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Neural differentiation of adipose-derived stem cells isolated from GFP transgenic mice

Juri Fujimura [a,c,*], Rei Ogawa [b], Hiroshi Mizuno [b], Yoshitaka Fukunaga [c], Hidenori Suzuki [a]

[a] Department of Pharmacology, Nippon Medical School, Tokyo, Japan
[b] Department of Plastic and Reconstructive Surgery, Nippon Medical School, Tokyo, Japan
[c] Department of Pediatrics, Nippon Medical School, Tokyo, Japan

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Abstract

Taking advantage of homogeneously marked cells from green fluorescent protein (GFP) transgenic mice, we have recently reported that adipose-derived stromal cells (ASCs) could differentiate into mesenchymal lineages in vitro. In this study, we performed neural induction using ASCs from GFP transgenic mice and were able to induce these ASCs into neuronal and glial cell lineages. Most of the neurally induced cells showed bipolar or multipolar appearance morphologically and expressed neuronal markers. Electron microscopy revealed their neuronal morphology. Some cells also showed glial phenotypes, as shown immunocytochemically. The present study clearly shows that ASCs derived from GFP transgenic mice differentiate into neural lineages in vitro, suggesting that these cells might provide an ideal source for further neural stem cell research with possible therapeutic application for neurological disorders.
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The identification of cell populations capable of neural differentiation has generated great interest [1,2]. Stem cells from embryonic tissue are able to expand and differentiate into multiple lineages including neural lineages in vitro and in vivo. However, there are still some limitations on the practical use of embryonic stem cells because of ethical questions [3,4]. Pluripotent mesenchymal stem cells have been detected in multiple tissues, including bone marrow [5–11]. Under appropriate conditions, bone marrow-derived stromal cells (BSCs) including mesenchymal stem cells selectively differentiate into neural lineages in vitro and in vivo [5–7,9,11–15].

In the last few years, adipose tissue has been identified as an alternative source of pluripotent stromal cells, sometimes referred to as processed lipoaspirate (PLA) cells [16,17]. Using a cell cloning technique, Zuk et al., isolated adipose-derived stem cells from a PLA cell population. Our group also isolated stromal cells from the adipose tissue without using any lipoaspirate techniques, and we therefore termed this cell population "adipose-derived stromal cells" (ASCs). ASCs including mesenchymal stem cells can be differentiated into various mesenchymal tissues such as chondrocytes, adipocytes, osteoblasts, and myocytes [16–18], and can also be differentiated into neural lineages [19–23].

Transgenic mice ubiquitously expressing green fluorescent protein (GFP) are very useful for cell transplan-

* Corresponding author. Fax: +81 3 5814 1684.
E-mail address: juri-f@nms.ac.jp (J. Fujimura).

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tation experiments, because the origin of GFP cells is easily found [24]. Recently, we reported that ASCs from GFP transgenic mice can differentiate into mesenchymal lineages including chondrocytes, adipocytes, and osteoblasts [25]. Here, we present the first report showing that ASCs from GFP transgenic mice can be differentiated into neural lineages presenting morphologic and immunochemical characteristics.

Materials and methods

Cell harvest. Primary culture of ASCs using GFP transgenic mice was performed as described in a previous study [25]. GFP transgenic mice aged 4 weeks were deeply anesthetized with diethyl ether. The inguinal fat pads were removed and extensively washed with phosphate-buffered saline (Gibco-BRL, Grand Island, NY, USA). They were finely minced and incubated on tissue culture plates (90 mm in diameter) (Sumilon, Tokyo, Japan) for 1 h with a control medium consisting of Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum (Gibco) and 1% antibiotic–antimycotic (Gibco). The cell suspension was centrifuged at 1300 rpm (260*g*) for 6 min in a 50-ml centrifuge tube. The cell pellet was then digested with 0.15% collagenase (Wako, Osaka, Japan) by vigorous shaking for 30 min at 37 °C, and an equal volume of control medium was added to inactivate the collagenase activity. The reconstituted cell suspension was centrifuged at 1300 rpm (260*g*) for 6 min, and the cell pellet was resuspended in the control medium. After cell counting using trypan blue staining, the viable cells were plated at a concentration of 10^5 cells per plate (90 mm in diameter) and maintained in the control medium at 37 °C in 5% humidified carbon dioxide. The culture media were changed every 3 days.

