A METHOD OF TRANSDUCING CELLS

A method of transducing a neural stem cell with a nucleic acid target sequence is disclosed. The method comprises providing a binding medium comprising at least a fragment of fibronectin. A viral vector containing the target sequence is then added to the binding medium such that the viral vector binds to the fragment. The cell is added to the binding medium such that the cell is bound to the fragment. The cell is then transduced with the target sequence.
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A METHOD OF TRANSDUCING CELLS

FIELD OF THE INVENTION

The present invention relates to a method of transducing stem cells and, in particular, a method of transducing neural stem cells. The present invention also relates to method of gene therapy and a method of culturing stem cells as well as to the products resulting from said methods.

BACKGROUND

Neural stem/progenitor cells (NSCs) carry the potential for the replacement of neurons lost to injury or disease, and are an important target for gene therapy of neurodegenerative syndromes. They can also be engineered to produce therapeutic molecules against brain cancer cells. To expand the potential of NSCs by introducing specific gene products in NSCs, efficient gene transfer is a prerequisite. However, it is also necessary that the gene transfer does not result in differentiation of the NSCs.

Several methods are used to introduce genetic information into NSCs. It is known to use protocols based on cationic lipids but the problem with these protocols is that they generally yield a low efficiency of transduction and stability of the transgene.

Protocols are also known which use viral vectors including adenovirus, recombinant adeno-associated virus, feline immunodeficiency virus and retrovirus. The viral vector contains the transgene. These protocols are more efficient than those based on cationic lipids and therefore more generally used. The protocols typically require that a tissue culture medium containing the viral vector is prepared and to this medium the cell requiring transduction is added. The problem with these protocols is that the stem cells must be taken from their specialised growth medium and
transferred to the tissue culture medium which often results in the stem cells irreversibly differentiating and losing their multi-potentiality. Thus the rate of transduction of such protocols is still relatively low.

Currently, fetal tissue is the only viable tissue source for human neural stem cells, raising important ethical issues as well as concern for heterologous transplantation. Therefore there is a need for alternative sources of stem cells. A potential alternative source of stem cells are skin derived neural stem cells (SSCs). SSCs may provide an accessible, autologous source of stem cells for neural transplantation and gene therapy. However, it has so far proved difficult to transduce SSCs efficiently and it is desired to find ways in which such stem cells can be transduced more readily.

A separate area of study has been the field of hematopoietic cells. Hematopoietic cells have been transduced on substrates having a layer of fibronectin located thereon (or, more usually, CH-296, a recombinant peptide of human fibronectin sold commercially as RetroNectin™). However, hematopoietic cells are very different from neural stem cells, not least because the respective cell types are selected using very different growth conditions. Furthermore, comparing the activity of the genes of the respective cell types reveals that, in many cases, expression is different. In addition, culturing neural stem cells on substrates is known to effect differentiation of the stem cells, and so it has been thought that it is not possible to maintain multi-potentiality of neural stem cells once they have been cultured on a substrate.

However, it has now surprisingly been found that neural stem cells can be adhered to substrates having a layer of fibronectin without causing the irreversible differentiation of the neural stem cells. Moreover, it has been found that by adhering neural stem cells (including skin-derived neural stem cells) in this way and then adding a viral vector containing a transgene to the substrate, the efficiency of transduction is greatly increased. It has also been found that such neural stem cells can be frozen and then thawed without loss of growth potential or loss of expression of any transgenes.
Therefore the present invention seeks to alleviate one or more of the above problems.

SUMMARY OF THE INVENTION

According to one aspect of the present invention there is provided a method of transducing a neural stem cell with a nucleic acid target sequence, the method comprising the steps of:

- providing a binding medium comprising at least a fragment of fibronectin;
- adding a viral vector containing the target sequence to the binding medium such that the viral vector binds to the fragment; and
- adding said cell to the binding medium such that the cell is bound to the fragment and the cell is transduced with the target sequence.

In some embodiments, the method is carried out "ex vivo", that is to say the method is carried out on an isolated cell or cells and is not practised on the human or animal body.

Conveniently the fragment of fibronectin comprises at least the cell-binding domain, the heparin-binding domain II and the CS-1 site of fibronectin.

Preferably, the fragment of fibronectin comprises the amino acid sequence of human fibronectin.

Advantageously the medium comprises a substrate, said at least a fragment of fibronectin forming a layer on the substrate.

Conveniently said cell is added to said binding medium in a growth medium, the growth medium comprising basic fibroblast growth factor and epidermal growth factor.

Preferably said viral vector is added to the binding medium by centrifugation.
Advantageously the viral vector is a retrovirus or a lentivirus.

Conveniently the nucleic acid target sequence is a bicistron.

5 Preferably the viral vector is added to the binding medium before the cell.

Alternatively the cell is added to the binding medium before the viral vector.

According to another aspect of the present invention there is provided a method of gene therapy in a patient comprising carrying out the method of any one of the preceding claims and then administering the transduced cell to the patient.

Conveniently, the method further comprises the step of, prior to adding the viral vector and the cell to the binding medium, obtaining the cell from the patient.

Preferably said viral vector and said cell are added to said binding medium with a multiplicity of infection of less then 1.

According to a further aspect of the present invention, there is provided a method of culturing neural stem cells comprising the steps of adding the cells to a binding medium comprising at least a fragment of fibronectin. The neural stem cells thus bind to the fragment of fibronectin.

Conveniently the binding medium is a substrate, said at least a fragment of fibronectin forming a layer on the substrate.

Preferably the neural stem cell is a brain derived stem cell, a skin derived stem cell or a mesenchymal cell.

