Reprogramming mechanisms influence the maturation of hematopoietic progenitors from human pluripotent stem cells

Hye-Ryeon Heo1, Haengseok Song2, Hye-Ryun Kim3, Jeong Eun Lee2, Young Gie Chung3, Woo Jin Kim1, Se-Ran Yang4, Kye-Seong Kim5, Taehoon Chun6, Dong Ryul Lee2,3 and Seok-Ho Hong1

Abstract

Somatic cell nuclear transfer (SCNT) or the forced expression of transcription factors can be used to generate autologous pluripotent stem cells (PSCs). Although transcriptomic and epigenomic comparisons of isogenic human NT-embryonic stem cells (NT-ESCs) and induced PSCs (iPSCs) in the undifferentiated state have been reported, their functional similarities and differentiation potentials have not been fully elucidated. Our study showed that NT-ESCs and iPSCs derived from the same donors generally displayed similar in vitro commitment capacity toward three germ layer lineages as well as proliferative activity and clonogenic capacity. However, the maturation capacity of NT-ESC-derived hematopoietic progenitors was significantly greater than the corresponding capacity of isogenic iPSC-derived progenitors. Additionally, donor-dependent variations in hematopoietic specification and commitment capacity were observed. Transcriptome and methylome analyses in undifferentiated NT-ESCs and iPSCs revealed a set of genes that may influence variations in hematopoietic commitment and maturation between PSC lines derived using different reprogramming methods. Here, we suggest that genetically identical iPSCs and NT-ESCs could be functionally unequal due to differential transcription and methylation levels acquired during reprogramming. Our proof-of-concept study indicates that reprogramming mechanisms and genetic background could contribute to diverse functionalities between PSCs.

Introduction

Human pluripotent ESCs, which are successfully derived by isolating an inner cell mass from a viable blastocyst, are allogeneic1. To overcome the issue of allogeneity, two innovative reprogramming approaches for converting somatic cells into PSCs were evaluated. The first approach involved the cellular reprogramming of somatic cells to pluripotency by the forced expression of four transcription factors (TFs), which resulted in the generation of iPSCs2,3. More recently, we and two other research groups independently reported the establishment of diploid pluripotent ESCs by transferring the nucleus of fetal and adult fibroblasts into enucleated oocytes4–6. These two reprogramming methods yield autologous PSCs, which could be suitable for the development of patient-specific cell therapies that do not cause immune rejection7. Thus, determining whether iPSCs and NT-ESCs are genetically safe and functionally competent is critical prior to their use in personalized regenerative medicine.

Recent achievements in the generation of human NT-ESCs have enabled the performance of detailed genetic and epigenetic comparisons between genetically matched human iPSCs and NT-ESCs, eliminating the genetic
heterogeneity among the PSC lines compared. These studies revealed that both cell types contained a similar number of coding mutations and variations in de novo copy number that were not detected in the donor somatic cells. Interestingly, Ma et al. reported the incomplete epigenetic reprogramming of iPSCs, and suggested that the transcriptional and epigenetic signatures of NT-ESCs are more similar to ESCs compared to iPSCs. Contrary to this finding, Johannesson et al. reported that the number of epigenetic changes between the two cell types was equivalent. The controversy between the two studies might be due to the use of different reprogramming methods or to the involvement of somatic cell donors with different potentials. Hence, understanding the fundamental states of NT-ESCs and iPSCs and determining the critical issues that must be addressed prior to their therapeutic application.

In this study, we generated isogenic sets of human NT-ESCs and iPSCs derived from different donors and compared their fundamental properties, including proliferation, clonogenicity, and heterogeneity in the undifferentiated state. Further, we first evaluated the in vitro potential of the isogenic pairs to differentiate into three germ layer lineages.

Materials and methods

Human SCNT-ESC and iPSC lines

CHA-hES NT2, 4, 5, and 8 (hereafter named NT, NT2, NT4, NT5, and NT8) for human SCNT-ESCs and iPS-NT2-S4, iP5-NT4-S1, iP5-NT4-E15, iP5-NT5-S9, and iP5-NT8-S1 (hereafter named iP52, iP54, iP54-Epi, iP55, and iP58) for isogenic iPSCs were used in this study. Human ESC line (CHA-hES 15, ESC) was used as a control. All these cell lines were initially produced in CHA Stem Cell Institute, CHA University, Seoul, South Korea. For human SCNT-ESC derivation, the procedures were described in the previous report. iPSC2, 4, 5, and 8 were generated using Sendai virus-based vectors, which express OCT4, SOX2, KLF4, and c-MYC (Cyto-Tune™.iPS Reprogramming kit; Invitrogen) according to the manufacturer’s protocol. Transgene and virus-free iPSC4-Epi was generated using episomal reprogramming vector, which express OCT4, SOX2, KLF4, and c-MYC (Epi5™. Episomal iPSC Reprogramming Kit; Invitrogen). Somatic donor for NT4 and iP54 was a healthy male donor (35 years old). Somatic donor for NT5 and iP58 was a female patient with age-related macular degeneration (73 years old).

