ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Acta Neurobiol Exp 2007, 67: 359–365

Human cord blood cells can differentiate into retinal nerve cells

Naoko Koike-Kiriyama,[1,2] Yasushi Adachi,[1,3] Keizo Minamino,[1,2] Masayoshi Iwasaki,[1] Keiji Nakano,[1] Yasushi Koike,[1] Haruhiko Yamada,[2] Hiromi Mukaide,[1] Akio Shigematsu,[1] Tomomi Mizokami,[1] Miyo Matsumura,[2,3] and Susumu Ikehara[1,3]

[1]First Department of Pathology, Kansai Medical University, Moriguchi City, Osaka, 570-8506, Japan; [2]Department of Ophthalmology, Kansai Medical University; [3]Regeneration Research Center for Intractable Diseases, Kansai Medical University

Abstract. Retinal degeneration and dystrophy are the major causes of blindness in the developed world. It has been reported that human cord blood cells (HCBCs) can differentiate into neuron-like cells *in vitro*. We have recently demonstrated that bone marrow cells (BMCs) of both mice and rats can differentiate into retinal nerve cells (RNCs). In the present study, we show the differentiation capacity of HCBCs into RNCs *in vivo*. We transplanted lineage-negative HCBCs into the subretinal space of severe combined immunodeficiency (SCID) mice. Two weeks after the transplantation, some of the transplanted cells expressed human nestin, human MAP2, human neuron specific enolase (NSE), β-III tubulin and also rhodopsin. These results indicate that HCBCs can differentiate into RNCs and suggest that our new strategy could be used for the regeneration of retinal nerve cells in degenerative or dystrophic diseases.

Correspondence should be addressed to S.Ikehara, Email: ikehara@takii.kmu.ac.jp

Key words: human cord blood, retinal nerve cell, stem cells, regeneration

INTRODUCTION

Retinal degeneration and dystrophy are the major causes of blindness in the developed world (Gavrieli et al. 1992). Several strategies, such as drug therapy, the transplantation of sensory retina, and electrical devices (Humayun et al. 1999, Turner and Blair 1986), to stimulate optic nerves or retinal neurons, have been proposed in an attempt to restore vision or at least retard the progression of diseases. However, no effective strategies for inhibiting or curing retinal degeneration and dystrophy have been developed. We and other researchers have recently reported that both neural stem cells and bone marrow cells (BMCs) can differentiate into retinal neural cells (Minamino et al. 2005, Nishida et al. 2000, Tomita et al. 2002), suggesting a new strategy for the regeneration of retinal nerve cells (RNCs) in degenerative or dystrophic diseases.

Cord blood is easily obtained and has been utilized for the transplantation for hematopoietic stem cells, mainly as the therapy for hematopoietic diseases (Broxmeyer et al. 1989, Gluckman et al. 1997). Recent reports suggest that human cord blood cells (HCBCs) can differentiate into several kinds of lineage cells; such as hepatocytes (Ishikawa et al. 2003), neural cells (Kogler et al. 2004), osteoblasts, chondroblasts, adipocytes (Kogler et al. 2004) and endothelial cells (Murohara et al. 2000). These results suggest the great potential of HCBCs for regeneration therapy of various organs. However, it has remained unclear whether HCBCs can differentiate into RNCs.

In the present study, we show that lineage-negative human cord blood nuclear cells (Lin^-HCBNCs) can differentiate into RNCs.

METHODS

The animals were handled in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the Kansai Medical University Guide for the Care and Use of Laboratory Animals.

