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Perivascular Mesenchymal Stem Cells From the Adult Human Brain Harbor No Intrinsic Neuroectodermal but High Mesodermal Differentiation Potential

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ABSTRACT

Brain perivascular cells have recently been identified as a novel mesodermal cell type in the human brain. These cells reside in the perivascular niche and were shown to have mesodermal and, to a lesser extent, tissue-specific differentiation potential. Mesenchymal stem cells (MSCs) are widely proposed for use in cell therapy in many neurological disorders; therefore, it is of importance to better understand the “intrinsic” MSC population of the human brain. We systematically characterized adult human brain-derived pericytes during *in vitro* expansion and differentiation and compared these cells with fetal and adult human brain-derived neural stem cells (NSCs) and adult human bone marrow-derived MSCs. We found that adult human brain pericytes, which can be isolated from the hippocampus and from subcortical white matter, are—in contrast to adult human NSCs—easily expandable in monolayer cultures and show many similarities to human bone marrow-derived MSCs both regarding both surface marker expression and after whole transcriptome profile. Human brain pericytes showed a negligible propensity for neuroectodermal differentiation under various differentiation conditions but efficiently generated mesodermal progeny. Consequently, human brain pericytes resemble bone marrow-derived MSCs and might be very interesting for possible autologous and endogenous stem cell-based treatment strategies and cell therapeutic approaches for treating neurological diseases. *STEM CELLS TRANSLATIONAL MEDICINE* 2015;4:1223–1233

SIGNIFICANCE

Perivascular mesenchymal stem cells (MSCs) recently gained significant interest because of their appearance in many tissues including the human brain. MSCs were often reported as being beneficial after transplantation in the central nervous system in different neurological diseases; therefore, adult brain perivascular cells derived from human neural tissue were systematically characterized concerning neural stem cell and MSC marker expression, transcriptomics, and mesodermal and inherent neuroectodermal differentiation potential *in vitro* and *in vivo* after *in utero* transplantation. This study showed the lack of an innate neuronal but high mesodermal differentiation potential. Because of their relationship to mesenchymal stem cells, these adult brain perivascular mesodermal cells are of great interest for possible autologous therapeutic use.

INTRODUCTION

Mesenchymal stem cells (MSCs) have received much interest regarding cell therapeutic approaches to treat neurological diseases because these cells are readily available for autologous cell therapy [1–3]. Several models of brain diseases have been investigated as potential targets including ischemic stroke, various neurodegenerative diseases, and brain tumors. MSCs transplanted into the brain have been demonstrated to migrate to the sites of brain lesions and

to promote functional recovery, the latter most likely due to their ability to produce trophic factors inducing survival and regeneration of host neurons (reviewed in [1–3]). The neuroectodermal differentiation potential of MSCs themselves, without genetically engineering the MSCs, seems to be rather limited [4–8]. Consequently, MSCs may be valuable for disease modulation, including trophic factor release, and immunomodulatory effects and as cellular vehicles for gene therapeutic approaches [1–3].

Although neural stem cells (NSCs) are also known to provide trophic factor support and to show high homing ability after transplantation [9], the first data of disease transmission to transplanted neuronal tissue limit their application [10, 11]. Pathological aggregates of brain diseases are not reported in non-neuronal tissue, thus non-neuronal cells might be better suited for cell therapies.

The identification of an MSC population originating from the perivascular space in multiple organs, including the brain, fosters additional interest in mesodermal cells [12]. Recently, such perivascular MSCs, or “fibroblast-like cells,” were also described in the adult human brain [13, 14]. Both reports described fibroblast-like cells deriving from the perivascular niche as harboring long-term expandability and mesodermal differentiation potential [13, 14]. Paul et al. noted no coexpression of neuro-precursor markers during expansion but neuronal differentiation potential of these MSCs [14], whereas Park et al. reported coexpression of neuroprecursor markers but a lack of neuronal differentiation potential [13]. Karow et al. recently reported the induction of fully mature neurons from human pericytes by overexpression of *SOX2* and *MASH1* [15, 16], supporting the interest in these cells for regenerative approaches to various neurological diseases. Nonetheless, proper characterization of these adult brain perivascular mesodermal cells (aBPMCs) and systematic comparison with human adult neuroprogenitor cells (aNPCs) is still lacking with regard not only to selected genes and proteins but also to whole transcriptomes and their innate neuroectodermal potential.

Although brain pericytes have long been known to play a major role in blood-brain-barrier function and dysfunction, their role in and potential for regeneration in neuronal diseases is limited [17]. Interestingly, a very recent report noted an increase of platelet-derived growth factor receptor beta-positive (PDGFR β +) cells in patients with temporal lobe epilepsy [18], suggesting that these cells might be activated for endogenous repair strategies.

In this study, we systematically characterized adult brain perivascular mesodermal cells (aBPMCs) derived from human adult neural tissue isolated from two different brain regions, the subcortical white matter and the hippocampus, concerning NSC/MSc marker expression and their mesodermal and inherent neuroectodermal differentiation potential in vitro and in vivo after in utero transplantation. Finally, we differentially characterized the whole transcriptome of human aBPMCs with human bone marrow-derived MSCs and human aNPCs and fetal human NSCs.

MATERIALS AND METHODS

Isolation and Propagation of Multipotent NPCs and BPMCs

Adult human hippocampal and subcortical white matter tissue was obtained from routine epilepsy surgery procedures following informed consent of the donors. All procedures were in accordance with the Helsinki convention and approved by the ethics committee of the University of Dresden (no. 47032006). All subjects underwent high-resolution magnetic resonance imaging excluding tumors and were screened for the presence of infectious disease. In all cases, the neuropathological examinations did not reveal evidence of tumor formation. Demographic

details of all donors used in this study, including diagnosis and anti-epileptic drug therapy, are given in Table 1. Of note, single experiments were not performed with aBPMCs from all 10 donors; we wanted to perform the experiments on similar passages, and there were not always enough cells for all experiments from one patient. Data were pooled from different donors because we aimed for biological replicates rather than technical replicates.

Tissue was taken and stored in ice-cold DPBS containing 10% glucose and 1% penicillin/streptomycin for transport (<1 hour). Tissue samples were cut into small pieces, incubated in trypsin (2.5 mg/ml; Sigma-Aldrich, St Louis, MO, <https://www.sigmaaldrich.com>) for 20 minutes at room temperature, incubated in DNase (40 μ g/ml; Sigma-Aldrich) for 15 minutes at 37°C, and homogenized to a quasi-single-cell suspension by gentle triturating. For expansion of neurospheres (aNPCs) or monolayer cultures (aBPMCs), the cells were either added to 25-cm² flasks for suspension cultures or seeded at a concentration of 35,000 cells/ml onto gelatin-coated 24-well plates in Knock-Out DMEM (Gibco BRL, Life Technologies; Thermo Fisher Scientific, Waltham, MA, <http://www.lifetechnologies.com/us/en/home.html>), supplemented with 10% serum replacement, 2% B-27 supplement, 1% GlutaMAX, 1% penicillin/streptomycin (all from Gibco), and 20 ng/ml of both EGF and FGF2 (both from Sigma-Aldrich) at 5% CO₂, 92% N₂, and 3% O₂, using an incubator equipped with an O₂-sensitive electrode system (Heraeus, Hanau, Germany, <http://www.heraeus.com>). Details of aNPC culture conditions and characterization are provided in [8, 19–22]. Neurospheres were cultured as previously described [8, 19–22]. For the monolayer cultures, medium was changed by only 50% during the first 2 weeks. Medium was routinely changed twice a week, and confluent cells were passaged 1:2 with Accutase (PAA Laboratories GmbH, Pasching, Germany, <http://www.atmp-ready.com>). All experiments were performed with cells from the second passage. Additional expansion protocols for the adherent human adult NPCs culture system are listed in supplemental online Table 1.

