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Human cord blood-derived multipotent stem cells (CB-SCs) treated with all-trans-retinoic acid (ATRA) give rise to dopamine neurons

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ABSTRACT

Parkinson's disease (PD) results from the chronic degeneration of dopaminergic neurons. A replacement for these neurons has the potential to provide a clinical cure and/or lasting treatment for symptoms of the disease. Human cord blood-derived multipotent stem cells (CB-SCs) display embryonic stem cell characteristics, including multi-potential differentiation. To explore their therapeutic potential in PD, we examined whether CB-SCs could be induced to differentiate into dopamine neurons in the presence of all-trans retinoic acid (ATRA). Prior to treatment, CB-SCs expressed mRNA and protein for the key dopaminergic transcription factors Nurr1, Wnt1, and En1. Following treatment with 10 μM ATRA for 12 days, CB-SCs displayed elongated neuronal-like morphologies. Immunocytochemistry revealed that 48 ± 11% of ATRA-treated cells were positive for tyrosine hydroxylase (TH), and 36 ± 9% of cells were positive for dopamine transporter (DAT). In contrast, control CB-SCs (culture medium only) expressed only background levels of TH and DAT. Finally, ATRA-treated CB-SCs challenged with potassium released increased levels of dopamine compared to control. These data demonstrate that ATRA induces differentiation of CB-SCs into dopaminergic neurons. This finding may lead to the development of an alternative approach to stem cell therapy for Parkinson's disease.

1. Introduction

Parkinson's disease (PD) results from the dysfunction and chronic degeneration of dopamine neurons in the substantia nigra of the mesencephalon. Dopamine neurons play key roles in controlling voluntary movements and regulating body gestures. To date, pharmacological agents have shown only limited therapeutic potential for slowing the progression of PD [1]. Therefore, finding alternative therapies may be necessary to provide a cure or a long-lasting, effective treatment of symptoms. Stem cells, which possess the ability to self-renew and give rise to different cell lineages to replenish the damaged and aged tissue cells, may provide a solution [1–4]. While embryonic stem (ES) cells have been successfully induced to generate dopamine neurons [5,6], ethical concerns have limited their practical application. Similarly, induced pluripotent stem cells (iPSCs) hold promise, but the use of lentiviral, retroviral, and reprogramming-protein approaches to develop iPSCs raise concerns about the safety and complexity of the technology [7]. Some evidence suggests that bone marrow-derived mesenchymal stem cells (MSCs) may differentiate into dopamine neurons [8,9], and autologous MSCs can be used to overcome concerns about rejection. However, MSCs from older PD patients may have reduced capacity for proliferation and differentiation [10–12], which limits their availability and utility as a source of stem cell derived dopamine neurons.

Stem cells derived from human cord blood have unique advantages over other stem cell sources including (1) a large source, (2) no ethical concerns, (3) no risk to the donors, and (4) low risk of graft-versus-host disease (GVHD) [2]. We identified a novel type of stem cells from human cord blood, designated cord blood multipotent stem cells (CB-SCs) [13]. CB-SCs display ES-like cell characteristics, including multi-potential differentiation [2,13]. Of particular relevance to neurodegenerative disease research, CB-SCs have been shown to differentiate into neurons when exposed

Abbreviations: ATRA, all-trans retinoic acid; CB-SCs, cord blood-derived multipotent stem cells; DA, dopamine; DAT, dopamine transporter; ES, embryonic stem cell; GFAP, glial fibrillary acidic protein; iPSCs, induced pluripotent stem cells; MAP2, microtubule-associated protein 2; MSCs, mesenchymal stem cells; PD, Parkinson's disease; TH, tyrosine hydroxylase.

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to neuronal growth factor (NGF) [13]. We recently demonstrated the safety and efficacy of CB-SCs in human patients in clinical trials evaluating a treatment for diabetes [14,15]. To explore the therapeutic potential of CB-SCs in PD, we examined whether CB-SCs could be induced to differentiate toward dopamine neurons in the presence of all-trans retinoic acid (ATRA), a well-established inducer involved in neuronal patterning, neural differentiation and axon outgrowth [16], and the differentiation of dopamine neurons [17]. We found that exposure to ATRA induced CB-SC differentiation into functional dopamine neurons.

