大いに力を発揮しており，厚生労働科研費・難治性疾患克服事業に於ける種々の難治性疾患由来iPS細胞樹立への取組みでは，我々は既に33症例からiPS細胞を樹立している（研究代表者：熊本大学・江良択実教授）。現在のところ，このCytoTune™-iPSを用いて数多くのヒト体細胞（新生児～成人由来，健常人～患者由来線維芽細胞，ケラチノサイト，活性化T細胞ほか）からiPS細胞の樹立実績がある。

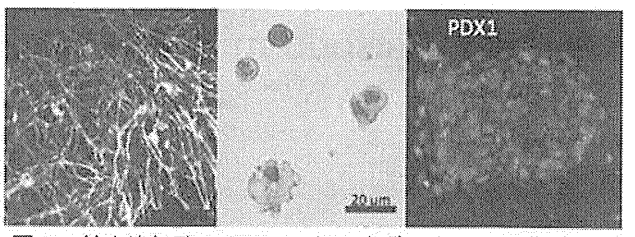

図3　外来遺伝子フリー・ヒトiPS細胞の優れた分化誘導法。左からドーパミン産生ニューロン（外胚葉由来），血球細胞（中胚葉由来），膵β細胞（内胚葉由来）。

■今後の展望

最近，海外のグループが改変RNAを持続的に添加することで，ヒト新生児細胞からiPS細胞の樹立に成功している[5]。効率という点では，センダイウイルスベクターによるiPS細胞樹立効率と同程度になるが，17日間という長期間のRNA持続的添加，インターフェロンの影響を避けるためのデコイ受容体の添加の必要性や，RNAが生体内で除去されないためのCap構造の付与などの工夫がなされている。しかしながらこれらの工夫は，本来SeVベクターが自分自身で持ち合わせている性質でもあるため，それの人工的な模倣に過ぎないとも言える。SeVベクターは，たった1回の感染で必要な遺伝子を持続的に発現し，かつ宿主ゲノムにも組み込まれず，iPS細胞化による増殖速度の上昇により緩やかに希釈され除去される。さらに細胞温度感受性ベクターの樹立により，ベクター除去が非常に容易になったことで，我々のSeVベクター法が現在最も簡便で効率のよい方法の１つと位置づけられると考えている。現在，上記細胞種以外に適用するための，より強力な発現ベクターを開発中であり，細胞種により選択できる幾つかの至適ベクターを，補助因子のラインナップと共に，次世代ベクターとして提供して行く予定である。

商品問い合わせ先
・株式会社 医学生物研究所 (MBL)
〒460-0008　愛知県名古屋市中区栄4-5-3 KDX名古屋栄ビル10階　support@mbl.co.jp
・ディナベック株式会社
〒300-2611　茨城県つくば市大久保6番
cytotune@dnavec-corp.com

文献

1) Nagai, Y., Takakura, A., Irie, T., *et al* (Norwick, The United Kingdom: Horizon Scientific Press), 2010, in press
2) Takahashi, K., Tanabe, K., Ohnuki, M., *et al*., 2007 Cell 131, 861-872.
3) Fusaki, N., Ban, H., Nishiyama, A., *et al*, 2009 Proc Jpn Acad Ser B Phys Biol Sci. 85, 348-362.
4) Seki, T., Yuasa, S., Oda, M., 2010 Cell Stem Cell 7, 11-14.
5) Warren, L., Manos, P.D., Ahfeldt, T., *et al*., 2010 Cell Stem Cell, 7, 1-13.

Mesoderm cell development from ES cells

Takumi Era MD, PhD

Department of Cell Modulation, Institute of Molecular Embryology and Genetics, Kumamoto University,
2-2-1 Honjo, Kumamoto 860-0811, Japan.

TEL: 81-96-373-6589 FAX: 81-96-373-6590
Email: tera@kumamoto-u.ac.jp

Keywords

Embryonic stem cell (ES cell), Induced pluripotent stem cell (iPS cell), Mesoderm, Vascular endothelial growth factor receptor 2 (VEGFR2), Platelet-derived growth factor receptor α (PDGFRα).

