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ORIGINAL ARTICLE

Transduction of human neural progenitor cells with foamy virus vectors for differentiation-dependent gene expression

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*Neural progenitor cells are potential vehicles for delivery of therapeutic agents into the brain. Differentiation-dependent promoters may be useful to target the therapeutic transgene expression to specific neural cell types. Here we explored the potential of vectors based on the foamy virus (FV) for genetic engineering of neural progenitor cells. We demonstrate that FV vectors can mediate stable long-term constitutive expression of the enhanced green fluorescent protein (EGFP) in neural progenitor cells. For differentiation-dependent gene expression, we constructed a FV vector with an internal expression cassette containing the human 2.2 kb promoter (Gfa2) of the astrocyte-specific glial fibrillary acidic protein (GFAP) and sequences encoding EGFP. We show FV-vector-mediated delivery of the Gfa2-*egfp *transgene into the human neural stem cell line HNSC.100 and differentiation-dependent expression in stably transduced cell populations. Differentiation of the FV-transduced HNSC.100 cells to astrocytes upregulated expression of both the Gfa2-*egfp *transgene and the native* gfap *gene, confirming differentiation-dependent activation of the transduced Gfa2 promoter. These results demonstrate that differentiation-dependent gene expression can be achieved by FV-vector-mediated gene transfer to neural progenitor cells. Our findings support the use of FV vectors for the genetic engineering of neural progenitor cells for therapeutic and research applications.*
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Keywords: *gene delivery; retrovirus vectors; foamy virus; neural progenitor cells; astrocytes; GFAP*

Introduction

Neural progenitor cells have recently received great interest as potential therapeutic agents for cell replacement and delivery of genes into injured or diseased areas of the brain. Neural progenitor cells are self-renewing populations in the fetal and adult brain. They hold great promise for gene therapy because they can be isolated, expanded, genetically manipulated and reintroduced into the central nervous system, where they are highly migratory and are attracted to areas of brain pathology.[1,2] The capacity of neural progenitor cells to generate neurons, oligodendrocytes and astrocytes makes them potential vehicles for the specific expression of genes in mature brain cells both for research and therapeutic purposes. This approach requires the availability of safe vectors that transduce neural progenitor cells and direct neural cell-specific gene expression.

Foamy viruses (FVs) are not known to be human pathogens, although there are some indications that the expression of viral gene products may be pathogenic in transgenic mouse models.[3] FVs are particularly suited for the development of effective and safe vector systems,[4–8] because they have broad host and tissue ranges,[9–11] are not transmitted between humans and have a large packaging capacity (up to 12 kb). Furthermore, FV vectors show lower preferences for integration within transcribed genes and within or near protooncogenes than other integrating retroviral vectors.[12–14]

The usefulness of FV vectors has recently been demonstrated by correcting a genetic defect in haematopoietic stem cells in a clinically relevant large animal model.[14] FV-based vectors have also been used to achieve constitutive expression of a transgene in mature rat neural cells.[15,16] In this study, we show that FV vectors have the capacity to transduce neural progenitor cells. Therefore, we investigated the feasibility of using FV vectors to achieve differentiation-dependent gene expression in transduced human neural progenitor cell populations. For differentiation-dependent expression, we chose a 2.2-kb promoter segment (Gfa2) of the human gene for the glial fibrillary acidic protein (GFAP), which is the most commonly used astrocyte marker. Expression of the *gfap* gene is activated during astrogenesis.[17–19] The Gfa2-promoter segment has been shown to direct astrocyte-specific expression in transgenic mice.[20–23]

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As an experimental model for human neural progenitor cells, we used the multipotent human neural stem cell line HNSC.100,[24] which can be induced to generate populations consisting of 95% GFAP-positive astrocytes under specific culture conditions.[25]

Here we demonstrate that a FV vector carrying a transgene with the Gfa2 promoter and sequences encoding the enhanced green fluorescent protein (EGFP) (Gfa2-*egfp*) can stably transduce HNSC.100 cells. We demonstrate that the expression of the Gfa2-*egfp* transgene is activated by astrogenesis of transduced HNSC.100 cells. These results provide proof-of-principle that FV vectors can be used to deliver genes into neural progenitor cells for differentiation-dependent expression.

Results

Differentiation of HNSC.100 progenitor populations into astrocyte populations

The cell line HNSC.100 is a nestin-positive, self-renewing human neural stem cell line capable of differentiating into neurons, oligodendrocytes and astrocytes under defined culture conditions.[24] We have demonstrated earlier that HNSC.100 populations cultured in the astrocyte-differentiation medium for 7 days adopt morphological features of astrocytes and show strongly increased immunoreactivity for GFAP antigen, compared with HNSC.100 populations cultured in the proliferation medium.[25] Here we show by real-time PCR analysis that levels of *gfap* RNA are strongly increased in HNSC.100 astrocyte populations compared with progenitor populations (66 784-fold increase) (Figure 1), confirming differentiation-dependent activation of the gfap promoter. To verify the differentiation of neural progenitor cells to astrocytes, a second astrocyte marker gene was analysed by real-time PCR analysis. Aquaporin 4 (AQP4) is expressed in astrocytes *in vivo*[26] and *aqp4* RNA levels have been shown to increase during astrocytic differentiation.[27] *Aqp4* RNA levels also strongly increased in HNSC.100 astrocyte populations compared with progenitor populations (982-fold increase) (Figure 1). This indicates that the HNSC.100 cell line is a useful experimental system to assay astrocyte-dependent gene expression.

