

RESEARCH ARTICLE

Astrocyte-like cells differentiated from a novel population of CD45-positive cells in adult human peripheral blood

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Abstract

We have previously reported a novel CD45-positive cell population called peripheral blood insulin-producing cells (PB-IPCs) and its unique potential for releasing insulin *in vitro*. Despite the CD45-positive phenotype and self-renewal ability, PB-IPCs are distinguished from hemopoietic and endothelial progenitor cells (EPCs) by some characteristics, such as a CD34-negative phenotype and different culture conditions. We have further identified the gene profiles of the embryonic and neural stem cells, and these profiles include Sox2, Nanog, c-Myc, Klf4, Notch1 and Mash1. After treatment with all-trans retinoic acid (ATRA) *in vitro*, most PB-IPCs exhibited morphological changes that included the development of elongated and branched cell processes. In the process of induction, the mRNA expression of Hes1 was robustly upregulated, and a majority of cells acquired some astrocyte-associated specific phenotypes including anti-gial fibrillary acidic protein (GFAP), CD44, Glutamate-aspartate transporter (GLAST) and S100 β . In spite of the deficiency of glutamate uptake, the differentiated cells significantly relaxed the regulation of the expression of brain-derived neurotrophic factor (BDNF) mRNA. This finding demonstrates that PB-IPCs could be induced into a population of astrocyte-like cells and enhanced the neurotrophic potential when the state of proliferation was limited by ATRA, which implies that this unique CD45+ cell pool may have a protective role in some degenerative diseases of the central nervous system (CNS).

Keywords: all-trans retinoic acid; astrocytes; differentiation; human peripheral blood; insulin-producing cells

Introduction

Regarding peripheral blood (PB) of adult humans, there are some types of cells, including mesenchymal stem cells (MSCs) and hemopoietic progenitor cells (HPCs), that have the potential to differentiate into neural cells (Mezey et al., 2003; Zhao et al., 2003; Cogle et al., 2004; Tondreau et al., 2005; Porat et al., 2006; Cesselli et al., 2009; Nichols et al., 2013). Therefore, the neural plasticity of this cell pool may have relevant clinical implications for the treatment of multiple nervous system diseases.

We have identified previously a novel type of cell from the PB of adult humans called peripheral blood insulin-producing cells (PB-IPCs) because of their potential for releasing insulin *in vitro* and migrating into the pancreatic islets in the diabetic mouse models (Zhao et al., 2007). PB-IPCs have embryonic stem (ES) cell-associated transcription factors, including Oct-4 and Nanog, and the leukocyte common antigen CD45, which distinguishes them from progenitor cells that migrate from bone marrow niches. PB-IPCs are also negative for CD34, CD14, CD80, CD86 and HLA-DR, which distinguishes them from hemopoietic and

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Abbreviations: ATRA, all-trans retinoic acid; BDNF, brain-derived neurotrophic factor; CB-SCs, cord blood-derived multipotent stem cells; CNS, central nervous system; EPCs, endothelial progenitor cells; ESCs, embryonic stem cells; G-CSF, granulocyte colony-stimulating factor; GFAP, glial fibrillary acidic protein; GLAST, glutamate-aspartate transporter; HPCs, hemopoietic progenitor cells; LIF, leukaemia inhibitory factor; MSCs, mesenchymal stem cells; NGF, nerve growth factor; NTC, no template control; NRT, no reverse transcriptase control; PB-IPCs, peripheral blood insulin-producing cells; PB, peripheral blood; PDC, L-trans-pyrrolidine-2, 4-dicarboxylic acid; SD, standard deviation; STAT3, signal transducer and activator transcription 3

endothelial progenitor cells (EPCs). Because the characteristics of PB-IPCs are similar to those of cord blood-derived multipotent stem cells (CB-SCs) with neural plasticity (Zhao et al., 2006), we hypothesised that PB-IPCs might have the potential to differentiate into neural cells. Here, the fate determination and differentiation of PB-IPCs treated with all-trans-retinoic acid (ATRA) *in vitro* were examined via morphological and immunocytochemical observations, and the gene expression of determinants of neural differentiation was further analysed.

Materials and methods

Cell preparations

With agreement from the ethics committee of Jinan Central hospital Affiliated to Shandong University, the principal investigator designed the experiment and received ethical approval. Written informed consent was obtained from each donor. Human peripheral blood samples (50–100 mL/unit) were collected from 26- to 42-year-old (averaged 31 ± 6) healthy donors (three male and two female) at Jinan Central Hospital Affiliated to Shandong University. Mononuclear cells were isolated with Ficoll-Hypaque ($\gamma = 1.077$, Sigma), and red blood cells were removed using red blood cell lysis buffer (eBioscience). The remaining mononuclear cells were washed three times with PBS and seeded in 150×15 or 60×15 mm Petri dishes (BD FalconTM) at $1\text{--}2.5 \times 10^6$ cells/mL. Cells were cultured in serum-free culture medium (Lonza, Allendale, NJ) and incubated at 37°C with 8% CO_2 in air (Zhao et al., 2007; Li et al., 2012).

Cell differentiation

When the PB-IPCs proliferated to 70–80% confluence, they were treated with ATRA (Acros Organics) at 1, 5 or $10 \mu\text{M}$. The CO_2 concentration was lowered to 5% to partially depress proliferation and maintain a proper condition for neural cell growth.

Immunocytochemistry

Cells were fixed with 4% paraformaldehyde and permeabilised with 0.5% Triton X-100 (Invitrogen, USA) for 15 min. We used 3% H_2O_2 to block endogenous peroxidase activity for 10 min, and blocked unspecific binding of the antibodies with 2.5% horse serum for 20 min. For fluorescence-labelled immunostaining, the step of 3% H_2O_2 block was omitted. The primary antibody, mouse anti-glial fibrillary acidic protein (GFAP; Abcam, UK), was applied overnight at 4°C . The samples were washed thoroughly and incubated with secondary antibodies (anti-mouse-labelled polymer HRP; OriGene, USA) for 30 min at room temperature. After

adding DAB (diaminobenzidine), specimens were ready for visualisation and positive cells counting. Images were acquired using an Olympus IX710 Camera with the manufacturer's software and edited using Adobe Photoshop CS6.

