JSHC
Japan Society of Histochemistry and Cytochemistry
Established 1959

Acta Histochem. Cytochem. 43 (6): 123–130, 2010
doi:10.1267/ahc.10015

Transplantation of Adipose Derived Stromal Cells into the Developing Mouse Eye

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Received May 4, 2010; accepted September 29, 2010; published online December 3, 2010

Adipose derived stromal cells (ADSCs) were transplanted into a developing mouse eye to investigate the influence of a developing host micro environment on integration and differentiation. Green fluorescent protein-expressing ADSCs were transplanted by intraocular injections. The age of the mouse was in the range of 1 to 10 days postnatal (PN). Survival dates ranged from 7 to 28 post transplantation (DPT), at which time immunohistochemistry was performed. The transplanted ADSCs displayed some morphological differentiations in the host eye. Some cells expressed microtubule associated protein 2 (marker for mature neuron), or glial fibrillary acid protein (marker for glial cell). In addition, some cells integrated into the ganglion cell layer. The integration and differentiation of the transplanted ADSCs in the 5 and 10 PN 7 DPT were better than in the host eye the other age ranges. This study was aimed at demonstrating how the age of host micro environment would influence the differentiation and integration of the transplanted ADSCs. However, it was found that the integration and differentiation into the developing retina were very limited when compared with other stem cells, such as murine brain progenitor cell.

Key words: retina, adipose derived stromal cell, transplantation, integration, differentiation

I. Introduction

Mesenchymal stem cells (MSCs) represent a population of adult stem cells. MSCs have a limited ability for differentiation in comparison to embryonic stem cells. However, unlike the the latter, they do not have the ethical problem, immune rejection response or tumor formation. Further, they also possess the advantages of efficiency and stability [8, 24, 46, 52]. MSCs were first found to originate in osteoprogenitor cells. Subsequently, the differentiation of MSCs into adipocytes, chondrocytes, osteoblasts, and myoblasts *in vitro* and *in vivo* has been diversely reported. MSCs are also viewed as promising candidates for mesodermal defect repair and disease management [6, 13, 33]. In addition, various researches have proven that MSCs can differentiate into neuronal cells [25, 45, 50]. However, this approach faces some difficulties in linical application as it involves painful surgical procedures for the donor to obtain the bone marrow MSCs (BM-MSCs). Further, the BM-MSCs present problems of morbidity and have a low number of cells upon harvest [34, 52]. These limitations have facilitated the investigation of alternative sources of BM-MSCs.

Adipose derived stromal cells (ADSCs) have gained considerable attention as a new cell line for surmounting the limitations of BM-MSCs. Some advantages of ADSCs in comparison to BM-MSCs are as follows: 1) the surgical procedures for obtaining ADSCs are less invasive than those for BM-MSCs; 2) ADSCs can be obtained 100,000 cells per 1 g of adipose, whereas BM-MSCs are obtained only per 0.01% of bone marrow nucleated cells; and 3) massive multiplication of ADSCs is possible [34, 40]. A recent comparative study between ADSCs and BM-MSCs reported that the features of ADSCs are by and large similar to those of BM-MSCs. However, the ability of ADSCs to proliferate is better than that of BM-MSCs. It was also reported that the acquisition process of ADSCs is simpler because they

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can be easily separated by enzymes and their morbidity is also lower than that of BM-MSCs [21, 23, 35]. In view of these advantages, ADSCs are now being recognized as a potentially good material for stem cell transplantation therapy.

The retina, which is a representative of the central nervous system, contains various neurons. Diseases caused by damaged and extinct retinal neurons, such as, macular degeneration, glaucoma, and retinitis pigmentosa, are classified as incurable retinal diseases. A practical strategy is considered which involves transplanting the stem/progenitor cells that support the cells involved in neuronal protection to replace the damaged retinal neurons caused by such diseases as well as for other reasons. The results obtained from research [18, 43] indicated that the neuronal cells survived and integrated into the retina when they were transplanted into the vitreous chamber or the subretinal space using the neuronal stem/progenitor cells derived from the brain and the retina. Interestingly, the researchers also found that the BM-MSCs differentiated into photoreceptors under a specific environment [17, 19, 43, 44].

