

# Generation of Induced Pluripotent Stem Cells from Human Nasal Epithelial Cells Using a Sendai Virus Vector

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## Abstract

The generation of induced pluripotent stem cells (iPSCs) by introducing reprogramming factors into somatic cells is a promising method for stem cell therapy in regenerative medicine. Therefore, it is desirable to develop a minimally invasive simple method to create iPSCs. In this study, we generated human nasal epithelial cells (HNECs)-derived iPSCs by gene transduction with Sendai virus (SeV) vectors. HNECs can be obtained from subjects in a noninvasive manner, without anesthesia or biopsy. In addition, SeV carries no risk of altering the host genome, which provides an additional level of safety during generation of human iPSCs. The multiplicity of SeV infection ranged from 3 to 4, and the reprogramming efficiency of HNECs was 0.08–0.10%. iPSCs derived from HNECs had global gene expression profiles and epigenetic states consistent with those of human embryonic stem cells. The ease with which HNECs can be obtained, together with their robust reprogramming characteristics, will provide opportunities to investigate disease pathogenesis and molecular mechanisms in vitro, using cells with particular genotypes.

**Citation:** Ono M, Hamada Y, Horiuchi Y, Matsuo-Takasaki M, Imoto Y, et al. (2012) Generation of Induced Pluripotent Stem Cells from Human Nasal Epithelial Cells Using a Sendai Virus Vector. PLoS ONE 7(8): e42855. doi:10.1371/journal.pone.0042855

**Editor:** Andrea Barbuti, University of Milan, Italy

**Received:** April 9, 2012; **Accepted:** July 12, 2012; **Published:** August 13, 2012

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**Funding:** This study was supported by research grants from the Japan Science and Technology Agency, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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## Introduction

Induced pluripotent stem cells (iPSCs) are generated from somatic cells by transducing them with reprogramming factors [1]. Initially, human dermal fibroblasts were used to derive human iPSCs (hiPSCs) [2,3], and the majority of iPSC research in humans has focused on fibroblasts as a source of somatic cells. However, recent studies have shown that other human somatic cells can be used to generate iPSCs such as those from blood [4,5,6,7], teeth [8], adipose tissues [9], and oral mucosa [10]. Obtaining these cells from the aforementioned sources, except blood, requires biopsy with local anesthesia, making it cumbersome for generating patient-specific stem cells. Additionally, although obtaining blood cells does not require local anesthesia, the rearrangement of the T-cell receptor (TCR) chain genes in T cells and the VDJ region in B cells means that they are not identical to naïve lymphocytes at the genomic level.

In the present study, we generated iPSC cells using human nasal epithelial cells (HNECs). This is a less invasive method to obtain human somatic cells, since neither anesthesia nor biopsy are required. In addition, we used Sendai virus (SeV) vectors to introduce reprogramming factors. SeV is an important respiratory pathogen of rats and mice, and it has been reported that SeV

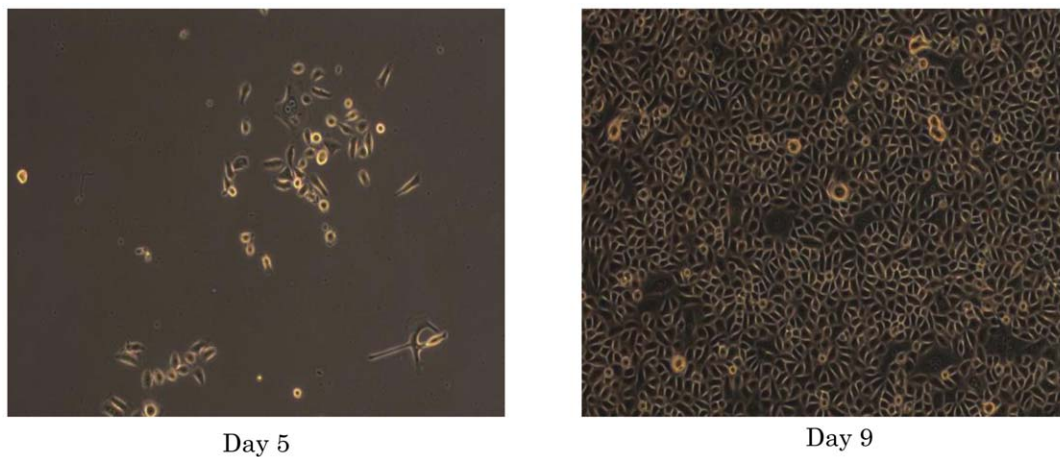
vectors efficiently transduce the respiratory tract of mice as well as humans [11]. Therefore, we speculated that HNECs would be highly amenable to efficient gene transduction with SeV vectors.

