Prolonged survival and expression of neural markers by bone marrow-derived stem cells transplanted into brain lesions

Esteban Alberti¹, Marek Los²,³,⁴, Rocio García¹, Jorge L. Fraga³, Teresa Serrano¹, Elizabeth Hernández¹, Thomas Klonisch², René Macías¹, Lísis Martínez², Lázara Castillo¹, Karelys de la Cuétara¹

¹ Department of Neurobiology, International Center of Neurological Restoration, CIREN, Havana, Cuba
² Department of Human Anatomy and Cell Science, Univ. Manitoba, Winnipeg, Canada
³ Manitoba Institute of Cell Biology, and Department of Biochemistry and Medical Genetics, Univ. Manitoba, Winnipeg, Canada
⁴ BioApplications Enterprises, Winnipeg, Manitoba, Canada
⁵ Department of Parasitology, "Pedro Kouri" Institute, Havana, Cuba

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Summary

Background:
Bone marrow-derived stem cell transplantation is a potentially viable therapeutic option for the treatment of neurodegenerative disease.

Material/Methods:
We have isolated bone marrow stem cells by standard method. We then evaluated the survival of rats’ bone marrow mononuclear cells implanted in rats’ brain. The cells were extracted from rats’ femurs, and marked for monitoring purposes by adenoviral transduction with Green Fluorescent Protein (GFP). Labeled cells were implanted within the area of rats’ striatum lesions that were induced a month earlier employing quinolinic acid-based method. The implants were phenotyped by monitoring CD34; CD38; CD45 and CD90 expression. Bone marrow stromal cells were extracted from rats’ femurs and cultivated until monolayer bone marrow stromal cells were obtained. The ability of bone marrow stromal cells to express NGF and GDNF was evaluated by RT-PCR.

Results:
Implanted cells survived for at least one month after transplantation and dispersed from the area of injection towards corpus callosum and brain cortex. Interestingly, passaged rat bone marrow stromal cells expressed NGF and GDNF mRNA.

Conclusions:
The bone marrow cells could be successfully transplanted to the brain either for the purpose of trans-differentiation, or for the expression of desired growth factors.

key words: bone marrow stromal cells • CD34 • CD38 • CD45 • CD90 • hematopoetic stem cells • trans-differentiation • transplantation

Abbreviations:
CNS – central nervous system; GDNF – glial-derived neurotrophic factor; GFP – green fluorescent protein; NGF – nerve growth factor; PBS – phosphate buffered saline; RT-PCR – reverse transcriptase-polymerase chain reaction

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Author's address: Marek Los, BioApplications Enterprises, Winnipeg, MB, R2V 2N6, Canada, e-mail: bioappl@gmail.com
BACKGROUND

Apoptosis is the common mechanism of cell demise in central nerve system and in other organs, and apoptosis inhibition is the principle of several experimental methods of treatment [1–3]. Transplantation of pluripotent stem cells that have the capacity to differentiate into neural tissue components is a viable treatment alternative for neurodegenerative diseases. Embryonic stem cells have been used in a regenerative medicine with variable success, but their use attracts significant ethical controversy in many countries [4]. Currently, pluripotent neural stem cells are not available, and surgical removal of neural tissues for the sole purpose of isolation of neural stem cells is not a feasible option. The aim of the described experiments was to test if bone marrow derived cells could technically be used to treat neurodegenerative lesions. The stem cells are an attractive cellular source for the treatment of illnesses of the Central Nervous System (SNC). Under certain conditions they are capable of self-renewal for long periods of time – remain in totipotent state until they receive appropriate differentiation signals [5,6].

Bone marrow is the only well-known organ containing a heterogeneous mixture of different types of stem cells [6,7]. These populations include the hematopoietic system and stromal cells that interact in a reciprocal way through intercellular contacts and secretion of cytokines or growth factors [8]. The hematopoietic system contains stem cells that are responsible for the formation of all the types of blood cells. Bone marrow stromal cells generate bone, cartilage, adipocytes, connective fibers and a reticular net that constitutes the formation of these cells [7,9,10].