The developing mouse was selected as the animal model in the present study. This is because the mouse breeds easily and is also amenable to genetic analyses. In addition, the mouse has incomplete eyes after birth and its retina formation for functional vision is in progress [7]. The eye development of a mouse is also relatively well known. In view of these facts, the mouse is the best animal model for stem cell research into the developing eye. In developing stages, various signaling factors involved in neuronal differentiation are activated in the retina and vitreous chamber [1–4, 9, 29, 32, 49]. Accordingly, it was known that multiple factors at each specific developing step would influence the differentiation into the different cell type and that they help to maintain a proper micro environment. Several researches have reported on the importance of specific micro environment for stem cell therapy [37, 48] and, especially, some signaling factors relating to developing stage are already known as key factors for stem cell therapy [26, 39, 47]. For these reasons, we supposed that some special micro environments during developing stages aided the differentiation and integration of the transplanted stem cells into the developing retina.

The goal of the present study was 1) to suggest the usefulness of the ADSCs for stem cell therapy in the developing eye, replacing BM-MSCs because of their several problems as mentioned above, and 2) to provide certain helpful tips to achieve the best micro environment for stem cell transplantation therapy by determining the degrees of the differentiation and integration of stem cell according to the different developing stages in the mouse eye.

II. Materials and Methods

Experimental animals

The guidelines of the National Institutes of Health regarding the Care and Use of Laboratory Animals were diligently followed in all experimental procedures. Mice (*C57/BL6*) were used in this study. Pups of ages 1, 5, and 10 days PN were used. Pups receiving transplantation were allowed to survive for 7, 14, and 28 DPT. Fifteen pups were allocated at each survival time so that 45 pups in total were used at each developing stage.

Cell culture and labeling

Human ADSCs (Cat# SV30102, Cellular Engineering Technologies Inc., Coralville, IA, USA) were used. ADSCs were obtained from the laboratory of Dr. D-H Hwang (CHA University). The cells were cultured in a medium containing α-MEM (Invitrogen Corp., Carlsbad, CA, USA), 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-Glutamine (Invitrogen), and 1% Antibiotic-Antimycotic (Invitrogen). ADSCs were labeled with Lenti hCMV-GFP-IRES puro (Macrogen Inc., Seoul, Korea) in order to identify the cell after transplantation. ADSCs were infected with a stock lenti-virus concentration of 2×10^6 infection units/μl. The infection efficiency is approximately more than 80% (Fig. 1).