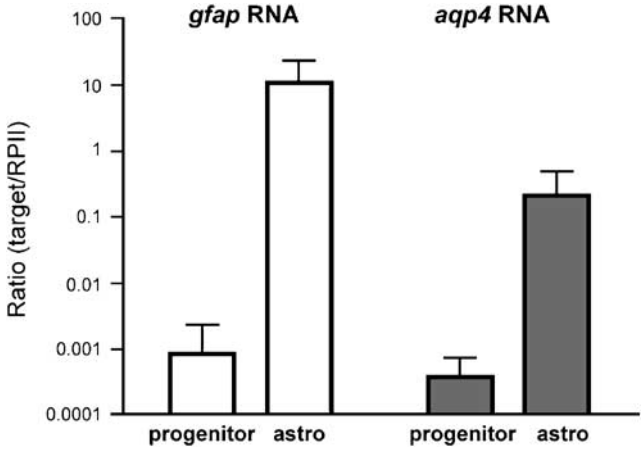

Figure 1 Upregulated expression of astrocyte-specific genes by differentiation of HNSC.100 neural progenitor cells into astrocytes. RNA expression levels of the *gfap* (glial fibrillary acidic protein) and *aqp4* (aquaporin 4) genes were analysed in HNSC.100 cell populations cultured as neural progenitor cells ('progenitor') or as astrocytes ('astro'), by real-time PCR analysis of cDNA. Data are presented as the ratio of the RNA levels of target genes (*gfap* and *aqp4)* to the internal reference gene *RPII*. Error bars represent the standard deviation of six analyses.

Establishment of FV-vector-transduced neural progenitor cell populations

Although FVs are known to infect various types of cells from different species,[10,11] it was necessary to prove that FV particles can also infect neural progenitor cells. Recombinant FV particles were produced in 293T cells cotransfected with the three packaging constructs and the pDM9 vector construct (for details see Materials and methods). The pMD9 vector[28] (Figure 2a) contains a cassette for the expression of EGFP under the transcriptional control of a constitutive promoter consisting of the U3 region of the LTR of the spleen focus-forming virus (SFFV). A single round of exposure to virus particles at an MOI of 0.15 resulted in the production of EGFP in 5% of the HNSC.100 cell population (data not shown). Subsequent enrichment of EGFP-positive cells by fluorescence-activated cell sorting (FACS) resulted in the establishment of an HNSC.100 population (HNSC.FV.MD9) in which the majority of cells showed marked production of EGFP (Figure 2b).

To confirm the infection of neural progenitor cells by FV vectors for another cell line, cultures of the mouse neural stem cell line C17.2[29] were exposed to pMD9 FV particles under the same conditions used for the transduction of the HNSC.100 cells. Again, this resulted in about 6% of EGFP-positive cells (data not shown), which could be enriched by FACS to generate an FV-transduced cell population (Figure 2b, C17.2.FV.MD9).

As transgene downregulation is an often-described phenomenon after gene transfer,[30] we analysed EGFP production in the HNSC.FV.MD9 population at two different cell passages (Figure 2c). Flow cytometry analysis revealed a moderate decrease in the proportion of EGFP-positive cells from 80 to 60% at the later passage. However, the levels of EGFP production in the EGFP-positive cell populations remained unchanged (same median fluorescence intensities). This indicates that long-term marker gene expression can be achieved by FV transduction of HNSC.100 neural progenitor cells.

Establishment of FV-vector-transduced HNSC.100 cell populations harbouring a transgene controlled by the astrocyte-specific Gfa2 promoter

For the transduction of a gene specifically expressed in astrocytes, the SFFV-U3 promoter in pMD9 was replaced with the astrocyte-specific Gfa2 promoter, generating the pFVGfa2-egfp vector (Figure 3a) (for details see Materials and methods). Transduction of HNSC.100 populations and FACS enrichment led to the establishment of HNSC.100 populations (HNSC.FV.Gfa2-EGFP), which displayed only very weak EGFP production (Figure 3b), indicating the low basal activity of the transduced Gfa2 promoter in undifferentiated neural progenitor cells. Stable transduction with the full-length Gfa2-*egfp* transgene was confirmed by PCR analysis (Figure 3c). Primers flanking the entire expression cassette