Characterization of human NT-ESCs and iPSCs

Immunocytochemistry (ICC) and reverse transcription-polymerase chain reaction (RT-PCR) were performed to confirm hESC-specific marker expression. For ICC, antibodies against OCT3/4 (Santa Cruz, 1:100), SSEA-4 (Cell Signaling, 1:100), TRA 1-60 (Millipore, 1:100), TRA 1-81 (Millipore, 1:100), and Alexa Flour® 555 goat anti-mouse IgG antibody (Molecular probes, 1:200) were used, and cell nuclei were co-stained with DAPI (Vector Laboratories). For RT-PCR, we confirmed the expression of OCT4, NANOG, and SOX2 genes using following primer sequences: OCT4 (F) 5′-GCAATT TGCCAAGCTCCTGAGACAG-3′, (R) 5′-CATAGCCT GGGTACCAAAAT GGGG-3′ (536 bp); NANOG (F) 5′-TGAACCTCAGCTACAAACAG-3′, (R) 5′-TGG TGAGGAAGAGAAAG-3′ (153 bp); SOX2 (F) 5′-A GCTACAGCATGATGCAGGA-3′, (R) 5′-GGTGACTGAGTCTGACTGCA-3′ (125 bp); and GAPDH (F) 5′-T GAAGG TCGGAGTCAACGGATTTGGT-3′, (R) 5′-C ATGTGGCCATGAGGTCCACCAC-3′ (983 bp)/(F) 5′- AGAAGGCTGGGCCCTCATTTGG-3′, (R) 5′-AGGGGCC ATCCACAGTCTCT-3′ (258 bp) as an internal control.

The differentiation capacity of NT-ESC and iPSC lines was confirmed by embryoid body (EB) formation in vitro and teratoma formation in vivo. For EB formation, NT-ESCs and iPSCs were cultured in suspension without human bFGF for 2 weeks, and then the differentiation of EBs into three germ layers was confirmed by immunohistochemistry (IHC) and RT-PCR. For IHC, antibodies against AFP (Alpha-1-Fetoprotein; Dako, 1:100) for endoderm, SMA (alpha smooth muscle actin; Abcam, 1:100) for mesoderm, and TUJ1 (B-tubulin; Covance, 1:100) for ectoderm were used. Alexa Flour® 555 goat anti-rabbit IgG antibody (Molecular probes) was used as a secondary antibody (1:200) and nuclei were stained with DAPI (Vector Laboratories). Primer sequences for RT-PCR are listed in Supplementary Table 1. For teratoma formation, approximately 1×10⁵ of undifferentiated NT-ESCs and iPSCs were injected into a testicle of NOD/SCID male mouse. For each NT-ESC and iPSC line, at least three animals were used. After 12–16 weeks, teratomas were excised, fixed in parafomaldehyde, embedded in paraffin, sectioned, and then analyzed histologically after staining.

Chromosome analysis

Chromosome analyses for both NT-ESCs and iPSCs were performed using a standard protocol. Metaphase spreads were stained with a GTG (G-bands by trypsin using Giemsa)-banding technique. Twenty metaphases were analyzed and karyotyped by two cytogenetics experts. The ideogram was produced by the Ikaros karyotyping system (MetaSystems, Germany).

Genomic DNA fingerprinting

DNA fingerprinting of donor fibroblasts, donor oocytes, and NT-ESCs was performed by using AmpFISTR® Identifier kit (Applied Biosystems). PCR reactions were
performed according to the manufacturer’s protocol. The PCR products were loaded on an ABI 3130 genetic analyzer (Applied Biosystems) and analyzed by the GeneMapper® ID Software v3.2.1 (Applied Biosystems).

**Maintenance of human NT-ESCs and iPSCs**

All human PSC lines used in this study were maintained in mTeSR1 serum-free medium (Stem Cell Technologies) on Matrigel (BD Biociences)-coated tissue culture plates. They were subcultured every 5 days by mechanical dissociation.

**BrdU incorporation and cell cycle analysis**

Undifferentiated human ESCs, iPSCs, and NT-ESCs were incubated with 10 μM of bromodeoxyuridine (BrdU) for 4 h at 37 °C and then fixed with 70% ethanol containing 50 mM glycine for 20 min. Cell cycle analysis was performed using aliphosphoryethrin (APC)-BrdU Flow Kit (BD Pharmingen) following the manufacturer’s instructions. Cells were stained with APC-conjugated anti-BrdU antibody and 7-amino actinomycin (7ADD). Flow cytometric analysis was performed by using a FACSCanto II flow cytometer (BD Biosciences), and FlowJo software (Tree Star) was used to derive the fraction of cells distributed throughout various phases of the cell cycle.