Human fetal NSCs were generated as described previously [23].

Differentiation

Adherent BPMCs were plated onto gelatin coated dishes in expansion medium without any mitogens. Further protocols included different media compositions, mitogens, and culturing surfaces. Details are shown in supplemental online Table 2.

Neurosphere cultures of aNPCs were plated onto poly-D-lysine chamber slides in expansion medium. Induction of differentiation of fetal NSCs was initiated by plating them onto poly-L-ornithine and fibronectin-coated dishes in neurobasal medium (Gibco) containing, 2% B-27 supplement, 1% GlutaMAX, 1% penicillin/streptomycin, 10 μ M forskolin (Sigma-Aldrich), and 100 μ M cAMP (Sigma-Aldrich). Cells were differentiated for 14 days prior to immunocytochemical analysis.

Fluorescence-Activated Cell Sorting

Flow cytometric analysis was performed with a FACSCalibur (BD Biosciences, Heidelberg, Germany, <http://www.bdbiosciences.com>) using BD CellQuest Pro and FlowJo software. At subconfluence, cells trypsinized and 3×10^5 cells were stained for CD29-PE

Table 1. Donor characteristics

| No. | Age (years) | Sex | Donor tissue type | Diagnosis/indication/surgery | Antiepileptic medication (daily dose) |
|-----|-------------|--------|---------------------------|--|---------------------------------------|
| 1 | 38 | Male | White matter | Angioma | LEV 1,500 mg |
| 2 | 49 | Female | White matter | AHS | LTG 500 mg |
| 3 | 7 | Female | White matter | FCD 2 | OXC 720 mg, TPM 200 mg, LTG 200 mg |
| 4 | 53 | Male | White matter | Astrogliosis, residues from brain bleeding | LTG 300 mg |
| 5 | 33 | Male | White matter | Cavernoma | LEV 2,000 mg |
| 6 | 61 | Female | White matter | Cavernoma | TPM 300 mg, PHT 225 mg, OXC 1,200 mg |
| 7 | 55 | Female | White matter, Hippocampus | FCD 1 + 2 or anterior temporal lobectomy and AHE | LTG 400 mg |
| 8 | 29 | Male | White matter, Hippocampus | FCD 1b | OXC 2,100 mg, LCM 400 mg |
| 9 | 49 | Male | Hippocampus | AHS, FCD 1 + 2 | LCM 500 mg, LEV 1500 mg |
| 10 | 32 | Male | Hippocampus | AHS, FCD 2 | LTG 350 mg |
| 11 | 50 | Male | Hippocampus | AHS, FCD 1 | LCM 300 mg, LEV 3,500 mg |
| 12 | 44 | Male | Hippocampus | Cavernoma | LTG 300 mg |
| 13 | 20 | Female | Hippocampus | AHS, FCD 1 | LEV 3,000 mg |
| 14 | 58 | Female | Hippocampus | AHS, MCD 1 + 2 | LEV 4,000 mg |
| 15 | 27 | Female | White matter | MCD 1 + 2, FCD | OXC 1,200 mg |
| 16 | 46 | Female | White matter | FCD 2 | OXC 2,850 mg |
| 17 | 23 | Male | Hippocampus | DNET 1° | OXC 2,400 mg |
| 18 | 56 | Female | Hippocampus | AHS, FCD 1 + 2 | LEV 3,500 mg |

Depicted are characteristics of all donors from which aBPMCs were derived and used in this study. Details of donors of adult neuroprogenitor cells are given in Lojewski et al. [21]. FCD is classified according to Blümcke et al. [35].

Abbreviations: AHE, amygdalohippocampectomy; AHS, Ammon's horn sclerosis; DNET, dysembryoplastic neuroepithelial tumor; FCD, focal cortical dysplasia; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; MCD, mild cortical dysplasia; OXC, oxcarbazepine; PHT, phenytoin; TPM, topiramate.

(BD Biosciences), CD73-PE (BD Biosciences), CD90-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany, <http://www.miltenyibiotec.com>), CD105-FITC (AbD Serotec; Bio-Rad Laboratories, Inc., Hercules, CA, <https://www.abdserotec.com>), CD146-APC (Miltenyi Biotec), and CD166-PE (BD Biosciences) at 1:100 each.

Mesodermal Differentiation

To determine trilineage mesodermal differentiation potential, aBPMCs from human hippocampus and subcortical white matter were differentiated into the osteogenic, adipogenic, and chondrogenic cell fates.

Osteogenic Differentiation and Analyses

Cells were plated in 6-well plates at a density of 5×10^3 cells per cm^2 in triplicate and cultured in expansion medium under hypoxia. Osteogenic differentiation was initiated at 60%–80% confluence with osteogenic supplements containing 100 nM dexamethasone, 10 mM β -glycerophosphate, and 50 μM ascorbic acid-2-phosphate (Sigma-Aldrich) in Dulbecco's modified Eagle's medium (DMEM; low glucose, Life Technologies; Thermo Fisher Scientific) supplemented with 10% fetal calf serum (FCS; Biochrom, Cambridge, UK, <http://www.biochrom.co.uk>) and 1% penicillin/streptomycin (Life Technologies; Thermo Fisher Scientific) under normoxic conditions for up to 21 days. Medium was changed three times a week. Undifferentiated cells cultured in basic medium without osteogenic supplements served as control.

After 14 days of differentiation, cells were cytochemically stained for the osteogenic marker alkaline phosphatase (ALP) with the leukocyte ALP staining kit (Sigma-Aldrich), according

to the manufacturer's instructions. In addition, matrix mineralization was determined after 21 days by alizarin red staining (Sigma-Aldrich). Briefly, cells were fixed for 10 minutes with 70% ice-cold ethanol, washed, and stained with alizarin red solution (40 mM in H_2O) for 10 minutes at room temperature. Images were taken by a Sony NEX-5 (Sony, Tokyo, Japan, <http://www.sony.net>) connected to a light microscope (Zeiss, Jena, Germany, <http://www.zeiss.com>). To quantify the amount of stained Ca^{2+} , cells were destained with 20% methanol and 10% acetic acid in distilled H_2O for 15 minutes. Lysate was centrifuged at full speed for 1 minute, and absorption was measured at 450 nm of 200 μl in triplicate in a 96-well plate by a plate reader (Sunrise; Tecan, Crailsheim, Germany, <http://www.tecan.com>).

Adipogenic Differentiation and Analyses

Cells were plated in 6-well plates at a density of 1×10^4 cells per cm^2 in triplicate and cultured in expansion medium under hypoxia. Adipogenic differentiation was initiated at 100% confluence with 1 μM dexamethason, 0.5 mM 3-isobutyl-1-methylxanthin (IBMX), 100 μM indomethacin, and 1 $\mu\text{g/ml}$ insulin (Sigma-Aldrich) in DMEM-low glucose (Life Technologies; Thermo Fisher Scientific) supplemented with 10% FCS (Biochrom) and 1% penicillin/streptomycin (Life Technologies; Thermo Fisher Scientific) under normoxic conditions for 21 days. Medium was changed three times a week. Undifferentiated cells cultured in basic medium served as control.

After 21 days, Oil Red O staining was performed to detect whether lipid droplets formed. Cells were washed with phosphate-buffered saline (PBS) and fixed for 20 minutes with 1%

paraformaldehyde (PFA) at room temperature. After two washing steps with distilled H₂O, cells were incubated for 3 minutes in 100% propylene glycol (Carl Roth, Karlsruhe, Germany, <https://www.carlroth.com>) and stained for 10 minutes at 60°C with pre-warmed 0.5% Oil Red O solution (Sigma-Aldrich) in propylene glycol. Afterward, cells were incubated at room temperature for 5 minutes with 1.5 ml of 85% propylene glycol and washed twice with distilled H₂O before analyzing the samples using an upright light microscope (Zeiss).