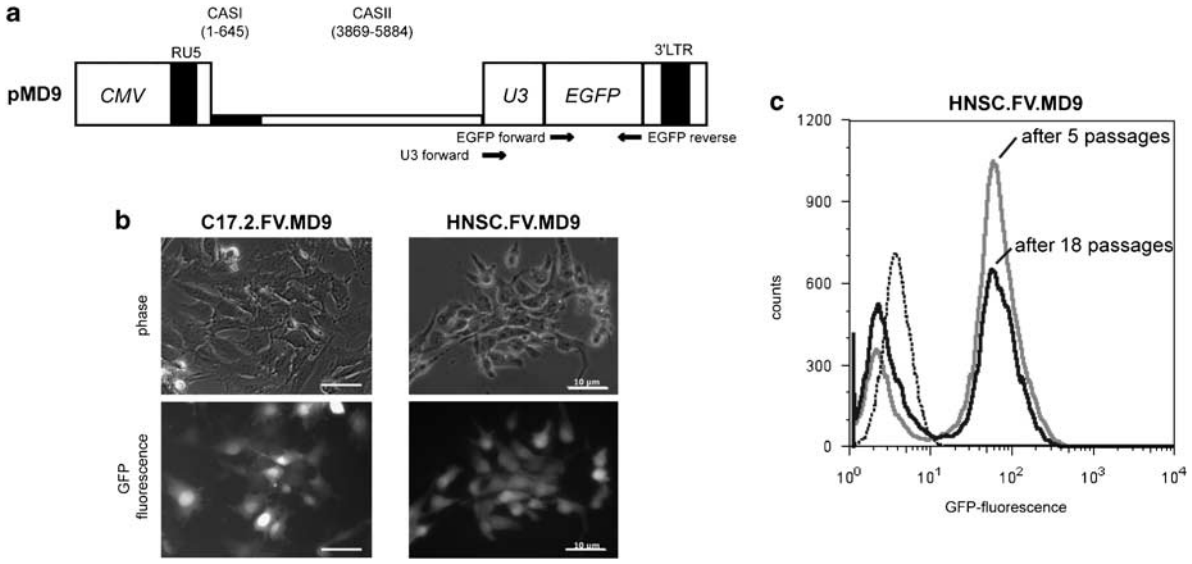

Figure 2 Stable transduction of neural progenitor populations by FV-mediated gene transfer. (**a**) Schematic structure of the FV vector pMD9. The pMD9 vector[28] contains an internal gene that uses the U3 region of the SFFV as a constitutive promoter for the expression of EGFP. Arrows indicate the binding sites of primers used for PCR analysis of the integrated vector (Figure 3c). (**b**) Production of EGFP in two cell populations generated by the transduction of different neural progenitor cell lines with the pMD9 vector and subsequent enrichment by FACS. The C17.2.FV.MD9 population was generated with the murine cell line C17.2 and the HNSC.FV.MD9 population with the human cell line HNSC.100. Scale bars: 10 µm. (**c**) Flow cytometry analysis of long-term EGFP production in HNSC.FV.MD9 cell populations. FACS plots are shown of EGFP production in progenitor populations after five (grey lines) and 18 passages (black lines). Untransduced HNSC.100 cells were analysed as control (dotted line). EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; FV, foamy virus; SFFV, spleen focus-forming virus.

(Figure 3a) yielded an amplification product of the expected size (2990 bp). Primers flanking the egfp sequence also yielded an amplification product of the expected size (706 bp product). This amplicon was also obtained with genomic DNA for HNSC populations transduced with pMD9 (HNSC.FV.MD9). As expected, PCR with a primer that binds to the U3 region in pMD9 (Figure 2a) yielded the expected amplification product (1186 bp) with DNA from the HNSC.FV.MD9 but not from the HNSC.FV.Gfa2-EGFP cell population. No smaller amplicons were observed in any of the PCRs.

*Expression of the Gfa2-*egfp *transgene is upregulated by the differentiation of FV-vector-transduced HNSC.100 progenitor populations into astrocytes*

Our next goal was to investigate the influence of astrogenesis on expression of the Gfa2-*egfp* transgene in the FV-vector-transduced HNSC.100 population (HNSC.FV.Gfa2-EGFP). Expression of the Gfa2-*egfp* transgene was also investigated in an HNSC.100 population established by the transfection of the pGfa2-egfp expression plasmid and the selection of antibiotic-resistant cells (HNSC.pGfa2-EGFP) (Figure 4a).

Both populations showed a substantially increased production of EGFP after culture in astrocyte-differentiation medium for 7 days (Figure 4a), which was confirmed by flow cytometry (Figure 4b). Median fluorescence intensity was increased 16-fold in FV-vector-transduced astrocyte populations, but only about threefold in the stably transfected population. This difference was partly due to the increased background production of EGFP in the transfected progenitor populations, compared with the FV-transduced progenitor populations. As expected, astrocyte differentiation did not increase the production of EGFP in the HNSC.100 populations transduced with the pMD9 vector (HNSC.FV.MD9) (Figure 4b).

*Coregulation of the transduced Gfa2-*egfp *and the native* gfap *genes*

To determine whether the transduced Gfa2-*egfp* transgene and the native *gfap* gene are coregulated, levels of RNAs produced from both genes in progenitor and astrocyte populations were quantified by real-time PCR analysis. As control, e*gfp* and *gfap* RNA levels were analysed in the HNSC.100 population transduced with the pMD9 vector (HNSC.FV.MD9).

For both the FV-vector-transduced HNSC.100 populations, expression of the native *gfap* gene was low in progenitor populations and strongly increased after culture in astrocyte-differentiation medium (Figures 5a and b). *Gfap* expression was similar in FV-vector-transduced and -untransduced HNSC.100 populations (compare Figures 1 and 5a), indicating that FV-vector transduction did not affect the regulation of the native gfap promoter. In contrast, the transfected HNSC.100 population (HNSC.pGfa2-EGFP) showed a much higher basal expression of the native *gfap* gene, which was only moderately elevated after culture in astrocyte-differentiation medium (Figures 5a and b).

Astrogenesis also increased RNA levels produced from the Gfa2-*egfp* transgene in both the transduced (HNSC.FV.Gfa2-EGFP) and the stably transfected HNSC.pGfa2-EGFP populations (Figure 5c). This increase was more pronounced in the FV-transduced than in the FV-transfected cell population. Background levels of *egfp* RNA in the undifferentiated progenitor cells were lower in the FV-transduced cell population than in the