For double staining assay, the following primary antibodies were used: mouse or rabbit GFAP (Abcam, UK); rabbit anti-Glutamate-aspartate transporter (GLAST; Abcam, UK); mouse anti-CD44 (Becton Dickinson, USA); mouse anti-S100 β (SANTA CRUZ, USA); mouse anti-O4 (R&D SYSTEMS, USA); and mouse anti-NeuN (FOX3; Abcam, UK). After the primary antibodies had been applied overnight at 4°C , the appropriate secondary antibodies (anti-mouse IgG-FITC/TRITC, anti-rabbit IgG-FITC/TRITC; Jackson ImmunoResearch Laboratories, USA) and mounting medium with DAPI (Invitrogen, USA) were used for detection and visualisation. Images were acquired by using a ZEISS LSM780 confocal microscope with the ZEN2010 software and edited with Adobe Photoshop CS6.

The percentage of positive cells was determined by counting in five random fields (50 or more cells in each field). The results were presented as the means \pm standard deviation (SD).

Quantitative real-time PCR

Quantitative real-time PCR was used to quantify the mRNA expression of genes associated with stem cell plasticity and neural differentiation and genes of several neurotrophins. Total RNA was extracted using a Qiagen kit (Valencia, CA). First-strand cDNAs were synthesised with a High-Capacity cDNA reverse transcription kit (including RNase inhibitor, random primers, dNTP mix and reverse transcriptase; Applied Biosystems). Real-time PCR was performed on each sample in triplicate with the ABI 7300 Real-Time PCR System according to the following protocol: 50°C , 2 min; 95°C , 2 min; 95°C , 15 s and 65°C , 1 min (40 cycles). The levels of gene expression were determined relative to β -actin which served as an internal control. Wells of no template control (NTC) and no reverse transcriptase control (NRT) were the negative controls to assess whether there was a DNA contamination in the PCR preparation. The validated PCR primer sets for each gene were designed and purchased from Takara Biotechnology (Dalian, China). Information about these primer sets is provided in Table 1.

Measurement of glutamate uptake

The method of measuring the ability of PB-IPCs for uptaking glutamate was modified from Krencik et al. (2011) and Abe et al. (2000). At day 14, PB-IPCs in the ATRA and Control group were incubated in the HBSS \pm L-trans-pyrrolidine-2, 4-dicarboxylic acid (PDC, 1 mM, Sigma, USA) containing $50 \mu\text{M}$ L-glutamate

Table 1 Primers pairs used for real-time polymerase chain reaction.

Gene	Accession number/position	Primer sequence	Predicted Size
c-Myc	NM_002467.4, +511/+646	5' GCAGCTGCTTAGACGCTGGA 3' 5' CGCAGTAGAAATACGGCTGCAC 3'	136 bp
K1f-4	NM_004235.4, +1819/+1909	5' AAGAGTTCCCATCTCAAGGCACA 3' 5' GGGCGAATTCCATCCACAG 3'	91 bp
Nanog	NM_024865.2, +603/+772	5' CAACATCCTGAACCTCAGCTACAA 3' 5' GGCATCCCTGGTGGTAGGAA 3'	170 bp
Sox2	NM_003106.3, +1041/+1122	5' GTGAGCGCCCTGCAGTACAA3' 5' GCGAGTAGGACATGCTGTAGGTG 3'	82 bp
Mash1	NM_004316.3, +804/+902	5' GTCACAAGTCAGCGCCCAAG 3' 5' TGTAGCCAAAGCCGCTGAAG3'	99 bp
Notch1	NM_017617.3, +6118/+6273	5' AATGTGGATGCCGAGTTG 3' 5' ATCCGTGATGTCCCGTTG 3'	156 bp
Ngn1	NM_006161.2, +1383/+1524	5' TTTGAGCCGGCTGACAATACA 3' 5' CTGTGCCTGAATAGCTAAGCAAGAG 3'	142 bp
Ngn2	NM_024019.3, +1243/+1384	5' CCTGGAAACCATCTCACTTCACA 3' 5' TTGGCTTTGACAATAAGCTCCATC 3'	142 bp
Oligo1	NM_138983.2, +1088/+1174	5' TGGGACGTTAAAGTGACCAGAG 3' 5' TCTCCCTAAAGOCGCTGGA3'	87 bp
Oligo2	NM_005806.3, +1703/+1787	5' GGGCCACMGTTAGTTGGAAG 3' 5' GTGTGGATTGACCCAGATATTGA 3'	85 bp
Hes1	NM_005524.3, +473/+559	5' GGACATTCTGGAATGACAGTGA 3' 5'AGCACACTTGGGTCTGTGCTC 3'	87 bp
GFAP	NM_001131019.2, +476/+610	5' TGGAGGTTGAGAGGGACAATCTG 3' 5' CCAGGGTGGCTTCATCTGCT 3'	135 bp
BDNF	NM_001143805.1, +2828/+2954	5' GTCAAGTTGGGAGCCTGAAATAGTG 3' 5' AGGATGCTGGTCC A AGTGGTG 3'	127 bp
GDNF	NM_000514.3, +3308/+3387	5' AACTGTCTTGGGATCTTGCTGA 3' 5' TGCTCCACGAATGACTGGATG 3'	80 bp
NGF	NM_002506.2, +75/+197	5' ATGCTGGACCCAAGCTCA 3' 5' TGATCAGAGTGTAGAACAACATGGA 3'	123 bp
β-actin	NM_001101.3, +1043/+1228	5' CACCCAGCACAAATGAA 3' 5' CTAAGTCATAGTCCGCCTAGAAGCA 3'	183 bp

(Sigma, USA). After incubation at 37°C for 1 h, the media of two groups was collected, and the glutamate concentrations were measured using the Glutamine/Glutamate determination kit (Sigma, USA). After subtraction of the blanks (without glutamate), glutamate concentrations in the medium of each group were assayed.

Statistical analysis

The data were analysed with two-tailed Student's *t*-tests to determine statistical significance. Values are given as the mean ± SD.

Results

PB-IPCs have some characteristics similar with those of CB-SCs and displayed some neural-like morphological changes under the treatment of ATRA

After isolation and culture for 10–14 days, PB-IPCs proliferated to 70–80% confluence. We found that the PB-

IPCs had characteristics that were similar to those of CB-SCs (Zhao *et al.*, 2006), i.e., round or oval morphology and tight adherence to the bottom of the Petri dish.

The responses of PB-IPCs exposed to different ATRA concentrations showed some morphological changes in the 5 and 10 μM ATRA groups at 7–9 days, which were followed by the formation of elongated and branched cell processes (Figure 1C and D). In comparison, more significant morphological changes and the typical star-shape of astrocytes occurred in the PB-IPCs at 10 μM ATRA (Figure 1D, indicated by black arrows). In the Control and 1 μM ATRA group, the majority of cells (>90%) showed no neural morphological changes and maintained their original PB-IPCs appearances (Figure 1A and 1B).