Abstract

Pluripotent stem cells such as embryonic stem (ES) and induced pluripotent stem (iPS) cells have attractive attention as a source of cells for use in therapeutic application. However, as the *in vitro* differentiation culture does not provide usefully positional information for cell type definition, this system definitely requires visible markers to identify and monitor the intermediates that present on the way of differentiation. We have been developing the cell surface markers against the various types of mesoderm in the ES cell culture. Using it, we have identified the intermediates of mesoderm and dissected their differentiation pathways in ES cell differentiation. The method described here could be useful for inducing and purifying mesoderm cells from iPS as well as ES cell cultures.

1. Introduction

1.1. Mesoderm development in the embryos

In mouse embryo, mesoderm development starts at E6.5 and, for a short time, dramatically produces three major types of mesoderm; organizer, embryonic mesoderm and extra-embryonic mesoderm (*1*, *2*). The most initial mesoderm appears at a proximal region in epiblasts of embryo as an early gastrula organizer (EGO) (*3*). EGO migrates into anterior part of embryo and become to mid gastrula organizer (MGO) that contributes to axial mesoderm. While organizer migrates, the epiblast at posterior region subsequently begin to transform to second type of mesoderm; embryonic mesoderm, in primitive streak (*4*). Along with the elongation of primitive streak distally, embryonic mesoderm become to diversify region-specifically two types of mesoderms, paraxial and lateral mesoderm, which eventually forms a majority of mesoderm progenies such as bone and blood cells, respectively. The epiblasts at the proximal part of embryo also produce a third type of mesoderm; extra-embryonic mesoderm. The precursors of this mesoderm move into the nascent streak and migrate to extra-embryonic part in which they mainly give rise to primitive hematopoietic cells and endothelial cells (*5*, *6*).

1.2. Flat culture for *in vitro* ES cell culture

The major aim of in vitro ES cell culture is to establish the culture condition that induces ES cell to the efficient differentiation for specific cell lineages (*7*). Embryoid-body (EB) formation method seemed to be suitable for attaining this. The method is based on the idea that ES cell differentiation requires the environments which are similar to those present in the actual embryo. However, EB exhibits a complex structure that disturbs the cells inside to meet the appropriate signals from outside. As

a result, the culture conditions around EB are not able to exclude differentiation into unnecessary lineages. Previous our study demonstrated that EB culture is less efficient in inducing mesendoderm cells expressing *Goosecid*, which is one of the markers for EGO, than the two-dimensional (2D) culture on collagen IV-coated dishes (***8***). This result indicates an inherent limitation of EB system in guiding ES cell differentiation, as uncontrollable complexity is inevitably associated with three-dimensional architecture in EB. To overcome the obstacle of EB formation, we prefer to use the flat culture system rather than EB formation method for an *in vitro* ES cell differentiation.

1.3. Differentiation of mesoderm cells in *in vitro* ES cell culture

ES cell have the multiple potentials to give rise to a whole cell types in mouse body and to undergo unlimited symmetrical divisions with maintaining its pluritotency (***9***). The high ability for differentiation and unlimited growth capacity lead us to expect to utilize it as the source of cell therapies such as transplantation. Moreover, the forced differentiation system of ES cell in vitro has been expected to use as a good tool to find the developmental pathways into the specific cell lineage and to dissect them from others. However, as ES cell differentiation culture does not provide usefully positional information for cell type definition, this system definitely requires visible markers to identify and monitor the intermediates that present on the way of differentiation. In fact, availability of Vascular Endothelial Growth Factor Receptor 2 (VEGFR2, FLK1) that marks the subtypes of mesoderm cells with a potential to give rise to hematopoietic cells (HPCs) and endothelial cells (ECs) facilitates our understanding on the developmental pathways of these lineages (***10-12***). Another important surface marker involving in mesoderm development is Platelet-derived growth factor receptor alpha (PDGFRα) that is mainly expressed in paraxial mesoderm during mouse embryogenesis (***13-15***). We have exploited these markers for dissecting the differentiation course of ES cell-derived mesoderm cells. Our previous results obtained from in vitro ES cell culture shows that PDGFRα^{+}VEGFR2^{+} cell (DP) that initially appears at day 3.5 ES cell culture is a common precursor for PDGRFRα^{+}VEGFR2^{-}(PSP) and PDGFRα^{-}VEGFR2^{+}(VSP) cells (***16***). Based on the results of in vitro fate analysis, we found a new differentiation pathway in which the DP gives rise to both the PSP and the VSP that eventually differentiate into bone and cartilage cells, and HPCs and ECs, respectively (**Fig. 1**)(***16***). These indicate that PSP and VSP populations represent the paraxial and lateral mesoderm populations in actual mouse embryo, respectively. The analyses for gene expression in both populations also support the hypothesis that PSP and VSP correspond to paraxial and lateral mesoderms, respectively (**Fig. 2**).

