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Towards a stem cell-based intraocular delivery system for therapeutically relevant gene products

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Introduction

Stem cells are defined as pluri- or multipotent cells that display the capability of self-renewal. Because of these properties, stem cells are considered as candidate cells to establish cell replacement strategies for the treatment of a variety of diseases. Stem cells are also amenable to genetic modifications, and thus of potential interest for the development of *ex vivo* gene therapies (Gaillard and Sauve, 2007; Gamm et al., 2007; Gregory-Evans et al., 2009; Lamba et al., 2009). The long-term aim of this work is to establish a stem cell-based intraocular delivery system for continuous and long-lasting application of therapeutically relevant gene products, such as neuroprotective or anti-angiogenic factors, to diseased retinas. To this end, we have established neural stem cell cultures (Conti et al., 2005) that can be effectively expanded *in vitro*. To genetically modify these stem cells, we have used novel lentiviral vectors (Lentiviral „Gene Ontology" (LeGO) vectors) that are based on the third-generation lentiviral vector LentiLox 3.7 (Weber et al., 2008, 2009). We have generated vectors encoding a reporter gene (enhanced green fluorescent protein (EGFP) or tdTomato) in fusion with a resistance gene (blasticidin-S deaminase (BSD) or neomycin (Neo)) under control of a strong and ubiquitously active promoter (CMV immediate enhancer/chicken ß-actin (CAG) or spleen focus-forming virus U3 promoter (SFFV)). Furthermore, we have generated bicistronic lentiviral vectors to simultaneously express a ´gene of interest´ (glial cell line-derived neurotrophic factor; GDNF) and a reporter/resistance fusion gene. Using these vectors, it is possible (i) to rapidly and effectively modify neural stem cells, (ii) to select positive cells by application of antibiotics or fluorescence activated cell sorting (FACS), (iii) to establish genetically engineered clonal stem cell lines, (iv) to simultaneously express more than one ´gene of interest´ in the same stem cell population and (v) to track modified stem cells after intraocular transplantations.

Methods

I. Neural stem cells:

Neural stem cells were isolated from the striatum, the cortex or the spinal cord of embryonic wild-type mice or transgenic mice ubiquitously expressing EGFP under control of a chicken ß-actin promoter. Cells were cultivated in adherent conditions in NS-A medium supplemented with N2, epidermal growth factor (EGF) and fibroblast growth factor-2 (FGF-2), and expanded over multiple passages (Conti et al., 2005; Weber et al., 2008). Directed differentiation protocols were employed to differentiate neural stem cells into a neuronal, astrocytic or oligodendrocytic lineage. Terminally differentiated neural cell types were identified by immunocytochemistry using antibodies to cell type-specific antigens (nerve cells: ß-tubulin III; oligodendrocytes: O4, MAG or MBP; astrocytes: GFAP; Pressmar et al., 2001; Bartsch et al., 2008).

II. Lentiviral vectors:

Novel lentiviral vectors (Lentiviral „Gene Ontology" (LeGO) vectors), based on the third-generation lentiviral vector LentiLox 3.7, were generated (Weber et al., 2008, 2009) to genetically engineer murine neural stem cells. Vectors were generated that encode a tdTomato or EGFP reporter gene in fusion with a BSD or Neo resistance gene under control of the SFFV or CAG promoter. Furthermore, bicistronic vectors were cloned that encode a „gene of interest" (GDNF), the internal ribosome entry site (IRES) of the encephalomyocarditis virus, and a tdTomato/BSD fusion gene under control of the CAG promoter.

III. Transduction of neural stem cells:

Neural stem cells were transduced by spinoculation in the presence of polybrene. Positive cells, identified by expression of the reporter genes, were either selected by application of antibiotics or by FACS. To establish genetically engineered clonal stem cell lines, selected cells were again expanded and cloned using FACS. Directed differentiation protocols were employed to analyze the differentiation potential of engineered cells after a culture period of up to five months. The expression of reporter genes in the terminally differentiated cell types was studied by fluorescence microscopy. Co-expression of two transgenes in the same stem cell population was achieved by transducing cells with lentiviral vectors that encode different combinations of reporter/resistance fusion genes and subsequent selection of positive cells with the respective antibiotics. Expression of GDNF by modified neural stem cells was verified by immunocytochemistry and immunoblot analysis of culture supernatants.

IV. Intraocular transplantations of neural stem cells

Modified stem cells were grafted subretinally, intraretinally or intravitreally (Bartsch et al., 1995, 2008; Ader et al., 2000) into adult wild-type mice or intraretinally into young postnatal *rd1* mutant mice. One month after transplantation, host eyes were cryosectioned and analyzed for the presence of grafted cells, and for expression of transgenes in donor-derived cells. Host retinas were also analyzed for adverse effects potentially associated with the transplantation procedures.

