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Expression of FGF-2 in neural progenitor cells enhances their potential for cellular brain repair in the rodent cortex

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Strategies to enhance the capacity of grafted stem/progenitors cells to generate multipotential, proliferative and migrating pools of cells in the postnatal brain could be crucial for structural repair after brain damage. We investigated whether the over-expression of basic fibroblast growth factor 2 (FGF-2) in neural progenitor cells (NPCs) could provide a robust source of migrating NPCs for tissue repair in the rat cerebral cortex. Using live imaging we provide direct evidence that FGF-2 over-expression significantly enhances the migratory capacity of grafted NPCs in complex 3D structures, such as cortical slices. Furthermore, we show that the migratory as well as proliferative properties of FGF-2 over-expressing NPCs are maintained after *in vivo* transplantation. Importantly, after transplantation into a neonatal ischaemic cortex, FGF-2 over-expressing NPCs efficiently invade the injured cortex and generate an increased pool of immature neurons available for brain repair. Differentiation of progenitor cells into immature neurons was correlated with a gradual down-regulation of the FGF-2 transgene. These results reveal an important role for FGF-2 in regulating NPCs functions when interacting with the host tissue and offer a potential strategy to generate a robust source of migrating and immature progenitors for repairing a neonatal ischaemic cortex.

Keywords: Brain repair; neonatal ischemia; neural progenitors; transplantation; migration; FGF-2

Abbreviations: DCX = doublecortin; DIV = days *in vitro*; GABA = (γ-aminobutyric acid; GAD-67 = glutamic acid decarboxylase 67; NPCs = neural progenitor cells; SVZ = subventricular zone; TU = transducing units

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Introduction

The postnatal cortex has a very limited ability to regenerate neural tissue after brain insults. This is due in part to the lack of a resident population of neural progenitor cells (NPCs) responsive to signals derived from the damaged tissue. Compensatory cortical neurogenesis has been reported after induced apoptotic degeneration (Magavi *et al*., 2000) or after stroke (Jiang *et al*., 2001; Jin *et al*., 2003), but the number of newly generated neurons, if present at all (Arvidsson *et al*., 2002), remains insufficient to restore a normal cortical function (Bjorklund and Lindvall, 2000). To overcome this limitation, the manipulation of NPCs has developed into a key strategy for brain repair. Attempts have been made to stimulate the postnatal subventricular zone (SVZ) with growth hormones in order to recruit a population of NPCs towards the lesioned cortex. These approaches have been supported by the finding that growth hormones such as the basic fibroblast growth factor (FGF-2) are capable of increasing the proliferation of SVZ progenitors and promote olfactory bulb neurogenesis (Kuhn *et al*., 1997). Furthermore, chronic infusion of FGF-2 and the epidermal growth factor in the lateral ventricles has been shown to be critical

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in regenerating new hippocampal neurons after global ischaemia (Nakatomi *et al.*, 2002). However, successful replacement of damaged cortical neurons using NPCs has not been reported. Among the multiple factors limiting the efficiency of NPC transplantation in the postnatal cortex is the fact that grafted NPCs rapidly lose their immature, proliferative and migratory properties. It would therefore be of considerable interest to supply a source population of multipotential, proliferative and migrating NPCs competent to respond to chemoattractant cues secreted by the site of injury. Support for this hypothesis derives from *in vitro* work showing that FGF-2-stimulated NPCs can respond to a chemoattractant cue such as vascular endothelial growth factor (Zhang *et al.*, 2003).

Autocrine/paracrine signalling of FGF-2 appears to play a key role in sustaining self-renewal of neural progenitor/stem cells *in vitro* (Maric *et al.*, 2003) and maintaining immature proliferative populations in neurogenic niches *in vivo* (Zheng *et al.*, 2004). We therefore tested the hypothesis that over-expression of FGF-2 in transplanted NPCs may provide robust sources of migrating NPCs for tissue repair after brain damage.

Materials and methods

All animal experiments were conducted in accordance with Swiss laws, previously approved by the Geneva Cantonal Veterinary Authority.

Isolation, cultures and differentiation of NPCs *in vitro*

The SVZ from coronal slices of newborn rat brains were dissected, mechanically dissociated and trypsinized. NPCs were purified using a 22% Percoll gradient centrifugation as previously described (Lim *et al.*, 2000; Zhang *et al.*, 2003) and seeded at 4×10^5 cells/dish concentration onto matrigel-coated dishes (1:500). Cells were allowed to expand in neurobasal medium (Invitrogen) supplemented with 20 ng/ml FGF-2 (human recombinant, R&D), 2% B27 supplement (Invitrogen), 2 mM L-glutamine, 1 mM sodium pyruvate, 2 mM N-acetyl-cysteine and 1% penicillin–streptomycin. At DIV3, half of the medium was replaced with fresh medium containing 20 ng/ml of FGF-2 with either the control lentiviral vector or FGF-2 lentiviral vector. For FGF-2 deprivation experiments, the dishes were washed and 2 ml of fresh medium was added with or without FGF-2 (20 ng/ml). To induce differentiation, NPCs were trypsinized at DIV5, seeded at 5×10^4 cells/dish concentration onto matrigel-coated dishes (1:500), allowed to expand during 48 h in the presence of 20 ng/ml FGF-2. Differentiation was induced by removing FGF-2 and adding 20 ng/ml BDNF and 1% fetal calf serum (Invitrogen).

Design and production of lentiviral vectors

The pWPI_SPbFGF lentiviral vector coding for FGF-2 was constructed as follows. A cDNA coding for the 18 kDa form of the human FGF-2 (basic FGF) fused to an immunoglobulin signal peptide facilitating the secretion of FGF-2 (Rinsch *et al.*, 2001) was cloned into the pWPI bicistronic lentiviral vector. pWPI is an HIV-1 derived SIN vector containing the EF1 alpha promoter and an EMCV-IRES-GFP cistron (http://tronolab.epfl.ch/). FGF-2 was cloned in the PmeI site located between the EF1 alpha promoter and the IRES_GFP sequences. Control lentiviral vectors were the following: pFUGW contains the ubiquitin promoter controlling the expression of GFP, pWPXL contains the EF1 alpha promoter controlling the expression of GFP (http://tronolab.epfl.ch/) and RIX-PGK-Tom-W vector was constructed by inserting the tdTomato gene (Shaner *et al.*, 2004) downstream of the hPGK promoter, in place of the GFP gene of the RIX-PGK-GFP-W vector. EF1 alpha, ubiquitin and PGK promoters are ubiquitous and active in neural cells. Details on pWPI plasmid and on the RIX-PGK-Tom-W vector can be obtained at http://www.medecine.unige.ch/~salmon/.

Lentiviral vectors were produced, concentrated and titrated according to standard protocols. Details on procedures can be obtained at http://www.medecine.unige.ch/~salmon/. Control and FGF-2 lentiviral vectors had titers ranging from 10^8 to 10^9 transducing units (TU)/ml. Transduction of NPCs was done at DIV3 except at DIV2 for the BrdU deprivation study. NPCs (~50 000–75 000 cells per 35 mm culture dish) were transduced using doses ranging from 5×10^4 to 5×10^5 TU of either control or FGF-2 lentiviral vectors.

FGF-2 secretion in the medium

To measure the secretion of FGF-2 by NPCs, cultures were transduced at DIV3 with the GFP control lentiviral vector and the FGF-2 lentiviral vector (1.5×10^5 TU/ml). At DIV6, cultures were washed with fresh medium containing no FGF-2. At DIV8, the medium was removed and the amount of FGF-2 secreted in the medium was quantified using a standard ELISA detection method (Quantikine, R&D). Cells were trypsinized, counted and the percentage of GFP positive cells was analysed by FACS.

Tissue processing and immunohistochemistry

Cultures and cortical slices were fixed overnight at 4°C with cold 4% paraformaldehyde (PFA) (pH 7.4). Rats were anesthezed by pentobarbital and sacrificed by intra-cardial perfusion of 0.9% saline followed by 4% PFA (pH 7.4). Brains were extracted from the skull and post-fixed in 4% PFA (pH 7.4) at 4°C overnight and cryoprotected with sucrose 30% if cut on a cryostat. For histology processing, 20 μm thick sections were cut on a cryostat or 60 μm thick sections were cut on a Vibratome 1500; sections or slices were washed three times with 0.1 M (PBS); incubated overnight at 4°C with a primary antibody diluted in PBS/0.5% bovine serum albumine (BSA)/0.3% Triton X-100; washed in PBS; incubated with the secondary antibodies against the appropriate species; nuclear counterstained with 33 258 bisbenzimide (Invitrogen) or TO-PRO-3 (Invitrogen). The following primary antibodies were used: monoclonal mouse anti-FGF-2 (1:250; Upstate), monoclonal mouse anti-nestin (1:1000, Chemicon), polyclonal rabbit anti-NCAM (1:1000) (Zhang *et al.*, 2003), polyclonal rabbit anti-NG2 (1:250; Chemicon), polyclonal rabbit anti-GFAP (1:500; Dakopatts), polyclonal goat anti-doublecortin (1:100; Santa Cruz), monoclonal mouse anti-GAD67 (1:1000; Chemicon), polyclonal rabbit anti-GFP (1:1000; Molecular Probes, Invitrogen), monoclonal mouse anti-NeuN (1:250; Chemicon), monoclonal mouse anti-BrdU (1:100; Boehringer-Mannheim), monoclonal rat anti-BrdU (1:100; Oxford Biotech. Ltd.), polyclonal rabbit anti-calretinin (1:1000; Swant, Switzerland), monoclonal mouse

anti-calbindin (1:5000; Swant, Switzerland), goat anti-parvalbumin (1:5000; Swant, Switzerland), mouse anti-FGFR1(1:100; Upstate), rabbit anti-FGFR2 (SC-122) (1:100; Santa-Cruz). The following secondary antibodies were used: anti-rabbit Alexa-568 and Alexa-488, anti-mouse Alexa-488 and Alexa-568, anti-goat Alexa-555 and Alexa-647 (Invitrogen). For BrdU labelling, cultures and cortical slices were incubated 30 min in 2 N HCL for DNA denaturation followed by standard incubation.

