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20. 中畑龍俊：iPS 細胞の小児がん治療への様々な応用．東京都福祉保健局委託事業小児がん早期診断推進研修会　公開シンポジウム「小児がん治療の現在」2015 年 2 月 11 日　聖路加国際大学アリス・C・セントジョンメモリアルホール

G. 知的財産権の出願・登録状況
1. 特許取得
特になし

2. 実用新案登録
特になし

3. その他
特になし

厚生労働科学研究委託費（免疫アレルギー疾患等実用化研究事業）
委託業務成果報告（業務項目）

CINCA症候群の化合物スクリーニング

担当責任者　太田　章　京都大学iPS細胞研究所　教授

研究要旨：本研究の目的は、iPS由来単球株を用いて、汎用性の高い化合物スクリーニングプラットフォームを確立し、横断的探索により新規創薬シーズを得ることである。本年度はCINCA症候群患者由来iPS細胞より分化させた単球系細胞を株化し、マクロファージへ分化させたものを持ちて、既知の化合物約4905種のスクリーニングを行った。384穴プレート16枚全てでZ 'factorは0.5以上であった。プライマリスクリーニングとしてIL-1b分泌抑制率>30%を基準としたところ、238化合物(4.9%)がヒットした。さらに、これらのうちIL-6産生を抑制しなかったものを抽出すると、36化合物(0.7%)が抽出された。

A. 研究目的

本研究の目的は、iPS由来単球株を用いて、汎用性の高い化合物スクリーニングプラットフォームを確立し、横断的探索により新規創薬シーズを得ることである。
炎症の制御はヒト疾患の制御において重要な課題である。ヒトと他の動物種では炎症制御経路が異なることがあり、ヒト試料による探索は有用であるが、患者由来サンプルを用いた免疫疾患の研究では、得られる細胞数が限られることや、生体内のサイトカイン環境などに影響されることもあり、治療薬候補をスクリーニングする試みはあまり行われていない。ヒト人工多能性幹細胞（iPS細胞）の樹立が解決策となり得るが、目的の分化細胞の純度・収量や成熟度が不十分でバッチ毎にばらつくこと、分化のコストが高いことなどの問題がある。そこで、本研究では（遺伝子改変）疾患iPS細胞より分化誘導した単球を株化し、大量に増幅することにより、ヒト患者由来の細胞を用いた、安定かつ高スループットの化合物探索を行う。得られたヒット化合物を創薬シーズとして、製薬企業との共同研究へ移行する。平成25年度は、1～複数の単球株（単一遺伝子疾患）を作成し、うち1疾患程度でスクリーニングを開始する。

なお、分担研究者は、京都大学iPS細胞研究所にて創薬ユニットを運営しており、各種化合物ライブラリと機器の管理を行っている。

B. 研究方法

平成26年度はCINCA症候群について、検討を行った。

化合物スクリーニング

研究所にて所持している活性既知の化合物ライブラリを用いて、IL-1b産生を抑制する化合物のスクリーニングを行う。化合物ライブラリには、既存薬、キナーゼ阻害剤などが含まれる。

（倫理面への配慮）
本研究は、疾患を有する患者さんから検体を頂きiPS細胞を作成して行う研究であるため、患者さんの同意・協力を必要とする研究である。また、作成するiPS細胞を用いた疾患解析においては、遺伝子解析が必須であり、個人情報の取り扱いの配慮を必要とする研究である。この2点に対して、京都大学医学部医の倫理委員会に、ヒトを対象とした医学の研究および臨床応用実施申請書として「ヒト疾患特異的iPS細胞の作成とそれを用いた疾患解析に関する研究」
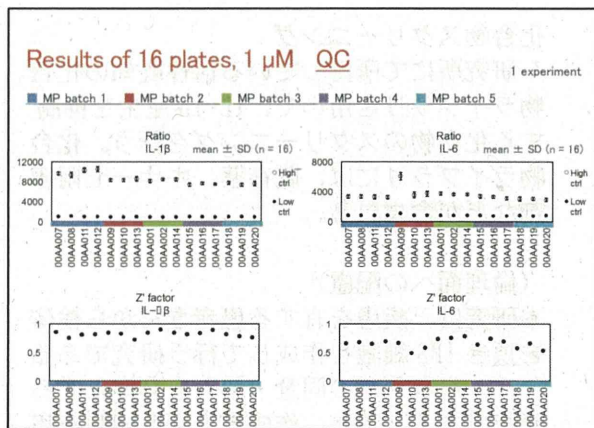
およびヒト遺伝子解析申請書として「ヒト疾患特異的 iPS 細胞を用いた遺伝子解析研究」の２申請を行った。その後、京都大学医学部医の倫理委員会での審査を頂き、平成２０年６月４日付けで、実施に関して承認を頂いた。本研究においては、その内容を忠実に順守して行った。また、組み替え遺伝子指針、ヒト ES 指針に則って実験計画を提出し、それを遵守して研究を行っている。
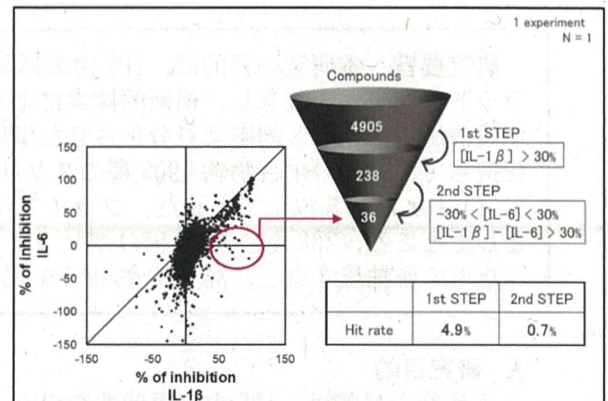
C. 研究結果
CINCA-iPSMLMP を用いた活性既知化合物スクリーニング

研究グループで開発されたプロトコル CINCA-iPS 細胞由来単球株より分化させたマクロファージを用いて、活性既知の化合物約 4905 種のスクリーニングを行った。

384 穴プレート 16 枚全てで Z'factor は 0.5 以上であった。

プライマリスクリーニングとして IL-1b 分泌抑制率>30%を基準としたところ、238 化合物（4.9%）がヒットした。さらに、これらのうち IL-6 産生を抑制しなかったものを抽出すると、36 化合物（0.7%）が抽出された。

D. 考察

疾患特異的 iPS 細胞を用いた創薬開発、患者細胞を用いた免疫疾患の新規治療法開発、はいずれも多大な成果が見込まれる領域であるが、様々な問題点のため、世界的に見ても有用なスクリーニング系は確立していない。本研究提案では、iPS 細胞の遺伝子改変による「厳密な対照細胞」「レポーター」作成、及び単球株化による「均質かつ大量の疾患特異的分化細胞の取得」を組み合わせ、大幅に汎用性の高く、低コストのスクリーニング系を確立できると期待される。

