Adult neurogenesis of epidermal neural crest stem cells (EPI-NCSC) in hippocampus of Alzheimer's rat model

Banafshe Esmaeilzade, Maliheh Nobakht, Seyed Mohammad Hosseini, Pejman Mortazavi, Mahmood Barati, Soraya Mehrabi, Leila Beygom H[illegible]an,

Comparative Clinical Pathology

ISSN 1618-5641

Comp Clin Pathol
DOI 10.1007/s00580-013-1690-z

Springer

Your article is protected by copyright and all rights are held exclusively by Springer-Verlag London. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your work, please use the accepted author's version for posting to your own website or your institution's repository. You may further deposit the accepted author's version on a funder's repository at a funder's request, provided it is not made publicly available until 12 months after publication.

Springer

Author's personal copy

Comp Clin Pathol
DOI 10.1007/s00580-013-1690-z

ORIGINAL ARTICLE

Adult neurogenesis of epidermal neural crest stem cells (EPI-NCSC) in hippocampus of Alzheimer's rat model

Banafshe Esmaeilzade · Maliheh Nobakht · Seyed Mohammad Hosseini · Pejman Mortazavi · Mahmood Barati · Soraya Mehrabi · Leila Beygom Hejazian · Fatima Moghani Ghoroghi

Received: 10 June 2012 / Accepted: 15 January 2013
© Springer-Verlag London 2013

Abstract Alzheimer's disease (AD) is characterised by progressive neuronal loss in the hippocampus. Our aim was to evaluate the effects of transplanting epidermal neural crest stem cells (EPI-NCSC) into the hippocampus in vivo and to assess adult neurogenesis and total granule cell number in the hippocampus of an Alzheimer's rat model after a single injection of EPI-NCSCs. Fourteen days after a bilateral injection of β-amyloid 1–40 into the hippocampus, 10 AD model rats received an intra-hippocampal injection of EPI-NCSCs; the cells were obtained from the vibrissa hair follicle of the rat, cultured, labelled with bromodeoxyuridine (BrdU) and suspended in normal saline. Y-Maze and single trial passive avoidance tests were used to show any learning and memory deficit. Nestin staining was performed in vitro. Double staining of BrdU–GFAP and BrdU–βIII was undertaken to study survival and differentiation of the grafted cells. Cell proliferation and differentiation were observed in all part of hippocampus in the double-stained histological sections. Total granular cell number was estimated to be more per hippocampus in the rats receiving the transplanted cells compared to the AD control group. We observed that rats with hippocampal damage made significantly more errors than control rats on the Y-maze. We showed that transplanted EPI-NCSCs survived and differentiated into neurons and glial cells. Total granule cell number in the treatment group was equal to the control group. Cell proliferation and migration tends to end in the dentate gyrus and the other part of hippocampus. Transplantation of EPI-NCSCs into the hippocampus might differentiate into neurons or induce neurogenesis.

Keyword EPI-NCSCs · Alzheimer's disease · Granule cell number · Neurogenesis · Hippocampus

B. Esmaeilzade · M. Nobakht · S. M. Hosseini · P. Mortazavi
Iran National Science Foundation, Tehran, Iran

B. Esmaeilzade
Department of Anatomy, School of Medicine,
Bushehr University of Medical Sciences, Bushehr, Iran

B. Esmaeilzade · M. Nobakht · L. B. Hejazian · F. M. Ghoroghi
Department of Anatomy, School of Medicine,
Tehran University of Medical Sciences, Tehran, Iran

M. Nobakht (✉)
Anti-microbial Resistance Research Center,
Tehran University of Medical Sciences, Tehran, Iran
e-mail: nobakht@yahoo.com

S. M. Hosseini
Department of Pathology, Veterinary Faculty,
Azad University of Babol, Babol, Iran

P. Mortazavi
Department of Pathology, Faculty of Specialized Veterinary Science, Science and Research Branch Islamic Azad University, Tehran, Iran

M. Barati
Department of Pharmaceutical Biotechnology,
School of Pharmacy,
Shahid Beheshti University of Medical Sciences, Tehran, Iran

S. Mehrabi
Department of Neurosciences, School of New Technology,
Tehran University of Medical Sciences, Tehran, Iran

Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder in the elderly. Typical pathological changes include abundant age pigments caused by aggregation of β-amyloid, neurofibrillary tangles, and the absence of neurons caused by abnormal phosphorylation of tau protein in the brain. Patients with AD suffer memory impairment as well as deterioration of their cognitive abilities including a dramatic

Published online: 15 February 2013

Author's personal copy

change in personality. These changes are due to the progressive loss of neuronal function and the death of neurons that are responsible for the storage and processing of information. Cholinergic neurons show significant pathology in AD (Xuan et al. 2009).

Although in AD massive neuronal loss only occurs in very few brain structures, such as the hippocampal CA1 and CA2 regions, the entorhinal cortex and the locus coeruleus, large parts of the brain are affected by pathological alterations and decreased neuronal metabolism(Givens and Olton 1994). Current treatments bring only temporary symptomatic relief and do not halt the progression of this disease (Korecka et al. 2007). AD is a prime candidate for the development of cell-replacement therapy, since the transplantation of cells lacking the AD-causing mutation can replace lost neurons and reconstitute damaged neuronal connections (Tsai et al. 2007).

Today, adult neurogenesis is well documented in the published literature. Numerous experiments have shown that, in adulthood, neurogenesis operates mainly in two areas of the mammalian central nervous system, in the anterior part of the subventricular zone (SVZ) along the lateral ventricles and in the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus (Abrous et al. 2005; Colucci-D'Amato et al. 2006) In both areas, neurogenesis progresses as a complex multi-stage process, which starts with the proliferation of neural precursors residing in the SVZ and the SGZ (Abrous et al. 2005; Rodriguez and Verkhratsky 2011). The hippocampus is affected early in AD. As mentioned, the hippocampus is also one of only two neurogenic niches of the adult brain (Taupin 2006). Therefore, the pathological process associated with AD is likely to affect neurogenesis. Impaired neurogenesis, in turn, can be relevant for disease progression arguably being involved in the cognitive impairments linked with neuro-degeneration (Verret et al. 2007; Rodriguez et al. 2009; Zhao et al. 2008). The majority of studies performed on transgenic animals expressing the mutant amyloid precursor protein demonstrate decreased neurogenesis either in the DG or in both the DG and the SVZ (Rodriguez and Verkhratsky 2011; Zhang et al. 2007).