Plasticity of bone marrow stem cell populations gives them the capacity to generate specialized cells of various tissues [5,6]. Thus, bone marrow hematopoietic stem cells are able to differentiate to microglia and macroglia once transplanted into the brain of mature mice [9]. In another experimental system when transplanted into the para-ventricular area of neonatal mice, they differentiated to astrocytes and neurons [11]. Furthermore, when these cells were given intravenously to rats with traumatic brain injury, they migrated toward the damaged brain area within 15 days, some of them differentiated to neurons, and improved neural functions [12].

The development of transplants in experimental models demands implanted cells to be detected in situ once the animal is sacrificed. One of the more frequently used methods for marking of these cells is genetic modification that may be carried out in vitro and in situ [14]. Femurs were used for cell extractions. Bone marrow was obtained by passing with a syringe and flushing through the femur. The mononuclear cells were isolated using Ficoll-Hypaque±Plus gradient (Amersham-Pharacia Biociences, Sweden) and then cultured in DMEM supplemented with 20% FCS, and 50 mg/mL gentamicine. Cell viability was evaluated by staining with Trypan Blue [22,23]. For immunocytochemical evaluation, the isolated cells were seeded in a 12-well-plates, fixed in 4% paraformaldehyde in PBS, pre-incubated in a blocking solution (5% skim milk in PBS for 1 h at RT) and then incubated on ice with monoclonal antibodies against CD34, CD38, CD45 and CD90 (Sigma-Aldrich, Oakville, ON, Canada). Cells were then washed 3 times with cold PBS and incubated with anti-mouse IgG and IgM, biotinylated secondary antibodies for 1 h at RT, then washed 3 times in cold PBS and finally the staining was developed using the ABC system and diamino-benzidine (DAB) substrate reagent (PA-ABC system, DAKO, Mississauga, ON, Canada). The staining was detected by light microscopy. Cells stained with secondary anti-

Neurotrophic factors promote neuronal survival and stimulate axonal growth. It has been proposed that under some clinical conditions, neurons fail to obtain a sufficient quantity of necessary neurotrophic factors and die by apoptosis [16–18]. Trophic support provided by transplanted cells could counteract cell death in damaged tissue. Therefore, we have tested whether bone marrow stem cells (both hematopoietic and stromal) could differentiate and produce neurotrophic factors. Furthermore, using bone-marrow derived stem cells, tagged by two different methods we have tested the survival and migratory potential of these cells once implanted into brain. Our paper communicates for the first time that rat bone marrow derived stromal cells could produce GDNF and NGF.

MATERIAL AND METHODS

Laboratory animals

Male rats of the Sprague-Dawley line were used with a body weight between 200–250 g (CENPALAB, Havana, Cuba). Experimental animals were kept in cages with free access to water and standard chow, with light/obscured cycles of 12 h. The animals were randomly divided into three experimental groups: (i) animals that received the transplant of mononuclear cells genetically modified to express GFP (GFP, n=10), (ii) animals that received the transplant of Hoechst labeled mononuclear cells (HOE, n=10), and (iii) n=10 animals used as control (mock-transplanted with cell culture medium DMEM).

Quinolinic acid induced neuronal lesions

The animals were anesthetized with chloral hydrate (420 mg/kg of body weight) and placed in the stereotactic surgical devices for rodents. Lesion inductions were carried out with 1.2 μl of quinolinic acid solution (112.5mM, pH=7.4) in the right striatum. (AP=+1.2; L=±2.8; V=5.5) volume was injected at 1 μl/min [19–21].

Isolation and immunocytochemical characterization of bone marrow-derived cells

The animals (20 Sprague-Dawley rats) weights between 250 and 300 g, were anesthetized with chloral hydrate and sacrificed [14]. Femurs were used for cell extractions. Bone marrow was obtained by passing with a syringe and flushing with sterile phosphate buffered saline (PBS) (NaCl 8 g/L; KCl 0.2 g/L; Na(HPO4) 1.09 g/L; KHPO4 0.26 g/L, pH 7.2) through the femur. The mononuclear cells were isolated using Ficoll-Hypaque±Plus gradient (Amersham-Pharacia Biociences, Sweden) and then cultured in DMEM supplemented with 20% FCS, and 50 mg/mL gentamicine. Cell viability was evaluated by staining with Trypan Blue [22,23]. For immunocytochemical evaluation, the isolated cells were seeded in a 12-well-plates, fixed in 4% paraformaldehyde in PBS, pre-incubated in a blocking solution (5% skim milk in PBS for 1 h at RT) and then incubated on ice with monoclonal antibodies against CD34, CD38, CD45 and CD90 (Sigma-Aldrich, Oakville, ON, Canada). Cells were then washed 3 times with cold PBS and incubated with anti-mouse IgG and IgM, biotinylated secondary antibodies for 1 h at RT, then washed 3 times in cold PBS and finally the staining was developed using the ABC system and diamino-benzidine (DAB) substrate reagent (PA-ABC system, DAKO, Mississauga, ON, Canada). The staining was detected by light microscopy. Cells stained with secondary anti-