次年度は、CINCA 症候群のスクリーニングで同定された候補化合物のバリデーション、コア・ジェネラルライブラリのスクリーニングを進める。

E. 結論

活性既知化合物ライブラリのスクリーニングにより、プライマリヒットを得た。次年度以降、研究を進める予定である。

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学会発表
なし

G．知的財産権の出願・登録状況
1．特許取得
特になし

2．実用新案登録
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3．その他
特になし

学 会 等 発 表 実 績

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機関名：国立大学法人京都大学

１．学会等における口頭・ポスター発表

発表した成果（発表題目、口頭・ポスター発表の別）	発表者氏名	発表した場所（学会等名）	発表した時期	国内・外の別
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RESEARCH ARTICLE

Derivation of Mesenchymal Stromal Cells from Pluripotent Stem Cells through a Neural Crest Lineage using Small Molecule Compounds with Defined Media

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Abstract

Neural crest cells (NCCs) are an embryonic migratory cell population with the ability to differentiate into a wide variety of cell types that contribute to the craniofacial skeleton, cornea, peripheral nervous system, and skin pigmentation. This ability suggests the promising role of NCCs as a source for cell-based therapy. Although several methods have been used to induce human NCCs (hNCCs) from human pluripotent stem cells (hPSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), further modifications are required to improve the robustness, efficacy, and simplicity of these methods. Chemically defined medium (CDM) was used as the basal medium in the induction and maintenance steps. By optimizing the culture conditions, the combination of the GSK3β inhibitor and TGFβ inhibitor with a minimum growth factor (insulin) very efficiently induced hNCCs (70–80%) from hPSCs. The induced hNCCs expressed cranial NCC-related genes and stably proliferated in CDM supplemented with EGF and FGF2 up to at least 10

passages without changes being observed in the major gene expression profiles. Differentiation properties were confirmed for peripheral neurons, glia, melanocytes, and corneal endothelial cells. In addition, cells with differentiation characteristics similar to multipotent mesenchymal stromal cells (MSCs) were induced from hNCCs using CDM specific for human MSCs. Our simple and robust induction protocol using small molecule compounds with defined media enabled the generation of hNCCs as an intermediate material producing terminally differentiated cells for cell-based innovative medicine.

Introduction

In order to apply human pluripotent stem cells (hPSCs) to innovative medicine, such as cell therapy, disease modeling, and drug discovery, robust and efficient methods to produce the desired cell types without contaminating undesired cells are indispensable [1]. Since the contamination of hPSCs, in particular, may cause serious adverse effects, careful monitoring, which requires a considerable amount of time and cost, has to be conducted. Therefore, it would be beneficial to have intermediate cells between hPSCs and terminally differentiated cells, which are proved to have no contaminated hPSCs, contain limited but multiple differentiation properties, and stably proliferate without phenotypic changes. One of the promising candidates with such features is the neural crest cell (NCC) [2].

The neural crest emerges at the border of the neural and non-neural ectoderm in gastrula embryos during vertebrate development [3]. Cells in the neural crest, and later in the dorsal part of the neural tube, eventually delaminate and migrate throughout the body while retaining their characteristic phenotype [4]. When they reach their target tissues, NCCs differentiate into specific cell types depending on the location [5]. NCCs give rise to the majority of cranial bone, cartilage, smooth muscle, and pigmented cells in the cranial region, as well as neurons and glia in the peripheral nervous system [3–5]. Cardiac NCCs are known to contribute to valves in the heart, while vagal NCCs differentiate into enteric ganglia in the gut [6]. NCCs give rise to neurons and glia in the peripheral nervous system in the trunk region, secretory cells in the endocrine system, and pigmented cells in the skin.

Using a lineage-tracing system, rodent neural crest-derived cells were detected in adult tissues such as bone marrow, and still retained multipotent differentiation properties, which indicated that these cells are one of the cell-of-origin of multipotent mesenchymal stromal cells (MSCs) [7, 8]. Therefore, the production of human MSCs (hMSCs) from hPSCs via NCC lineage is a promising approach for the use of hPSCs in innovative medicine [9, 10]. A considerable number of studies have been dedicated to establishing robust and efficient induction methods from hPSCs to hNCCs in the past decade [11–13]. However, most of these studies used non-human stromal feeder cells or only achieved low induction
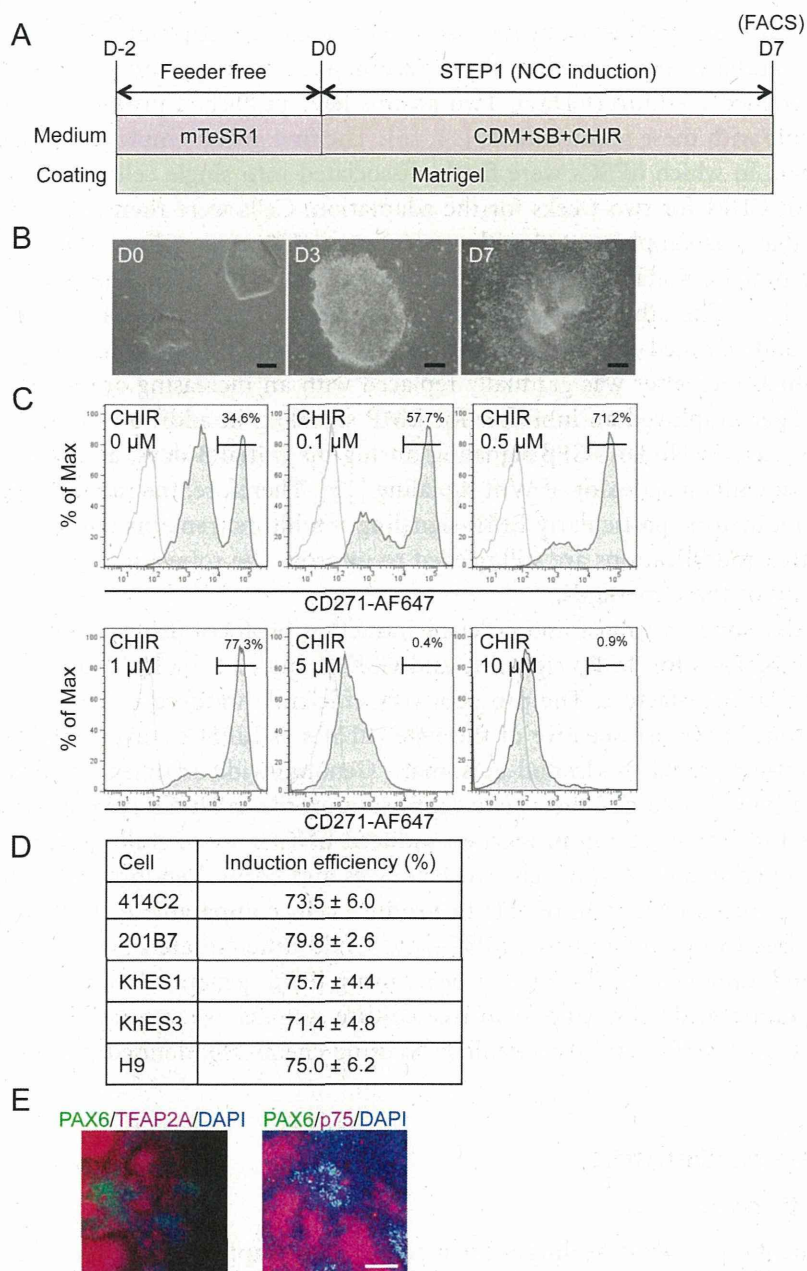
A