ATRA triggered PB-IPCs to acquire some specific markers of astrocyte

At day 14, after more cells exhibited significant morphological changes in the 5 and 10 μM ATRA groups, the cells were fixed and immunostained for the astrocytic specific marker

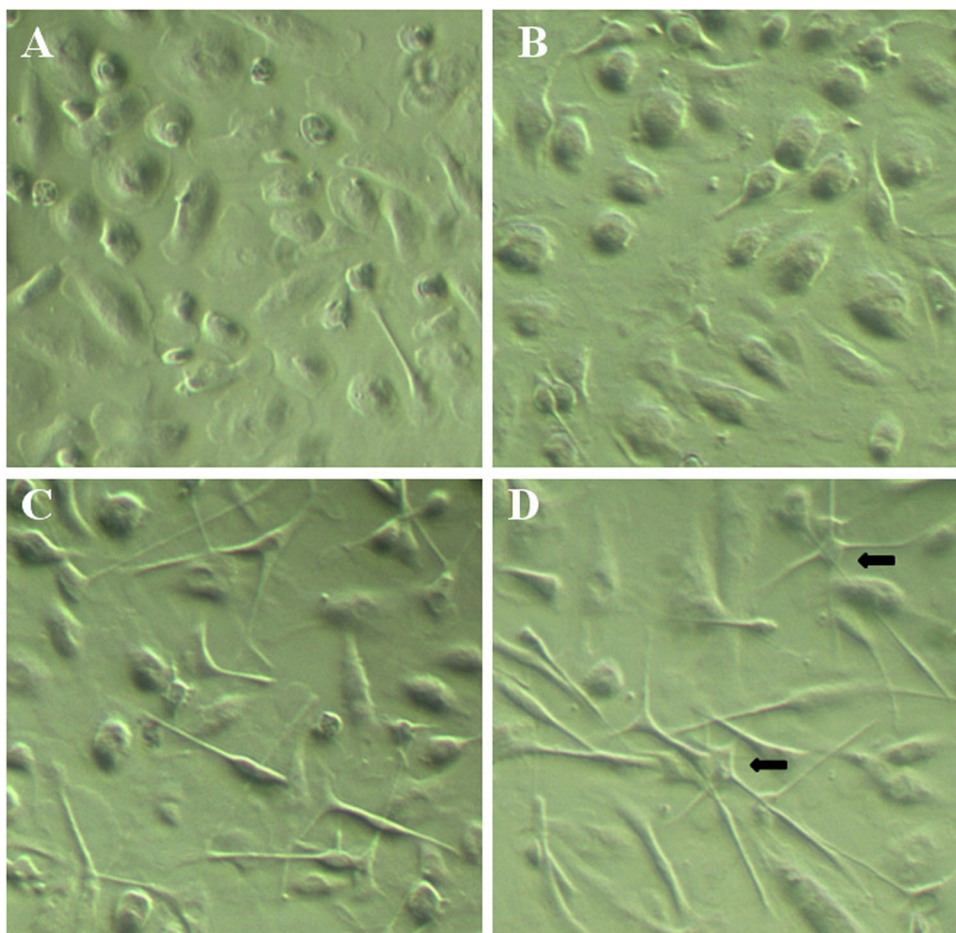


Figure 1 Morphological changes of PB-IPCs under the treatment with ATRA. In the Control (A) and 1 μ M ATRA (B) group, most cells (>90%) maintained the original appearances of PBIPCs. (C): Apparent morphological changes of elongated and branched cell processes were observed in PB-IPCs under the treatment with 5 μ M ATRA at the 9th day. (D): In the 10 μ M ATRA group, the induced cells displayed more significant morphological changes, and some cells exhibited the typical star-shape of astrocytes (indicated by black arrows).

GFAP. Most cells were positively stained for the GFAP marker (Figure 2C and D). Conversely, the GFAP marker was only slightly expressed in the minority of cells in the Control and 1 μ M ATRA groups (Figure 2A and B). Thus with a higher concentration (5 and 10 μ M) of ATRA incubation, the expression level of the GFAP⁺ phenotype increased more significantly in the PB-IPCs (Figure 2E).

To confirm whether the PB-IPCs had differentiated into astrocytes, at day 1 the cells from both the 10 μ M ATRA and Control groups were fixed and immunostained for other astrocytic specific markers, including CD44, S100 β and GLAST. Expression of NeuN (a marker of mature neurons) and O4 (a marker of oligodendrocytes) was also investigated. A majority of GFAP⁺ cells in the ATRA group positively expressed the CD44 marker (55 \pm 8%) (Figure 3A) and GLAST marker (90 \pm 9%) (Figure 3B), while all of the GFAP⁺ cells were co-labelled with the S100 β marker (Figure 3C). In the Control group, the CD44 marker was

expressed in the minority of cells (29 \pm 3%) (Figure 3A), and the expression of GLAST marker was at a background level (Figure 3B), while all cells positively expressed the S100 β marker (Figure 3C). Both induced and uninduced cells had little expression of O4 (\approx 5%), and were negative for NeuN (data not shown). These results suggest that, after incubation with 10 μ M ATRA for 14 days, most PB-IPCs differentiated into astrocyte-like cells.

Genetic expression profile of PB-IPCs shows pluripotentiality, and an increased tendency for astrocytic differentiation and unregulated neurotrophic potential during the process of ATRA induction

PCR results established that PB-IPCs expressed *c-Myc*, *Klf4*, *Nanog* and *Sox2* mRNA, which are related to pluripotentiality (Cartwright *et al.*, 2005; Li *et al.*, 2005; Das and Lavoie, 2013; Saunders *et al.*, 2013), and *Notch1* mRNA