ENVIRONMENTAL

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bodies only were used as negative control [24,25]. The primary antibodies were diluted as follows: CD34 1:40, CD38 1:200, CD45 1:50, and CD90 of 1:20 (Table 1). The optimal working dilutions for our experimental purposes were obtained empirically.

**Bone marrow stromal cells culture**

Bone marrow stromal cells were obtained from the femurs of adult male Sprague-Dawley rats (as described above). The extraction was carried out using MEM. The cells were then cultured in the same medium supplemented with 20% FCS, 2 mM L-glutamine and 100 μg/ml streptomycin. Non-adherent cells were removed by 3× washing with PBS and subsequent replacement of the medium. Bone marrow stromal cells were subcultured 6 times prior to performing experiments [26].

**Labeling of bone-marrow-derived cells with GFP or with Hoechst 33342**

The bone marrow-derived cells were infected with an adenovirus type 5 (MOI=10) carrying a GFP under a human cytomegalovirus early promoter. Cells were then cultured for 5 days at 37°C, 5% CO₂. Next, the cells were washed with PBS, cell viability was determined by Trypan blue exclusion, and used for transplantation [22,23]. Hoechst 33342 labeling (1 μg/ml) was carried out in DMEM medium. The cells were incubated for 24 h at 37°C and 5% CO₂. The cellular viability was determined by MTT-assay as described previously [27].

**Stereotactic cell transplantation**

The transplant was carried out by stereotactic surgery into the lesions area, one month after lesion induction, following the same procedure as described previously for the lesion development. The cells were grafted into two sites in the striatum (AP=+0.7; L=+2.8; V=-5.5 and AP=+0.7; L=+2.8; V=-4.6) [28]. The cellular sample had a concentration of 1.5x10⁶ cells/μl and 1 μl was infused in each site. The maximum surgical time was 5 h and during this time; the cellular suspensions remained at 4°C. The cellular viability was determined/confirmed again after the surgical procedure was concluded [19,20,22].

**Histologic evaluation of transplantation results**

The animals that were transplanted with cells genetically modified to express GFP were sacrificed (n=4 each time) at day 10, 20 and 30 post-transplantation. The animals were anesthetized with chloral hydrate prior to killing and then perfused with 4% paraformaldehyde (PFA) through the ascending aorta. The extracted brains were then maintained for 2 h at 4% PFA and then passed through sucrose gradient solutions at 15 and 30% respectively for 24 h and subsequently frozen. The specimens were sectioned at 20 μm thickness according to the stereotactic atlas [28], and were randomly selected for further study [22]. GFP- and Hoechst-labeled cells were then detected by fluorescence (Hoechst λ exc=420 nm, GFP λ exc=590 nm), by microscopy (Digital Microscope Leica DM4000/5000). Some sections were processed for conventional violet cresil histology-staining to identify glial and astrocyte cells by immunocytochemistry [19,20].