2. Materials and methods

2.1. CB-SC preparations

Human umbilical cord blood samples (50–100 ml/unit) were collected from healthy donors at Jinan Central Hospital. 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 mm Style Petri dishes (Becton Dickinson Labware, Franklin Lake, NJ) at 1×10^6 cells/ml. Cells were cultured in serum-free culture medium (Lonza, Allendale, NJ) and incubated at 37 °C with 8% CO_2 [13].

2.2. Cell differentiation

CB-SCs grown to 70% confluence were treated with 5 μM or 10 μM ATRA (Acros Organics) in the presence of Neurobasal-A medium (Invitrogen) combined with 1 × B27 supplement (Invitrogen). CB-SCs cultured in Neurobasal-A medium with 1 × B27 supplement served as control. CB-SCs cultured in serum-free culture medium served as an additional control. After treatment for 12 days at 37 °C, 8% CO_2 conditions, cells were subjected to examination for specific markers of dopamine neurons.

2.3. Quantitative real-time PCR

Quantitative real-time PCR was used to quantify mRNA expression for specific markers of dopamine neurons. Total RNA was extracted using a Qiagen kit (Valencia, CA). First-strand cDNAs were synthesized from total RNA using a QuantiTect Reverse Transcription kit according to the manufacturer's instructions (Qiangen, Valencia, CA). Real-time PCR was performed on each sample in triplicate with the ABI Prism 7900HT Fast Real-Time PCR System (Applied Biosystems, CA) using the following protocol: 95 °C for 10 min, 40 cycles of (95 °C for 15 s, 60 °C for 60 s). The validated gene-specific RT2 PCR Primer sets for each gene were designed and purchased from SABiosciences (Frederick, MD). Expression levels were determined relative to β-actin as an internal control [18].

2.4. Western blot analysis

To determine the expression of dopaminergic transcription factors, we performed Western blotting as previously described [14,19]. Briefly, cells were washed with cold PBS and solubilized with a lysis buffer (20 mM pH 7.5 Tris, 150 mM NaCl 1 mM EGTA, 1 mM EDTA, 1% TritonX-100, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate, 1 μg/ml leupeptin, 1 mM Na_3VO_4) and a cocktail of protease inhibitors (Sigma). Samples (20 μg protein each) were mixed with a loading buffer (2% SDS, 10% glycerol, 62.5 mM Tris–HCl (pH 6.8), 2 mg Bromophenol blue, 50 mM DTT) at a volume ratio of 1:1, then boiled, loaded, and separated by electrophoresis on a 10% SDS gel. The separated proteins were transferred to a nitrocellulose membrane and blocked with 5% non-fat dry milk in TBST for 1 h followed by incubation with rabbit anti-human Nurr1, En1, or Wnt1 Abs (Abcam, Cambridge, MA) at 1:1000 dilutions. β-Actin served as an internal loading control.

2.5. Immunocytochemistry

The ATRA-treated CB-SCs and cells from control groups were fixed with 4% paraformaldehyde and subsequently permeabilized with 0.5% Triton X-100 (Invitrogen) for 15 min, incubated with 3% hydrogen peroxide for 10 min, and blocked with 2.5% horse serum for 20 min. Fixed cells were immunostained with rabbit anti-human TH Ab, rat anti-DAT Ab, mouse anti-NeuN (Neuronal nuclear) Ab, mouse anti-GFAP (glial fibrillary acidic protein) Ab, mouse anti-β III tubulin Ab (Abcam), or mouse anti-MAP-2 (microtubule-associated protein-2) Ab (Millipore, Billerica, MA). Appropriate secondary antibodies anti-rabbit/rat IgG-TRITC, anti-mouse IgG-FITC (Jackson ImmunoResearch Laboratories), and mounting medium with DAPI (Invitrogen) were used for detection and visualization. Images were acquired using an Olympus IX710 Camera with the manufacturer's software and edited using Adobe Photoshop CS3.

2.6. Dopamine enzyme-linked immunosorbent assay

Following 12 days of treatment with ATRA or control medium, cells were examined for dopamine release. Dopamine levels were quantitated using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). Briefly, cells were washed twice with PBS to remove all culture medium and then placed in Hank's balanced salt solution (Solarbio, Beijing, China) containing 5.33 mM K^+ or 56 mM K^+ concentration. High concentrations of potassium induce secretion of dopamine through cell depolarization [17]. After incubation for 5 min at 37 °C, supernatants were collected for ELISA following the manufacturer's instructions.