Advantageously, the method further comprises the step of, after the cell or cells are bound to the fragment of fibronectin, freezing the cell or cells.
Conveniently, the method further comprises the step of, after the cell or cells are bound to the fragment of fibronectin and prior to freezing the cell or cells, detaching the cell or cells from the fragment of fibronectin. Indeed, in certain embodiments, the cell or cells need not have been bound to the fragment of fibronectin prior to freezing, provided that the cell or cells are in a single-cell suspension prior to freezing.

Preferably, the method further comprises the step of, after freezing the cell or cells, thawing the cell or cells.

Advantageously, the method further comprises the step of, after thawing the cell or cells, adding the cell or cells to a second binding medium comprising at least a fragment of fibronectin such that the cell or cells are bound to the fragment in the second binding medium. In some embodiments, the second binding medium is the same as the binding medium used initially.

According to another aspect of the present invention, there is provide a stem cell culture comprising: a binding medium comprising at least a fragment of fibronectin; and at least one neural stem cell.

Conveniently the binding medium is a substrate, said at least a fragment of fibronectin forming a layer on the substrate.

According to a further aspect of the present invention, there is provided an optionally transduced stem cell produced according to the method as described above. For example in one embodiment there is provided a frozen single-cell suspension of neural stem cells.

Advantageously the transduced stem cell is for use in a method of treatment, preferably gene therapy.

In this specification, the word "comprising" is used to mean "including" or "consisting of" and the word "comprises" is used to mean "includes" or "consists of".
BRIEF DESCRIPTION OF THE DRAWINGS

In order to describe embodiments of the present invention, and examples thereof, further, reference will be made to the following figures.

Figure 1 is a graph showing the kinetics of retroviral vector transduction. NSC/TCP (●) and NSC/rFN (■) were exposed to SF91-EGFP retroviral vector with or without polybrene, respectively, for the indicated times, and passed for an additional 48 h on tissue culture plastic. Transduction efficiency was measured by flow cytometry.

Figure 2A is a graph showing proliferation of NSCs under different conditions. The growth rate of GFP- (■) and GFP+ (▲) NSC/rFN was compared with that of GFP-NSC/TCP (●) in parallel cultures. Cells were detached from rFN-coated dishes or dissociated from neurospheres, and their number was assessed by an electronic cell counter. All data are mean values ± s.d. from three to four independent experiments. Significant differences between NSC/TCP and NSC/rFN (p<0.01, t test) are indicated by an asterisk.

Figure 2B is a graph showing proliferation of NSCs under different conditions. The growth rate of SSC/rFN (■) was compared with that of SSC/TCP (●) in parallel cultures. Cells were detached from rFN-coated dishes or dissociated from neurospheres, and their number was assessed by an electronic cell counter. All data are mean values ± s.d. from three to four independent experiments. Significant differences between NSC/TCP and NSC/rFN (p<0.01, t test) are indicated by an asterisk.

Figure 3A shows a scatter graph of gene expression patterns of NSCs exposed to different growth conditions. The scatter graphs show the correlation for the intensities of all present transcripts obtained for NSC grown first as a monolayer on rFN-coated plates in the absence of the viral vector SF91-EGFP and re-grown in suspension as floating neurospheres for another 6 days (RF-RNA) versus control cells which were
grown only in suspension as floating neurospheres (C-RNA). The lines flanking the diagonal denote difference factors of 2, 3 and 10.

Figure 3B is a scatter graph in the same form as Figure 3A except that it shows a correlation for the intensities of all present transcripts obtained for NSC grown first as a monolayer on rFN-coated plates in the presence of the viral vector SF91-EGFP, and re-grown in suspension as floating neurospheres for another 6 days (T-RNA) versus C-RNA.

Figure 3C shows images of cells after immunostaining:

i. Neuronal staining of transduced cells: red, anti-β-tubulin III (Sigma; T8660), blue, DAPI (Roche), green eGFP;

ii. Glial staining of transduced cells: red, anti-GFAP polyclonal antibody (Sigma; G3893), blue, DAPI, green eGFP; and

iii. Neural and Glia double staining of non-transduced NSCs previously grown on retronectin: red, anti-GFAP, green anti-MAP2 (Chemicon, AB5622), blue, DAPI.

Figure 4A shows a graph of flow cytometric analysis of EGFP fluorescence of SSC transduced with SF91-EGFP on rFN at MOI of 2 (middle panel) or 0.3 (right panel). The left panel is a control showing analysis of untransduced NSC. A.U. means arbitrary fluorescence units.

Figure 4B shows a phase contrast micrograph (left panel) and a fluorescence micrograph (right panel) of transduced SSC. SSC were transduced with SF91-EGFP at an MOI of 2 on rFN for 24 h and subsequently cultured on tissue culture plastic for 7 days.

Figure 4C shows phase contrast micrographs (left panels) and fluorescence micrographs (right panels) of NSCs. NSCs were transduced with SF91 GCSH-EGFP on rFN for 24 h (upper panels) and subsequently cultured on tissue culture plastic for 96 h (lower panels).
Figure 4D shows graphs of EGFP expression in NSC. The left panel is the control in which the NSC are untransduced. The middle panel relates to NSC transduced with SF91-EGFP and the right panel NSC transduced with SF91 GCSh-EGFP. M1 is the gate for EGFP-expressing cells.