B

C

D

Cell	Induction efficiency (%)
414C2	73.5 ± 6.0
201B7	79.8 ± 2.6
KhES1	75.7 ± 4.4
KhES3	71.4 ± 4.8
H9	75.0 ± 6.2

E

Figure 1. Induction of p75^{high} cells from hPSCs. A) Schematic representation of the protocol. B) Morphology of colonies during the induction. Phase contrast images were taken on days 0, 3, and 7. Scale bar, 200 μm. C) The fraction of p75-positive cells in 201B7 cells was treated with SB431542 (SB) (10 μM) and CHIR99021 (CHIR) (indicated concentration) for seven days, stained with an anti-p75 antibody, and analyzed by FACS. D) Fraction of the p75^{high} population induced by SB (10 μM) and CHIR (1 μM) from hESCs (KhES1, KhES3, H9) and hiPSCs (414C2, 201B7). Average ± SD. N=3, biological triplicate. E) Immunocytochemical analyses of colonies on day 7 (201B7). Cells were stained with antibodies against PAX6, TFAP2A, and p75. Scale bar, 100 μm.

efficiencies. An ideal method from the standpoint of clinical applications is free from xeno-materials, such as feeder cells or serum, and can be performed using a chemically defined medium (CDM). Two groups have published protocols that are compatible with these requirements [14, 15]. The first group employed a two-step approach, in which hPSCs were firstly dissociated into single cells and cultured with CDM for two weeks for the adaptation. Cells were then cultured with CDM that was supplemented with an activator of Wnt signaling and inhibitor of Activin/Nodal/TGFβ signaling, but was free from BMP signaling modulation [14]. The other group used MEF-conditioned hESC media for the initial step, and replaced it with knockout serum replacement (KSR)-based medium, which thereafter was gradually replaced with an increasing amount of N2 media. They employed an inhibitor for BMP signaling in addition to an inhibitor for Activin/Nodal/TGFβ signaling during the initial 3 days, and then replaced them with an activator of Wnt signaling [15]. Therefore, the requirement for signal modulators, particularly BMP signaling inhibitors, remains controversial. Further modifications are still needed to improve the robustness, efficacy, and simplicity of these methods.

We here developed a robust and efficient induction protocol using CDM containing inhibitors for TGFβ signaling and GSK3β, but not for BMP signaling with minimal growth factors. The protocol very efficiently induced hNCCs (70–80%) from hPSCs irrespective of the type (hESCs vs hiPSCs) or generating method (viral-integrated vs plasmid-episomal). Genome-wide analyses revealed that induced hNCCs retained their gene expression profile as NCCs even after 10 passages. As for differentiation properties, induced hNCCs successfully differentiated into peripheral neurons, glia, melanocytes and corneal endothelial cells. In addition, induced hNCCs were able to produce cells comparable to hMSCs, which were free from contaminated hPSCs and could differentiate into osteo-, chondro-, and adipogenic cells. Furthermore, using iPSCs generated and maintained under feeder-free and xeno-free culture systems, we successfully induced hNCCs, hMSCs, and osteogenic cells using chemically defined media.

Materials and Methods

Ethics statement

The experimental protocols dealing human subjects were approved by the Ethics Committee of the Department of Medicine and Graduate School of Medicine, Kyoto University. Written informed consent was provided by each donor.

Cell lines

hESCs (H9, KhES1, and KhES3) and hiPSCs (414C2 and 201B7) were used in this study [16–19] They were maintained on SNL feeder cells [20] in Primate ES cell medium (ReproCELL, Tokyo, Japan) supplemented with 4 ng/ml recombinant human FGF2 (WAKO, Osaka, Japan). 987A3, hiPSCs generated and maintained

under feeder-free and xeno-free culture systems from human primary fibroblasts, were maintained on iMatrix-551 (rLN511E) (Nippi, Tokyo, Japan)-coated cell culture plates with StemFit (Ajinomoto, Tokyo, Japan) as described previously [21]. Bone marrow derived hMSCs were obtained from donors and used in our previous study [22]. Human corneal endothelial cells were isolated from human corneal tissues obtained for research purpose from SightLife (Seattle, WA, USA).

Culture media and reagents

mTeSR1 medium (STEMCELL Technology, Vancouver, Canada) was used for the feeder-free culture of PSCs. The induction and maintenance of hNCCs were performed using previously reported CDM [23], which contains Iscove's modified Dulbecco's medium/Ham's F-12 1:1, 1x chemically defined lipid concentrate (GIBCO, Grand Island, NY, USA), 15 µg/ml apo-transferrin (Sigma, St. Louis, MO, USA), 450 µM monothioglycerol (Sigma), 5 mg/ml purified BSA (99% purified by crystallization; Sigma), 7 µg/ml Insulin (WAKO), and penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Culture dishes were coated with growth factor-reduced Matrigel (BD, Bedford, MA, USA) or fibronectin (Millipore, Bedford, CA, USA). EGF (R&D, Minneapolis, USA) and FGF2 were used to maintain hNCCs [24]. SB431542 (SB) (Sigma), CHIR99021 (CHIR) (WAKO), BMP4 (R&D), DMH1 (Tocris, Bristol, UK), LDN193189 (Stemgent, Cambridge, MA, USA), and recombinant human Noggin (R&D) were used to modulate growth factor signals. Retinoic acid (RA) (Sigma) was used to modulate hNCCs.

Fluorescence-Activated Cell Sorting (FACS)

FACS was performed by AriaII (BD) according to the manufacturer's protocol. The antibodies used in FACS were listed in Table S1. In all experiments, FACS histograms of isotype controls were similar to those without antibodies; therefore, histograms without antibodies were used as control populations.

