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UNIVERSITÉ DE GENÈVE

Département de Biologie et Zoologie Animale

FACULTÉ DES SCIENCES
Professeur Ivan Rodriguez

Département des Neurosciences Fondamentales

FACULTÉ DE MÉDECINE
Professeur Jozsef Z. Kiss

A neural stem/progenitor cell based approach for brain repair

THÈSE

présentée à la Faculté des sciences de l'Université de Genève,

pour obtenir le grade de Docteur ès sciences, mention biologie

par

Benoît Jenny

de

Fribourg

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RÉSUMÉ

Le cortex cérébral adulte a une capacité régénérative très limitée après ischémie cérébrale. L'une des raisons principales est le manque d'une quantité suffisante de cellules neurales progénitrices endogènes disponibles pour réparer le tissu cérébral endommagé. Pour compenser cette défaillance, nous avons évalué l'hypothèse que la transplantation de progéniteurs neuronaux directement dans le cortex cérébral d'un model d'hypoxie/ischémie cérébrale périnatal chez le rat, représente une approche intéressante à investiguer parmi les stratégies régénératives du cortex cérébral.

Après la transplantation de progéniteurs neuronaux dans le cortex cérébral du rat, le nombre de cellules qui survivent et se différencient en neurones est extrêmement bas, ceci essentiellement en raison du fait que les progéniteurs neuronaux se différencient rapidement, perdant ainsi leurs propriété immatures, proliférative et migratoires. La manipulation génétique de progéniteurs neuronaux avant transplantation par des facteurs de croissances représente une stratégie innovatrice et essentielle pour augmenter l'efficacité de cette approche régénérative. Pour ce travail de thèse, nous évaluons la possibilité que la surexpression du facteur de croissance « fibroblast growth factor-2 » (FGF-2) par les progéniteurs neuronaux avant leurs transplantation, permette de significativement augmenter leurs potentiels réparateurs après transplantation dans le cortex cérébral.

Dans la première partie de ce travail de thèse, nous démontrons que par l'utilisation de la technologie lentiviral, la surexpression de FGF-2 dans les progéniteurs neuronaux augmente significativement leurs capacités prolifératives et migratoires après transplantation dans le cortex cérébral de rat. Aussi dans un contexte d'ischémie/hypoxie cérébrale la quantité de neurones immatures ainsi générés est significativement augmentée.

Dans la deuxième partie de ce travail de thèse, nous démontrons que les progéniteurs neuronaux surexprimant FGF-2 ont une tendance très significative de s'associer aux vaisseaux de l'hôte transplanté et surtout de former des clusters neurovasculaires artificiels dans le cortex cérébral. Ensemble, ces résultats suggèrent un rôle primordial de la signalisation de FGF-2 dans les progéniteurs neuronaux pour les stratégies de transplantation. La démonstration que la manipulation des progéniteurs neuronaux avant transplantation permette d'augmenter l'efficacité des cellules transplantées pour repeupler les zones cérébrales atteintes par l'ischémie, représente une étape convaincante dans le développement des stratégies de réparation cérébrale.

SUMMARY

The postnatal mammalian cortex has a very limited capacity for neuronal replacement after brain injury. One of the reasons for this restricted repair might be the lack of available neural progenitors. A promising approach is to transplant new cells in the rat cerebral cortex aiming at reconstructing the damaged brain. However the efficiency of these approaches for neuronal replacement *in vivo* remains very limited. After transplantation of NPCs into the cortex, the number of cells that survive and differentiate into new neurons is exceedingly low. Among the multiple factors limiting the efficiency of NPCs after transplantation in the postnatal cortex is the fact that grafted NPCs rapidly lose their immature, proliferative and migratory properties.

A major challenge is to develop pre-transplant cell manipulations that may promote the survival, engraftment and differentiation of transplanted cells. For this purpose, we investigated the possibility that over-expression of fibroblast growth factor-2 (FGF-2), a strong niche factor, in NPCs could improve their potential for structural brain repair.

In the first part of the thesis we showed that lentiviral overexpression of FGF-2 in NPCs provides a source of multipotential, proliferative and migrating NPCs that can efficiently invade the injured cortex and generate an increased pool of immature neurons available for brain repair.

In the second part of the thesis, we demonstrated that FGF-2 overexpressing NPCs associate preferentially with host blood vessels, generating neurovascular clusters. The close interaction between transplanted NPCs and the host vasculature appeared to be critical in maintaining NPCs in an undifferentiated and proliferative state. Furthermore, these neurovascular ectopic “clusters” seemed to preserve the potential to generate new neurons after being challenged by an ischemic insult.

Together, these datas reveal an essential role for FGF-2 in regulating NPCs functions when interacting with the host tissue, essentially by generating “ectopic” neurovascular clusters and offer a novel strategy to generate a robust source of migrating and immature progenitors for repairing an ischemic cortex.

INTRODUCTION

The development of new efficient treatments for ischemic or degenerative central nervous diseases is one of the leading challenges of this century. The old concept that the brain is in post-mitotic state and not able to compensate for neuronal loss has changed since Altman revealed in the 1960s, the proliferation of cells in the brain of cats and dogs *(Altman, 1969)*. This has raised new hopes for the treatment of neurological disease. Although neurogenesis occurs in the adult brain, it is still not sufficient to re-generate the injured areas. The possibility that neural stem/progenitor cells could be a therapeutic answer for patients suffering life-long debilitating brain injuries has opened an extraordinary field in neuroscience for the search of better brain repair strategies. There are two main strategies to achieve cellular brain repair: 1) One approach is the activation of the endogenous potential, by stimulating the germinative niches of the brain 2) Another approach is the direct transplantation of neural stem cells to repair the injured brain.

Although recruiting new neurons form the endogenous germinative zones of the brain is a potential approach, the transplantation of cells to replace loss or damaged neural cells appears to be a promising strategy for brain repair. While relevant progress has been made in understanding the behaviour and potential of stem cells, the efficiencies of neural stem/progenitor cell based therapies are still very limited. This is mainly due to poor grafting, deficient maintenance and survival of neural stem into the brain parenchyma. With this work we attempt to gain more insight into the mechanisms used by neural progenitor cells (NPCs) to integrate the host brain parenchyma *(Dayer et al., 2007)* with the possibility that transplanted NPCs could reproduce ectopic neurogenic niches available to replace lost cells after brain damage *(Jenny et al., 2009)*.

