Adult Stem Cells from the Hyaluronic Acid-Rich Node and Duct System Differentiate into Neuronal Cells and Repair Brain Injury

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The existence of a hyaluronic acid-rich node and duct system (HAR-NDS) within the lymphatic and blood vessels was demonstrated previously. The HAR-NDS was enriched with small (3.0–5.0 μm in diameter), adult stem cells with properties similar to those of the very small embryonic-like stem cells (VSELs). Sca-1⁺Lin[−]CD45[−] cells were enriched approximately 100-fold in the intravascular HAR-NDS compared with the bone marrow. We named these adult stem cells “node and duct stem cells (NDSCs).” NDSCs formed colonies on C₂C₁₂ feeder layers, were positive for fetal alkaline phosphatase, and could be subcultured on the feeder layers. NDSCs were Oct4⁺Nanog⁺SSEA-1⁺Sox2⁺, while VSELs were Oct4⁺Nanog⁺SSEA-1⁺Sox2[−]. NDSCs had higher sphere-forming efficiency and proliferative potential than VSELs, and they were found to differentiate into neuronal cells in vitro. Injection of NDSCs into mice partially repaired ischemic brain damage. Thus, we report the discovery of potential adult stem cells that may be involved in tissue regeneration. The intravascular HAR-NDS may serve as a route that delivers these stem cells to their target tissues.

Introduction

A NUMBER OF ADULT non-hematopoietic stem cells have been identified in the bone marrow (BM) to date. These include very small embryonic-like stem cells (VSELs) [1], multipotent adult stem cells [2], multipotent adult progenitor cells [3], marrow-isolated adult multilineage inducible cells [4], mesenchymal stem cells [5], and endothelial progenitor cells [6].

VSELs have been described as rare, small, pluripotent stem cells found in murine and human BM [7,8]. They are lineage- and CD45-negative, express stem-cell markers, and give rise to cells corresponding to all three germ layers in vitro [9]. In addition, Kassmer et al. [10] reported that they differentiate into lung epithelial cells in vivo.

We recently described a hyaluronic acid-rich node and duct system (HAR-NDS) [11,12] also known as the Bonghan (or Primo) vascular system [13–16]. We referred to the nodes and ducts as hyaluronic acid-rich node and hyaluronic acid-rich duct, respectively, and to the two together as hyaluronic acid-rich node(s) and duct(s) (HAR-ND) [12]. We detected frequent, small, immature cells in the HAR-NDS by both light and electron microscopy [11,12]. Here, we employed a purification method typically used to purify BM VSELs, to demonstrate these VSEL-like cells in the

HAR-NDS. Because the cells were enriched in the HAR-NDS located inside the blood and lymph vessels, we named them “node and duct stem cells (NDSCs).” We describe the similarities and differences between the BM-derived VSELs and the HAR-NDS-derived NDSCs. We also demonstrate that the NDSCs have the potential to differentiate into neuronal cells, and to repair ischemic injury in the brain.

Materials and Methods

Mice

Imprinting control region (ICR) mice were purchased from Orient Bio, Inc. (Sungnam, Korea). All mice were kept in specific, pathogen-free conditions in a dedicated vivarium at the National Cancer Center, Korea. All animal experiments were reviewed and approved by the Animal Care and Use Committee of the National Cancer Center, and performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Extraction of the HAR-ND

Pathogen-free, 1 to 10-week-old male ICR mice were anesthetized by intramuscular injection of Zoletil (2.5 mg/kg;

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Virbac S.A.) and Rompun (0.5 mg/kg; Bayer Korea). The anesthetized mice were then injected intramuscularly at the right and left base of the tail [17] with 1% alcian blue solution (Sigma-Aldrich) to visualize HAR-NDS components inside the lymphatic vessels, and into the left tail-vein with 1% alcian blue to visualize them inside the veins. An incision was made along the abdominal linea alba. A blue line was visible inside the clear lumbar, sciatic, and/or caudal lymph vessels. A longitudinal incision was made along the lymph vessels before extracting the HAR-ND. To extract the HAR-ND from the veins, the blood was first drained through an incision along the vein, with the top and bottom of the lumbar vein clamped by forceps. The HAR-ND was carefully lifted out from all vessels by holding both ends of each vessel under a stereomicroscope (Zeiss Stereo Discovery V20) with a camera (Zeiss AxioCamHRc).