2.7. Statistical analysis

Data were analyzed with a two-tailed Student's *t*-test to determine statistical significance. Values are given as mean ± SD (standard deviation).

3. Results

3.1. CB-SCs possess the potential for dopaminergic differentiation

CB-SC cultures were established from multiple human cord blood preparations. To evaluate the CB-SCs' potential for differentiation into dopamine neurons, we examined basal expression of the dopamine neuron-specific transcription factors Nurr1, Wnt1, and En1. Real time PCR analysis showed that untreated CB-SCs expressed Nurr1, Wnt1, and En1 mRNA (Fig. 1(A)). Western blot analysis further confirmed protein expression of all three (Fig. 1(B)). Real-time PCR analysis also revealed that CB-SCs displayed a low level of TH mRNA, a key enzyme responsible for catalyzing the conversion of the tyrosine to dihydroxyphenylalanine (DOPA, a precursor for dopamine). The presence of these markers indicated that CB-SCs possess the potential for dopaminergic differentiation.

3.2. Differentiation of CB-SCs into neuron-like cells after treatment with ATRA

We evaluated the response of CB-SCs to exposure to two ATRA concentrations. Both concentrations induced morphological changes consistent with differentiation to neurons, and the higher concentration induced changes in a larger proportion of cells. Most

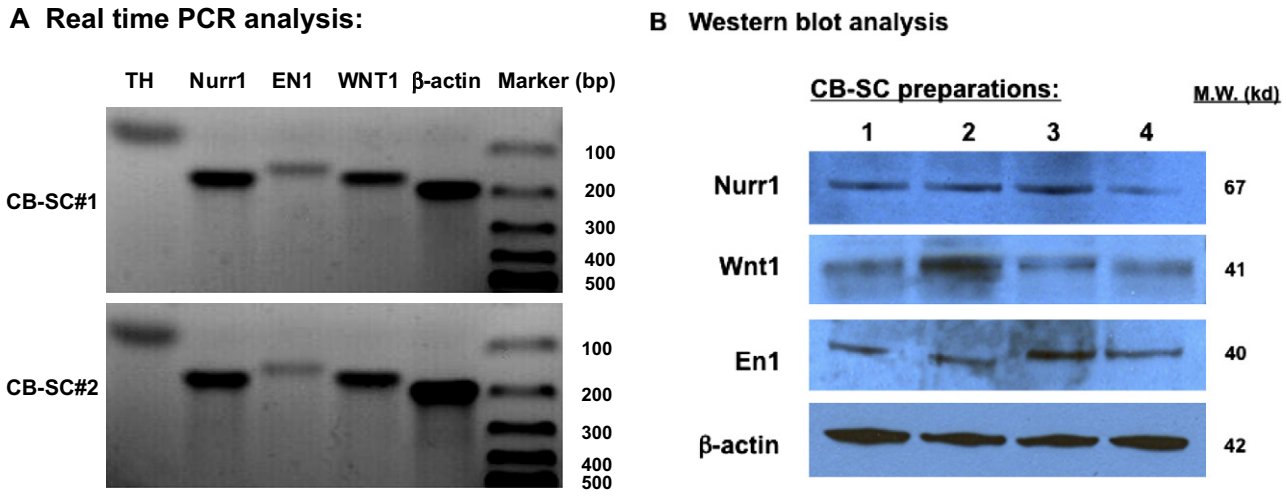

Fig. 1. CB-SCs display dopaminergic transcription factors. (A) Real-time PCR analysis for mRNA expression of transcription factors, followed by electrophoresis in 2% agarose gel. Data are representative of two CB-SC preparations. (B) Western blot analysis showing expression of transcription factors at the protein level. Data are representative of four CB-SC preparations. β-Actin served as an internal control. Data represent one of three experiments.