**Reverse transcriptase-polymerase chain reaction (RT-PCR)**

To detect mRNA expression, total cellular RNA from bone-marrow-derived cells at passage 7, was isolated using Trizol method (Gibco). For GDNF RT-PCR amplification, the following primers were used: 5'-gactctaagactcacagg-3' and 5'-cgcggatctgggtcagatacatccacaccgtttagc-3' primers. To amplify the NGF message we used 5'-ttggctacccagctgtgcggac-3' and 5'-ctcttcgatattctcacagccttcctgctgag-3' primers. To amplify the BDNF message we used 5'-tggctacccagcttggtcagacaccgtttagc-3' and 5'-ctcttcgatattctcacagccttcctgctgag-3' primers. The RT-PCR was carried out using “Access RT-PCR Systems” (Promega). The reaction included 10 mM of a dNTP mix, 50 pmol of each primer, 25 mM MgSO₄, 31 ng of RNA, and 3U DNase. The reaction mixtures were maintained for 45 min at 48°C, 2 min at 94°C, then cycled 40 times through a program of 30 sec at 94°C, 1 min at 62°C for GDNF or 1 min at 50°C for NGF and 1 min at 60°C for BDNF, and 2 min at 68°C, finally the reaction was incubated for an extra 7 min at 68°C. The RT-PCR DNA products were electrophoresed on 1.8% agarose gel and stained with ethidium bromide. To confirm the integrity of isolated mRNA a fragment of the β-actina mRNA was amplified (primers: 5'-tca cgc aag att tcc ttc tca g-3' and 5'-att tgg cac cac act ttc tca a-3'). The PCR was carried out as described above, except that the annealing temperature was 51°C [29,30].

**RESULTS**

**Characterization of bone marrow derived cells used for transplantation**

Bone marrow cells were extracted from femurs of 20 Sprague-Dawley rats. We next used hematopoietic stem cell markers (CD38, CD45), stromal cells marker CD90, and CD34 that

<table>
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<th>CD</th>
<th>Marker distribution</th>
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<th>(%) of expression</th>
<th>SD</th>
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<td>3.119</td>
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<tr>
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<td>2.88</td>
<td>IgG2 (1:50)</td>
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<tr>
<td>CD90</td>
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<td>23.5%</td>
<td>3.58</td>
<td>IgG1 (1:20)</td>
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Table 1. Detection of surface markers on bone marrow derived cells (see also Figure 1).
is expressed on subpopulations of both cell types, to assess the composition of the bone marrow-derived cells. For each case, the corresponding negative control was utilized (Figure 1A–E). The mean percentages of cells positive for each marker were as follows: CD34=19%, CD38=21%, CD45=17% and CD90=24% (Table 1).

Bone marrow stromal cells have significant trans-differentiation capacity and migratory potential, even in CNS [11,12,18]. Thus, using RT-PCR we next tested these cells for the expression of neural mRNAs. We extracted the total RNA by Triazol method, and assessed its quality by RNA denaturating gel electrophoresis. The electrophoregram shown in figure 2A shows good quality of RNA preparation (quality control). RT-PCR signals indicate that bone marrow stromal rat cells, after 7 passages, expressed NGF, (395 bp) (Figure 2B, panel 2, lane 3), BDNF (Figure 2B, panel 3, lane 3), and GDNF (648 bp) amplified message fragments (Figure 2B, bottom, panel lane 3).

Transplanted bone-marrow-derived stem cells remain viable within neural tissue for at least 30 days

To evaluate the experimental model, we microscopically assessed the degree of brain tissue injury induced by the injection of quinolinic acid (Figure 3A–C). The injured animals showed loss of neuronal cells and abundant gliosis. The injured groove differed from the intact groove; it was thicker in the right lateral ventricle ipsilateral area.

Figure 1. Detection of CD34, CD38, CD45, and CD90 markers in bone marrow derived mononuclear cells. (A) Negative control (secondary antibody only), (B) CD34 positive cells, (C) CD38 positive cells, (D) CD45 positive cells, and (E) CD90 positive cells. Triplicate samples for each staining were blindly evaluated by two independent scientists. At least 400 cells per each sample were counted. Mean values of the percentage of positive cells for each one of the antigens are indicated in Table 1.

The cells meant for transplantation were marked with two different methods (GFP, Hoechst 33342). Unless indicated differently, the viability of bone marrow-derived cells was above 90% during all experimental procedures. The adenoviral transduction to express GFP showed maximum (~50%) infection efficiency after 5 days of incubation with the virus (Figure 4). The prolonged incubation caused 25–43% of cell death within the virus treated cell population. However, when the bone marrow-derived cells were stained with Hoechst 33342 (24 h incubation in Hoechst-containing medium), 100% of cells had incorporated the nuclear marker and cell viability continued to be always above 90%.