Figure 4E shows immunoblot analyses of γ-GCSh (upper panel) and β-actin (lower panel). Expression of γ-GCS in NSC transduced with SF91-EGFP or SF91 GCSh-EGFP was analysed. Protein immunoblot analyses were performed using polyclonal antibodies to γ-GCSh and β-actin. The protein marker sizes are indicated. Total tissue proteins (4 and 12 μg per γ-GCS-GFP NSC and EGFP-NSC, respectively) were size fractionated in an SDS/7.5% polyacrylamide gel and transferred to nitrocellulose by electroblotting. The gel was loaded with a higher amount of total proteins from EGFP-NSC cells to allow the visualization of the γ-GCS band. Binding of antibody was visualized by enhanced chemiluminescence (Amersham). kD is an abbreviation for kilodaltons.

DETAILED DESCRIPTION

The present invention relates, in general, to a method of transducing neural stem cells. The term "transducing" is used herein to mean that the cell is transformed with an exogenous nucleic acid target sequence carried by a viral vector. In many embodiments, the target sequence is an entire gene and is thus referred to as a transgene. However, in other embodiments, the target sequence consists of only a portion of a gene such as a promoter or some other nucleic acid sequence.

In some embodiments, particularly those in which transduction is effected as part of a gene therapy, the co-expression of two separate genes, such as a potentially therapeutic gene and a selectable marker, is required. In these embodiments, the target sequence is a bicistron, separated by an internal ribosome entry site (IRES). Thus, after transcription of the bicistron, translation of the downstream gene is initiated at the IRES.
The neural stem cells used include brain derived neural stem cells; skin derived neural stem cells and mesenchymal cells. In particular, it has been found that the transduction method of the present invention provides a high efficiency of transduction of skin derived neural stem cells. In some embodiments of the invention, the neural stem cells are obtained by carrying out the protocol that is provided in Example 1.

In the transduction method, a binding medium is provided that comprises at least a fragment of fibronectin. Fibronectin is a large glycoprotein whose structure is known in the art. In some embodiments the binding medium comprises the complete fibronectin molecule. However, it has been found that the complete fibronectin molecule is not essential to the invention and that transduction will take place when only certain portions of the glycoprotein are provided. In particular, in some embodiments, the recombinant human fibronectin CH296 is used which contains only the cell-binding domain, the heparin-binding domain II and the CS1 site of fibronectin. In further embodiments, the fragment of fibronectin that is used is only a portion of CH296. Indeed, a range of possible fragments may be used within the scope of the present invention. In some embodiments, substitutions of amino acids or insertions into the fibronectin sequence are also effected to form the fragment. What is essential, however, is that the fragment has the capacity to bind the viral vector and the neural stem cell in order to locate the vector and cell adjacent one another to allow transduction to take place. In order to determine whether or not a particular fragment can be used, an assay is carried such as that in Example 1 to determine whether transduction occurs using that fragment of fibronectin. In particularly preferred embodiments, the fragment is taken from human fibronectin.

In preferred embodiments of the invention, the binding medium is a substrate such as a culture plate. The substrate has a layer of the fibronectin or fibronectin fragment disposed on it such that the viral vector and the neural stem cell bind to the fibronectin. The vector and the cell are thus located adjacent, which enables the vector to transform the cell with the target sequence. It is to be appreciated that, in other embodiments, the binding medium is a liquid culture rather than a solid substrate. However, a liquid culture operates in much the same way by binding the
viral vector and the neural stem cell in close proximity so that transduction can take place.

The viral vector that is used is, in some embodiments, a retrovirus and, in other embodiments, a lentivirus. In preferred embodiments, the viral vector is Friend mink cell focus forming/murine embryonic stem cell virus (FMEV). The viral vector contains the nucleic acid target sequence as well as the any other sequences required for the transformation process.

The viral vector is added to the binding medium and bound thereto. In preferred embodiments, this is achieved by centrifugation of the viral vector. Any excess of the viral vector is washed off. The neural stem cell is then also added to the binding medium and bound to it. It is to be appreciated that the neural stem cell is, in preferred embodiments, added to the binding medium in a standard growth medium for such stem cells. Typically, such a medium comprises basic fibroblast growth factor and epidermal growth factor. The advantage of adding the neural stem cell in such a medium is that differentiation of the stem cell is not induced. Furthermore, it has been found that adding the neural stem cell to a substrate coated in a layer of fibronectin (or a fragment thereof) does not induce differentiation either.

Once the viral vector and the neural stem cell are bound to the binding medium, the close proximity of the vector and the cell allows transduction to take place at a high efficiency. Since the important factor is that the vector and the cell are located adjacent one another, this can be achieved in more than one way. For example, in some alternative embodiments, the neural stem cell is added to the binding medium before the viral vector is added. In other embodiments, the viral vector and the neural stem cell are added to the binding medium simultaneously.

In preferred embodiments, substantial numbers of viral vector particles and neural stem cells are added to the binding medium to ensure that the probability of each stem cell being adjacent a viral vector particle, and thus the probability of transduction taking place, is acceptably high. In different embodiments, the proportion of viral vector particles to neural stem cells (i.e. the multiplicity of infection
(MOI) is different. In preferred embodiments, the MOI is between 0.3 and 4, preferably 2.

The above described embodiments take advantage of the finding that NSCs grow faster on rFN than as neurospheres on tissue culture plastic and do not lose their stem cell nature or multipotentiality. Furthermore, retroviral-mediated transgene expression can be sustained with time in culture and upon differentiation of NSCs into neurons and astrocytes.