Neural induction. Neural induction was performed after two passages. At 40–60% confluence, the medium was replaced by a neural induction medium prepared according to a previous report [22] but without fetal bovine serum: Dulbecco's modified Eagle's medium containing 1% antibiotic–antimycotic (Gibco), 5 μg/ml insulin (Wako), 200 μM indomethacin (Sigma, St. Louis, USA), and 0.5 mM isobutylmethylxanthine (Sigma). At several time points from 6 to 48 h following the neural induction, the cells were fixed and assessed.

Electron microscopy. Twenty-four hours after the neural induction, the cultured cells were fixed with 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide. After dehydration in ethanol, the cells were embedded in resin according to standard techniques. Ultrathin sections were cut on an ultramicrotome with a diamond knife and were then stained with uranyl acetate and lead citrate. The sections were examined under an electron microscope.

Immunocytochemistry. ASCs were grown on a circular cover glass of 9 mm in diameter (Matsunami, Osaka, Japan). After the neural induction, the cells were fixed with 4% buffered paraformaldehyde, and incubated with each primary antibody at 4 °C overnight and subsequently with appropriate secondary antibody conjugated with Alexa fluor 594 for 1 h at room temperature. The primary antibodies used in the present study were mouse monoclonal anti-nestin (1:100) (Chemicon, Temecula, CA, USA), mouse monoclonal anti-microtubule-associated protein 2 (MAP 2a + 2b) (neuronal lineage) (1:500) (Sigma), mouse monoclonal anti-70-kDa-neurofilament protein (NF-70) (Dako, Carpinteria, CA, USA), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) (astrocyte lineage) (Dako), synaptophysin (Chemicon), and $GABA_A$ receptor α1 subunit ($GABA_A$-R α1) (Upstate, Lake Placid, NY, USA). The staining was visualized under a fluorescent microscope (Olympus, Tokyo, Japan).

Results

Isolation of adipose-derived stem cells from GFP transgenic mice

The inguinal fat pads from each mouse yielded approximately 5×10^5 nucleated cells. When plated, the ASCs from these mice exhibited a heterogeneous population of fibroblast-like cells morphologically (Fig. 1A), which is consistent with the results of previous reports. These ASCs expressed bright green fluorescence under a fluorescent microscope (Fig. 1B).

Neural induction

After the neural induction, the morphology of ASCs began to change within a few hours. The cells developed characteristic round cell bodies with several branching extensions (Fig. 2A), concomitantly expressing GFP fluorescence (Fig. 2B). At most 60% of the total cell population were bipolar or multipolar in shape. Some of