Immunocyto- and immunohistochemistry

Prior to performing immunostaining with antibodies, cells on plates were fixed with 4% paraformaldehyde at 4°C for 15 minutes, washed two times with PBS, and incubated with 0.3% TritonX100 at 4°C for 30 minutes as the surface-active agent for penetration processing, and any nonspecific binding was blocked with 2% skim milk/PBS at 4°C for 1 hour. Cornea samples obtained from rabbits euthanized three days after the injection of cells were fixed with 4% paraformaldehyde and incubated in 1% bovine serum albumin (BSA) (Sigma) to block other bindings. DAPI (1:5000; Sigma) was used to counterstain nuclei. The primary antibodies used in this study were summarized in Table S1. The observation and assessment of samples were performed with BZ-9000E (Keyence, Osaka, Japan).

RT-PCR and qPCR

Total RNA was purified with the RNeasy Mini kit (Qiagen, Valencia, CA, USA) and treated with the DNase-one kit (Qiagen) to remove genomic DNA. One microgram of total RNA was reverse transcribed for single-stranded cDNA using a random primer and Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. PCR was performed with ExTaq (Takara, Shiga, Japan). Quantitative PCR with the Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan) was performed using the StepOne real-time PCR system (Applied Biosystems, Forester City, CA, USA) in duplicate or triplicate. Primer sequences were listed in Table S2.

cDNA microarray

Total RNA was prepared using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the GeneChip WT (Whole Transcript) Sense Target Labeling and Control Reagents kit as described by the manufacturer (Affymetrix, Santa Clara, CA, USA). Hybridization to the GeneChip Human Gene 1.0 ST expression arrays, washing, and scanning were performed according to the manufacturer's protocol (Affymetrix). Expression values were calculated using the RMA summarization method and the data obtained were analyzed by GeneSpring GX 11.5.6 (Agilent Technologies, Santa Clara, CA, USA) for correlation coefficients, scatter plots, a volcano plot, heat maps, and hierarchical clustering (Distance metrics: Pearson's Centered, Linkage rule: Average). Differentially expressed genes were identified by statistical analyses and fold changes. Statistical analyses were performed using a one-way ANOVA with a Benjamini and Hochberg False Discovery Rate (BH-FDR =0.01) multiple testing correction followed by Tukey HSD post hoc tests (GeneSpring GX). Microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI, and the accession number is GSE 60313. Data for hBM90, 91, and 94 have already been described [22]. Data from GSE44727 and GSE45223 were used for a comparison analysis in Figure S3.

Differentiation of hPSC-derived hNCCs

Peripheral neuronal differentiation

Sorted hNCCs were cultured in CDM supplemented with 10 µM SB and 1 µM CHIR as a sphere using the hanging drop technique (1×10^4 cells per sphere) as previously described [25]. Twenty-four hours after the hanging drop culture, spheres were plated onto Polyornithine/laminin/fibronectin (PO/Lam/FN)-coated plates in DMEM/F12 (Invitrogen) supplemented with 1 x N2 supplement (GIBCO), 1 x GlutaMAX (Invitrogen), 20 ng/ml FGF2, and 20 ng/ml EGF. These cells were cultured for two days under these conditions and the medium was then replaced with DMEM/F12 supplemented with 1 x N2 supplement, 1 x GlutaMAX, and 10 ng/ml BDNF (R&D), GDNF (R&D), NT-3 (R&D), and NGF (R&D). The medium was changed every 3 days and passages were performed every week [26].

Differentiation was confirmed by immunostaining for peripherin, Tuj-1, and GFAP 3 weeks after induction.

Melanocyte differentiation

Cells were plated onto fibronectin-coated dishes in CDM supplemented with 10 μM SB and 1 μM CHIR. Melanocyte induction was performed the next day with CDM supplemented with 1 μM CHIR, 25 ng/ml BMP4, and 100 nM endothelin-3 (American Peptide Company, Sunnyvale, CA, USA) [15, 27, 28]. The medium was changed every other day. Differentiation was confirmed by induction of the *MITF* and *c-KIT* genes on day 7.

Corneal endothelial cell differentiation

Cells were induced to corneal endothelial cells with corneal endothelial cell-conditioned CDM. Conditioned CDM was derived by collecting medium from cultured human corneal endothelial cells [29]. The selective ROCK inhibitor Y-27632 (WAKO) was used on the first day of the induction. The medium was changed every two days, and cells were analyzed by immunocytochemistry after twelve days. RT-qPCR was performed 3, 5, and 8 days after the induction.

Induction of hMSCs from hNCCs

Cells were plated onto tissue culture dishes (BD) at a density of 6.5×10^4 cell/cm^2 in CDM supplemented with 10 μM SB and 1 μM CHIR. The medium was replaced the next day with αMEM (Nacalai Tesque, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (Nichirei Inc., Tokyo, Japan) [14, 26]. The morphology of cells started to change approximately 4 days after the induction. Passages were performed every week using 0.25% trypsin-EDTA (GIBCO) at a density of 1×10^4 cells/cm^2. hMSC markers (CD73, CD44, CD45 and CD105) were analyzed by FACS 14 days after the hMSC induction. We used STK2 (DS Pharma Biomedical, Osaka, Japan) as the MSC medium and tissue culture dishes coated with fibronectin for the hMSC induction under chemically defined media conditions.

Differentiation of hNCC-derived hMSCs

Osteogenic differentiation

A total of 2.5×10^5 induced hMSCs/well were seeded on 6-well dishes (BD) and cultured in osteogenic induction medium, αMEM, 10% FBS, 0.1 μM dexa-methasone, 50 μg/ml ascorbic acid, and 10 mM β-glycerophosphate for 2 weeks for osteogenic differentiation [24]. STK3 (DS Pharma) was used as the osteogenic induction medium instead of the osteogenic induction medium to achieve osteogenic differentiation under chemically defined media conditions. The culture medium was changed every other day for 1 week. Differentiation properties were confirmed by the formation of calcified nodules, as detected with Alizarin Red staining. Briefly, culture wells were washed twice in phosphate-buffered saline (PBS) and fixed for 10 minutes at room temperature in 100% ethyl alcohol. The

Alizarin Red solution (40 mM, pH 4.2) was applied to the fixed wells for 10 min at room temperature. Non-specific staining was removed by several washes with water.