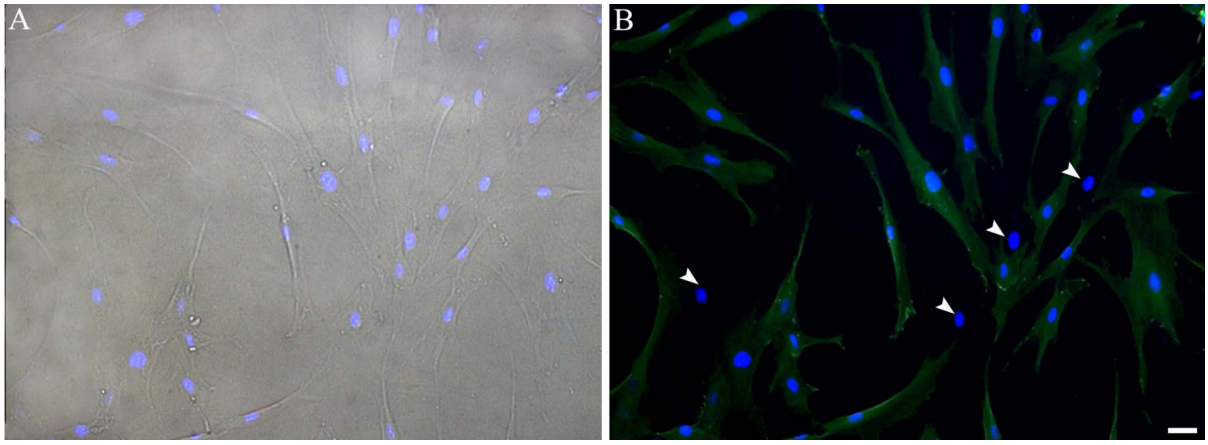

Fig. 1. The transduction efficiency of lentiviral vector in the adipose derived stromal cells. (**A**) Merged image of the differential interference micrograph and DAPI staining picture of adipose derived stromal cells. (**B**) Merged image of the GFP positive cells and DAPI staining of the adipose derived stromal cells. Arrowheads indicate cells that are not GFP positive. Bar=50 μm.

Transplantation of ADSCs into the developing retina

Animals were anesthetized using a mixture of ketamine hydrochloride (30–40 mg/kg) and xylazine (3–6 mg/kg). Pups of 1, 5, and 10 PN received intraocular ADSCs injected through the dorsolateral aspect of the eye using a 30 gauge Hamilton syringe (Hamilton Co., Reno, NV, USA). One μL of cell suspension (~50,000 cells/μl) was slowly injected into the vitreous chamber of the pups. Pups receiving the transplanted ADSCs were allowed to survive for 7, 14, and 28 DPT.

Tissue preparation and immunohistochemistry

Tissue fixation, sectioning, and immunohistochemistry were performed, as previously described [48]. The heads (pups) and eyes (young mice) were quickly removed, fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, and then cryoprotected in a series of 10, 20, and 30% sucrose in 0.1 M phosphate buffer. The tissue was embedded (Tissue-Tek; OCT compound; VWR International, West Chester, PA, USA), frozen, and sectioned coronally at 20 μm thickness using a cryostat. The sections were thaw-mounted onto Superfrost microscope slides (Fisher Scientific, Pittsburgh, PA, USA) and stored at –20°C until further processing. The primary antibodies and the concentration were as follows; mouse anti-MAP2ab antibody (mature neuronal cell marker, 1:300, Abcam Inc., Cambridge, MA, USA), mouse anti-GFAP antibody (glial cell marker, 1:300, Abcam), and rabbit anti-GFAP antibody (glial cell marker, 1:500, Sigma, St Louis, MO, USA). The standard immunocytochemical techniques and immunohistochemical methods have been described previously in detail [48]. For detection by immunofluorescence, the secondary antibody Cy3 conjugated anti-mouse (1:200, Jackson ImmunoResearch Lab., West Grove, PA, USA) and Cy3 conjugated anti-rabbit (1:200, Jackson ImmunoResearch Lab.) were used. After immunohistochemistry, the tissues were stained with DAPI (1:500, Sigma). The labeled tissues were coverslipped with a Vectashield mounting medium (Vector Lab. Burlingame, CA, USA).

Data analysis

The retina tissues transplanted with ADSCs were compared with the control retina tissues and the normal development of the retina transplanted ADSCs was examined. The labeled tissues were examined and photographed using a Zeiss Axioplan microscope with high power differential interference contrast (DIC) optics. The images were viewed on a computer monitor using a Zeiss Plan-Apochromat 100× objective and a Zeiss AxioCam HRc digital camera (Carl Zeiss Meditec Inc., Jena, Germany).

III. Results

Cellular differentiation of the transplanted ADSCs in the developing mouse eye

Several transplanted ADSCs were found in the host eye of 5 and 10 PN 7 DPT. In Figures 2A, C and E, they showed DAPI labeling images. The transplanted ADSCs at 5 PN 7 DPT are illustrated in Figures 2B and D. In Figure 2B, it can be observed that the ADSCs differentiated morphologically into the cell with bidirectional dendrites (arrow) and multipolar processes (arrowhead). In Figure 2D, it can be seen that the transplanted ADSCs displayed morphological differentiation with the branched processes emerging from the primary process. The transplanted ADSCs at 10 PN 7 DPT are illustrated in Figures 2E and G. In Figure 2E, it can be seen that the transplanted ADSCs differentiated morphologically into the multipolar stellate cell with the processes oriented in several directions in the ganglion cell layer (GCL). In Figure 2G, it can be observed that the transplanted ADSCs displayed morphological differentiation into the cell with horizontally-oriented long bidirectional processes.