Neurogenesis for brain repair

Neurogenesis in the post natal brain

Neural stem cells play a fundamental role for brain development. They persist in the mammalian and human brain throughout postnatal development and into adulthood while continuing to generate neurons. "Neural stem cells" are defined as cells that 1) generate neural tissue or are derived from the nervous system, 2) have some capacity for self-renewal, and 3) produce cells other than themselves through asymmetric cell division *(Gage, 2000)*. In the adult mammalian brain, neural progenitor cells reside and give rise to either: neurons, astrocytes and oligodendrocytes. The generation of new neurons (neurogenesis) occurs mainly in two regions: the subventricular zone of the lateral ventricle (SVZ) and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus *(Bjorklund et al., 2000)*. In these germinative niches, there is a continuous production of new neurons to replace dying cells, although the exact function of newly generated neurons in these regions is not completely understood *(Jin et al., 2007)*. The SVZ lies laterally to the lateral ventricles and its postnatal neurogenic potential has been highlighted initially by Altman *(Altman, 1969)*. The cytoarchitecture of the SVZ has been further well characterized in rodents *(Lim et al., 1999; Alvarez-Buylla et al., 2002)*, and recently in humans *(Quinones-Hinojosa et al., 2006)*. It has been established in rodents that chains of young neurons or neuroblasts (type A cells) are surrounded by SVZ slow dividing astrocytes (Type B cells), which have astrocytes characteristics, forming tube like structures, and clusters of proliferative immature precursors (Type C cells, transit amplifier). Ependymal cells form an epithelial layer that separates the SVZ from the ventricle (Figure 1B), and are also considered to have a neural stem potential. Recently, by tangential examination of the ventricular surface, it was demonstrated a striking "pinwheel" pattern specific to regions of adult neurogenesis. The

pinwheel's core contains the apical endings of B1 cells (Figure 1 B1) *(Mirzadeh et al., 2008)*. The adult SVZ maintains a continuous production of young new neurons which divide and migrate along the rostral migratory stream (RMS) as chains of migrating cells surrounded by glial cells forming tunnels *(Doetsch et al., 1996; Doetsch et al., 1997; Alvarez-Buylla et al., 2002; Doetsch, 2003)*, towards the olfactory bulb where they preferentially integrate into granular interneurons and functionally integrate into local circuit *(Carleton et al., 2003)* (Figure 1C). Estimations have suggested that about 30 to 60'000 new neurons may be generated each day in the adult rodent olfactory bulb *(Basak et al., 2009)*. Neurogenesis occurs also in the SGZ of the dentate gyrus (DG) in the hippocampus. In distinction with the migration undertaken by neuroblasts along the rostral migratory stream, the progeny of proliferative cells accumulates within the SGZ *(Doetsch, 2003)*, where astrocytes give rise to progenitor cells which mature into granule cells, and integrate into the DG granule cell layer (Figure 1D) *(Alvarez-Buylla et al., 2002; Alvarez-Buylla et al., 2004)*. In other parts of the central nervous system, neural progenitors contribute to the formation of new non-neuronal cells, which are the astrocytes and oligodendrocytes and participate in the glial reaction seen after injury to the brain *(Rolls et al., 2009)*.

Interestingly, neurogenesis is present also directly within the cortex itself, although at a low level. This has been shown in the intact neocortex of adult monkeys but the newly generated neuron-like cells appear to survive only transiently *(Gould et al., 1999)*. Moreover, in the intact cortex of the adult mouse, a continuous formation of new cells has been observed. Out of these newly formed cells, only 1-2% express neuronal markers two weeks after cortical injury induced by experimental apoptosis *(Magavi et al., 2000)*. Furthermore, it has been shown that neural progenitor cells can be isolated from the early postnatal rat cortex *(Marmur et al., 1998)*, and

also from the adult human brain *(Arsenijevic et al., 2001)*. Very recently, the presence of neural progenitor cells in cortical layer I have been demonstrated *(Ohira et al., 2010)*, thus suggesting that resident stem cells within cortical regions of the brain might be an additional source for neurogenesis.

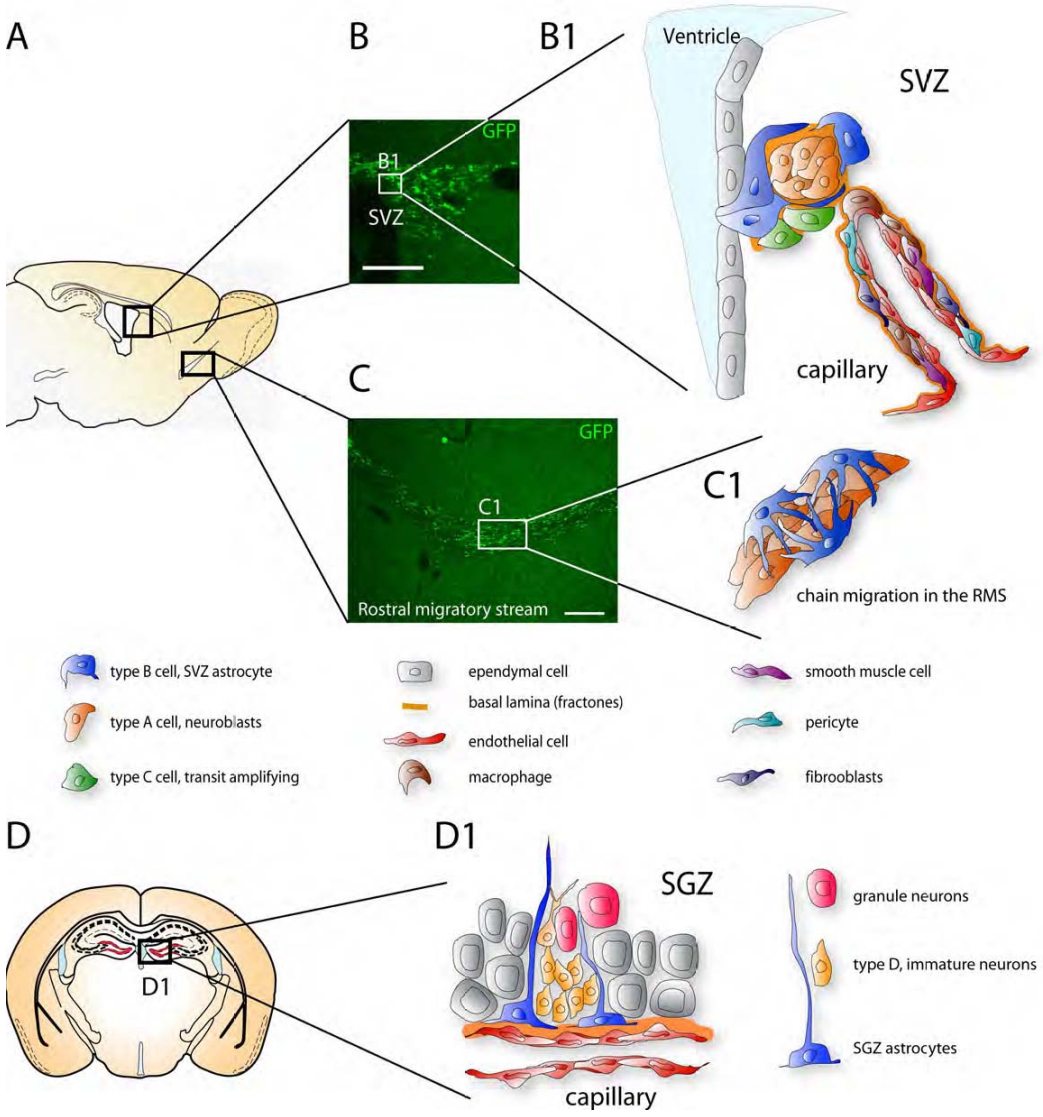

Figure 1: The neurogenic vascular niches of the rodent brain

The neurogenic niche of the SVZ and SGZ in the rodent brain is located on a sagittal (A) and coronal (D) views. The SVZ lies anterior to the lateral ventricles and provide the constant production of new immature neurons that migrate along the rostral migratory stream (RMS), while the SGZ of the dentate gyrus of the hippocampus is a thin laminae that lies between the granule cell layer and the hilus (B) Epifluorescence image showing the labelling of SVZ neural progenitors after intraventricular injection of a lentivirus carrying the green fluorescent protein GFP. (B1) Illustration of the cellular composition of the neurogenic niche composed of ependymal cells, astrocytes (type B cells), transit amplifying cells (type C cells) and neuroblasts (type B cells). The neurogenic niche is intimately associated with the vascular network of the SVZ via the basal lamina surrounding capillaries and