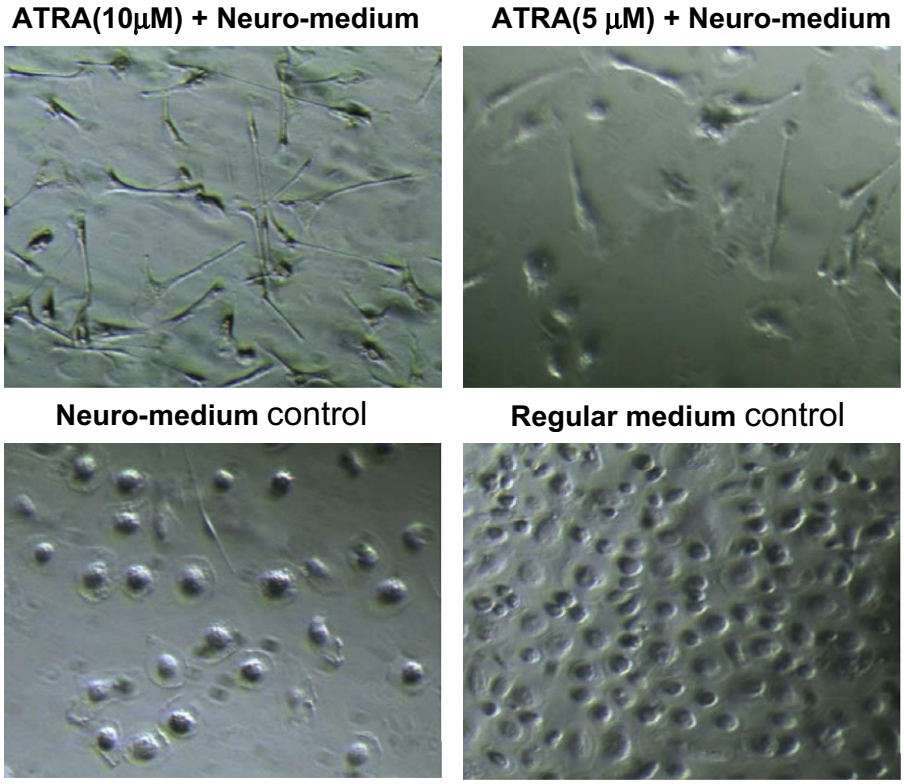

Fig. 2. Phase-contrast microscopy shows the differentiation of CB-SCs into neuron-like cells after treatment with ATRA. CB-SCs were treated with ATRA at 5 μM or 10 μM in Neurobasal-A medium with 1 × B27 supplement. CB-SCs cultured in Neurobasal-A medium with 1 × B27 supplement served as control (bottom left panel). CB-SCs cultured in serum-free culture medium served as an additional control (bottom right panel). Cells were examined for neuronal differentiation after 12 days of treatment. Original magnification, ×100.

cells (~90%) exposed to 10 μM ATRA displayed typical neuronal morphologies within 5–8 days, followed by the formation of more elongated and branched cell processes after 10–12 days (Fig. 2, top left panel). In contrast, 35% of cells in 5 μM ATRA group also turn into neuron-like morphologies after 10–12 days, but with relatively shorter cell processes (Fig. 2, top right panel). Most of the Neuro-medium control cells (>95%) failed to display neuronal differentiation (Fig. 2, bottom left panel). CB-SCs cultured in serum-free culture medium continued to grow with round morphologies at high confluence (Fig. 2, bottom right panel).

Cells from each group were immunostained for specific neuronal markers including βIII tubulin, microtubule-associated protein 2 (MAP2) (structural proteins of neurons), NeuN (mature neuronal marker), and glial fibrillary acidic protein (GFAP, an astrocyte marker). Immunostaining showed that 90 ± 4% of ATRA-treated cells strongly expressed βIII tubulin (Fig. 3(A), top panels) and MAP-2