Preparation of alcian blue

Alcian blue 8-GX was purchased from Sigma-Aldrich. One percent alcian blue solution was prepared in phosphate-buffered saline (PBS, pH 7.2) at room temperature and filtered by using a 0.22 μ m membrane filter immediately before use (Merck Millipore) with a 1 mL-syringe with 26-gauge needle (BD).

Antibodies and fluorescence-activated cell sorting analysis

VSELs and NDSCs were isolated from a suspension of total nucleated cells from the BM and HAR-NDS, respectively, by live sterile cell sorting. Briefly, the BM- or HAR-NDS-derived mononuclear cells were resuspended in cell-sort medium (CSM) composed of 1% heat-inactivated fetal bovine serum (Gibco), 1 mM EDTA, and 25 mM HEPES in PBS, pH 7.4 (Ca^{2+} / Mg^{2+} -free). The following mAbs were used to stain these cells: anti-Ly-6A/E (Sca-1)-PE (clone E13-161.7), anti-CD45-PE-cy5 (clone 30-F11) and biotinylated lineage cocktail, anti-CD45R/B220-biotin (clone RA-3 H57-597), anti-Gr-1-biotin (clone RB6-8C5), anti-TCR $\alpha\beta$ -biotin (clone H57-597), anti-TCR $\gamma\delta$ -biotin (clone GL-3), anti-CD11b-biotin (clone M1/70), and anti-Ter-119-biotin (clone TER-119). Streptavidin-FITC was used to detect the primary mAbs. All mAbs were added at saturating concentrations, and the cells were incubated for 30 min on ice and washed twice with PBS (pH 7.4), then resuspended for sorting in CSM. All antibodies were purchased from BD Pharmingen. Cell sorting was performed on a FACSaria flow cytometer (BD Biosciences), and the analysis was performed on a FACSCalibur (BD Biosciences).

Electron microscopy of NDSCs from the HAR-NDS

Sorted NDSCs were fixed in Karnovsky's fixative (2% paraformaldehyde and 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2) at 4°C for 2 h. For transmission electron microscopy (TEM), the NDSCs were post-fixed in 1% osmium tetroxide (EMS) at 4°C for 2 h, dehydrated in an ethanol series, embedded in SURR resin (EMS), and polymerized at 70°C overnight. Ultra-thin sections (0.5–1 μ m thick) were cut using an ultramicrotome (RMC MTX; Boeckeler Instruments, Inc.) with a histo-jumbo-diamond knife (Diatome AG), and stained with uranyl acetate (EMS)

for 20 min followed by lead citrate for 10 min. The sections were examined under a transmission electron microscope (JEM1010; JEOL) at an accelerating voltage of 80 kV. For scanning electron microscopy (SEM), the fixed NDSCs were washed with 0.05 M sodium cacodylate buffer (pH 7.2, 4°C) thrice for 10 min. They were post-fixed in 1% osmium tetroxide in 0.05 M sodium cacodylate buffer (pH 7.2) and then briefly washed with distilled water twice at room temperature. The cells were then dehydrated in an ethanol series at room temperature, 10 min for each step. The cells were stiffened with 100% isoamyl acetate twice for 10 min at room temperature and dried at the critical point with liquid carbon dioxide. They were then mounted on metal stubs and coated with gold using a Sputter Coater (SCD 005; BAL-TEC), and examined under a field-emission scanning electron microscope (Carl Zeiss SUPRA 55VP).

In vitro expansion of VSELs and NDSCs

Freshly sorted VSELs and NDSCs were plated (1×10^3 cells/well) over an irradiated (at 40 Gy) C₂C₁₂ murine myoblast feeder layer in DMEM-F12 medium (Sigma-Aldrich) supplemented with 20% knockout serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 100 μ M MEM NEAA (Invitrogen), 100 μ M β -mercaptoethanol (Sigma-Aldrich), and 4 ng/mL human basic FGF (Sigma-Aldrich) for 7 days. The cells were then harvested and replated on C₂C₁₂ cells in the same medium (first passage) and cultured to form new spheres for 6–7 days. The medium was replaced every 24 h, and the cells were cultured under the same conditions during the second to fourth passages. To demonstrate their stem cell-like features, a sample of the spheres was fixed in 4% paraformaldehyde for 15 min, washed twice in TBST (0.15 M NaCl, 0.05% Tween-20 in 20 mM Tris-HCl, pH 7.4), and stained using an alkaline phosphatase (AP) detection kit (Millipore).