**Clonogenic assay**

Dissociation of human iPSCs and NT-ESCs were performed as previously shown. For quantitative comparison of clonogenic potential between iPSCs and NT-ESCs, we seeded 10,000 cells into a six-well tissue culture plate coated with Matrigel in mTeSR1 medium supplemented with 10 μM Rho-associated protein kinase (ROCK) inhibitor for the initial 24 h. On the next day, all non-adherent cells were removed and fresh medium without ROCK inhibitor was added. The numbers of colonies were counted after 7 days of cultivating.

**Alveolar epithelial progenitor cell differentiation**

The stepwise alveolar epithelial progenitor cell (AEC) differentiation was performed as previously described with minor modification. Briefly, undifferentiated iPSC and NT-ESC colonies were prepared with low density (less than 10 colonies per well of a six-well tissue culture plate coated with Matrigel. When the colonies grew up to approximately 1 mm in diameter, the mTeSR1 medium was replaced by RPMI1640 medium containing 1× B27 supplement, activin A (100 ng/mL; R&D systems), 1 μM of CHIR99021 (Axon Medchem), and 0.125 mM sodium butyrate (Sigma) and cultured for 6 days to induce definitive endoderm (DE) cells. The DE cells were differentiated to ventralized anterior foregut endodermal (VAFE) cells as a AECs via AFE cells by sequential exposure to two different combinations of external factors for 8 days (100 ng/mL of noggin and 10 μM SB-431542 for 4 days, followed by 100 ng/mL of BMP4, 1 μM all-trans retinoic acid, and 1.5 μM CHIR99021 for 4 days). On day 14, VAFE cells were further differentiated to alveolar and distal airway epithelial (ADAE) cells by culturing in Ham’s F12 medium supplemented with KGF10 (100 ng/mL), 50 nM dexamethasone, 0.1 mM 8-br-cAMP, and 0.1 mM 3-isobutyl-1-methylxantine. Induction efficiency was assessed by observing the localization and frequencies of AEC and ADAE cell markers (NKKX2.1, EPCAM, CPM, and SFTP-B) on days 14 and 25 using immunostaining and flow cytometry.

**Neural differentiation**

Human iPSCs and NT-ESCs were induced to neural progenitor cells (NPCs) using serum-free STEMdiff® Neural Induction Medium (Stem Cell Technologies) according to the manufacturer’s instructions. Briefly, undifferentiated iPSC and NT-ESC colonies were prepared with low density of less than 10 colonies per well of a six-well tissue culture plate coated with Matrigel. After incubation for 4 days, followed by treatment with SCF (50 ng/mL) and VEGF (40 ng/mL) for 2 days. On day 6, the cultures were exposed to Stemline II serum-free medium supplemented with Insulin-Transferrin-Selenium and BMP4 (20 ng/mL) for 4 days, followed by treatment with SCF (50 ng/mL) and VEGF (40 ng/mL) for 2 days. On day 11, all cytokines were purchased from R&D Systems. The commitment capacity was assessed by measuring the frequencies of CD34- and CD45-positive cells on day 17 by flow cytometry.

**Hematopoietic differentiation**

A stepwise direct hematopoietic differentiation was performed as previously described with slight modifications. Briefly, undifferentiated iPSC and NT-ESC colonies were prepared with low density (less than 10 colonies in a single well of six-well tissue culture plate). When the colonies grew up to approximately 1 mm in diameter, the hemogenic specification began with them exposed to Stemline II serum-free medium supplemented with Insulin-Transferrin-Selenium and BMP4 (20 ng/mL) for 4 days, followed by treatment with SCF (50 ng/mL) and VEGF (40 ng/mL) for 2 days. On day 6, the cultures were given fresh hematopoietic induction medium supplemented with hematopoietic cocktail (50 ng/mL SCF, 10 ng/mL TPO, 50 ng/mL IL3, 50 ng/mL FLT-3L, and 50 ng/mL G-CSF) as described in Fig. 4a and cultured for 11 days. All cytokines were purchased from R&D Systems. The commitment capacity was assessed by measuring the frequencies of CD34- and CD45-positive cells on day 17 by flow cytometry.

**Colony-forming units assay**

Colony-forming units (CFU) assay was performed by plating 10,000 hematopoietic progenitor cells into methylcellulose H4434 (Stem Cell Technologies) supplemented with SCF (50 ng/mL), IL3 (10 ng/mL), EPO (3 U/mL), and GM-CSF (10 ng/mL). After incubation for
14 days at 37 °C in 5% CO₂, hematopoietic cell clusters were counted using standard morphological criteria.