Amyloid beta-peptide disrupts neurogenesis in SVZ and hippocampus in a mouse model of AD (Haughey et al. 2002a, b), but the status of neurogenesis in neurodegenerative disorders in humans is unknown. As a corollary, measures designed to enhance neurogenesis could have therapeutic value in AD (Jin et al. 2004).

Hair follicle stem cells, located in the hair follicle bulge, possess stem cell characteristics, including multi-potency, high proliferative potential and ability to enter quiescence (Cotsarelis 2006; Cotsarelis and Lavker 1990). Neural crest stem cells (NCSCs) have been isolated from rodent embryonic and postnatal tissue: sciatic nerve (Morrison et al. 1999), gut (Bixby et al. 2002; Kruger et al. 2002), dorsal root ganglion (Li et al. 2007), heart (Tomita et al. 2005) and cornea (Yoshida et al. 2006). NCSCs have also been obtained from murine and human epidermis and/or dermis (Fernandes et al. 2004; Amoh et al. 2005; Sieber-Blum et al. 2004; Yu et al. 2010).

For the first time, Sieber-Blum et al. (2004) described NCSCs in the inner layer of the bulge region of the adult murine whisker follicle and designated them epidermal neural crest stem cells (EPI-NCSCs). The cells were isolated from the explanted bulge region emigrated out and shown to express Sox10, a marker of NC (Britsch et al. 2001; Rehberg et al. 2002) intermediate filament protein nestin, a marker of immature and undifferentiated cells (Lendahl et al. 1990; Lothian and Lendahl 1997; Mokry et al. 2004). Nestin is also expressed in some cells in the bulge of the hair follicle (Li et al. 2003). EPI-NCSCs have been serially cultured and under conditions that favoured differentiation showed a broad potential for generating cells expressing markers appropriate for neurons, glia, smooth muscle cells, chondrocytes, melanocytes and osteocytes (Krejci and Grim 2010; Clewes et al. 2011).

In the present study, we aimed to assess whether EPI-NCSCs grafted in the hippocampus of a rat model of Alzheimer's disease could differentiate into neurons or induce adult neurogenesis in the hippocampus to replace neuronal loss. Additionally, we focused on spontaneous alternation of rat model of Alzheimer's disease in the Y-maze, which evaluates spatial working memory.

Methods and materials

Animals and Alzheimer's model

Adult male Wistar rats weighing 250–300 g (9–10 weeks of age, $n=40$) were purchased from the Central Animal Laboratory, Pastor Institute, Iran. Animal were kept in the central animal house of the Tehran University of Medical Science under a 12-h light/12-h dark regime at 22–24 °C, fed with commercial standard rodent feed and allowed free access to water. All animal experimental protocols were approved by the Animal Care and Use Committee of Tehran University of Medical Science. The rats were randomly allocated into four groups: A, control group (intact); B, AD control group (injected with Aβ1-40 amyloid protein); C, sham group (injected with water instead of Aβ1-40 amyloid protein); and D, the EPI-NCSC treated group (AD rat receiving EPI-NCSC).

Synthetic Aβ1-40 amyloid protein Sigma, St. Louis, MO, USA) dissolved in water at 2 nmol/μl (4 μl) was injected bilaterally into the CA1 area of the hippocampus with characterisation depth of 2.6 mm, 2.0 mm lateral and −3.8 mm

Author's personal copy

anterior to posterior fontanel using a stereotaxic apparatus (Stoelting Co., USA), based on the atlas by Paxinos and Watson (2007). In the AD sham group (group C), 4 μl of water was bilaterally injected over a period of 12 min into the hippocampus using a 26-gauge needle connected to a Hamilton syringe. The needle was slowly withdrawn after the injection (Azad et al. 2011; Rasoolijazi et al. 2007)

Bulge explants and EPI-NCSC culture

Bulge explants from the whisker follicles of 7–10 week-old Wistar rats were prepared. Whisker follicles were dissected and cleaned, and 20 isolated bulges were cut into small pieces, plated in six-well culture plates and immersed in a 3:1 supplemented mixture medium DMEM/F12 containing 5 % foetal bovine serum, antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin and 0.5 μg/ml fungizon), 10 ng/ml epidermal growth factor (EGF, Sigma-Aldrich, USA), 10^{-9}M cholera toxin (Sigma-Aldrich, USA), 0.5 mg/ml hydrocortisone and 5 μg/ml insulin. Bulges adhered to the collagen substratum and within 3–4 days started to release migratory cells that proliferated. Primary culture was carried out for 8 days, after two passages, the cells were labelled with BrdU and grafted to AD model (Nobakht et al. 2011a).

Immunocytochemistry

Characterisation of the expanded EPI-NCSC was performed by immunocytochemistry 10 days after isolation and cell culture. Cells passaged and plated on collagen-coated coverslips overnight were washed three times with phosphate-buffered saline (PBS) and fixed in 4 % paraformaldehyde for 10 min. Fixed cells were then washed three times with PBS and incubated with a blocking buffer (10 % goat serum, Sigma, USA; 0.3 % Triton X-100, Fluka) for 30 min at room temperature. This was followed by overnight incubation at 4 °C with the following primary antibodies: mouse anti-nestin monoclonal antibody (1:200; Millipore, USA) and mouse anti-Sox10 monoclonal antibody (1:100; Chemicon, USA). The next day, they were incubated for 2 h at room temperature with the following secondary antibodies: fluorescein isothiocyanate (FITC) (goat polyclonal antibody to mouse IgG; 1:4,000; Abcam, UK), Alexa Fluor 546-conjugated goat anti-mouse (1:400; Invitrogen, USA). Cell nuclei were counterstained with 1 μg/ml DAPI (Sigma, USA) in PBS for 1 min at room temperature in the dark. Slides were then coverslipped and visualised using a fluorescence microscope.