showing elongated processes extending from blood vessels and engulfing all cells of the neurogenic niche. Scale bar = 100 um (C) Epifluorescence images showing the migration of endogenous neural progenitors from the SVZ along the RMS. Neural progenitors are labelled after intraventricular injection of a GFP lentiviral vector. (C1) Illustration of the chain migration in the RMS inside channels formed by astrocytes. (D) Illustration of a coronal section of the rat brain showing (in red) the DG of the hippocampus was the SGZ localizes. (D1) Illustration of the cellular composition of the SGZ. Type B cells, SGZ astrocytes give rise to type D immature neurons that will migrate and differentiate into granule neurons to form the SGZ. This niche is also intimately associated with the BL of the vascular network.

(Figures adapted from Doetsch, 2003; Alvarez-Buylla et al., 2004)

Signalling in the adult germinative niche

Active neurogenesis under physiological conditions is restrited within these two specific germinative regions of the adult brain, the SVZ and the SGZ of the hippocampus, where highly specialized molecular signalling directs the proliferation and fate of neural stem cells. In these regions, neural stem cells reside in a very unique niche microenvironment which allows neurogenesis. In the SVZ, the slow dividing radial glia-like progenitors (type B cells) which express the Glial Fibriallry acid protein (GFAP) and CD133 are presumed to be the primary neural stem cells *in vivo.* They generate rapidly dividing transit amplififier progenitors (type C cells), which are positive for the homeobox Dlx2, the proneuronal basic-helix-loop-helix transcription factors Mash1 and the epidermal growth factor (EGFR). These intermediate progenitors further give rise neuroblasts (type A) cells expressing doublecortin (DCX+) and the Poly-Sialated Neural Cell Adhesion Molecule (PSA-NCAM). These neuroblasts migrate into the rostral migratory stream and integrate into interneurons expressing γ-Aminobutyric acid (GABA) into the olfactory bulb. In the SGZ of the dentate gyrus in the hippocampus, neural stem cells express GFAP, Sox2, also known as (sex determining region Y)-box 2, and express also Nestin. Sox2 is a transcription factor which is essential for the maintenance and self renewal of

undifferentiated embryonic stem cells. These neural stem cells known as radial cells (type 1 cells) give rise to self-renewing non-radial progenitor cells (type 2 cells) expressing Sox2 and Nestin but not GFAP. These type 2 cells give rise to DCX+ neuroblasts which mostly differentiate into local glutamatergic dentate granule cells (DGCs)*(Mu et al., 2010)*.

The differentiation of neural stem cells to mature depends on well synchronized molecular mechanisms which are only partially elucidated. Important signalling molecules during embryonic development are also involved in adult neurogenesis, modulating proliferation, fate commitment, migration and integration of NSCs and their progeny. Known key regulators of NSCs proliferation are the Wnt pathway, Sonic Hedghhog (Shh) and the Sox family of genes encoding for transcription factors.

Wnt is involved in proliferation and differentiation during embryonic development, but also in hippocampal neurogenesis *(Lie et al., 2005; Toledo et al., 2008)*. It keeps the balance between NSC maintenance and differentiation *(Wexler et al., 2009)*. New evidence shows that Wnt signalling is involved in synaptic plasticity *(Inestrosa et al.)*. In adult neurogenesis activation of the Wnt pathway together with the removal of Sox2 increases the expression of NeuroD1 a transcription factor that is essential for the development of the CNS especially the generation of granule cells in the hippocampus and cerebellum *(Kuwabara et al., 2009)*.

Shh is known for its role in the developing central nervous system and also for the regulation of hippocampal NSCs proliferation *in vitro* and *in vivo*. This correlates with a high expression of Patched the receptor for Shh, in neural progenitor cells isolated from the adult rat hippocampus *(Lai et al., 2003)*.

Morever, transcription factors like the Sox family of gene and especially Sox2 are critical for neural stem cell proliferation *(Chen et al., 2007)*. The deletion of Sox2 in mice results on several brain development anomalies *(Ferri et al., 2004)*.

Fate determination in neurogenesis is regulated mainly by transcription factors, such as basic helix-loop-helix (bHLH) transcription factor or Ascl1 (achaete-scute complex homolog-like 1, also known as Mash1), which can redirect the fate of adult hippocampal neural stem/progenitor cells towards an oligendendrocytic phenotype *in vivo (Jessberger et al., 2008)*. Other proneuronal (bHLH) transcription factors like Neurog2 *(Ozen et al., 2007)*, or Tbr2 (a T-domain transcription factor) *(Brill et al., 2009)* could be involved in adult fate determination and influence neural stem/progenitor cells to produce glutamatergic juxtaglomerular neurons *(Mu et al.)*. Interestingly, recent data suggests that epigenetic mechanisms such as DNA methylation, chromatin remodelling histone modification are relevant contributors to neurogenesis regulation *(Zhao et al., 2003; Li et al., 2008; Sanosaka et al., 2009; Covic et al., ; Liu et al., ; Szulwach et al.)*.

The mechanisms involved in the regulation of adult neurogenesis are only partially revealed and understanding how new neurons are born from the specialized niche microenvironement of endogenous neurogenic niche is essential to elaborate new strategies for brain repair.

Neurovascular niche in the germinative zones

Neurogenesis occurs in dens cluster around blood vessels

An interesting characteristic of the cytoarchitecture of the SVZ is a specialized basal lamina (BL), which extends from blood vessels in the SVZ, terminates in small bulbs adjacent to ependymal cells and contacts the SVZ cell types, named fractones *(Mercier et al., 2002)*.

Fractones are contacting local blood capillaries and one individual fractone engulfs numerous processes of adult neural stem cells, neuroblasts and epdendymocytes. Fractones may represent channels of communication as they contact with the CSF space at the surface of ependymal cells *(Mercier et al., 2003)*. The stems of extravascular BL are connected to blood vessels measuring 15-20µm in diameter. Blood vessels are localized usually at a short distance from the ependyma and consist of three cell layers: (1) an inner endothelial layer, (2) a layer of pericytes or smooth muscle cells and (3) a perivascular layer consisting of macrophage and fibroblast, which are delimited by BL (Figure 1 B1) *(Dore-Duffy et al., 2006)*. Recently, the 3-dimensional configuration of the SVZ with cell-cell interaction was analysed, showing that a rich vascular network was closely associated with neuroblastic chains *(Shen et al., 2008)*.