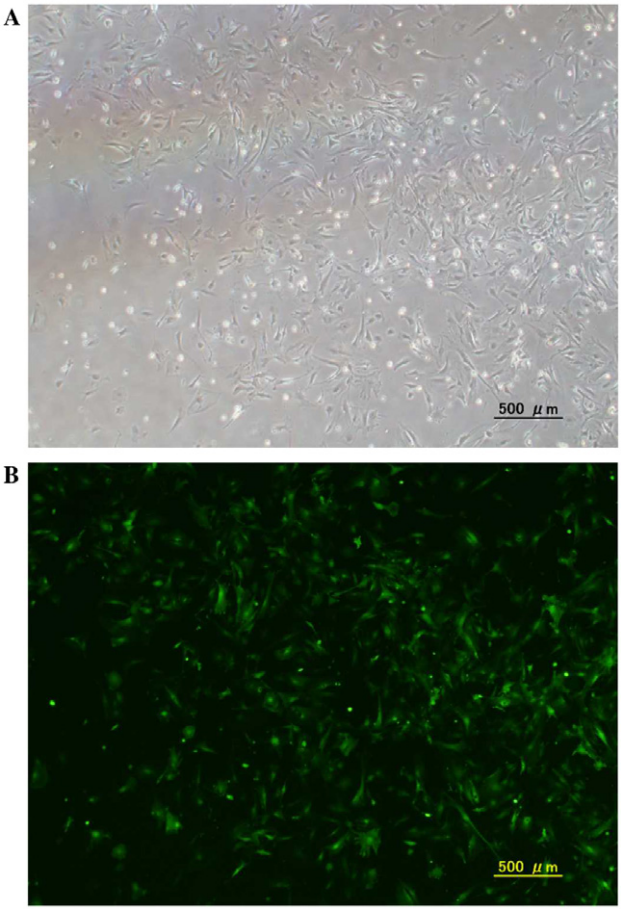

Fig. 1. Primary culture of ASCs harvested from GFP transgenic mice in the control medium after 7 days. (A) A light microscopic image. (B) A fluorescent microscopic image. The cells show an elongated fibroblastic appearance with concomitant GFP fluorescence.

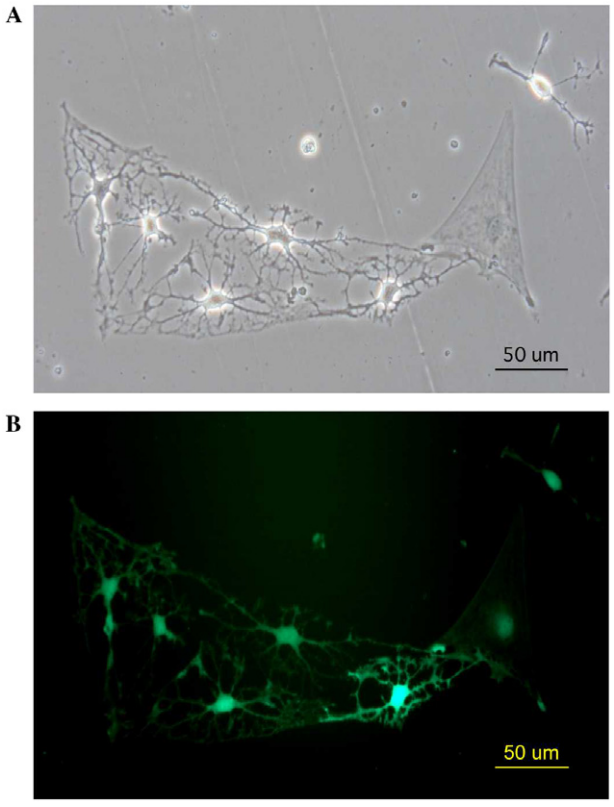

Fig. 2. ASCs 24 h after incubation with neural induction medium. (A) A light microscopic image. (B) A fluorescence microscopic image. The cells developed characteristic round cell bodies with several branching extensions retaining GFP fluorescence.

them appeared to make contact with their neighboring cells, as shown in Fig. 2.

Electron microscopy

After the neural induction (Figs. 3A and B), the multipolar cells exhibited many microtubules (arrow M in Fig. 3B) and thinner actin filaments (arrow A in Fig. 3B) in their soma and processes. The microtubules were long, smooth-walled hollow cylindrical structures, several micrometers long and around 20 nm in diameter, and they occurred frequently in the dentrites and axons of neurally induced cells. The non-induced cells had no microtubules (Fig. 3C). This observation suggests that these multipolar cells have the characteristics of neurocytes.

Immunocytochemistry

After the neural induction, most of the ASCs with bipolar processes expressed nestin (Fig. 4), while those with multipolar processes tended to be stained with MAP2 or neurofilament-70, but not nestin (Figs. 5 and 6). There were some GFAP-positive cells (Fig. 7), but this study indicated rare occurrence. Neither synaptophysin nor $GABA_A$-R $\alpha 1$ was detected in neurally induced cells (data not shown), even when the differentiated cells appeared to be in contact with each other.