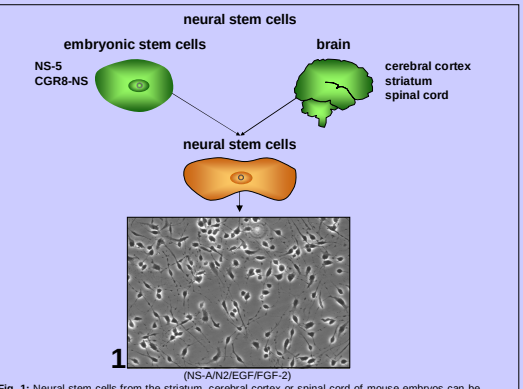

Fig. 1: Neural stem cells from the striatum, cerebral cortex or spinal cord of mouse embryos can be effectively expanded *in vitro*.

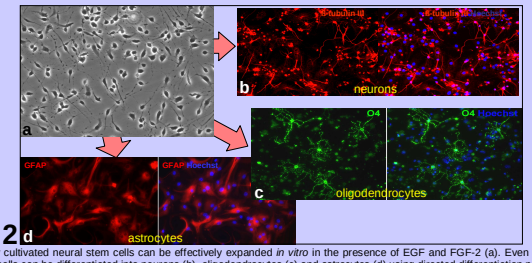

Fig. 2: Adherently cultivated neural stem cells can be effectively expanded *in vitro* in the presence of EGF and FGF-2 (a). Even after extended passaging, these cells can be differentiated into neurons (b), oligodendrocytes (c) and astrocytes (d) using directed differentiation protocols.

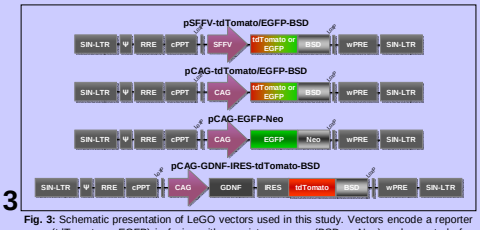

Fig. 3: Schematic presentation of LeGO vectors used in this study. Vectors encode a reporter gene (tdTomato or EGFP) in fusion with a resistance gene (BSD or Neo) under control of a ubiquitous promoter (SFFV or CAG; pSFFV-tdTomato/EGFP-BSD, pCAG-tdTomato/EGFP-BSD, pCAG-EGFP-Neo). Bicistronic vectors were designed to simultaneously express a ´gene of interest´ (GDNF) and tdTomato in fusion with BSD under control of the CAG promoter (pCAG-GDNF-IRES-tdTomato-BSD).

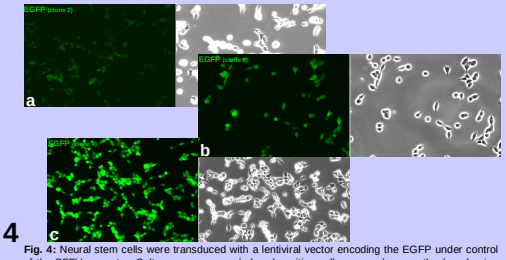

Fig. 4: Neural stem cells were transduced with a lentiviral vector encoding the EGFP under control of the SFFV promoter. Cultures were expanded and positive cells were subsequently cloned using FACS. Three neural stem cell clones with weak (a), intermediate (b) and strong (c) expression of the reporter gene are shown.

Results

- **We have isolated neural stem cells from the CNS of embryonic mice that can be effectively expanded *in vitro* (Figs. 1,2).**
- **Novel lentiviral vectors („LeGO" vectors) were designed to genetically engineer neural stem cells. Vectors were generated that encode a reporter gene (EGFP or tdTomato) in fusion with a resistance gene (BSD or Neo) under control of a strong ubiquitous promoter (SFFV or CAG). In addition, bicistronic vectors were cloned to simultaneously express a „gene of interest" (GDNF) and a tdTomato/BSD fusion gene under control of the CAG promoter (Fig. 3).**
- **Neural stem cells could be effectively modified with the novel lentiviral vectors. Positive cells were either selected by application of antibiotics or by FACS, and expanded again to establish genetically modified bulk cultures or clonally derived stem cell lines (Figs. 4-8).**
- **Coexpression of two transgenes in the same stem cell population was achieved by transducing cells with lentiviral vectors encoding different combinations of reporter/resistance fusion genes, followed by selection of positive cells with the respective antibiotics (Fig. 6).**
- **Neural stem cells transduced with bicistronic vectors expressed ´genes of interest´ and reporter genes over extended periods of time, both *in vitro* and after intraocular transplantation *in vivo* (Figs. 7,8).**

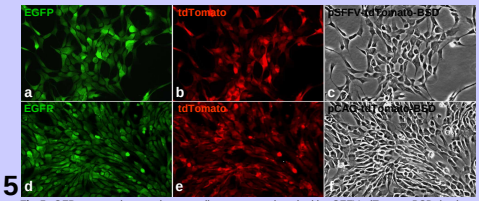

Fig 5: GFP-transgenic neural stem cells were transduced with pSFFV-tdTomato-BSD (a-c) or pCAG-tdTomato-BSD (d-f), and positive cells were selected with blasticidin. Analysis of cultures five months after transduction demonstrates expression of tdTomato in apparently all cells (compare (a) and (b); (d) and (e)).