Preparation of cells for grafting

Human cord blood was obtained after approval of a local Ethics Committee and the informed consent of the mothers. The blood was collected into a blood collection bag (Terumo, Tokyo, Japan) containing anticoagulant. Within 12 hours after acquisition of the blood, the mononuclear fraction was isolated using Lymphoprep solution (Nycomed Pharma; Oslo, Norway). Lin^-HCBNCs were enriched by negative selection using anti-human antibodies (Abs) and the immunomagnetic beads were coated with anti-mouse IgG (Dynabeads, Oslo, Norway). The anti-human Abs used were anti-CD3, anti-CD8, anti-CD4, anti-CD56, anti-CD11c, anti-CD11b, anti-CD14, anti-CD15, anti-CD16, anti-CD19, anti-CD20, anti-Glycophorin A Abs (BD Biosciences Pharmingen, San Jose, CA, USA). Lin^-HCBNCs obtained in this way consisted of $CD34^+$ cells (51.7 ± 12.7%) and $CD34^-$ cells. The cells were labeled using PKH26 (red fluorescence; MINI-26; Sigma; Saint Louis, MO, USA), and the cells were injected into the murine eyes immediately after labeling.

Animal preparation and transplantation procedure

Severe combined immunodeficiency (SCID) mice were obtained from Shimizu Laboratory Supplies (Kyoto, Japan). The mice were injected with anti-asialo GM1 Ab to kill NK cells. Five microliters of the cell suspension, containing 2×10^5 cells, was slowly injected into the subretinal space with the injector, as previously described (Minamino et al. 2005, Tomita et al. 2002).

Tissue sectioning

Two weeks after transplantation, the animals were deeply anesthetized by inhalation of diethyl ether, and were then sacrificed. Ten-micrometer frozen sections of the eyes were prepared in a cryostat with sections including the injury site for hematoxylin-eosin (HE) staining or immunohistochemical staining.

Immunohistochemistry

The specimens, fixed with 4% paraformaldehyde, were stained with primary antibodies. Antibodies (Abs) used in this study were as follows: rabbit polyclonal anti-human nestin (h nestin) Ab for immature cells containing neural stem cells (1:200,Chemicon, Temecula, California, USA), and rabbit polyclonal anti-human microtubule-associated protein 2 (h MAP2) Ab, mouse monoclonal anti-human neuron

specific enolase (NSE) Ab and mouse monoclonal anti-β-III tubulin Ab for neural cells (1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), and mouse monoclonal anti-rhodopsin Ab and rabbit polyclonal anti-rhodopsin Ab for the rod photoreceptors (1:200, Cosmo bio, Tokyo, Japan). After conjugation with rabbit primary antibodies, the specimens were incubated with FITC-labeled goat anti-rabbit Abs (secondary antibody). To label mouse Abs, we used a "Zenon Labeling Kit" (Molecular Probes, Eugene, OR). Nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI)-containing mounting medium (Vector Laboratories, Ins., Burlingame, CA, USA). The stained specimens were observed using a laser-scanning confocal microscope (LSM510-META, Carl Zeiss, Oberkochen, Germany).

RT-PCR and real time PCR

cDNA from each eyes was prepared as previously described (Adachi et al. 1999). We performed real time PCR on the cDNA using OPTICON2 (MJ Research, Waltham, MA, USA), QuantiTect SYBR Green PCR Kit (QIAGEN, Hilden, Germany) and human GAPDH-specific primers (QIAGEN) or murine GAPDH-specific primers (QIAGEN). RT-PCR were performed using human rhodopsin-specific primers designed and synthesized by Sigma (F: catgctcaccaccatctgctg, R: taagacctgcctaggactctgtg) with Takara PCR Thermal Cycler MP (Takara). PCR products were separated on a 1.2% agarose gel (Gibuco BRL, Rockville, MD, USA) and visualized by ethidium bromide (Nakarai, Kyoto, Japan) staining. To estimate the expression of RNA of human G3PDH, we prepared a standard curve of human G3PDH, using cDNA from human retina RNA. Human retina cDNA was determined as 100 relative units and units of human G3PDH of other cDNA were estimated. To measure relative units of G3PDH of murine G3PDH, we prepared a standard curve using cDNA from the eye of a SCID mouse (cDNA of SCID mouse as 100 relative units). And we estimated the expression of murine G3PDH of other cDNA. We repeated the experiment 3 times.

