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ScienceDirect
Neuroscience Letters 421 (2007) 185–190
Neuroscience Letters
www.elsevier.com/locate/neulet

Genetic modification does not affect the stemness of neural stem cells in nestin promoter-GFP transgenic mice

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Received 12 December 2006; received in revised form 31 January 2007; accepted 19 February 2007

Abstract

Because nestin promoter-GFP mice have frequently been used in neural stem cell (NSC) research, it is essential to prove that there is no alteration in the stemness of NSCs derived from this transgenic model for the interpretation and validity of the data. We compared the stemness of NSCs derived from transgenic mice expressing GFP driven by the nestin enhancer with those from wild-type (C57BL/6) mice with respect to the general gene expression profile, expression of neural stem cell markers as nestin and Sox2, and responsiveness to neurotrophins (BDNF, PDGF-BB, and NT-3). The gene expression profile analysis showed that the coefficient of correlation between the two groups was very high ($r = 0.9865$) in the total genes. We found that 23 genes were either up- or down-regulated more than two-fold in the NSCs from the transgenic mice ($p < 0.05$), without any obvious functional relatedness among them. Likewise, there was no difference between the two mouse groups in the expression of nestin or Sox2, the ability to form neurospheres and the neuronal differentiation of NSCs by neurotrophins. Taken together, the self-renewal and neuronal differentiation ability of NSCs from the transgenic mice showed the great similarity to those from wild-type mice. Such information will be useful when the properties of NSCs are evaluated following genetic modification in such a nestin-GFP Tg model.

Keywords: Transgenic; Microarray; Nestin; Sox2; Neurotrophin; Neural stem cell

The discovery of neural stem cells (NSCs) opened the door to a novel therapeutic approach for incurable neuronal diseases [8]. A variety of methods including fluorescence tagging have been developed for the prospective characterization of NSCs [5,11]. Nestin is a class IV intermediate filament protein expressed in NSCs and progenitor cells [10]. Although there are several transgenic (Tg) mice expressing marker proteins such as green fluorescent protein (GFP) under the control of nestin gene regulatory regions [2,7,13,19], there have been no studies in which NSCs derived from this type of Tg mice were compared with those derived from wild-type (WT) mice in terms of their stemness (self-renewal, differentiation, etc.).

In this study, we used one of the nestin-GFP Tg models, which has been used to isolate NSCs and has been conducted functional analyses for NSCs [3,16,18,21]. We tried to investigate the alteration of the nature of the NSCs in the nestin-GFP Tg mice and provide useful information to verify the usefulness of such a model in NSC research.

For the assessment of stemmness, we determined the neurosphere formation ability, gene expression profile and expressions of stem cell markers in the primary neurospheres. In addition, we evaluated the differentiation of the neurosphere forming cells after the treatment of various neurotrophins, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) or platelet-derived growth factor (PDGF-BB).

Nestin-GFP Tg mice were kindly provided by Yamaguchi [20]. Inbred SPF C57BL/6 mice (Samtako, Seoul, Korea) were randomly housed at the animal facility of National Institute of Toxicological Research. All animal procedures were done in accordance with a program which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). For dating the pregnancies, the date when the

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doi:10.1016/j.neulet.2007.02.069

vaginal plug was observed was defined as embryonic day 0.5 (E0.5).

Cells from the forebrain of E11.5 embryo were triturated in cold Hank's Balanced Salt Solution (HBSS). Viable cells were counted by the trypan blue exclusion method and plated at a density of 4×10^5 cells/ml. For neurosphere formation, the cells were incubated in a stem cell culture medium (DMEM:F12 = 1:1, N2 supplement, 20 ng/ml EGF, 20 ng/ml bFGF) at 37 °C with a 5% CO_2 atmosphere. EGF and bFGF were added to the culture medium every 2 days.

The cells were subcultured as previously described with a slight modification [17]. Briefly, the neurospheres were collected by centrifugation for 5 min at $110 \times g$. The pellets were resuspended in accutase (Innovative Cell Technologies Inc.) and triturated using pipette. After centrifugation, the viable cells were resuspended with culture media and neurosphere conditioning media (1:1 cocktail).

Total RNA was isolated from the primary neurospheres using an RNeasy kit (Qiagen). Five micrograms of total RNA from each sample were used to synthesize cRNA and were hybridized onto Applied Biosystems mouse genome survey microarrays representing 30,000 transcripts following the manufacturer's protocol (Applied Biosystems).

The microarrays were analyzed using ABI1700 Chemiluminescent Microarray Analyzer. The differences in the intensity of the microarrays were normalized. And those values for which the signal to noise ratio was less than two were ruled out and grouped using the Avadis Prophetic program version 3.3 (Strand Genomics Pvt. Ltd.).