Chondrogenic Differentiation and Analyses

For chondrogenic differentiation, cells were cultured in high-density pellets, as described by Johnstone et al. [24]. Briefly, 1.2×10^6 cells were pelleted at 400g for 10 minutes in 15-ml Falcon tubes (Greiner, Frickenhausen, Germany, https://www.gbo.com/de_DE.html) and cultured for 14 days in 1 ml of DMEM-high glucose (Life Technologies; Thermo Fisher Scientific) supplemented with 1% penicillin/streptomycin (Life Technologies; Thermo Fisher Scientific), 1% insulin–transferrin–selenium (ITS-X; Life Technologies; Thermo Fisher Scientific), 10 ng/ml human transforming growth factor β 3 (Miltenyi Biotec), 100 nM dexamethasone (Sigma-Aldrich), and 130 μ M ascorbic acid (Sigma-Aldrich) under normoxic conditions. Caps of the tubes were not entirely closed to allow gas exchange. Medium was changed three times a week.

After 14 days, pellets were analyzed immunohistologically and stained for chondrogenic markers. Pellets were washed with PBS and fixed for 10 minutes in 10 ml 1.5% PFA at room temperature. After dehydration, pellets were embedded in paraffin, sectioned (2- μ m thickness), and stained with hematoxylin, Alcian blue (Sigma-Aldrich), COL1A1 (Clone: COL-1, number C2456, 1:200; Sigma-Aldrich), COL2A1 (mouse monoclonal antibody (Clone 2B1), 1:200; LabVision Corp., Fremont, CA), SOX9 (rabbit anti-Sox9 affinity-purified polyclonal antibody, 1:100; Chemicon International, Merck Millipore, Darmstadt, Germany, <http://www.merckmillipore.com>) COL10A1 (mouse monoclonal anti-collagen X, 1:500; Sigma-Aldrich). After detection with the appropriate biotinylated secondary antibody (ABC Standard Kit; Linaris, Dossenheim, Germany, <http://www.linaris.de>), the Romulin AEC Chromogen kit (Zytomed, Berlin, Germany, <http://www.zytomed-systems.com>) was used for staining. Images of stained slices were acquired in bright-field modus using a Keyence microscope (Keyence, Neu-Isenburg, Germany, <http://www.keyence.eu>) at $\times 40$, $\times 100$, $\times 200$, and $\times 400$ magnification.

Immunocytochemistry

Cell cultures were fixed with Accustain (Sigma-Aldrich Inc.) for 30 seconds, washed with PBS, and blocked for 2 hours with PBS containing 0.2% Triton-X 100 and 3% donkey serum. Cultures were incubated with primary antibodies overnight at 4°C followed by secondary fluorescence-conjugated antibodies for 1 hour at room temperature. Cell nuclei were counterstained with Hoechst 33342 (Invitrogen; Thermo Fisher Scientific). The following primary antibodies were used: mouse anti-A2B5 1:250, mouse anti-GalC 1:750, mouse anti-nestin 1:500, mouse anti-NG2 1:100, rabbit anti-NG2 1:200, rabbit anti-Olig2 1:500 (all from Merck Millipore); rabbit anti-CD133 1:500, rabbit anti-PDGFR α 1:500, chicken anti-glia fibrillary acidic protein (anti-GFAP) 1:1000, rabbit anti-S100A4 1:250 (all from Abcam,

Cambridge, UK, <http://www.abcam.com>); mouse anti-MAP2ab 1:400, mouse anti-PECAM 1:100 (both from BD Biosciences); mouse anti-Tuj1 1:500, rabbit anti- β -III tubulin 1:2,000 (both from Covance, Richmond, CA, <http://www.covance.com>); mouse anti-SOX2 1:500 (R&D Systems, Minneapolis, MN, <https://www.rndsystems.com>), goat anti-DCX 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, <http://www.scbt.com>), mouse anti- α -smooth muscle actin (anti- α -SMA; 1:100; Dako, Glostrup, Denmark, <http://www.dako.com>), rabbit anti-PDGFR β 1:100 (Cell Signaling Technology, Beverly, MA, <http://www.cellsignal.com>), and rabbit anti-Ki67 1:500 (Novocastra, Newcastle, U.K.). The secondary antibodies conjugated to Alexa 488, 555, 594, and 647 1:500 were obtained from Invitrogen (Thermo Fisher Scientific), and those conjugated to Rhodamine Red-X and DyLight 649 1:200 were obtained from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA, <https://www.jacksonimmuno.com>). Images were captured using the Leica DM IRE2 (Leica Biosystems Inc., Buffalo Grove, IL, <http://www.leicabiosystems.com>).

Cell Counting and Statistics

The number of cells immunoreactive for a given marker was determined by counting the number of positive cells in relation to the number of Hoechst-stained nuclei. In a typical experiment, 500–1,000 cells were counted within randomly selected fields per well. Statistical comparisons were made using the analysis of variance model and post hoc *t* test with Bonferroni adjustment, as appropriate. All data are presented as mean \pm SEM.

RNA Extraction and Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis

For gene expression analysis, a small piece of the sample was removed before starting the tissue preparation and immersed into RNAlater solution (Ambion; Thermo Fisher Scientific). Total tissue RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany, <https://www.qiagen.com>), according to the manufacturer's protocol. Total cellular RNA was extracted from human neural stem and progenitor cells during expansion using the RNeasy total RNA purification kit followed by treatment with RNase-free DNase (Qiagen). Quantitative real-time one-step reverse transcription polymerase chain reaction (RT-PCR) was carried out using the LightCycler System from Stratagene and the QuantiTect SYBR Green PCR kit from Qiagen. PCR conditions were as follows: 25 minutes at 50°C; 15 minutes at 95°C; 45 cycles of 15 seconds at 94°C, 20 seconds at 55°C, 30 seconds at 72°C, and 1 minute at 95°C; and 1 cycle of 30 seconds at 55°C. To demonstrate product specificity, melting curves of the RT-PCR products were acquired, and agarose gel electrophoresis was performed to confirm the product length. Primer sequences and lengths are available from the authors on request. The results are expressed relative to the housekeeping gene *HMBS* (hydroxymethylbilane synthase).

Gene Expression Microarray Analysis

For the gene expression microarray analysis, we used Affimetrix U133A chips containing 22,215 probe sets representing at least 12,905 individual genes. The whole procedure was performed following the manufacturer's standard protocol (Affimetrix, Santa Clara, CA, <http://www.affymetrix.com>). For data processing,