One application of the transduction method of the present invention is for use as part of a method of therapy, in particular cell or gene therapy, of a patient. Gene therapy is used, for example, when the patient suffers from a genetic condition owing to a chromosomal mutation. In particular embodiments, the gene therapy comprises autologous therapy in which a neural stem cell (usually a skin derived neural stem cell) is removed from the patient. In some embodiments, the specific cell removed from the patient is then transduced but in other embodiments, the cell is first cloned so that a substantial number of cells are transduced at the same time. After transduction of the cell or cells is complete, the transduced cell or cells are returned to the patient. Again, in preferred embodiments, the transduced cell or cells are cloned prior to implantation in the patient in order to provide a substantial number of transduced cells. Because the cell or cells were taken from the patient or are derived from a cell taken from the patient, the cell or cells are recognised as “self” by the immune system of the patient and immune rejection does not occur. Furthermore, the transduced cell or cells maintain multipotentiality so that a single implantation of the cell or cells can achieve therapy of the disorder.

In the transduction of neural stem cells it is recognised that, in order to keep the probability of insertional mutagenesis to a minimum, it is preferable that there is only one integration site in the transduced cell. In order to achieve this, in preferred embodiments, the multiplicity of infection in the transduction method is less then 2, more preferably less than 1 and more preferably around 0.3 (i.e. 3 viral vector particles for every 10 neural stem cells).
In another aspect of the present invention, a method of culturing neural stem cells is provided. This has arisen from two findings. Firstly, it has been found that culturing neural stem cells on a substrate having a layer of fibronectin or a fragment thereof does not necessarily result in differentiation of the stem cells. Secondly, it has been found that culturing neural stem cells on such a substrate results in a rapid proliferation of the neural stem cells. The method of culturing the neural stem cells comprises adhering the stem cells to a binding medium (in particular a substrate) comprising at least a fragment of fibronectin as described above. In preferred embodiments, the neural stem cell is provided in a growth medium suitable for such cells, preferably one as described above. In order to study neural stem cells, it is useful to have the cells adhered to a flat substrate and thus by using this culturing method, the stem cells are adhered in this way without the cells differentiating. Thus the rFN coating required for reaching high transduction rates in NSCs not only dramatically improve transduction rates, but also offers a tool for growing NSCs as a monolayer without impairing their differentiation potential.

The present invention provides almost complete retroviral gene transfer without intrinsic alterations of the differentiation potential of the transduced cells. The method of the invention represents an important tool to study gene function in NSCs and, provides a valuable tool towards manipulating NSCs for cellular therapy.

In some embodiments, neural stem cells bound to fibronectin are frozen and, optionally, subsequently thawed. This can be done whether or not the neural stem cells have been transduced.

One method of carrying this out is to detach the neural stem cells from the fibronectin by incubation with standard trypsin-EDTA. The resulting suspension of single cells is then frozen in neurobasal medium with 7% dimethylsulfoxide (to prevent the formation of ice crystals) under liquid nitrogen. It has been found that if the cells are subsequently thawed then they have a viability of over 90% and it is possible to regrow the cells on fibronectin and have them re-form neurospheres on tissue culture plastic. The expression of any transgene is maintained indefinitely, and the
freezing/thawing process is more efficient than if the neural stem cells were growing as neurospheres.

In certain embodiments, the neural stem cells are frozen and thawed a plurality of times, being bound to fibronectin on each thawing. It has been found that by doing this there is no loss of growth potential of the cells nor loss of expression of any transgenes.

The present invention also provides the products of the above described methods. In particular, there are provided neural stem cells transduced according to the method described above and there are provided stem cell cultures comprising a binding medium as described above to which one or more neural stem cells are bound.
EXAMPLES

The following examples are provided in order to describe embodiments and aspects of the present invention further.

Example 1

In this example, a retroviral vector was used which was derived from the improved FMEV (Friend mink cell focus forming/murine embryonic stem cell virus) retroviral backbone in order to transduce neural stem cells. This has previously been shown to be highly efficient in hematopoietic stem/progenitor cells, even when co-expressing two distinct genes.\textsuperscript{8, 9, 10, 11}

In particular, NSCs were exposed to the SF91-EGFP retroviral vector with polybrene. The efficiency of FMEV-mediated gene transfer (% enhanced green fluorescence protein positive (EGFP\textsuperscript{+}) cells) was measured by flow cytometry (the protocol for which is described in greater detail below). The optimal conditions for transduction of neurospheres on tissue culture plastic (TCP) were firstly determined using the protocol that is described in detail below.

NSCs are commonly grown as floating neurospheres (that is to say clonal expansions of 1000 or more cells) in bFGF (basic fibroblast growth factor) and EGF (epidermal growth factor). Under these conditions, about 1% of the cells retain multipotentiality. The highest transduction efficiency was achieved when neurospheres were enzymatically disaggregated, and cells transduced by low-speed centrifugation in the presence of retroviral vector-containing tissue culture medium and 8 \textmu g/ml polybrene. Under these conditions, with a multiplicity of infection (MOI) of 2 transducing particles per cell, the rate of transduction was time-dependent, reaching a maximum of 7.5 % at 20 h. The results are shown in Figure 1, with transduction of NSC on TCP indicated as (●).

Having determined the optimal conditions for transduction on TCP, transduction on recombinant fibronectin (rFN) plates was investigated. The retroviral vector was pre-
loaded onto rFN-coated culture plates and subsequently the NSCs were added in polybrene-free culture medium at the same MOI of 2. Again, transduction efficiency was measured by flow cytometry and the results are shown in Figure 1 in which transduction of NSC on rFN is shown as ■. It was found that the efficiency of gene transfer was increased by an order of magnitude, with no apparent change in the growth rate compared to untransduced NSC/rFN (see Figure 2A, which is described in greater detail in Example 2). It was also found that EGFP expression was sustained with time in culture: when NSC were grown for 1-7 days on rFN, no changes in the level of EGFP fluorescence were observed (not shown). Moreover, when NSC grown on rFN were transferred to TCP and grown for up to two months as neurospheres, up to 92% of cells were EGFP+ as measured by flow cytometry.