The neurospheres or dissociated cells were plated onto poly-L-ornithine (PLO) coated cover slips, and then fixed with 4% paraformaldehyde in PBS at room temperature for 10 min. The cells were incubated with blocking solution containing a specific primary antibody at room temperature for 3 h at the following dilutions; a mouse monoclonal anti-nestin antibody (1:100, Chemicon), a rabbit polyclonal anti-Sox2 antibody (1:100, Stem cell Co.), and a monoclonal anti-tubulin βIII (1:100, Covance). After washing with TPBS (PBS containing 0.1% tween 20), the cells were probed with FITC or texas-red conjugated secondary antibody (1:100, Vector Lab.), and then with DAPI. The fluorescence images were acquired with a fluorescent microscope (Leica Microsystems).

The single cells which were dissociated from the primary neurospheres were plated and fixed at a density of 5×10^5 cells/ml in 96 well plates. After washing with PBS, the pellet was resuspended in SAP buffer (HBSS containing 0.1% saponin and 0.05% sodium azide) and was incubated with a mouse monoclonal anti-nestin antibody (1:100) or a rabbit polyclonal anti-Sox2 antibody (1:100). After the removal of the primary antibody, the cells were incubated with FITC-conjugated anti-mouse IgG (1:100, Vector Lab.) or PE-conjugated anti-rabbit IgG (1:100, Abcam). The flow cytometric analysis was conducted using FACSCalibur™ (Becton Dickinson).

From passage 2 to 4, the NSCs were plated at a density of 5×10^4 cells/well onto PLO-coated glass cover slips in 24 well plates and cultured with media for 48hrs. Differentiation was induced by withdrawal of EGF and bFGF and following treatment with each neurotrophin (BDNF, NT-3, or PDGF-BB 50 ng/ml, Chemicon) for 6–8 days.

To quantify the number of neurons and nuclei, five representative fields per well were randomly selected and digitalized with 20× objective using a cooled CCD digital camera (Leica Microsystems).

The statistical analysis was carried out using Sigma Stat (version 3.01). The significance was tested by two-way analysis of variance, followed by the Bonferroni *post hoc* test with $p < 0.05$.

To determine the gene expression of the NSCs by nestin prometer-GFP transgene, we compared the gene expression profiles in the primary neurospheres from the Tg mice with those from the WT mice using microarrays. Four independent experiments were performed and the statistical analysis was carried out by the *t*-test.

The genes showing more than two-fold expression changes ($p < 0.05$) are total 21 genes. Fifteen genes were down-regulated and six genes were up-regulated in the primary neurospheres derived from the Tg mice as compared to those from the WT mice. Among them, seven genes were known genes which serine/threonine kinase, C-type lectin like receptor-1 and prostaglandin F2 receptor were down-regulated and NADH dehydrogenase (ubiquinone) 1 beta subcomplex, S100 calcium binding protein A4, torsin family 3, member A and ribosomal protein L14 were up-regulated. However, there were no functional relatedness among them. Furthermore, the gene expression profile in the primary neurospheres from each group was similar and the coefficient of correlation was very high ($r = 0.9865$) in the total genes (Fig. 1).

Also, there were no differences in the expression of Sox2 and nestin positive cells between the neurosphere forming cells derived from the Tg and WT mice. Most nestin positive cells also express GFP but nestin expression did not show perfect coincidence with GFP expression in neurosphere forming cells derived from Tg mice (Fig. 2A).

To investigate the ratio of each stem cell marker expression, and to confirm the expression of nestin and GFP in neurospheres forming cells, we also compared the nestin and Sox2 positive

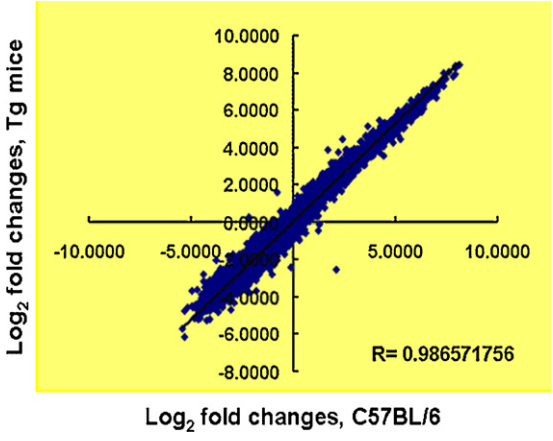

Fig. 1. Expression profile and correlation coefficient for total genes of neurospheres in Tg and WT mice.