Neuronal differentiation

To generate neuronal derivatives (neurons, oligodendrocytes, and glial cells), cells from 10 VSEL or NDSC spheres were cultured in NeuroCult[®] basal medium (Stem Cell Technologies) supplemented with 10 ng/mL rhEGF, 20 ng/mL FGF-2, and 20 ng/mL NGF on eight-well culture slides (SPL Life Science). All growth factors were purchased from R&D Systems. The growth factors were added every 24 h, and the medium was replaced every 3 days. The cultures were examined after 21–25 days.

A murine model of cerebral ischemia

Seven-week-old male ICR mice were anesthetized, and a small incision was made on the right side of the neck. The right carotid artery was exposed and double-ligated with 7-0 silk sutures (Ethicon LCC). The incision was closed with 5-0 nylon sutures (Ethicon LCC), and the animals were allowed to recover with access to food and water for 2 h. Systemic hypoxia was induced by exposure to 8% O₂, balance N₂, in a temperature-controlled chamber for 20 min. Animals were then returned to their cages with free access to food and water [18–21]. The following day, they were heated in a water-bath at 38°C for 5 h [22,23]. This process was repeated every 2 days. The combination of unilateral

carotid artery ligation with systemic hypoxia produces ischemia and heat stroke, which triggers reproducible brain damage affecting both brain hemispheres. After 7 days, sorted and CM-DiI-labeled NDSCs were adoptively transferred (5×10^3 cells by intravenous injection) into ischemic mice. Control animals received the same volume of $1 \times \text{PBS}$.

The mice were killed on day 35 following the NDSC injection, and the brains were removed from the skulls and soaked in 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich) at 37°C for 30 min. Brain images were obtained with a dissecting microscope. To determine the infarct area, both the contralateral and ipsilateral brain hemispheres were analyzed with NIH ImageJ software (version 1.47; NIH Image) [18]. Infarct volume was calculated as the ratio of the damaged area (white area) to the total area of a hemisphere: infarct volume (%) = [damaged area (ipsilateral area + contralateral area)/(total ipsilateral area + contralateral area)] $\times$ 100. For immunohistochemical staining, the brains were post-fixed, and embedded in paraffin. Coronal sections (10 μm thick) through the infarct were cut with a microtome and mounted on microscope slides.

Immunofluorescence staining

The sections were fixed with 4% paraformaldehyde for 20 min. Nonspecific binding sites were blocked for 30 min with 2% bovine serum albumin (BSA) in PBS, pH 7.4 for extracellular epitopes, or with 2% BSA in 0.1% Triton X-100 for intracellular epitopes. The specimens were incubated with primary antibodies at 4°C overnight. Excess primary antibodies were removed the following day by washing with PBST for 5 min three times. For staining with secondary antibodies, we used anti-rabbit IgG-DyLight[®] 650 (ab96922; Abcam), anti-rabbit IgG-DyLight 488 (ab96883; Abcam), or anti-mouse IgG-Alexa Fluor 546 (Invitrogen). Nuclei were stained with 0.5 $\mu\text{g}/\text{mL}$ 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). The following antibodies were used: anti-Oct4 (ab18976), anti-Sox2 (ab59776), anti-Nanog (ab80892), anti-NeuN (ABN78; Millipore), or anti-MAP-2 (ab32454; Abcam). All antibodies to stem cell markers were from Abcam. Primary and secondary antibodies were diluted with 1% BSA at, respectively, 1:100 and 1:1,000. Specimens were visualized by confocal microscopy (LSM 510; Zeiss).

Reverse transcriptase–polymerase chain reaction and western blot analysis

VSEL and NDSC spheres, differentiated cells, ES-D3 cells, C₂C₁₂ feeder cells, or mouse brain cells were harvested and washed once with chilled $1 \times \text{PBS}$, and total RNA was extracted using the TRIzol reagent (Invitrogen). cDNA was synthesized from 2 μg total RNA and oligo (dT) primers (Promega) with M-MLV reverse transcriptase, according to the manufacturer's protocol. Twenty units of RNase inhibitor (Ambion, Inc.) were added to each reaction. Polymerase chain reaction (PCR) amplifications were performed with PyroHotStart Taq (Bioneer, Inc.), with 10 pmol of appropriate oligonucleotide primers. The PCR primers used were as follows: *Oct4*, forward 5'-TGG AAAGCAACTCAGAG GGAACCT-3', reverse 5'-ATTGAGAACCGTGTGAGGT GGAGT-3'; *Sox2*, forward 5'-AACATGATGGAGACGGA GCTGAAG-3', reverse 5'-TACAG CATGTCCTACTCGC