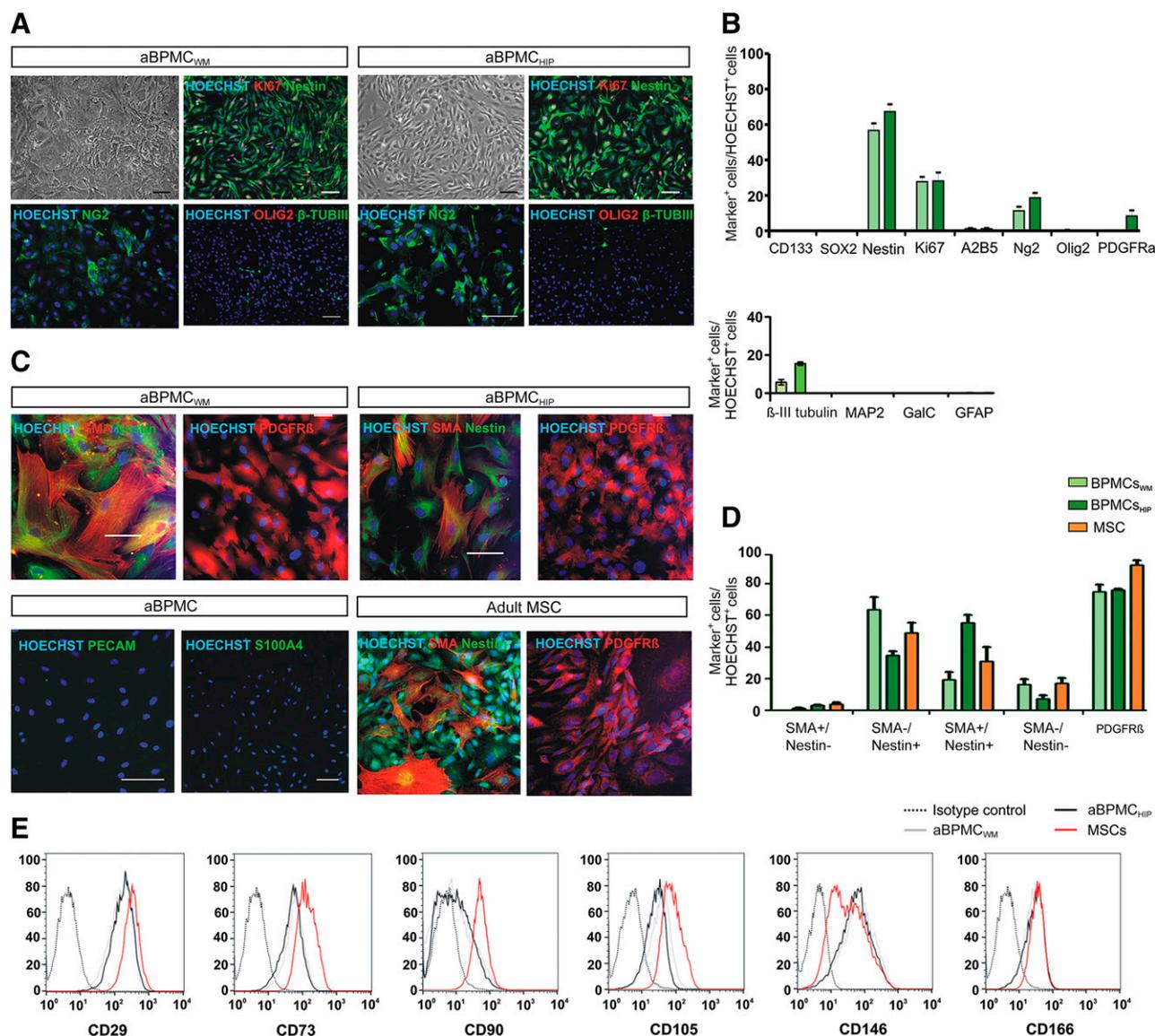


Figure 1. Expansion of human aBPMCs from white matter and hippocampal tissue. Human aBPMCs from white matter and hippocampus were cultured as monolayer for up to 4 weeks and stained for various marker proteins. Representative selection of stainings for typical neuroprogenitor or oligodendrocyte progenitor cells (**A**) and pericyte (**B**) marker proteins in aBPMc_{WM} and aBPMc_{HIP}. Nuclei were counterstained with Hoechst dye. Scale bars = 100 μ m. The only significant difference was in expression of β -tubulin, $p < .001$ compared with aBPMc_{WM} (**C, D**): Quantitative analysis of marker proteins. Results are mean \pm SEM from at least three independent experiments. One-way analysis of variance with post hoc Bonferroni adjustment was used to calculate the effects of cell types on marker-positive percentages in Nestin, α -SMA, and PDGFR- β stainings. There were no significant effects of cell types on SMA+/Nestin- ($F_2, 6 = 3.14, p = 0.116$) and SMA-/Nestin- ($F = 2.76, p = 0.141$) stainings (first and fourth bar sets). aBPMc_{WM} and aBPMc_{HIP} showed significant effects on SMA-/Nestin+ ($F = 5.69, p = .041$) and SMA+/Nestin+ stainings ($F = 7.86, p = .021$). aBPMc_{WM} showed significantly higher expression of SMA-/Nestin+ in comparison to aBPMc_{HIP} ($p = .045$), whereas SMA+/Nestin+ was higher in the latter ($p = .024$). Significantly higher expression of PDGFR- β was noticed in MSCs in comparison with aBPMc_{WM} as well as aBPMc_{HIP} ($F = 9.7, p = .013$), with differences from aBPMc_{WM} at $p = .023$ and from aBPMc_{HIP} at $p = .031$. No differences were observed within the aBPMc_{WM} and aBPMc_{HIP} for PDGFR- β . (**E**): Fluorescence-activated cell sorting analysis comparing aBPMc_{WM} and aBPMc_{HIP} with MSCs. Note the similarity of both aBPMc_{WM} to bone marrow-derived MSCs. Abbreviations: aBPMc_{WM}, adult brain perivascular mesodermal cells derived from cortical white matter; aBPMc_{HIP}, adult brain perivascular mesodermal cells derived from the hippocampus; MSC, mesenchymal stem cell; PDGFR, platelet-derived growth factor receptor; SMA, smooth muscle actin.

normalization was calculated with the GCRMA (GC content corrected robust multiarray analysis) algorithm [25]. Data post-processing and graphics was performed with functions developed in-house in Matlab (MathWorks Inc., Natick, MA, <http://www.mathworks.com>). Hierarchical clustering of genes and samples was performed with the one minus correlation metric and the

nonweighted average distance (UPGMA; also known as group average) linkage method. The gene ontology terms were taken from the AMIGO gene ontology database [26].

The data discussed in this publication have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (GEO) system [27] and are accessible through