BrdU incorporation experiments

For culture experiments, NPCs were expanded in FGF-2 (20 ng/ml) during two days. At DIV2, the medium was removed and replaced by (i) medium supplemented with FGF-2 (20 ng/ml) and containing control lentiviral vector (1.5×10^5 TU/ml) (ii) medium containing control lentiviral vector (1.5×10^5 TU/ml) without exogenous FGF-2 (iii) medium containing the FGF-2 lentiviral vector at two different doses (0.75×10^5 TU/ml and 1.5×10^5 TU/ml) without exogenous FGF-2. At DIV6, BrdU (10 µM) was added to the medium during 16 h before PFA fixation. For BrdU incorporation experiments on cortical slices, BrdU (10 mM) was added to the medium during 6 h before PFA fixation either at DIV1 or at DIV2 after NPC deposition. For *in vivo* experiments, intraperitoneal BrdU (50 mg/kg) was injected twice daily from P7 to P9 or from P37 to P39.

Cortical slice preparation and *in vitro* NPC transplantation

The brains of P0 Sprague Dawley pups were dissected; 200 µm coronal sections were cut on a Vibratome in ice-cold Hanks medium and cultured on porous nitrocellulose filters (Millicell-CM); Details for cortical slice cultures and *in vitro* NPC transplantation can be found in the Supplementary Material. For time-lapse imaging slices were placed in a microscope chamber maintained at 37° and 5% CO_2. For *ex–in vivo* slices, rats were sacrificed at P5 after *in vivo* transplantation at P3 and slices were cut as described above.

In vivo NPC transplantation

For *in vivo* transplantation in the intact cortex, Wistar pups at postnatal day 3 (P3) were anesthetized with a mixture of Isofluran (Foren® 100%), O_2 30% and air 70%, and maintained in a stereotaxic frame. Hypoxia-ischaemia injury was performed at P3 as described previously (Sizonenko *et al*., 2003). Details on the number of animals and the survival time points can be found in the Supplementary Material.

Image processing

Epifluorescent time-lapse images were acquired with a digital camera (Retiga EX; Qimaging) linked to a fluorescent microscope (Eclipse TE2000-U; Nikon Corp.) equipped with Nikon Plan Fluor 4×/0.13, 10×/0.30 objectives and Nikon Plan Apo 60× A/1.40/oil objective. Time-lapse images were quantified with the Openlab software (version 3.1.2) and *post hoc* images were transferred to image J (NIH) software for quantification. Confocal images were acquired with a LSM 510 confocal microscope using a Plan-Neofluar 40×/1.3 oil objective. Unpaired *t*-test and χ^2 test were performed using the software SigmaStat® 3.1. Details on quantification of cells can be obtained in the Supplementary Material.

Results

Effects of FGF-2 over-expression on NPCs in culture

To study the effects of FGF-2 over-expression in multipotential NPCs, SVZ cells were isolated from newborn rats and expanded as described previously (Zhang *et al*., 2003). Cultures were transduced after 3 days *in vitro* (DIV3) with either the control green fluorescent protein (GFP) lentiviral vector or the FGF-2-GFP lentiviral vector. At DIV6, moderate FGF-2 immunoreactivity was detected in control NPCs whereas a much stronger signal was detected in FGF-2-transduced NPCs, showing that FGF-2 transduction can efficiently increase FGF-2 expression (Fig. 1A). Elisa quantification demonstrated that FGF-2 transduction increased the amount of FGF-2 into the culture media by ~10-fold (Fig. 1B). Control and FGF-2-transduced NPCs expressed the FGF receptor 1 (FGFR1) and the FGF receptor 2 (FGFR2) at the mRNA level and protein level (Supplementary Fig. 1), demonstrating that NPCs could bind secreted FGF-2. Quantification of the mRNA levels using real-time PCR revealed that FGFR1 and FGFR2 mRNA levels were not significantly modified after FGF-2 transduction (Supplementary Fig. 1). To investigate if FGF-2 over-expression could modify proliferation, cultures were exposed to the S-phase marker bromodeoxyuridine (BrdU) at DIV6. Proliferation significantly decreased in cultures deprived of FGF-2, but could be restored after FGF-2 transduction in a dose-dependent manner (Fig. 1C and D). Taken together, these experiments demonstrate that the FGF-2 transduction of NPCs increases the secretion of FGF-2 and maintains the proliferation of NPCs in the absence of exogenous FGF-2.

To study *in vitro* the effects of FGF-2 over-expression in NPCs undergoing differentiation, we applied a protocol that has been shown to induce neurogenesis (Zhang *et al*., 2003). After 6 days of differentiation, we observed that FGF-2-transduced cells had a strikingly different morphology compared to control cells: the vast majority of FGF-2-transduced cells displayed elongated processes strongly immunoreactive for nestin and a cell body faintly GFAP positive, whereas a high proportion of control cells displayed a much larger cell body strongly positive for GFAP and variable levels of nestin immunoreactivity (Fig. 1E, F and H). Furthermore, a significantly higher proportion of control cells expressed the immature neuronal marker doublecortin compared to FGF-2-transduced cells, indicating that FGF-2 transduction maintained the cells in an undifferentiated phenotype (Fig. 1H). Only a small fraction of cells expressed the oligodendrocyte precursor marker NG2 in both conditions (Fig. 1H). FGF-2 immunohistochemistry revealed a robust expression of FGF-2 in the cytoplasm and nucleus of almost all FGF-2-transduced NPCs, whereas FGF-2 immunoreactivity was located only in a fraction of control NPCs (Fig. 1G and H). These experiments indicate that *in vitro* FGF-2 over-expression

