Neurospheres From Human Adipose Tissue Transplanted Into Cultured Mouse Embryos can Contribute to Craniofacial Morphogenesis: A Preliminary Report

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Adipose-derived stromal cells (ASCs) are one of the most promising stem cell populations that differentiate into the mesodermal as well as neural lineages in vitro. In this study, we examined the neural differentiating potential of human ASCs by a neurosphere culture method. Neurospheres derived from human ASCs expressed *Nestin* and *Musashi-1* genes, which are marker genes for neural stem cells. When these cells were labeled with *green fluorescent protein* gene transfection by Sendai virus vector and transplanted into the head region of mouse embryos using a whole embryo culture system, these cells were incorporated into the craniofacial development. Some transplanted cells appeared to migrate along the second branchial arches, implicating some similarity to the cranial neural crest cells. Although preliminary, our results support an idea that ASC-derived neurospheres have properties of neural progenitors in vitro and in vivo.

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Adipose-derived stromal cells (ASCs) were originally reported as a subtype of the mesenchymal stem cells (MSCs) isolated from liposuction aspirates differentiating into the mesodermal tissues such as bone, cartilage, and adipose tissue.[1] Characterization of ASCs has recently been studied world wide by many groups, including ours.[2–4] ASCs are now regarded as one of the most promising adult stem cells for regenerative medicine because they can be harvested safely by liposuction, and a good yield can be anticipated.

Advances in stem cell research have resulted in a novel concept of cellular plasticity of differentiation beyond the boundary of germ layers. MSCs and ASCs can differentiate into neuronal (and thus ectodermal) derivatives, although these cells are primarily mesodermal.[5,6] Recent reports further indicate that stem cells with neural characteristics can be isolated from the mesodermal tissues such as the dermis and the heart.[7–9] In these cases, the cells were harvested by a neurosphere method, which was originally developed as a culture method of isolating spheres of neural stem cells from the embryonic and adult brain.[10–12] However, it is to be elucidated whether this method is also applicable for obtaining neural stem cells from the adipose tissue or ASCs.

In this study, the neurospheres expressing neural stem cell marker genes were obtained from human ASCs. We also transplanted these cells into mouse embryos cultured in vitro to examine whether these cells behave similar to neuronal cells in vivo.

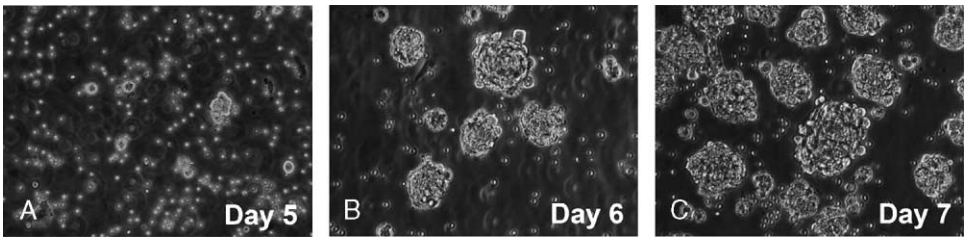

Fig 1 Neurosphere formation of adipose-derived stromal cells cultured in neurosphere medium for 5 days (A), 6 days (B), and 7 days (C) (magnification ×200).

Materials and Methods

Isolation of Human ASCs and Neurosphere Cell Culture

ASCs were isolated from the human liposuction aspirates as reported previously.[3] The suctioned fat was digested with 0.075% collagenase in phosphate-buffered saline (PBS) for 30 minutes on a shaker at 37°C. Mature adipocytes and connective tissues were eliminated by centrifugation. Blood cells were also eliminated by treating with erythrocyte lysis buffer, and resultant ASC pellets were obtained. Alternatively, ASCs could be isolated from the fluid portions of liposuction aspirates by treating with erythrocyte lysis buffer and density gradient centrifugation with Ficoll (GE Healthcare Bio-sciences, Piscataway, NJ).

Neurosphere culture was performed as described previously with slight modification.[12] Freshly isolated ASCs were plated at a density of 2×10^7 cells in 10 cm uncoated dishes and cultured in the neurosphere culture medium at 37°C in an atmosphere of 5% CO_2 in humid air. The neurosphere medium was a Dulbecco's Modified Eagle's Medium/F12 (1:1)-based medium supplemented with human recombinant epidermal growth factor (EGF, 20 ng/mL, PeproTech, Rocky Hill, NJ), human recombinant basic fibroblast growth factor (FGF, 20 ng/mL, Kaken Pharmaceutical, Tokyo, Japan), 2% B27 supplement (Gibco, Carlsbad, CA), 100 U/mL penicillin, and 100 μg/mL streptomycin. Half of the medium was replaced with a fresh medium on the fourth to fifth day, and the passaging was performed on the eighth day.