Most importantly, in the SGZ of the dentate gyrus (DG) in the hippocampus, it was shown that neurogenesis occurs in dense clusters associated with blood vessels. In a set of experiments, Palmer showed that two hours after BrdU injection, most labelled cells were found as single or doublets of synchronously dividing cells *(Palmer, 2002)*. Two days after BrdU injection, doublets and small group of cells were frequently found near small capillaries. Clusters were mainly nestin positive but some cells were immature neuroblasts (β-Tubulin or PSA-NCAM positive) or glial cells. Interestingly, it was found that 7 days after BrdU injection, most BrdU labelled cells were found in large groups, with a smaller population distributed as doublets or individual cells between the larger clusters, with on average 5 proliferative cluster cells directly in contact with blood capillaries *(Palmer et al., 2000)*. These results suggested that a mutual co-regulation of endothelial and neural cell precursors exists and that endothelial cells are an important source of signals for neurogenesis *(Doetsch, 2003)*.

The role of the ECM and BL as a supportive niche environment

The basal lamina (BL) is an important component of the neural stem/endothelial cell interaction within physiological neurogenic niches. Laminin, the essential component of the basal membrane has been shown to be particularly important in organ morphogenesis and development *(Miner et al., 2004)*. In different organs, the basal lamina has been identified to be a common and conserved feature of the "vascular niches", providing micro-environmental support for the development and proliferation of progenitor/stem cells *(Nikolova et al., 2007)*. In the SVZ, signalling growth factors, cytokines and extracellular matrix (ECM) protein with a molecular mass less than 50 kDa can cross the basal lamina *(Brightman, 1968)* and exert their effects on neural stem cells proliferation and differentiation *(Kuhn et al., 1997; Arsenijevic et al., 1998)*. The BL contains an abundance of heparin sulphate glyocasaminoglycan (HSPG) with a strong affinity for growth factors such as fibroblast growth factor-2 (FGF-2). Cytokines and growth factors can concentrate in the BL and subsequently bind to cellular receptors present on the cells in direct contact with the BL *(Yayon et al., 1991)*. Also carbohydrates can attach to the BL and ECM and potentiate ligand activity *(Doetsch, 2003)*. Moreover, in a 3-D systematic analysis of the SVZ it was shown that adult SVZ progenitor cells express the laminin receptor α6β1 integrin, and blocking this integrin inhibits their adhesion to endothelial cells, altering their position and proliferation *in vivo*, suggesting that it plays a significant role in binding neural stem cells to the vascular system *(Shen et al., 2008)*. BL acts as a strong structural and molecular support for the homeostasis of neural stem cells within the neurovascular niche, and FGF-2 has an important role in this phenomenology.

Endothelial/neural stem cells signalisation within the neurovascular niche environment

The molecular mechanisms that regulates neurogenesis within the neurovascular niche is mostly unknown, but a few studies have shown direct evidence for specific molecular signalization between endothelial and neural stem cells that could serve as niche signals and play a role for the maintenance, self-renewal and differentiation of neural stem cells. Most of these pathways like FGF-2 have a role both for angiogenesis and neurogenesis (Figure 2).

Endothelial cells in a co-culture system release soluble factors that stimulate Notch signalling, and thereby maintains the self-renewal of neural stem cells, inhibiting their differentiation and enhancing their neuron production *(Shen et al., 2004)*. An important role is played by the ECM and fractones. In germinative niches, most mitosis occurs near fractones immunoreactive for N-sulphate heparan sulphate proteoglycan (HSPG), a molecule that can bind heparin-binding growth factor like FGF-2. It was demonstrated that these structures can capture FGF-2 from the extracellular milieu after intracerebroventricular injection, suggesting that it represents a niche structure controlling neural stem cells proliferation via the presentation of the growth factor to contacting neural stem cells *(Kerever et al., 2007)*.

In the adult rat brain, within the SGZ of the hippocampus, clusters of proliferative cells reside in close association with capillaries. Within these clusters of dividing neural progenitors, some cells are positive for the major receptor for vascular endothelial growth factor (VEGF), VEGFR-2 (flk-1). Also, VEGF immunoreactivity is seen in the surrounding cells *(Palmer et al., 2000)*, highlighting the potential effect of VEGF for neural stem cells maintenance, and suggesting an

important role for VEGF in mediating both angiogenesis and neurogenesis within the microenvironment of the neurovascular niche.

Endothelial cells secrete also pigment epithelium-derived factor (PEDF), which is secreted by a variety of cells and expressed in various regions of the brain *(Tombran-Tink et al., 2003)*. In the mouse adult SVZ, PEDF is expressed by ependymal and endothelial cells. This promotes self-renewal but not proliferation of adult stem cells isolated from the SVZ. PEDF treatment also induces molecular changes that are associated with neural stem cells multipotentiality *(Ramirez-Castillejo et al., 2006)*, thus identifying PEDF as a niche-derived regulator of adult neural stem cells *(Pumiglia et al., 2006)*.

In the microenvironmental niche of the higher vocal cord center (HVC) in the adult songbird neostriatum, neurogenesis occurs throughout life. Interestingly, testosterone treatment of the adult female canary can trigger primitive song in an otherwise non singing bird, by increasing the rapid production of both VEGF and VEGFR-2/KDR in the nucleus HVC, thus inducing rapid endothelial cell division and expansion, causing the vasculature substantially to increase its production and release of brain derived neurotrophic factor (BDNF). This induction was associated with the recruitment of new neurons to HVC from the overlying ventricular zone *(Louissaint et al., 2002)*. Interestingly, in the same setting, testosterone induced also the secretion of (metalloproteinase-2) MMP2 by endothelial cells, mediated by VEGF, identifying androgenic induced MMP2 as a critical regulator of neuronal addition in the adult HVC *(Kim et al., 2008)*. The above studies highlight important endothelial dependant molecular pathways in adult neurogenesis, which could play a significant role in the maintaining neural stem cells.