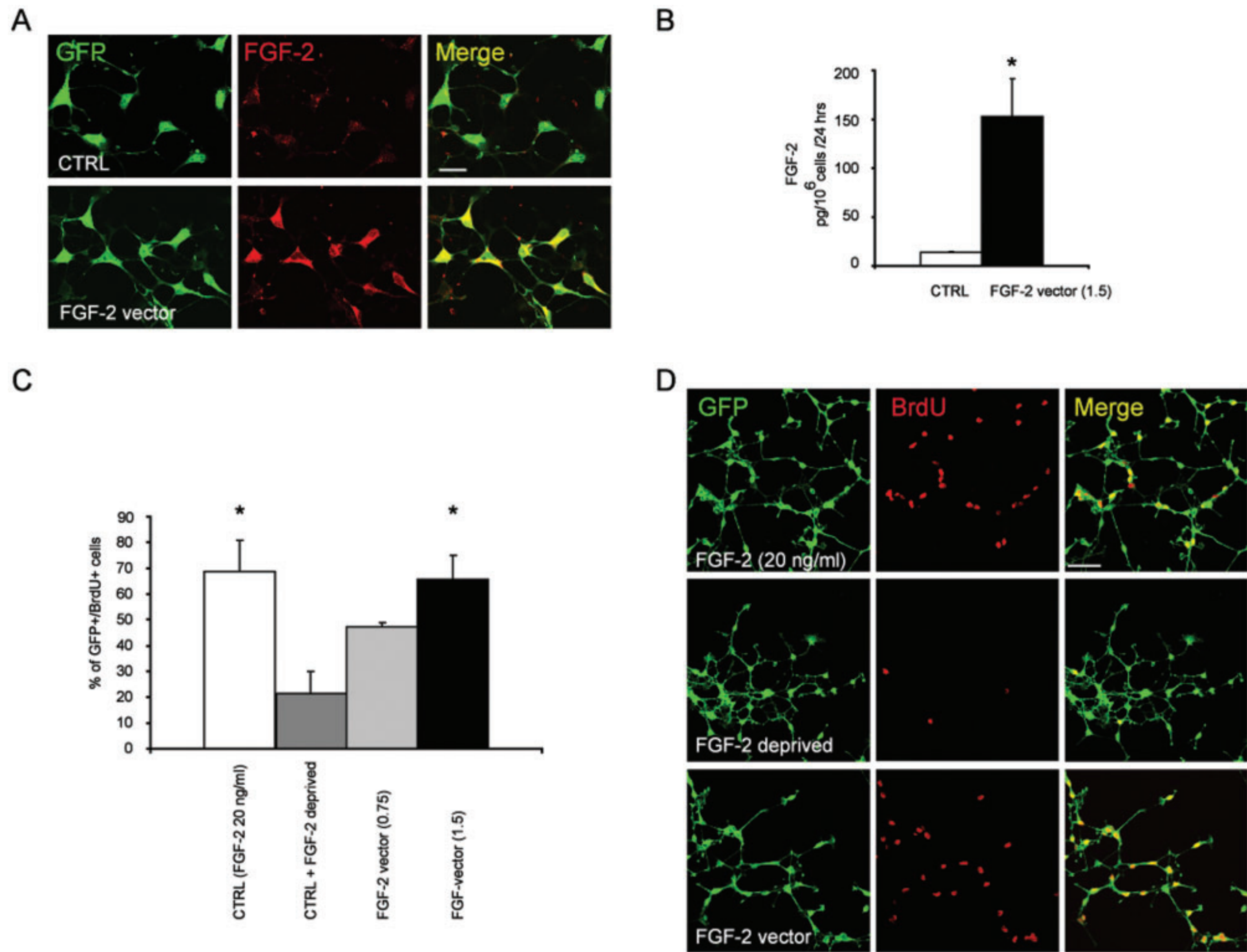


Fig. 1 FGF-2 transduction of neural progenitor cells (NPCs) maintains a pool of proliferative and immature cells *in vitro*. (**A**) Confocal images showing FGF-2-transduced NPCs displaying an intense FGF-2 cytoplasmic immunoreactivity compared to control NPCs. (**B**) Elisa quantification demonstrating a significant increase in the amount of secreted FGF-2 after FGF-2 transduction compared to control transduction (*$P<0.05$, *t*-test). (**C**) Dose-dependent increase in BrdU incorporation after FGF-2 transduction compared to control transduction. After transduction at DIV2, NPCs were either maintained in 20 ng/ml FGF-2 or deprived of exogenous FGF-2 (CTRL + FGF-2 deprived and FGF-2 vector). BrdU incorporation was performed at DIV6 (*$P<0.05$, One Way ANOVA). (**D**) Confocal images showing that in the presence of exogenous FGF-2 (20 ng/ml), a large fraction of control NPCs co-localize for BrdU. After 4 days of FGF-2 deprivation, few control NPCs co-localize for BrdU whereas FGF-2 transduction increases the amount of NPCs co-localizing for BrdU. (**E** and **F**) Confocal image showing that 6 days after induction of differentiation, a large fraction of control NPCs have differentiated into strongly GFAP positive astrocytes displaying variable levels of nestin immunoreactivity (left panel). In contrast, FGF-2-transduced NPCs display low levels of GFAP immunoreactivity and strong nestin immunoreactivity (right panel). (**G**) Confocal image showing that after induction of differentiation a fraction of cells do not display FGF-2 immunoreactivity (arrow-head) whereas FGF-2-transduced NPCs are strongly immunoreactive for FGF-2. (**H**) After 6 days of differentiation, the percentage of doublecortin positive neurons and strongly GFAP positive astrocytes increase in the control condition whereas the vast majority of FGF-2-transduced NPCs remain immature and express FGF-2 immunoreactivity (*$P<0.05$, *t*-test). CTRL = control GFP-vector transduction at a dose of 1.5×10^5 TU/ml, FGF-2 vector (0.75) = FGF-2-GFP vector transduction at a dose of 0.75×10^5 TU/ml, FGF vector (1.5) = FGF-2-GFP vector transduction at a dose of 1.5×10^5 TU/ml. Scale bar = 20 μm.

can efficiently maintain NPCs in an immature state and prevent them from undergoing differentiation.

FGF-2 over-expression promotes the proliferation and migration of transplanted NPCs on brain slices

To explore the behaviour of control and FGF-2-transduced NPCs in a complex environment such as brain tissue, we deposited small islets of NPCs on the surface of sub-acute cortical slices (Fig. 2A). Twenty-four hours after transplantation, FGF-2 transduction significantly increased the size of transplanted islets compared to controls (Fig. 2D). A combination of both proliferation and migration could explain this increase. To investigate these possibilities, BrdU pulse labelling was performed and revealed a significantly higher proliferation rate in FGF-2-transduced NPCs compared to control cells (Fig. 2B and

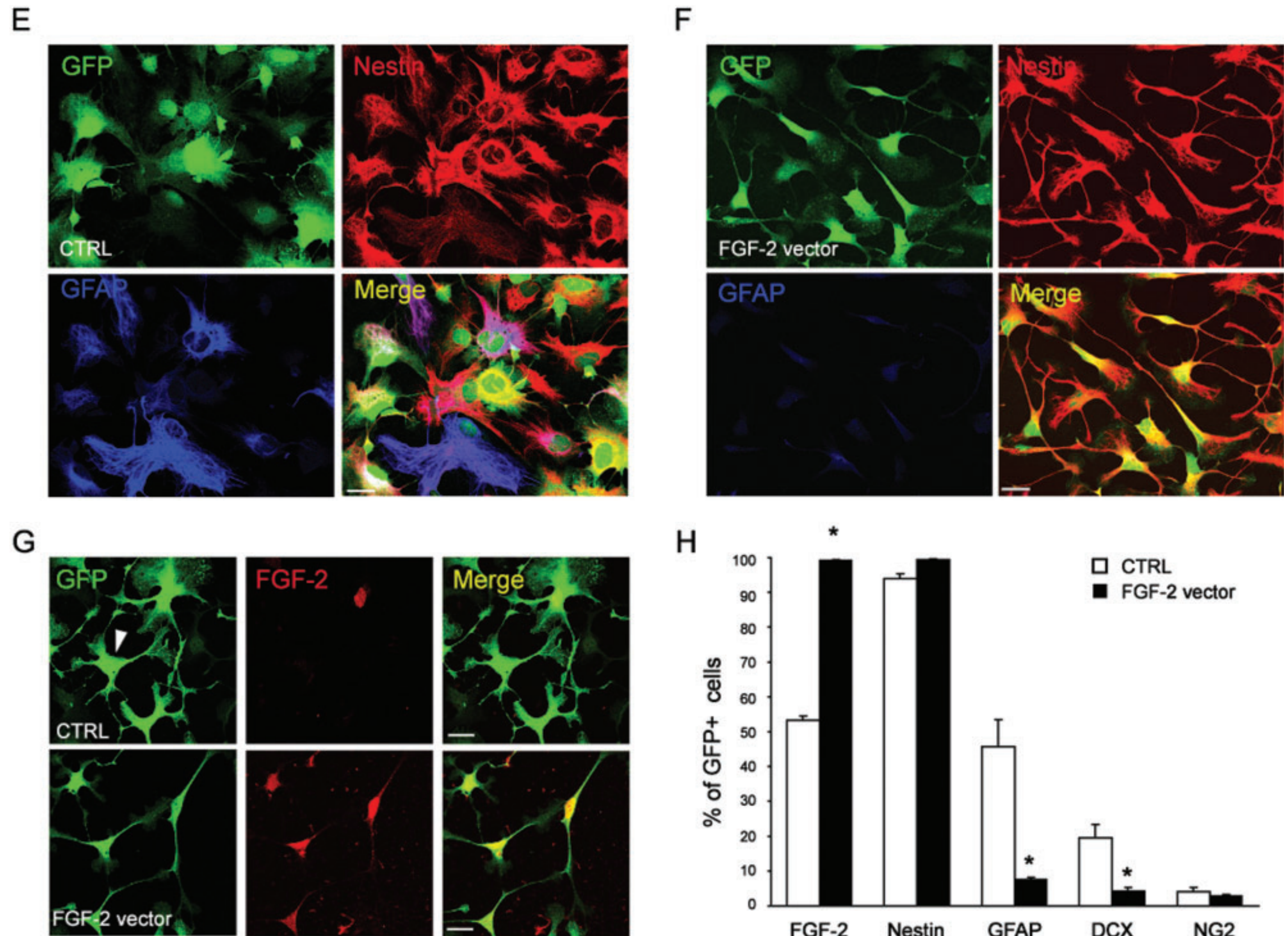


Fig. 1 Continued.

E). Furthermore, live-imaging of transplanted cells revealed that FGF-2 transduction induced a significant shift in the migration velocity (distance traveled per hour) of FGF-2-transduced NPCs compared to controls (Fig. 2F).

To explore whether this pool of FGF-2-transduced NPCs displayed enhanced invasive capacities, cortical slices were fixed at DIV2 and analysed with a confocal microscope (Fig. 3). Z-sectioning revealed a significant shift in the depth migration between the two populations indicating that FGF-2 transduction enables NPCs to invade more efficiently cortical slices. These results indicate that FGF-2 lentiviral transduction not only maintains a pool of immature proliferative NPCs but also promotes migratory properties and enhances the invasion of cortical tissue.

Transplantation of FGF-2-transduced NPCs *in vivo*

To further assess the effects of FGF-2 over-expression, NPCs were transplanted in the cortex of postnatal day 3 (P3) pups. Two days later, animals were sacrificed and cortical slices were prepared. Comparison of images taken at DIV0 and DIV2 in both conditions revealed that FGF-2-transduced NPCs could efficiently disperse in the surrounding cortical tissue whereas the majority of control NPCs remained near the locus of transplantation (Fig. 4A). Time-lapse recordings done over a period of 12 h revealed that control cells displayed limited migratory capacities. (Fig. 4B, F1, Supplementary movie 1). In contrast, the majority of FGF-2-transduced NPCs displayed a robust migratory activity over the 12 h of time-lapse recordings. (Fig. 4C, F2, Supplementary movie 2). Single cell tracking analysis revealed that NPCs adopted a migratory strategy characterized by the dynamic extension and retraction of several processes allowing cells to switch from a bipolar morphology with a leading process exhibiting lamellipodia activity to a transient multipolar morphology (Fig. 4C, Supplementary movie 2). Time-lapse recordings at DIV1 revealed a significant shift in the migration velocity of control versus FGF-2-transduced NPCs (Fig. 4E). Furthermore, we observed that a small fraction (1.66%) of FGF-2-transduced cells divided en route after an initial phase of migration, whereas this phenomenon was less frequent (0.33%) in the control situation (Fig. 4C).