AGCA-3'; *Nanog*, forward 5'-TCGAATTCTGGGAACG CCTC ATCA-3', reverse 5'-AACCAAAGGATGAAGTG CAAGCGG-3'; *GFAP*, forward 5'-GGAGCTCAATGACC GCTTTG-3', reverse 5'-TCCAGGAAGCGAACCTTCTC-3'; *Nestin*, forward 5'-CCCTGATGATCCATCCTCCTT-3', reverse 5'-CTGGAATATGCTAGAAACTCTAGACTCAC T-3'; and β -III tubulin, forward 5'-TCCGTTTCGCTCAGG TCCTT-3', reverse 5'-CCCAGACTGACCGAAAACGA-3'. Reactions were carried out in a thermocycler, beginning with a hot start at 94°C for 5 min, followed by 25–40 cycles consisting of 94°C for 30 s, 52–62°C for 30 s, and 72°C for 30 s. The final extension was performed at 72°C for 10 min. The PCR products were analyzed by 1% agarose gel electrophoresis. For western blotting, the cells were lysed in RIPA buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% DOC, 1 mM PMSF, and 25 mM MgCl₂) supplemented with a phosphatase inhibitor cocktail. The cell lysates were resolved by 8%–12% SDS-polyacrylamide gel electrophoresis and subjected to immunoblotting using antibodies to Oct-4 (ab18976; Abcam), Sox-2 (ab59776; Abcam), Nanog (ab80892; Abcam), GFAP (12389; Cell Signaling Technology), Nestin (ab27952; Abcam), or β -III tubulin (ab18207; Abcam). As secondary antibodies we used either anti-rabbit IgG-HRP (Invitrogen) or anti-mouse IgG-HRP (Invitrogen). The blots were stripped and reprobed with anti- β -actin antibody to confirm equal loading. Protein concentration was determined by the BCA assay (Bio-Rad Laboratories).

Statistical analysis

All data were analyzed using the Prism 5.0 GraphPad statistical package. Student's *t*-test was used to determine statistically significant differences between groups.

Results

Extraction of HAR-ND from veins and lymph vessels

We injected 1% alcian blue at the right and left base of the tail to stain the intralymphatic HAR-ND, and a blue line formed inside the clear lumbar, sciatic, and caudal lymphatic vessels (Fig. 1A-a,b). The stained HAR-ND inside could be extracted by clamping the vessels at both ends. HAR-ND isolated from the lymph vessels were often observed to roll up rapidly, which suggests considerable elasticity (Fig. 1A-c). To isolate HAR-ND from veins, we injected 1% alcian blue into the left tail-vein, which resulted in a blue line forming inside the lumbar vein (Fig. 1B-a). To drain the blood and facilitate extraction a longitudinal incision was made along the vein after clamping it at both ends (Fig. 1B-b). An example of a node inside a vein is shown in Fig. 1B-c.

Purification of Sca-1⁺ Lin[−] CD45[−] cells from the HAR-NDS

We employed a method previously used to purify VSELS [7] to purify small Sca-1⁺ Lin[−] CD45[−] cells from the HAR-NDS. To compare their properties, we purified bone marrow VSELS and the HAR-NDS small Sca-1⁺ Lin[−] CD45[−] cells side by side. Briefly, we selected a population of cells between 2–5 μm in size (Fig. 2A-a, b) and, from this population, those that were positive for Sca-1 and negative for

FIG. 1. HAR-NDS inside veins and lymphatic vessels of a mouse. ICR mice (~ 8 weeks old) were anesthetized and the HAR-ND inside their lymph and blood vessels was stained with 1% alcian blue. **(A)** Intralymphatic HAR-ND can be seen as a blue line inside the central lymph vessel (arrowhead). Yellow broken lines indicate the boundary of a vein (**a**, $\times 100$; **b**, $\times 225$). HAR-ND separated from lymph vessels rolled up rapidly (**c**, $\times 225$). **(B)** Intravenous HAR-NDs are shown as a blue line inside a lumbar vein (arrowhead). Yellow broken lines indicate the boundary of a vein (**a**, $\times 100$). The HAR-NDs were exposed by draining the blood (**b**, $\times 150$). Example of a HAR-N is shown (**c**, $\times 100$). BV, blood vessel; HAR-N, hyaluronic acid-rich node; HAR-ND, hyaluronic acid-rich node(s) and duct(s); HAR-NDS, hyaluronic acid-rich node and duct system; LV, lymph vessel.