2. Materials

2.1. ES cell lines

With numerous ES cell lines currently available, we recommend feeder-free ES cell lines such as CCE, EB3, EB5 and E14tg2a (*17*, *18*). Before real experiments, each cell line should first be examined for the its ability to generate $VEGFR2^+$ and $PDGFR\alpha^+$ cells. CCE is usually analyzed in our laboratory.

2.2. Reagents for ES cell maintenance

1. KNOCKOUT-Dulbecco's modified Eagle's medium (KO-DMEM, Invitrogen cat. no. 10829-018) is stored at 4℃.
2. Leukemia inhibitory factor (LIF) (Chemicon International, cat. no. ESG1107) was purchased as $1x10^7$ U/ml in a rubber-capped vial. Use a 1-ml syringe with a needle to push 1 ml of air into the bottle and pull out all the liquid. Aliquots of 100μl each ($1x10^6$ U) are stored in sterilized cryotubes at -80℃.
3. Fetal bovine serum (FBS) pretested for ES cells. (see **Note 1**).
4. 0.1% (w/v) Gelatin (Sigma cat. no.G2500)

Add 0.5g Gelatin into 500ml deionized water (culture-grade) and autoclaved. Store at room temperature.

5. Dulbecoco's Phosphate Buffered Saline without calcium and magnesium chloride (D-PBS; Invitrogen cat. no. 14190-250).
6. 2-Mercaptoethanol (2-ME) (Sigma cat. no. M7522): stock solution: 1000X (*0.1M*). Add 70 μl 2-ME to 10 ml PBS and sterilized by 0.2-μm filter. Store up to 4 weeks at 4℃. Final concentration in medium: 10^{-4} *M*.
7. L-Glutamine (200mM; 100X Invitrogen cat. no. 25030-081) and Penicillin-streptomycin (P/S; 100X Invitrogen cat. no. 15140-122) are stored in 15-ml centrifuge tubes as 5 ml aliquots ar -20℃.
8. Non-Essential Amino Acids (NEAA; 100X Invitrogen cat. no. 11140-050) is stored at 4℃.
9. 0.25% (w/v) trypsin-EDTA (Invitrogen cat. no. 25200-072) is stored in 10 ml aliquots at -20℃.
10. 6 cm and 10 cm culture dishes (Becton Dickinson cat. no. 353802 and 353003))
11. ES cell culture medium: KO-DMEM + 15%FBS + 10^{-4} 2-ME + 2mM L-glutamine + 1XP/S + 0.1 mM NEAA + 1000 U/ml LIF. Store up to 4 wk at 4℃.

2.3. Reagents for OP9 stromal cell maintenance

1. Minimum essential medium α medium (αMEM) with ribonucleosides and deoxyribonucleosides (Invitrogen cat. no. 12571-063)

2. FBS pretested for OP9 cells. (see **Note 1**).

3. D-PBS

4. L-Glutamine and Penicillin-streptomycin

5. 0.05% (w/v) trypsin-EDTA (Invitrogen cat. no. 25300-062)is stored in 10 ml aliquots at -20℃.