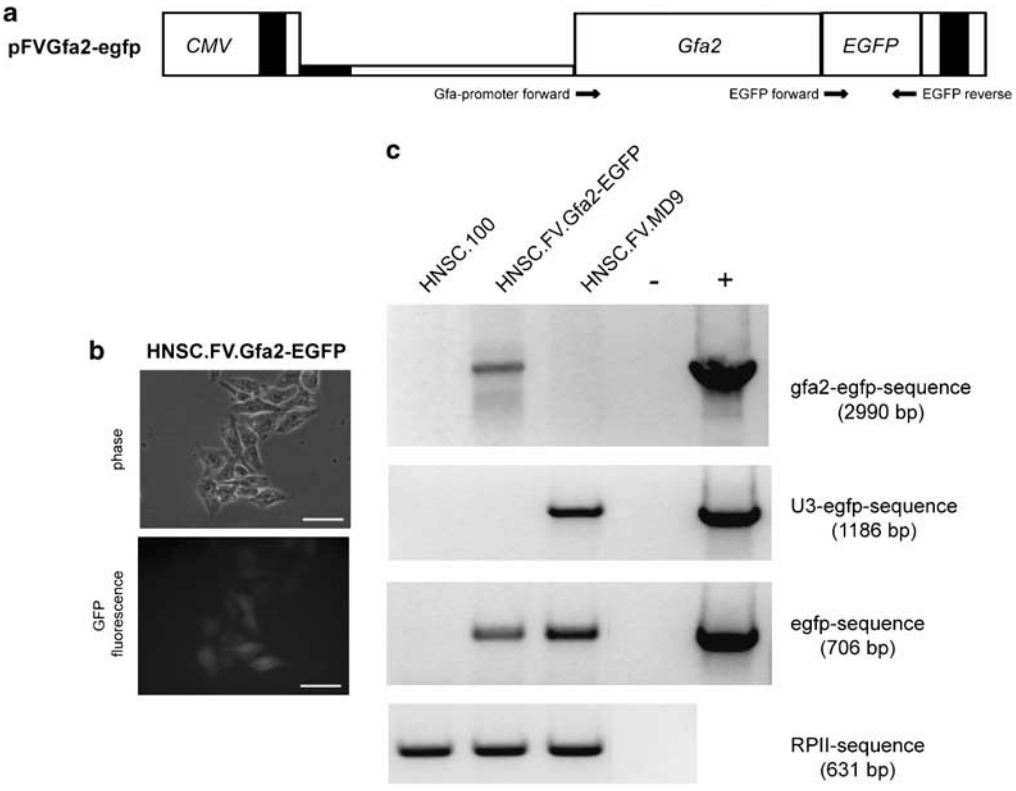

Figure 3 FV transduction of neural progenitor populations with a transgene controlled by the astrocyte-specific Gfa2 promoter. (**a**) The pFVGfa2-egfp vector was generated by replacing the U3 promoter in pMD9 with the 2.2 kb astrocyte-specific human Gfa2 promoter. Arrows indicate the binding sites of primers used for PCR analysis of the integrated vector (Figure 3c). (**b**) Low EGFP production in the HNSC.100 neural progenitor cell population transduced with the pFVGfa2-egfp vector (HNSC.FV.Gfa2-EGFP). Scale bars: 10 μm. (**c**) Verification of integration of the pFVGfa2-egfp vector by PCR amplification of different vector segments in genomic DNA from the HNSC.FV.Gfa2-EGFP cell population. Genomic DNAs from untransduced HNSC.100 cells and the HNSC.FV.MD9 cell population were analysed as negative and positive controls, respectively. The presence of genomic template DNA was verified by the amplification of a segment of the RNA polymerase II (RPII) gene. PCRs lacking DNA served as reagent controls (−). Amplicons of the expected sizes were generated by PCRs with pFVGfa2-egfp or pMD9 plasmid DNAs (+). EGFP, enhanced green fluorescent protein; FV, foamy virus.

stably transfected population (data not shown). This agrees with the lower background production of the EGFP protein in the FV-transduced progenitor cell population (HNSC.FV.Gfa2-EGFP, Figure 4b). As expected, *egfp* RNA levels were not elevated in astrocyte populations of pMD9-transduced HNSC.100 cells (HNSC.FV.MD9, Figure 5c).

In agreement with the results of the analysis of RNA levels, western blot analysis confirmed the upregulated production of both EGFP and GFAP proteins in astrocyte populations of HNSC.FV.Gfa2-EGFP cells, whereas only production of GFAP but not EGFP was upregulated in HNSC.FV.MD9 cells (Figure 6).

Discussion

The availability of versatile and safe vectors for stable transfer of genes to neural progenitor cells is a prerequisite for the use of this cell type in gene therapy of central nervous system diseases. In this study, we demonstrate that FV vectors can deliver genes into human and mouse neural progenitor cells for expression. Furthermore, we provide evidence for the long-term expression of a transgene with a constitutive promoter in transduced populations of the human neural progenitor cell line HNSC.100.

Foamy virus vector-mediated delivery of a transgene controlled by the astrocyte-specific human Gfa2 promoter resulted in the establishment of HNSC.100 populations that upregulated the expression of the transduced gene upon astrogenesis. Coregulation of the FV-vector-transduced and the native gfap promoter provided further evidence for the astrocyte-specific regulation of the transduced Gfa2 promoter. This indicates that FV vectors are capable of delivering large segments of regulatory sequences for differentiation-dependent gene expression. An important prerequisite for the design of such inducible transgenes in neural progenitor cells is the availability of transcriptional regulatory sequences that retain the capacity to confer neural cell-specific gene expression out of the context of their natural gene locus. Indeed, several promoters have been identified that mediate the expression of transgenes in other neural cell types in rodent brains.[32,33] Future studies will address the capacity of these and other promoters to direct differentiation-dependent gene expression in human neural progenitor cells transduced with the appropriate FV vectors.