BrdU labelling

BrdU was used to label the cells 48–72 h before cell transplantation. BrdU (5 μmol/ml; Sigma, USA, St. Louis, MO, USA) was added to flask of cultured cells. To check whether cells were labelled with BrdU after 48 h, the labelled cells that had been on collagen-coated coverslips were washed three times with PBS and fixed in 4 % paraformaldehyde for 10 min. The fixed cells were washed three times with PBS and incubated in 2 N HCL for 45 min at 60 °C and washed twice in 0.1 M borate buffer (pH8.3). After washing, cells were incubated in a blocking buffer (10 % goat serum, 0.3 % Triton X-100 and 1 % bovine serum albumin) for 60 min at room temperature, then incubated overnight at 4 °C with the primary antibody anti-BrdU (1:500; Sigma, USA). The next day, the cells were rinsed three times in PBS and incubated for 1 h at room temperature with the secondary antibody: FITC (goat polyclonal antibody to mouse IgG; 1:4,000; Abcam, UK). Subsequently, they were washed three times in PBS, mounted and visualised using a fluorescence microscope.

EPI-NCSC transplantation

Y-maze analysis was performed 14 days after the induction of AD in order to verify the presence of an AD model. EPI-NCSC (200–300,000 cells in a total of 4 μl solution) was injected bilaterally into the hippocampus of the EPI-NCSC-treated group. The site of injection was in the CA3 region with depth of 3.8 mm, 2.6 mm lateral and −4.30 mm anterior to posterior fontanel, based on the atlas by Paxinous and Watson (2007).

Behavioural measurement

Y-Maze task was done twice. It was initially undertaken14 days after Aβ injection and then 4 weeks after cells transplantation, before killing the animals. Rats were examined in the behavioural assessment Y-maze test. All tests were carried out from 3 to 6 P.M.

Behavioural testing was conducted in an enclosed Plexiglas Y-maze. The Y-maze is a three-arm horizontal maze, 40 cm long, 30 cm high and 15 cm wide in which the arms are symmetrically apposed at 120° angles from each other. The maze floor and walls are constructed from dark opaque polyvinyl plastic (Nobakht et al. 2011b). Rats were initially placed within one arm, and the sequence and number of arm entries were recorded manually for each rat over an 8-min period. Air circulation equipment in continuous operation provided masking noise of 40 dB. Alternation was defined as successive entries into the three arms on overlapping triple sets. The alternation percentage was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus two)×100. The number of arm entries serves as an indicator of locomotor activity (Roghani et al. 2006)

Author's personal copy

Single trial passive avoidance test This test was performed 16 days post-surgery and 4 weeks after cell delivery.

Apparatus The apparatus for the step-through passive inhibitory avoidance test (BPT Co., Tehran, Iran) consists of an illuminated (Base side; 20×20 cm, floor side; 13.5×10 cm; height, 30 cm) and a dark (base side; 20×20 cm, floor side; 15.5×10 cm; height, 30 cm) compartment. These two compartments are divided by a wall that has either a guillotine door or a hole (5×10 cm) connecting them. The dark compartment has a removable cover made of the same material. A lamp (20 W, positioned 20 cm above the apparatus) is used to illuminate the side of the light compartment.

Procedure The test was conducted on four consecutive days. All rats were allowed to adapt to the apparatus on the first and second days of testing for 5 min in the acquisition trial; the rats were gently placed into the illuminated compartment, facing away from the dark compartment. The door to the dark compartment was then opened, and the rat was allowed stepping with all four paws into the dark compartment. On the third day, the rats independently entered the light compartment and remained there for 2 min; then, the guillotine door was opened, and the rat entered the dark compartment and received a 2 s, 1 mA electric footshock. To retest, 24 h later, each rat was again placed in the light compartment. The latency to step through the dark compartment (maximum, 600 s) was measured and recorded as index for the passive avoidance behaviour. The behavioural observations were carried out between 1200 midday to 1500 hours (Roghani et al. 2006).

Histological procedure

Four weeks after cell transplantation, animals were perfused transcardially with 0.9 % saline, followed by 4 % paraformaldehyde (PFA) under anaesthesia [ketamine (100 mg/kg) and xylazine (20 mg/kg) mixture]. Brains were post-fixed in 4 % PFA in phosphate buffer overnight. Following routine processing in paraffin, a total of 16 coronal brain sections (8 μm) were collected from each brain with a rotary microtome (Leitz, 1512, Germany); every section collected was spaced by five sections not collected to ensure that the examined sections were at a similar level between control and experimental rats. One in five sections from each rat was subjected to haematoxylin and eosin (H&E), modified Bielschowsky and Nissl dye staining.

Neuron numbers were counted on Nissl-stained slides (light microscope at ×40 magnification) in the molecular layer in the CA1 area of hippocampus. The results were expressed as density (number/square millimetre) and were calculated as percentage of control rats. Results were analysed using the Student's *t* test.

Immunohistochemistry

Paraffin sections were stained with EnVision G12 double stain system kit (DAKO, USA) after antigen retrieval, according to the kit protocol. The following primary antibodies were used in this protocol: monoclonal anti-βIII-tubulin antibody (1:400,Sigma, USA), monoclonal anti-GFAP antibody (Invitrogen,1:1,000), monoclonal anti-BrdU antibody (1:500; Sigma, USA) and monoclonal anti-ChAT antibody (1:200, Sigma, USA).The secondary antibodies in this protocol were horse radish peroxidase and alkaline phosphatase (AP) detected using by diaminobenzidine (DAB) and Permanent Red staining.

Statistical analysis Data were analysed by one-way analysis of variance (ANOVA) followed by post hoc analysis and Student's *t* test where appropriate. Differences were considered significant at a level of $P<0.05$.

Results

EPI-NCSC characterisation with immunocytochemistry

EPI-NCSC were isolated from the vibrissa follicular bulge area of adult rats, cultured and propagated in vitro (4 days post explanation) (Fig. 1a, b) and formed colonies. Fluorescent cell sorting at passage 1 demonstrated that the cells were positive for nestin (Fig. 1c) and Sox10 (Fig. 1d) and negative for βIII-tubulin and GFAP at this stage (8 days post-explanation) (Fig. 1e). These data suggest that these cells were primitive stem cells. Cells were appropriately BrdU-labelled prior to transplantation (Fig. 1f).