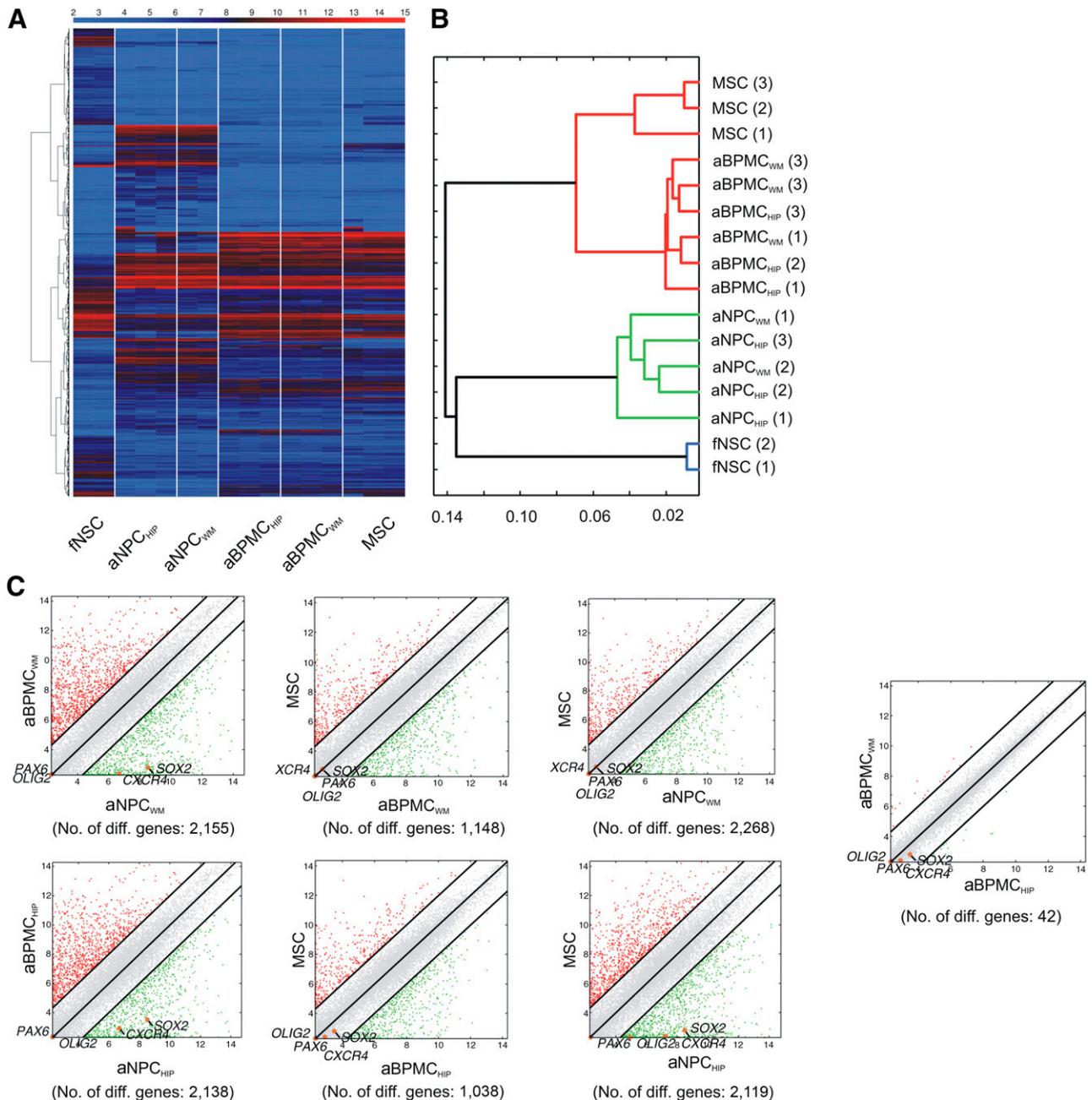


Figure 2. Transcriptome analysis of aBPMCs from white matter and hippocampal tissue compared with adult NPCs, fetal NSCs, and adult MSCs. **(A):** Heat map from microarray data demonstrating global gene expression pattern of white matter and hippocampal aBPMCs in comparison with adult white matter and hippocampal NPCs, fetal NSCs, and adult bone marrow-derived MSCs. The color bar at the top indicates gene expression in \log_2 scale. Red and blue represent higher and lower gene expression levels, respectively. **(B):** Hierarchical clustering of the cell lines based on the gene expression profiles in panel A. **(C):** Pairwise scatter plot analysis of the global gene expression profiles of aBPMCs, aNPCs, and MSCs. Black lines indicate boundaries of twofold difference in gene expression levels. The positions of some markers are shown as orange dots. Gene expression levels are depicted in \log_2 scale. The number of differentially expressed genes is indicated under each scatter plot. Abbreviations: aBPMCs, adult brain perivascular mesodermal cells; diff., different; fNSC, fetal neural stem cells; hip, derived from the hippocampus; MSC, mesenchymal stem cells; No., number; NPC, neuroprogenitor cells; wm, derived from cortical white matter.

GEO Series accession number GSE62505 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE62505>).

In Vivo Studies

Cells were dissociated and resuspended in DMEM Nutrient Mixture F-12 to a high-density single-cell suspension. Ultrasound imaging and guided microinjections were performed, as described

by Neumeister et al. [28]. Host embryos were collected 7 days after transplantation (embryonic day 17.5 [E 17.5]). Embryonic brains were isolated and fixed in 4% paraformaldehyde overnight. Dehydrated brains were snap frozen and coronally sectioned into 14- μ m slices using a cryostat (Leica). Immunohistochemical staining was performed in a humidified chamber. The slices were fixed on object slides, incubated for 1.5 hours in blocking

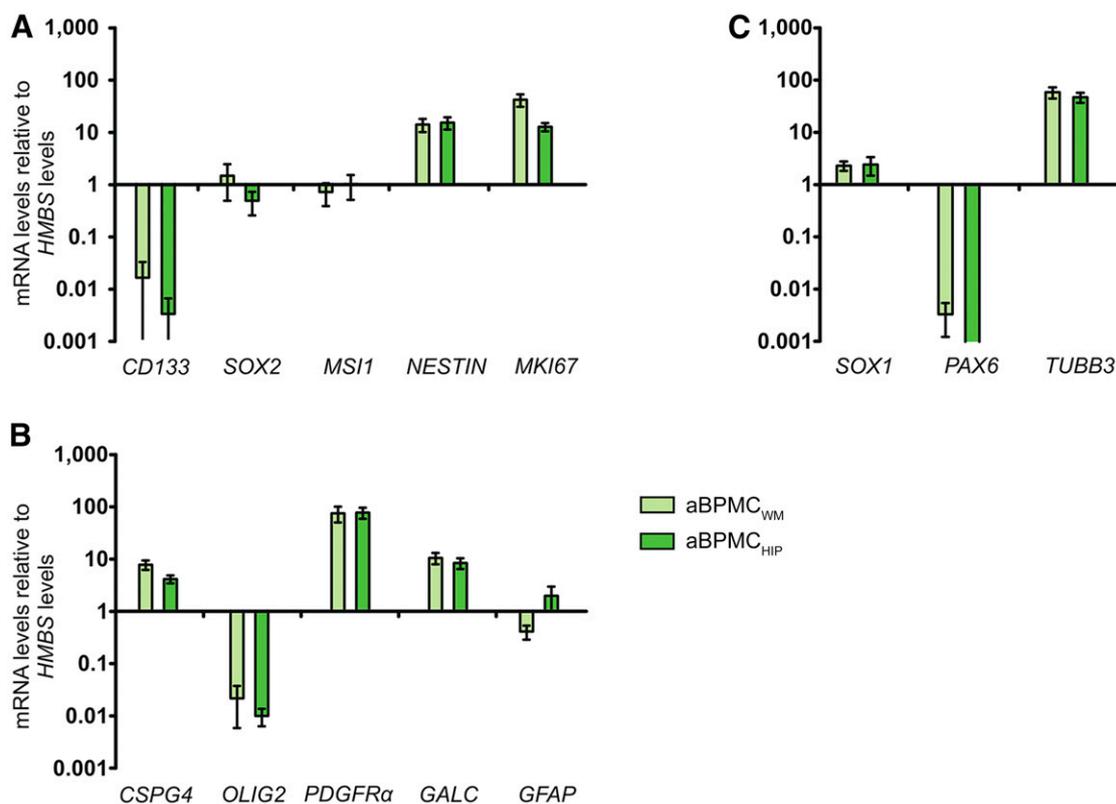


Figure 3. Quantitative reverse transcription polymerase chain reaction (qRT-PCR) characterization of aBPMCs from white matter and hippocampal tissue. Gene expression analysis of marker genes for neural stem/progenitor cells (**A**), glial lineage restricted cells (**B**), and neuronal restricted progenitor cells (**C**) in aBPMCs during expansion using qRT-PCR. Results are mean \pm SEM from at least three independent experiments. Abbreviations: aBPMC_{WM}, adult brain perivascular mesodermal cells derived from cortical white matter; aBPMC_{HIP}, adult brain perivascular mesodermal cells derived from the hippocampus.

solution (96.8% DPBS, 0.2% Triton-X 100, and 3% donkey serum), and immunostained at 4°C overnight by primary antibodies diluted as follows in blocking solution: Sox2 1:1,000 (ab97959, rabbit; Abcam), human nuclei 1:1,000 (MAB 1281, mouse; Merck Millipore), Olig2 1:500 (AB9610, rabbit; Merck Millipore), Dcx 1:400 (SC-8066, goat; Santa Cruz Biotechnology), and Map2 1:2,000 (Abcam, ab5392, chicken). Nuclei were stained by Hoechst dye. Images were collected by conventional (Leica DM IRE2) and confocal (Leica TSC SP5 I/II) fluorescence microscopy.