Basal expression of the Gfa2-*egfp* transgene was higher in HNSC.100 cell populations established by transfection/antibiotic-selection procedures than in the FV-vector-transduced HNSC.100 populations (Figures 4 and 5). Interestingly, basal activation in the transfected

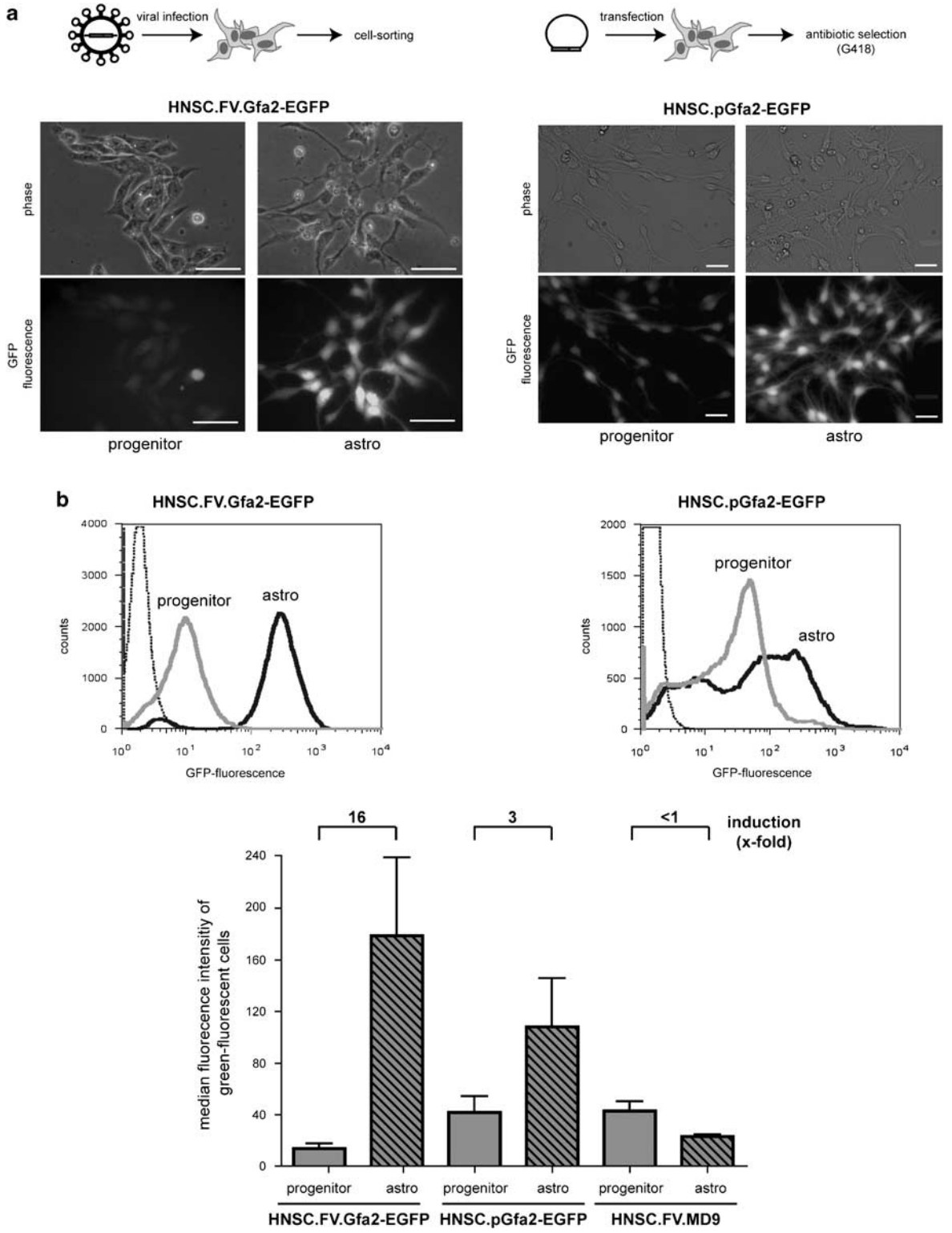

Figure 4 Astrogenesis-dependent upregulation of the expression of the Gfa2-egfp transgene. The influence of astrogenesis on the expression of the Gfa2-egfp transgene was analysed in HNSC.100 populations established either by FV transduction and cell sorting (HNSC.FV.Gfa2-EGFP) or by stable transfection of the pGfa2-egfp plasmid and selection of G418-resistant cells (HNSC.pGfa2-EGFP). (**a**) EGFP production in single cells. Astrogenesis increased EGFP production in both HNSC.100 populations. Background expression in progenitor cells was lower for FV-transduced cells than for the transfected cells. Scale bars: 10 μm. (**b**) Flow cytometry analysis of EGFP production in cell populations. Representative FACS plots of EGFP production in astrocyte (astro; black lines) and progenitor (grey lines) populations are shown. Untransduced HNSC.100 cells were analysed as control (dotted line). The bar graph shows the median fluorescence intensities of the EGFP-expressing populations, with numbers above the bars indicating the *x*-fold induction of EGFP expression in astrocyte populations relative to progenitor populations. Error bars represent the standard deviation of three independent analyses. EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorting; FV, foamy virus.