Statistical analyses

The results are represented as mean ± SD. The significance of the data was determined by a Student's *t*-test.

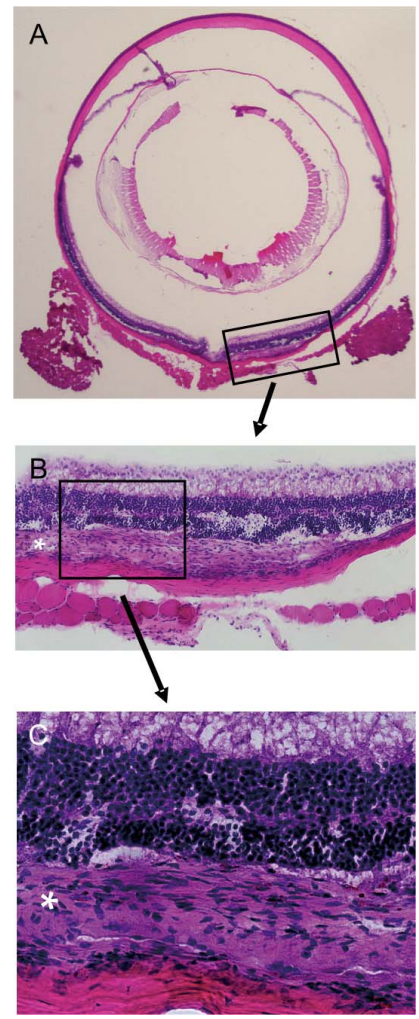

Fig. 1. Lin$^-$HCBNCs were transplanted into the subretinal space of SCID mice that had been treated with anti-asialo GM1 Ab. Two weeks after injection, morphological changes of the Lin$^-$HCBNCs-injected eyes were examined using HE staining. (A), (B), and (C) show a low-power view (original magnification: ×10), a mid-power view (original magnification: ×25) and a high-power view (original magnification: ×100) of the Lin$^-$HCBNC-injected eye. (*) thickened subretinal space. Representative data are shown from 4 independent experiments.

RESULTS

Morphological changes induced by subretinal injection

As shown in Fig. 1, a thickened subretinal space was detected in the Lin$^-$HCBNC-injected eyes. The cells in the subretinal space were morphologically heterogeneous; some showed large cytoplasm with round

nucleus, and some showed a spindle shape with an oval nucleus. No such morphological changes were observed in the eyes injected with saline subretinally as a control (data not shown). These results suggest that injected Lin$^-$HCBNCs existed in the subretinal space, and that they differentiated into several kinds of cells since they showed a variety of morphologies.

Differentiation from Lin$^-$HCBNCs into RNCs

We next examined whether the injected Lin$^-$HCBNCs differentiated into RNCs. As shown in Fig. 2, PKH 26-positive Lin$^-$HCBNCs were detected in the injected retina (Fig. 2A, D, G, J, M, and P). Next, we stained the retinas with anti-h nestin Ab (which reacts only with human nestin), anti-h MAP2 Ab (which reacts only with human MAP2), anti-h neuron specific enolase (NSE) Ab (which reacts only with human NSE), anti-β-III tubulin Ab (which reacts with both human and murine β-III tubulin), monoclonal anti-rhodopsin (which reacts with both human and murine rhodopsin) and polyclonal anti-rhodopsin Ab (which reacts with both human and murine rhodopsin); no Abs for only human β-III tubulin, or