Antibodies directed against MAP2ab and GFAP were used to determine whether the transplanted ADSCs expressed differentiated markers within the environment of the eye. A subpopulation of the GFP immunoreactive cells was clearly found to co-express MAP2ab (microtubule-associated protein 2 which is found in mature neurons) and GFAP (glial fibrillary acid protein present in glial cells). The transplanted ADSCs at 10 PN 7 DPT are illustrated in Figures 3A–L, and the transplanted ADSCs at 5 PN 7 DPT are illustrated in Figures 3M–O.

In Figures 3A–F, the transplanted ADSC in the GCL expressed MAP2ab, while the transplanted ADSC in the vitreous chamber expressed MAP2ab in Figures 3G–I. Lastly, it can be seen that in Figures 3J–L, the transplanted ADSC in the GCL expressed GFAP, while in Figures 3M–O, the transplanted ADSC in the vitreous chamber expressed GFAP.

Integration of the transplanted ADSCs into the developing mouse retina

The transplanted ADSCs integrated into the developing mouse retina are illustrated in Figure 4. In Figures 4A, C and E, they showed the DIC images which correspond to each fluorescent image. The transplanted ADSCs at 5 PN 7 DPT retina are illustrated in Figures 4B and D. In Figure 4B, it can be seen that the ADSCs integrated into the GCL and morphologically differentiated with horizontally-oriented long processes. In Figure 4D, the transplanted ADSCs integrated into the GCL and morphologically differentiated into a horizontally-oriented, spindle shaped cell body, with long branched processes. In Figure 4F, it can be seen that the transplanted ADSCs differentiated morphologically into the multipolar stellate cell.

In the present study, there was a limited availability of ADSCs to integrate into the retina. Thus, it was not possible to determine whether the transplanted ADSCs integrated into the inner nuclear layer (INL) and the outer nuclear layer (ONL). The most effective transplantation stage and the duration time of the transplanted ADSCs in the retina are shown in Table 1. It can be observed that the differentiation and integration of ADSCs mainly occurred at 5, 10 PN