6. OP9 culture medium: αMEM + 20%FBS + 2mM L-glutamine + 1XP/S.

2.4. Reagents for *in vitro* ES cell differentiation without OP9

1. αMEM

2. FBS pretested for in vitro ES cell differentiation. (see **Note 2**).

3. 2-ME: Final concentration in medium: $5X10^{-5}$ *M.*

4. L-Glutamine (200mM 100X)

5. Penicillin-streptomycin (100X)

6. BIOCOAT Collagen IV-coated 10cm dish (Becton Dickinson cat. no.35 4453)

7. Differentiation culture medium: αMEM + 10%FBS + $5X10^{-5}$M 2ME + 2mM L-Glutamine + 1X P/S (see **Note 3**)

2.5. Reagents for *in vitro* ES cell differentiation with OP9

1. αMEM

2. FBS pretested for in vitro ES cell differentiation. (see **Note 2**).

3. L-Glutamine (200mM 100X)

4. Penicillin-streptomycin (100X)

5. OP9 differentiation culture medium : αMEM + 20%FBS + 2mM L-Glutamine + 1X P/S

2.6. Reagents for Purification of mesoderm cells

1. D-PBS

2. Cell dissociation buffer (Invitrogen cat. no. 13150-016)

3. 0.25% (w/v) Trypsin-EDTA (Invitrogen cat. no. 25200-072)

4. Neutralization buffer for Cell dissociation buffer and Trypsin-EDTA: D-PBS + 10%FBS

5. Normal mouse serum (NMS). NMS can be prepared from in-house or can be purchased (Chemicon international cat. no. S25-10ML). Sterilized by 0.2-μm filter and aliquots of 500 μl each are stored in sterilized tubes at -20℃.

6. Hank's balanced Salt Solution (HBSS) (10X Invitrogen cat. no. 14185-052)
HBSS/BSA: 1X HBSS + 1% bovine serum albumin (BSA) (Sigma cat. no. A-1253)

7. HBSS/BSA/PI: HBSS/BSA with 5mg/ml propidium iodide (Sigma cat. no. P-4170)

8. Anti-VEGFR2 (AVAS12): Phycoerythrin-conjugated (eBioscience cat. no. 12-5821) and Allophycocyanin-conjugated (eBioscience cat. no. 17-5821)

9. Anti-PDGFRα (APA5)(eBioscience cat. no. 13-1401) Biotin-conjugated
10. Allophycocyanin-conjugated Streptavidin (SAV-APC eBioscience 17-4317)

2.7. Reagents for bone cell differentiation from ES cell-derived mesoderm cells

1. Dulbecco's modified Eagle's medium (DMEM Invitrogen cat. no. 10569-010)
2. FBS pretested for in vitro ES cell differentiation. (see **Note 2**).
3. L-Glutamine (200mM 100X)
4. Penicillin-streptomycin (100X)
5. Dexamethasone (Sigma cat. no. D8893)
6. Ascorbic acid 2-phosphate (Sigma cat. no. 49752)
7. β-glycerophosphate (Sigma cat. no. G6251)
8. Recombinant Human BMP4 (R&D systems cat. no. 314-BP)
9. 24-well culture plate (Becton Dickinson, Falcon 353047)
10. Bone cell differentiation medium : DMEM + 10%FBS + 2mM L-Glutamine + 1X P/S + 0.1 μM Dexamethasone + 50μM ascorbic acid 2-phosphate + 10mM β-glycerophosphate + 10 ng/ml BMP4
11. 4% Paraformaldehyde solution (PFA) (Sigma cat. no. 158127)
12. Alizarin red S (Sigma cat. no. A5533)
13. Ammoniun hydroxide solution (28%, Sigma cat. no. 338818)
14. Alizarin red staining solution:

Solution A: 1g Alizarin red S + 100 mL Distilled water

Solution B: 0.1 ml Ammoniun hydroxide solution + 100 mL Distilled water

Mix solution A well. Adjust the pH 6.36-6.40 with solution B. The pH is critical, so make fresh or check pH if the solution is more than one month old. Keep at room temperature upto 6 mo.

2.8. Reagents for cartilage 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. Dexamethasone
6. Ascorbic acid 2-phosphate
7. Recombinant Human TGF-β3 (R&D systems cat. no. 243-B3)
8. Recombinant Human BMP2 (R&D systems cat. no. 355-BM)
9. 24-well culture plate (Becton Dickinson, Falcon 353047)
10. Cartilage cell differentiation medium: αMEM + 10%FBS + 2mM L-Glutamine + 1X P/S + 0.1 μM Dexamethasone + 170μM ascorbic acid 2-phosphate

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 $2x10^6$ 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: $7-8x10^5$ cells/6-cm dish and $1.2-1.6x10^6$/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. $Gr\text{-}1^+$ 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*).