Chondrogenic differentiation

Two-dimensional chondrogenic induction was performed as previously described [30]. Briefly, cells (1.5×10^5) that induced hMSCs were suspended in 5 µl of chondrogenic medium (DMEM: F12 (Invitrogen), 1% (v/v) ITS1 mix (BD), 0.17 mM AA2P, 0.35 mM Proline (Sigma), 0.1 mM dexamethasone (Sigma), 0.15% (v/v) glucose (Sigma), 1 mM Na-pyruvate (Invitrogen), 2 mM GlutaMax, and 0.05 mM MTG supplemented with 40 ng/ml PDGF-BB and 1% (v/v) FBS (Nichirei)), and were subsequently transferred to fibronectin-coated 24-well plates (BD). A total of 1 ml of the chondrogenic medium was added after 1 hour. TGFβ3 (R&D) was subsequently added at 10 ng/ml on days 3 to 6, and BMP4 was added to a concentration of 50 ng/ml on day 10. Micromass cultures were maintained at 37°C under 5% CO_2 and 5% O_2 for 16 days. Differentiation properties were confirmed by Alcian Blue staining. Briefly, induced cells were fixed for 30 minutes with 10% formalin (Sigma) and rinsed with PBS. These cells were then stained overnight with Alcian Blue solution (1% Alcian Blue (MUTO PURE CHEMICAL CO., LTD, Tokyo, Japan) in 3% glacial acetic and 1% HCl, pH 1) and destained with the acetic acid solution.

Adipogenic differentiation

Cells were seeded onto 6-well tissue culture dishes at a density of 5.0×10^5 cells/well for adipogenic differentiation, and were cultured in αMEM containing 10% FBS, 1 mM dexamethasone, 10 mg/ml insulin, and 0.5 mM isobutylxanthine for 3 weeks [31]. Induced cells were fixed in 10% formalin for 1 hour at room temperature, followed by 20 minutes in 0.3% Oil Red O staining solution (Sigma).

Results

Derivation of p75^{high} cells from hPSCs

To transfer hPSCs from feeder to feeder-free culture conditions, colonies were dissociated into small cell clumps (about 10 cells/clumps) by pipetting several times, seeded on matrigel-coated dishes (2–4 clumps/cm^2), and cultured with mTeSR1 medium for two days. hNCC induction was then initiated by substituting CDM supplemented with chemicals (Figure 1A). Cells gradually migrated from colonies and proliferated during the induction (Figure 1B). Cells were harvested after 7 days of being induced, and were subsequently sorted according to the expression of p75 (Figure 1C). We detected two peaks in p75-positive populations, designated p75^{low} and p75^{high}, and the efficiency of hNCC induction was evaluated based on the fraction of p75^{high} cells.