The protocols used in this example are as follows.

For the preparation of NSC, neonatal forebrains (P0 stage) were dissected with a razor blade and cells mechanically dissociated in serum-free Neurobasal-A medium containing B27 (Gibco-BRL), 2 mM glutamine and penicillin/streptomycin (referred to as basal medium). An incubation of the dissected tissue in 0.05% trypsin with 0.02% EDTA for 10 min at 37°C was performed prior to dissociation of the cells in the basal medium. Where indicated, additional recombinant growth factors were added in the following concentrations: basic fibroblast growth factor (bFGF) 10 ng/ml, epidermal growth factor (EGF) 20 ng/ml (both R&D Systems). Low passage (passage 2-5) NSC and SSC (for Example 6) were used throughout all the experiments. Untreated tissue culture 24-well plates were coated with 10 μg/cm² rFN (Retronectin, Takara Shuzo Co.), according to the manufacturer's instructions. For culture on rFN, neurospheres growing on TCP were incubated in the presence of 0.05% trypsin with 0.02% EDTA for 10 min at 37°C. The single cell suspensions were then pelleted by centrifugation at 400xg for 3 min, resuspended in basal medium additioned with 10 ng/ml bFGF and 20 ng/ml EGF, filtered through a 70-μm filter to remove eventual remaining cell clumps, and added to rFN-coated plates. The cells were then incubated at 37°C in an atmosphere of 5% CO₂ in air.
Fluorescent cell sorting (FACS) was carried out as follows. Dissociation to single-cell suspension was achieved by incubation in 0.05% trypsin and 0.02% EDTA for 10 min at 37°C. The fluorescence of EGFP was measured using a FACStar Flow cytometer (Becton-Dickinson, San Jose, CA). Emission was collected in the wavelength range 500-560 nm. In order to standardize the assay, the mean fluorescence of Hoechst UV calibration beads (Flow Cytometry Standards Corp., Research Triangle Park, NC) was recorded and the mean cellular fluorescence compared to that of the beads. A minimum of 20,000 cells was analyzed for each sample.

Example 2

It is known that coated dishes such as L-polylysine or polyornithine support differentiation of NSCs. To determine whether the culture of NSC on rFN-coated plates pre-loaded with retroviral vector supernatant could trigger irreversible differentiation and loss of the property of self-renewal, the rate of growth of untransduced NSC as floating neurospheres on TCP (NSC/TCP) and as monolayers on rFN (NSC/rFN) was compared.

The details of the experiment are as follows. The growth rate of GFP- (■) and GFP+ (▲) NSC/rFN was compared with that of GFP- NSC/TCP (●) in parallel cultures. Cells were detached from rFN-coated dishes or dissociated from neurospheres, and their number was assessed by an electronic cell counter. All data are mean values ± s.d. from three to four independent experiments. The results are shown in Figure 2A. Significant differences between NSC/TCP and NSC/rFN (p<0.01, t test) are indicated by an asterisk.

After a lag phase of 24 h, common to both substrates, the rate of growth was found to be considerably faster for NSC/rFN than for NSC/TCP (p<0.01).

The number of neurospheres formed on TCP from GFP- and GFP+ NSC pre-cultured for 1 to 3 days on rFN was then compared with that of GFP- NSC/TCP in parallel cultures. 10,000 cells detached from rFN-coated dishes or dissociated from
neurospheres were replated on TCP and grown for 4 days as floating neurospheres. Their number was counted by an automated colony counter. The results (not shown) indicated that NSC grown on rFN produced a 4 to 8-fold increase in numbers of neurospheres formed, when passaged to TCP culture conditions, as compared with NSC grown as floating culture. This indicates that an expansion of the stem cell/progenitor cell pool occurs when NSCs are grown on rFN. No significant changes in apoptosis rates as measured by TUNEL assay were observed between NSC/rFN and NSC/TCP (2.5-3%, not shown).

Thus this example showed that culturing of NSCs on rFN does not result in irreversible differentiation of the NSCs. Furthermore, the example showed that culturing of NSCs on rFN results in rapid proliferation of NSCs. The data also suggest that rFN may increase the efficiency of gene transfer in NSC by promoting proliferation, a known requisite for retroviral transduction\textsuperscript{12,13}.

Example 3

It was examined whether NSC passaged on rFN, and transduced NSC retained their potential to differentiate into neurons and glia.

GFAP, β-tubulin III and MAP2 immunostaining of NSC derived Gila and neurons was conducted after growth in differentiation medium containing basic FGF. 5-7 passage transduced and non-transduced neurospheres were cultured for 7 days as floating cultures in proliferation medium (containing EGF and bFGF). Subsequently, the neurospheres were trypsinised and plated as single cells onto poly-L-lysine coated plates in differentiation medium (without EGF) containing basic FGF (20 ng/ml, R&D Systeme). The cells were incubated for 4 days prior to immunostaining. The cultures were processed for immunocytochemistry as previously described in Example 1. (Machon, O., van den Bout, C. J., Backman, M., Rosok, O., Caubit, X., Fromm, S. H., Geronimo, B., Krauss. S. (2002) Neuroscience.112 (4), 951-66). The results are shown in Figure 3C as follows:
i) Neuronal staining of transduced cells: red, anti-β-tubulin III (Sigma; T8660), blue, DAPI (Roche), green eGFP

ii) Glial staining of transduced cells: red, anti-GFAP polyclonal antibody (Sigma; G3893), blue, DAPI, green eGFP

iii) Neural and Glia double staining of non-transduced NSCs previously grown on retronectin: red, anti-GFAP, green anti-MAP2 (Chemicon, AB5622), blue, DAPI.