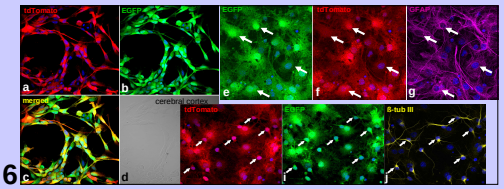

Fig. 6: Co-expression of two transgenes in neural stem cells and their differentiated progeny. Neural stem cells were transduced with pCAG-tdTomato-BSD and pCAG-EGFP-Neo. Double positive cells were selected by simultaneous application of blasticidin and G418. Note co-expression of both reporter genes in virtually every stem cell (a-d), and in stem cell-derived astrocytes (e-g) and neurons (h-j).

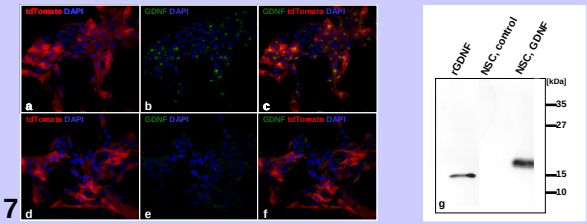

Fig 7: Expression of GDNF in neural stem cells. Neural stem cells (a-f) were transduced with pCAG-GDNF-IRES-tdTomato-BSD (a-c) and a control vector (pCAG-IRES-tdTomato-BSD; d-f). Positive cells were selected with blasticidin and immunostained with GDNF antibodies. Neural stem cells transduced with pCAG-GDNF-IRES-tdTomato-BSD co-expressed tdTomato and GDNF (a-c), whereas cells transduced with the control vector expressed tdTomato but no detectable levels of GDNF (d-f). Immunoblot analysis of culture supernatants (g) from neural stem cells transduced with pCAG-GDNF-IRES-tdTomato-BSD revealed secretion of GDNF into the supernatant whereas cells transduced with the control vector did not express detectable levels of GDNF. control: pCAG-IRES-tdTomato-BSD; GDNF: pCAG-GDNF-IRES-tdTomato-BSD; NSC: neural stem cells; rGDNF: recombinant GDNF.

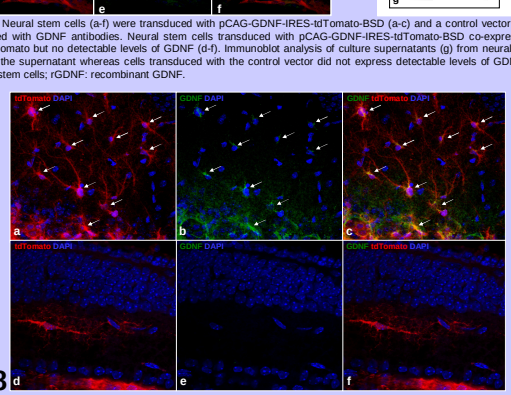

Fig. 8: Neural stem cells were transduced with pCAG-GDNF-IRES-tdTomato-BSD or pCAG-IRES-tdTomato-BSD, positive cells were selected with blasticidin and used for intraretinal transplantations into juvenile *rd1* mice. Analysis of retinas 13 days after transplantation reveals the presence of tdTomato-positive donor cells in different retinal layers (a, d). Immunostainings with antibodies to GDNF demonstrate co-expression of GDNF and tdTomato in cells transduced with pCAG-GDNF-IRES-tdTomato-BSD (arrows in a-c). Cells transduced with the control vector pCAG-IRES-tdTomato-BSD, in contrast, lack detectable levels of GDNF expression (d-f).

Summary

- **with the long-term aim to develop a stem cell-based intraocular delivery system for continuous and long-lasting applications of therapeutic gene products to the diseased retina, we have used novel lentiviral vectors (LeGO vectors) to genetically modify neural stem cells. Experiments of this study demonstrate that:**
- ***the bicistronic vectors allow robust expression of a ´gene of interest´ and a reporter gene.***
- ***the novel vectors encoding reporter/resistance fusion genes allow efficient selection of positive stem cells by application of antibiotics or FACS.***
- ***the expression of reporter genes enables rapid derivation of genetically modified clonal stem cell lines by FACS.***
- ***two or more transgenes can be co-expressed in the same stem cell population by transducing cells with vectors encoding different combinations of reporter/resistance fusion genes and subsequently selecting positive cells with the respective antibiotics.***
- ***neural stem cells show strong expression of transgenes over extended periods of time, both* in vitro *and after intraocular transplantations* in vivo**
- **intraocular transplantations of neural stem cells engineered to express therapeutic gene products (e.g. neuroprotective or anti-angiogenic factors) into mouse models of retinal disorders will reveal the therapeutic potential of this stem cell-based intraocular delivery system.**

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