Behavioural tests We determined cognition deficit in rats with well-established plaque and tangle pathology and behavioural deficits. In this study, short-term spatial memory was examined using the Y-maze task. AD group showed a significant reduction in alternation behaviour compared to control and sham groups ($P<0.05$, Fig. 2). There was no significant difference between sham and control groups. As a second test, the passive avoidance test, which demonstrates step-through latency, was used. The AD group rats indicated significant damage in retention in the passive avoidance test. The mean acquisition in passive avoidance test showed a non-significant decrease in the sham group compared to the control group. However, there was a significant decrease in acquisition of passive avoidance response in the AD group compared to control and sham groups ($P<0.05$, Fig. 3). One month after EPI-NCSC transplantation, rats were habituated, trained and tested on two hippocampal-dependent behavioural tasks again.

Author's personal copy

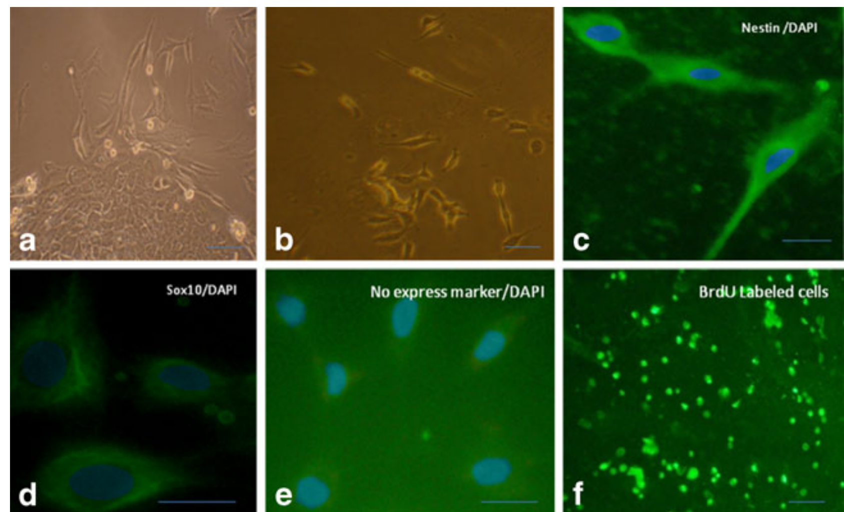

Fig. 1 Immunocytochemical characterisation of EPI-NCSCs. **a**, **b** EPI-NCSCs emigrating from hair follicle explant 8 days post-explantation. **c** Expansion of marker of immature and undifferentiated cells nestin. **d** Expression of NC marker SOX10. **e** Cells did not express βIII-tubulin and GFAP in this stage (eighth day). Nuclei were counterstained using DAPI (*blue*) in **c**–**e**. **f** BrdU-labelled cells (12 days post-explantation, exactly before transplantation). Scale bars=100 μm

As shown in Figs. 2 and 3, the learning and memory impairments, recovered in the treatment group, was significant compared to the AD group. However, there was no significant difference among sham, control and treatment groups. The mean acquisition in passive avoidance test showed a non-significant decrease in sham and treatment groups compared to the control group.

Histological procedure A neuronal population reduction in the hippocampus was observed. Nissl-stained neuronal densities indicated that AD group had consistently reduced neuron density 55.6 % in the CA1 (Fig. 4b). The number of cells in CA1 area (per square millimetre) were 6.350 in control, 6.250 in sham, 2.920 in AD and 6.290 in treatment group. An increase in the number of neurons was observed after EPI-NCSCs transplantation, approximately up to normal levels. Result from the cell counts was not significant ($p>0.05$) between the control group compared with sham and treatment group but was significant ($p<0.05$) between AD group compared with the treatment, control and sham groups. Using Bielschowsky silver staining, amyloid plaques and neurofibrillary tangles were detected in the cerebral cortex and hippocampus of AD and transplantation groups but were not seen in the other groups (Fig. 4c, d). In the sections stained with H&E, no tumour formation was seen in the transplanted group compared to the control group, since there were no hypertrophic and dividing nuclei in any of the transplanted rats 4 weeks after transplantation (Fig. 4e–h).

Immunohistochemistry Using double-staining procedure, we were able to detect numerous glial cells in and around thetransplanted site that were BrdU–GFAP positive; additionally, cells positive for BrdU–βIII-tubulin and BrdU-ChAT were also observed (Fig. 5a–c). Using the double staining EnVision kit, we were able to stain the nuclei of differentiated cells that were BrdU positive as brown and the

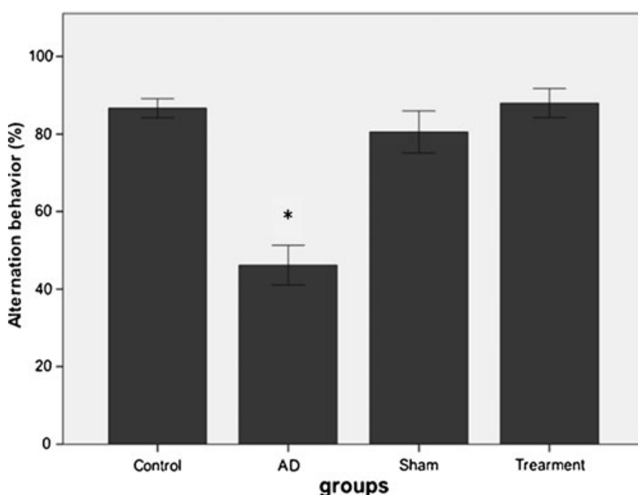

Fig. 2 Per cent of alternation behaviour in Y-maze task in studied group (mean±SEM). *$P<0.05$ as compared to sham, control and treatment groups