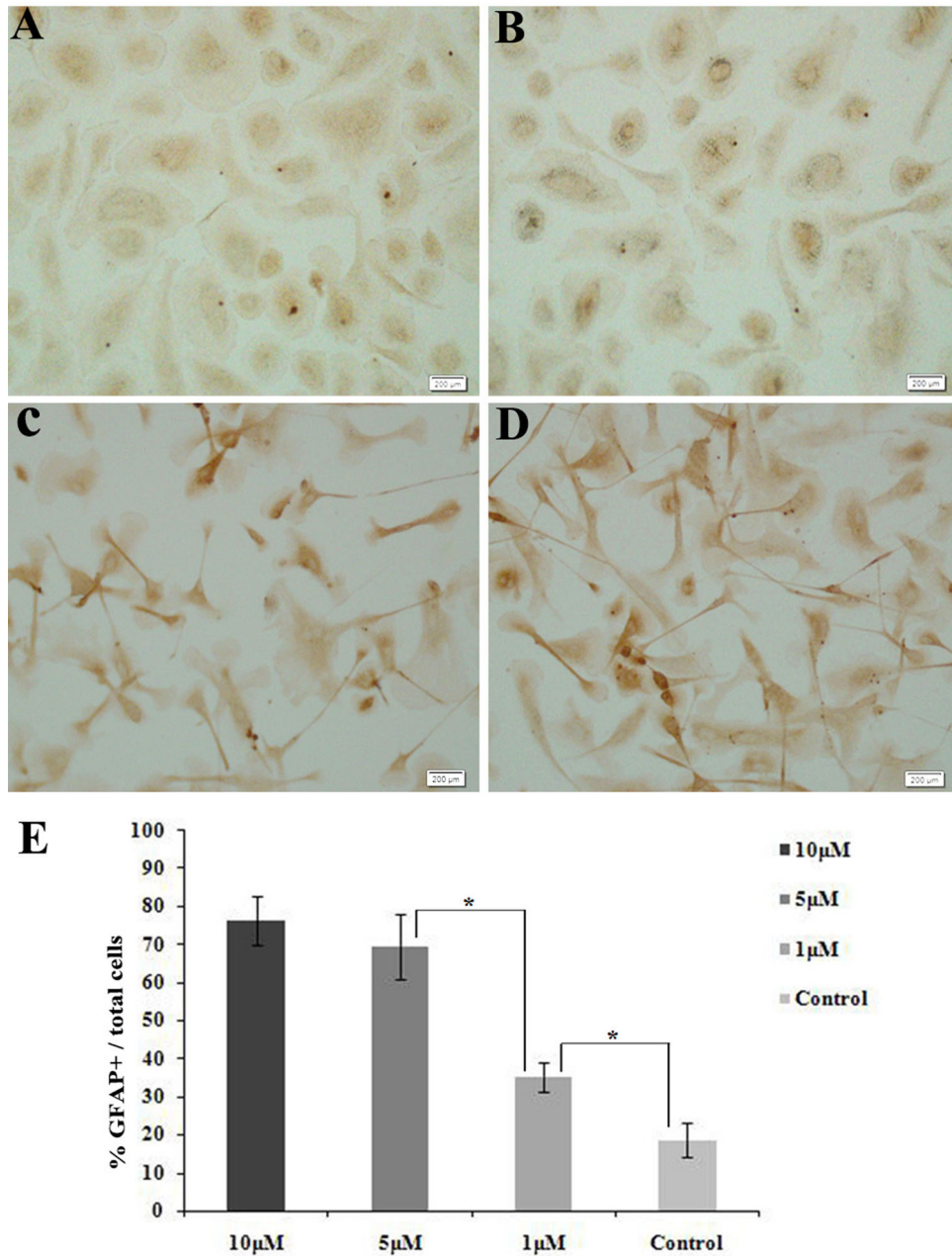


Figure 2 Exposure to ATRA increased the expression level of GFAP phenotype of PB-IPCs. At day 14 in the Control (A) and 1 μM ATRA (B) group, immunocytochemical analysis revealed that the GFAP+ cells accounted for only 19 ± 4% and 35 ± 4% independently. In the 5 μM (C) and 10 μM (D) ATRA group, a majority of neural-like cells were stained for the GFAP marker by 71 ± 6% and 76 ± 6% independently. (E): By statistical analysis of counting data, there is no significant difference in the percentage of GFAP+ cells between the 5 and 10 μM ATRA group ($P=0.2$). The differences among the 1, 5 μM ATRA and Control groups were significant ($P < 0.01^*$). Data represented one of three experiments.

which is associated with gliogenesis (Taylor *et al.*, 2007), but small amounts of Mash1 mRNA which is associated with neurogenesis (Casarosa *et al.*, 1999; Parras *et al.*, 2007) were also detected. By day 6, the levels of Nanog, Klf4 and Notch1 mRNA expression were more significantly decreased in the induced cells than the uninduced cells. There were no

significant differences between the 10 μM ATRA and Control groups for the Sox2 and Mash1 mRNA expression (Table 2).

We followed the expression of mRNA for GFAP, and several important bHLH factors involved in progenitor fate commitment to various neural lineages and neurotrophic