In order to quantify results, an analysis of GFAP (+) and β-tubulin III (+) differentiated cells generated from NSCs grown as floating culture or grown on retronectin, and virus transduced NSCs was carried out. For differentiation, cells were plated and maintained for 4 days on poly-L-lysine-coated dishes in serum-free neurobasal medium + B27 and basic FGF. To estimate the percentage of each cell type 1,000-1,500 cells in at least 8 different microscope fields were counted. Positive cells were reported with respect to the total cell number, as detected by DAPI nuclear staining. Two overlapping single colour images of each field (red, blue) were captured digitally using the AxioVision software (Zeiss Axiocam, Germany). The results are shown in Table 1.

Table 1

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<thead>
<tr>
<th></th>
<th>GFAP(+) %</th>
<th>β-tubulin III (+) %</th>
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<tbody>
<tr>
<td>wt as floating culture</td>
<td>3.6 ± 0.5</td>
<td>97.7 ± 1.0</td>
</tr>
<tr>
<td>wt on retronectin</td>
<td>3.2 ± 0.5</td>
<td>96.7 ± 0.5</td>
</tr>
<tr>
<td>wt transduced</td>
<td>3.6 ± 0.3</td>
<td>97.3 ± 0.3</td>
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Comparing NSC cultured on rFN with parallel cultures of NSC/TCP plated on poly-L-lysine under identical conditions, no difference in the percentage of neurons (97.2%±0.5%) and glia (3.4%±0.2%) was observed.
Thus this example showed that culturing of NSCs on rFN does not significantly reduce their potential to differentiate into neurons and glia.

Example 4

In this example, microarray analysis was performed to analyze whether the transient growth of NSCs on rFN-coated plates for transduction, could permanently alter patterns of gene expression.

The microarray analysis was carried out as follows. After 7 days of growth as monolayer on rFN-coated plates in the presence or absence of the viral vector SF91-EGFP, NSC (passage n. 4) were trypsinised and re-grown in suspension as floating neurospheres for another 6 days before cells were harvested for RNA isolation (T-RNA and RF-RNA, respectively). At least 80 % of NSC were EGFP-positive at the time of cell harvest for RNA isolation. RNA from non-transduced and floating neurospheres of the same batch was used as control (C-RNA). RNA was isolated according to a protocol from an RNA isolation kit (Ambion). Microarray experiments were carried out at the Affymetrix unit of the National hospital of Norway, using the mouse chip 430A which consists of 22645 murine gene sequences and the software Microarray Suite 5.0 for data analysis. The calls (present, marginal and absent) and the intensities of the individual transcripts were based on affymetrix algorithms with following settings: alpha1 of 0.05, alpha2 of 0.065, tau of 0.15. Only signals defined as present calls were used to plot scatter graphs.

RF-RNA were plotted versus C-RNA to generate the scatter graph of Figure 3A. The RF-RNA pool showed an expression pattern highly similar to that of the control C-RNA pool, thus indicating that the transient growth on rFN-coated plates did not cause significant changes in gene expression of the resulting neurospheres. Thus pre-cultivation of NSC on rFN did not impair their multilineage potential or cause irreversible gene expression.
The expression profile from FMEV (EGFP+) transduced neurospheres (T-RNA) was also analysed by microarray. Figure 3B is a scatter graph of T-RNA versus C-RNA and shows only a moderate difference between the two populations, with only one signal that shows a higher that ten-fold change in intensity. It has been suggested that EGFP alters the growth potential of Neural stem cells (Martinez-Serrano A, Villa A, Navarro B, Rubio FJ, Bueno C. Nat Med 2000 May;6(5):483-4) and therefore minor alterations in the expression profile of EGFP expressing cells are to be expected.

Thus this example shows that transient growth of NSCs on rFN-coated plates did not significantly alter patterns of gene expression.

Example 5

This example was carried out in order to confirm that after differentiation of transduced NSCs, expression of the transgene still occurred.

NSCs transduced with SF91-EGFP on rFN were transferred to poly-L-lysine-coated tissue culture plates under conditions of growth factor deprivation. The percentage of fluorescent cells was measured (using the protocol as described in Example 1) and remained constant. The intensity of fluorescence was maintained compared with parallel undifferentiated cultures, with a ratio of the mean fluorescence intensity between differentiated and undifferentiated NSC of 0.92 ± 0.15.

Thus significant EGFP expression was observed after NSC differentiation, confirming that expression of the transgene occurred even after differentiation of the transduced cell.
Example 6

In order to investigate efficacy for clinical applications, the efficiency of the rFN-based retroviral transduction protocol from Example 1 was tested on skin derived neural stem cells (SSC) obtained from newborn mice\(^\text{17}\).

The growth rate of SSC/rFN was compared with that of SSC/TCP in parallel cultures. Cells were detached from rFN-coated dishes or dissociated from neurospheres, and their number was assessed by an electronic cell counter. The results are shown in Figure 2B. All data are mean values ± s.d. from three to four independent experiments. Significant differences between NSC/TCP and NSC/rFN (p<0.01, t test) are indicated by an asterisk.

As can be seen from Figure 2B, low passage skin-derived SSC/TCP displayed a much slower growth rate than brain-derived SSC/TCP; however, when cultured on rFN, the rate of growth was significantly faster (p<0.01).