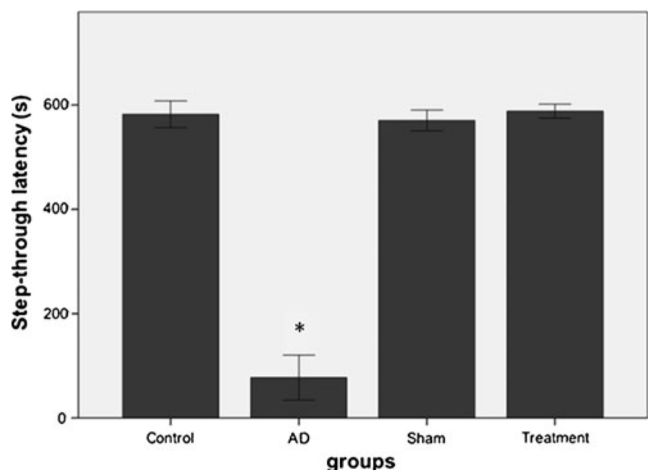

Fig. 3 Single-trial passive avoidance test. Step-through latency of control, sham and treatment rats compared to AD group, *$P<0.05$

Author's personal copy

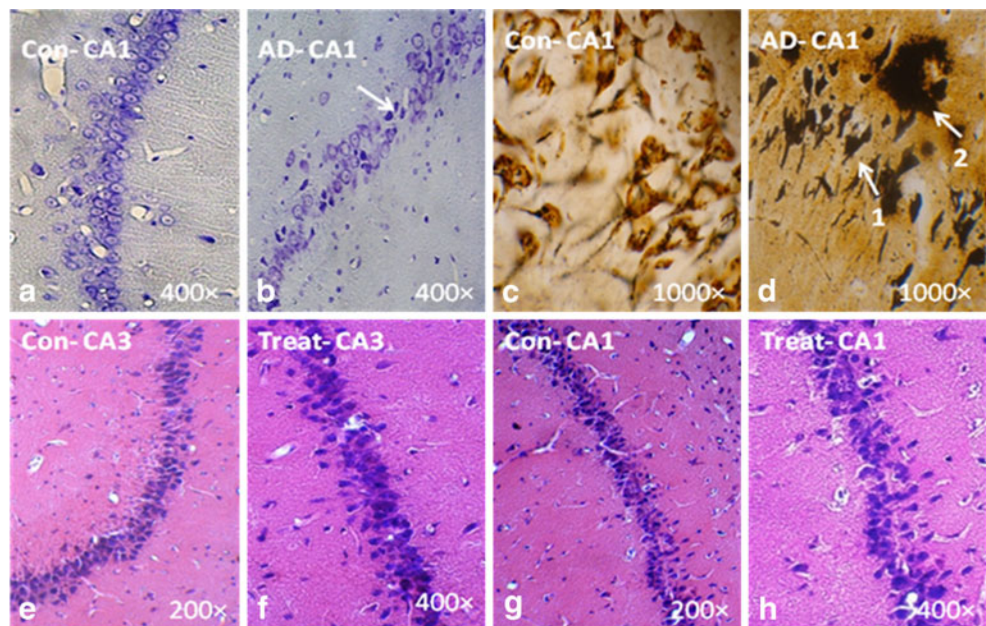

Fig. 4 Histology study of hippocamp. Nissl staining for cell in CA1 area. **a** Control. **b** *Arrow* shows necrotic nuclei in AD model. Bielschowsky staining: **c** no amyloid plaques and neurofibrillary tangle in group and **d** neurofibrillary tangles (*arrow 1*) and amyloid plaque (*arrow 2*) in CA1 area in the AD group. H&E staining, cells transplanted in CA3 area: **e** control group and **b** treatment group, CA1 area. Did not see hypertrophic and dividing nuclei 4-week cells transplantation in any part of hippocamp (tumour formation)

βIII-tubulin or GFAP in the cytoplasm as red. However, for the detection of differentiated cholinergic cells, we used DAB staining for ChAT in the cytoplasm and Permanent Red staining for BrdU in the nuclei; hence, differentiated cholinergic neurons were viewed as having a brown-coloured cytoplasm and red nuclei. We did not count differentiated cells because they were diffused and present not only in hippocampus but also in other parts of the brain.

Discussion

This experiment was performed on a recently developed AD rat model. We improved the AD model by focusing on the production of Aβ plaques and neurofibrillary tangles and decreasing the number of neurons and deficits in memory and learning abilities. We found many amyloid plaques and neurofibrillary tangles in the hippocampus, cortex and white matter. In addition, our findings showed that AD rats have a reduction in the number of neurons in the CA1 zone. Using Y-maze and passive avoidance tests, we showed that the ability of spatial learning and memory in AD rats was significantly decreased compared to the control and sham groups. Improvement in test performance was found between the transplanted and AD control group. This study is similar to other studies in this field and confirmed that deposition of Aβ in AD brains impairs learning and memory (Tsai et al. 2007; Wang et al. 2006; Wu et al. 2007; Nobakht et al. 2011b). The passive avoidance test has been widely used to evaluate rodent working memory ability in association with cortical and hippocampal functions (Shimizu-Sasamata et al. 1992). In our previous study, latency

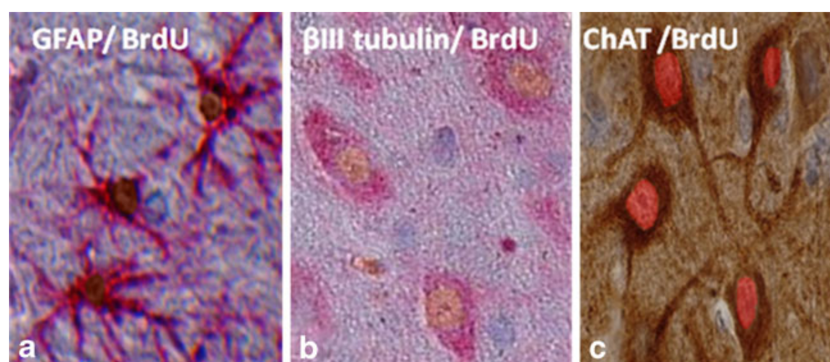

Fig. 5 Differentiation of EPI-NCSC after grafted into rat hippocampus. BrdU. Incorporation and antigenic markers expression in hippocampus using EnVision kit. **a** The cells incubated with BrdU 48 h before transplantation, then paraffin embedded sections immunostained with antibodies against BrdU (DAB; *brown*) and GFAP (Permanent Red; *red*). **b** Neurons like, determined by their expression of βIII-tubulin (Permanent Red; *red*), had incorporated BrdU (DAB; *brown*). **c** ChAt-positive neuron (DAB; *brown*) had incorporated BrdU (Permanent Red; *red*) as determined by double labeling with antibodies against ChAt and BrdU (×400)

Author's personal copy

periods of passive avoidance response in rats were evaluated and showed that the retention of memory was disturbed significantly in the AD control group (Nobakht et al. 2010). In the treatment group, memory improved, and there was no significant difference between the treatment group and the sham and control groups. Moreover, the cognitive ability partially improved in the transplanted group, indicating that the grafted EPI-NCSCs in the AD model might play an important role in this improvement.