lineage marker expression (Fig. 2A-c). Next, we displayed the cells according to their CD45 expression levels. Eventually, we obtained a rare fraction of small cells with Sca-1⁺Lin⁻CD45⁻ characteristics both from the BM and the HAR-NDS (Fig. 2A-d).

VSELs accounted for $\sim 0.02\%$ of total BM cells, while the VSEL-like cells accounted for $\sim 2.32\%$ of total HAR-NDS cells. In other words, the intravascular HAR-ND contained approximately 100-fold more VSEL-like cells than the BM (Fig. 2B). When we counted the fluorescence-activated cells sorted from ~ 1 million input cells, there were $\sim 1,200$ BM VSELs compared with $\sim 25,000$ VSEL-like cells from the HAR-NDS, showing that about 20-fold more VSEL-like cells could be obtained from the HAR-NDS than from the BM (Fig. 2C). We named the HAR-NDS-derived cells “node and duct stem cells (NDSCs)” because they were enriched in the HAR-ND inside lymphatic and blood vessels. The majority of the purified NDSCs were negative for both 7AAD and annexin V, indicating that they were not undergoing apoptosis [24] (Fig. 2D).

Electron microscopic morphology of NDSCs

SEM revealed that NDSCs were round and small ($3.5\text{--}4.5\text{ }\mu\text{m}$ in diameter) (Fig. 3A-a-d). TEM, showed that they contained a large nucleus with a narrow rim of cytoplasm (Fig. 3B-a). We also identified a prominent nucleolus (Fig. 3B-b), nuclear membrane (Fig. 3B-c), heterochromatin and euchromatin (Fig. 3B-d), mitochondria and vacuoles (Fig. 3B-e), and an endoplasmic reticulum with scattered ribosomes (Fig. 3B-f).

In vitro expansion of NDSCs

VSELs and NDSCs were cocultured on a C₂C₁₂ murine myoblast feeder layer. Both cell types formed spheres re-

sembling embryoid bodies (Fig. 4A) that were positive for fetal AP, indicating that they might contain pluripotent stem cells (Fig. 4B). We compared the efficiency with which the two cell types formed spheres and found that the NDSCs produced ~ 176 spheres per 1,000 cells, compared with ~ 14 spheres per 1,000 cells for the VSEL; thus, NDSCs were approximately 12.5-fold more efficient than VSELs at forming spheres (Fig. 4C). To examine their proliferative potentials, we replated the sphere-forming cells on C₂C₁₂ feeders every 7 days and counted fetal AP-positive spheres. The number of AP-positive spheres derived from the NDSCs increased after repeated replating, while the number of VSEL-derived spheres remained almost constant (Fig. 4D-a, b).

NDSCs express pluripotent stem cell markers

Using reverse transcriptase-PCR and western blotting, we investigated whether VSELs and NDSCs expressed the pluripotent stem cell markers such as Oct4, Sox2, SSEA-1, and Nanog. NDSCs were found to express both mRNA and protein corresponding to all three markers, at levels similar to those found in a murine embryonic stem cell line (ES-D3). However, VSELs expressed only Oct4 and Nanog, but not Sox2 (Fig. 5A-a, b). In parallel colony-staining experiments, we confirmed that NDSC spheres were positive for Oct4, Sox2, Nanog, and SSEA-1, while VSELs were positive for Oct4, Nanog, and SSEA-1, but negative for Sox2 (Fig. 5B).