Quantitative Real-Time Reverse-Transcriptase Polymerase Chain Reaction

Total mRNA was extracted using RNeasy-mini kit (Qiagen, Hilden, Germany) from the neurosphere cells derived from passage one ASCs, which were precultured in the normal medium containing M199 medium and 10% fetal bovine serum (FBS). The preculturing was necessary for reducing the contamination of blood cells. Control mRNA was also extracted from the passage one undifferentiated ASCs cultured in M199 plus 10% FBS.

Expressions of undifferentiated neural stem cell marker genes *Nestin* and *Musashi-1*[13] and adipogenic differentiation marker *Leptin* were analyzed by real-time quantitative reverse-transcription polymerase chain reaction (RT-PCR) using an ABI PRISM 7700

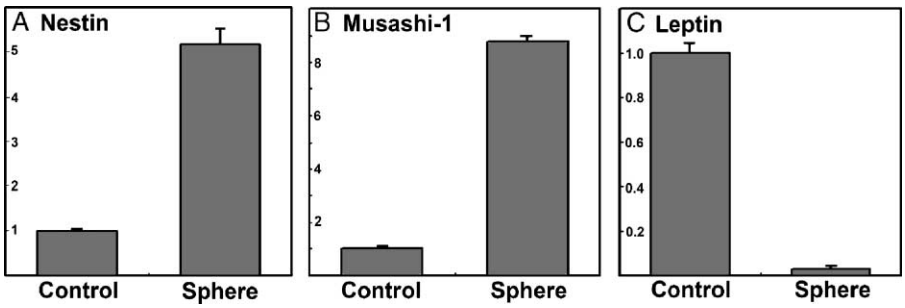

Fig 2 Quantitative real-time reverse-transcription polymerase chain reaction analysis of gene expressions of neural stem cell marker *Nestin* (A), *Musashi-1* (B), and adipogenic differentiation marker *Leptin* (C). Control = undifferentiated adipose-derived stromal cells; Sphere = neurospheres. Assays were performed in triplicate, and standard errors are indicated by error bars.

(Applied Biosystems, Foster City, CA), as reported previously. Gene expression of the target sequence was normalized to that of the housekeeping gene *β-actin.* Transcript level in the control (undifferentiated ASC) group was arbitrarily expressed as 1. TaqMan chemistry and assay by design primers and probe sets were used for human *Nestin*, *Musashi-1, Leptin*, and *β-actin*. All the primers and probe sets were purchased from Applied Biosystems.

Mouse Whole Embryo Culture and Transplantation of Neurosphere-Like Cells

Neurospheres derived from human ASCs were transfected with *green fluorescent protein* (*GFP*) gene using the Sendai virus vector (DNAVEC Corp. Tsukuba, Japan), as reported previously.[14,15] The original vector SeV/ΔF lacks the F gene encoding fusion protein necessary for penetration of ribonucleoprotein complex into infected cells, and is thus nontransmissible and nonpathogenic.[14] The modified SeV/ΔF vector has additional mutations to reduce its cytotoxicity,[15] and we used the modified vector in the present study. Neurospheres were incubated for 1 hour in the medium with the modified SeV/ΔF carrying the *GFP* gene at a multiplicity of infection of 250 and rinsed with PBS.

Mouse whole embryo culture was performed as reported previously.[16–19] Nine mouse embryos at embryonic day (E) 8 were dissected out without damaging yolk sacs, and the GFP-transfected neurosphere cells were transplanted using micropipettes into the head region of the embryos. The embryos were cultured for approximately 40 hours, and presence or absence of the GFP-positive transplanted cells was investigated under a fluorescent dissecting microscope. All experimental procedures were performed at the University of Tokyo under approval of the ethical committee.

RESULTS

We first cultured human ASCs in the neurosphere culture medium containing EGF and basic FGF without serum. On the third day of culture of freshly prepared ASCs, the floating ASCs started to form small masses (data not shown). The neurosphere-like cellular aggregates were clearly observed on the fifth day (Fig 1A). The number and the size of the spheres became increasingly larger within the next 2 days (Fig 1, B and C). The passaging was performed on the eighth day when the spheres were dissociated and resuspended in the new medium. The spheroids were newly formed after culturing again for several days (data not shown), suggesting self renewal of the neurosphere cells.

To characterize the neurosphere cells, we next examined expressions of neural stem cell marker *Nestin* and *Musashi-1* genes and adipocyte marker *Leptin* by quantitative real-time RT-PCR. Expressions of *Nestin* and *Musashi-1* genes were remarkably upregulated in the neurosphere cells compared with the control ASCs without culturing in the neurosphere medium (Fig 2, A and B), suggesting characteristics of neural progenitor. Conversely, *Leptin* expression