Stereotactic transplantations were carried out one month after the injury induction, and it was done as described in the method section. Microscopy studies of tissues derived from animals transplanted with bone marrow derived-GFP-expressing cells, transplanted into groove-area, were performed 3 days, and 30 days post transplantation. After 3 days, the GFP-expressing cells were easily detected within striatum, but not in other areas of the brain. 30-days post-transplantation many cells still expressed the GFP, but their number diminished considerably (Figure 5A). The cell viability within the transplanted areas, as assessed morphologically, was about 70–75%.

The implanted bone marrow-derived cells marked with Hoechst 33342 were easily and abundantly detectable at the site of injury, by fluorescent microscopy between 10–30 days after transplantation to the groove (Figure 6A–C). The anteroposterior transplant’s extension was 2–2.2 mm as de-
The transplanted cells spread markedly from the point of injection, indicating significant migratory potential of these cells. The viability of transplanted cells at day 30 after transplantation was above 75% as assessed morphologically. Cells that shrunk and had condensed nuclei were considered apoptotic.

**DISCUSSION**

This project was aimed to test the feasibility and migratory potential of bone marrow derived stem cells as donor cells for the treatment of brain lesions. We have used a quinolinic acid based method to induce neuronal lesions. Upon stereotactic injection of quinolinic acid into the brain a notorious gliosis was evident in the corresponding groove in the ipsilateral ventricle beside the lesion (product of the shrinkage of the groove). The characteristics described in this report coincide with those reported by other authors within the pattern of striatal lesions induced with quinolinic acid [20].

For tagging of the cells that we have transplanted, we have used either adenoviral-vector based GFP gene transfer to mark the bone marrow-derived mononuclear cells [22,25,29] or a Hoechst 33342 based tagging (see below). The adenoviral tagging was only moderately effective; upon 5 days incubation with the adenovirus carrying the GFP-gene, only about 50% of cells expressed the tag. Furthermore, the adenovirus genome is not integrated to the genome of the host cell, thus each cell division dilutes the adenoviral gene and the marker gene (GFP). The Hoechst 33342 re-

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**Figure 2.** Expression of neurotrophic factors by bone marrow-derived stem cells. (A) mRNA isolated from rat bone marrow stromal cells in passage 7 (quality control). (B) Detection of NGF, BDNF and GDNF mRNA by RT-PCR from bone marrow stromal cells. β-actina (top panel) was used as an amplification control. Lane 1: Molecular weight marker (BlueScript/HpaII, sizes 713/ 489/ 404/ 367/ 242/ 190/ 157/ 147/ 110/ 57/ 34 and 26 pb). Lane 2: Reaction without reverse transcriptase (negative control). Lane 3: Reaction with reverse transcriptase.

**Figure 3.** Quinolinic acid induced neuronal lesions. (A) Quinolinic acid neuronal lesion was induced within the right striatum, (1 day after induction) see the left, healthy striatum for comparison (magnification: 50×). (B) Normal (control) striatum (magnification: 100×). (C) Quinolinic acid neuronal lesion was induced within the right striatum, (1 month after induction), see (B) for comparison to non-damaged striatum (magnification: 100×).
agent, the other method that we used for cell tagging, is a live DNA-dye that preferentially stains A- and T-bases within DNA. In our case, the cells stained 100%, and cell viability measured at 24 h of incubation with Hoechst 33342 was above 90%. When comparing both methods of cell tagging, the Hoechst 33342 staining appears to be superior to the virus-based tagging. The Hoechst 33342 labeling method has been successfully applied in the past to follow cells transplanted into CNS [31].

The in vivo survival of GFP-tagged grafts was diminished considerably as compared to those tagged with Hoechst 33342. The lower survival of GFP-tagged cell is likely due to host’s immune response mounted against the adenovirus and/or the GFP. Such interpretation is supported by the presence of yellow bodies in the striatal area that was transplanted with GFP-tagged cells. Such yellow bodies are typically associated with the inflammatory reaction within the neural tissue. These results are in agreement with observations of other authors that noted adenoviral system-induced inflammation, thus limiting the expression of the desired transgene, (in our case the GFP) [32]. Contrary to the results reported by some authors, the infused cells did not aggregate, as it has been reported with the infusion of fibroblasts and also some types of stem cells [33]. Instead, in our experimental model, the cells integrated well into the new environment, and even migrated 2–3 mm from the injection site. The same patterns of integration and migration have been reported by some other authors in both rat and human bone marrow stem cells infused into the rats’ brains [34].