Flow cytometric analysis was also carried out on SSC transduced with SF91-EGFRP on rFN at MOI of 2 and this was compared with untransduced NSC as a control. The results are shown as a graph in Figure 4A, in which the left panel is the control, and MOI of 2 is the middle panel. As can be seen from Figure 4A, pre-loading the monocistronic retroviral vector on rFN-coated culture plates in the absence of polybrene, and subsequently adding SSC enzymatically dissociated from skin-derived neurospheres (MOI = 2), resulted in 66.1 % of cells being transduced, as detected by flow cytometric analysis of EGFP fluorescence.

These data indicate that it is possible to achieve on rFN fast growth rates and high levels of gene transduction for both brain-derived and skin-derived NSC.

The experiment was then repeated using a very low MOI (0.3 functional viral particles per cell) to ensure that the results could be transferred to the clinical setting. The results are shown in the right hand panel of Figure 4A. In a clinical setting, the number of integration sites per cell should be kept as close as possible to 1 to reduce
the risk of insertional mutagenesis. Under these conditions, 20.2% of SSC were EGFP+.

An example of EGFP+ SSC regrown into neurospheres is shown in Figure 4B. The left hand panel is imaged as a phase contrast micrograph whereas the right hand panel is imaged as a fluorescence micrograph. The SSC were transduced with SF91-EGFP at an MOI of 2 on rFN for 24 h and subsequently cultured on tissue culture plastic for 7 days.

Thus this example confirms that the transduction protocol can be used in a clinical setting because the transduction is effective for skin derived neural stem cells even using a very low MOI.

Example 7

Most gene therapy applications require the co-expression of two separate genes, such as a potentially therapeutic gene and a selectable marker. Thus, in this example, the efficiency of transduction of NSCs with a bicistronic gene on rFN was investigated.

An SF91-derived bicistronic vector co-expressing the catalytic (heavy) subunit of γ-glutamyl cysteine synthetase (γ-GCSH) and EGFP (SF91GCSH-EGFP) was used. The γ-GCS gene, in association with L-buthionine S,R-sulfoximine, is used for in vivo selection of retrovirally transduced hematopoietic stem cells18, 19 (Rappa, G. and Lorico, A., unpublished data). In the bicistronic vector, the IRES sequence (internal ribosomal entry site) is able to promote ribosome binding and re-initiate translation at an internal site within the mRNA, thus allowing coordinate expression of both resistance genes.

The retroviral vector was constructed as follows. A 1923-bp NotI fragment of the human full-length γ-GCS heavy cDNA was obtained by subjecting pSF91GCS-MRP18 to the polymerase chain reaction. The primer sequences were: 5'ATA-AGA-ATG-
CGG-CCG-CCA-TGG-GGC-TGC-TGT-CCC-AG-3' (SEQ ID NO:1) and 5'-ATA-GTT-TAG-CCG-CCG-CTA-GTT-GGA-TGA-GTC-AGT-TTT-AC-3' (SEQ ID NO: 2). The fidelity of the γ-GCS sequence was confirmed by DNA sequence analysis. The retroviral plasmid pSF91-GCSh-i-EGFP-WPRE (pSF91-GCSh-EGFP) was cloned by introducing the 1923-bp γ-GCS heavy cDNA fragment into the plasmid pSF91-I-EGFP-WPRE, digested with NotI. pSF91-I-EGFP-WPRE is a derivative of pSF91<sup>9</sup> containing the enhanced green fluorescent protein cDNA (EGFP) under control of the internal ribosomal entry site (IRES) of poliovirus and the woodchuck hepatitis posttranscriptional regulatory element (WPRE) as an EcoRI fragment 3' of EGFP.

Retroviral producers were generated as follows. The Phoenix-gp packaging cell line was transfected by the calcium phosphate/chloroquine method<sup>24</sup> with the pSF91-EGFP or the pSF91 GCSh-EGFP retroviral plasmids and a plasmid expressing the ecotropic glycoprotein. Culture supernatants containing viral particles were collected at 24-48 h after transfection, passed through 0.22 μm Millex GP filters (Millipore Co., Bedford, MA, USA) and stored at −80°C.

NSCs were transduced on rFN for 1 to 3 days at an MOI of 2, and subsequently passaged on TCP, and reformed as neurospheres, 90% of which expressed high levels of EGFP. The results are shown in Figure 4C in which the upper panels show the cells after 24 h and the lower panels after 96 h. The left hand panels are imaged as phase contrast micrographs whereas the right panels are imaged as fluorescence micrographs.

