DIFFERENTIATION OF NEURAL STEM CELLS TRANSDUCED „IN VIVO” WITH LENTIVIRAL VECTORS

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INTRODUCTION

Until recent times, it was believed that the regeneration mechanisms of the central nervous system (CNS) would be limited to the postmitotic state and would imply the following mechanisms: the increase of axonal terminations and the synaptic reorganization. Nowadays, we can demonstrate the presence and functionality of neurogenesis and we can isolate neural precursor cells from the brain of adult mammals and human beings too, which offers good...
development perspectives of some new and veridical strategies of curing degenerative diseases of the CNS.1

Stem cells are characterized by their own regeneration capacity and by their ability to differentiate into multiple phenotypes. Neural stem cells (NSC) differentiate into neuroectodermal progenitors and also into neurons, astrocytes, oligodendrocytes, not only in vivo but also in vitro.2,3 In mice, during their lifetime, adult neural stem cells are localized in two neurogenic cerebral zones: the subventricular zone (SVZ) of the lateral cerebral ventricles and the subgranular layer of the dentate gyrus (SL).4

The subventricular zone (SVZ), located along the lateral wall of each lateral cerebral ventricle (LV), hosts the largest population of proliferating cells from the brain of the majority of adult mammals, including humans.5 These proliferating cells can be divided into 4 types:

1. Multiciliated ependymal cells (MEC) – Type E cells, which separate the ventricular lumen from the subjacent cerebral parenchyma and induce the cerebrospinal fluid circulation.
2. Slowly proliferating cells – astroglial cells – Type B cells, which generate:
3. Actively proliferating cells – Type C cells, also called transitory progenitors, which give birth to
4. Migratory neuroblasts – Type A.

Type A neuroblasts migrate in chains along the rostral migratory stream (RMS) to the olfactory bulb (OB). Type C cells form agglomerations between the migratory chains, along the SVZ. When reaching the OB, the neuroblasts detach from the chain structure and migrate into the granular and periglomerular layers, where up to 75-99 percents of them turn into periglomerular neurons (GABA-ergic or tyrosine-hydroxylase). Although they have the same cellular origin, the formation of the two neuron types takes place in two different zones: for example, periglomerular neurons are born in the RMS and granular neurons are born in the SVZ, their differentiation being controlled by different transcription factors.6

The structure and organization of the SVZ in adult human beings is different from the one present in other mammals, to a certain extent. The lateral ventricular wall consists of four layers with different thicknesses and cellular densities: a monolayer of ependymal cells, a hypocellular layer, which contains mainly the astrocytic prolongations, the astrocytic layer and a transitional zone to the cerebral parenchyma. The migratory chains could not be identified in the SVZ in humans. Although, some young cells have been found in the granular and periglomerular layer of the OB, one cannot demonstrate a so-called migratory stream from the SVZ.7

The second region where neurogenesis is continuously present in mice, monkeys and humans is the subgranular layer (SL) of the dentate gyrus of the hippocampal formation. Neural stem cells migrate over a short distance into the molecular layer and give birth to mature granular cells, which send their dendritic terminations to the external molecular layer of the cerebral structure.5 These cells show electrophysiological properties similar to those of mature granular neurons. Still it is not well known to what extent these newly generated neurons contribute to the functionality of the OB and of the hippocampal formation, but researches underline their importance in the acquisition of the olfactory memory.

MATERIAL AND METHODS

Lentiviral vectors

The lentiviral vector particle was produced by derivating the HIV-1 virus8,9 following the conjugation of three plasmids: 10 the transfer plasmid (pCH-GFP-WPRE), which contains the green fluorescent protein (eGFP) as a reporter gene, a plasmid which encodes the envelope of VSV-G (pMDG) and a second generation plasmid (pCMV-GFP). The central polypurine tract (cPPT) was inserted by blunted digestion in the PpuMI and BamHI sites, for the nuclear import facilitation. The vector also includes the cytomegalic virus promoter (CMV) and the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and the final construction is pCH-cPPT-CMV-GFP-WS. (Fig. 1)