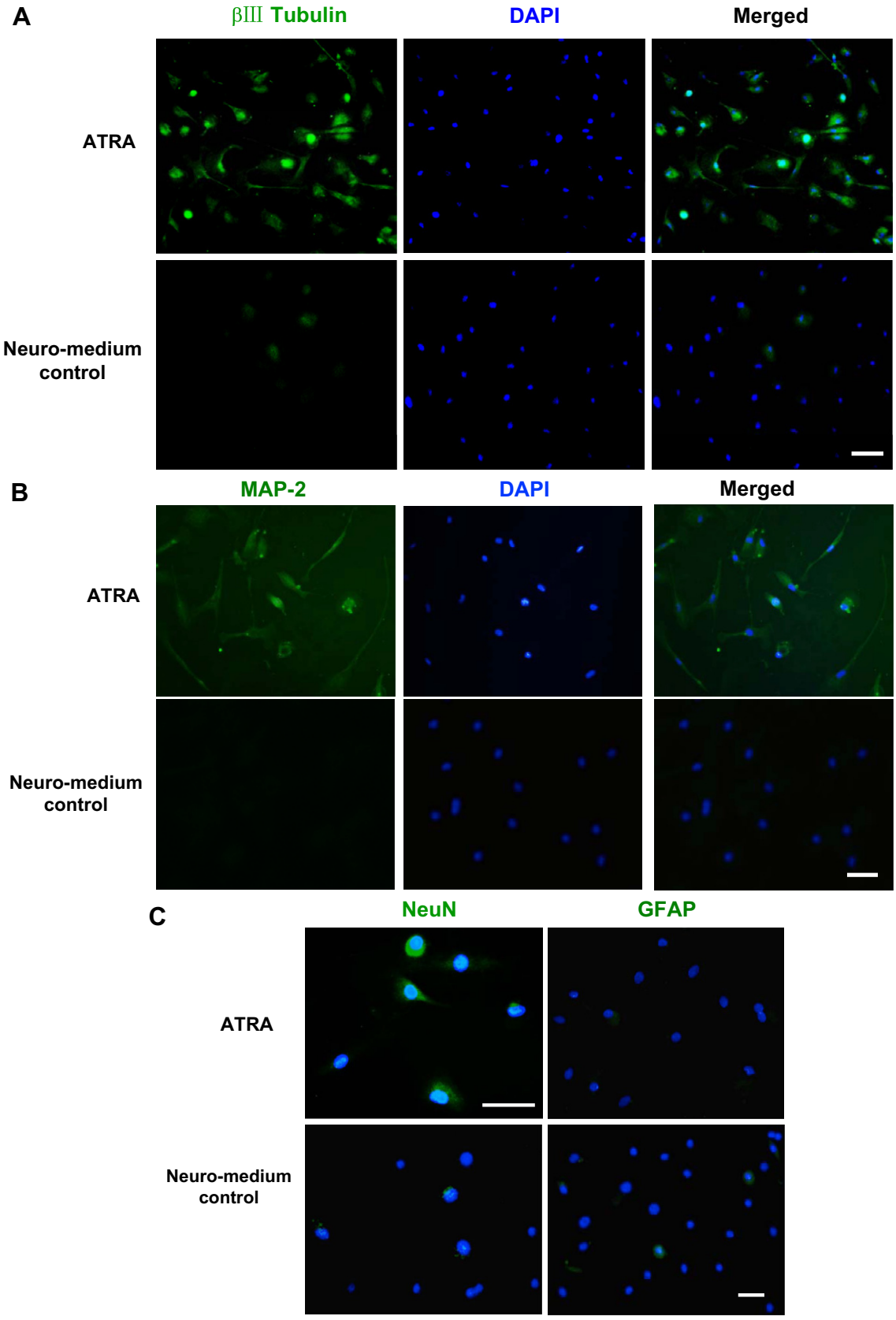

Fig. 3. Characterization of ATRA-treated CB-SCs with different neuronal markers. After treatment with 10 μM ATRA + Neurobasal-A medium with 1 × B27 supplement for 12 days, cells were immunostained with different neuronal markers (green), followed by nuclear counter-staining with DAPI (blue). CB-SCs cultured in Neurobasal-A medium with 1 × B27 supplement served as control. (A) Immunostaining for βIII tubulin. (B) Immunostaining for MAP-2. (C) Immunostaining for NeuN and GFAP. Isotype-matched IgGs served as controls for immunostainings. Scale bar, 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