## Results

Freshly obtained HNECs were maintained on collagen-coated matrix, and they got attached within 4–6 hours, forming small colonies. The HNECs reached confluence within 2 weeks (Figure 1) with typical epithelial morphology. We also confirmed that primary HNECs can be cultured and expanded after cryopreservation in liquid nitrogen.

We first determined the infection efficiency using a SeV vector that expressed green fluorescent protein. HNECs seeded at  $1.0 \times 10^5$  cells per 35-mm dish were infected by green fluorescent protein vectors over a range of multiplicities of infection (MOI, number of viral particles per cell; Figure 2). We determined that a MOI of 3 or 4 was sufficient to induce the transgenes for HNECs.

The scheme for generation of iPSC from HNECs is presented in Figure 3. We observed the appearance of colonies with an embryonic stem (ES) cell-like morphology at 20 days after infection of SeV vectors carrying 4 reprogramming factors

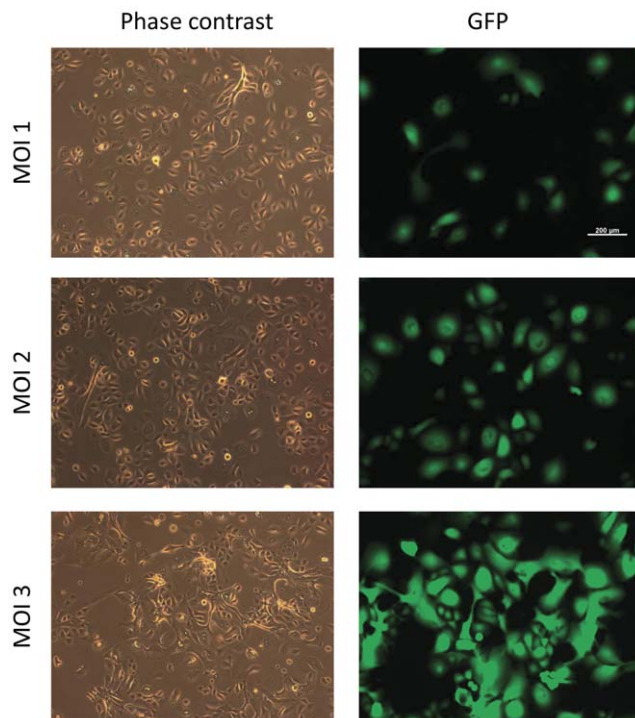


**Figure 1. Primary culture of human nasal epithelial cells (HNEC).** Bright-field images 5 days after cell sampling (left), and 9 days after sampling with early epithelioid morphology (right).  
doi:10.1371/journal.pone.0042855.g001

(Figure 4A), and reprogramming efficiency was 0.1% at MOI 4, and 0.075% at MOI 3 (Figure 4B).