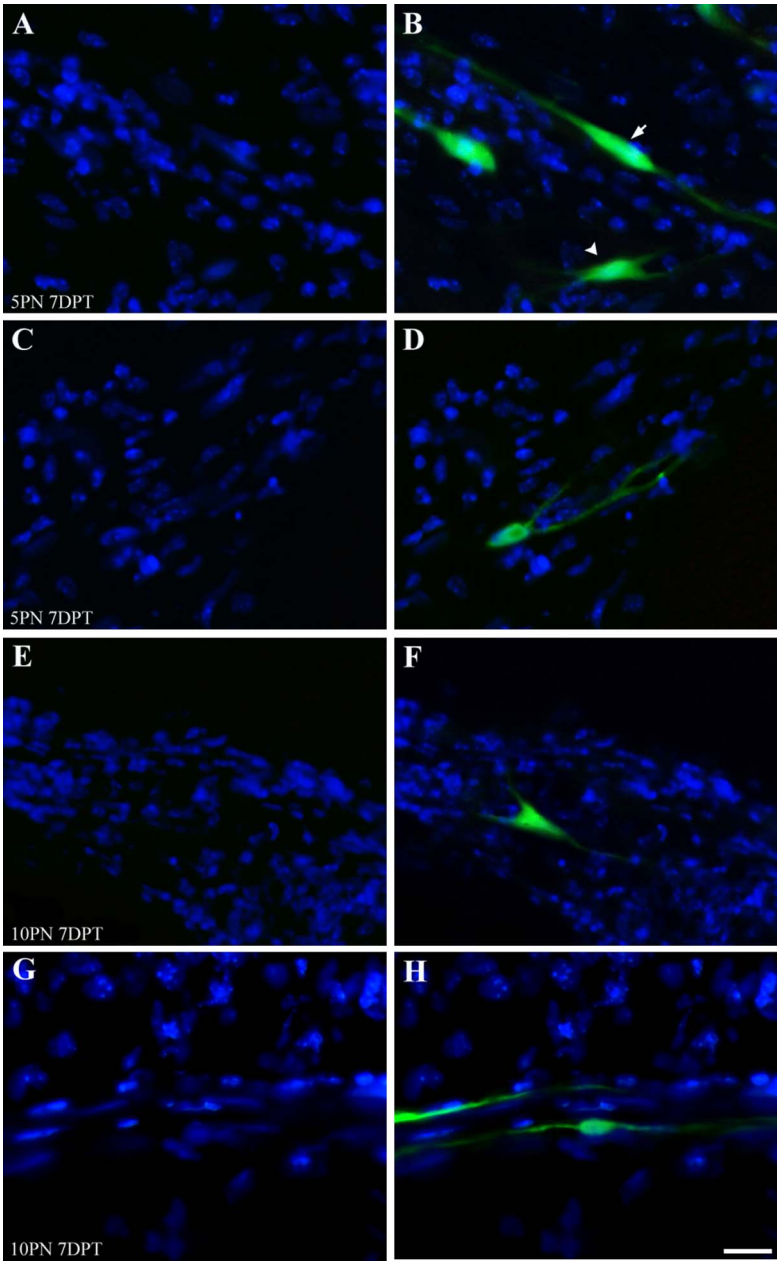

Fig. 2. Morphological differentiation of transplanted adipose derived stromal cells. (**A, C, E, G**) Images of DAPI staining (**B**) The transplanted adipose derived stromal cells morphologically differentiated into the cell with bidirectional dendrites (arrow) and multipolar processes (arrowhead) in the vitreous chamber. (**D**) The transplanted adipose derived stromal cell illustrated the morphological differentiation into cells with branched processes in the vitreous chamber. (**F**) The transplanted adipose derived stromal cell morphologically differentiated into the multipolar cell in the ganglion cell layer. (**H**) The transplanted adipose derived stromal cell illustrated the morphological differentiation into the cell with bidirectional dendrites in the vitreous chamber. (**A–D**) 5 PN 7 DPT, (**E–H**) 10 PN 7 DPT. Bar=20 μm.

rather than 1 PN. These results indicate that the host micro environment, depending on the development stage, influences the transplantation and integration of ADSCs. In addition, except for 7 DPT, the differentiation and integration of the transplanted ADSCs were rarely observed in the case of 14, 28 DPT. Thus, it was concluded that the transplanted ADSCs did not survive for a long duration of time.

IV. Discussion

The present study was carried out to determine whether the transplanted ADSCs can be morphologically integrated and molecularly differentiated into the retina of a developing mouse. Further, the study also examined how the host micro environment in the developing mouse eye would influence the transplanted ADSCs.

Some researches have demonstrated ADSCs to exhibit the potential for stem cell therapy. For example, ADSCs transplanted into the myocardial scar tissue formed cardiac islands and vessel-like structures that induced angiogenesis, thereby improving the cardiac function and vasculogenesis [28, 51]. In addition, the ADSCs also differentiated into the neuronal cells. Although studies about ADSCs differentiated into the neuronal cells *in vivo* are not sufficient, some researches suggest that ADSCs exhibited a capability of differentiation into the neuronal cells *in vitro* [10, 36]. In addition, it was also reported that the intracerebral transplantation of ADSCs after cerebral ischemia in rats could improve the neurological deficit [15]. In the present study