To explore the long-term effects of FGF-2 over-expression on the dispersion of grafted NPCs, we

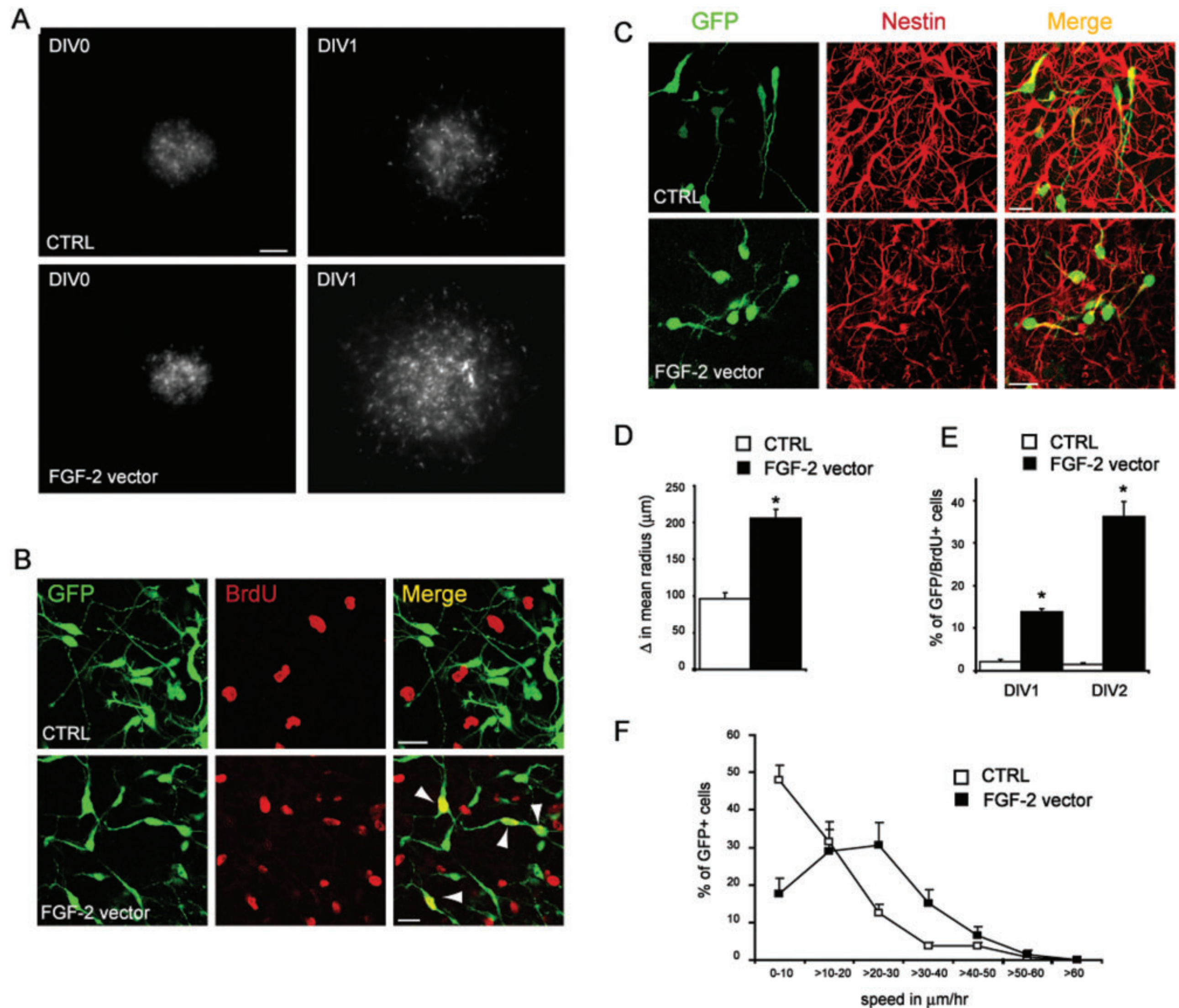


Fig. 2 FGF-2 transduction maintains a pool of proliferative and migrating NPCs after transplantation on cortical slices. (**A**) Epifluorescence images showing a larger increase in the size of a FGF-2-transduced GFP positive islet between DIV0 and DIV1 compared to a control islet. (**B**) Confocal images showing that at DIV1 the proportion of GFP + cells co-localizing for BrdU (arrow-heads) is higher after FGF-2 transduction compared to the control condition. (**C**) Confocal images showing that at DIV2 the majority of control and FGF-2-transduced NPCs express nestin, a marker of immature neural progenitors. (**D**) Graph showing that between DIV0 and DIV1, the mean radius difference of FGF-2-transduced islets is increased compared to controls (*$P<0.001$, *t*-test). (**E**) Graph showing that the BrdU proliferation rate of FGF-2-transduced NPCs is increased at DIV1 and DIV2 compared to controls (*$P<0.001$, Mann-Whitney). (**F**) Graph showing a shift in the migration speed of FGF-2-transduced NPCs compared to controls ($P<0.001$, Chi-square test). CTRL = control GFP-vector transduction, FGF vector = FGF-2-GFP vector transduction. Scale bar = 100 µm in **A** and 20 µm in **B** and **C**.

transplanted control versus FGF-2-transduced NPCs in the cortex of rat pups and analysed their brains at several survival time points. Two weeks after transplantation, FGF-2-transduced cells were found at increasing distances from the center of the cortical transplantation site compared to control cells (Fig. 5A and B). To know if the local secretion of FGF-2 by FGF-2-transduced NPCs could be sufficient to increase the dispersion of co-transplanted control NPCs, we transplanted a mixture of the same amount of control tomato-labelled NPCs and FGF-2-GFP-transduced NPCs in the cortex of neonatal rats. Analysis of brains 8 days after transplantation revealed that a significant proportion of GFP-FGF-2-transduced cells had dispersed in the surrounding cortex, whereas co-transplanted tomato-labelled cells mainly remained at the locus of transplantation, indicating that FGF-2-transduction did not significantly modify the behaviour of co-transplanted control NPCs (Fig. 5C and D).

To investigate whether the increased dispersion of FGF-2-transduced NPCs was linked to a change in their proliferation index, BrdU labelling was performed between P6 and P9 and animals were sacrificed at P10. Interestingly,