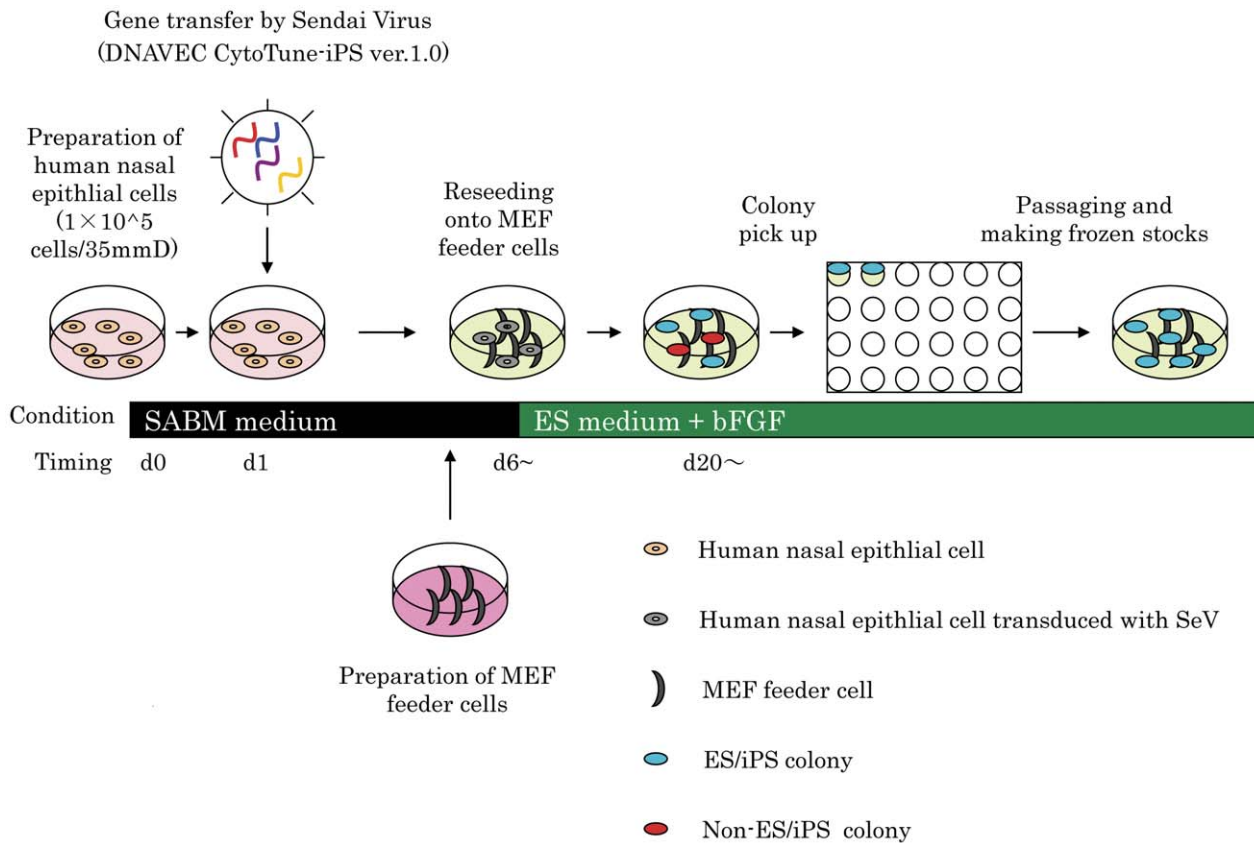
To characterize colonies generated from SeV-infected HNECs, we picked a total of 74 colonies, and randomly chose 7 lines (iPS-B2, iPS-B3, iPS-C6, iPS-2B1, iPS-2B6, iPS-3A1, and iPS-4B1). We first performed a high-density single nucleotide polymorphism (SNP) genotyping assay to evaluate structural variations of HNEC-derived colonies. As shown in Figure S1, chromosomal duplications were observed on chromosome 2p in iPS-2B6 and

chromosome 12p in iPS-C6. We excluded these colonies from further analysis. None of the other 5 colonies (iPS-B2, iPS-B3, iPS-2B1, iPS-3A1, and iPS-4B1) harbored duplications or deletions on chromosomes, and the genotype concordance rate in each colony with those of original HNECs was greater than 99.99%. This value was similar to the rate of technical replicates (i.e., concordance rate of the same genomic DNA), showing that colonies were derived from parental HNECs. Then, we examined the gene expression of the reprogramming factors and the expression of SeV vectors in HNEC-derived colonies (Figure 5). We used a temperature-sensitive mutant SeV vector in these experiments in order to shut off transgenes efficiently by temperature shift [12]. Colonies generated from HNECs showed endogenous *OCT4*, *SOX2*, *KLF4*, and *c-MYC* gene expression levels that were similar to those of human ES cells, and we confirmed that SeV-derived gene expressions were not detected by reverse transcription-polymerase chain reaction (RT-PCR) using SeV-specific primers (Figure 5A). Protein expression of pluripotency markers was confirmed by immunofluorescence staining (Figure 5B and Figure S2). DNA methylation analysis revealed that CpG dinucleotides at the *OCT4* and *NANOG* promoter region in the HNEC-derived colonies were demethylated while those in original HNECs were mostly methylated (Figure 6). Furthermore, global gene expression pattern in the HNEC-derived cell lines strongly correlated with that of human ES cells ( $r = 0.99$ , Figure 7), while correlation of gene expression between HNEC-derived cell lines and parental HNECs was weak ( $r = 0.87$  in iPS-B3 and  $r = 0.86$  in iPS-2B1).



**Figure 2. Expression of GFP in HNECs following various multiplicities of infection (MOI).** Induction of GFP protein with MOI=1 (top), MOI=2, (middle), and MOI=3 (bottom). GFP expression was observed when MOI=1 and 2, but the expression was stronger when MOI=3.  
doi:10.1371/journal.pone.0042855.g002

In order to evaluate the differentiation potential of HNEC-derived iPSCs, we performed *in vitro* differentiation using HNEC-derived cell lines. We generated embryoid bodies (EBs), which were spontaneously differentiated for 10~13 days. RT-PCR analysis revealed that these cells were positive for three germ cell markers (Figure S3), although the gene expression pattern of these cells varied in different HNEC-derived iPS cell lines. Next, in order to investigate *in vivo* differentiation, we injected HNEC-derived cell lines into immunocompromised mice and evaluated their ability to form teratomas. In the experiments, we used 4 cell lines (iPS-B2, iPS-B3, iPS-2B1, and iPS-3A2). Histological examination of the teratomas revealed that tissues were from the endoderm, mesoderm, and ectoderm lineages, and we observed neural and epithelial tissues, muscle, cartilage, bone, gut-like structures, and various glandular structures (Figure S4).