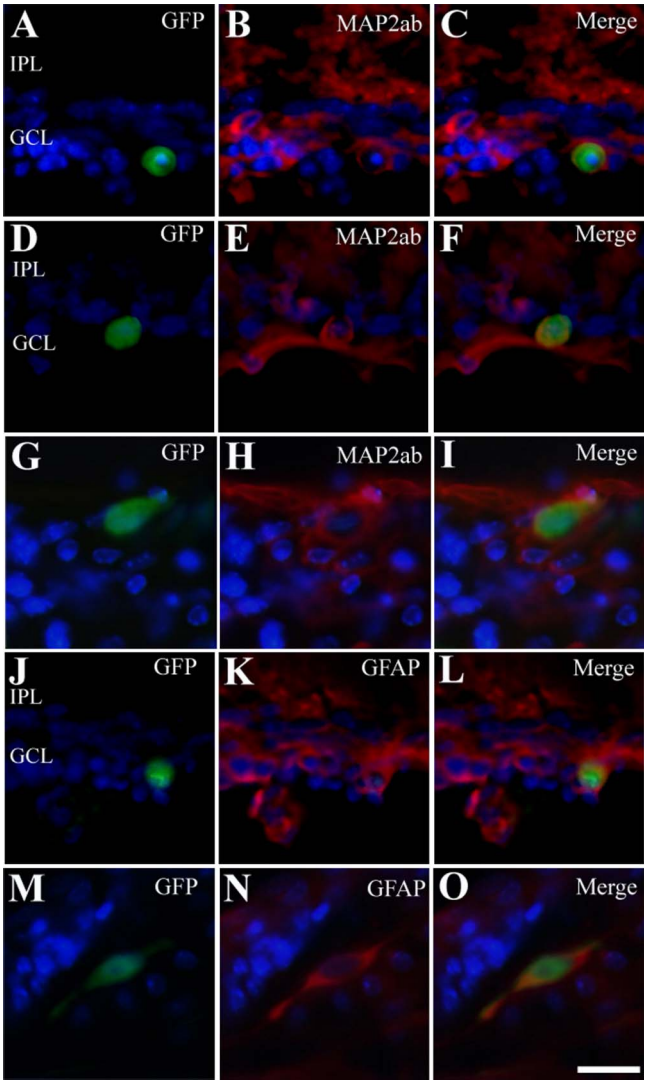

Fig. 3. Cellular differentiation of transplanted adipose derived stromal cells. (**A, D, J**) GFP-expressing human adipose derived stromal cells in the ganglion cell layer. (**G, M**) GFP-expressing human adipose derived stromal cells in the vitreous chamber. (**B, E, H**) MAP2ab-immunoreactive adipose derived stromal cells. (**K, N**) GFAP-immunoreactive human adipose derived stromal cells. (**C, F, I, L, O**) Merged images (**A–L**) 10 PN 7 DPT, (**M–O**) 5 PN 7 DPT. Bar=20 μm.