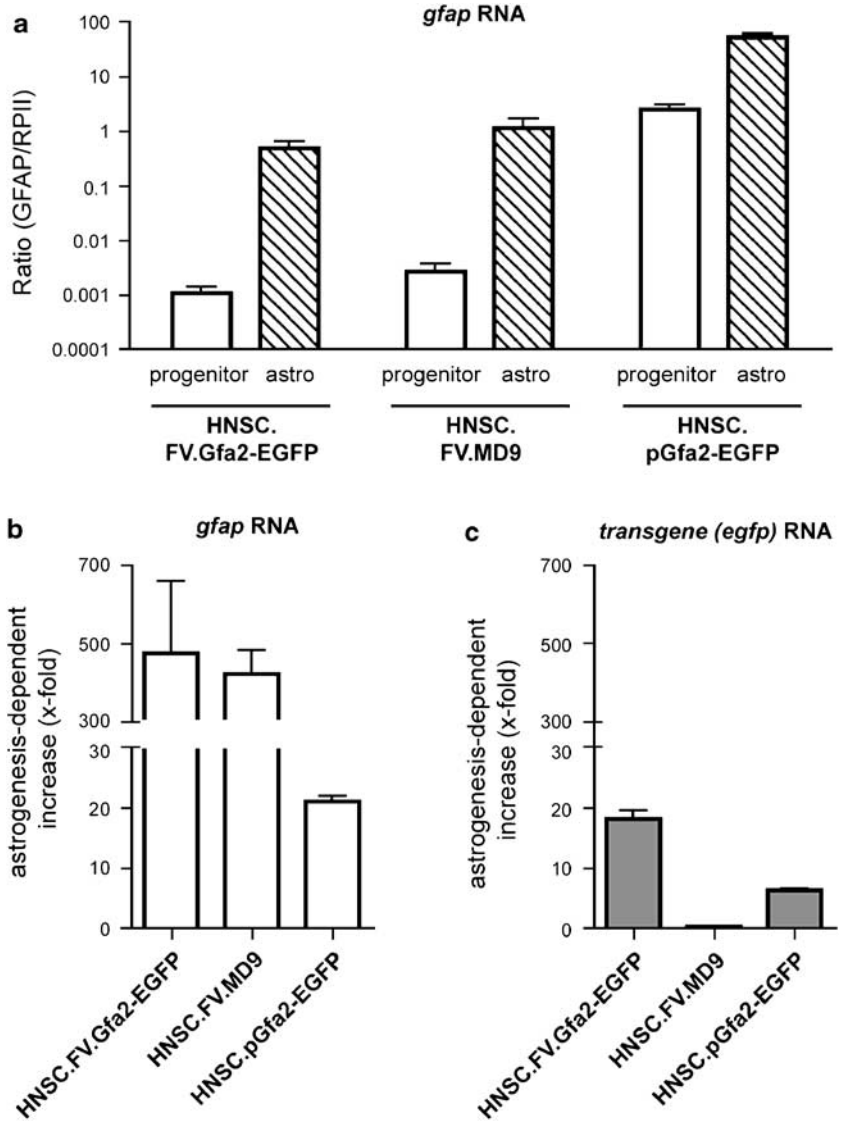

Figure 5 Influence of astrogenesis on the expression of the native *gfap* gene and the Gfa2-*egfp* transgene in HNSC.100 populations stably transduced with FV vectors or stably transfected with the vector plasmid. (**a**) Expression of the native *gfap* gene. *gfap* RNA levels were quantified in astrocyte and progenitor populations by real-time PCR. Data are shown as the ratio of the *gfap* target gene to the internal reference gene *RPII*. Error bars represent the standard deviation of three analyses. Astrogenesis increased the expression of *gfap* in all HNSC.100 populations. Levels of *gfap* RNA in undifferentiated progenitor cells were lower in FV-transduced cell populations (HNSC.FV.Gfa2-EGFP and HNSC.FV.MD9) than in stably transfected HNSC.100 populations (HNSC.pGfa2-EGFP). (**b**) Astrogenesis-dependent increases in RNA levels of the native *gfap* gene. The data generated for the graph shown in (**a**) were used to calculate the increases in relative *gfap* RNA levels in the astrocyte populations versus progenitor cell populations by the $2^{-\Delta\Delta CT}$ method[31] (see Materials and methods). (**c**) Astrogenesis-dependent increases in RNA levels of the transduced *egfp* transgene. Astrogenesis increased relative levels of *egfp* RNA in the HNSC.100 populations containing the Gfa2-*egfp* transgene (HNSC.FV.Gfa2-EGFP and HNSC.pGfa2-EGFP) but not in the HNSC.100 populations with the U3-*egfp* transgene (HNSC.FV.MD9). EGFP, enhanced green fluorescent protein; FV, foamy virus; GFAP, glial fibrillary acidic protein.

cells was not limited to the transgene but was also apparent for the native (that is, cellular) *gfap* gene. This suggests that the transfection/selection procedure is associated with cellular changes that increase the activity of the gfap promoter in neural progenitor cells. These changes would also be expected to increase the basal activity of the transduced Gfa2 promoter. In contrast to the transfected cell populations, the FV-vector-transduced cell populations show a similar low expression of the native *gfap* gene as the untransduced neural progenitor cell populations. This suggests that the procedures for FV-vector-mediated gene delivery are less invasive and therefore safer for the generation of genetically modified neural progenitor cell populations than transfection/selection procedures.

Foamy virus vector-mediated transfer of genes to neural progenitor cells has several potential applications aiding the experimental evaluation and the potential development of gene therapy approaches based on neural progenitor cells. One methodical potential application could be the labelling of neural progenitor cells by transgenes encoding fluorescent marker proteins. Line-

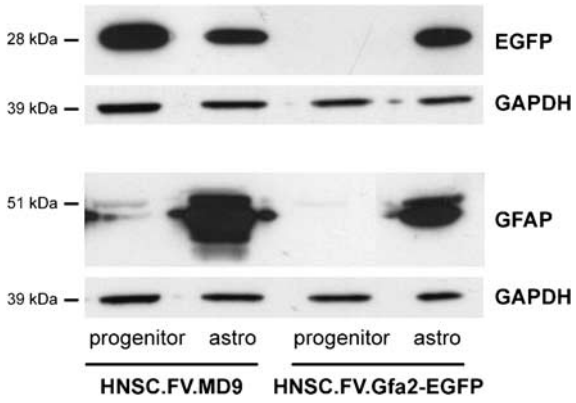

Figure 6 Western blot analysis of the influence of astrogenesis on the production of GFAP and EGFP proteins in FV-transduced HNSC.100 populations. Astrogenesis increased the production of both EGFP and GFAP in HNSC.FV.Gfa2-EGFP populations. In HNSC.FV.MD9 populations, astrogenesis selectively increased the production of GFAP but not EGFP. GAPDH was used as loading control. EGFP, enhanced green fluorescent protein; FV, foamy virus; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein.