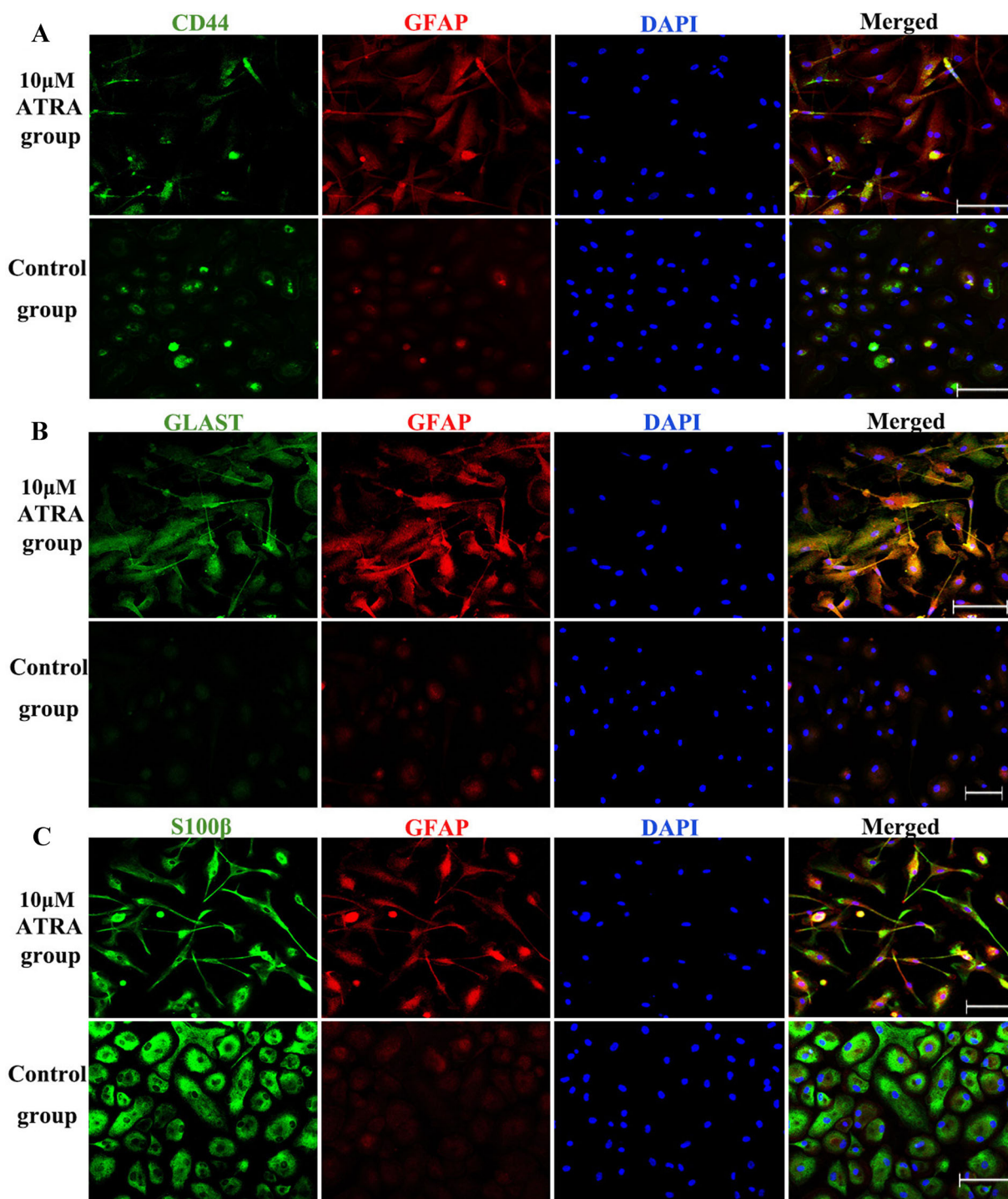


Figure 3 Confocal microscopy analysis of the expressions of astrocytic specific markers for PB-IPCs in the 10 μ M. ATRA and Control group (A, B): At day 14 in the 10 μ M ATRA group, a majority of GFAP+ cells made a positive expression of the GLAST marker ($90 \pm 7\%$) and CD44 marker ($55 \pm 8\%$), while in the Control group, the CD44 marker was expressed in the minority of cells ($29 \pm 3\%$) and the expression of GLAST was just at a background level. (C): Both all of the induced and uninduced cells made an obvious immunofluorescent expression of the S100 β marker. Data were representative of three sample preparations. Scale bar, 100 μ m.

Table 2 Gene expression of several pluripotentiality-associated, bHLH and neurotrophic factors and GFAP at day 0 and 6.

Primer	PB-IPCs		iPB-IPCs			uPB-IPCs			iPB-IPCs vs. uPB-IPCs
	Day 0		Day 6			Day 6			Day 6
	Mean Ct (SD)	Delta Ct	Mean Ct (SD)	Delta Ct	Fold change (SD)	Mean Ct (SD)	Delta Ct	Fold change (SD)	<i>P</i> for Fold change
Nanog	27.8 (0.05)	14.0	30.4 (0.13)	16.9	0.1 (0.01)	28.3 (0.89)	14.8	0.6 (0.04)	<i>P</i> < 0.01
c-Myc	18.6 (0.08)	5.1	19.4 (0.07)	5.8	0.6 (0.03)	19.3 (0.12)	5.8	0.6 (0.05)	<i>P</i> = 0.96
K1f4	23.6 (0.14)	9.9	25.6 (0.19)	12.1	0.2 (0.03)	23.9 (0.17)	10.4	0.7 (0.08)	<i>P</i> < 0.01
Sox2	25.5 (0.47)	11.8	28.2 (0.76)	14.7	0.2 (0.08)	27.2 (0.03)	13.6	0.3 (0.01)	<i>P</i> = 0.08
Notch1	25.0 (0.07)	11.2	28.6 (0.50)	15.1	0.1 (0.03)	27.2 (0.03)	13.7	0.2 (0.01)	<i>P</i> < 0.01
Mash1	36.6 (0.24)	22.9	36.1 (0.43)	22.6	1.3 (0.38)	35.5 (0.12)	21.3	1.9 (0.16)	<i>P</i> = 0.1
Ngn1	N/A	N/A	36.5 (0.39)	22.8	N/A	N/A	N/A	N/A	N/A
Ngn2	29.6 (0.47)	15.9	29.4 (0.27)	15.9	1.0 (0.19)	30.7 (0.62)	17.2	0.4 (0.18)	<i>P</i> = 0.04
Oligo1	NA	NA	38.7 (0.38)	25.2	N/A	37.7 (0.79)	24.2	N/A	N/A
Oligo2	30.3 (0.01)	16.6	28.0 (0.05)	14.6	4.0 (0.15)	30.6 (0.08)	17.0	0.7 (0.04)	<i>P</i> < 0.01
Hes1	28.7 (0.42)	15.0	24.8 (0.24)	11.3	12.9 (2.16)	27.8 (0.37)	14.3	1.6 (0.40)	<i>P</i> < 0.01
GFAP	29.0 (0.29)	15.3	27.6 (0.03)	14.1	2.3 (0.04)	31.3 (0.16)	17.8	0.2 (0.02)	<i>P</i> < 0.01
BDNF	28.1 (0.22)	14.4	25.1 (0.05)	11.6	7.0 (1.92)	27.0 (0.34)	13.5	1.9 (0.48)	<i>P</i> < 0.01
GDNF	32.5 (0.17)	18.8	32.2 (0.18)	18.7	1.1 (0.14)	33.8 (0.12)	20.3	0.4 (0.03)	<i>P</i> < 0.01
NGF	N/A	N/A	35.4 (0.10)	21.9	N/A	35.6 (0.75)	22.1	N/A	N/A

N/A: not applicable; iPB-IPCs: induced PB-IPGs; uPB-IPCs: uninduced PB-IPCs; SD: Standard Deviation.

factors that are important in protection of some CNS degenerative diseases. Quantitative analyses indicated that the GFAP mRNA expression was significantly upregulated in the 10 μ M ATRA group compared to the Control group at day 6. Ngn1 mRNA could not be detected in the uninduced cells, and made a slight expression in the induced cells at day 6. Both Oligo1 and nerve growth factor (NGF) mRNA were not detected in PB-IPCs at day 0, and were at a low level of expression either in the induced or uninduced cells at day 6. Otherwise, the mRNA expression of glial-derived neurotrophic factor (GDNF) was weak in the PB-IPCs, but there was a significant difference derived from an upregulation of GDNF expression in the induced cells rather than in the uninduced cells at day 6 (Table 2).