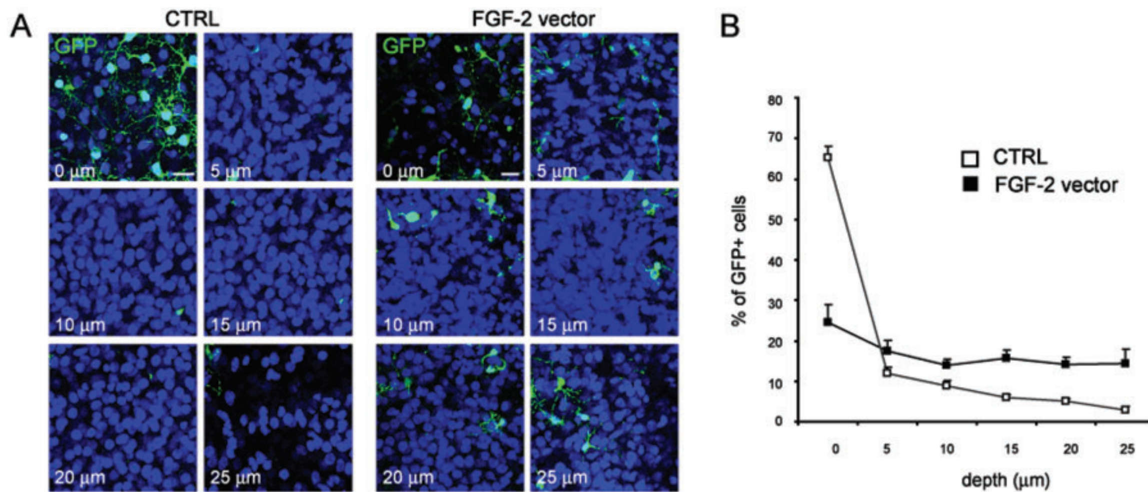


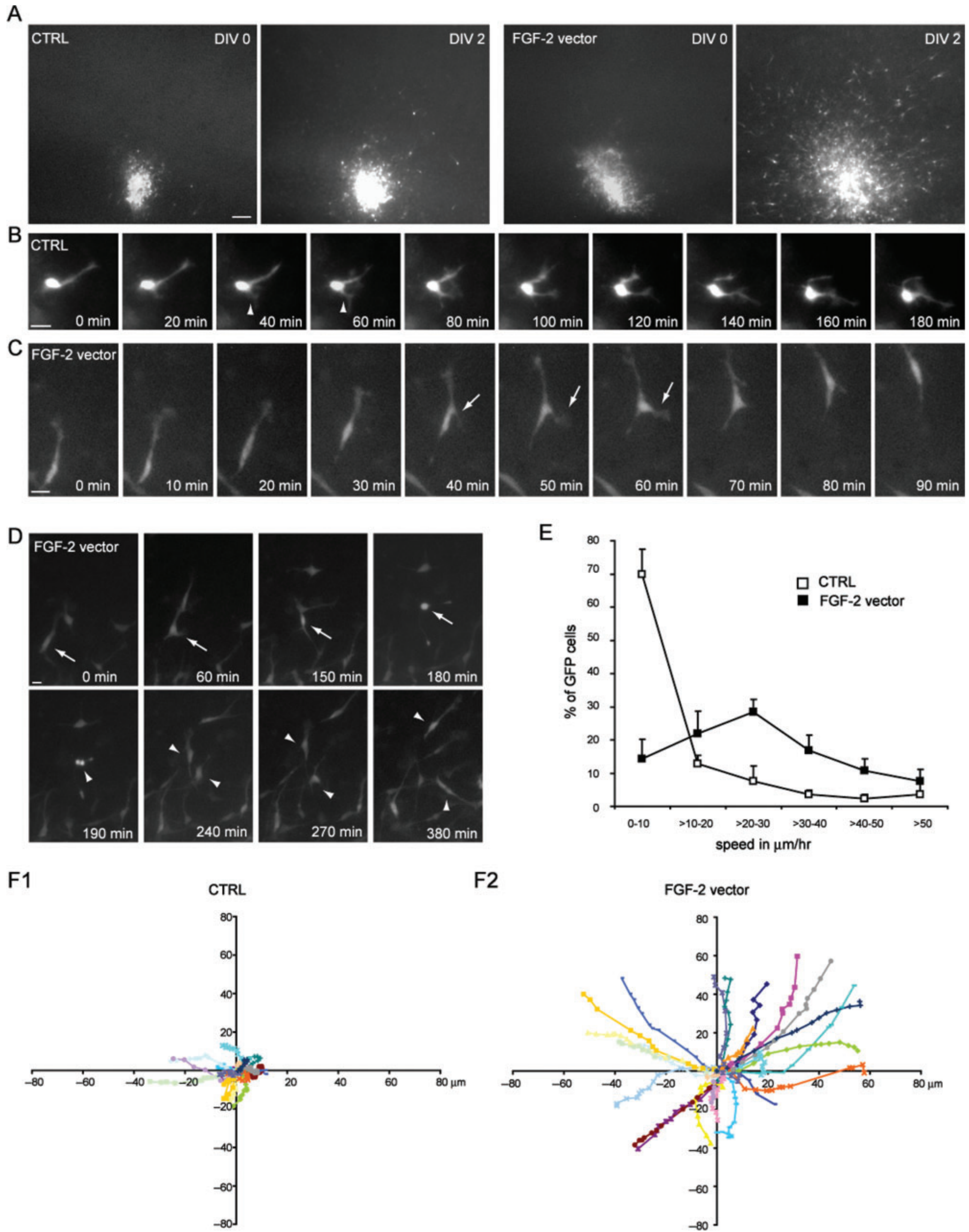
Fig. 3 FGF-2 transduction increases the invasiveness of NPCs in cortical slices.(**A**) Confocal Z-stacks at 5 µm interval showing that FGF-2-transduced NPCs invade the whole thickness of the slice, whereas control NPCs are mainly located at the surface of the slice. Nuclear counterstain To-Pro-3 (blue). (**B**) The majority of control NPCs remains at the surface of the cortical slice whereas FGF-2-transduced NPCs invade the whole thickness of the slice ($P < 0.001$, Chi-square test). CTRL = control GFP-vector transduction, FGF vector = FGF-2-GFP vector transduction. Scale bar = 20 µm.

at this time point a significantly higher proportion of FGF-2-transduced NPCs had incorporated BrdU compared to controls (Fig. 5E and G). To explore if the phenotype of transplanted NPCs was modified by FGF-2 transduction, nestin staining was performed 2 weeks after transplantation and revealed that a significant proportion of FGF-2-transduced cells remained nestin positive whereas very few control cells expressed this immature neural marker (Fig. 5F and G). In contrast, staining for NG2, a marker for oligodendrocyte precursors but also endothelial cells, revealed that the majority of control cells expressed NG2 compared to only a small amount of FGF-2-transduced cells. Staining for neuronal markers such as doublecortin and NeuN revealed that only a very limited number of control or FGF-2-transduced NPCs (<0.5%) differentiated into immature or mature neurons after transplantation in the normal cortex. These results indicate that FGF-2-transduction of NPCs increases their cortical invasiveness *in vivo* and that this phenomenon is associated with the preservation of an immature and proliferative state.

Constitutive FGF-2 over-expression in NPCs raises the concern of potential tumour formation. To evaluate the fate of FGF-2-transduced NPCs at longer survival time points, we sacrificed rats 40 days after transplantation in the intact cortex. At this time point, grafted FGF-2-transduced NPCs were still more dispersed than control cells and no tumour formation was detected. Immunohistochemistry quantification revealed that the majority of GFP positive cells had differentiated into NG2 positive cells (73.3 ± 3.4% mean ± SEM) or GFAP positive astroctyes (5.1 ± 1.0% mean ± SEM). Only a very small fraction of grafted NPCs maintained an immature nestin phenotype (0.7 ± 0.3% mean ± SEM). To confirm that grafted cells had lost their proliferative properties, BrdU injections were performed three days before sacrifice. Only 2.7 ± 0.8% (mean ± SEM) of FGF-2-transduced cells still incorporated BrdU.

To investigate if the transient proliferative and migratory properties of FGF-2-transduced cells could be correlated with a change in the levels of FGF-2 production, we tracked FGF-2 protein expression at different survival time points after transplantation. We observed that at the early survival time points during which FGF-2-transduced NPCs displayed proliferative and migratory properties, the majority of FGF-2-transduced NPCs were strongly immunoreactive for FGF-2, whereas at longer survival time points this

Fig. 4 FGF-2 transduction increases the dispersion and migration speed of NPCs in cortical slices after *in vivo* transplantation. (**A**) Epifluorescence images of sagittal slices cut at P5 after *in vivo* transplantation at P3 showing that between DIV0 and DIV2, the dispersion of FGF-2-transduced NPCs in the cortex is increased compared to controls. (**B**) Time-lapse sequence showing a control NPC actively extending (arrow-head) and retracting processes while the soma remains stationary. (**C**) Time-lapse sequence showing a FGF-2-transduced NPC migrating while switching from a bipolar to a multipolar morphology. Note the appearance of a transient lateral process (arrow). (**D**) Time-lapse sequence showing the migration and division of a FGF-2-transduced NPC. The migrating cell (arrows) divides into two daughter cells (arrow-heads) that continue to migrate. (**E**) Graph showing a shift in the migration speed of FGF-2-transduced NPCs compared to controls ($P < 0.001$, Chi-square test). (**F**) Representative migration tracks of 25 control NPCs compared to 25 FGF-2-transduced NPCs during a time-lapse sequence of 130 min. Only a small fraction of control NPCs migrate distances higher than 20 µm in contrast to the majority of FGF-2-transduced NPCs. The starting point for each cell is the interaction between the X and Y axes (0,0). CTRL = control GFP-vector transduction, FGF vector = FGF-2-GFP vector transduction. Scale bar = 100 µm in **A**, 20 µm in **B**–**D**.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CTRL
DIV 0
DIV 2
FGF-2 vector
DIV 0
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B
CTRL
0 min
20 min
40 min
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80 min
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160 min
180 min
C
FGF-2 vector
0 min
10 min
20 min
30 min
40 min
50 min
60 min
70 min
80 min
90 min
D
FGF-2 vector
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60 min
150 min
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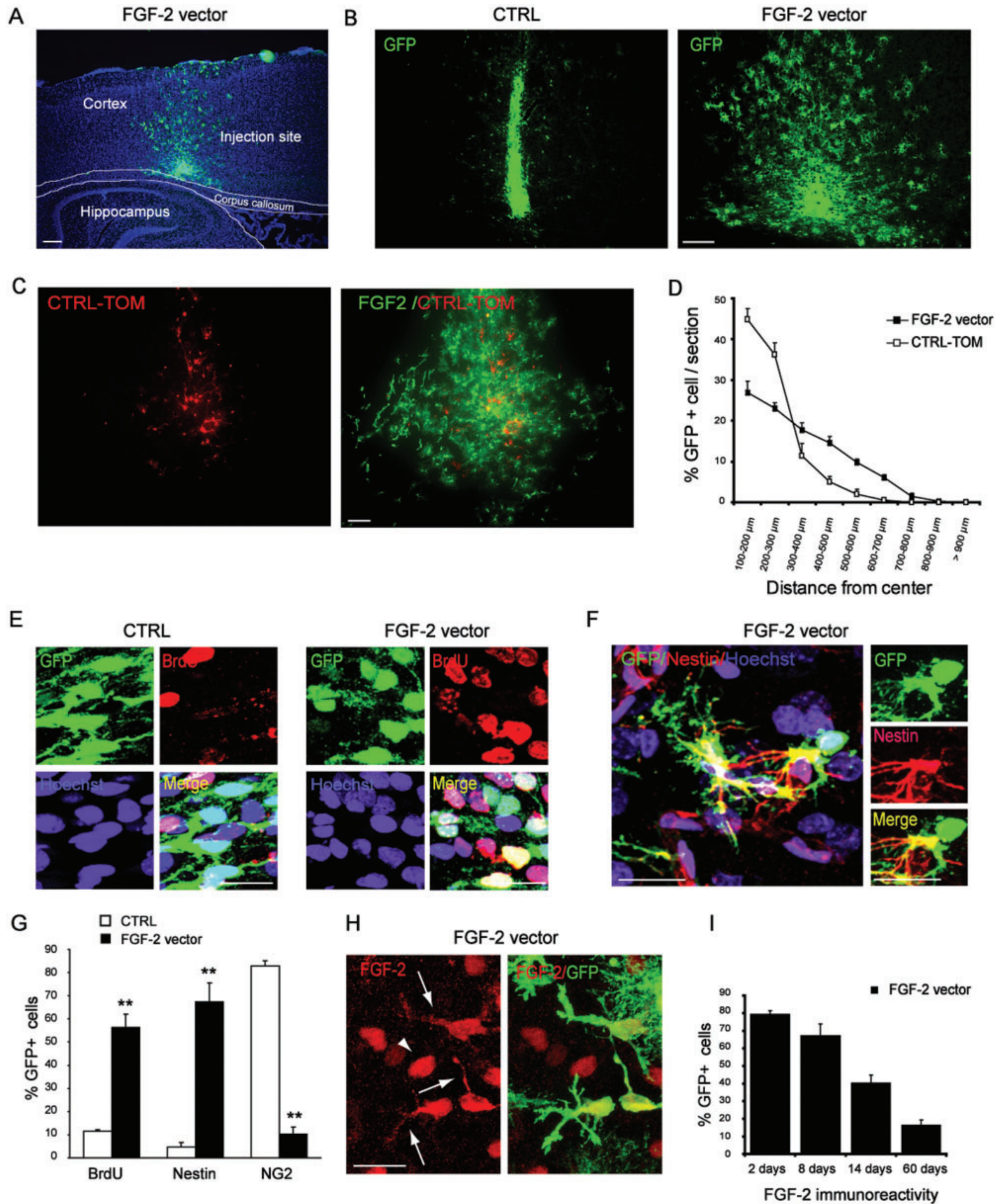