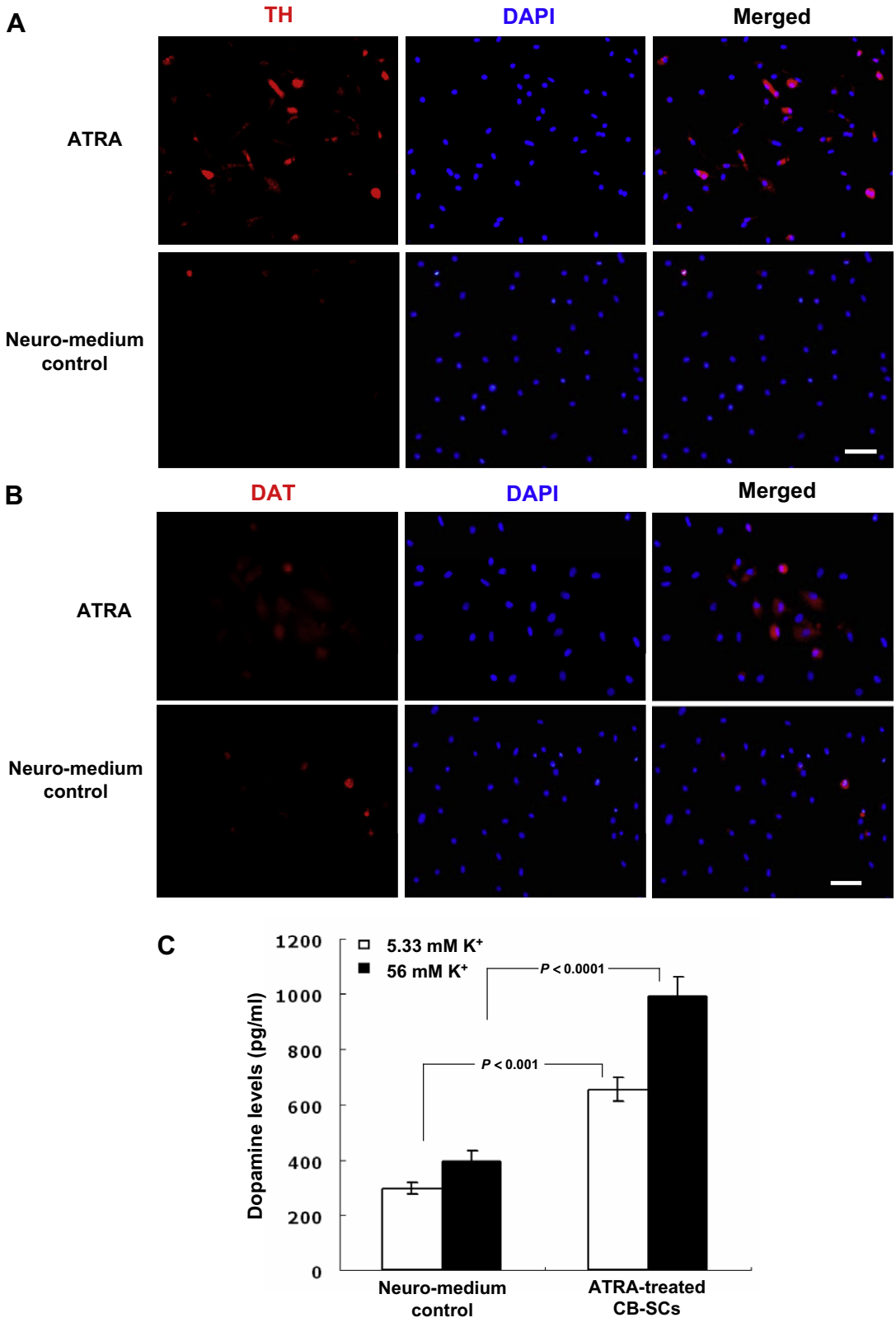

Fig. 4. Characterization of ATRA-treated CB-SCs with DA neuron-specific markers. After treatment with 10 μM ATRA + Neurobasal-A medium with 1 × B27 supplement for 12 days, cells were immunostained with different DA neuron-specific markers (red), followed by nuclear counter-staining with DAPI (blue). CB-SCs cultured in Neurobasal-A medium with 1 × B27 supplement served as control. (A) Immunostaining for TH. (B) Immunostaining for DAT. Isotype-matched IgGs served as controls for immunostainings. Scale bar, 50 μm. (C) Functional analysis of dopamine release by ATRA-treated CB-SCs and control cells in the presence of potassium at different concentrations as described in methods. Data represent mean (±SD) of three experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3(B), top panels), about 70 ± 7% of ATRA-treated cells were positive for NeuN (Fig. 3(C), top left panel), and only a few cells (5 ± 2%) expressed GFAP (Fig. 3(C), top right panel). In contrast, a few cells (~5%) in the Neuro-medium-treated group displayed only background levels of βIII tubulin, MAP-2, NeuN, and GFAP (Fig. 3(A)–(C)). These data indicate that treatment with 10 μM ATRA induces differentiation of CB-SCs into neuronal-like cells.