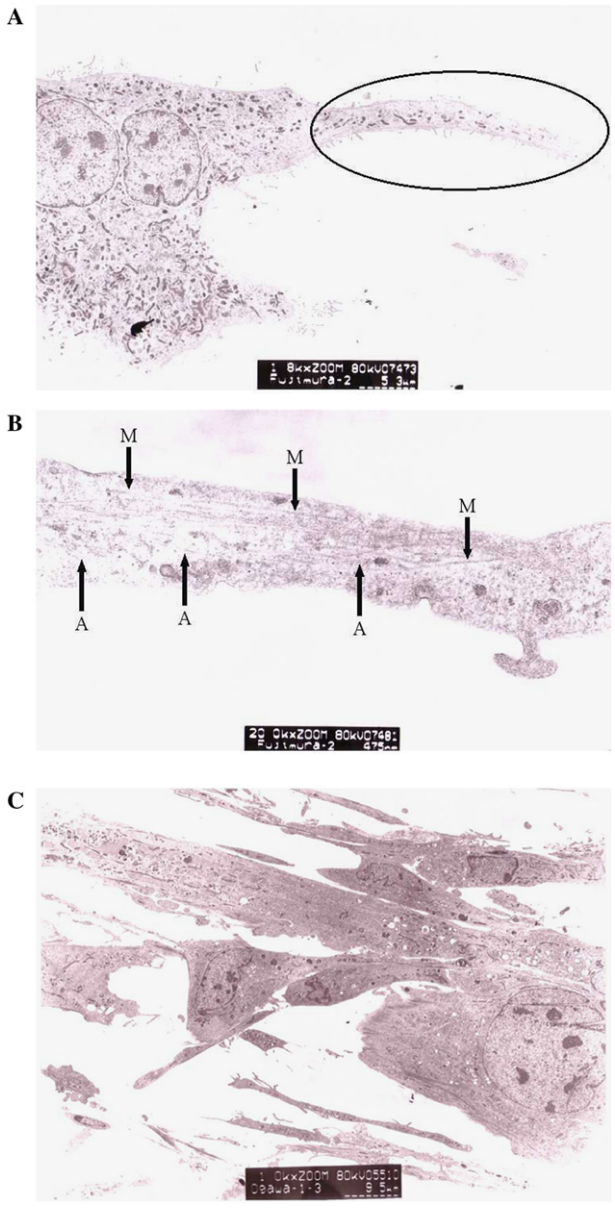

Fig. 3. Electron micrographs of a neurally induced cell 24 h after induction (A,B: circle of A) A multipolar cell had many microtubules (arrow M) and thinner actin filaments (arrow A) in its soma and processes. (C) A non-induced cell: there are no microtubules in the cell structure.

Discussion

Our findings demonstrate that ASCs isolated from GFP transgenic mice possess the ability to undergo morphologic and phenotypic changes consistent with

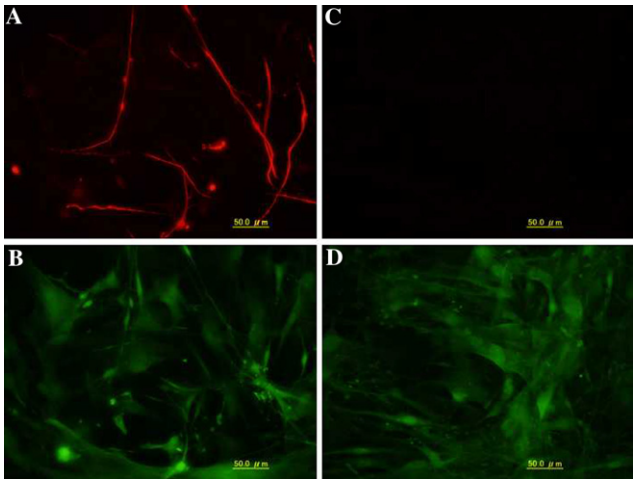

Fig. 4. Immunocytochemical stainings for nestin (red fluorescence) and their GFP fluorescent view (green). (A,B) Induced cells (cells were fixed at 6 h following induction). (C,D) Non-induced cells. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