Fig. 5 FGF-2 transduction increases the dispersion of NPCs and maintains their proliferative immature phenotype after *in vivo* transplantation. (**A**) Epifluorescent image showing the site of transplantation in the cortex (left). (**B**) Dispersion of grafted control NPCs (middle) and FGF-2-transduced NPCs (right) 2 weeks after transplantation. (**C**) Epifluorescent images showing the increased dispersion of grafted FGF-2-transduced NPCs compared to control tomato-labelled NPCs 8 days after transplantation. (**D**) Increased dispersion of FGF-2-transduced NPCs compared to tomato-labelled NPCs 8 days after transplantation ($P < 0.001$, Chi-square test.) (**E**) Confocal images showing a higher fraction of BrdU positive FGF-2-transduced NPCs (right) compared to control NPCs (left) 1 week after transplantation. (**F**) FGF-2-transduced NPCs expressing the immature neural marker nestin 2 weeks after transplantation. (**G**) One week after

percentage progressively decreased (Fig. 5I). Taken together, these results indicate that the proliferative and migratory properties of FGF-2-transduced NPCs as well as their immature phenotype is transient and that the loss of these properties is strongly correlated with a gradual down-regulation of FGF-2 production.

FGF-2 transduction increases the pool of grafted olfactory bulb neurons

To test the behaviour of FGF-2-transduced NPCs in a physiological neurogenic system, we transplanted tomato-labelled control NPCs and FGF-2-transduced NPCs in the P0 anterior subventricular zone (SVZ). Brains analysed at different time points after transplantation revealed that both FGF-2-transduced NPCs and control NPCs were found in the SVZ, along the rostral migratory stream and in the olfactory bulb where they had differentiated into immature neurons expressing doublecortin (Fig. 6A1,A2 and C). Six weeks after transplantation, numerous GFP positive neurons with well-developed processes could be observed in the olfactory bulb. These cells extended typical dendrites, expressed the more mature neuronal marker NeuN and displayed the characteristic morphology of granular interneurons (Fig. 6D). Quantification of the ratio of GFP versus tomato-labelled neurons at 4 weeks in the olfactory bulb revealed that neurons derived from FGF-2-transduced NPCs were 4 times more abundant than control neurons indicating that FGF-2 transduction could efficiently increase the amount of transplanted neurons in the olfactory bulb (Fig. 6B). To further confirm that the loss of an immature phenotype strongly correlates with a gradual down-regulation of the levels of FGF-2, we quantified the amount of GFP positive cells immunoreactive for FGF-2 in the rostral migratory stream and in the olfactory bulb 1 month after transplantation. We found that only a very small fraction of FGF-2-transduced neuroblasts in the rostral migratory stream ($1.8 \pm 0.6\%$ mean $\pm$ SEM) and FGF-2-transduced neurons in the olfactory bulb ($4.4 \pm 0.7\%$ mean $\pm$ SEM) continued to express FGF-2, indicating that the loss of an immature phenotype is strongly correlated with FGF-2 down-regulation.

Transplantation of FGF-2-transduced NPCs after neonatal ischaemia

To study the behaviour of FGF-2-transduced NPCs in an ischaemic environment, we used an animal model of neonatal ischaemia. In this model, rats at postnatal day 3 undergo right carotid artery coagulation followed by 6% hypoxia for 30 min. This moderate hypoxic-ischaemic injury leads to selective neuronal loss in the infragranular layers of the somatosensory cortex (Sizonenko *et al.*, 2005). After grafting NPCs at the base of the cortex, close to the ischaemic sites (Fig. 7A), the migration pattern of labelled cells was determined in fixed tissues at different intervals. Two weeks after transplantation, we observed that both control and FGF-2-transduced NPCs, had survived, migrated out of the injection site and dispersed in a region that included the infragranular ischaemic cortex and the margin between the cortex and the corpus callosum (Fig. 7A). When comparing the two groups, we observed that FGF-2 transduction significantly increased the number of FGF-2-transduced cells located in the ischaemic regions compared to control cells. Quantification of the ratio of GFP versus tomato-labelled cells at 2 weeks in the ischaemic cortex revealed that FGF-2-transduced NPCs were 2.4 times more abundant than control NPCs indicating that FGF-2 transduction could efficiently increase the pool of grafted NPCs in the ischaemic cortex (Fig. 7I). At this survival time point, only $31.0 \pm 4.4\%$ (mean $\pm$ SEM) of FGF-2-transduced NPCs still expressed FGF-2, indicating that FGF-2 down-regulation also occurred in an ischaemic environment and was correlated with neural differentiation. The differentiation process was similar in both the control condition and after FGF-2 transduction (Fig. 7J). A large fraction of grafted cells remained nestin positive (Fig. 6D), while a proportion of them were found expressing the astrocytic marker GFAP or the oligodendrocytic marker NG2 (Fig. 6J). Most importantly ~50% of grafted cells expressed the immature neuronal marker doublecortin (DCX) (Fig. 7B,C and J). Quantification revealed that the number of grafted immature doublecortin positive neurons was significantly increased after FGF-2 transduction (447.1 ± 96.6 (mean $\pm$ SEM) cells/mm^2) compared to the control condition (135.3 ± 19.4 (mean $\pm$ SEM) cells/mm^2) ($P < 0.05$, *t*-test), indicating that the ischaemic environment could promote neurogenesis, in contrast to the non-ischaemic cortex where very few neurons were observed. In the ischaemic lesion, grafted cells could differentiate into neurons expressing the γ-aminobutyric acid (GABA) synthesizing enzyme glutamic acid decarboxylase 67 (GAD67) (Fig. 7F) and a later survival time points the calcium-binding protein calretinin (Fig. 7G). No FGF-2-transduced cells were immunoreactive for calbindin or parvalbumin. Some FGF-2-transduced cells had acquired a more complex neuronal morphology and expressed the neuronal marker NeuN (Fig. 7H). These results indicate

transplantation the percentage of BrdU positive NPCs is increased after FGF-2-transduction compared to controls. Two weeks after transplantation, the majority of FGF-2-transduced NPCs remain nestin positive compared to controls, whereas the majority of control NPCs differentiate into NG2 positive cells. ($^{**}P < 0.005$, *t*-test). (**H**) Confocal images showing cytoplasmic and nuclear FGF-2 immunoreactivity in FGF-2-transduced NPCs (arrows) and nuclear FGF-2 immunoreactivity in resident cells (arrow-head). (**I**) Graph showing a time-dependent FGF-2 down-regulation in grafted FGF-2-transduced NPCs. CTRL = control GFP-vector transduction, CTRL-TOM = control tomato-vector transduction, FGF vector = FGF-2-GFP vector transduction. Scale bar = 200 μm for epifluorescence images and 20 μm for confocal images.