age-specific marker transgenes would allow the tracking of mature cell types originating from the engrafted progenitor cells, providing distinct advantages over current methods like directing labelling of engrafted cells with dyes[34–37] and immunohistochemical staining of fixed cells.[38–41]

Another potential application of FV vectors is their use in the *ex vivo* genetic engineering of neural progenitor cells for the expression of therapeutically relevant genes. The FV vector system has several advantages over other retroviral vectors for potential gene therapy approaches. Thus, it is based on a virus without any known pathogenicity in humans. Furthermore, FV particles can directly deliver genes into neural progenitor cells without the need for incorporation of foreign glycoproteins, whereas the human immunodeficiency virus-based gene delivery into central nervous system cells requires pseudotyping of virus particles.[42,43] In addition, the results of this study show that FV vectors can transfer genes with large regulatory DNA segments and mediate inducible gene expression.

HNSC.100 cells and other clonally derived immortalized neural progenitor cell lines have been shown to survive engraftment, refrain from forming tumours and to retain the capacity to migrate and to differentiate into various cell types in rodent brain tissues.[44,45] FV transduction of genes for lineage-specific expression has been demonstrated only for the HNSC.100 human neural progenitor cell line so far. Nevertheless, these results raise the exciting possibility that FV vectors may allow the generation of transduced neural progenitor cell populations, for use as delivery vehicles for regulated expression of therapeutic molecules *in vivo*.

Neural progenitor cells can migrate and differentiate into astrocytes in response to factors produced at sites of brain pathology.[46–48] This raises the possibility that engrafted FV-vector-transduced neural progenitor cells transduced with genes controlled by the Gfa2 promoter could migrate to diseased areas of the brain, where differentiation into astrocytes could induce the expression of therapeutically relevant gene products.

Our study implicates FV vectors as promising tools for the genetic engineering of neural progenitor cells for potential therapies of central nervous system diseases. However, it is important to consider that at this stage it is far from clear whether and how gene therapy concepts based on neural progenitor cell can be developed into clinically useful therapeutic options for neurodegenerative diseases in humans. FV vectors have a high potential to contribute to the clarification of this important issue.

Materials and methods

Cell culture

All cells were cultured in an H_2O-saturated atmosphere with 7% CO_2 at 37 °C. All cell cultures with the human immortalized neural stem cells HNSC.100[24] were carried out on poly-D-lysine-coated plastic ware in a basal medium containing Dulbecco's modified Eagle's medium:F12, 0.5% fetal calf serum, N2 supplement, 1% bovine serum albumin, 1% penicillin/streptomycin and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer. HNSC.100 cells were maintained in a proliferating and self-renewing state (that is, progenitor cultures) by supplementing the basal medium with 20 ng ml^{-1} of epidermal growth factor and 20 ng ml^{-1} of basic fibroblast growth factor. HNSC.100 progenitor cultures were passaged by splitting cells 1:10 after 4 days of culture and re-plating. For differentiation into astrocytes, HNSC.100 were cultured in basal medium supplemented with 100 ng ml^{-1} of ciliary neurotrophic factor (CNTF) (that is, astrocyte-differentiation medium) for at least 7 days.[25] Basic fibroblast growth factor, epidermal growth factor and CNTF were purchased from Tebu-Bio (Offenbach, Germany) and all other cell culture reagents from Invitrogen (Karlsruhe, Germany).

Human embryonic kidney cell line 293T and human HT1080 fibrosarcoma cells were maintained in Dulbecco's modified Eagle's medium containing 2 nM GlutaMAX I supplemented with 10% fetal calf serum and 1% penicillin/streptomycin.

Mouse neural stem cell line C17.2[29] was cultured in Dulbecco's modified Eagle's medium containing 2 nM GlutaMAX I supplemented with 10% fetal calf serum, 5% horse serum and 1% penicillin/streptomycin.

HNSC.pGfa2-EGFP populations were established by transfecting HNSC.100 cells with the pGfa2-egfp plasmid (500 ng) (see section Plasmids and FV vectors) using Fugene6 transfection reagent (Roche Diagnostics, Mannheim, Germany) and subsequent G418 selection (500 μg ml^{-1}).

Plasmids and FV vectors

The pMD9 FV vector[28] harbours a human cytomegalovirus immediate-early gene enhancer/promoter, sequences from the genome of the primate FV type 1 required for vector transfer (for details see Heinkelein *et al*.[28]) and a cassette for the expression of EGFP under the control of the SFFV-U3 promoter. The 2.2 kb promoter sequence of the human *gfap* gene was isolated from the plasmid pGfa2-lacZ[49] by digestion with *Eco*RI and introduced into the multiple cloning site of pEGFP-1 (Clontech, Saint-Germain-en-Laye, France) to generate pGfa2-egfp. Plasmid pGfa2-egfp contains a separate expression cassette for neomycin resistance. The promo-

ter–reporter gene cassette was isolated from pGfa2-egfp by digestion with *Bgl*II, followed by fill-in of 5′ overhangs to form blunt ends with Klenow DNA polymerase and subsequent digestion with *Not*I. pFVGfa2-egfp was generated by inserting the Gfa2-egfp segment into plasmid pMD9 digested with *Bsm*BI and *Not*I to remove the SFFV-U3-egfp expression cassette.