Flow cytometric analysis

Single-cell suspensions were harvested from undifferentiated or differentiated iPSCs and NT-ESCs. Undifferentiated cultures were dissociated with cell dissociation medium (CDM; Invitrogen) for 20 min at 37 °C. Differentiated cells were treated with collagenase IV for 2 h, followed by treatment with CDM for 20 min in a 37 °C incubator. The cells were filtered through a 70 μm cell strainer and incubated for 30 min at 4 °C with following anti-human antibodies: OCT4 (BD Biosciences), SSEA-3 (BD Biosciences), SSEA-4 (BD Biosciences), CD34 (Miltenyi), CD45 (BD Biosciences), E-Cadherin (BD Biosciences), SOX1 (BD Biosciences), SOX2 (BD Biosciences), NESTIN (BD Biosciences), GFAP (BD Biosciences), NKKX2.1 (Abcam), CPM (Abcam), and EPCAM (Santa Cruz). For intracellular staining of OCT4, SOX1, SOX2, and NKKX2.1, the cells were fixed and permeabilized using the Cytofix/Cytoperm buffer (BD Biosciences). Dead cells were excluded by 7AAD staining. Flow cytometric analysis was performed by using a FACS Canto II flow cytometer and acquired data were analyzed by the FlowJo software.

Immunofluorescence staining

Undifferentiated PSCs and AECs were rinsed with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde (Sigma) for 20 min at room temperature. After washing with PBS, cells were permeabilized with 0.5% saponin (Sigma) in PBS containing 1% bovine serum albumin for 10 min and then blocked with 10% normal rabbit or donkey serum for 30 min. The following primary and secondary antibodies were used: goat anti-OCT3/4 (Santa Cruz), mouse anti-CPM (Abcam), rabbit anti-NKKX2.1 (Abcam), mouse anti-EPCAM (Santa Cruz), rabbit anti-goat Alexa Fluor 594 (Invitrogen), goat anti-mouse Alexa Fluor 594 (Invitrogen), donkey anti-mouse Alexa Fluor 594 (Invitrogen), and donkey anti-rabbit Alexa Fluor 488 (Invitrogen). Nuclei were stained with prolong gold DAPI with antifade (Invitrogen). Immunofluorescence signals were observed and photographed with an Olympus IX51 microscope equipped with a photomemix Cool Snap HQ2 camera using Image-Pro 3DA vs. 6.0. software.

qRT-PCR

Total RNA was extracted from undifferentiated PSCs and their derivatives using RNeasy kit (Qiagen). First strand cDNA was synthesized from 500 ng of total RNA using TopScript™ RT DryMIX kit (Enzynomix). The cDNA was then subjected to qPCR using AccuPower® PCR PreMix reagents (Bioneer Corp). Primer sequence is listed in Supplementary Table 1. Melting curve analyses were performed to confirm the presence of a single amplicon with correct size and absence of nonspecific bands. The expression levels of each gene were normalized to GAPDH and the relative quantification was conducted using the comparative CT method according to the manufacturer’s instructions (Applied Biosystems).

Microarray analysis

Total RNA was extracted with TriZol from undifferentiated hESC, hiPSC, and hNT-ESC lines. The quality of total RNA was evaluated using an Agilent 2100 Bioanalyzer. Agilent Human Gene expression 4x44K V2 microarray (Agilent Technologies, Santa Clara, CA, USA) was hybridized with appropriate probe at the facility of E-Biogen (Seoul, Korea). The hybridization images were analyzed by an Agilent’s DNA microarray Scanner and normalization steps were performed using GeneSpring GX 7.3 software. Hierarchical clustering with the average linkage method was performed with MeV4.9.0 software. Genes were classified into specific biological processes by gene ontology and expression profiles of gene clusters were visualized as heat maps with the MultiExperiment Viewer program. The heat map signals were generated by the log2 transformed human iPSC/human NT-ESC intensity ratio to compare expression level of genes.

Methylation analysis

Genomic DNA extracted from human iPSC and NT-ESC lines was assessed for DNA methylation using Human CpG island 244k microarray. The array was entrusted by E-Biogen (Seoul, Korea). The raw intensities of the scanned image for both human iPSC-Cy5 and NT-ESC-Cy3 channels were extracted and normalized according to the manufacturer’s instructions (Agilent Technology, USA). The heat maps and boxplots show the signal intensity ratios generated by the log2 transformed human iPSC/human NT-ESC intensity to compare methylation landscapes of CpG sites on the promoter region of genes classified into specific biological processes between isogenic cell lines.

Statistical analysis

Values for all measurements were presented as mean ± SD. Statistical significance was determined using Student’s t-test and p < 0.05 was considered statistically significant.