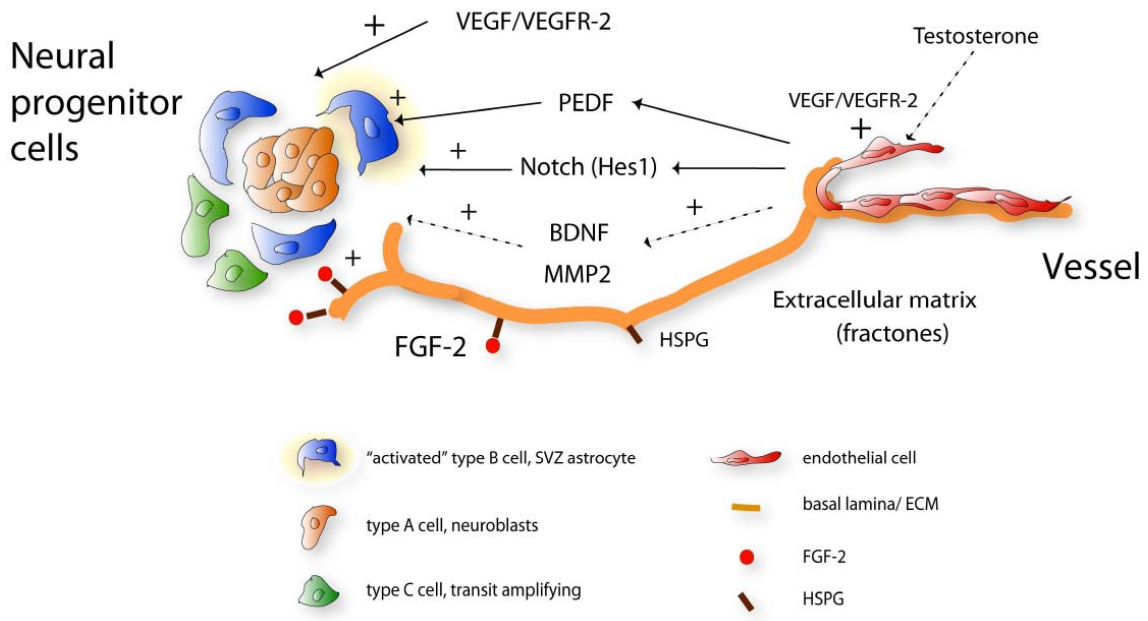

Figure 2 : Signalization within the germinative neurovascular niche of the SVZ

Essential signalling pathways shown to play a role for the homeostasis of the neurovascular niche. Endothelial cells provide trophic factors that serve as niche signals and maintain the self-renewal, proliferation and differentiation of neural stem/progenitor cells. The angiogenic factor VEGF and its receptor VEGFR-2 expressed on neural stem cells induces the proliferation and survival of neural stem cells. PEDF has been demonstrated to influence directly slow dividing astrocytes. Endothelial /neural stem cell contacts upregulates the Notch effector (Hes 1) in neural stem cells. VEGF pathway induction upregulates the secretion of endothelial derived BDNF and MMP2 which increases neurogenesis and neuronal recruitment (in higher vocal center of the songbird). Finally, fractones can capture FGF-2 via the attachment with HSPG and present FGF-2 to close neural stem cells which will be stimulated.

What should we expect from neural stem cells to achieve efficient brain repair?

One of the fundamental concepts for neural repair lies in the replacement of specialized cells that are lost as a result of disease or injury. To achieve this task, neural stem cells have to overcome different challenges to accomplish a multiple step process essential to replace missing glial or neuronal cells (Figure 3):

1) Neural stem cells must maintain immature and proliferative properties, fundamental to sustain a constant pool of cells available for repair. The maintenance of neural stem cells within the SVZ or the SGZ depends on a specific cellular/molecular microenvironment and "niche" signals provided within the germinative zones. In addition, preservation of resident stem cells in cortical regions may also rely on local cues provided by the surrounding milieu.

2) Neural stem cells must keep strong migratory properties and attachment capacities to the extracellular matrix. This is a critical issue especially to travel long distance across the diseased brain parenchyma and replenish the site of lost neurons. An important aspect in the directed migration of neural progenitor cells is the up-regulation of chemoattractant factor by the regions of damaged brain.

3) An essential feature once neural stem cells have reached the damage cortex is the determintation of their fate. This is crucial to promote efficient cell replacement and integration of cells into a fully functional network, and raises also several questions: Do neural progenitor cells differentiate in glial or neuronal cells? Do molecular changes induced by the ischemic regions influence the fate of neural progenitors?

4) Moreover, to sutain long term brain repair, neural stem cells must preserve extended survival period within the brain parenchyma to ensure a sustainable function into an integrated network.

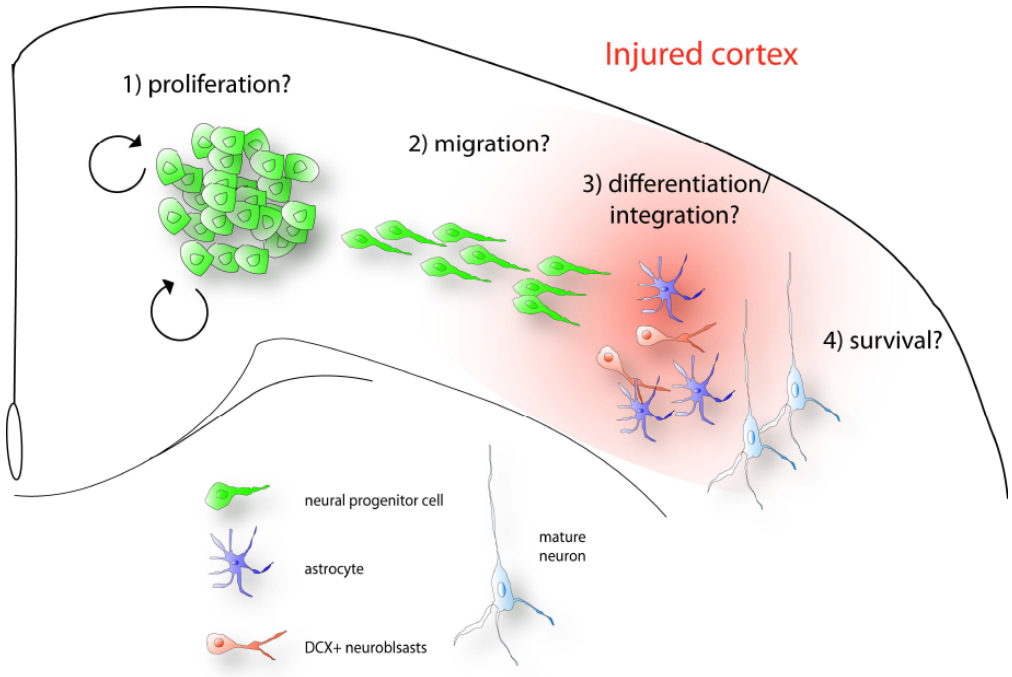

Figure 3: Neural stem cells potential for brain repair

For efficient brain repair capacities, neural stem cells must maintain specific features:

1) Proliferation and maintenance

2) Migration through the brain parenchyma towards the sites of injuries

3) Neuronal or glial fate determination and integration into the cortical neuronal network

4) Survival in the injured areas of the brain.

Can sufficient new neurons be recruited from the physiological neurogenic sties?

Cerebral ischemia can mobilise endogenous repair mechanisms by inducing neurogenesis. This has given in the recent decade many hopes for brain repair strategies. Unfortunately scientific evidence suggests that, although neurogenesis can be stimulated in the SVZ and the DG after

cerebral ischemia, it is not sufficient to replenish areas of damaged neural cells and integrate them into fully functional cortical circuits *(Plane et al., 2004)*. Evidence shows that an increased cell proliferation in the SVZ after cerebral ischemia has been demonstrated, showing that endogenous neural stem cells are able to respond to the microenvironmental changes induced by cerebral ischemia and increase the pool of cells available for brain repair *(Parent et al., 2002; Parent, 2003; Plane et al., 2004)*. Many studies have shown that stroke activates endogenous neural stem cells in the SVZ contributing to neurogenesis and neuroblasts migration towards the infarct boundary of the striatum in adult rats *(Parent et al., 2002; Zhang et al., 2004)*. After induced synchronous apoptotic degeneration of neurons in the anterior cortex of adult mice, close to the SVZ, it was found that only about 1-2% of the newly formed cells in the damage necrotic express neuronal markers *(Magavi et al., 2000)*. In a model of focal right middle cerebral artery occlusion in adult rats, it was found that neuronal precursor cells seem to migrate from the SVZ towards the site of injury, playing a potential role for repair, although the amount of newly formed neurons around the ischemic core was very low *(Jin et al., 2003)*. This shows that the number of newly generated neurons, if present at all *(Arvidsson et al., 2002)*, remains insufficient to restore normal cortical function *(Bjorklund et al., 2000)*. Although ischemic stimulation of endogenous pool of neural progenitor cells of the SVZ is able to provide immature neurons which migrate towards the site of injury in the striatum, efficient cortical neurogenesis is difficult to achieve and the capacity of self-repair is clearly not sufficient *(Jin et al., 2007)*.