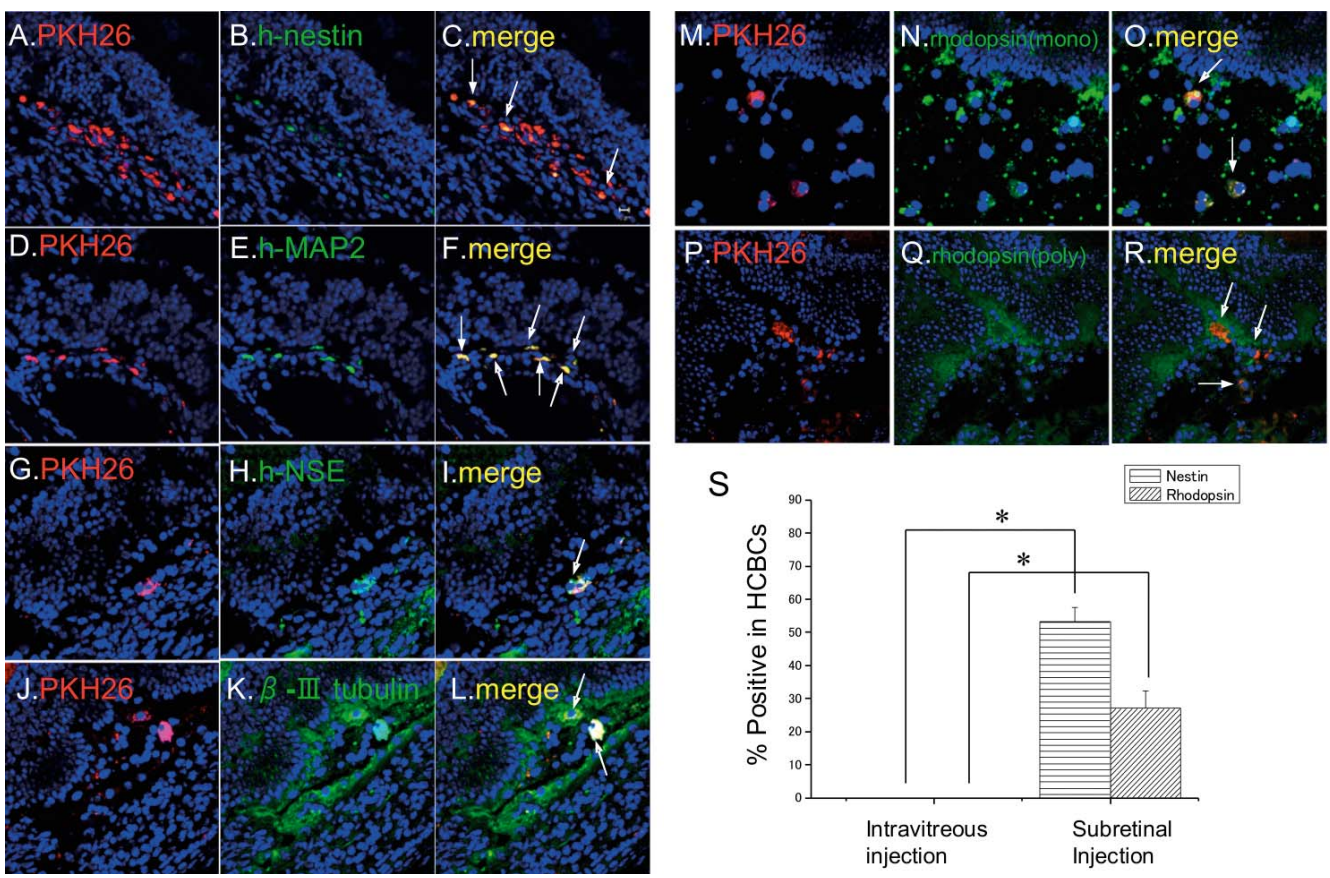

Fig. 2. PKH 26-labeled Lin$^-$HCBNCs can differentiate into RNCs in the mouse retina. Lin$^-$HCBNCs were transplanted into the subretinal space of SCID mice that had been treated with anti-asialo GM1 Ab. Two weeks after treatment, the mice were sacrificed and the eyeballs were collected for examination. The specimens were stained with anti-h nestin (B, C), anti-h MAP2 (E, F), anti-h NSE (H, I), anti-β-III tubulin (K, L), rhodopsin (monoclonal Ab) (N, O) or rhodopsin (polyclonal Ab) (Q, R) Abs. Lin-HCBNC-derived cells that were labeled with PKH 26 are shown in red (A, C, D, F, G, I, J, L, M, O, P, R). H nestin-positive cells (B, C), h MAP2-positive cells (E, F), h NSE-positive cells (H, I), β-III tubulin-positive cells (K, L), rhodopsin-positive cells (detected by monoclonal Ab) (N, O) and rhodopsin-positive cells (detected by polyconal Abs) (Q, R) are shown in green. In (C), (F), (I), (L), (O), and (R), the Lin$^-$HCBNC-derived cells expressing each neurogenic marker are shown in yellow (arrows). Representative data are shown from 4 independent experiments. Quantitative analyses of Lin$^-$HCBNCs into nerve cells were performed. In the anti-h-nestin stained section or the anti-rhodopsin-stained section of eyes injected with PKH-labeled Lin$^-$HCBNCs subretinally or intravitreously, nestin-positive or rhodopsin-positive cells in PKH-positive cells were counted in high power view, and the numbers of a high power field were counted. Means and SDs were shown in (S) (n=4).