Figure 1. Overview of the lentiviral vector construct: (A) cPPT-CMV fragment was extracted from pTO-cPPT-CMV-eGFP (Molecular Virology Dept., KUL) by digestion with PpuMI and BamHI sites, for the nuclear import facilitation. The vector also includes the cytomegalic virus promoter (CMV) and the Woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and the final construction is pCH-cPPT-CMV-GFP-WS. (B) represents the schematic construct of the lentiviral vector pCH-cPPT-CMV-GFP-WS. (Fig. 1)
Eagle Medium, Invitrogen) with 10% FCS (Fetal Calf Serum). The next day, 20 μg of transfer plasmid, 10 μg of packaging construct and 5 μg of envelope plasmid were mixed in NaCl-PEI (polyethylemine) enriched solution. After 15 min incubation at room temperature, the DNA-PEI complex was added to the 293T cells in DMEM with 1% FCS solution and placed under 37°C in a 5% CO₂ humidified atmosphere for 5 hours, after which the medium was replaced with DMEM, 10% FCS and 25 mM HEPES buffer. The supernatant was harvested 2 and 3 days post-transfection, purified and concentrated by tangential flow filtration (TFF). The filtered vector particles were further concentrated by low-speed centrifugation (5 hrs, 26,000 g) at 4°C and re-dissolved afterwards in PBS, resulting in an 800-fold concentration, which was stored at -70°C. The vectors titers were determined by functional titration methods after transduction on cells, namely by expression of the eGFP transducing units (TU/ml) quantified by FACS (fluorescence-activated cell sorting) analysis and were ranging between 10⁹–10¹⁰ (TU/ml).

In order to make a comparison between the efficiency of vector transfection and transduction, retroviral vectors have been used in parallel. A 750-b fragment containing the entire coding region of the GFP gene was picked up by HpaI and HindIII digestion from the p-eGFP-N1 vector (Clontech Labs, Inc, CA). The eGFP fragment was then inserted into the HpaI – HindIII multiple cloning site of the retrovirus pLNCX (Clontech Labs). The eGFP gene is regulated by the cytomegalovirus (CMV) promoter and the final vector construction is pLNC-GFP.

**Figure 2.** Marking of dividing cells in the SVZ-RMS-OB pathway by vector injection in the SVZ: a. eGFP expression was detected in the SVZ and the RMS 2 days after the retroviral injection. b. eGFP positive cells were detected in the striatum around the site of injection with lentiviral vectors, after 2 days.

**Figure 3.** Evidence of eGFP marked cells after vector injection in the SVZ: a. eGFP expression in the OB 1 week after the retroviral injection. b. eGFP expression in the OB 1 week after lentiviral injection.

**Histology**

For these experiments we have used a number of 36 C57BL/6 mice, 8-9 weeks old, complying with the bioethical requirements on animal testing. Mice received intraperitoneal anesthesia with ketamine (75µg/g) and medetomidine (1µg/g) and they were prepared for stereotactic surgery, with the purpose of injecting vectors in the SVZ and LV.
A number of 12 mice were injected with 1.4x10⁸ RNA copies in the right SVZ and the same number in the LV respectively. Two animals for each experiment have been sacrificed at different intervals: 2 days, 1 week, 2 weeks, 1 month, 2 months and 7 months, for the identification of GFP expression in the migratory stream consisting in the subventricular zone – the rostral migratory stream – the olfactory bulb (SV-RMS-OB) with the help of immunohistology.

For control purposes, 6 mice having the same characteristics were injected with the retroviral vector, 2.8x10⁸ RNA copies, in the right SVZ and respectively in the LV and then sacrificed after 2 days, 1 week, 2 weeks, 1 month, 2 months and 7 months after injection.

In order to mark cell division from the cerebral substance, we used Bromodeoxyuridine (BrdU, 50µg/g) which was injected in the intraperitoneal region of the operated mice, for 5 consecutive days following surgery, with the purpose of obtaining the desired histological material.