Results

Intrinsic proliferative and clonogenic potentials of isogenic PSC pairs are not altered by the reprogramming process

To determine if genetically matched human iPSCs and NT-ESCs are functionally equivalent, we generated four pairs of isogenic iPSCs (iPS2, 4, 5, and 8) and NT-ESCs
isogenic iPSCs and NT-ESCs exhibit similar alveolar epithelial cell and neuronal differentiation capacities

Next, we explored whether isogenic human iPSCs and NT-ESCs possess comparable potentials for in vitro differentiation into the three specific germ layer lineages. First, we evaluated the in vitro potential of the isogenic pairs to differentiate into an AEC lineage to determine endodermal potential. Using a previously reported step-wise induction protocol\(^{16}\), we compared the induction efficiency in VAFE and ADAE cells on days 14 and 25, respectively, using several specific markers (NKX2.1, CPM, EPCAM, and SFTP-B) (Fig. 2a). Following immunofluorescence staining, we found that AEC markers (NKX2.1, CPM, and EPCAM) were strongly expressed on day 14 (Fig. 2b). The frequencies of both VAFE and ADAE cells did not differ significantly between the isogenic iPSCs and NT-ESCs on days 14 and 25 (Fig. 2c, d). AEC differentiation resulted in the time-dependent down-regulation of OCT4 and the upregulation of AEC-related genes including GATA6 and NKX2.1 (Fig. 2e), indicating that the AEC lineage commitment of iPSCs and NT-ESCs followed similar trajectories in response to external stimuli. We then explored the fundamental nature of lung
Fig. 1 (See legend on next page.)
developmental-related genes in the isogenic pairs in the undifferentiated state at both the transcriptional and epigenetic levels. The mRNA expression profiles (Fig. 2f) and promoter methylation levels (Fig. 2g) of genes involved in lung development in the undifferentiated isogenic PSCs did not indicate a bias for the AEC lineage (Supplementary Excel file 1). These results indicated that isogenic iPSCs and NT-ESCs in undifferentiated cultures possess similar AEC commitment and maturation capacities in the absence of a genetic or epigenetic predisposition to the AEC lineage.

We likewise compared the differentiation potential of isogenic iPSCs and NT-ESCs to NPCs to determine ectodermal lineage potential using an established serum-free neural induction protocol (Fig. 3a). All of the undifferentiated cell line colonies exhibited a similar morphology to NPCs during differentiation (Fig. 3b). Isogenic iPSCs and NT-ESCs likewise displayed similar NPC marker frequencies (SOX2, SOX1, NESTIN, and GFAP) on day 9 of differentiation. In addition, no variation in neural differentiation potential was observed in the PSCs derived from two donors (Fig. 3c). NPC differentiation resulted in the concurrent upregulation of neural-related genes (*NEUROG2* and *SOX1*) and the time-dependent downregulation of *OCT4* (Fig. 3d), indicating that the induction of neural lineage commitment in iPSCs and NT-ESCs using specific soluble stimuli was similar. Furthermore, the mRNA expression profiles (Fig. 3e) and promoter methylation levels (Fig. 3f) of genes involved in neuronal differentiation in the undifferentiated isogenic iPSCs and NT-ESCs indicated the absence of a genetic or epigenetic predisposition to the neural lineage (Supplementary Excel File 1).

**Donor-dependent variations in hematopoietic commitment capacity between human PSC lines**

Lastly, we attempted to induce the differentiation of the isogenic pairs to the hematopoietic lineage to determine mesodermal potential. We employed a directed, stepwise differentiation protocol as previously described with some modifications17 (Fig. 4a). Following hematopoietic induction using BMP4, colonies from all of the cell lines exhibited a similar plateau-like central area surrounded by monolayer cells. Floating blood cells emerged from the hematopoietic cell clusters along the plateau area margins during the hematopoietic commitment phase (Fig. 4b). We assessed the commitment capacity of all of the cell lines by measuring the primitive hematopoietic progenitor (CD34+CD45+ or CD34+CD43+) and mature hematopoietic (CD34−CD45+) cell frequencies on day 17. CD45 was previously identified as a hematopoietic progenitor marker for human ESC-derived hematopoiesis in vitro18. No significant differences in the CD34+CD45+, CD34+CD43+, or CD34−CD45+ subset frequencies were detected between the isogenic iPSCs and NT-ESCs (Fig. 4c, d and Supplementary Figs. 7a, b). However, NT4 and iP54 yielded significantly more hematopoietic progenitors and mature blood cells than NT5 and iP55. NT2 and iP52 generated higher levels of hematopoietic progenitors than NT8 and iP58. In addition, both NT2/iP52 and NT8/iP58 pairs exhibited significantly higher frequencies of CD45+ mature blood cells compared with NT5 and iP55. This finding was consistent with those from recent studies demonstrating donor-dependent variations in hematopoietic differentiation potential among hPSC lines19,20. To further understand the underlying mechanisms that influence donor-dependent variations in hematopoietic differentiation at the molecular level, we compared the expression profiles of hematopoietic-related genes in undifferentiated NT4 and NT5 and iP54 and iP55. We found that several genes were up or downregulated in NT4 and iP54 compared to NT5 and iP55, and the observed differences in expression levels were validated using qPCR (Supplementary Figs. 7c, d). In PSCs, RUNX1 expression, a master regulator of hematopoietic stem cell emergence, enhances the conversion of undifferentiated PSCs to hematopoietic progenitor cells21. GYPA (CD235) is a specific marker for human erythroid precursors22. These findings suggest that the increased expression of hematopoietic-related genes such as RUNX1 and GYPA in NT4 and iP54 may prime undifferentiated cells toward hematopoietic or erythrocyte lineage commitment or maturation.
Fig. 2 (See legend on next page.)
Differential transcription and methylation levels acquired during reprogramming may influence the hematopoietic maturation capacity of isogenic NT-ESCs and iPSCs