NDSCs differentiate into neuronal cells in vitro

We investigated whether NDSCs differentiated into neuronal cells under in vitro conditions. As before, we performed the same experiments on VSELs for comparison. Both NDSCs and VSELs differentiated into NeuN-positive,

FIG. 2. Purification of VSELs from the BM and NDSC from the HAR-NDS. **(A)** FACS of VSELs and NDSCs. **(a)** Granular events between 2–5 μm in diameter were assigned to gate R1 after comparison with beads of a predefined size (Invitrogen). **(b)** Nucleated cells from BM and HAR-ND were visualized by dot plots representing FSC versus SSC signals, related to the size and complexity of the cells, respectively. **(c)** Cells from region R1 were further analyzed for expression of Sca-1 and Lin markers. The population of Sca-1⁺/Lin⁻ objects was included in region R2. **(d)** Cells from the BM and HAR-ND in region R2 were subsequently sorted into CD45⁻ (R3) and CD45⁺ (R4) subpopulations. Sca-1⁺/Lin⁻/CD45⁻ cells (VSELs from BM, NDSC from HAR-ND) were sorted as cells from regions R1, R2, and R3, while Sca-1⁺/Lin⁻/CD45⁺ cells (HSCs) were from R1, R2, and R4. Percentages show the average number in each cell population ($\pm$ standard error of the mean) among total BM or HAR-ND nucleated cells. **(B)** Frequency of small Sca-1⁺Lin⁻CD45⁻ cells in BM and HAR-ND. Percentages of Sca-1⁺Lin⁻CD45⁻ (2–5 μm in diameter) cells among total nucleated BM and HAR-ND input cells were calculated in 10 independent experiments. Horizontal bars indicate mean $\pm$ standard error of the mean. $*P < 0.05$. **(C)** Small Sca-1⁺Lin⁻CD45⁻ cells recovered by FACS. When we sampled approximately 1×10^6 BM or HAR-ND cells for FACS, we recovered the numbers of Sca-1⁺Lin⁻CD45⁻ cells shown in the graph. These cells were used in further experiments. $*P < 0.05$. **(D)** Frequency of apoptotic Sca-1⁺Lin⁻CD45⁻ cells. FACS-sorted Sca-1⁺Lin⁻CD45⁻ (2–5 μm in diameter) cells from HAR-ND were stained with 7-AAD and annexin V. The numbers of NDSCs negative for 7-AAD and annexin V were measured by flow cytometry. Numbers in each panel represent percent values ($\pm$ SD) from three independent experiments. Fifteen mice were used in each experiment. BM, bone marrow; FACS, fluorescence-activated cell sorting; FSC, forward scatter; Lin, lineage; NDSC, node and duct stem cell; SSC, side scatter; VSEL, very small embryonic-like stem cell.

MAP-2-positive neuronal cells with dendrites, when cultured under neuronal differentiation conditions for 25 days (Fig. 6A-a, b). As in the case of ES-D3 cells, neither VSELs nor NDSCs were found to express the neuronal cell markers such as GFAP, nestin, and β -III tubulin (Fig. 6B). However, the VSEL- and NDSC-derived neuronal cells expressed all of those markers at both mRNA and protein levels (Fig. 6C).

Transplantation of NDSCs into mice partially repairs ischemic brain injury

We induced cerebral hypoxic ischemia by permanent unilateral ligation of the right common carotid artery, a short period of systemic hypoxia (8% oxygen for 20 min), and heat stroke. This model generated cell death in the cortex, striatum, and hippocampus in the hemisphere contralateral and ipsilateral to the ligation. We intravenously injected $\sim 1 \times 10^3$ NDSCs labeled with CM-DiI into the ischemic mice. After 5

weeks we removed the brains to visualize the infarct area by TTC staining (Fig. 7A-a) and to determine its volume. In the NDSC injection group, infarct volume was significantly smaller (15.8%) than in the PBS group (47.5%) (Fig. 7A, b) and CM-DiI-positive cells were detected in the dentate gyrus (DG) of the hippocampus (Fig. 7B-a). Some of the DiI⁺ cells also expressed the NeuN marker and had spread throughout the ischemic brain, indicating that the NDSCs were undergoing neuronal differentiation (Fig. 7B-b). CM-DiI⁺ cells were also detected in the cornu ammonis 1 (CA1) and cornu ammonis 3 (CA3) areas of the hippocampus in the ischemic mice. CM-DiI⁺ cells in these regions expressed NeuN weakly, suggesting that they might be in process of differentiating into neuronal cells (Fig. 7C-a, b).