**Figure 3. Schema of generation of iPS from HNECs.**  
doi:10.1371/journal.pone.0042855.g003

## Discussion

In the present study, we demonstrated that efficient reprogramming of HNECs can be achieved without integration of the reprogramming transgenes or a viral genome. iPS cells generated in the present study showed characteristics similar to human ES cells, and had the potential to differentiate into the 3 germinal layers of endoderm, mesoderm, and ectoderm in a teratoma formation assay *in vivo*.

One of the many benefits that iPS technology offers is the development of robust and personalized models of human disease. iPSCs can be generated from subjects with various genetic diseases [13,14]. Skin fibroblasts are frequently used to generate iPSCs. However, because this requires skin biopsy with local anesthesia, it can be challenging to recruit donors. An alternative is to use blood cells as a source for iPSCs. This has been performed using both peripheral B cells [15], and T cells [5,7], but in this scenario, the genomic region of TCR chain genes in T cells and the VDJ region in B cells are rearranged. Therefore, iPSCs derived from T and B cells can exhibit only 1 pattern of the TCR gene and VDR genomic regions, respectively. CD34-positive cells have also been used to generate iPSCs [6,16,17]. This has a potential advantage over using the mature T and B cells, since CD34-positive cells do not have their genomes rearranged. However, these cells comprise only a small proportion (0.1%) of total peripheral hematopoietic cells, which poses a challenge for the separation of pure CD34-positive cells. Our current method using HNECs maintains the genome in the same state as the original somatic cells, does not require complicated methods for culture or a high MOI for gene transduction, and achieves reprogramming efficiencies similar to

those found using skin fibroblasts (0.08–0.13% [12]). Because of the minimal invasiveness involved in obtaining these somatic cells and their amenability to reprogramming, using SeV vectors to reprogram HNECs may be a more suitable approach for generating patient-specific iPSCs. This will provide opportunities to investigate disease pathogenesis and molecular mechanisms *in vitro*, using cells with particular genotypes.

## Materials and Methods

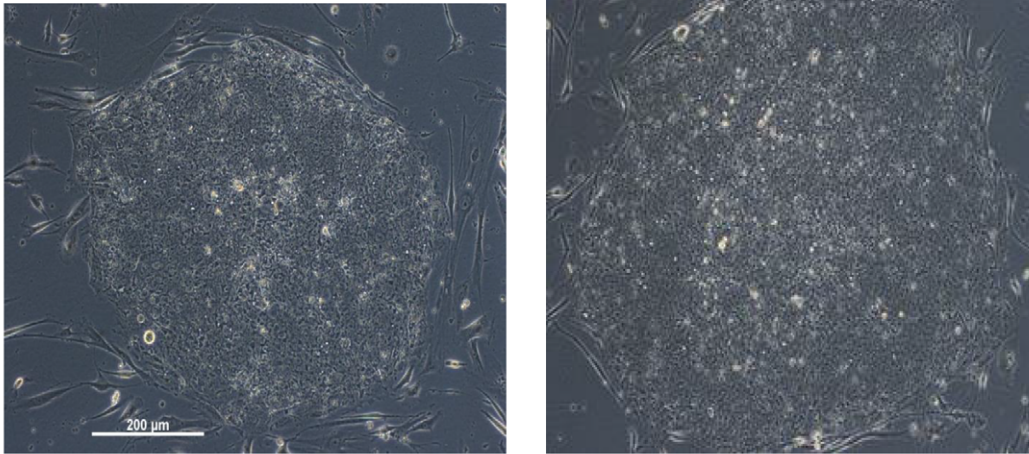
### Ethics statement

This study was approved by the Ethical Committee of University of Tsukuba and followed International guidelines (i.e., the Helsinki Declaration). Informed written consent was taken from a tissue sample donor. All animal work was conducted according to university and international guidelines.