For the immunofluorescence evaluation, the sections have been treated with the following primary antibody dilutions: Rat anti-BrdU – for marking cells in division; Goat anti-double cortin – for mature neurons; Mouse Anti-Polysialic Acid (Anti-PSA) – for migratory neuroblasts; Mouse anti-neuronal nuclear antigen – for mature neurons; Mouse anti-GFAP (glial fibrillary acidic protein) – for glial cells, astrocytes and B type stem cells; Rabbit anti-eGFP - for marking transduction. The day after, the sections have been washed and incubated into the following secondary antibody dilutions: Alexa Fluor donkey anti-rabbit; Alexa Fluor donkey anti-mouse; Alexa Fluor donkey anti-goat; Alexa Fluor donkey anti-rat. The sections treated in this manner have been analyzed through confocal laser scanning microscopy.

**Histological analysis**

In order to analyze the efficiency of proliferating cells transduction by lentiviral vectors, in the SVZ, the double-labeled cells for eGFP and cortin have been identified and quantified through confocal scanning laser microscopy with x40 objective lens. Seven microscopic regions have been analyzed for each animal at intervals of 300µm, starting with the dorsolateral region towards the peak of the LV and SVZ, until the vicinity of the injection point.

For the estimation of the total number of eGFP-positive cells from the OB, we have used the random sampling method with the help of the optical fractionator. For each animal seven regions were selected, at intervals of 300µm. With the help of a micrometric scale, the eGFP-positive cells were quantified inside the selected square by a tridimensional count, which lead to an estimation of the total number of cells from the desired area.

**RESULTS**

**eGFP - positive cells identification**

Following the gene transfer through the lentiviral vector, we noticed an increased in time of the GFP-positive cells expression in the OB, by using methods of immunohistology and effective quantification of the OB cells. In order to determine the efficiency of neuroblasts transduction by the lentiviral vectors, compared to the retroviral ones, equal doses of vectors have been injected in the SVZ and LV, which have been analyzed at identical time intervals.

For the immunofluorescence analysis, the seriated sections obtained through the method previously described have been double-labeled for the visualization of cortin and eGFP for the confocal microscopic analysis. Therefore, the majority of the cells from or around the subependymal layer (SL) of the OB have been eGFP-cortin labeled, respectively identified as mature interneurons. In the granular layer of the OB, there has been identified the biggest number of eGFP-positive neurons, which must have been newly generated, because they have been labeled at the same time as positive in the BrdU too (cells in division). They started to be detected one month after injection and they originate in proliferatory and migratory cells, transduced with the lentiviral vector.

While the cells labeled for the retroviral vector are just temporarily present in the SVZ for a little more than a month, the lentivirally transduced cells remained present in the RMS and their number remained increased along the 7 months, under control. (Fig. 4) It confirms the hypothesis that the gene transfer into neural stem cells is superior in the case of lentiviral vectors.

In order to study if eGFP-positive cells from the SVZ are migratory neuroblasts (Type A) or slowly-dividing stem cells (Type B), a double labeling for eGFP–BrdU has been used concomitantly with the labeling with anti-PSA antibodies (polysialic acid), for the migratory neuroblasts and respectively anti-GFAP (glial acidic fibrillary protein) for the slowly-dividing or glial cells. Because positive cells in eGFP-GFAP (Type B) have been detected in all the reference points of the migratory stream and at all time intervals, it means that stem cells continuously generate positive progenitory cells, which migrate to the OB.

The lentiviral transduction of mitotic cells in the SVZ illustrated the migration of neuroblasts...
from the SVZ to the subependymal layer of the OB. This migration requires between 2 and 7 days and it is followed by 5-7 days of radial migration to the granular layer. In this location takes place the cellular maturation, from the stage of simple non-ramified cells to the stage of mature neurons with their dendritic ramifications located in the external plexiform layer, a process which requires 20-30 days.

Transduced cells morphology, migrated in the OB

The integration of lentiviral vectors into the neural stem cells genoma and respectively of their derivatives is stable and for a long term, and its gene expression is not diminished through cell division. Due to the specificity of vectors for neural stem cells, their transduction can be used, besides the long-term analysis of the migration and differentiation of stem cells or their derivatives in vivo, also in order to describe the typology and morphology of lentivirally transduced cells from the OB.