Here, we report the isolation and characterisation of rat EPI-NCSCs using the method based on the migratory ability of these cells. A similar method for bulge region isolation and the same culture conditions as described by Sieber-Blum et al. (2004) was used. They have shown that explantation of the bulge region of whisker follicle changes the environment of the stem cell niche in a way that activates proliferation and emigration of NCSCs, so that more than 80 % of emigrated cells are multi-potent (Sieber-Blum et al. 2004; Krejci and Grim 2010). We obtained a population of cells that expressed both NC marker SOX10 and immature cell marker nestin. Thus, glial and neuronal markers were not co-expressed at this stage. Close inspection of the cultures revealed that all cells did not express both markers at low levels. No cells with expression of either marker were detected, supporting the notion that neither matures glia nor differentiated neurons were presented in the grafted cell population.

Moreover, EPI-NCSCs grow rapidly in culture, precluding the need for immortalisation, and differentiate into neurons exclusively with the use of a simple protocol that we used in our previous study (Nobakht et al. 2010) and used by others (Sieber-Blum et al. 2006; Jaks et al. 2010). Furthermore, these cells did not possess the potential for tumour formation (Jaks et al. 2010). In this study, grafted cells did not form tumours since no hypertrophic and dividing nuclei were seen 4 weeks after cell transplantation in H&E-stained sections. In other studies, tumour formation after stem cells transplantation was evaluated at the same duration (Wu et al. 2007; Homayouni Moghadam et al. 2009). Although significant progress has been reported towards stem cells therapy, stem cell sourcing remains a major problem (El Seady et al. 2008). Transplantation of pluri-potential embryonic stem cells into the mammalian brain has seen the development of uncontrolled growth (Roy et al. 2006) and teratoma formation (Wang et al. 2006). Hence, EPI-NCSCs are a favoured source for stem cell therapy.

The hippocampus is affected early in Alzheimer's disease; impaired memory related to hippocampal damage may be associated with deregulations of neurogenesis (Rodriguez and Verkhratsky 2011; Verret et al. 2007). Adult hippocampal neurogenesis may play a role in normal brain functions, such as memory formation (Zhao et al. 2008), and accumulating evidence has shown that neurogenesis is impaired in animal models of AD (Lazarov and Marr 2010), although the mechanism by which adult neurogenesis is impaired in AD remains ill-defined (Se and Yun 2011). In this study, using double-labelling immunohistochemistry, we demonstrated changes in cell proliferation, differentiation and neurogenesis in the hippocampus of AD rat model. Numerous cells were positive for BrdU–βIII-tubulin, BrdU–ChAT and BrdU–GFAP. This showed that the transplanted cells survived and migrated in the host tissue and differentiated into neuron-like and GFAP-positive cells. It has been demonstrated that EPI-NCSs can differentiate into neurons and neuroglia in vivo. Other studies have indicated that these cells can differentiate into neurons and glial cells in vitro (Nobakht et al. 2010, 2011a). Furthermore, these cells can differentiate into Schwann cells that improve movement in peripheral nerve and spinal cord injury models (Sieber-Blum et al. 2006; Hu et al. 2010). We also noted cells positive for BrdU but not co-labelled with the other markers, suggesting that these cells have not differentiated yet. Cells count showed an increase in the number of hippocampal neurons in the grafted animals compared to AD control group, but there were many neurons that were not BrdU positive, suggesting that neurogenesis might be occurring. EPI-NCSCs integrated with the brain tissue and their differentiation improved the microenvironment to induce host neural stem cells. In addition, grafted EPI-NCSCs are either able to prevent cell death or promote neurogenesis in the host. There are other studies that have administered stem cells to improve cognitive recovery in AD and recovery was associated with increase in numbers of granular cells of the hippocampus (Xuan et al. 2009; Wu et al. 2007; Homayouni Moghadam et al. 2009; Wu et al. 2008). While these studies did not quantitate the extent of hippocampal neurogenesis, it is not clear whether the cognitive improvements seen in these AD models involved increased hippocampal neurogenesis. In addition, the improvement of cognitive ability may be due to differentiation of grafted cells to ChAT-positive cells. We found cholinergic differentiation after transplantation of EPI-NCSC. Other studies have shown that generation of cholinergic neurons from other cells in experimental animals is possible (Wu et al. 2007; HomayouniMoghadam et al. 2009). The beneficial effect of the grafted cells could be attributed to one or both of two functions of the cells in vivo: (1) simple secretion of acetylcholine from transplanted cells or (2) actual functional integration into the host tissue (Nikolic et al. 2008). The improvement in reference memory function in grafted animals suggests that transplanted cells exert their effect by generating new synapses as well as acetylcholine secretion.

Conclusion

In the present study, we have showed that transplantation of EPI-NCSC into the hippocampus of a rat model of AD

Author's personal copy

resulted in the recovery of memory and learning compared to AD control group. These rats express many features of AD; especially, neuronal loss in the brain is comparable to AD brain. We have also demonstrated that neuro-degeneration triggers adult neurogenesis in the dentate gyrus. This effect is dynamically associated with different neurodegenerative stages. Most importantly, the dynamic involvement of neurogenesis in the dentate gyrus is correlated to the degree of neuronal loss, providing the first evidence that the endogenous neurogenesis may functionally work as a self-repairing mechanism to compensate, at least partially, for the neuronal loss-induced by neurodegeneration. As the hippocampus is one of the regions in the brain most vulnerable to AD neurodegeneration and this vulnerability may contribute to the earliest symptoms, learning and memory deficit, the demonstration of the dynamic changes of neurogenesis in this region suggests that enhancing the endogenous adult neurogenesis may be a valuable strategy to delay or stop the neurodegenerative progression (Tchantchou et al. 2007).