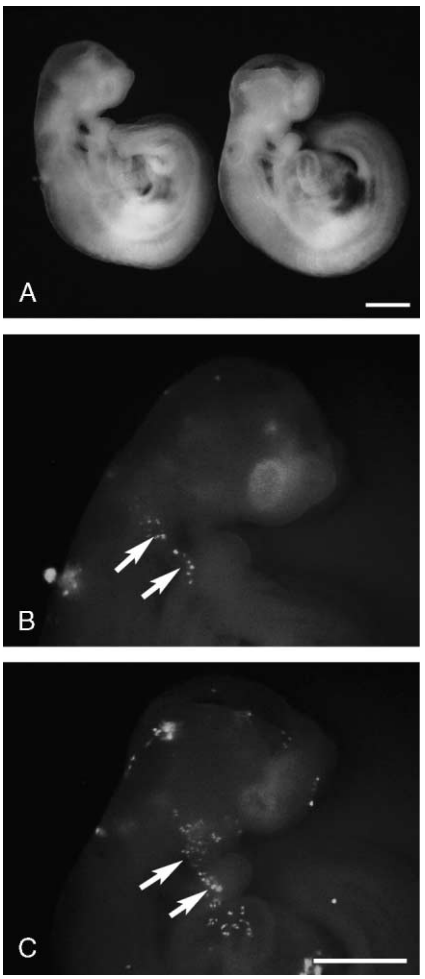

Fig 3 Neural crest-like migrations of green fluorescent protein (GFP)-transfected, adipose-derived stromal cell-derived neurospheres grafted into mouse embryo cultured in vitro. (A) Appearances of mouse embryos cultured for 40 hours from embryonic day 8. (B and C) Fluorescent views of embryos. GFP-positive neurosphere cells were arranged in a row (arrows), suggesting their migration along second branchial arch. Bars = 500 μm.

was dramatically reduced in the neurospheres (Fig 2C), indicating loss of adipogenic potential.

To investigate functions of the neurosphere cells in vivo, we labeled these cells by the modified Sendai virus vector carrying the *GFP* gene and transplanted them into the head region of the E8 mouse embryos. After the embryos were cultured for approximately 40 hours in vitro, the transplanted GFP-positive cells were clearly observed and appeared viable in only two embryos of the nine cultured embryos. The GFP-positive cells were incorporated into the craniofacial region as well as the heart and the trunk in these two embryos (Fig 3). Notably, the transplanted cells were arranged in a row along the second branchial arch (arrows in Fig 3, B and C) in a quite similar pattern to the neural crest cells migrating within the second branchial arch. Although not confirmatory, this result suggests a intriguing possibility that neurosphere cells derived from ASCs have neural crest-like properties.

Discussion

ASCs are probably one of the most well-known stem cells among plastic surgeons. ASCs were originally reported by Zuk et al[1] from the clinical samples of liposuction aspirates. According to their broad spectrum of differentiation potential, ASCs have been used in a number of preclinical animal studies of in vivo regeneration of a various tissues such as bone,[20,21] cartilage,[22] vessels,[4,23,24] soft tissue,[4] bone marrow,[25] and so on. Even a clinical case was reported, in which a calvarial defect was repaired by ASCs combined with scaffold.[26] Several groups reported neural differentiation of ASCs in vitro,[5,6,27] and Kang et al[28] reported functional recovery of the rat model with cerebral infarction after ASC transplantation in vivo.

The neurosphere method was originally reported by Reynolds et al[10,11] and is one of the most frequently used methods for isolating neural stem cells from the embryo or from the adult central nervous systems. However, this method has not yet been applied for obtaining neural stem cells from adipose tissue or the ASC population. In this preliminary study, we obtained neurospheres from the ASCs in human liposuction aspirates. Proliferation of these cells was quite rapid, possibly faster than other neurospheres from various tissue origins such as the dermis and the heart,[7–9] suggesting advantages of ASCs as a origin of neuronal progenitors for regenerative medicine. These neurosphere cells expressed *Nestin* and *Musashi-1*, marker genes for neural stem cells, probably reflecting their tendency of differentiating into neuronal progenitors. This view is further supported by inhibition of their expression of *Leptin*, a marker for adipogenic differentiation and maturation.

Do the ASC-derived neurosphere cells behave as neuronal progenitors in vivo? Our attempt of grafting these cells into the cultured mouse embryo revealed that some of the cells migrate along the second branchial arch and contribute to craniofacial morphogenesis. Their migratory pattern is quite similar to that of cranial neural crest cells, as we reported previously.[16] The neural crest cells are an embryonic cellular population characterized by extensive migration and a unique repertoire of differentiation.[29] The neural crest cells are often regarded as stem or progenitor cells for peripheral neurons and Schwann cells, and the craniofacial skeletal mesenchyme is also neural-crest derived.[17,19,29,30] Recent studies indicate that the neural crest stem cells can be harvested from the seemingly ''mesodermal'' tissues of adult animals, such as the dermis,[7] the hair follicular dermal papilla,[8] or the heart,[9] by means of the neurosphere method, implicating that it is also the case in the adipose tissue. Because our data are preliminary and we have a small sample size, further studies such as those with detailed expression analysis of neural/neural crest marker genes and large-scale in vivo grafting are necessary to confirm this interesting idea.

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