A

B

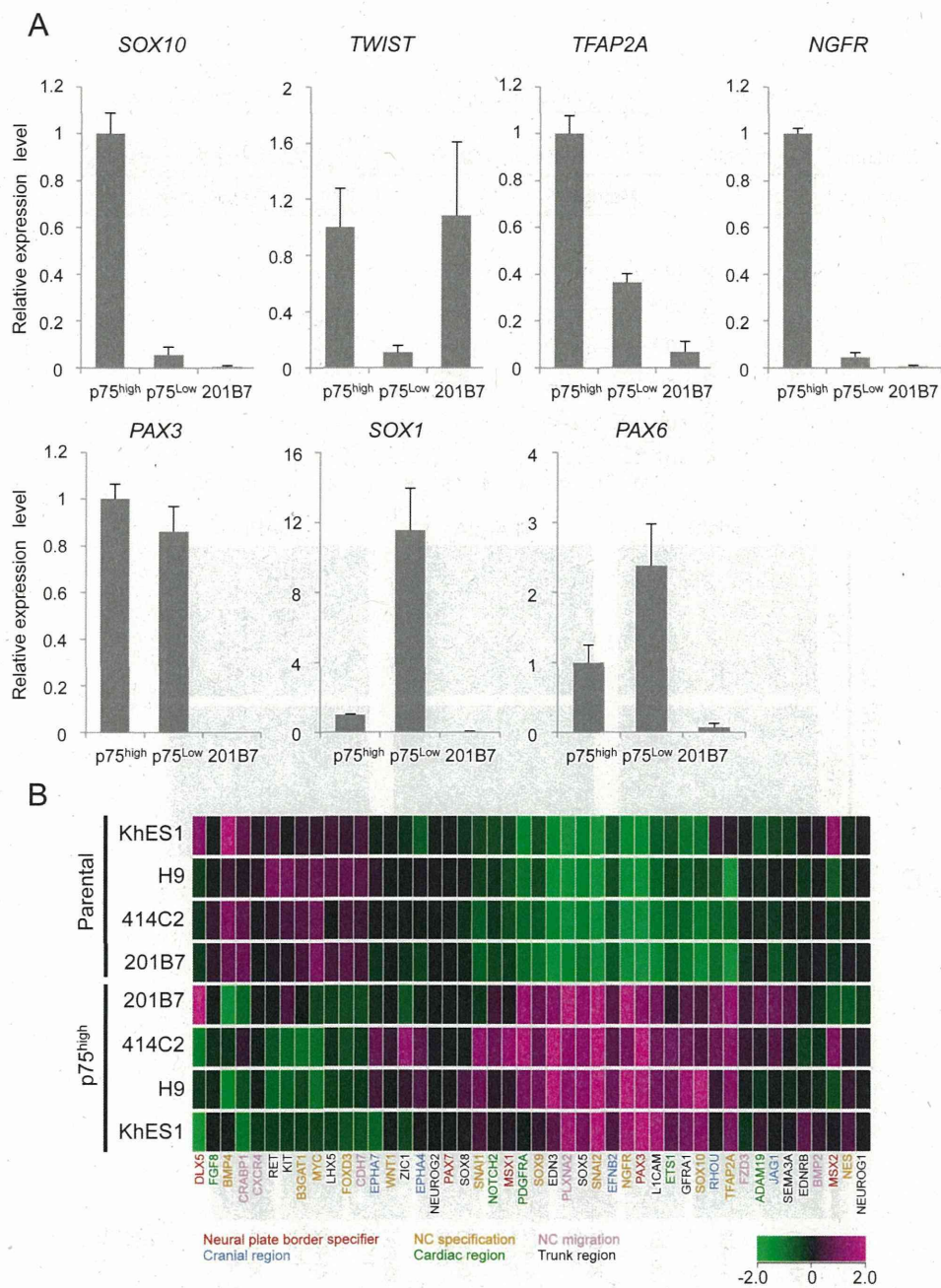**Figure 2. Expression profiles of sorted p75^high cells.** A) The expression of marker genes in sorted p75^high and p75^low cells. The mRNA expression of each gene was analyzed by RT-qPCR in undifferentiated 201B7 (hiPSCs) and sorted p75^low and p75^high cells, and was shown as a relative value using the level in sorted p75^high cells as 1.0. Average ± SD. N=3, biological triplicates. B) Clustering analyses of NCC markers in p75^high populations from several hESC and hiPSC lines. Marker genes for each sub-population of NCCs were labeled using the indicated colors.
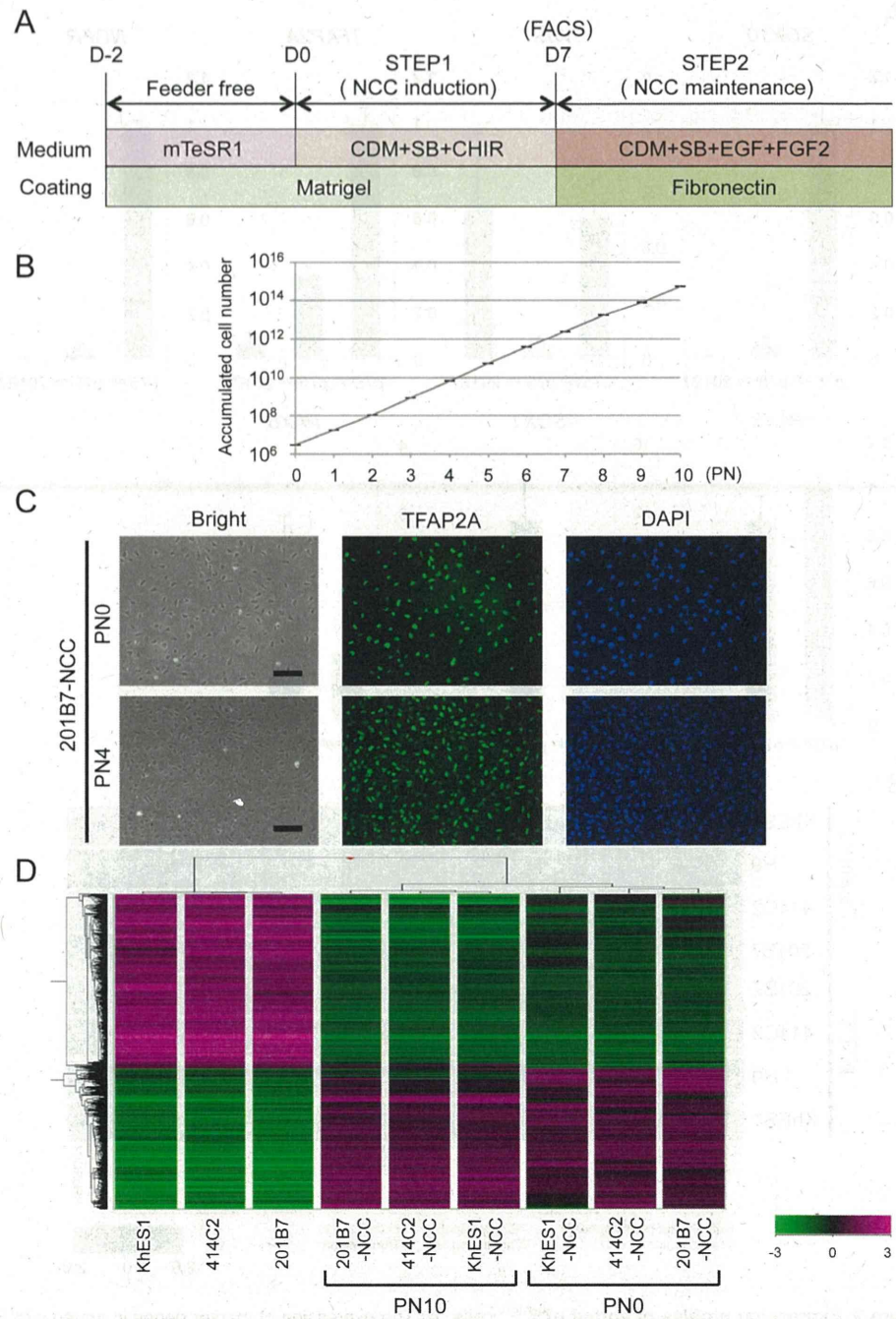
Figure 3. Sustained expansion of hNCCs with original characteristics. A) Schematic representation of the culture conditions. B) Growth profile of 201B7-derived hNCCs. Average ± SD. N=3, biological triplicate. C) Phase contrast images and immunostaining of TFAP2A in 201B7-derived hNCCs at PN0 and PN4, Scale bar, 200 μm. D) Hierarchical clustering analyses of hPSCs and hPSC-derived hNCCs at PN0 and PN10.
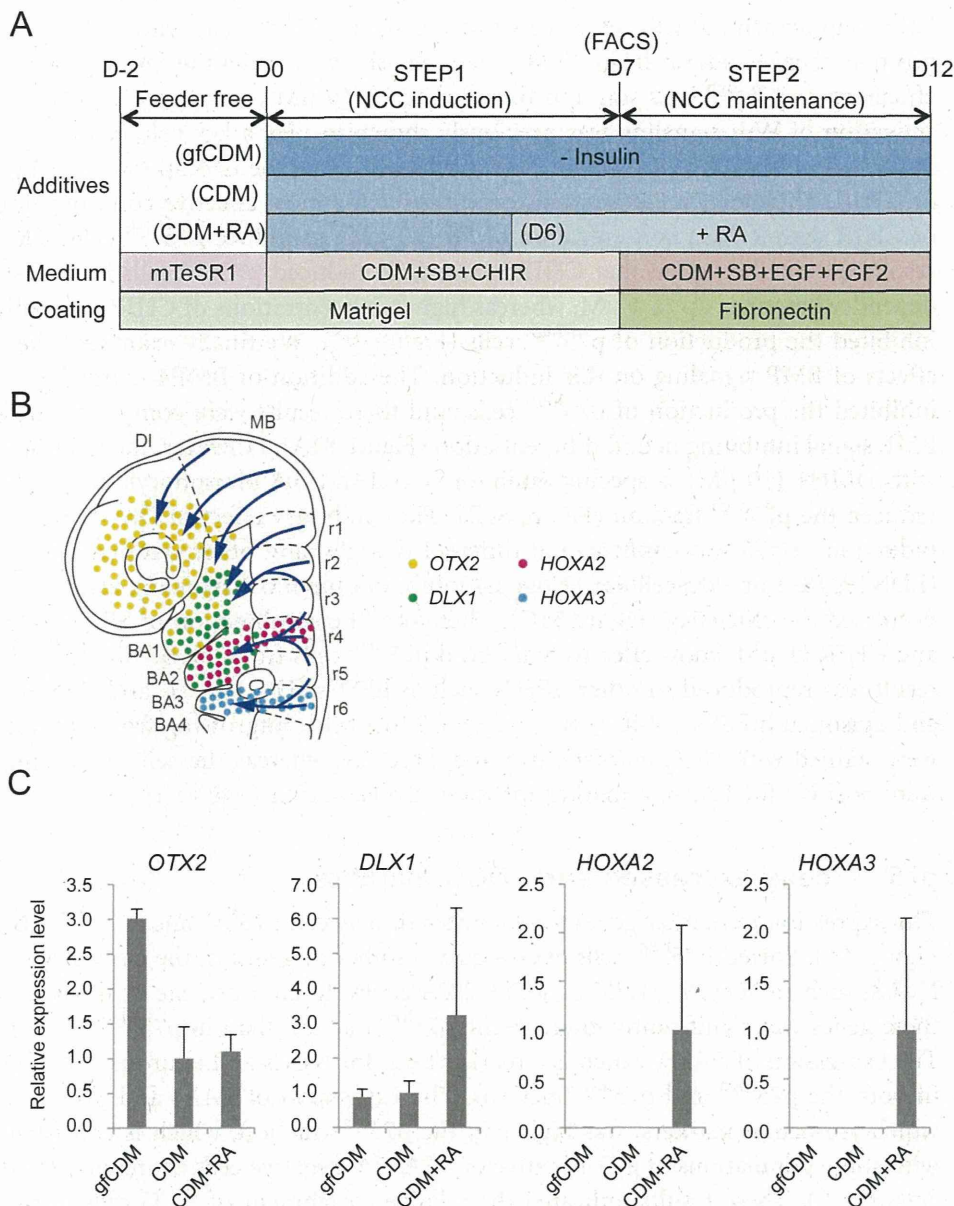
A