Following the lentiviral transduction in the SVZ, the observation of the cellular migration and differentiation showed an equal distribution of cells in the entire OB and for a long time. The cells which have been identified in the granular layer have been described as being in close contact or in small groups with the typical morphology of granular interneurons: round cellular body, little cytoplasm and pluri-ramified terminations, long oriented towards the external plexiform layer. The cellular body is often located around the neurites of other local cells, which can suggest their guiding role for the new cells in the process of adhesion and fixation in the OB.

Together with these granular neurons, we have identified other cellular morphological types with different levels of GFP expression: triangular cells; big cells with much cytoplasm and big vesicular nucleus, dark colored; round cells with very rich dendritic ramifications and clearly emphasized, located in the superficial part of the granular layer; apoptotic cellular fragments. For the different types of observed morphologies, we cannot characterize the maturation stages or cell functionality, but we can state that numerous long and vigorous ramifications, especially from the superficial part of the layer, are in contact with the nervous terminations of the nasal mucosa olfactory neurons, participating probably in the transmission of the olfactory sense.

B type stem cells transduction from the LV and SVZ

It has been previously proved that the authentic stem cells from the LV are the astroglial cells (B Type). These cells have been described as having uni- or bipolar morphology, with a main or unique prolongation in contact with the ventricular lumen. The majority of the LV cells eGFP-labeled had the typical morphology of slowly-proliferating cells, but, besides them, one could identify other cellular types too, especially multiciliated ependymal cells, 2 days after injection and for a maximum period of 7 months, the last point in time for surveillance. (Fig. 4)
after injection, eGFP-positive cells of the granular interneurons type, could be identified in the ipsilateral and contralateral OB, in animals injected in the LV, fact which could not be illustrated by injecting the right SVZ, situation in which cells have been identified only in the ipsilateral OB. This leads to the important conclusion that injection in the LV leads to the B type cells transduction, which are in contact not only with the left SVZ but also with the right SVZ. Seven months after injection, the number of eGFP-positive cells remained significantly big, and the majority of cells have been identified in the granular layer.

In comparison with the cellular transduction in the case of lentiviral vectors injection in the SVZ, in animals injected in the LV there have been identified only few eGFP-positive cells in the RMS, in all the investigated intervals. The confocal microscopy emphasized double-labeled cells for eGFP-GFAP (B Type) but negative in BrdU (proliferating cells marker), and also type A cells and other cellular types, along the stream of the RMS and in the SVZ. In conclusion, the lentiviral vector injected in the LV transduced the ependymal cells, the mature glial cells and the B type cells, and the transduced cells spread not only in the ipsilateral ventricle but also in the contralateral one. Nevertheless, the number of cells identified along the SVZ-RMS-OB stream was significantly lower than in the case of injecting the vector in the SVZ.

**DISCUSSION**

The in vivo marking of neural stem cells is of considerable interest, not only in the study of cellular migration and differentiation towards the mature tissue but especially for the research and therapeutic applications potential. The analysis of the SVZ-RMS-OB migratory chain, at different time intervals following the stereotactic injection of lentiviral vectors, illustrated the presence of positive cells with a stable and long term gene expression and their accumulation in the OB. The increase of the number of positive cells in the OB can be explained only through the migration and continuous differentiation of cells coming from the SVZ and marked following lentiviral transfection.

It is well known that the most important cells in the OB, particularly the neurons and mitral cells derive from the SVZ and begin to form in the prenatal period. In concordance with this fact, we could identify the gene expression of transfection especially in the cells from the granular layer, from the periglomerular layer and in some cells from the mitral and internal plexiform layer.