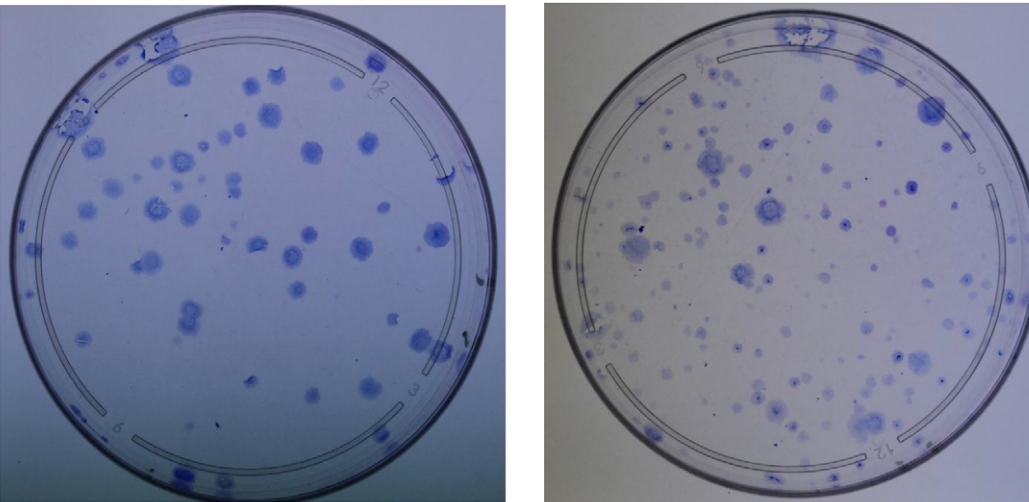
### Primary cell culture

HNECs were obtained from a healthy female donor by the brushing technique. Nasal brushing was performed by an otolaryngologist using a soft sterile brush (Medical Packaging Camarillo, CA). A brushing was performed in both nostrils by a gentle circular movement. Brushes were immersed in 3 mL of small airway epithelial cells basal medium (SABM) (Lonza, Basel, Switzerland) with small airway epithelial cell growth medium (SAGM) SingleQuots (Lonza, Basel, Switzerland) and 100 U penicillin and 0.1 mg/mL streptomycin (Sigma-Aldrich, St. Louis, MO), followed by a manual shake and pipetting. SABM containing HNECs were seeded on type I collagen-coated 35-mm dishes (BD Biocoat, BD, Franklin Lakes, NJ), and cultured at

A



B



**Figure 4. Morphologies of HNEC-derived iPS.** A. Colonies generated from HNECs show a round shape with large nucleoli and scant cytoplasm, similar to the morphology of human ES cells. B. Crystal violet staining of growing cells. Efficiency of iPS cell generation is 0.75% at MOI 3 (left), and 0.1% at MOI 4 (right).

doi:10.1371/journal.pone.0042855.g004

37°C in an atmosphere of 5% CO<sub>2</sub>. After 24 and 48 h of culture, non-adherent HNECs were collected and transferred to a new type I collagen-coated dish. SABM was changed every 2 days, and cells were cultured with SABM with SAGM SingleQuots and Reagent Pack (Lonza, Basel, Switzerland).

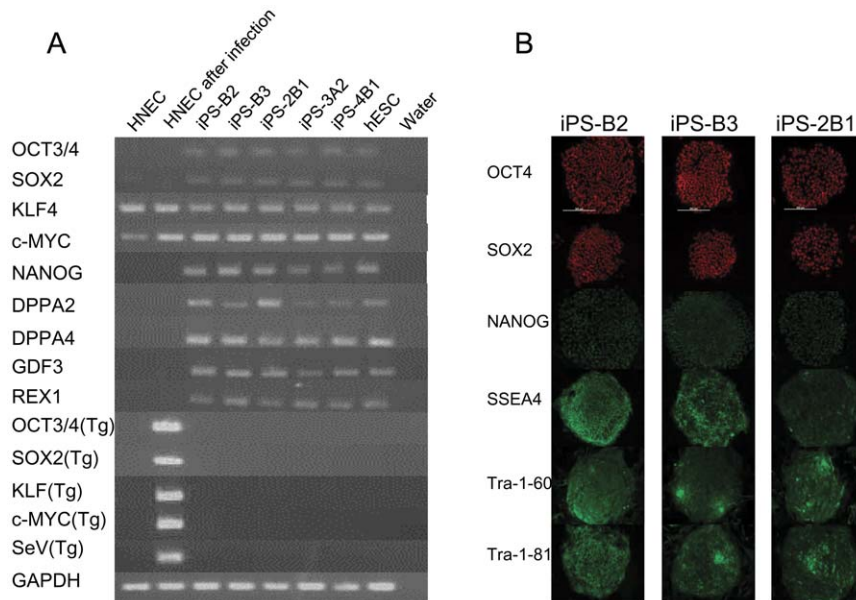
#### Induction of pluripotent stem cells