RESULTS

aBPMCs Express Markers of Neuroprogenitor Cells and Pericytes

Human aBPMCs cultured as monolayers were used to compare aBPMCs from two different brain regions, namely, from cortical white matter tissue (aBPMC_{WM}) and the hippocampus (aBPMC_{HIP}). All experiments were performed with cells at passage 2. Monolayer cultures were analyzed at the protein and mRNA expression levels (Figs. 1–3). We initially analyzed protein expression of established neuroprogenitor, oligodendrocyte progenitor, and neuroectodermal differentiation markers. More than 50% of the cells were positive for nestin (57% \pm 10% aBPMC_{WM}; 67% \pm 11% aBPMC_{HIP}; $n = 6$), and \sim 25% expressed the proliferation marker Ki67 (27% \pm 7% aBPMC_{WM}; 28% \pm 13% aBPMC_{HIP}; $n = 7$) (Fig. 1A, 1B). Next we analyzed the oligodendrocyte progenitor markers A2B5, NG2, OLIG2, and PDGFR α . Some cells expressed NG2 (11% \pm 8% aBPMC_{WM}; 18% \pm 8% aBPMC_{HIP}; $n = 6$) and PDGFR α (0% aBPMC_{WM}; 8% \pm 5%

aBPMC_{HIP}; $n = 3$) but not OLIG2 and A2B5. aBPMCs did not express the NSC-associated proteins SOX2 and CD133.

We found that 20% \pm 8% of the aBPMC_{WM} and 57% \pm 9% of the aBPMC_{HIP} expressed the pericyte marker α -SMA, and 73 \pm 8% of the aBPMC_{WM} and 76 \pm 2% of the aBPMC_{HIP} were positive for PDGFR β (Fig. 1C, 1D), both very similar to human bone marrow-derived MSCs (Fig. 1C, 1D). In addition, 19% \pm 8% of the aBPMC_{WM} and 54% \pm 9% of the aBPMC_{HIP} were double positive for α -SMA and nestin. This is in striking contrast to adult human NPCs from the white matter or the hippocampus, grown in suspension cultures, for which expression of pericyte markers is not observed [21]. Fluorescence-activated cell sorting (FACS) analysis also showed striking similarities among aBPMC_{WM}, aBPMC_{HIP}, and MSCs for the expression of typical MSC surface markers (Fig. 1E). In detail, aBPMCs expressed several MSC markers: aBPMC_{WM} CD29 99% \pm 0.6%, CD73 94% \pm 1%, CD90 8% \pm 10%, CD105 75% \pm 17%, CD146 91% \pm 5%, CD166 60% \pm 12% positive cells, aBPMC_{HIP} CD29 99% \pm 0.4%, CD73 96% \pm 2.5%, CD90 10% \pm 11%, CD105 88% \pm 9%, CD146 91% \pm 5%, and CD166 78% \pm 18%. We did not observe contamination of the monolayer cultures with endothelial cells (PECAM) or fibroblasts (S100A4) (Fig. 1C).

aBPMC_{WM} and aBPMC_{HIP} Have Very Similar Transcriptome Expression Profiles and Resemble MSCs Rather Than aNPCs or fetal NSCs

We comparatively analyzed the whole-genome gene expression profile of aBPMCs from both white matter and hippocampus to

evaluate differences in gene expression pattern. The global gene expression heat map indicated a close transcriptomics fingerprint for both aBPMC types (Fig. 2A). Accordingly, both hierarchical clustering analyses and pairwise scatter plots showed that aBPMC_{WM} closely resemble aBPMC_{HIP}, but both aBPMCs clearly differed from adult human brain-derived NPCs (aNPC) and fetal human brain-derived NSCs (Fig. 2B, 2C). Nonetheless, the transcriptomes of both aBPMC_{WM} and the aBPMC_{HIP} had much more in common with that of the MSCs (Fig. 2A–2C). aBPMC_{WM} and aBPMC_{HIP} differed only in 42 transcripts (Table 2). Of note, expression levels of NSC markers such as *OLIG2* or *SOX2* were similar in both aBPMC cell types compared with MSCs but markedly reduced compared with the different aNPCs (Fig. 2C).

Quantitative RT-PCR analyses of known NSC markers and for genes encoding for the glial and neuronal lineage confirmed these findings (Fig. 3; supplemental online Fig. 1). We were unable to detect relevant mRNA amounts of the NSC marker genes *CD133*, *SOX2*, *PAX6*, and *MSI1* in both aBPMC types, whereas *SOX1* mRNA was significantly expressed and *NES* and *MKI67* were highly expressed (Fig. 3). Glial and oligodendrocyte precursor cell marker expression confirmed the results seen with immunostaining (Figs. 1, 3).

aBPMCs Lack In Vitro Neuroectodermal Differentiation Potential but Have High Mesodermal Differentiation Capacity

Long-term expanded aBPMC_{WM} and aBPMC_{HIP} (up to 12 weeks in vitro) were unable to differentiate into immature or mature neurons (<1% β -III tubulin or MAP2+ neurons; $n \geq 3$) or oligodendrocytes and astrocytes (<1% GFAP+ or GalC+ cells; $n \geq 3$) (Fig. 4A, 4B). We tested several differentiation conditions (supplemental online Table 2), but none of them were capable of inducing an increase in the number of mature neuronal cells. Furthermore, using BrdU labeling of aBPMCs during expansion, we could not detect double-positive cells (BrdU+ and neuroectodermal marker-positive; data not shown).

In contrast, when culturing under conditions known to induce mesodermal differentiation of MSCs, aBPMCs efficiently differentiated into cells expressing key markers of osteogenic, adipogenic, and chondrogenic tissue formation (Fig. 4; supplemental online Fig. 2). Both aBPMC_{WM} and aBPMC_{HIP} were positive for the osteogenic marker enzyme ALP and showed a strong mineralization potential, as seen with alizarin red stain after osteogenic differentiation (Fig. 4C). aBPMCs were able to differentiate into the adipogenic lineage, as seen by lipid formation, although no typical droplet formation, as expected for mesodermal-derived stromal cells, could be detected by Oil Red O staining (Fig. 4C). The chondrogenic marker and transcription factor *SOX9* was expressed after pellet culture (Fig. 4C), as was the extracellular matrix protein *COL2A1* and the hypertrophic marker *COL10A1* (supplemental online Fig. 2). *COL2A1* was expressed more in aBPMCs from white matter compared with hippocampus-derived cells.

Xenotransplantation Did Not Increase the Innate Neuroectodermal Differentiation Potential of aBPMCs

We wanted to assess whether aBPMCs harbored any innate neuroectodermal differentiation potential if transplanted into a strong neurogenic environment of the developing fetal brain.

Table 2. The 43 genes differentially expressed aBPMC_{WM} and aBPMC_{HIP}

| Category | Genes |
|------------------------------|---|
| Signaling (5.1%) | <i>STC1</i> ; <i>CXCL6</i> |
| Extracellular matrix (33.3%) | <i>LAMB1</i> ; <i>NID1</i> ; <i>COL1A1</i> ; <i>COL1A2</i> ; <i>FBLN1</i> ; <i>LOXL1</i> ; <i>NID2</i> ; <i>DCN</i> ; <i>COL4A2</i> ; <i>COL4A1</i> ; <i>COL5A1</i> ; <i>COL3A1</i> ; <i>CD248</i> |
| Cell membrane (20.5%) | <i>IFITM1</i> ; <i>PDGFRA</i> ; <i>ATP1A2</i> ; <i>RGS4</i> ; <i>IFITM3</i> ; <i>TFPI</i> ; <i>ITGA8</i> ; <i>FLVCR2</i> |
| Cytoskeleton (5.1%) | <i>FHL2</i> ; <i>PDLIM1</i> |
| Receptors (7.7%) | <i>PDGFRA</i> ; <i>AKR1C2</i> ; <i>ITGA8</i> |
| Metabolism (43.6%) | <i>RPS4Y1</i> ; <i>COL1A1</i> ; <i>IGF2</i> ; <i>FHL2</i> ; <i>PDGFRA</i> ; <i>ATP1A2</i> ; <i>LOXL1</i> ; <i>ASS1</i> ; <i>AKR1C2</i> ; <i>CYR61</i> ; <i>DCN</i> ; <i>COL4A2</i> ; <i>COL5A1</i> ; <i>TFPI</i> ; <i>CFH</i> ; <i>COL3A1</i> ; <i>COL11A1</i> |
| Development (48.7%) | <i>LAMB1</i> ; <i>RPS4Y1</i> ; <i>COL1A1</i> ; <i>COL1A2</i> ; <i>IGF2</i> ; <i>FHL2</i> ; <i>PDGFRA</i> ; <i>CXCL1</i> ; <i>STC1</i> ; <i>ASS1</i> ; <i>CYR61</i> ; <i>DCN</i> ; <i>COL4A2</i> ; <i>COL4A1</i> ; <i>COL5A1</i> ; <i>ITGA8</i> ; <i>COL3A1</i> ; <i>OLFML3</i> ; <i>COL11A1</i> |
| Cell adhesion (23.1%) | <i>LAMB1</i> ; <i>NID1</i> ; <i>PDGFRA</i> ; <i>NID2</i> ; <i>CYR61</i> ; <i>COL5A1</i> ; <i>ITGA8</i> ; <i>COL3A1</i> ; <i>COL11A1</i> |
| Apoptosis (2.6%) | <i>CYR61</i> |
| Others (12.8%) | <i>TFF3</i> ; <i>CP</i> ; <i>FSTL1</i> ; <i>TM4SF1</i> ; <i>KIAA1199</i> |