B

C

Figure 4. Modulation of regional characteristics of hNCCs. A) Schematic representation of culture conditions for the induction and maintenance of hNCCs. RA, retinoic acid (100 nM). B) Schematic distribution of marker-positive cells in the murine embryo. DI, diencephalon; MB, midbrain; BA1 to BA4, branchial arch 1 to branchial arch 4; r1 to r6; rhombomere 1 to rhombomere 6. C) The mRNA expression of regional specifier genes in hNCCs. p75^{high} cells were collected at the end of the hNCC induction by FACS and seeded onto fibronectin-coated dishes. RNAs were extracted when cells reached a semi-confluent state and used for RT-qPCR. The relative expression level of each gene was demonstrated using the value of cells cultured in CDM (OTX2 and DLX1) or CDM + RA (HOXA2 and HOXA3) as 1.0. Average ± SD. N=3, biological triplicate.

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The effects of SB, which has been shown to inhibit Activin/Nodal/TGFβ signaling and induce neural cells and hNCCs from hPSCs without the help of other chemicals, were firstly evaluated [14]. In accordance with the reported data,

CDM supplemented with SB successfully delivered p75^{high} cells with a PAX6-positive neuroectoderm from 201B7 (date not shown), while the induction efficiency of p75^{high} cells was approximately 35% (0 μM in Figure 1C). The activation of Wnt signaling was previously shown to play a key role in the induction of hNCCs [14, 15], and can be achieved using the GSK3β inhibitor BIO or CHIR. Therefore, we attempted to determine the most effective concentration of CHIR with a fixed concentration of SB (10 μM) to induce p75^{high} cells. The results obtained revealed that CHIR successfully induced p75^{high} cells in a dose-dependent manner up to 1 μM, whereas higher concentrations of CHIR markedly inhibited the production of p75^{high} cells (Figure 1C). We finally examined the effects of BMP signaling on this induction. The addition of BMP4 markedly inhibited the production of p75^{high} cells, and these results were compatible with BMP signal inhibiting neural differentiation (Figure S1A). However, the treatment with DMH1 (10 μM), a specific inhibitor of SMAD1/5/8 phosphorylation, also reduced the p75^{high} fraction (Figure S1B). The inhibitory effect of DMH1 on the induction of p75 was confirmed at different dosages, and other cytoplasmic (LDN193189) or extracellular (Noggin) inhibitors for BMP signaling also decreased the efficiency (Figure S1C). Therefore, the combination of SB (10 μM) and CHIR (1 μM) most effectively induced p75^{high} cells from 201B7 hiPSCs. This result was reproduced in other hPSCs such as hESCs (H9, KhES1, and KhES3) and episomal hiPSCs (414C2) (Figure 1D). Most cells outgrowing from colonies were stained with NCC markers, p75 and TFAP2A, whereas the cells in colonies were positive for PAX6, a marker for the neuroectoderm (Figure 1E).

p75^{high} cells expressed early NCC markers

The expression of marker genes were compared between p75^{high} and p75^{low} cells (Figure 2A). Sorted p75^{high} cells expressed a number of genes in the early stage of NCCs, such as *SOX10*, *TWIST*, and *TFAP2A* genes. In contrast, the expression of these genes was significantly lower in the p75^{low} fraction than in p75^{high} fraction. The expression of *PAX3*, which is a marker both for NCCs and neurons, was high in both the p75^{high} and p75^{low} fractions. The expression of *PAX6* and *SOX1*, which are neural markers, was higher in the p75^{low} fraction, which is consistent with some populations of p75-negative or TFAP2A-negative cells expressing *PAX6* (Figure 1E). These results indicated the relative enrichment of NCC cells in the p75^{high} cell population.