After 2 weeks of culture, HNECs were collected and  $1.0 \times 10^5$  cells were transferred to a new 35-mm dish coated with type I collagen (BDBiocoat, BD, Franklin Lakes, NJ), and cultured at 37°C in an atmosphere of 5% CO<sub>2</sub> overnight. HNECs were infected with SeV vectors (Dनावेक, Tsukuba, Japan) that individually carried each of *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC* at an MOI of 3 or 4. Infected cells were cultured in SABM supplemented with SAGM SingleQuots. After 24 h of infection, the medium was changed to fresh medium. At 6–7 days after infection,  $1.0$ – $2.0 \times 10^5$  cells were collected and transferred to a 10 cm-dish containing mitomycin C-inactivated mouse embryonic fibroblast (MEF) feeder cells ( $1.5 \times 10^6$  cells per dish). After an additional 24 h of incubation, the medium was changed to hiPSC

medium, which consisted of Dulbecco's modified Eagle medium/F12 medium (Life Technologies, Carlsbad, CA) supplemented with 20% Knock-out Serum Replacement (KSR; Life Technologies, Carlsbad, CA), 2 mM L-glutamine (Life Technologies, Carlsbad, CA), 1 mM non-essential amino acids (Sigma-Aldrich, St. Louis, MO), 0.1 mM β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), and 4 ng/mL basic fibroblast growth factor (WAKO, Osaka, Japan). The hiPSC medium was changed every day. The generated iPSCs were maintained on mitomycin C-inactivated MEF feeder cells in hiPSC 3 medium, and the cells were passaged with CTK solution consisted of 0.25% trypsin, 1 mg/mL collagenase IV, 20% KSR (all from Life Technologies, Carlsbad, CA), 20 mM CaCl<sub>2</sub> (Wako) in PBS(-) (Life Technologies, Carlsbad, CA).

#### Reprogramming efficiency

Reprogramming efficiency was calculated as the number of iPS colonies formed per number of infected cells seeded. iPS colonies were identified based on ES cell-like morphology and crystal violet staining. Crystal violet staining was performed with 0.1% crystal



**Figure 5. Gene/Protein expressions of HNEC-derived iPS.** A. RT-PCR analyses for the hESC marker genes *NANOG*, *OCT3/4*, *SOX2*, *KLF4*, *c-MYC*, *GDF3*, *REX31*, *DPPA2*, and *DPPA4* and the transgenes *OCT3/4*, *SOX2*, *KLF4*, and *c-MYC*. B. Immunofluorescence staining for pluripotency and surface markers (*OCT4*, *SOX2*, *NANOG*, *SSEA4*, *Tra-1-60*, and *Tra-1-81*). Scale bars indicate 500  $\mu$ m. Red: Alexa 568, Green: Alexa488. doi:10.1371/journal.pone.0042855.g005

violet (Nakaraitesk, Kyoto, Japan) in methanol, as described previously [18].

### SNP genotyping

Genomic DNA was isolated from HNEC-derived iPS cells by DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). High-density SNP genotyping was performed using Human610-Quad (Illumina, San Diego, CA) that contains more than 610,000 rationally selected tag SNPs and markers per sample, according to the manufacturer's protocol. The scanned image was imported into GenomeStudio (Illumina) for analysis. Genotype calls and analysis were performed by GenomeStudio (Illumina).

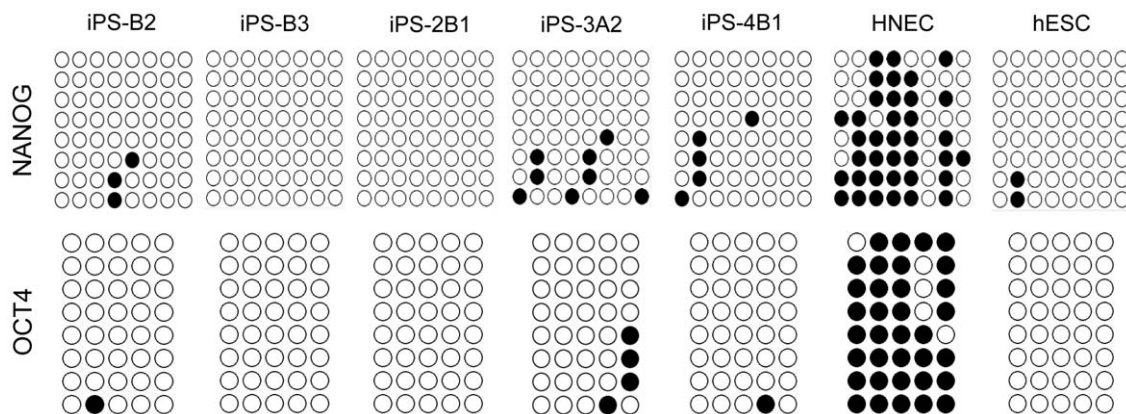
### Immunofluorescence staining

Immunofluorescence staining was performed using the following primary antibodies: anti-NANOG (Cell Signaling Technology,