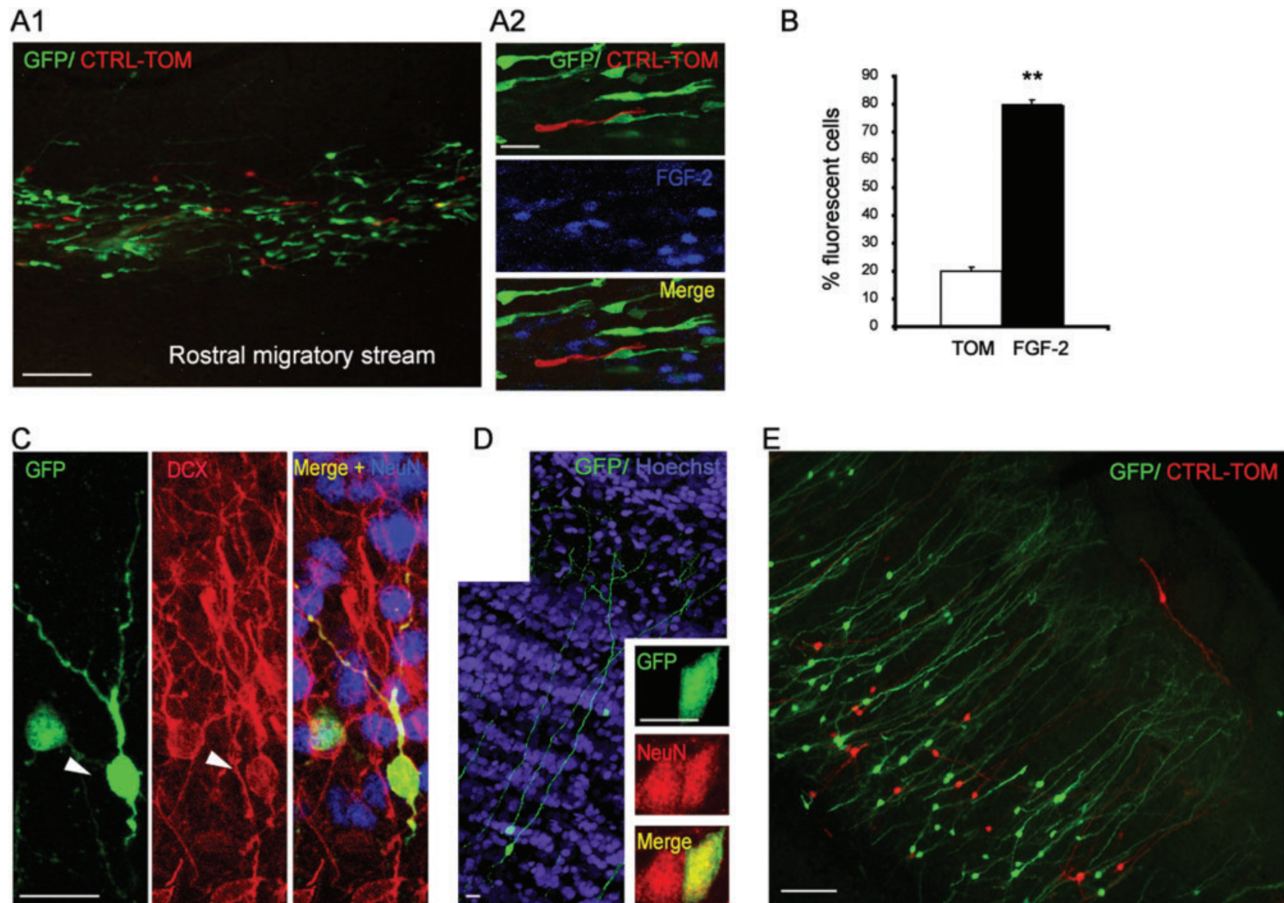


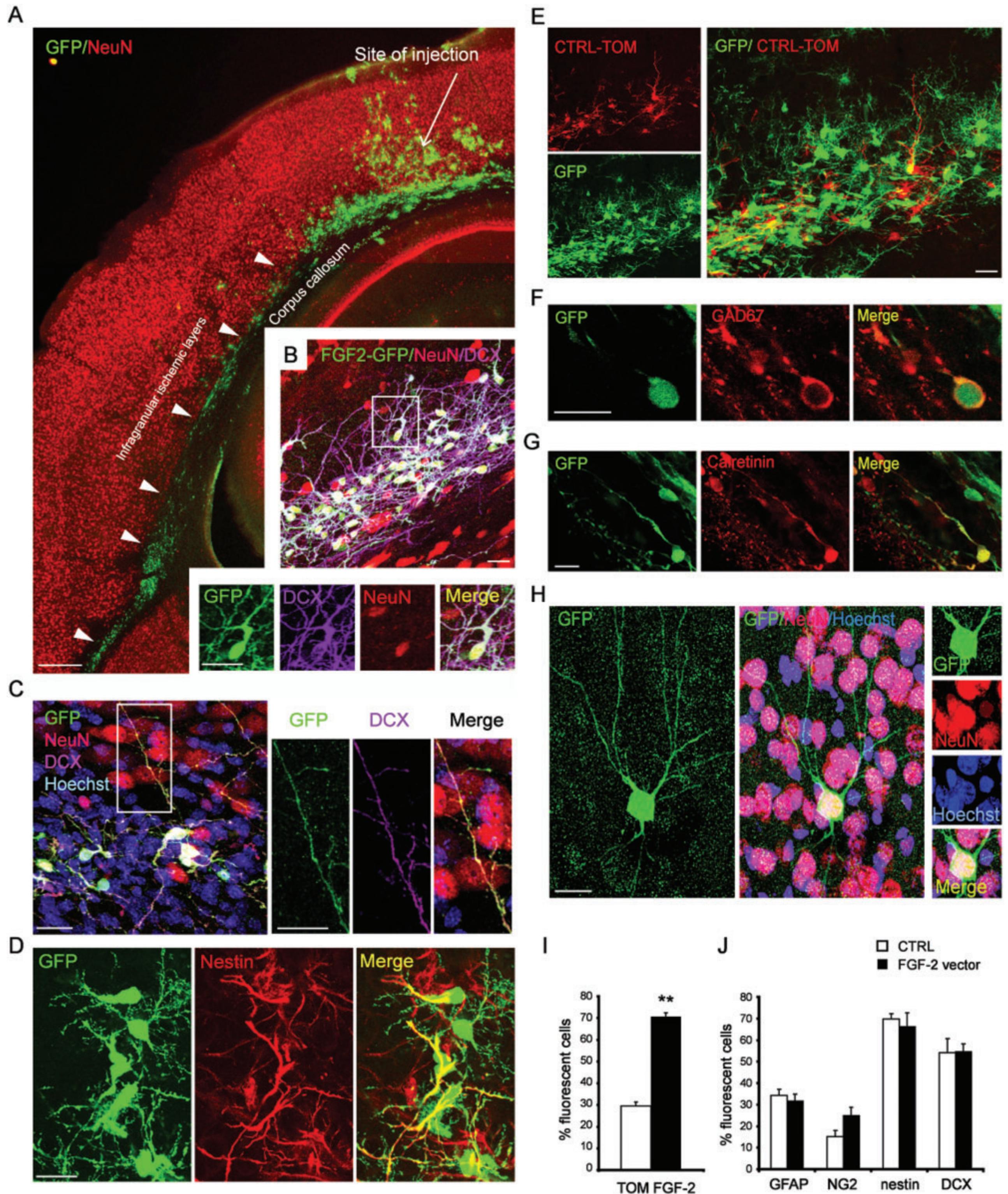
Fig. 6 FGF-2-transduction increases the pool of grafted neurons in the olfactory bulb. (A1) Epifluorescent image of a sagittal section showing FGF-2-transduced NPCs and tomato-labelled NPCs migrating along the rostral migratory stream 1 month after transplantation in the SVZ. (**A2**) Confocal images showing that both control and FGF-2-transduced neuroblasts do not express FGF-2. (**B**) The proportion of GFP-FGF-2-transduced neurons is significantly increased compared to control tomato-labelled neurons in the olfactory bulb 1 month after transplantation ($^{**}P < 0.001$, *t*-test). (**C**) FGF-2-transduced NPC (arrow-head) which has reached the granule cell layer of the olfactory bulb and is positive for DCX 1 week after transplantation. (**D**) FGF-2-transduced neuron extending a dendritic arbourization in the olfactory bulb 6 weeks after transplantation. Insert showing that the cell is immunoreactive for NeuN. (**E**) Epifluorescent image at low magnification showing a higher proportion of FGF-2-transduced neurons compared to tomato-labelled neurons in the olfactory bulb 1 month after transplantation. CTRL-TOM = control tomato-vector transduction, GFP = FGF-2-GFP vector transduction, Hst = Hoechst. Scale bar = 100 μm for epifluorescence images and 20 μm for confocal images.

that an ischaemic environment can dramatically change the phenotypic fate of both control and FGF-2-transduced NPCs and that FGF-2 over-expression increases the pool of NPCs available for brain repair.

Discussion

The identification of molecular signals that could enhance the capacity of grafted NPCs to invade an injured area in the brain is of key importance for neural cell replacement

Fig. 7 FGF-2 transduction increases the pool of grafted neural progenitors in the ischaemic cortex. (**A**) Epifluorescent composite images of a coronal section showing an ischaemic cortex 1 month after transplantation of FGF-2-transduced NPCs. Note the injection site (arrow) and the large dispersion of FGF-2-transduced NPCs at the margin between the corpus callosum and the ischaemic infragranular layers (arrow-heads). (**B**) Confocal images showing a pool of FGF-2-transduced NPCs positive for the immature neuronal marker doublecortin (DCX). Insert showing an FGF-2-transduced neuron positive for NeuN and DCX. (**C**) FGF-2-transduced cell extending a DCX immunoreactive process towards the ischaemic cortex. (**D**) FGF-2-transduced NPCs expressing nestin. (**E**) FGF-2-transduced NPCs form a larger pool compared to co-transplanted tomato-labelled NPCs 8 days after transplantation. (**F**) FGF-2-transduced NPC expressing the GABA synthesizing enzyme GAD67 2 weeks after transplantation (**G**) FGF-2-transduced NPC expressing the calcium-binding protein calretinin 6 weeks after transplantation. (**H**) FGF-2-transduced cell expressing NeuN and displaying a more mature neuronal morphology 4 weeks after transplantation. (**I**) The proportion of FGF-2-transduced NPCs is significantly increased compared to tomato-labelled NPCs 2 weeks after transplantation ($^{**}P < 0.001$, *t*-test). (**J**) Both control and FGF-2-transduced NPCs differentiate into GFAP positive cells, NG2 positive cells and immature neurons positive for doublecortin. A large proportion of grafted NPCs remain nestin positive. CTRL-TOM = control tomato-vector transduction, GFP = FGF-2-GFP vector transduction. Scale bar = 200 μm for epifluorescence images and 20 μm for confocal images.