Percentages indicate the number of differentially regulated genes belonging to each category.

Abbreviations: aBPMC_{WM}, adult brain perivascular mesodermal cells derived from cortical white matter; aBPMC_{HIP}, adult brain perivascular mesodermal cells derived from the hippocampus.

We grafted single cells, using ultrasound backscatter-guided real-time transplantation, into the brains of mouse embryos at E 10.5 [28]. The engraftments were analyzed 7 days after transplantation to avoid having to use immunosuppressive drugs or immunodeficient mice (Fig. 5).

aBPMCs survived in only one-fifth of transplanted brains. By analyzing the grafts, we discovered that the cellular implants formed spheres within the ventricular system. It seems that aBPMCs did not migrate into the host brains. Furthermore, we did not detect expression of the NSC marker *SOX2* or differentiation into immature (DCX+) or mature (MAP2+) neurons (Fig. 5); however, we rarely observed GFAP+ and human nuclei-positive cells suggestive of occasional astrocyte differentiation of aBPMCs. Consequently, we concluded that the neurogenic environment of mouse embryos at E 10.5 cannot increase the neural differentiation potential of transplanted aBPMCs underpinning our in vitro results.

DISCUSSION

We reported the detailed characterization of human aBPMCs derived from white matter and hippocampal tissue, showing that long-term expanded aBPMC_{WM} are nearly identical to aBPMCs isolated from the hippocampus. Neither aBPMC_{WM} nor aBPMC_{HIP} expressed neural stem cell markers, but they expressed classical pericyte markers and resembled bone marrow-derived MSCs rather than adult or fetal NPCs. aBPMCs do not harbor relevant intrinsic neuronal differentiation potential but have great potential to differentiate into mesodermal lineages. Whole-transcriptome analysis clustered aBPMCs close to MSCs.

Two recent reports provided the first hints of the existence of pericytes in the human adult brain [13, 14]; however, the groups

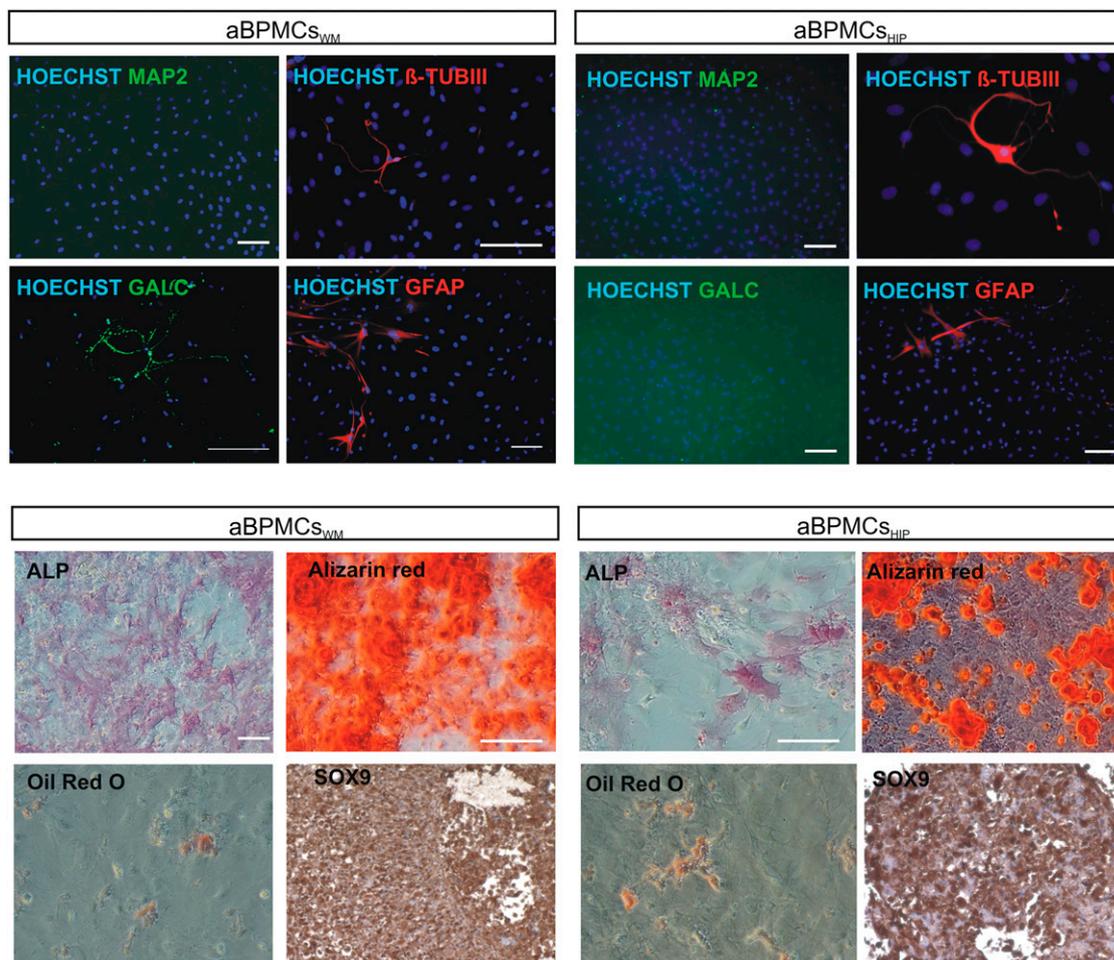


Figure 4. Differentiation of aBPMCs from white matter and hippocampal tissue. **(A):** Representative selection of stainings. Cells were stained against markers for neurons (β -III tubulin, MAP2ab), oligodendrocytes (GALC) and astrocytes (GFAP). Nuclei were counterstained with Hoechst dye. Scale bars = 100 μ m. **(B):** Mesodermal lineage differentiation potential is depicted. aBPMCs efficiently differentiated into osteogenic (ALP+, Alizarin red), adipogenic (Oil Red O), and chondrogenic (SOX9) progeny. Scale bars = 100 μ m. Other marker expression is shown in supplemental online Fig. 2. Abbreviations: aBPMC_{WM}, adult brain perivascular mesodermal cells derived from cortical white matter; aBPMC_{HIP}, adult brain perivascular mesodermal cells derived from the hippocampus; ALP, alkaline phosphatase; GFAP, glial fibrillary acidic protein.

reported different observations. Although Paul et al. reported no coexpression of NSCs and pericyte markers by aBPMCs but rather an intrinsic neuroectodermal differentiation potential [14], Park et al. reported coexpression of these markers but no neuroectodermal differentiation potential [13]. Furthermore, thorough characterization of these aBPMCs and systematic comparison with aNPCs are still lacking at the whole-transcriptome level and in terms of their innate neuroectodermal differentiation potential.