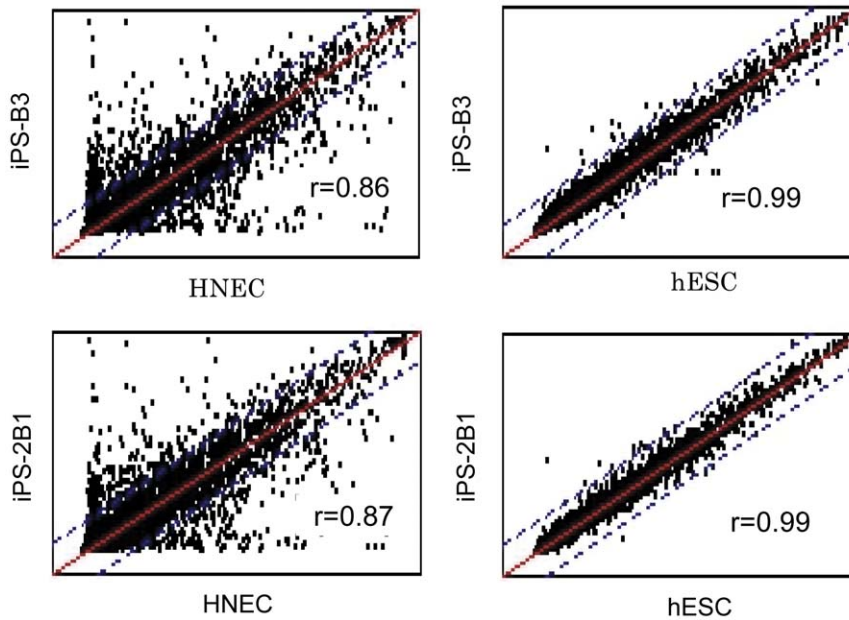
Danvers, MA), StemLite™ Pluripotency kit (anti-OCT4, anti-Sox2, anti-SSEA4, anti-TRA-1-60, anti-TRA-1-81; Cell Signaling Technology). The secondary antibodies used were: anti-rabbit IgG, anti-mouse IgG and IgM conjugated with AlexaFluor 488 or AlexaFluor 568 (Life Technologies, Carlsbad, CA) and anti-rabbit IgG(H+L), fragments conjugated with AlexaFluor 488 (Cell Signaling Technology). Cell nuclei were stained with DAPI (Vector Laboratories). The fluorescence signals were detected using a conventional fluorescence laser microscope (ZEISS-HBO-100; Carl Zeiss AG, Oberkochen, Germany).

### RT-PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen), according to the manufacturer's instructions. The concentration and purity of the RNA were determined using the ND-1000 spectrophotometer (Nanodrop, Wilmington, DE). The cDNA was



**Figure 6. Methylation analysis of the promoter region.** Bisulfite sequencing analysis of the *NANOG* and *OCT4* promoter regions in HNECs, hESCs, and HNEC-derived iPS cells B2, B3, 2B1, 3A2, and 4B1 are shown. Open circles indicate unmethylated CpGs, while closed circles indicate methylated CpGs. doi:10.1371/journal.pone.0042855.g006



**Figure 7. Global gene expression analysis.** Gene expression patterns were compared between HNEC-derived iPS cells (iPS-B3) and HNECs (left, top), between HNEC-derived iPS cells (iPS-2B1) and HNECs (left, bottom), between HNEC-derived iPS cells (iPS-B3) and human ES cells (hESC, right, top), and between HNEC-derived iPS cells (iPS-2B1) and hESCs (right, bottom). Blue dotted lines indicate 2-fold changes.  $r$  represents the correlation coefficient.

doi:10.1371/journal.pone.0042855.g007

synthesized using the T7 Oligo (dT) Primer (Ambion, Austin, TX), 1000 U/ $\mu$ L ReverTra Ace (TOYOBO, Osaka, Japan), 5000 U RNaseout<sup>TM</sup> recombinant ribonuclease inhibitor (Life Technologies). RT-PCR was performed with AmpliTaq Gold 360 Master Mix (Life Technologies). The primers used for RT-PCR are listed in Table S1.