A
GFP/NeuN
Site of injection
Corpus callosum
Infragranular ischemic layers
B
FGF2-GFP/NeuN/DCX
GFP
DCX
NeuN
Merge
C
GFP
NeuN
DCX
Hoechst
GFP
DCX
Merge
D
GFP
Nestin
Merge
E
CTRL-TOM
GFP/ CTRL-TOM
GFP
F
GFP
GAD67
Merge
G
GFP
Calretinin
Merge
H
GFP
GFP/NeuN/Hoechst
GFP
NeuN
Hoechst
Merge
I
% fluorescent cells
**
TOM FGF-2
J
CTRL
FGF-2 vector
% fluorescent cells
GFAP
NG2
nestin
DCX

and structural repair. Here we developed a novel system allowing the over-expression of FGF-2 in NPCs and studied its effects on several cellular functions after transplantation into the early postnatal rat cortex. We provide direct evidence for the first time that FGF-2 over-expression not only enhances the proliferative activity, as has been shown previously, but that it also dramatically enhances the migratory properties of grafted NPCs. When transplanted into a neurogenic region such as the SVZ, FGF-2-expressing NPCs gave rise to a significantly increased pool of new interneurons in the olfactory bulb, without altering their differentiation potential. A striking observation of the present study is that differentiation of progenitor cells into immature neurons was systematically correlated with a down-regulation of the FGF-2 transgene. Finally and most importantly, we show that after transplantation into a neonatal ischaemic cortex, FGF-2 over-expressing NPCs efficiently invade the injured cortex and generate an increased pool of immature neurons available for brain repair. These results reveal an important role for FGF-2 in regulating NPCs functions when interacting with the host tissue and offer a potential strategy to generate a robust source of migrating neural progenitors for repairing a neonatal ischaemic cortex.

Using a lentiviral gene transfer system, we successfully over-expressed FGF-2 in NPCs and showed that this technology could be a reliable and non-toxic tool to genetically engineer primary cultures of NPCs. FACS analysis allowed us to estimate the number of copies that transduced NPCs had incorporated in their genome. According to our calculations, using lentiviral vector doses of 1.5×10^5 TU/ml, 2–3 copies of the FGF-2 lentiviral vector were incorporated in the genome. With this range of copy number, FGF-2 transduction was able to produce a 10-fold increase in the amount of FGF-2 secreted in the culture medium. Our data suggest that *in vitro* this relatively low amount of secreted FGF-2 had a potent biological effect since it could maintain the proliferation of NPCs in culture at a level equivalent to that measured after the exogenous addition of FGF-2 at a dose of 20 ng/ml. Noteworthy that control NPCs secrete low but detectable amounts of FGF-2 into culture media hence confirming previous reports (Maric *et al*., 2003) and supporting the concept that autocrine/paracrine axes of FGF signalling might regulate biological properties of progenitor cells.

Previous studies demonstrated that FGF-2 signalling is a potent regulator of mammalian neurogenesis (Ghosh and Greenberg, 1995; Temple and Qian, 1995; Palmer *et al*., 1999; Ford-Perriss *et al*., 2001). FGF-2 appears to be a major determinant in maintaining proliferative and undifferentiated populations of NPCs *in vitro* (Maric *et al*., 2003) and in neurogenic zones such as the SVZ *in vivo* (Zheng *et al*., 2004). It has also been reported to be critical in the reprogramming of primordial germ cells into pluripotent stem cells (Durcova-Hills *et al*., 2006) and in maintaining pluripotency in human embryonic stem cells (Amit *et al*., 2000). However, its role in regulating dynamic interactions of grafted NPCs with the recipient host tissue was unknown. This is a critical issue since NPCs rapidly differentiate after grafting into postnatal brain tissue. Using a lentiviral-based approach, we demonstrated that FGF-2 over-expression in grafted NPCs was sufficient to maintain their immature phenotype as well as their proliferative and migratory properties in a tissue context. In striking contrast, grafted control cells rapidly lost their migratory and proliferative properties as well as their immature phenotype while differentiating into glial cells. Co-transplantation experiments with tomato-labelled control NPCs mixed with GFP-labelled FGF-2 over-expressing NPCs, revealed that the presence of FGF-2-transduced cells was not sufficient to confer increased migratory properties to control cells. Thus, cross-talk through paracrine signalling of FGF-2 between NPCs may not be sufficient to maintain an undifferentiated and migratory phenotype and we speculate that an autocrine signalling loop of FGF-2 might underlie the observed biological effects of FGF-2 transduction. The expression of FGF receptor 1 and FGF receptor 2 at the mRNA and protein level in FGF-2-transduced NPCs further support the hypothesis that over-secretion of FGF-2 in the extracellular compartment could maintain the biological properties of FGF-2-transduced NPCs by signalling through specific FGF-2 receptors. In addition to this mechanism, it remains possible that the secreted 18 kDa FGF-2 isoform after binding to its receptors could be internalized and reach the nucleus where it could regulate cellular processes in an intracrine fashion (Sorensen *et al*., 2006).

We systematically observed that the migratory and proliferative properties of FGF-2-transduced NPCs were transient and were lost at longer survival time points. We never detected tumour formation and the dispersion of grafted cells was limited to a few hundred micrometres from the injection site. Cell fusion events have been reported after transplantation of stem cells in various organs. However, several arguments indicate that in our system grafted NPCs do not appear to fuse to resident cells. Using time-lapse imaging, we were able to directly monitor the behaviour of GFP-labelled NPCs after engraftment in various brain regions. No cell fusion events were detected in time-lapse movies monitoring the migration of NPCs in the cortex, in the rostral migratory stream and in the olfactory bulb. Furthermore, no evidence for multinucleated cells was detected after confocal imaging of hundreds of grafted NPCs in various brain regions. Although we cannot totally exclude the possibility that rare cell fusion events could occur after transplantation, these rare events would not modify the results obtained in this study.

By tracking the level of FGF-2 expression in grafted cells at different survival time points and in different transplantation sites, we found that the loss of the proliferative and migratory properties of FGF-2 over-expressing NPCs was strongly correlated with a spontaneous and gradual

down-regulation of FGF-2 production. Furthermore, we found that FGF-2 down-regulation was also strongly correlated with the appearance of grafted cells capable of differentiating into a wide range of glial and neuronal cells displaying normal morphologies and expressing standard markers of differentiation. Several mechanisms could account for the observed FGF-2 down-regulation, such as decreased transcription levels, increased mRNA instability, decreased mRNA translation and increased protein degradation. Interestingly, the majority of FGF-2-transduced NPCs grafted in the anterior SVZ continued to express FGF-2 1 month after transplantation (unpublished data), suggesting that *in vivo* FGF-2 down-regulation may occur in a region-dependent fashion.

The combination of confocal video time-lapse microscopy and cortical slice preparations allowed us to directly observe the migration of FGF-2-transduced NPCs in a 3D tissue context. Imaging of FGF-2-transduced NPCs in cortical slices revealed an important pool of individually migrating NPCs. This pool of migrating cells was significantly reduced but not absent in the control situation, indicating that FGF-2 transduction increases the fraction of migrating cells but does not induce a mode of migration which is absent in the control situation. These observations are consistent with previous reports showing that FGF signalling stimulates the migration of astrocytes (Holland and Varmus, 1998; Sorensen *et al*., 2006), myoblasts (Allen *et al*., 2003), oligodendrocyte progenitors (Simpson and Armstrong, 1999) and germ cells (Takeuchi *et al*., 2005). Interestingly mitosis could be directly observed online after a phase of migration in a small fraction of FGF-2-transduced NPCs and more rarely with control NPCs. These time-lapse observations indicate that immature NPCs are able to migrate and divide in a complex 3D structure. *Post ho*c confocal Z-stacks taken throughout the cortical slice demonstrated that FGF-2-transduced NPCs had the ability to migrate inside the cortical tissue, whereas control NPCs mainly remained on the surface of the slice, further demonstrating the enhanced invasive migratory properties of FGF-2-transduced NPCs. The molecular mechanisms of this effect remain to be determined. One of the possibilities is that FGF-2 facilitates migration through stimulating the secretion of matrix degrading enzymes such as MMP 2 and 9 (Tsuboi *et al*., 1990; Kenagy *et al*., 1997).

Overall our results indicate that the over-expression of FGF-2 in NPCs prior to transplantation could be of considerable interest to generate a larger pool of proliferative and migrating neural progenitor cells available for brain repair (creation of a 'launch pad' *in situ*). Furthermore, we show that FGF-2-transduced NPCs can be recruited towards sites of brain injury where they generate dense clusters of immature neurons. The addition of a large pool of immature neurons in a damaged neonatal cortex represents a promising strategy to achieve efficient neuronal replacement. Additional studies are needed to determine if this strategy could lead to functional improvements in transplanted rats. Furthermore, the behaviour of FGF-2-transduced NPCs in an adult ischaemic cortex remains to be determined. Finally, it remains a long-term goal to understand the molecular mechanisms that regulate the survival and integration of immature neuronal precursors in a damaged cortex.

Supplementary material

Supplementary material is available at *Brain* online.

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