By the cellular co-labeling with specific markers, we could emphasize the lentiviral transduction of migratory neurons (Type A) in the SVZ and of astrocytic cells (Type B), at an interval of max. 7 months of surveillance. The retroviral vectors demonstrated the preferential transduction of rapidly-dividing cells compared to the slowly-dividing ones (Type B), emphasized through the progressive loss of eGFP-positive cells in the SVZ. The lentiviral vectors were more efficient in the global cellular transduction, in proportion of 70%, in comparison with the retroviral vectors, due to the capacity of lentiviral vectors of transducing not only cells in division but also non dividing cells. Since Type C cells are rapidly-dividing cells, we consider that the transduction of B type cells can lead to the ulterior and continuous marking of type A cells (migratory cells), which can be identified in the RMS and OB, taking into account the relatively long interval of 2-7 days necessary for the cells migration from the SVZ to the OB.

We also succeeded in proving the increase in time of lentivirally transduced cells number, from the SVZ. Because mature cells, double cortin-positive, derive not only from rapidly-dividing cells (type C) but also from slowly – dividing ones (type B) and since they migrate continuously to the OB, the increasing number of eGFP-positive mature cells which accumulate in the OB is based on the stable expression of B type cells transduction.

Since neural stem cells from the SVZ (Type B) are the preferred target of lentiviral vectors, we supposed that, through the continuous division of these cells, we could be able to identify an increased number of daughter cells which are positive too, at different time intervals. Following the appraisal of positive cells in the SVZ and the dorsolateral region of the LV, one could notice a four-time increase of their number, from 7.17 ± 2.04% after two weeks, to 29.21 ± 2.46%, 7 months after injection.

A disadvantage of lentiviral vectors use for transfection could be the lack of selectivity for neural stem cells (NSN), because not only the postmitotic neurons are transduced, but also the glial cells. This problem can be solved by the optimization of vectors either regarding target cells, by modifying or changing the tropism plasmid or by using other orientation promoters of the reporter gene.

**CONCLUSIONS**

Two days after injecting the retroviral vector, the cellular expression of the GFP could be identified in the VS and RMS. Nevertheless, 2 weeks after the
moment of injection, the number of eGFP-positive cells started to decrease. One and two months after injection, only few eGFP-labeled cells were still left in the SVZ (Fig. 3). Two days after injection, some eGFP-positive cells were identified in the subependymal layer of the OB (Fig. 2), which can be explained by the migration of transduced cells or their derivatives. One week after, the positive cells were present especially in the granular layer. These cells presented the morphological characteristics of mature granular interneurons. The number of positive cells in the OB was smaller and remained relatively constant in the interval 1 week – 2 months.

In mice injected with the lentiviral vector, the positive expression of GFP has been established for at least 7 months – the investigation period. Two days after the injection in the SVZ, the eGFP-positive cells were identified in the subependymal layer of the ipsilateral OB (Fig. 2).

One week after, the positive cells started to appear in the granular layer and some cells could also be identified in the glomerular layer. The GFP expression could be noticed in the RMS too and in the subependymal layer at different time intervals. The number of positive cells in the OB decreased in time until the last control interval of 7 months. Marked cells could be identified in the ipsilateral accessory olfactory bulb, but no cell could be detected in the contralateral olfactory bulb.

In the sagittal sections at the level of the OB and RMS we could identify the profile of neuroblastic cells, as prolonged cells, with the axonic termination oriented towards the bulb. The majority of cells identified in this manner were granular interneurons with the plexiform dendritic terminations situated in the external plexiform layer. Some positive cells have also been identified in the glomerular, internal plexiform layer and in that of mitral cells.

In conclusion, the long-term presence of eGFP-double-cortin positive cells and eGFP-GFAP in the SVZ-RMS-OB chain, along with the increasing number of time in position cells in the OB, proves the fact that it is possible to realize the stable transduction of the CNS by a unique in vivo lentiviral injection. In addition, our experiment could demonstrate the origin of B type cells from neural stem cells lentivirally transduced in the LV and also the fact that this transduction did not interfere with the processes of cellular division and differentiation. Since lentiviral vectors are very efficient in the mechanisms of cellular labeling and in vivo gene transfer into the adult neural stem cells, they can be used in the cerebral neurodegenerative pathology, and in future for the delineation of some therapeutic strategies for these degenerations, based on neural stem cells.

REFERENCES