The experiment was repeated with the monocistronic EGFP vector and a comparison of the results is shown as graphs in Figure 4D in which the left panel shows a control, the middle panel shows the results after transduction with the monocistron and the right hand panel shows the results after transduction with the bicistron. As is shown in Figure 4D, EGFP was expressed in NSCs transduced with the bicistron, although at lower levels than NSCs transduced with the monocistron. A reduced translation efficiency via the IRES is a frequent phenomenon (Hildinger et al., 1999a; Jelinek et al., 1999; Mizuguchi et al., 2000; Rappa et al., 2001).
The level of expression of the first transgene, γ-GCS, mediated by the bicistronic vector in NSCs was then investigated by immunoblotting. In particular, expression of γ-GCS in NSCs transduced with SF91-EGFP or SF91 GCSh-EGFP was analysed. Binding of antibody was visualized by enhanced chemiluminescence (Amersham). Immunoblotting was carried out as follows. The cells were mixed with lysis buffer (70μl per 1x10^6 cells) and protease inhibitors (1 mM PMSF, 15 μM Pepstatin A, 0.3 μM aprotinin, 2 μM leupeptin (all from Sigma, St. Louis, MO) and incubated on ice for 15 min. The samples were sonicated for 3x10sec at 1 min intervals and centrifuged at 3220xg at 4 °C. The protein concentration of the supernatants was determined by the BioRad Protein Assay. Total tissue proteins (4 and 12 μg per γ-GCS-GFP NSC and EGFP-NSC, respectively) were size fractionated in an SDS/7.5% polyacrylamide gel and transferred to nitrocellulose by electroblotting. The gel was loaded with a higher amount of total proteins from EGFP-NSC cells to allow the visualization of the γ-GCS band. The gel was electrophoresed for 1.5 hours at 30mA and transferred for 12 hours at 30V onto Immobilon-P membranes (Millipore, Bedford, MA). The blotted membrane was blocked and incubated with polyclonal mouse antibodies to γGCS-h diluted 1:80K (a gift from Dr. T. Kavanagh, University of Washington, Seattle) followed by a goat anti-rabbit antibody conjugated to horseradish peroxidase (DAKO, Glostrup, Denmark; dilution 1: 20K). Actin expression was detected using the same membranes after stripping them of bound antibodies. As a control, mouse monoclonal antibodies against actin (1:2K, Ab-5 from NeoMarkers, Fremont,CA) and rabbit anti-mouse (1:10K, DAKO) were used as primary and secondary antibodies, respectively. Chemiluminescent detection was performed by the ECL kit (Amersham, Little Chalfont, England) according to the manufacturer's instructions. Densitometry was performed using ImageQuant software (Molecular Dynamics Co., Sunnyvale, CA). The results are shown in Figure 4E, upper panel.

As can be seen from Figure 4E, in NSCs transduced with the bicistronic vector on rFN at a MOI of 2, the expression of the γ-GCS protein was increased 18.3-fold compared to the baseline expression of untransduced NSC/rFN or NSC/TCP (not shown). No changes in γ-GCS expression was observed in mock transductions (not shown).
Therefore, this example shows that expression of both genes in the bicistron occurred at reasonable efficiency.
REFERENCES


CLAIMS

1. A method of transducing a neural stem cell with a nucleic acid target sequence, the method comprising the steps of:
   providing a binding medium comprising at least a fragment of fibronectin;
   adding a viral vector containing the target sequence to the binding medium such that the viral vector binds to the fragment; and
   adding said cell to the binding medium such that the cell is bound to the fragment and the cell is transduced with the target sequence.

2. A method according to Claim 1, wherein the fragment of fibronectin comprises at least the cell-binding domain, the heparin-binding domain II and the CS-1 site of fibronectin.

3. A method according to Claim 2, wherein the fragment of fibronectin comprises the amino acid sequence of human fibronectin.

4. A method according to any one of the preceding claims, wherein the medium comprises a substrate, said at least a fragment of fibronectin forming a layer on the substrate.

5. A method according to any one of the preceding claims wherein said cell is added to said binding medium in a growth medium, the growth medium comprising basic fibroblast growth factor and epidermal growth factor.

6. A method according to any one of the preceding claims wherein said viral vector is added to the binding medium by centrifugation.

7. A method according to any one of the preceding claims wherein the viral vector is a retrovirus or a lentivirus.

8. A method according to any one of the preceding claims wherein the nucleic acid target sequence is a bicistron.
9. A method according to any one of the preceding claims, wherein the viral vector is added to the binding medium before the cell.

10. A method according to any one of Claims 1 to 8, wherein the cell is added to the binding medium before the viral vector.

11. A method of gene therapy in a patient comprising carrying out the method of any one of the preceding claims and then administering the transduced cell to the patient.

12. A method according to Claim 11, further comprising the step of, prior to adding the viral vector and the cell to the binding medium, obtaining the cell from the patient.

13. A method according to Claim 11 or 12 wherein said viral vector and said cell are added to said binding medium with a multiplicity of infection of less then 1.

14. A method of culturing neural stem cells comprising the steps of adding the cells to a binding medium comprising at least a fragment of fibronectin.

15. A method according to Claim 14 wherein the binding medium is a substrate, said at least a fragment of fibronectin forming a layer on the substrate.

16. A method according to any one of the preceding claims, wherein the neural stem cell is a brain derived stem cell, a skin derived stem cell or a mesenchymal cell.

17. A method according to any one of the preceding claims further comprising the step of, after the cell or cells are bound to the fragment of fibronectin, freezing the cell or cells.
18. A method according to claim 17 further comprising the step of, after the cell or cells are bound to the fragment of fibronectin and prior to freezing the cell or cells, detaching the cell or cells from the fragment of fibronectin.

19. A method according to claim 17 or 18 comprising the step of, after freezing the cell or cells, thawing the cell or cells.

20. A method according to claim 19 further comprising the step of, after thawing the cell or cells, adding the cell or cells to a second binding medium comprising at least a fragment of fibronectin such that the cell or cells are bound to the fragment in the second binding medium.

21. A stem cell culture comprising: a binding medium comprising at least a fragment of fibronectin; and at least one neural stem cell.

22. A stem cell culture according to Claim 21, wherein the binding medium is a substrate, said at least a fragment of fibronectin forming a layer on the substrate.

23. A transduced stem cell produced according to the method of any one of Claims 1 to 10 or 16 to 20.

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A Method of Transducing Cells

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PatentIn version 3.1

1
35
DNA
Artificial

ataagaatgc ggccgccatg gggctgctgt cccag

35

2
38
DNA
Artificial

atagtttagc ggccgctagt tggatgagtc agttttac

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