To examine the changes of mRNA expression over time for the induced and uninduced cells, Ngn2, Hes1 and O2 as key transcriptional factors involving in the differentiation program of neural lineages, and BDNF, all were expressed at a relatively higher level at day 0 and 6, before real-time PCR analysis at 0, 3, 6, 9 days. Hes1 expression of the induced cells began to be upregulated at day 3 and robustly increased by ~12-fold at day 6, remaining steady until day 9. In contrast, the expression of Hes1 was relatively stable in the non-induced cells (Figure 4A). Real-time PCR analyses showed that the expression of Oligo2 was somewhat upregulated during induction compared to the Control group (Figure 4B). At days 6 and 9, there was a significant upregulation of BDNF expression, and this change was more apparent in the induced cells than in the non-induced cells

(Figure 4C). There were no significant differences in Ngn2 mRNA expression between the 10 μ M ATRA and Control group at the 3th and 6th day (*P* = 0.02 and *P* = 0.04, respectively). By day 9, this difference between the two groups had gone (*P* = 0.1; Figure 4D). Real-time PCR experiments were repeated in triplicate, and there were no CT data reported in the reaction of NTC and NRT wells.

Clearance of glutamate was not detected in the astrocyte-like cells derived from PB-IPCs

At day 14, cells in the 10 μ M ATRA group and Control group were incubated with 50 μ M L-glutamate. The 10 μ M ATRA induced cells which were incubated with 50 μ M L-glutamate and 1 mM PDC, were designed as the PDC group. After 1 h at 37°C, supernatant in each group was collected and measured for concentration of L-glutamate. Due to blockade of glutamate uptake by PDC, the concentration of extracellular L-glutamate in the PDC group was set as the negative control. By statistical analysis, there was no significant concentration change of L-glutamate in the 10 μ M ATRA and Control group compared to in the PDC group (*P* = 0.55: the 10 μ M ATRA group vs. the PDC group; *P* = 0.21: the Control group vs. the PDC group).

Discussion

This study has characterised a novel population of CD45+ cells that circulate in the PB, called PB-IPCs. The cells share

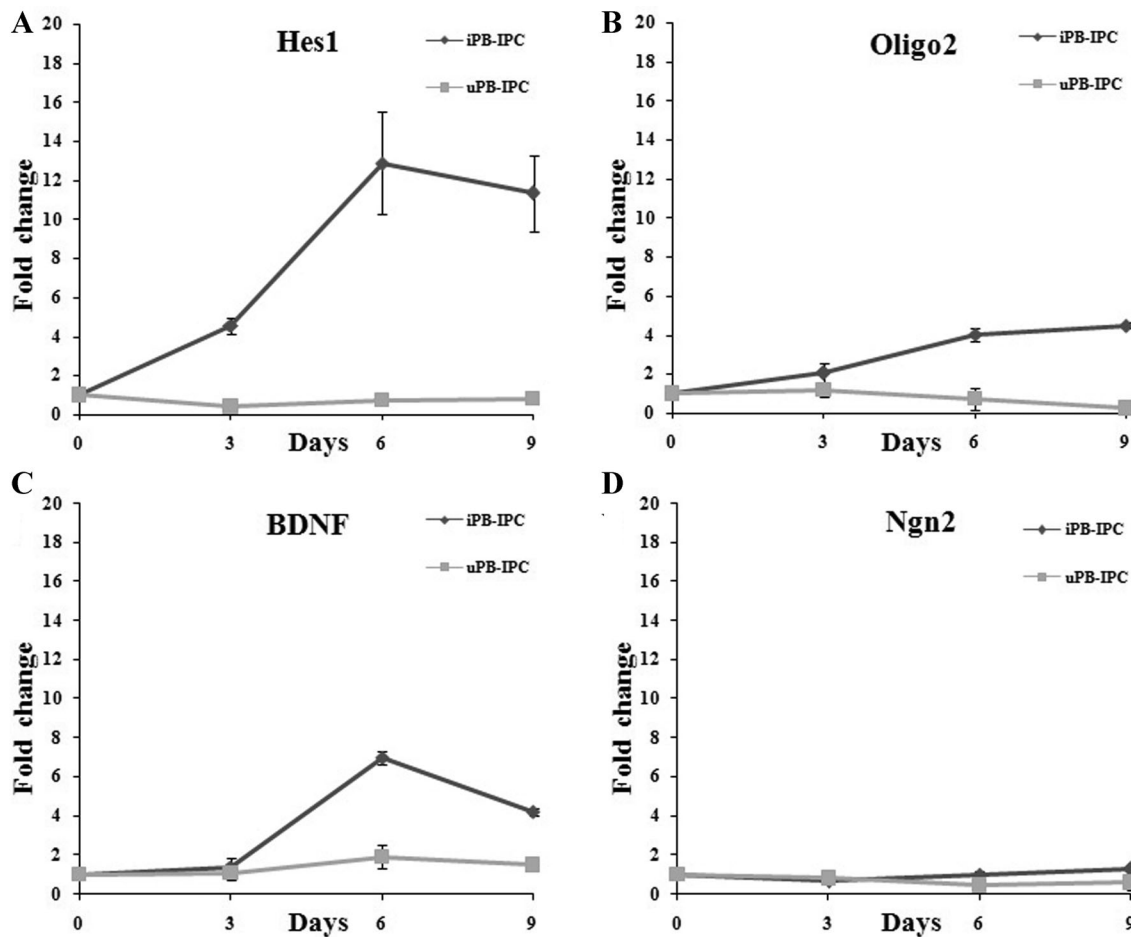


Figure 4 Real-time PCR analyses of Hes1, Oligo2, Ngn2 and BDNF mRNA expression in the process of induction. (A) ATRA induced PB-IPCs to express of Hes1 by the 3rd day ($P < 0.01$, compared to the Control group), exhibit a robust lack of regulation on the 6th day ($P < 0.01$, compared to the 3rd day), then maintain a stable expression on day 9 ($P = 0.46$, compared to the 6th day). In the Control group, the expression of Hes1 mRNA by the PB-IPCs was relatively stable in the process of culturing non-induced cells ($P = 0.03$, the 6th vs. 3rd day; $P = 0.78$, the 9th vs. 6th day). (B) The expression of Oligo2 was, to some extent, upregulated compared to the Control group at the 6th and 9th days ($P < 0.01$). (C) The induced PB-IPCs expressed BDNF in an unregulated manner compared to the Control group ($P < 0.01$ for the 6th and 9th days). (D) There were no significant differences in Ngn2 expression between the induced and non-induced cells ($P = 0.023$ on the 3rd day, $P = 0.04$ on the 6th day and $P = 0.09$ on the 9th day). The experiments were repeated in triplicate. Student's *t*-tests were used for statistical analyses.

the expression of multiple genes with ESCs, including *c-Myc*, *Klf4*, *Nanog* and *Sox2*. PB-IPCs also express *Notch1* and *Mash1*, which are associated with gliogenesis and neurogenesis, respectively. Under treatment with ATRA, the Hes1 and GFAP mRNA expression was upregulated; hence, a majority of the PB-IPCs displayed neural-like morphological changes and acquired some phenotypes of astrocyte, including GFAP, S100 β , GLAST and CD44. Despite apparent expression of GFAP and GLAST for the astrocyte-like cells differentiated from PB-IPCs, the undesirable results of glutamate clearance assay indicated that these cells maybe a population of immature astrocytes. Nevertheless, the BDNF mRNA expression of PB-IPCs was

at a high level and significantly upregulated during the process of ATRA induction; BDNF is an important neurotrophin that is secreted by astrocytes in general.