rhodopsin were available. Nestin has been reported as a marker for immature cells containing neural stem cells, immature neurons and immature glial cells (Kaneko et al. 2000), while MAP2, neuron specific enolase (NSE) and β-III tubulin have been reported as markers of neurons (Kaneko et al. 2000). Figure 2B and C, E and F, H and I, or K and L show cells expressing human nestin, human MAP2, human NSE or β-III tubulin in the retinas, and some of the cells were also positive for PKH26. These results suggest that Lin$^-$HCBNCs differentiated into neural cells. Next, we examined the expression of rhodopsin, which is expressed in photoreceptors. Some of the injected Lin$^-$HCBNC-derived cells expressed rhodopsin (Fig. 2N and O, Q, and R). These results suggest that Lin$^-$HCBNCs can differentiate into not only neural cells but also RNCs. We also performed quantitative analyses of the effects of subretinal injection of Lin$^-$HCBNCs on differentiation from Lin$^-$HCBNCs into nerve cells between subretinal injection and intravitreous injection. We prepared frozen samples from the eyes and we compared numbers of nestin-positive or rhodopsin-positive Lin$^-$HCBNCs in the eyes. As shown in Fig. 2S, many more Lin$^-$HCBNCs injected into the subretinal space differentiated into nestin-positive or rhodopsin-positive neural cells than those injected into the intravitreous space.

Eyes injected with Lin$^-$HCBNCs expressed human GAPDH and human rhodopsin mRNA

To confirm survival of human derived cells in transplanted eyes, we performed real time RT-PCR to detect human GAPDH and we also performed RT-PCR to detect human specific rhodopsin. As shown in Fig. 3A, mouse GAPDH was expressed in eyes from SCID mouse and the murine eyes injected with Lin$^-$-HCBNCs. Human GAPDH was expressed not only in Lin$^-$HCBNCs, human retina but also the murine eyes injected with Lin$^-$HCBNCs. Next we examined expression