Discussion

In this study we present evidence for the existence of a new anatomical structure that carries adult pluripotent

FIG. 3. SEM and TEM imaging of NDSCs. **(A)** SEM images of NDSCs purified by FACS (**a–d**). A scale bar is included in each figure and the estimated diameter of each cell is indicated. **(B)** TEM pictures of purified NDSCs. **(a)** Whole cell, nucleus, and cytoplasm ($\times 25,000$). **(b)** Nucleolus. **(c)** Nuclear membrane. **(d)** Euchromatin and heterochromatin. **(e)** Mitochondria and vacuoles. **(f)** Endoplasmic reticulum and ribosomes (**b–f**, $\times 150,000$). The scale bar represents 1 μm in the central figure and 200 nm in (**b–f**). SEM, scanning electron microscopy; TEM, transmission electron microscopy.

stem cells (Fig. 1). The name intravascular “node and duct stem cells (NDSCs)” reflects the locations of these cells. Elements of the intravascular HAR-ND are invisible without staining because typically they are very thin and transparent. The diameter of the intralymphatic HAR-ND was 15–20 times smaller than the diameter of the sur-

rounding lymphatic vessels [15]. The HAR-ND diameter became artificially enlarged when alcian blue was injected (Fig. 1).

NDSCs and VSELs possess similar properties, in that both are small, with immature morphologies in the EM, express stem cell markers, form spheres, and have the potential to

FIG. 4. Sphere formation from BM-derived VSEL and HAR-ND-derived NDSCs. **(A)** VSELs and NDSCs were co-cultured on C₂C₁₂ feeder cells. Spheres of similar morphologies formed from both VSELs and NDSCs. The image shown was taken on day 7 of coculture. Magnification is shown in each panel. **(B)** VSEL- and NDSC-derived spheres expressed fetal alkaline phosphatase (AP). Magnification is shown in each panel. **(C)** Frequency of sphere formation. Numbers of spheres were counted on day 7 after 1×10^3 VSELs or NDSCs were plated on C₂C₁₂ cells. * $P < 0.05$. **(D)** In vitro expansion of NDSCs. VSELs (**a**) and NDSCs (**b**) were replated every 7 days on C₂C₁₂ cells. Numbers of fetal AP-positive and -negative colonies were counted. * $P < 0.05$.

FIG. 5. NDSC spheres express pluripotent stem cell markers. (A) RT-PCR and western blot analysis. Expression of Oct-4, Sox-2, and Nanog was determined in spheres derived from embryonic stem cells (ES, VSEL, and NDSC) and from C₂C₁₂ cells by RT-PCR (a) and western blotting (b). GAPDH was used to control for equal loading. Numbers on right side indicate size markers. (B) Immunostaining of NDSC- and VSEL-derived spheres. Differential interference contrast images show the morphologies of the spheres, and white broken lines indicate sphere boundaries (a). Spheres were stained with antibodies against Oct-4, Sox-2, Nanog, and SSEA-1 (c; red) and with DAPI (b; blue). The fourth row (d) of each panel is a merged image of (b) and (c). Scale bar: 100 μ m. DAPI, 4',6-diamidino-2-phenylindole; RT-PCR, reverse transcriptase-polymerase chain reaction.

FIG. 6. Neuronal differentiation of VSELs and NDSCs. (A) VSEL- (a) and NDSC-derived (b) spheres were mechanically dissociated into single cells and cultured in neuronal differentiation medium for 25 days. Expression of neuron-specific nuclear proteins, NeuN (red) and microtubule-associated protein-2 (MAP-2, green), is shown. Nuclei were stained with DAPI (blue). Merge images (bottom right corner, both panels) include NeuN, MAP-2, and DAPI images. These figures are representative of at least three independent experiments. Magnification: $\times 100$. (B) Expression of neuronal markers in undifferentiated ES, VSEL, NDSC, and mouse brain cells. Results of RT-PCR (left panel) and western blotting (right panel) for GFAP (early marker of neuronal differentiation), Nestin, and β -III tubulin (both late markers of neuronal differentiation) are shown. GAPDH was used to control for equal loading. Numbers on right side indicate size markers. (C) Expression of neuronal markers in VSEL and NDSC cells induced to differentiate. Results of RT-PCR (left panel) and western blotting (right panel) for GFAP, Nestin, and β -III tubulin are shown. ES-D3 cells are undifferentiated. Mouse brain cells were used as positive controls. GAPDH was used to control for equal loading. Numbers on right side indicate size markers.