PB-IPCs with the CD45-positive phenotype can be obtained from adult human peripheral blood without mobilisation by granulocyte colony-stimulating factor (G-CSF). This unique population of cells exhibits round or oval morphologies that are similar to the cobblestone appearances of EPCs and distinguish them from MSCs. However, PB-IPCs require different culturing conditions of plating onto Petri dishes and higher CO₂ concentrations. The most significant distinctions between PB-IPCs and other stem cells or progenitors involve the surface epitopes:

PB-IPCs are positive for CD45 and negative for CD34, CD14 and CD11b (Zhao *et al.*, 2007). Based on their characteristics of self-renewal and their expression of pluripotent state-specific transcription factors including OCT-4 (Zhao *et al.*, 2007), *c-Myc*, *Klf4*, *Nanog* and *Sox2*, it is reasonable to assume that the PB-IPCs is a unique population of stem cells that exists in adult human peripheral blood.

ATRA, as an important factor that influences astroglialogenesis (Faigle *et al.*, 2008), can facilitate the leukaemia inhibitory factor (LIF)-induced astrocytic differentiation of neural precursor cells by activating signal transducer and activator transcription 3 (STAT3) (Asano *et al.*, 2009). During the process of ATRA induction, the expression of several transcription factors like *Nanog*, *Klf4* and *Notch1* was downregulated, which indicated that the ATRA treatment limited the pluripotentiality of PB-IPCs and raised its tendency of differentiation. The presence of *Notch1*, but not *Mash1*, expression that was apparent in PB-IPCs raised the possibility of differential fate commitment to an astrocytic lineage (Casarosa *et al.*, 1999; Taylor *et al.*, 2007). Based on time-course analyses of the real-time PCR results, *Hes1* (a transcription factor that is downstream of *Notch*-signalling and blocks neurogenesis and promotes gliogenesis) (Wu *et al.*, 2003; Taylor *et al.*, 2007; Kageyama *et al.*, 2008) and *GFAP*, were upregulated in the process of induction. Conversely, *Ngn1* and *Ngn2*, which are involved in initiating neurogenesis (Ma *et al.*, 1999; Ross *et al.*, 2003), were either not expressed or stably expressed during induction. Despite the apparent expression of *Oligo2* mRNA (Ross *et al.*, 2003), the majority of PB-IPC failed to display the special marker of oligodendrocytes (*O4*) during treatment with ATRA. This result can be explained by the facts that the induced *Oligo2* expression was not sufficient for the repression of astrogenesis (Du *et al.*, 2006) and that *Hes1* was robustly upregulated rather than *Oligo1* in PB-IPCs (Ross *et al.*, 2003). Moreover, oligo+ progenitors could also generate a subtype of astroglia (Jiang *et al.*, 2013).

A majority of induced PB-IPCs expressed astrocytic specific markers, including *GFAP*, *CD44*, *GLAST* and *S100 β* . Uninduced PB-IPCs also expressed the *S100 β* marker. This result may be explained by the facts that *S100 β* acts as a stimulator of cell proliferation and migration and an inhibitor of apoptosis and differentiation, and is expressed not only in astrocytes, but also in certain neuronal populations, melanocytes, chondrocytes, adipocytes, certain dendritic cell and lymphocyte populations and a few other cell types (Donato *et al.*, 2013). However, in spite of *GLAST* positive phenotype predomination, the induced PB-IPCs were not able to be detected for the ability of glutamate uptake in this experiment. This negative finding demonstrated that the astrocyte-like cells derived from PB-IPCs might be a population of immature astrocytes (Abe *et al.*, 2000; Roybon *et al.*, 2013).

To verify that PB-IPCs-derived astrocyte-like cells possess neurotrophic functions, we tested the mRNA expression of several neurotrophic factors, including *BDNF*, *NGF* and *GDNF*, which play important protective roles in some neurodegenerative diseases, such as Alzheimer's and Parkinson's (Allen *et al.*, 2013). *BDNF* mRNA is more strongly expressed in PB-IPCs than *GDNF* or *NGF* mRNA. During the process of induction, this upregulated mRNA expression of *BDNF* and *GDNF* further increased the neurotrophic potential of PB-IPCs-derived astrocyte-like cells.

In conclusion, a novel population of CD45+ cells, called PB-IPCs, shared some characteristics with ESCs that include self-renewal and the expression of pluripotent state-specific transcription factors was been reported. Treatment with ATRA allowed the PB-IPCs to be induced into a population of astrocyte-like cells with specific markers including *GFAP*, *GLAST*, *CD44* and *S100 β* . In spite of the deficiency of glutamate uptake, the upregulated mRNA expression of *BDNF* and *GDNF* increased the neurotrophic potential of PB-IPCs-derived astrocyte-like cells. Hemopoietic progenitors and stem cells obtained from bone marrow can migrate into the central nervous system (CNS) and differentiate into neural lineages after the CNS suffers lesions and the blood-brain barrier becomes leaky (Mezey *et al.*, 2003; Cogle *et al.*, 2004). Given that PB-IPCs exist in the peripheral blood, we speculate that PB-IPCs migrate into the CNS and have an important protective role. Thus, although the astrocyte-like cells derived from PB-IPCs were not demonstrated to be a population of mature astrocytes, the apparent advantages of PB-IPCs (they are easy to obtain, and have the tendency of astrocytic differentiation and enhanced neurotrophic potential under the state of limited proliferation) suggest that this cell pool may constitute an alternative source of autologous cell therapies for degenerative diseases in the CNS.

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References

- Abe K, Abe Y, Saito H (2000) Evaluation of L-glutamate clearance capacity of cultured rat cortical astrocytes. *Biol Pharm Bull* 23: 204–7.
- Allen SJ, Watson JJ, Shoemark DK, Barua NU, Patel NK (2013) *GDNF*, *NGF* and *BDNF* as therapeutic options for neurodegeneration. *Pharmacol Ther* 138: 155–75.