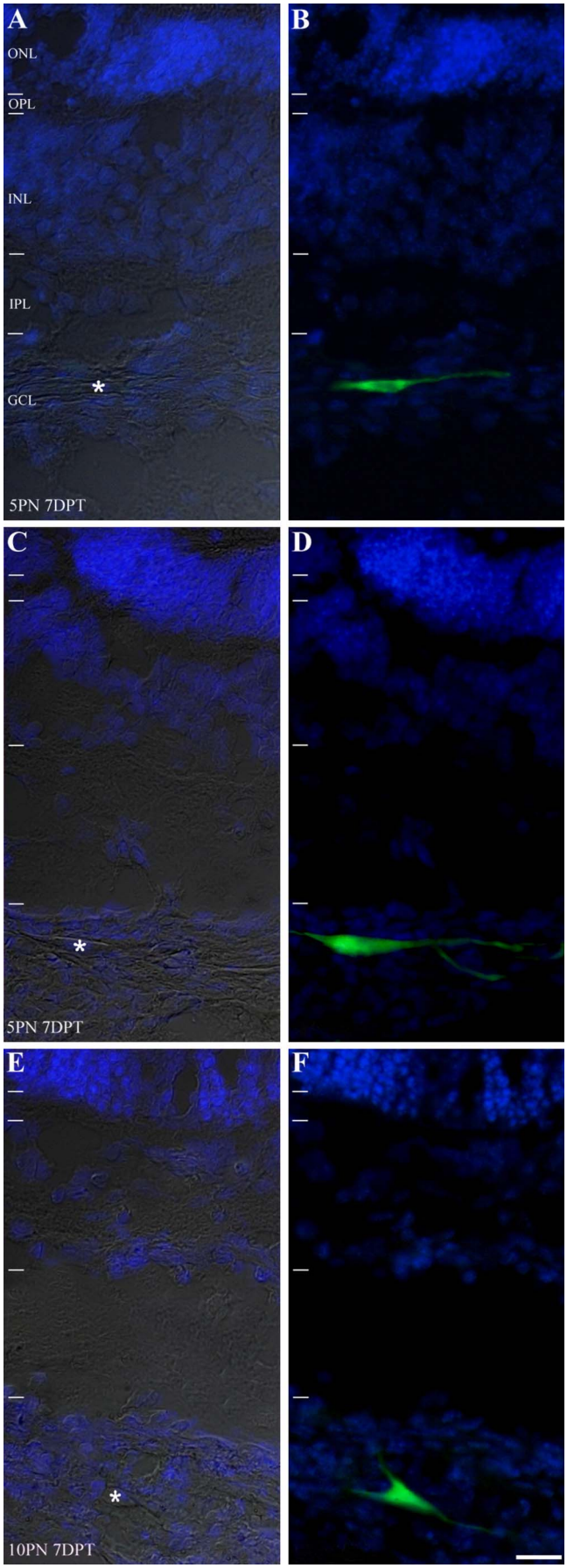

Fig. 4. Integration of transplanted adipose derived stromal cells. (**A, C, E**) Merged image of the differential interference micrograph and DAPI staining picture of the integrated adipose derived stromal cells in ganglion cell layer. Asterisks indicate GFP+ cell bodies. (**B** and **D**) The transplanted adipose derived stromal cell integrated into the ganglion cell layer at 5 PN 7 DPT retina. (**F**) The transplanted adipose derived stromal cell integrated into the ganglion cell layer at 10 PN 7 DPT retina. Bar=20 μm.

Table 1. *Morphological and phenotypic differentiation of human adipose derived stromal cells in the developing vitreous body and retina*

	1PN			5PN			10PN		
	7DPT	14DPT	28DPT	7DPT	14DPT	28DPT	7DPT	14DPT	28DPT
Morphological differentiation	–	–	–	+++	–	–	+++	++	–
Integration	–	–	–	+	–	–	+	–	–
Map2ab expression	–	–	–	++	–	–	++	–	–
GFAP expression	–	–	–	++	–	–	++	–	–

+++; ≥30%, ++; 10~30%, +; 1~10%, –; rare

%=Cells of Morphological differentiation, Integration or Expression/Total survival transplanted cells ×100

it was suggested that the transplanted ADSCs differentiated in the developing eye. It was observed that, although the transplanted ADSCs did not integrate into the other retinal cell layer, except in the GCL, the transplanted ADSCs morphologically differentiated and fully displayed an ability of differentiation into the various retinal cell types, thereby showing expressions of MAP2ab and GFAP.

Some transplantation researches have used the different cell lines into the eye. Lamba *et al.* (2009) demonstrated that the embryonic stem cell can differentiate into the retinal cells and be used as a therapy for retinal diseases [20]. However, the embryonic stem cell presents some problems which must be solved, such as ethical issues, immune rejection response, and tumor formation. Researches using the neural stem/progenitor cells such as mBPC (murine brain progenitor cell), mRPC (murine retinal progenitor cell) and AHPC (adult rat hippocampal progenitor cell), have also been published [31, 37, 38, 41, 48]. When the mBPCs were transplanted [48], they functionally integrated into several cell layers of the retina and morphologically differentiated into a retinal cell-like shape. In addition, mBPC expressed MAP2 which has been used to identify the neuronal cells, and calretinin which has been used as a marker of a subclass of horizontal, amacrine and ganglion cells. In addition, the mBPCs also expressed the recoverin protein. Further, BM-MSCs differentiated into the various retinal cells [44] and delayed the retinal degeneration in the RCS (Royal College of Surgeons) rat model of retinal degeneration [14]. When the BM-MSCs were transplanted into the rat eye, injured by ischemia/reperfusion, they did not migrate well. However, a few cells integrated into the GCL and lowered the reduction of the ganglion cell [22].