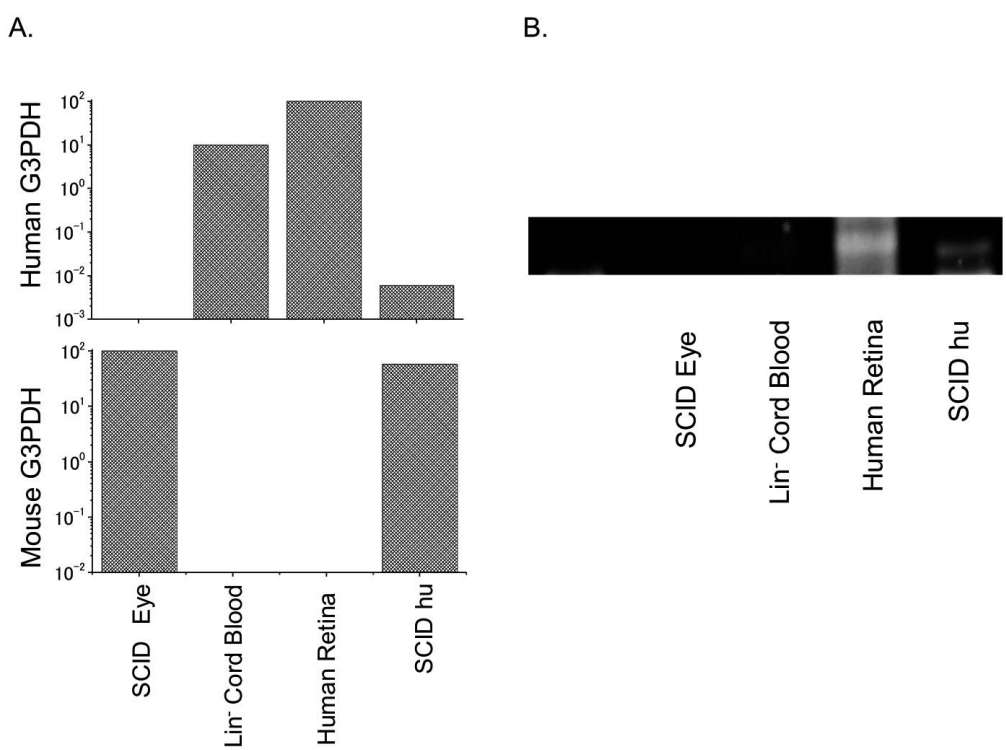

Fig. 3. Expression of mRNA of human GAPDH and human rhodopsin in eyes injected with Lin$^-$HCBNCs. cDNA was prepared from murine eyes that were injected with Lin$^-$HCBNCs. Each sample was prepared from a single eye. Controls were prepared from saline-injected eyes of SCID mouse and RNA of human retina. Real time PCR for detection of human GAPDH and murine GAPDH (A), and PCR for detection of human rhodopsin (B) were carried out using the cDNAs, described in the Methods. Y axis in (A) shows relative units of human GAPDH or murine GAPDH. We repeated the experiments 3 times, and the means and standard deviations are shown. In (A), we show means and standard deviations of the data, and we show the representative data in (B).

of human rhodopsin. We did not detect mRNA of human rhodopsin in eyes of the SCID mouse or Lin$^-$HCBNCs. However, we detected mRNA of human rhodopsin from both human retina and murine eyes injected with Lin$^-$HCBNCs. These results suggest that Lin$^-$HCBNCs can survive and differentiate into RNCs in the murine eyes.

DISCUSSION

Several sources of RNCs have been proposed, including neural stem cells (Nishida et al. 2000), cells in the ciliary marginal zone (CMZ) (Haruta et al. 2001, Hasegawa 1965), embryonal stem (ES) cells (Ikeda et al. 2005) and BMCs (Minamino et al. 2005, Tomita et al. 2002). Neural stem cells exist in the hippocampal region and subventricular zone, which is very difficult to reach (Eriksson et al. 1998). Cells in the mammalian CMZ have shown reduced regenerative ability (Haruta et al. 2001). ES cells have the great capacity to differentiate into various organs, but sometimes they produce teratoma (Fujikawa et al. 2005). Auto BMCs do not suffer in transplantation from the problem of the human leukocyte antigen (HLA) barrier, but, since these BMCs would also carry the same abnormal gene as the RNCs in hereditary diseases, the disease(s) could redevelop (Sugihara et al. 1999). These sources therefore suffer from several problems in their application in human retinal regeneration therapy, suggesting that HLA-matched cord blood from normal persons might be a suitable source of hematopoietic stem cells.

Recent data suggest that BMCs are sometimes fused with other type of cells, resulting in BMCs appearing to differentiate into some other type of cells (Wang et al. 2003). In our experiment, neither Lin$^-$HCBNCs nor cells in the murine retina express human specific neural marker, and neither express mRNA of human rhodopsin. After injection of Lin$^-$HCBNCs into the eyes of SCID mice, we found both human specific neural marker and mRNA of human rhodopsin. These suggest that the the injected cells expressing human neural cell specific marker and mRNA are really trans-differentiated into RNCs.