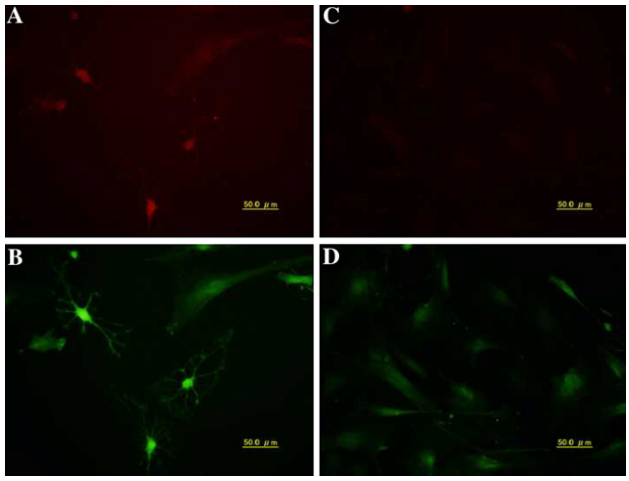

Fig. 6. Immunocytochemical stainings for neurofilament-70 (red fluorescence) and their GFP fluorescent view (green). (A,B) Induced cells (cells were fixed at 24 h following induction). (C,D) Non-induced cells. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

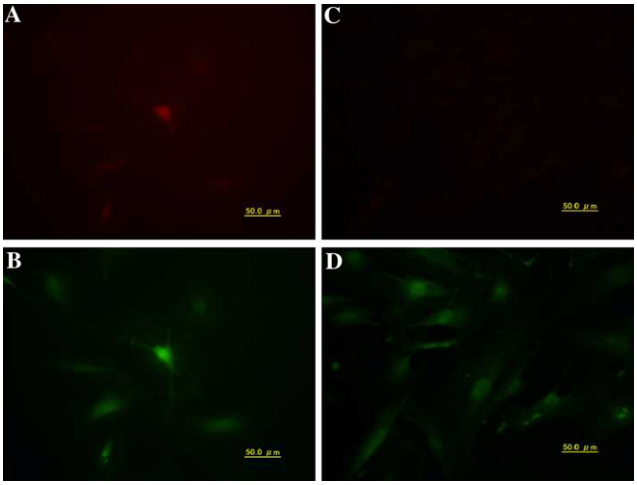

Fig. 5. Immunocytochemical stainings for MAP 2 (red fluorescence) and their GFP fluorescent view (green). (A,B) Induced cells (cells were fixed at 24 h following induction). (C,D) Non-induced cells. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

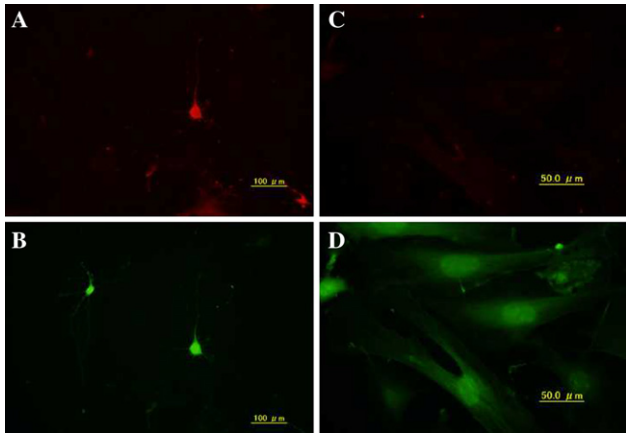

Fig. 7. Immunocytochemical stainings for GFAP (red fluorescence) and their GFP fluorescent view (green). (A,B) Induced cells (cells were fixed at 24 h following induction). (C,D) Non-induced cells. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

neural differentiation. As shown in Table 1, our results were in good agreement with those of previous reports, although there were some differences in the neurogenic media, mouse strains and species used.