3.3. CB-SCs gave rise to functional dopamine neurons after treatment with ATRA, and these cells release dopamine in response to stimulation

We performed immunocytochemical analysis to examine the expression of dopamine neuron-specific proteins including TH (a marker of dopamine neurons) and DAT (a dopamine transporter). Immunostaining results revealed that 48 ± 11% of ATRA-treated cells expressed TH (Fig. 4(A)), while 36 ± 9% of ATRA-treated cells were positive for DAT (Fig. 4(B)). In contrast, CB-SCs in the medium control groups only showed background levels of TH and DAT expression (5 ± 1%) (Fig. 4(A) and (B)). These data indicate that CB-SCs exposed to ATRA produce proteins indicative of dopamine neurons.

To examine whether ATRA-induced CB-SCs produce and are capable of secreting dopamine, we stimulated the cultured cells with potassium to initiate depolarization. Results from ELISA demonstrated that dopamine levels were markedly increased in the 10 μM ATRA-treated groups compared to the control groups (Fig. 4(C), $P < 0.001$ and $P < 0.0001$, respectively). These data confirm that CB-SCs can give rise to functional dopamine neuron-like cells after treatment with 10 μM ATRA in Neuro-medium.

4. Discussion

Reestablishing populations of DA neurons is essential for the reversal of PD. This study demonstrated that CB-SCs can be induced to differentiate into functional DA neurons in the presence of 10 μM ATRA combined with B27 + Neurobasal medium. These ATRA-induced CB-SCs displayed neuronal morphology, expressed tyrosine hydroxylase (TH) and other DA neuron-specific molecular markers, and secreted DA in response to potassium. These findings indicate ATRA-induced CB-SCs may provide an alternative to other stem cell-based approaches to generating DA neurons for the treatment of PD.

DA neurons are essential for the control of voluntary movement and the regulation of emotion [20]. The limited success of pharmacological treatments for PD suggest that only the replacement of lost DA neurons will provide a complete and lasting cure for the disease. To date, researchers have evaluated the replacement potential of DA neurons derived from embryonic stem (ES) cells [5,21], induced pluripotent stem cells (iPS) [7], bone marrow -derived mesenchymal stem cells (MSC) [8], and cord blood-derived unrestricted somatic stem cells, which are leukocyte common antigen CD45 negative and different from CB-SCs [2,22,23]. While the results of these studies are promising, the immunological, ethical, and safety concerns associated with these cells will likely limit their utility in PD treatment for the foreseeable future. CB-SCs derived from human cord blood are not limited by these concerns and have the additional advantages of very low immunogenicity [13] and plentiful worldwide availability. In addition, our clinical trials in diabetic patients have demonstrated that CB-SCs are safe for use in human subjects [2,14].

Notably, untreated CB-SCs possess the potential to differentiate into DA neurons, as confirmed by expression of dopaminergic transcription factors (e.g., Nurr1, Wnt1, and EN1 [20]). This study revealed that ATRA treatment can promote the differentiation of CB-SCs into functional DA neurons without the use of sonic hedgehog (SHH), fibroblast growth factor 8 (FGF8), or basic fibroblast growth factor (bFGF) [17]. This represents a significant advance in the development of usable sources of DA neurons, sidestepping concerns about the use of these factors to initiate differentiation in other types of stem cells [5,6,8,9,22–24]. We tested the combination of ATRA with FGF8 and/or bFGF but did not find synergistic effects on the differentiation of DA neurons (data not shown). Thus, treatment with ATRA is sufficient to provide the key signal or signals leading to the differentiation of CB-SCs into functional DA neurons. Interestingly, we found a low percentage of DA neurons in the B27 control group. The differentiation of a small number of cells in the population was probably due to the low concentration of all-trans retinol (about 6.7×10^{-3} μM) in B27 NeuroMix [25,26] and the basal expression of dopaminergic transcription factors in the CB-SCs. As expected, CB-SCs cultured in serum-free medium did not differentiate and proliferated constantly.

In this study, we demonstrated an efficient, effective approach for developing functional DA neurons from a stem cell source that does not carry the immunological, ethical, or safety concerns common to other stem cell sources. Further evaluation of CB-SC-derived DA neurons in animal models may facilitate the translation of this finding into a clinical treatment for PD.

Authors' contributions

Y. Z., X.L., and H.L. designed experiments. H.L. and J.B. were in charge of culturing and preparing cells. Laboratory experiments were performed by X.L., H.L., and S.J., and directed by Y.Z. Y.C. was in charge of collecting and providing cord blood samples. H.L. and X.L. collected experimental data. Y.Z. wrote the manuscript.

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