In the present study, it was demonstrated that the transplanted ADSCs morphologically differentiated and displayed the expression of the neuronal cell and glial cell markers and integrated into the GCL. The molecular and morphological differentiation and the retinal integration were limited in this study when compared to the embryonic stem cell and the neural stem/progenitor cell. However, the ability of molecular and morphological differentiation and integration in the retina was clearly demonstrated. Thus, ADSCs are considered as one of the candidates for effective therapeutic research in the future.

During the developing stage, diverse signaling is involved in regulation of the gene expression, formation and development of the necessary organ. The mouse has incomplete eyes after birth and its retina formation for functional vision is in progress [7]. Consequently, in the developing mouse eye, various signaling factors, such as Wnt, Shh, BMP and FGF, related with development are active [1, 9, 49]. The morphogenetic signals are recycled, which provide guidance information to retinal ganglion cell axons during their growth toward their synaptic targets [5]. FGF signaling also contributes in positioning the presumptive retinal cells in the right place at the right time [30]. In addition, even if the evidence is still insufficient for age-dependent expression profile of ocular growth factors or cytokines in the vitreous fluid, diverse factors such as VIGF, IGF-1, GH, TGFβ and EGF are involved with survival and differentiation of retinal cell in vitreous fluid [3, 4, 29, 32]. Thus, differentiation and integration of the transplanted ADSCs in the host environment is influenced by various growth factors in vitreous body and retina. In addition, the various factors also exist and influence differentiation and integration into the retinal specific cell. In the present research, the transplanted ADSCs at 5, 10 PN displayed better morphological and molecular differentiation and integration than that of 1 PN. The micro environment of the 5 and 10 PN retina is distinct from the differentiation of the bipolar cells and Müller cells in comparison to that of 1 PN [7]. The bipolar cells and Müller cells are mainly differentiated at 5 and 10 PN. Mash1, Math3 and Chx10 are involved in the differentiation of the bipolar cell in the developing retina. Rax, Hes1, Hes5, Hesr2 and notch1 promote the formation of the Müller cell [11, 12]. Thus, it is likely that the various factors involved in the differentiation of the bipolar and Müller cell can influence the differentiation and integration of the transplanted ADSCs into the developing retina. For better differentiation and integration of the ADSCs, the present study suggests that the host micro environment at 5, 10 PN eye should have specific signaling characters or a different timing and strength of each signal. For example, it was reported that one type of the matrix metalloproteinase (MMP), MMP2, raised the survival rate of the transplantation and 17 beta-estradiol and concanavalin A facilitated the movement of the transplanted cells [42]. In addition, retinoic acid (RA) [16], neurocytokine ciliary neurotrophic factor (CNTF) [35] and the glial cell line derived neurotrophic factor (GDNF) [27] are all related with the photoreceptor survivals.

The ADSCs survived after xenotransplantation and the

transplanted hosts had the absence of immunosuppression in the present research. These could be due to the relative purity of cultured ADSCs, which lack donor adipose antigen-presenting cells and have no passenger leukocyte induced transplant rejection [48].

To conclude, the present study demonstrated that the transplanted ADSCs survived and showed morphological and molecular differentiation with expressions of MAP2ab and GFAP. Some of the transplanted cells integrated into the host retina. The study also demonstrated how the developing stage of the host environment influenced the differentiation and integration of the transplanted ADSCs. The morphological and molecular differentiation and the integration into the retina were very limited in comparison to other stem cells. However, the study provided some evidence for better developmental stages and for the analysis of better micro environmental signaling conditions for transplantation in the future.

V. Acknowledgments

We wish to thank Language Edit for assistance with proofreading. This work was supported in part by the Korea Research Foundation Grant funded by the Korea Government, KRF-2008-313-E00367.

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