As has been previously reported, adipose tissue may include neuronal fibers [26]. However, unlike in brown adipose tissue, adipocytes are not directly in contact with nerve terminals in white adipose tissue [27]. Zuk et al. [16] showed that white adipose tissues contained stem cells as well as adipocytes, vasculature, and matrix. It is, therefore, unlikely that preexisting neural cells continue to grow in passaged cultures.

Recently, Lu et.al. and Neuhuber et al. [28,29] reevaluated the results of induction of BSCs to neurons in vitro and suggested the possibility that the morphological changes resulted from rapid disruption of the cytoskeleton due to the cytotoxic medium. However, using the electron microscopic images, we proved that neurally induced cells possessed microtubules in their soma and processes, which provides strong evidence that the neurally induced cells in the present study had neuronal structures.

Within a few hours of neural induction, the ASCs changed shape from a fibroblast-like appearance to

Table 1
A comparison of the characteristics of neurally induced ASCs as revealed in previous reports and in this study

	Murine			Human			
	Safford 2004	Safford 2002	This study	Safford 2002	Kand 2003	Ashjian 2003	Zuk 2002
Nestin	+	+	+	+			
NSE						+	+
Neu N	+	+		+		+	+
MAP 2	+		+		+		−
β-III tubulin	+						
Trk-A						+	
NF-70			+				−
IF-M				+		−	
Tau	+						
GFAP	+	+	+		+	−	−

bipolar and multipolar ones. Immunostaining revealed that the bipolar cells expressed nestin and that the multipolar ones were positive for MAP 2 or neurofilament-70. Nestin, an intermediate filament protein, is thought to be expressed at high levels in neural stem cells, although its expression has also been observed in myogenic cells, endothelial cells, and hepatic cells [13]. MAP 2 and neurofilament-70 are used as markers for mature neurons [19–23]. Therefore, these morphological changes from bipolar to multipolar cells may represent neuronal maturation. Immunochemical studies with GFAP as a marker for astrocytes demonstrated that there were far fewer glial cells than neurons among the induced cells. ASCs tend to differentiate into neurocytes rather than astrocytes under the condition in the present study.

When we used the previously reported neural induction medium containing serum [22], the cells gradually took on a neural morphology over a period of two weeks (data not shown). On the other hand, without the serum, the morphological changes were observed more rapidly in a higher proportion of the cells. This suggests that an environmental stressor, such as a low serum level, may be involved in the rapidity of neural differentiation.

Safford et al. [21] reported that murine adipose-derived adult stromal cells could be induced to express GABA, a synaptic vesicle protein synapsin I, *N*-methyl-D-aspartate (NMDA) receptor subunit, and voltage-gated calcium channel α1, and also to demonstrate decreased viability in response to NMDA. In our immunochemical experiment, however, we could not confirm that the neurally induced cells were stained for the neuronal presynaptic marker synaptophysin [30] or $GABA_A$-R α1. It still remains unclear whether the neurally induced cells in this study had synaptic connections and/or exerted neural functions.

ASCs are readily accessible in quite large quantities and with minimal morbidity, which overcomes the risks associated with obtaining neural stem cells from some regions of the brain such as the subventricular zone or of collecting stromal cells from bone marrow. ASCs also provide a renewable population of cells that can be easily expanded in culture media. They therefore provide significant potential benefits when it comes to the clinical use of neural cells derived from adipose tissue. Moreover, through fluorescent microscope, the origin of the GFP cells is easily found. Therefore, ASCs from GFP transgenic mice may be an ideal source for further experiments on neural differentiation and transplantation in animal models of neurological diseases.

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