By performing such whole transcriptome analysis, we found no expression of classical NSC markers, including SOX2, CD133, or MSI1, but noted markedly increased proliferation potential compared with human aNPCs [19, 21]. Adult human brain-derived NPCs are currently believed to be oligodendrocyte progenitor cells (OPCs) [19, 21]. Typical OPC markers including A2B5 and OLIG2 were not expressed by either aBPMC population, whereas pericyte markers were nearly as abundant in both aBPMC_{WM} and aBPMC_{HIP} as in MSCs. Despite using typical culture conditions for adult human [19, 21], fetal human [23], and murine NSCs [29–32], aBPMCs always overgrow without previous selection (e.g., by FACS). This suggests that adherent monolayer cultures do not

provide the needed cues for aNPC expansion. Consequently, unlike fetal human and adult murine NSCs [23, 29–32], aNPCs depend on niche factors of neurospheres to enable human aNPC expansion [7, 8, 19, 21].

Emphasis has been placed on the possible potential of tissue-specific progenitor cells to overcome lineage restriction [1, 2]. This potential was also reported for MSCs [4–6]. If analyzed more precisely, MSCs often retain mesodermal transcriptome characteristics after transgerminal conversion and thus lack full transdifferentiation characteristics [7, 8]. Consequently, aBPMCs could not overcome their mesodermal lineage commitment, even after in utero transplantation in the developing brain, which is likely the most powerful inductive environment for neuroectodermal differentiation (Fig. 5). We concluded that neither aBPMC_{WM} nor aBPMC_{HIP} harbor an innate neuronal differentiation potential; however, because of the low migration and integration of aBPMCs into the host brain, we cannot fully rule out that we missed such neuroectodermal differentiation behavior. Nevertheless, we believe that the lack of integration is a major finding because we previously succeeded by showing integration and classical NPC behavior of

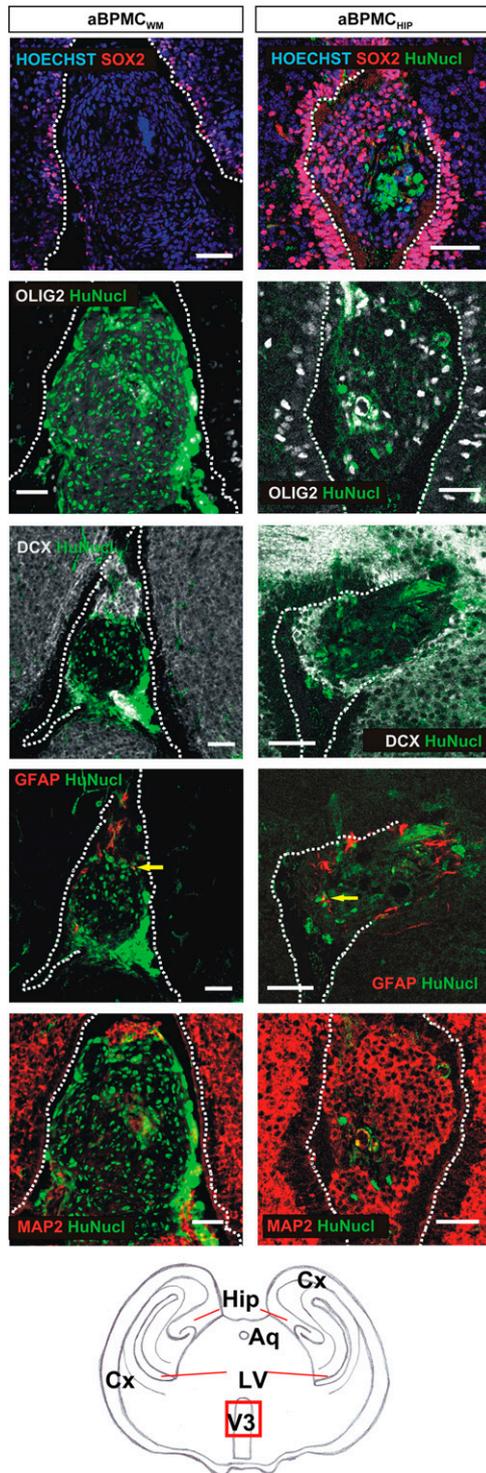


Figure 5. In utero transplantation of aBPMCs from white matter and hippocampal tissue. Human aBPMCs were transplanted in utero into mice at embryonic day 10.5, and brain sections were analyzed 7 days after engraftment. Brain section of the third ventricle showed a cluster of cells positive for human nuclei but negative for stem cell markers like nestin or differentiation markers like DCX or MAP2. Very few cells were GFAP⁺/HuNucl⁺ (arrow). Scale bars = 100 μ m. Abbreviations: aBPMC_{WM}, adult brain perivascular mesodermal cells derived from cortical white matter; aBPMC_{HIP}, adult brain perivascular mesodermal cells derived from the hippocampus; Aq, aqueduct; Cx, cortex; GFAP, glial fibrillary acidic protein; Hip, hippocampus; HuNucl, human nuclei; LV, lateral ventricle; V3, third ventricle.

implanted NPCs [28]. Although species differences could play a role, many reports show that in utero surgery is appropriate to prove the innate neuroectodermal potential from human NSCs [33]. Consequently, Karow et al. recently reported the induction of fully mature neurons from human pericytes only by overexpression of SOX2 and MASH1 [15, 16]. Even SOX2 alone was not efficient for deriving fully mature neurons from aBPMCs [15, 16]. We also did not succeed in inducing neurons from aBPMCs by retroviral human SOX2 overexpression (data not shown).

Although data exist showing a role of aBPMCs in blood-brain barrier function and dysfunction, their role in neurodegenerative or even neuroinflammatory diseases remains enigmatic [17]. Because mesenchymal stem cells are often discussed as cell vehicles for trophic support or modulatory effects on diseased neurons in many incurable brain diseases, aBPMCs should attract future work with the potential for endogenous cell therapy. Interestingly, a recent report noted an increase in PDGFR β ⁺ cells in patients with temporal lobe epilepsy [18], suggesting that these cells might be activated for endogenous repair strategies; however, it is not known yet whether these aBPMCs are activated during neurodegenerative diseases or are even part of the disease pathology. aBPMCs in our study were derived from epileptic surgery, thus we cannot fully exclude that our high yield of aBPMCs was the result of an increased cell population due to the patient's epilepsy or epileptic drugs.

Because tissue samples from the subcortical white matter tissue are relatively easy to obtain by neurosurgical intervention and the risk for the patients is much lower compared with surgeries on neurogenic brain regions such as the hippocampus, aBPMCs generated from subcortical white matter have a potential for translational medicine toward clinical applications. MSCs have already been shown to have many beneficial effects in different neurological diseases [1–3]; therefore, aBPMCs could serve as an endogenous cell source for restorative approaches in various neurological diseases [34].

CONCLUSION

We reported detailed characterization of adult human brain-derived perivascular mesenchymal cells, thereby clearly showing the lack of an innate neuronal differentiation potential. Due to their relationship to mesenchymal stem cells, which have various beneficial modular effects on diseased neurons of various conditions, these aBPMCs might prove beneficial for treating various neurological diseases.

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AUTHOR CONTRIBUTIONS

X.L.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; S. Srimasorn, J.R., S.F., M.W., V.T., M.J.A.-B., S.H.-E., M.K., S. Schwarz, J.S.: collection and assembly of data, data analysis and interpretation, manuscript writing; A.S.: conception and design, data analysis and interpretation, manuscript writing, financial support, administrative support;

A.H.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, financial support, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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