### Global gene expression analysis

Total RNA was isolated from HNEC-derived iPS cells using the RNeasy Mini Kit. We used Illumina Bead Array with single-color array (Illumina) as a microarray platform. For the Illumina BeadArray assay, cRNA was synthesized with an Illumina RNA Amplification kit (Life Technologies), according to the manufacturer's instructions. In brief, 500 ng of total RNA were reverse transcribed to synthesize first- and second-strand cDNA, purified with spin columns, and then *in vitro* transcribed to synthesize biotin-labeled cRNA. A total of 750 ng biotin-labeled cRNA was hybridized to each Illumina Human-Ref8 v3.0 BeadChip array (Illumina) at 55°C for 18 h. The hybridized BeadChip was washed and labeled with streptavidin-Cy3 (GE Healthcare, Buckinghamshire, UK) and then scanned with the Illumina BeadStation 500 System (Illumina). The scanned image was imported into GenomeStudio software (Illumina) for analysis. Twenty-two thousand transcripts representing 8 whole-genome samples can be analyzed on a single BeadChip. GenomeStudio output of the microarray data was processed with lumi package for the R language [19] on R version 2.10.0 (<http://www.R-project.org/>).

### Bisulfite sequencing

Genomic DNA was isolated from HNECs, human ES cells, and iPS cells derived from HNECs using DNeasy Blood & Tissue kit (Qiagen), and was treated with sodium bisulfite using EpiTect Bisulfite kit (Qiagen), according to the manufacturer's instructions. Converted DNA was used as the template for PCR using primer sets previously described to amplify the promoter regions of *OCT4*

[20] and *NANOG* [2]. The purified PCR products were TA-cloned into pCR4-TOPO vector using TOPO TA Cloning Kit for Sequencing (Life Technologies). The insert sequences of randomly picked clones were analyzed using the ABI 3130 DNA analyzer (Life Technologies).

### In vitro differentiation

iPS cells were cultured on Petri dishes for one days in hiPSC medium, then were cultured free floating to induce EBs for 10–13 days in EB medium, consisting of DMEM/Ham's F12 containing 5% KSR, 2 mM L-glutamin,  $1 \times 10^{-4}$  M nonessential amino acids, and  $1 \times 10^{-4}$  M 2-mercaptoethanol.

### Teratoma formation

All mouse procedures were conducted in compliance with institutional animal use guidelines. hiPSCs grown on MEF feeder layers were collected by CTK solution into tubes, and centrifuged, and the pellets were suspended in 10  $\mu$ M Y-27632 (Wako) in cold Hanks' Balanced Salt Solution (Life Technologies). The cells from a confluent 60-mm dish were injected ( $5 \times 10^6$  cells in 200  $\mu$ L per injection) into 1 testis of SCID mice (Japan Charles River, Yokohama, Japan) using a 1-mL syringe. At 8–10 weeks post injection, teratomas were dissected, fixed in 4% paraformaldehyde, and embedded in paraffin. The sections were stained with hematoxylin and eosin.

### Supporting Information

**Figure S1 Genome-wide SNP genotyping analysis.** A. Log R ratio and B allele frequency plots of chromosome 12 in iPS-C6. B. Log R ratio and B allele frequency of chromosome 2 in iPS-2B6. Red circles indicate duplicated chromosomal regions. (TIF)

**Figure S2 Protein expressions of HNEC-derived iPS.** Immunofluorescence staining for pluripotency and surface mark-

ers (OCT4, SOX2, NANOG, SSEA4, Tra-1-60, and Tra-1-81). All cells were also stained with DAPI and images were merged with lineage markers. Scale bars indicate 100  $\mu$ m. Yellow: Alexa 546, Green: Alexa488, Blue: Alexa460.

(TIF)

**Figure S3 RT-PCR analysis of EBs generated from HNEC-derived iPS cells.** RT-PCR results for ectoderm (paired box 6 (PAX6) and microtubule-associated protein 2 (MAP2)), mesoderm (BRACHYURY/T and homeobox 1 (MSX1)), and endoderm (SRY-box 17 (SOX17) and alpha-fetoprotein (AFP)) are presented. 1,2,3, and 4 correspond to iPS-B2, iPS-B3, iPS-2B1, and the negative control, respectively.

(TIF)

**Figure S4 Differentiation of 3 embryonic germ layers from HNEC-derived iPS cells.** Endoderm (left), mesoderm

(middle), and ectoderm (right) generated from iPS-B2, iPS-B3, and iPS-2B1 and iPS3A2, respectively.

(TIF)

**Table S1 Primers used for RT-PCR.**

(XLS)

## Acknowledgments

We thank Ms Yoshiko Tanabe and Ms Rieko Sato for the technical assistance. iPS cells derived from this study were deposited at RIKEN BioResource Center in Tsukuba-city, Japan.

## Author Contributions

Conceived and designed the experiments: Y. Horiuchi MMT TA EN. Performed the experiments: MO Y. Hamada YI KS TF. Analyzed the data: MO TH EN. Contributed reagents/materials/analysis tools: MMT YN MH. Wrote the paper: MO Y. Hamada MMT EN.

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