To evaluate the apparent functional inequality between the isogenic pairs with respect to the maturation capacity of the hematopoietic progenitors, an equal number of hematopoietic progenitors were plated on methylcellulose and the colony-forming potential was measured (Supplementary Fig. 7e). Interestingly, the hematopoietic progenitors derived from the NT-ESC lines produced more colony-forming units (CFUs) (Fig. 5a, b) and gave rise to a greater number of CFU-macrophage (CFU-M) and CFU-erythrocytes (CFU-E) than the iPSC lines (Fig. 5c, d). In addition, CFU assay showed a significant increase of the percentage of CFU-M colonies in the NT-ESC lines compared to the iPS lines (Fig. 5e, f).

The functional advantage of human NT-ESCs with regard to the maturation capacity of the hematopoietic progenitors in this study was reinforced by similar findings reported in mouse isogenic NT-ESCs and iPSCs.28 To investigate the molecular signatures associated with the greater maturation capacity of NT-ESCs, we analyzed the transcription profiles of hematopoietic-related genes in the isogenic pairs (NT4 vs iPS4 and NT5 vs iPS5), and detected higher (11 genes) and lower (8 genes) expression among the genes in NT-ESCs. Furthermore, a strong inverse correlation was detected between the gene expression profiles and the methylation status of their promoter regions (Fig. 5g–i). Numerous reprogramming methods have been developed to generate iPSCs and may lead to different transcript levels of the same genes which can have specific biological functions. Thus, we generated iPSCs (iPS4-Epi) with non-integrating episomal vectors from the donor cells for iPS4 generation and then compared the expression levels of the selected genes between iPS4-Epi and NT4. Interestingly, iPS4-Epi showed the similar expression patterns as seen in iPS4 (Supplementary Figs. 8a, b), indicating that this phenomenon may not reprogramming method-dependent. Taken together, our results indicate that hematopoietic commitment capacity is not positively correlated with maturation capacity, which might be due to the differential transcription and methylation of genes related to hematopoietic development during reprogramming.

Discussion

Recent reports have suggested that the cell fate potential and functional variations among human ESCs and iPSCs primarily depend on the donors rather than on the origin of the cell type19,20,25. In this study, we also determined that genetically identical NT-SCs and iPSCs are generally functionally equivalent. However, our data clearly demonstrated that stage-specific variations during differentiation, such as the maturation capacity of hematopoietic progenitors, might be due to different reprogramming systems. The predisposition potentials could be influenced by different genetic and/or epigenetic landscapes acquired during reprogramming. Therefore, monitoring transcriptome and epigenome alterations during reprogramming by NT or forced TF expression is required to understand the resulting differentiation capacity26.

In our study, we have identified a set of potential genes that might influence variations in hematopoietic maturation between isogenic NT-ESCs and iPSCs. Some of the genes upregulated in NT-ESCs are known to enhance self-renewal and the differentiation of murine and human hematopoietic stem/progenitor cells. For example, PRDM16 induced by HOXA9 enhances the self-renewal of hematopoietic and myeloid progenitor cells.27 HOXA9 also promotes hematopoietic differentiation in human ESCs, indicating that the HOXA9–PRDM16 axis may play a crucial role in hematopoietic differentiation. MDX1 serves as a transcriptional regulator during the megakaryocytic and granulocytic differentiation of hematopoietic and myeloid progenitors.29–31 Interestingly, the expression of NOG, a BMP4 antagonist, was notably downregulated in NT-ESCs, suggesting that increased BMP4 action may promote the self-renewal and maturation of hematopoietic progenitors in NT-ESCs. Thus, further genetic functional studies will be required to define the precise role of these genes in determining the differentiation propensity of human PSC lines toward hematopoietic lineage.
Although comparable biological features of undifferentiated isogenic NT-ESCs and iPSCs have been reported at the genetic and epigenetic levels, data evaluating their differentiation potentials are limited. A previous report indicated that the ability of mouse NT-ESCs to form hematopoietic colonies is greater than the corresponding capacity of isogenic iPSCs. Recently, Zhao et al. demonstrated the functional similarity of cardiomyocytes and endothelial cells derived from isogenic human iPSCs and NT-ESCs. However, both prior studies only evaluated the cell fate potential of the mesodermal lineage. Therefore, the precise evaluation of the functional equivalence of the isogenic pairs requires a comparison of a broad spectrum of fundamental features including the differentiation of three germ layer lineages and self-renewal capacity. To our knowledge, we are the first to evaluate...
Fig. 4 (See legend on next page.)
**Fig. 4** Donor-dependent variations in hematopoietic commitment capacity between human PSC lines. 