Acknowledgements This work was supported by grant no. 89000433 from Iran National Science Foundation (INSF). The authors would like to thank the Department of Pharmacology in Medicine faculty of Tehran University of Medical Science.

References

Xuan AG, Luo M, Ji WD, Long DH (2009) Effects of engrafted neural stem cells in Alzheimer's disease rats. NeurosciLett 450(2):167–171

Givens B, Olton D (1994) Local modulation of basal forebrain: effects on working and reference memory. J Neurosci 14(6):3578–3587

Korecka JA, Verhaagen J, Hol EM (2007) Cell-replacement and gene-therapy strategies for Parkinson's and Alzheimer's disease. Regen Med 2(4):425–446

Tsai KJ, Tsai YC, Shen CK (2007) G-CSF rescues the memory impairment of animal models of Alzheimer's disease. J Exp Med 204(6):1273–1280

Abrous DN, Koehl M, Le Moal M (2005) Adult neurogenesis: from precursors to network and physiology. Physiol Rev 85(2):523–569

Colucci-D'Amato L, Bonavita V, di Porzio U (2006) The end of the central dogma of neurobiology: stem cells and neurogenesis in adult CNS. Neurol Sci 27(4):266–270

Rodriguez JJ, Verkhratsky A (2011) Neurogenesis in Alzheimer's disease. J Anat 219(1):78–89

Taupin P (2006) Neurogenesis in the adult central nervous system. C R Biol 329(7):465–475

Verret L, Jankowsky JL, Xu GM, Borchelt DR, Rampon C (2007) Alzheimer's-type amyloidosis in transgenic mice impairs survival of newborn neurons derived from adult hippocampal neurogenesis. J Neurosci 27(25):6771–6780

Rodriguez JJ, Jones VC, Verkhratsky A (2009) Impaired cell proliferation in the subventricular zone in an Alzheimer's disease model. NeuroReport 20(10):907–912

Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132(4):645–660

Zhang C, McNeil E, Dressler L, Siman R (2007) Long-lasting impairment in hippocampal neurogenesis associated with amyloid deposition in a knock-in mouse model of familial Alzheimer's disease. Exp Neurol 204(1):77–87

Haughey NJ, Liu D, Nath A, Borchard AC, Mattson MP (2002a) Disruption of neurogenesis in the subventricular zone of adult mice, and in human cortical neuronal precursor cells in culture, by amyloid beta-peptide: implications for the pathogenesis of Alzheimer's disease. Neuromolecular Med 1(2):125–135

Haughey NJ, Nath A, Chan SL, Borchard AC, Rao MS, Mattson MP (2002b) Disruption of neurogenesis by amyloid beta-peptide, and perturbed neural progenitor cell homeostasis, in models of Alzheimer's disease. J Neurochem 83(6):1509–1524

Jin K, Peel AL, Mao XO, Xie L, Cottrell BA, Henshall DC et al (2004) Increased hippocampal neurogenesis in Alzheimer's disease. ProcNatlAcadSci U S A 101(1):343–347

Cotsarelis G (2006) Epithelial stem cells: a folliculocentric view. J Invest Dermatol 126(7):1459–1468

Cotsarelis GST, Lavker RM (1990) Label-retaining cells reside in the bulge area of pilosebaceous unit: implications for follicular stem cells, hair cycle, and skin carcinogenesis. Cell 61:1329–1337

Morrison SJ, White PM, Zock C, Anderson DJ (1999) Prospective identification, isolation by flow cytometry, and in vivo self-renewal of multipotent mammalian neural crest stem cells. Cell 96:737–749

Bixby S, Kruger GM, Mosher JT, Joseph NM, Morrison SJ (2002) Cell-intrinsic differences between stem cells from different regions of the peripheral nervous system regulate the generation of neural diversity. Neuron 35(4):643–656

Kruger GM, Mosher JT, Bixby S, Joseph N, Iwashita T, Morrison SJ (2002) Neural crest stem cells persist in the adult gut but undergo changes in self-renewal, neuronal subtype potential, and factor responsiveness. Neuron 35(4):657–669

Li HY, Say EH, Zhou XF (2007) Isolation and characterization of neural crest progenitors from adult dorsal root ganglia. Stem cells 25(8):2053–2065

Tomita Y, Matsumura K, Wakamatsu Y, Matsuzaki Y, Shibuya I, Kawaguchi H et al (2005) Cardiac neural crest cells contribute to the dormant multipotent stem cell in the mammalian heart. J Cell Biol 170(7):1135–1146

Yoshida S, Shimmura S, Nagoshi N, Fukuda K, Matsuzaki Y, Okano H et al (2006) Isolation of multipotent neural crest-derived stem cells from the adult mouse cornea. Stem cells 24:2714–2722

Fernandes KJ, McKenzie IA, Mill P, Smith KM, Akhavan M, Barnabé-Heider F et al (2004) A dermal niche for multipotent adult skin-derived precursor cells. Nat cell biol 6:1082–1093

Amoh Y, Li L, Katsuoka K, Penman S, Hoffman RM (2005) Multipotentnestin-positive, keratin-negative hair-follicle bulge stem cells can form neurons. ProcNatlAcadSci USA 102(15):5530–5534

Sieber-Blum M, Grim M, Hu YF, Szeder V (2004) Pluripotent neural crest stem cells in the adult hair follicle. DevDyn 231(2):258–269

Yu H, Kumar SM, Kossenkov AV, Showe L, Xu X (2010) Stem cells with neural crest characteristics derived from the bulge region of cultured human hair follicles. Dermatol 130:1227–1236

Britsch S, Goerich DE, Riethmacher D, Peirano RI, Rossner M, Nave KA et al (2001) The transcription factor Sox10 is a key regulator of peripheral glial development. Genes Dev 15:66–78