There are many reasons why endogenous neurogenesis is not satisfactory to obtain adequate brain repair. One explanation is that the pool of neural stem cells available to replace lost cells is insufficient and that most cells are not able to reach the areas of cortical injury. Furthermore,

cortical resident stem cells might be very rare and have limited potential to ensure even local adequate cell replacement near the sites of injury. A reasonable alternative would be to generate in the cortex near the sites of brain damage an ectopic pool of dormant cells that would maintain neural stem cells characteristics and be available for brain repair.

Cell based strategies for brain repair

In the recent decade cell therapies have shown promising results and the idea that the engraftment of neural stem /progenitor cells could supply a pool of cells able to replace damaged cells has generated an enormous hope in neuroscience *(Chang et al., 2007)*. Cell replacement therapies for central nervous system disorders have shown some interesting results so far, initially in Parkinson disease with well characterized rodent and primate models and furthermore with clinical trials, strongly suggesting that cell based therapy may also work in other disease of the central nervous system *(Bjorklund et al., 2000; Olanow et al., 2009)*.

In stroke therapies, cultured stem cells, thanks to their self-renewal ability and their potential to differentiate into many cell types of the CNS, can be transplanted aiming to repair the damaged brain. The behaviour of transplanted cells depends primarily on the source of cells and the animal model used for transplantation. Stem cells are the most primordial cells of the organism or can originate from a given organ, thus defining two major categories of stem cells: 1) embryonic and 2) somatic stem cells *(Singec et al., 2007)*. These two categories constitute the two main sources of stem cells used for cell replacement strategies. Recently the development of induced pluripotency and transdifferentation, have added new, interesting options for cell replacement therapies.

Embryonic stem cells

Embryonic stem cells (ESCs) derive from blastocysts which are pluripotent and have unlimited renewing capacities. The concept that a pluripotent cell can be perpetuated indefinitively and induced to differentiate, is based on pioneering work in 1964 from teratocarcinomas and embryonic carcinoma cells, with the demonstration that a single embryonic carcinoma cell could form a tumour and also give rise to differentiated cell types *(Kleinsmith et al., 1964)*. Thus, although progress has been made in controlling the use of embryonic carcinomas cells, they are still cancer cells and not applicable for transplantation therapies.

Later in 1981, two important studies *(Evans et al., 1981; Martin, 1981)* showed the successful derivation of pluripotent embryonic ESCs from the inner cells mass of blastocyst stage mouse embryos. These ESCs were still capable of generating teratomas when ectopically injected into mice, but were easier to control in terms of growth and differentiation compared to embryonic carcinomas cells. The hope to improve embryonic stem cell based therapies increased with the successful isolation of human embryonic stem cells (hESCs) *(Thomson et al., 1998)*. The differentiation potential of ESCs is theoretically unlimited and cell types successfully derived from hESCs are continuously being discovered. One of the first developmental steps that can be recapitulated during ESCs differentiation *in vitro* is the formation of the three germ layers (ectoderm, mesoderm, endoderm), a process called "gastrulation" *in vivo*. The most widely used method to induce germ layer cells is the differentiation of ESC into three dimensional free floating structures, the embryoid bodies (EBs) *(Singec et al., 2007)*. For cell therapy, ESCs can be used directly for transplantation or differentiated into neural progenitors *in vitro* prior to transplantation, thus limiting undesirable effects such as the formation of teratomas.

Somatic stem cells

Although continuous development is made with ESCs, the potential of somatic stem cells has opened remarkable hopes in the field. Somatic stem cells derive from specific tissue in the postnatal period and can include the bone marrow, central nervous system, peripheral blood, adipose tissue and skeletal muscle. In fact the CNS became the first solid organ in which the existence of stem cells was recognized. Compared to the CNS, the hematopoietic system has a rapid turnover within a few weeks to months, and the existence of stem cells was therefore not unexpected. The concept that both organs, the CNS and the hematopoeitic system, store stem cells with quite similar features has generated enormous hope for regenerative medicine. In contrast to "pluripotent" ESCs, which can give rise to all cell types of the body, somatic stem cells are believed to be capable of generating only the major cell types of their tissue origin and are therefore "multipotent" (Figure 4). It is suggested that during development, a "pluripotent" stem cell give rise to a somatic, tissue specific stem cell which then participates in organogenesis and persists throughout life in a specialized microenvironment "the stem cell niche". This specialized milieu supports cell turnover as well as contributes to the production of their factors crucial for the homeostasis of stem cells. Neural stem cells (NSCs) or neural progenitor cells (NPCs) which are immature cells with a more restricted neurodevelopmental potential than NSCs, can be generated form ESCs or directly isolated from the developing CNS and from germinative regions of the adult brain *(Reynolds et al., 1992).* As already mentioned above, in the adult mammals, NSCs reside in specific germinative niches, comprising the SVZ of the lateral ventricles but also the DG of the hippocampus where neurogenesis occurs throughout life. In cortical regions also, dormant stem cells might participate in local neurogenesis and cell

turnover. NSCs give rise to the three major cell types which are neurons, astrocytes and oligodendrocytes. NSCs should be able to generate all cell types of the nervous system during development as well as to reconstitute those regions following their destruction. Neural stem/progenitor cells can be cultured as monolayer or as free floating aggregates called "neurospheres" *(Reynolds et al., 2005)*, and will be propagated for extended periods of time in the presence of mitogenic niche factors such as basic fibroblast growth factor (bFGF/FGF-2) or epidermal growth factor (EGF).

Induced pluripotency

In the recent years, a new technical advancement in stem cells research has been the generation of induced pluripotent stem cells (iPS){Patel, 2010 #635}. Induced pluripotent cells are generated by differentiating somatic cells into pluripotent ES cell. It was demonstrated that a fertile mouse can be derived entirely form iPS generated from inducible genetic reprogramming of mouse embryonic fibroblasts *(Boland et al., 2009)*. iPS can also be produced from adult neural stem cell by reprogramming with two factors, exogenous Oct4 together with either Klf4 or c-Myc *(Kim et al., 2009)*. These reports show the possibility to generate pluripotent stem cells from somatic stem cells thus opening a new field in human cell replacement strategies without the need for embryonic stem cells which use is still much debated around the world. This would also bypass the oncogenic risk associated with the use of embryonic stem cells.

Transdifferentiation

Another novel technology in stem cells research is the concept of transdifferentiation. This implies that adult differentiated cells can be directly and efficiently converted into a functional

cell of another type of cells within an organism without going through the steps of reprogramming into an ES-cell-like state a process called dedifferentiation/differentiation *(Krabbe et al., 2005)*. Using this technology it was shown that functional neurons could be converted directly from fibroblasts *in vitro*, using only a combination of three neural-lineage-specific transcription factors (Ascl1, Brn2 and Myt1l)*(Vierbuchen et al., 2010)*. The generation of induced neurons (iN) from non-neuronal lineage opens also significant opportunites in cell replacement strategies for brain repair, bypassing the use of embryonic or another source of stem cells. Moreover this allows differentiated cells to be an interesting source for effective neuronal replacement.