**a** Schematic diagram of serum- and feeder-free stepwise hematopoietic induction of human iPSCs and NT-ESCs. Flow cytometry analysis of committed hematopoietic progenitors (CD34^+CD45^+) and mature blood (CD34^−CD45^+) cells on day 17. 

**b** Representative images at different stages of hematopoietic differentiation. Scale bar, 100 μm. 

**c, d** Flow cytometry analysis of cells harvested on day 17 showing the frequencies of hematopoietic progenitor and mature blood cells. \( p < 0.05 \) (vs NT5), \( ^{*}p < 0.05 \) (vs IPS5), \( ^{*}p < 0.05 \) (vs IPS8), \( **p < 0.01 \) (vs NT8). Both floating and attached cells were harvested to measure the efficiency of hematopoietic differentiation. All bars indicate the mean±SD from three independent experiments.

**Fig. 5** Differential transcription and methylation levels in genes related to hematopoietic development during reprogramming may influence the maturation capacity of isogenic NT-ESCs and iPSCs. 

**a–f** Assessment of hematopoietic progenitor capacity by counting the number (a–d) and distribution of CFU subtypes (e, f). CFU-E, erythroid; CFU-M, macrophage; and CFU-G, granulocytes; CFU-GM, granulocytes/macrophage; CFU-GM, erythroid. \( ^{*}p < 0.05 \). 

**g** Heat maps representing up- and downregulated genes in NT lines compared with iPSC lines and the promoter methylation status of corresponding genes. 

**h, i** Verification of the transcriptomic and methylocmic analysis of selected genes using qPCR. \( ^{*}p < 0.05 \), \( **p < 0.01 \). All bars indicate the mean±SD from three independent experiments.
the functional equivalence of isogenic human NT-ESCs and iPSCs regarding the lineage specificity of all three germ layers, and to provide evidence that reprogramming mechanisms could contribute to a functional bias toward differentiation lineage specificity between PSCs. The isogenic iPSC and NT-ESC pairs used in this study were derived from one cell type (dermal fibroblast). Thus, it is critical to note that future studies involving the establishment and testing of large cohorts of isogenic pairs derived from various cell types are required to strengthen our findings. More importantly, in vivo studies involving humanized animal and disease models will be required to determine the regenerative potential and immunogenicity of isogenic NT-ESCs and iPSCs.

Acknowledgements
This study was supported by grants from the Ministry of Science, ICT and Future Planning (2017M3A9B3061838, 2017M3A9B4029250, and 2015M3A9C6028961).

Author details
1Department of Internal Medicine, School of Medicine, Kangwon National University, Chuncheon, Republic of Korea. 2Department of Biomedical Science, College of Life Science, CHA University, Seongnam, Republic of Korea.
3Research Institute for Stem Cells, CHA Health Systems, Los Angeles, CA 90036, USA. 4Department of Thoracic and Cardiovascular Surgery, School of Medicine, College of Medicine, Kangwon National University, Chuncheon, Republic of Korea. 5Department of Biomedical Science, College of Medicine, Hanyang University, Seoul 04763, Republic of Korea. 6Department of Biotechnology, College of Life Science and Biotechnology, Korea University, Seoul, Republic of Korea.

Author contributions
D.R.L. and S.-H.H. conceived and designed the research. H.-R.H., W.J.K., T.C., S.-R.Y., K.-S.K., J.E.L. and Y.G.C. performed the experiments. H.-R.H., W.J.K., T.C., S.-R.Y., K.-S.K., D.R.L. and S.-H.H. analyzed the data and wrote the manuscript.

Competing interests
The authors declare no conflict of interest.

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Supplementary Information
accompanies this paper at (https://doi.org/10.1038/s41419-018-1124-6).

Received: 10 April 2018 Revised: 26 August 2018 Accepted: 1 October 2018 Published online: 24 October 2018