The mechanism underlying the differentiation into RNCs from Lin$^-$HCBNCs is not clear. However, it has been already reported that the environment of stem cells has a profound effect on the differentiation of the stem cells (Bratincsák et al. 2007, Metallo et al. 2007). In our experiment, the freshly isolated Lin$^-$HCBNCs did not express rhodopsin but Lin$^-$HCBNCs injected into the eyes of SCID mice expressed not only neural specific marker but also rhodopsin. These results suggest that the tissues of the eyes surrounding Lin$^-$HCBNCs promoted the differentiation of the Lin$^-$HCBNCs into RNCs.

To our knowledge, this is the first report showing the differentiation of cord blood cells into RNCs. These results suggest that cord blood is a potential candidate for retinal regeneration therapy.

CONCLUSION

We transplanted Lin$^-$HCBNCs into the subretinal layer of severe combined immunodeficiency (SCID) mice that had been treated with anti-asialo GM1 antibody. Two weeks after the transplantation, some of the transplanted cells expressed human nestin, human MAP2, h NSE, β-III tubulin, and also rhodopsin. These results indicate that HCBCs can differentiate into RNCs.

ACKNOWLEDGMENTS

We thank Ms. Y. Tokuyama, Ms. M. Murakami-Shinkawa, Ms. S. Miura, Ms. K. Hayashi and Ms. A. Kitajima for their expert technical assistance, and also Mr. Hilary Eastwick-Field and Ms. K. Ando for the preparation of this manuscript. Grant support: a grant from The 21st Century COE Program of the Ministry of Education, Culture, Sports, Science and Technology, the Department of Transplantation for Regeneration Therapy (Sponsored by Otsuka Pharmaceutical Company, Ltd.), Molecular Medical Science Institute, Otsuka Pharmaceutical Co., Ltd, Japan Immunoresearch Laboratories Co., Ltd (JIMRO), and Scientific research (C) 18590388.

REFERENCES

Adachi Y, Taketani S, Oyaizu H, Ikebukuro K, Tokunaga R, Ikehara S (1999) Apoptosis of colorectal adenocarcinoma induced by 5-FU and/or IFN-gamma through caspase 3 and caspase 8. Int J Oncol 15: 1191–1196.

Bratincsák A, Lonyai A, Shahar T, Hansen A, Tóth ZE, Mezey E (2007) Using brain slice cultures of mouse brain to assess the effect of growth factors on differentiation of bone marrow derived stem cells. Ideggypgy Sz 60: 124–129.

Broxmeyer HE, Douglas GW, Hangoc G, Cooper S, Bard J, English D, Arny M, Thomas L, Boyse EA (1989) Human umbilical cord blood as a potential source of transplantable hematopoietic stem/progenitor cells. Proc Natl Acad Sci U S A 86: 3828–3832.

Eriksson PS, Perfilieva ET, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nat Med 4: 1313–1317.

Fujikawa T, Oh SH, Pi L, Hatch HM, Shupe T, Petersen BE (2005) Teratoma formation leads to failure of treatment for type I diabetes using embryonic stem cell-derived insulin-producing cells. Am J Pathol 166: 1781–1791.

Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119: 493–501.

Gluckman E, Rocha V, Boyer-Chammard A, Locatelli F, Arcese W, Pasquini R, Ortega J, Souillet G, Ferreira E, Laporte JP, Fernandez M, Chastang C (1997) Outcome of cord-blood transplantation from related and unrelated donors, Eurocord Transplant Group and the European Blood and Marrow Transplantation Group. N Engl J Med 337: 373–381.

Haruta M, Kosaka M, Kanegae Y, Saito I, Inoue T, Kageyama R, Nishida A, Honda Y, Takahashi M (2001) Induction of photoreceptor-specific phenotypes in adult mammalian iris tissue. Nat Neurosci 4: 1163–1164.