Rehberg S, Lischka P, Glaser G, Stamminger T, Wegner M, Rosorius O (2002) Sox10 is an active nucleocytoplasmic shuttle protein, and shuttling is crucial for Sox10-mediated transactivation. Mol Cell Biol 22:5826–5834

Lendahl U, Zimmerman LB, McKay RD (1990) CNS stem cells express a new class of intermediate filament protein. Cell 60:585–595

Author's personal copy

Lothian C, Lendahl U (1997) An evolutionarily conserved region in the second intron of the human nestin gene directs gene expression to CNS progenitor cells and to early neural crest cells. Eur J Neurosc 9:452–462

Mokry J, Cizkova D, Filip S, Ehrmann J, Osterreicher J, Kolar Z et al (2004) Nestin expression by newly formed human blood vessels. Stem Cells Dev 13(6):658–664

Li L, Mignone J, Yang M, Matic M, Penman S, Enikolopov G et al (2003) Nestin expression in hair follicle sheath progenitor cells. ProcNatlAcadSci USA 100:9958–9961

Krejci E, Grim M (2010) Isolation and characterization of neural crest stem cells from adult human hair follicles. Folia Biol (Praha) 56(4):149–157

Clewes O, Narytnyk A, Gillinder KR, Loughney AD, Murdoch AP, Sieber-Blum M (2011) Human epidermal neural crest stem cells (hEPI-NCSC)-characterization and directed differentiation into osteocytes and melanocytes. Stem Cell Rev 7(4):799–814

Azad N, Rasoolijazi H, Joghataie MT, Soleimani S (2011) Neuroprotective effects of carnosic acid in an experimental model of Alzheimer's disease in rats. Cell Journal (Yakhteh) 13(1):39–44

Rasoolijazi H, Joghataie MT, Roghani M, Nobakht M (2007) The Beneficial Effect of Epigallocatechin-3-Gallate in an Experimental Model of Alzheimer's disease in Rat: a Behavioral Analysis. Iran Biomed J 11(4):237–243

Nobakht M, Asalgoo S, RahbarRooshandel N, Mousavizadeh K, Najafzadeh N (2011a) Effects of silibinin on hair follicle stem cells differentiation to neural-like cells. Am J BiochemMolBiol 1(2):212–222

Roghani M, Joghataie MT, Jalali MR, Baluchnejadmojarad T (2006) Time course of changes in passive avoidance and Y-maze performance in male diabetic rats. Iran Biomed J 10:99–104

Wang Q, Matsumoto Y, Shindo T, Miyake K, Shindo A, Kawanishi M et al (2006) Neural stem cells transplantation in cortex in a mouse model of Alzheimer's disease. JMI 53:61–69

Wu QY, Li J, Feng ZT, Wang TH (2007) Bone marrow stromal cells of transgenic mice can improve the cognitive ability of an Alzheimer's disease rat model. NeurosciLett 417:281–285

Nobakht M, Hoseini SM, Mortazavi P, Sohrabi I, Esmailzade B, RahbarRooshandel N et al (2011b) Neuropathological changes in brain cortex and hippocampus in a rat model of Alzheimer's disease. Iran Biomed J 15(1 & 2):51–58

Shimizu-Sasamata M, Yamamoto M, Okada M, Yamaguchi T, Tamura A (1992) Effects of indeloxazine hydrochloride on behavioral and biochemical changes in chronic phase of focal cerebral ischemia in rats. Arch Int Pharmaco dyn Ther 314:74–89

Nobakht M, Najafzadeh N, Safari M, RahbarRoshandel N, Delaviz H, Joghataie MT et al (2010) Bulge cells of rat hair follicles: isolation, cultivation, morphological and biological features. Cell journal (Yakhteh) 12(1):51–58

Sieber-Blum M, Schnell L, Grim M, Hu YF, Schneider R, Schwab ME (2006) Characterization of epidermal neural crest stem cell (EPI-NCSC) grafts in the lesioned spinal cord. Mol Cell Neurosci 32:67–81

Jaks V, Kasper M, Toftgård R (2010) The hair follicle—a stem cell zoo. Exp Cell Res 316(8):1422–1428

Homayouni Moghadam F, Alaie H, Karbalaie K, Tanhaei S, Nasr Esfahani MH, Baharvand H (2009) Transplantation of primed or unprimed mouse embryonic stem cell-derived neural precursor cells improves cognitive function in Alzheimerian rats. Differentiation 78(2–3):59–68

El Seady R, Huisman MA, Löwikc WGM, Frijns JHM (2008) Uncomplicated differentiation of stem cells into bipolar neurons and myelinating glia. BiochemBiophys Res Commu 376:358–362

Roy NS, Cleren C, Singh SK, Yang L, Beal MF, Goldman SA (2006) Functional engraftment of human ES cell-derived dopaminergic neurons enriched by coculture with telomerase-immortalized midbrain astrocytes. Nat Med 12(11):1259–1268

Lazarov O, Marr RA (2010) Neurogenesis and Alzheimer's disease: at the crossroads. ExpNeurol 223:267–281

Se HC, Yun L (2011) Elevated levels of BMP6 impair neurogenesis in Alzheimer's disease. jneurosci 31(2):371–372

Hu YF, Gourab K, Wells C, Clewes O, Schmit BD, Sieber-Blum M (2010) Epidermal neural crest stem cell (EPI-NCSC)-mediated recovery of sensory function in a mouse model of spinal cord injury. Stem Cell Rev 6(2):186–198

Wu S, Sasaki A, Yoshimoto R, Kawahara Y, Manabe T (2008) Neural stem cells improve learning memory in rats with Alzheimer's disease. Pathobiology 75(3):186–194

Nikolic WV, Hou H, Town T, Zhu Y, Giunta B, Sanberg CD et al (2008) Peripherally administered human umbilical cord blood cells reduce parenchymal and vascular B-amyloid deposits in Alzheimer mice. Stem Cells Dev 17:1–17

Tchantchou F, Xu Y, Wu Y, Christen Y, Luo Y (2007) EGb 761 enhances adult hippocampal neurogenesis and phosphorylation of CREB in transgenic mouse model of Alzheimer's disease. FASEB J 21(10):2400–2408

Paxinos G, Watson C (2007) The rat brain in stereotaxic coordinates, 6th edn. Academic, London