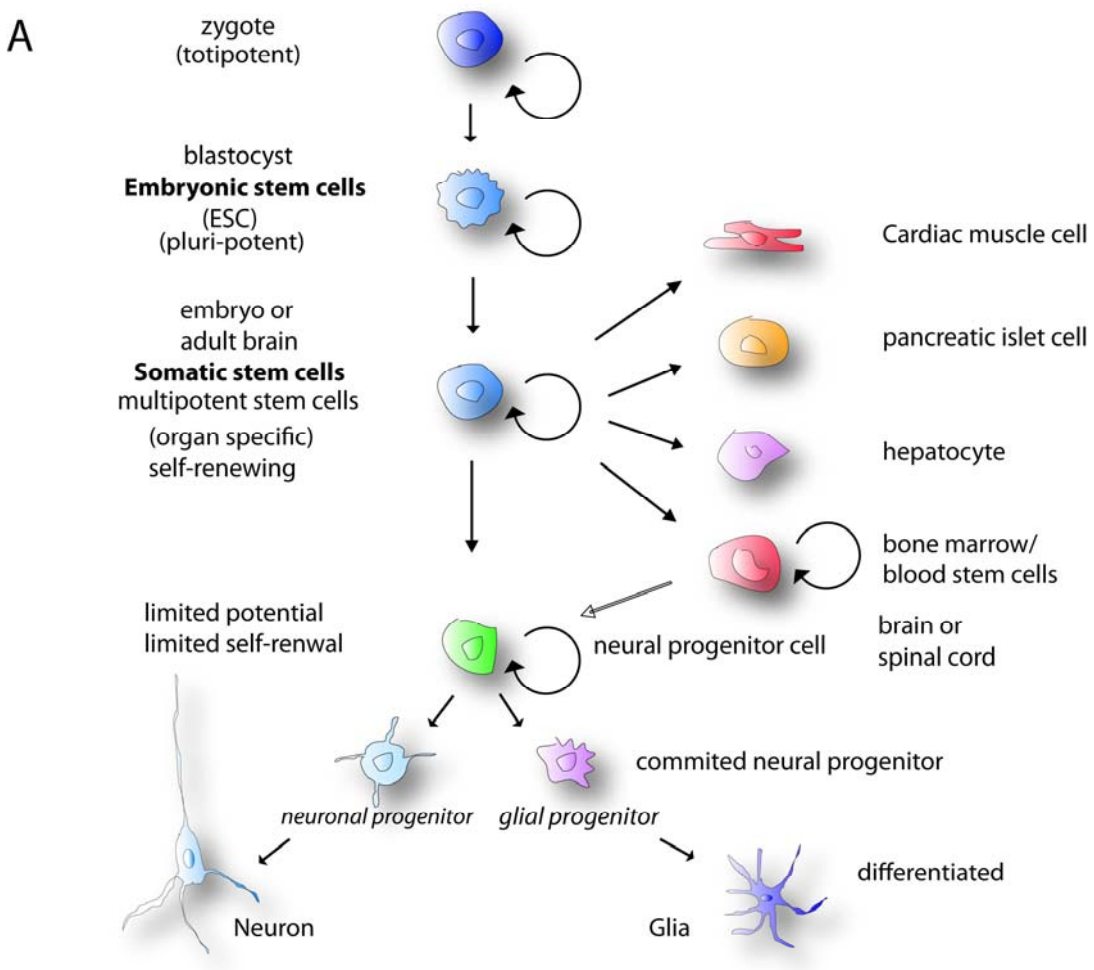

Figure 4: Differentiation potential of stem cells.

Embryonic stem cells can differentiate into almost all cells of our body. With ongoing maturation, their ability to differentiate into different type of cells becomes more and more restricted. The differentiation capacity of neural progenitor cells is restricted to the neuronal and glial lineage. Bone marrow stem cells have the capacities to transdifferentiate into neural stem cells. Stem cells have an unlimited potential for self-renewal.

What has been achieved so far in cell replacement therapies?

The concept of cell replacement strategies raises a series of fundamental questions: 1) Which type of cells should be used (embryonic, somatic stem cells, induced neurons), 2) What type of cell replacement should be achieved, dopaminergic neurons for Parkinson's disease, pyramidal neurons and/or supportive cells for stroke, 3) Are the cells used from autologous (same subject) heterologous (different species) or homologous (same species) origin? 4) This further raises the question of immune reaction and rejection from the host to the graft as it is well known for organ transplants. Experiments for cell replacement in brain disorders have flourished with the concept that the brain is an "immunological privileged site" allowing cell survival without rejection, although this view can be wrong and a significant degree of immune response of the brain influence the survival of transplanted cells *(Barker et al., 2004; Mueller et al., 2005)*. 5) Prior to transplantation, cells can be genetically modified by the use of different transfection technologies which raises the question of long term stability of the transgene after engraftment. 6) Finally, the risk of long term tumour formation from the grafted cells, especially from ESC origin or modified with a retroviral based technology needs to be addressed and carefully monitored before using any of these approaches in clinical trials.

Strategies for repair in acute injury (stroke, trauma) and neurodegenerative disorders (Alzheimer's, Parkinson's and Huntington's disease) include the use of either embryonic stem cells or somatic stem cells. Initial work showing the use of stem cells for cell replacement therapies in a solid organ have originated more than a decade ago with the transplantation of multipotent neural precursors in an adult mouse model of experimentally induced apoptosis of pyramidal neurons. In this model transplanted cells responded to environmental cues and as

much as 15% of the transplanted cells specifically differentiated into neurons with many characteristics of the degenerated pyramidal neurons. Interestingly, in control adult neocortex, transplanted precursors differentiated exclusively into glial, suggesting that microenvironmental alterations induced neuronal precursors to undergo neuronal differentiation *(Snyder et al., 1997)*.

In Parkinson disease, experimental evidence has shown that dopaminergic neurons can be generated directly from embryonic stem cells by neuronal induction, differentiation and selection, or from neural progenitors isolated from the subventricular zone and induced *in vitro (Lindvall et al., 2004)*. Also, clinical trials have shown that the transplantation of human fetal dopaminergic neurons can produce major, long-lasting improvements in some patients *(Kordower et al., 1995; Piccini et al., 1999)*.

For the treatment of stroke, transplantation of stem cells has generated increasing hopes but still lacks evidence for efficient approaches. To date no reliable cell based regenerative therapy can be proposed to patients suffering consequences of ischemic brain injury. At present, few reported clinical trials in patients with stroke have been made, thus with no proven clinical improvement *(Kondziolka et al., 2000)*, thus experimental essays have flourshied with the aim to improve the potential of this approach for stroke or brain injury.