Particle production and infection

For the production of FV particles, 1.6×10^6 293T cells were seeded in 6-cm diameter dishes 1 day before they were cotransfected with 1.5 μg each of the packaging constructs pCIgag-2, pCpol-2 and pCenv-1[28,50,51] and 1.5 μg of the vector construct pFVGfa2-egfp or pMD9.[28] Transfections were performed with PolyFect according to the manufacturer's protocol (Qiagen, Hilden, Germany). Twenty-four hours after transfection, the cells were treated with 10 mM sodium butyrate for 8 h to stimulate the expression from the cytomegalovirus enhancer/promoter contained in all constructs. Cell culture supernatants (2.5 ml) were harvested 48 h after transfection, purified by filtration through filters (pore size 0.45 μm) and used for infection of target cells.

Titres of the pMD9 vector were determined by monitoring transduction efficiencies of HT1080 cells by flow cytometry 4 days after infection as described.[28]

For infection with FVs, HNSC.100 progenitor populations were seeded at a density of 3×10^5 cells in six-well plates. Twenty-four hours after plating, the culture medium in each well was replaced with 1 ml of cell-free supernatant from 293T FV-producer cells. Infections with the pMD9 FV vector were performed at an MOI of 0.15. After incubation for 48 h, the 293T supernatant was removed, cells were washed and culture of HNSC.100 cells continued in basal medium containing epidermal growth factor and basic fibroblast growth factor. Flow cytometry analysis of EGFP expression in HNSC.100 progenitor populations 10 days after infection revealed about 5% EGFP-positive cells. Cell cultures were expanded and enriched populations of green fluorescent cells obtained by FACS.

Quantitative and qualitative PCR

Total RNA was extracted by Qiagen RNeasy Mini Kit, subjected to DNAse digestion (Promega, Mannheim, Germany) and first-strand cDNA generated from 1 μg of total RNA with M-MLV reverse transcriptase RNase H Minus, Point Mutant, followed by RNase H digestion step (Promega). Quantitative real-time PCR was performed with the Roche LightCycler 1.5 System, using LightCycler FastStart DNA Master SYBR Green I-Kit and standard LightCycler protocol (Roche Diagnostics).

For the relative quantification of *gfap*, *aqp4* and *egfp* RNA levels, real-time PCRs were performed with the following primers: hGFAP forward 5′-GCTTCCTGGAA CAGCAAAACAAGGC-3′, hGFAP reverse 5′-GTCTAT AGGCAGCCAGGTTGTTC-3′; hAQP4 forward 5′-GG AATCCTCTATCTGGTCACA-3′, hAQP4 reverse 5′-TGT TTGCTGGGCAGCTTTGCT-3′; and EGFP (light cycler) forward 5′-ACGTAAACGGCCACAAGTTC-3′, EGFP (light cycler) reverse 5′-AAGTCGTGCTGCTTCATGTG-3′. RNA polymerase II transcripts (RPII) were analysed as internal reference, using primers given in Radonic *et al*.[52] The ratio of a target gene relative to RPII was calculated as $2^{CT(RPII)}/2^{CT(target)}$. The *x*-fold induction of *gfap* and *egfp* expression in astrocyte populations relative to expression in progenitor populations was analysed using the $2^{-\Delta\Delta C_T}$ method.[52] The ΔC_T value was calculated as C_T (target)$-C_T$ (RPII) and the $\Delta\Delta C_T$ value as ΔC_T (astrocyte populations)$-\Delta C_T$ (progenitor populations).

Genomic DNA was extracted using QIAamp DNA MiniKit (Qiagen) according to the manufacturer's protocol. PCRs to detect integrated foamy proviral sequences were performed with the Expand High Fidelity PCR System (Roche Diagnostics) using the following primers: hGfa promoter forward 5′-TCTC TGTGCTGGGACTCACA-3′, SFFV-U3 promoter forward 5′-TGCGGGAGACGTCGAGTCCA-3′, EGFP forward 5′-AAGGGCGAGGAGCTGTTCA-3′ and EGFP reverse 5′-TTGTACAGCTCGTCCATGCC-3′. A segment of the RPII gene using the primers given in Radonic *et al*.[52] was amplified as control for genomic DNA.

Fluorescence microscopy

Microscopy of cells expressing EGFP protein was performed with a computer-controlled Zeiss Axiovert 200M research microscope (Carl Zeiss, Goettingen, Germany) and Software AxioVision 4.6 (Carl Zeiss Imaging Solutions, Hallbergmoos, Germany), using a Zeiss AxioCam HRm CCD Camera with full resolution. For comparative purposes, fluorescent images were acquired with identical exposure times.

Flow cytometry

Harvested cells were analysed with a FACScalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Green fluorescence of EGFP was detected in channel FL-1. To gate the non-fluorescent cell population, non-transduced HNSC.100 cells were analysed as control. Median fluorescence intensity of green fluorescent cells was measured in histogram plots.

Western blot

For western blot analysis, equal amounts of cells were lysed with lysis buffer containing protease inhibitors. Whole-cell lysates were separated on pre-cast 4–12% Bis–Tris gradient acrylamide gels (Invitrogen) and transferred onto nitrocellulose membranes (Biorad, München, Germany). The following primary antibodies were used for protein detection with the indicated dilutions: rabbit anti-GFAP (DAKO, Hamburg, Germany), 1:5000; monoclonal rat antibodies against GFP contained in hybridoma supernatants (kindly provided by Elisabeth Kremmer, Helmholtz Center Munich), 1:50; and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (Millipore, Schwalbach, Germany), 1:10 000. Horseradish peroxidase-conjugated goat anti-rabbit, anti-rat or anti-mouse antibodies were used as secondary antibodies (1:10 000; Dianova, Hamburg, Germany). Protein bands were detected by an enhanced chemoluminescence system (Perbio Science, Bonn, Germany).

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