References
1. Thomson, J. A. et al. Embryonic stem cell lines derived from human blastocysts. Science 282, 1145–1147 (1998).
2. Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676 (2006).
3. Takahashi, K. et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861–872 (2007).
4. Chung, Y. G. et al. Human somatic cell nuclear transfer using adult cells. Cell Stem Cell 14, 777–780 (2014).
5. Tachibana, M. et al. Human embryonic stem cells derived by somatic cell nuclear transfer. Cell 153, 1228–1238 (2013).
6. Yamada, M. et al. Human oocytes reprogram adult somatic nuclei of a type 1 diabetic to diploid pluripotent stem cells. Nature 510, 533–536 (2014).
7. Wolf, D. P. et al. Concise review: Embryonic stem cells derived by somatic cell nuclear transfer: a horse in the race? Stem Cells 35, 26–34 (2017).
8. Ma, H. et al. Abnormalities in human pluripotent cells due to reprogramming mechanisms. Nature 511, 177–183 (2014).
9. Johansson, B. et al. Comparable frequencies of coding mutations and loss of imprinting in human pluripotent cells derived by nuclear transfer and defined factors. Cell Stem Cell 15, 634–642 (2014).
10. Colman, A. & Burley, J. Human somatic cell reprogramming: does the egg know best? Cell Stem Cell 15, 531–532 (2014).
11. Chung, Y. G. et al. Histone demethylase expression enhances human somatic cell nuclear transfer efficiency and promotes derivation of pluripotent stem cells. Cell Stem Cell 17, 758–766 (2015).
12. Singh, A. M. & Dalton, S. The cell cycle and Myc interact with mechanisms that regulate pluripotency and reprogramming. Cell Stem Cell 5, 141–149 (2009).
13. Stewart, M. H. et al. Clonal isolation of hESCs reveals heterogeneity within the pluripotent stem cell compartment. Nat Methods 3, 807–815 (2006).
14. Hong, S. H. et al. Cell fate potential of human pluripotent stem cells is encoded by histone modifications. Cell Stem Cell 9, 24–36 (2011).
15. Li, L., Bennett, S. A. L. & Wang, L. S. Role of E-cadherin and other cell adhesion molecules in survival and differentiation of human pluripotent stem cells. Cell Adh. Migr. 6, 59–70 (2012).
16. Gottoh, S. et al. Generation of alveolar epithelial spheroids via isolated progenitor cells from human pluripotent stem cells. Stem Cell Rep. 3, 394–403 (2014).
17. Nwa, A. et al. A novel serum-free monolayer culture for orderly hematopoietic differentiation of human pluripotent cells via mesodermal progenitors. PLoS ONE 6, e22286 (2011).
18. Vodyanyk, M. A., Thomson, J. A. & Suluken, I. I. Leukosialin (CD43) defines hematopoietic progenitors in human embryonic stem cell differentiation cultures. Blood 108, 2095–2105 (2006).
19. Feraud, O. et al. Donor dependent variations in hematopoietic differentiation among embryonic and induced pluripotent stem cell lines. PLoS ONE 11, e0149291 (2016).
20. Kyttilä, A. et al. Genetic variability overrides the impact of parental cell type and determines PSC differentiation potential. Stem Cell Rep. 6, 200–212 (2016).
21. Sugimura, R. et al. Haematopoietic stem and progenitor cells from human pluripotent stem cells. Nature 545, 432–438 (2017).
22. Jackson, M. et al. Enforced expression of HOXB4 in human embryonic stem cells enhances the production of hematopoietic progenitors but has no effect on the maturation of red blood cells. Stem Cells Transl. Med. 5, 981–990 (2016).
23. Kim, K. et al. Epigenetic memory in induced pluripotent stem cells. Nature 467, 285–290 (2010).
24. Trevisan, M. et al. Reprogramming methods do not affect gene expression profile of human induced pluripotent stem cells. Int. J. Mol. Sci. 18, 206–219 (2017).
25. Kajiwara, M. et al. Donor-dependent variations in hepatic differentiation from human-induced pluripotent stem cells. Proc. Natl Acad. Sci. USA 109, 12538–12543 (2012).
26. Nishizawa, M. et al. Epigenetic variation between human induced pluripotent stem cell lines is an indicator of differentiation capacity. Cell Stem Cell 19, 341–354 (2016).
27. Yu, H. et al. Downregulation of Prdm16 mRNA is a specific antileukemic mechanism during HOX84-mediated HSC expansion in vivo. Blood 124, 1737–1747 (2014).
28. Ramos-Mejia, V. et al. HOX9A promotes hematopoietic commitment of human embryonic stem cells. Blood 124, 3065–3075 (2014).
29. Fuhrken, P. G. et al. Gene Ontology-driven transcriptional analysis of CD34+ cell-initiated megakaryocytic cultures identifies new transcriptional regulators of megakaryopoiesis. Physiol. Genomics 33, 159–169 (2008).
30. Zin, R. et al. miR-382-5p controls hematopoietic stem cell differentiation through the downregulation of MJD1. Stem Cells Dev. 25, 1433–1443 (2016).
31. Zada, A. A. et al. Proteomic discovery of Max as a novel interacting partner of C/EBPalpha: a Myc/Max/Max link. Leukemia 20, 2137–2146 (2006).
32. Kim, K. et al. Epigenetic memory in induced pluripotent stem cells. Nature 467, 285–290 (2010).
33. Zhao, M. T. et al. Molecular and functional resemblance of differentiated cells derived from isogenic human iPSCs and SCNT-derived ESCs. Proc. Natl Acad. Sci. USA 114, E11111–E11120 (2017).