The cells tagged with Hoechst 33342 survived much better the engraftment as compared to GFP-tagged cells. The transplanted cells were located around the line of the needle transition through the brain tissue, and they dispersed toward the cortex and corpus callosum. The migration dispersion occurred to a similar extend as for GFP-tagged cells and it was on average of 2–2.2 mm from the injection site. Interestingly, the difference in migration distance among the animals sacrificed at 10 days and those sacrificed at 20 days after transplantation was not significant, thus the migration pattern and distance were likely established in an early time window after transplantation. We observed similar migration routes of rat femur-derived bone marrow cells toward the corpus callosum, cerebral cortex and ipsilateral temporal lobe, as it was previously reported for bone marrow stem cells that had been transplanted directly into the striatum [33,34]. The migration of grafted cells in the injured brain is likely due to the action of chemotactic factors produced by the damaged brain. These factors create an attraction of implanted bone marrow mononuclear cells to the site of injury [22,23,25,29]. The extent of migratory behavior may depend on the cell types transplanted and the age of the animal these cells are implanted into. Much stronger migratory activities were reported if transplanted stromal cells were implanted in the lateral ventricle of neonatal mice. These cells were even detected in the contralateral hemisphere of brain and cerebellum without causing any disruption of cerebral architecture [25]. Similarly as in our experimental system, some other authors have reported that the migration took place toward the corpus callosum, cerebral cortex and ipsilateral temporal lobe, when bone marrow stem cells were implanted directly in the grooved body [33,34]. In contrast to our rat brain injury model, which did not utilize neonatal rats, the mice studies were performed on neonatal brains and this may encourage stronger cell migration.

Figure 4. Staining of bone marrow derived mononuclear cells by infection with type-5 adenoviral vector carrying GFP cDNA. Cells were incubated with the vector (MOI=10) for 5 days (see method section for details). (A), magnification 100×, (B,C) magnification 1000×.

Figure 5. Brain sections with implanted bone marrow mononuclear cells that were labeled by the infection with adenoviral vector carrying GFP. Brain sections at day (A) 3, and (B) 30 post-transplantation (magnification ×200). See method section for experimental details. The yellow signal in (A) represents a high intensity fluorescence of GFP.

Figure 6. Brain sections with implanted bone marrow mononuclear cells that were label with Hoechst 33342. Brain sections at day (A) 10, (B) 15, and (C) 30 post-transplantation (magnification ×200). See method section for experimental details.
Our results show that the bone marrow derived stem cells are able to survive at least for one month upon transplantation into brain. Thus, our data encourage the use of bone marrow derived stem cells in the development of experimental therapies of neurodegenerative diseases.

Bone marrow is a rich source of stem cells. Besides, the hematopoietic and mesenchymal cells discussed before, it includes a subpopulation of multipotent adult stem cells [35,36]. Our study supports the present concept of the heterogeneity of adult stem cell populations within bone marrow. This is further confirmed by the presence of mRNAs from both hematopoietic and neurotrophic factors in the tested bone marrow derived stem cells. For example, we detected a GDNF signal, and a NGF mRNA signature. The level of production of NGF by bone marrow derived stromal stem cells was similar to the expression of this neurotrophic factor by murine stromal cell line MS-5 and other stromal cell lines [11,37]. Our paper communicates for the first time that rat bone marrow derived stromal cells produce GDNF and NGF. Interestingly, recently it has been communicated that fresh murine bone marrow derived stem cells cannot produce GDNF [38]. Our tests in the rat model were conducted after passage 7 of cell culture that may allow some trans-differentiation. Thus, it is possible that simple passaging of stromal cells under the described in method section condition, prompts some cells into differentiation towards neural phenotypes.

CONCLUSIONS

In summary, our experiments demonstrate the feasibility of the usage of bone marrow derived stem cells for the development of cellular therapies of brain lesions. The use of the Hoechst reagent for tagging of cells to be implanted into the brain is much more advantageous as compared to GFP-tagging by adenoviral infection. The bone marrow derived stem cells survive as transplants for at least a month, and they express neural growth- and survival factors like NGF and GDNF. Further research is needed to define bone marrow derived cell sub-populations best suited as a source for cell-based therapies of degenerative disorders, strokes and traumatic lesions.

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