- Asano H, Aonuma M, Sanosaka T, Kohyama J, Namihira M, Nakashima K (2009) Astrocyte differentiation of neural precursor cells is enhanced by retinoic acid through a change in epigenetic modification. *Stem Cells* 27: 2744–52.
- Cartwright P, Mclean C, Sheppard A, Rivett D, Jones K, Dalton S (2005) LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* 132: 885–96.
- Casarsa S, Fode C, Guillemot F (1999) Mash1 regulates neurogenesis in the ventral telencephalon. *Development* 126: 525–34.
- Cesselli D, Beltrami AP, Rigo S, Bergamin N, D'aurizio F, Verardo R, piazza S, Klaric E, Fanin R, Toffoletto B, Marzinotto S, Mariuzzi L, Finato N, Pandolfi M, Leri A, Schneider C, Beltrami CA, Anversa P (2009) Multipotent progenitor cells are present in human peripheral blood. *Circ Res* 104: 1225–34.
- Cogle CR, Yachnis AT, Laywell ED, Zander DS, Wingard JR, Steindler DA, Scott EW (2004) Bone marrow transdifferentiation in brain after transplantation: a retrospective study. *Lancet* 363: 1432–7.
- Das S, Levasseur D (2013) Transcriptional regulatory mechanisms that govern embryonic stem cell fate. *Methods Mol Biol* 1029: 191–203.
- Donato R, Cannon BR, Sorci G, Riuzzi F, Hsu K, Weber DJ, Geczy CL (2013) Functions of S100 proteins. *Curr Mol Med* 13: 24–57.
- Du ZW, Li XJ, Nguyen GD, Zhang SC (2006) Induced expression of Olig2 is sufficient for oligodendrocyte specification but not for motoneuron specification and astrocyte repression. *Mol Cell Neurosci* 33: 371–80.
- Faigle R, Liu L, Cundiff P, Funa K, Xia Z (2008) Opposing effects of retinoid signaling on astroglialogenesis in embryonic day 13 and 17 cortical progenitor cells. *J Neurochem* 106: 1681–98.
- Jiang P, Chen C, Wang R, Chechneva OV, Chung SH, Rao MS, Pleasure DE, Liu Y, Zhang Q, Deng W (2013) HESC-derived Olig2⁺ progenitors generate a subtype of astroglia with protective effects against ischaemic brain injury. *Nat Commun* 4: 2196–212.
- Kageyama R, Ohtsuka T, Kobayashi T (2008) Roles of Hes genes in neural development. *Dev Growth Differ* 50 Suppl 1: S97–103.
- Krencik R, Weick JP, Liu Y, Zhang ZJ, Zhang SC (2011) Specification of transplantable astroglial subtypes from human pluripotent stem cells. *Nat Biotechnol* 29: 528–34.
- Li X, Li H, Bi J, Chen Y, Jain S, Zhao Y (2012) Human cord blood-derived multipotent stem cells (CB-SCs) treated with all-trans-retinoic acid (ATRA) give rise to dopamine neurons. *Biochem Biophys Res Commun* 419: 110–6.
- Li Y, McClintick J, Zhong L, Edenberg HJ, Yoder MC, Chan RJ (2005) Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. *Blood* 105: 635–7.
- Ma Q, Fode C, Guillemot F, Anderson DJ (1999) Neurogenin1 and neurogenin2 control two distinct waves of neurogenesis in developing dorsal root ganglia. *Genes Dev* 13: 1717–28.
- Mezey E, Key S, Vogelsang G, Szalayova I, Lange GD, Crain B (2003) Transplanted bone marrow generates new neurons in human brains. *Proc Natl Acad Sci U S A* 100: 1364–9.
- Nichols JE, Niles JA, Dewitt D, Prough D, Parsley M, Vega S, Cantu A, Lee E, Cortiella J (2013) Neurogenic and neuroprotective potential of a novel subpopulation of peripheral blood-derived CD133⁺ ABCG2⁺ CXCR4⁺ mesenchymal stem cells: development of autologous cell-based therapeutics for traumatic brain injury. *Stem Cell; Res Ther* 4: 3.
- Parras CM, Hunt C, Sugimori M, Nakafuku M, Rowitch D, Guillemot F (2007) The proneural gene Mash1 specifies an early population of telencephalic oligodendrocytes. *J Neurosci* 27: 4233–42.
- Porat Y, Porozov S, Belkin D, Shimoni D, Fisher Y, Belleli A, Czeiger D, Silverman WF, Belkin M, Battler A, Fulga V, Savion N (2006) Isolation of an adult blood-derived progenitor cell population capable of differentiation into angiogenic, myocardial and neural lineages. *Br J Haematol* 135: 703–14.
- Ross SE, Greenberg ME, Stiles CD (2003) Basic helix-loop-helix factors in cortical development. *Neuron* 39: 13–25.
- Roybon L, Lamas NJ, Garcia-Diaz A, Yang EJ, Sattler R, Jackson-Lewis V, Kim YA, Kachel CA, Rothstein JD, Przedborski S, Wichterle H, Henderson CE (2013) Human stem cell-derived spinal cord astrocytes with defined mature or reactive phenotypes. *Cell Rep* 4: 1035–48.
- Saunders A, Faiola F, Wang J (2013) Concise review: pursuing self-renewal and pluripotency with the stem cell factor nanog. *Stem Cells* 31: 1227–36.
- Taylor MK, Yeager K, Morrison SJ (2007) Physiological Notch signaling promotes gliogenesis in the developing peripheral and central nervous systems. *Development* 134: 2435–47.
- Tondreau T, Meuleman N, Delforge A, Dejeneffe M, Leroy R, Massy M, Mortier C, Bron D, Lagneaux L (2005) Mesenchymal stem cells derived from CD133-positive cells in mobilized peripheral blood and cord blood: proliferation, Oct4 expression, and plasticity. *Stem Cells* 23: 1105–12.
- Wu Y, Liu Y, Levine EM, Rao MS (2003) Hes1 but not Hes5 regulates an astrocyte versus oligodendrocyte fate choice in glial restricted precursors. *Dev Dyn* 226: 675–89.
- Zhao Y, Glesne D, Huberman E (2003) A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. *Proc Natl Acad Sci U S A* 100: 2426–31.
- Zhao Y, Huang Z, Lazzarini P, Wang Y, Di A, Chen M (2007) A unique human blood-derived cell population displays high potential for producing insulin. *Biochem Biophys Res Commun* 360: 205–11.
- Zhao Y, Wang H, Mazzone T (2006) Identification of stem cells from human umbilical cord blood with embryonic and hematopoietic characteristics. *Exp Cell Res* 312: 2454–64.

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