Hasegawa M (1965) Restitution of the eye from the iris after removal of the retina and lens together with the eye-coats in the newt, triturus pyrrhogaster. Embryologia (Nagoya) 61: 362–386.

Humayun MS, de Juan E Jr, Weiland JD, Dagnelie G, Katona S, Greenberg R, Suzuki S (1999) Pattern electrical stimulation of the human retina. Vision Res 39: 2569–2576.

Ikeda H, Osakada F, Watanabe K, Mizuseki K, Haraguchi T, Miyoshi H, Kamiya D, Honda Y, Sasai N, Yoshimura N, Takahashi M, Sasai Y (2005) Generation of Rx+/Pax6+ neural retinal precursors from embryonic stem cells. Proc Natl Acad Sci U S A 102: 11331–11336.

Ishikawa F, Drake CJ, Yang S, Fleming P, Minamiguchi H, Visconti RP, Crosby CV, Argraves WS, Harada M, Key LL Jr, Livingston AG, Wingard JR, Ogawa M (2003) Transplanted human cord blood cells give rise to hepatocytes in engrafted mice, Ann N Y Acad Sci 996: 174–185.

Kaneko Y, Akakibara S, Imai T, Suzuki A, Nakamura Y, Sawamoto K, Ogawa Y, Toyama Y, Miyata T, Okano H (2000) Musashi1: an evolutionally conserved marker for CNS progenitor cells including neural stem cells. Dev Neurosci 22: 139–153.

Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N, Liedtke S, Sorg RV, Fischer J, Rosenbaum C, Greschat S, Knipper A, Bender J, Degistirici O, Gao J, Caplan AI, Colletti EJ, Almeida-Porada G, Muller HW, Zanjani E, Wernet P (2004) A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. J Exp Med 200: 123–135.

Metallo CM, Mohr JC, Detzel CJ, de Pablo JJ, Van Wie BJ, Palecek SP (2007) Engineering the stem cell microenvironment. Biotechnol Prog 23: 18–23.

Minamino K, Adachi Y, Yamada H, Higuchi A, Suzuki Y, Iwasaki M, Nakano K, Koike Y, Mukaide H, Kiriyama N, Shigematsu A, Matsumura M, Ikehara S (2005) Long-term survival of bone marrow-derived retinal nerve cells in the retina. Neuroreport 16: 1255–1259.

Murohara T, Ikeda H, Duan J, Shintani S, Sasaki K, Eguchi H, Onitsuka I, Matsui K, Imaizumi T (2000) Transplanted cord blood-derived endothelial precursor cells augment postnatal neovascularization. J Clin Invest 105: 1527–1536.

Nishida A, Takahashi M, Tanihara H, Nakano I, Takahashi JB, Mizoguchi A, Ide C, Honda Y (2000) Incorporation and differentiation of hippocampus-derived neural stem cells transplanted in injured adult rat retina. Invest Ophthalmol Vis Sci 41: 4268–4274.

Sugihara A, Adachi Y, Inaba M, Hisha H, Sugiura K, Miyashima S, Amoh Y, Taketani S, Oyaizu H, Ikebukuro K, Kawamura M, Genba H, Horio T, Ikehara S (1999) Age-dependent abnormalities of hematopoietic stem cells in (NZW x BXSB) F1 mice. Stem Cells 17: 357–365.

Tomita M, Adachi Y, Yamada H, Takahashi K, Kiuchi K, Oyaizu H, Ikebukuro K, Kaneda H, Matsumura M, Ikehara S (2002) Bone marrow-derived stem cells can differentiate into retinal cells in injured rat retina. Stem Cells 20: 279–283.

Turner JE, Blair JR (1986) Newborn rat retinal cells transplanted into a retinal lesion site in adult host eyes. Brain Res 391: 91–104.

Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M (2003) Cell fusion is the principal source of bone-marrow-derived hepatocytes. Nature 422: 897–900.

Received 26 April 2007, accepted 7 August 2007