FIG. 7. NDSC transplantation in a mouse ischemic brain injury model. (A) Ischemic brain injury was induced in ICR mice. CM-Dil-labeled NDSCs or PBS were injected through the tail vein. Mouse brains were stained with 2,3,5-triphenyltetrazolium chloride (TTC) to reveal the infarct area (arrows) (a). Magnification: $\times 14$. Infarct volume was calculated as percentages of the contralateral and ipsilateral hemispheres (b). The figures are representative of three independent experiments. $*P < 0.05$. (B) Transplanted NDSCs (red) are present in the dentate gyrus (DG) of the hippocampus. Brain sections of the DG are shown (a). NeuN-positive (green), DiI-positive (red) cells can be seen in the DG. Some NeuN-positive, DiI-positive, and DAPI-positive cells are detected in the DG (b). (C) Transplanted NDSCs (red) can be seen in areas CA1 and CA3 of the hippocampus. DiI-positive cells are present in both the CA1 (a) and CA3 (b) areas. Magnifications are shown in each panel. The figures are representative of three independent experiments. CA1, cornu ammonis 1; CA3, cornu ammonis 3; PBS, phosphate-buffered saline.

differentiate into neuronal cells (Figs. 2 and 3). NDSCs, however, differ from VSELs in that NDSCs have a higher replating efficiency (Figs. 4D and 5). Another difference is that NDSCs strongly express Sox2, while VSELs do not express it at all. The absence of Sox2 in VSEL may be due to its heterogeneous nature. Shin et al. [25] prepared several Oct4-positive cDNA libraries from 20 BM-sorted Sca-1⁺Lin⁻CD45⁻ VSELs and reported that these libraries differ in terms of the expression of some crucial stemness genes. They identified three patterns of gene expression: (i) the first group expresses both stemness (eg, Oct4, Sox2, or Nanog) and germ line markers (eg, Stella and Prdm14), (ii) the second group expresses only stemness genes (eg, Oct4, Sox2, or Nanog), and lacks germ line markers (eg, Stella and Prdm14), and (iii) the third group expresses Oct4 and lacks several genes that are present in other VSELs. Hence, in this study absence of Sox2 in VSEL is a minor variation or may be due to cellular heterogeneity.

An intriguing finding is that the HAR-NDS is enriched almost 100-fold more in NDSCs than the BM is in VSELs. If NDSCs and VSELs belong to the same type of stem cells (ie, VSEL-like stem cells), the HAR-NDS appears to be the main site where they perform their biological functions, and may be the system responsible for transporting VSEL-like adult stem cells to their target tissues.

Two different surface appearances of NDSCs in SEM (Fig. 3A) seem to be due to a procedural artefact in the steps of cell fixation of sample preparation. We do not know whether NDSCs circulates only through HAR-NDS or they also can be detected in the peripheral blood. Because we can identify HAR-ND within both veins and arteries, our current hypothesis is that NDSC circulates only through HAR-NDS. This remains to be determined.

We demonstrated that NDSCs could differentiate into neuronal cells (Fig. 6), with an efficiency similar to that of VSELs in vitro. To take this line of inquiry further, it may

be worth investigating whether NDSCs can differentiate into cell lineages characteristic of all three germ layers, as previously demonstrated for VSELs [7].

Ratajczak et al. [26,27] reported the presence of VSELs in the major organs of adult mice. In the light of our results, their findings are intriguing. The HAR-NDS is thought to form a network that spreads throughout the body including organ surfaces, inside the organs, under the skin, inside the lymphatic and blood vessels, and along the nervous system [14]. Therefore, the VSELs demonstrated by Ratajczak et al. could be the NDSCs residing in the HAR-NDS within those organs.

When we injected CM-DiI-labeled NDSCs into the tail veins of mice with brain ischemia, the cells were observed in the CA1, CA3, and DG regions of the damaged hippocampus. We also found evidence of NDSC differentiation in the same regions of the hippocampus. Other investigators have shown that DiI-labeled stem cells can differentiate into neuronal cells in the brain [28–30]. As indicated by the smaller infarct volume in the NDSC-treated mice (Fig. 7A-a, b), NDSCs may have therapeutic value in brain injury. If further studies confirm that NDSCs are a type of pluripotent adult stem cells, the HAR-NDS may prove to be an important pathway that delivers stem cells to areas of damaged tissue.

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Author Disclosure Statement

The authors have no conflicts of interest to disclose.

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