In an attempt to further characterize p75^{high} cells, genome-wide expression profiles were compared between sorted p75^{high} cells and their corresponding hPSCs using a cDNA microarray (Affymetrix Gene 1.0 ST), and we found that the overall profiles of p75^{high} cells derived from several PSCs were similar to each other (Figure S2). Based on the previous report [32], 46 genes were selected as markers for distinct NC-subpopulations and the expression level of these genes were compared between hNCCs and parental PSCs (Figure 2B). hNCCs in this study highly expressed early stage-related genes such as neural plate border specifier (PAX3) and NC specification (SNAI2, NGFR, TFAP2A, SOX9, and
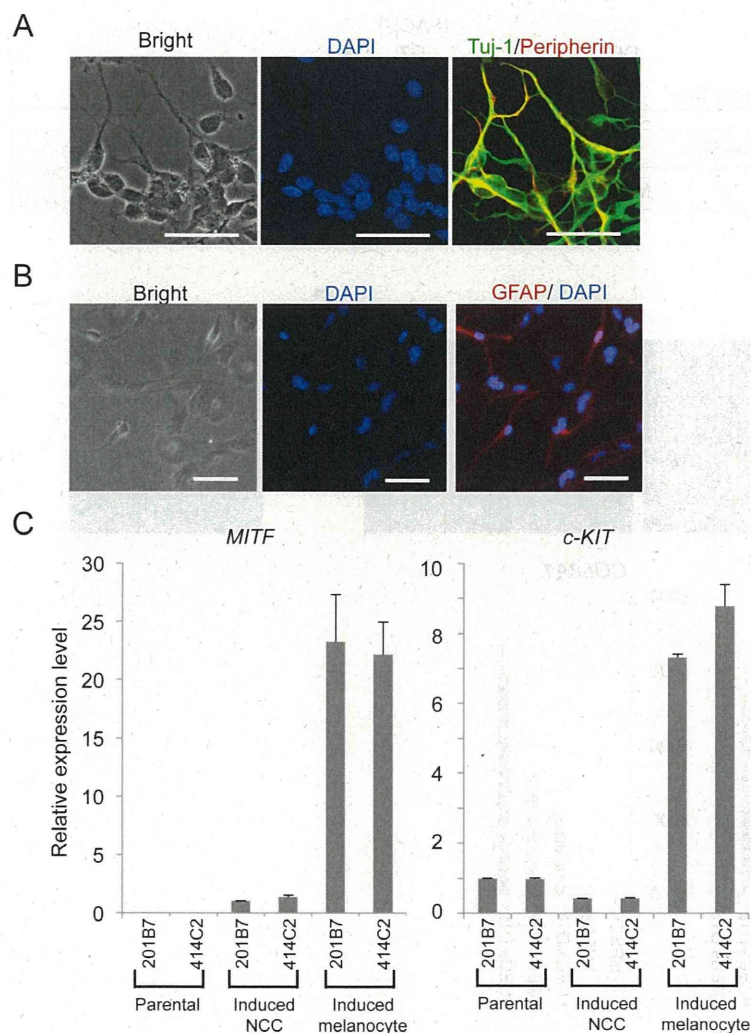
Figure 5. Derivation of peripheral neural cells, glia, and melanocytes from hNCCs. A) Neuronal differentiation of 201B7-derived hNCCs. Cells were stained with an antibody against peripherin (red) and Tuj-1 (green). B) The glial differentiation of 201B7-derived hNCCs. Cells were stained with an antibody against GFAP. Scale bar, 50 μm. C) Melanocyte differentiation of 201B7-derived hNCCs. The mRNA expression levels of *MITF* and *c-KIT* genes were shown as a relative value using the value in 201B7-derived hNCCs and 201B7 as 1.0, respectively. Average ± SD. N=3, biological triplicates.

SOX10), but also some region-specifying genes (EFNB2 for cranial, PDGFRA for cardiac, and SOX5 for trunk region), suggesting the heterogeneous population of p75^{high} cells, which were designated hNCCs hereafter. The profiles of the current hNCCs were compared with those of two PSC-derived NCCs, which were induced by different protocols in previous studies [15, 32] (Figure S3). Although the three types of PSC-derived NCCs all highly expressed some genes, such as SNAI2, their
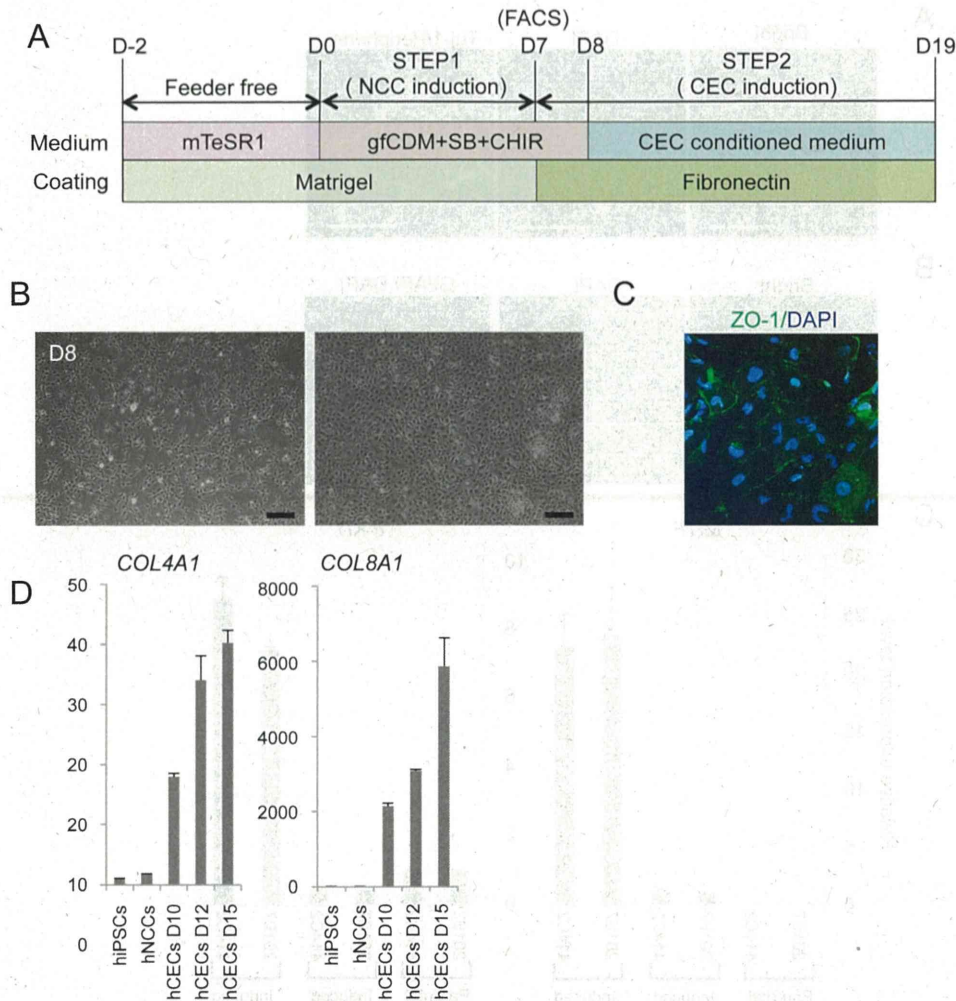
Figure 6. Derivation of corneal endothelial cells from hNCCs. A) Schematic protocol for the induction of corneal endothelial cells. B) Phase contrast images of cells before (D8) and after (D19) the induction. Scale bar, 200 μm. C) The expression of ZO-1 in cells at D12. Cells were stained with an antibody against ZO-1. D) The mRNA expression of corneal endothelial cell marker genes. RNAs were extracted from cells at D10, D12, and D15. The expression level of each gene was demonstrated as a relative value using the value in human primary corneal endothelial cells as 1.0. Average ± SD. N=3, technical triplicate. We performed this CEC induction twice and confirmed its reproducibility.

expression profiles were considerably different, suggesting the protocol-dependent heterogeneity of PSC-derived NCCs.

Sustained expansion of hNCCs with original characteristics

We investigated whether hNCCs could be stably expanded. The growth of hNCCs cultured in the hNCC induction medium (CDM with SB and CHIR) was very slow (data not shown). We employed a cultured condition using CDM supplemented with SB, EGF (20 ng/ml), and FGF2 (20 ng/ml) based on the