Cells from different sources have been tested for their ability to reconstruct the forebrain and improve function after transplantation in animals subjected to stroke *(Lindvall et al., 2004)*. Some studies have used NPCs derived form ESCs and transplanted them into ischemic mouse brains, showing that they could incorporate in the ischemic brain parenchyma and expressed

mature neuronal markers. Moreover, many of the grafted ESC-derived NSCs survived in the infarct core up to 12 weeks *(Takagi et al., 2005)*. When NPCs derived from monkey's ESCs, were transplanted into the ischemic rodent striatum at 24 hours after reperfusion, cells were observed to migrate into the area surrounding the ischemic lesion and differentiated into various types of neurons and glia *(Hayashi et al., 2006)*, showing also some degree of motor improvement when injected in an hemiplegic mouse model of experimental brain injury *(Ikeda et al., 2004)*. However in some cases undifferentiated ESCs formed teratomas when transplanted into the brain *(Liu et al., 2009)*. When embryonic mice NPCs were administrated into the lateral ventricle of post-ischemic rats, they showed some degree of migration into the striatum, ipsilateral to the ischemic side after middle cerebral artery occlusion. Also 6 days after direct striatal injection, cells showed some degree of migration towards the ischemic striatum whereas in control rats, most transplanted cells remained at the injection site and most of these died within one week *(Jin et al., 2005)*. When injected into an ischemic rodent brain, human fetal NPCs migrated to the infarct area adjacent to the injection site, expressed neuroepithelial or neuronal markers such as Nestin and NeuN, this compared to control rats where transplanted cells stayed stuck into the needle track injection site *(Kelly et al., 2004)*.

Stem cells based therapies have shown attractive development in the last ten years but the many issues obeserved with present models warrant significant improvement for this approach to be applied to the care of patients. Many challenging issues remain to be addressed essentially in regards to the efficient use of a stem cell based approach in stroke therapies. In fact, with the use of ESCs, the risk of developing teratomas is a major drawback which precludes a safe use of this approach. On the opposite, the use of NPCs, has shown from our own preliminary experience,

and in other studies that most of the grafted cells differentiate rapidly in glial cells, losing their stem properties after transplantation, thus making them not available to migrate and repopulate the different sites of brain damage where they should differentiate into the various cell types needed to reconstruct the damaged brain.

Goal to achieve for cell replacement brain repair strategies

The repair of the stroke induced damaged brain appears to be difficult to obtain and many issues identified after the transplantation of stem cells need to be addressed. Stroke induces most commonly an undefined lesion leading to the destruction of many different cell types (neuron, glial cells and vessels). The goal of any brain repair strategies using stem cells would be to engraft next to the cortical ischemic lesion, a pool of cells which would keep neural stem cells properties and repopulate the sites of lost cells.

These properties must include 1) maintaining an immature, proliferative and self-renewing population of neural stem cells that can act as a launch pad available for brain repair, 2) keeping strong migratory properties allowing transplanted cells to be recruited by the sites of injuries and travel through the injured brain parenchyma, 3) differentiating into the adequate cell types required once established in the area of injury and 4) surviving for a long period after transplantation.

Hypothesis and objectives of the project

As a first approach to improve the potential of neural stem cells after transplantation in cerebral ischemia, we considered the possibility that, growth hormones such as fibroblast growth factor-2 (FGF-2) could be determinant in maintaining the stem properties of neural progenitor cells after

engraftment. FGF-2 is a key niche factor expressed in the physiological niche of the SVZ *(Frinchi et al., 2008)*, mainly released by GFAP-positive cells *(Mudo et al., 2009)* and is a member of a large family of proteins which binds heparin and heparin sulfate, and modulates the function of a wide range of cell types.

FGF-2 signalling in the brain

Biological activity of FGF-2

FGF-2 also known as basic fibroblast factor belongs to the fibroblast growth factor (FGF) superfamily consisting of 22 members encoding for 22 genes in mice and in humans. One of the members, FGF-2 is expressed as five different isoforms in humans (18, 22, 22.5, 24 and 34kDa) and three different isoforms in rodent (18, 21 and 23 kDa). The 18kDa FGF-2 form, usually known as low molecular weight (lmw), is mainly cytoplasmic and can be secreted using unconventional secretion mechanisms, where it binds to and activate cell-surface receptors. The other forms are known as high molecular weight (hmw) FGF-2 (22-34 kDa), are not secreted from the cell but are transported to the nucleus, due to N-terminal extensions containing several GR (Glu/Arg) repeats that act as nuclear localizing sequence (NLS) for FGF-2. They regulate cell growth or behaviour in an intracrine fashion. Normally FGF posseses a signal sequence for secretion through the Golgi system into the extracellular space, the 18kDa isoform of FGF-2 is synthesized without a signal sequence and is transported out of the cells by mechanisms bypassing the ER-Golgi secretory route *(Sorensen et al., 2006).* FGF-2 binds to four cell surface receptors expressed as a number of splice variants (FGFR-1,2,3 and 4). They act via receptor tyrosine kinase signalling which activates downstream transcription in the nucleus. Ligand binding to FGF receptors leads to the formation of receptor complex consisiting of two FGF

molecules bound to a receptor, which are linked by heparan sulfate proteoglycan molecules. Many of the biological activities of FGF-2 have been found to depend on intrinsic tyrosine kinase receptors activity and second messengers such as the mitogen activated protein kinases *(Nugent et al., 2000)*. Activation of FGFRs triggers several intracecellular sgnaling cascades and signals to the nucleus by receptor tyrosine kinase (RTK) mediated phosphorylation of downstream effectors (via MAPK, p38, PLCγ, PI3K), thus regulating gene transcription *(Reuss et al., 2003)*. As another cellular signalling route, FGF-2 bound to FGFRs can be internalized by endocytosis to the cytosol. Within the endosome, the 18kDa form of FGF-2 is processed to a 16kDa form and translocated to the cytosol from where it reaches the nucleus bound to translokin which is involved in nuclear import of FGF-2. Once it reaches the nucleus, FGF-2 can bind to CK2 (a serine/threonine kinase) and regulate ribosomal biogenesis. FGF-2 can also bind to the upstream binding factor (UBF) and regulate rRNA transcription and also to the ribosomal S6 protein kinase 2 (RSK2), thus involved via phosphorylation of histone H3 during G0-G1 transition *(Sorensen et al., 2006)*.

Intracellular FGF-2 has been found to have biological effects in a wide range of cell types, with specific effects of intracellular hmw FGF-2 or intracellular 18kDa FGF-2, the most common effects relates to cell survival and growth. Extracellular FGF-2 can mediate various biological responses such as cell growth and proliferation, migration and differentiation, by interaction with FGFRs.

FGF-2 during brain development

Members of the FGFs family are expressed in the central nervous system as significant signal during development and repair mechanisms. FGF-1 and FGF-2 are expressed in the early stages of brain development *(Ford-Perriss et al., 2001)*. FGF-2 is expressed highly in the SVZ until E17.5 in rat and in the meninges and the choroid plexus *(Iwata et al., 2009)*. FGF-2 has been found to be expressed not only by neurons but also by glial cells. At an mRNA level, FGF-2 is expressed in the cerebral cortex, hippocampus and brain stem along with the FGFRs at a various level during brain development and adulthood *(Ford-Perriss et al., 2001)*. Some studies suggest that FGF-2 is primarily expressed by astrocytes and some groups of neurons, also FGFR-2 seemed to be preferentially expressed at a high level by astrocytes from the neurogenic zones of the SVZ and the DG suggesting its important role in neurogenesis *(Chadashvili et al., 2006)*. Recent evidence has shown an important role of FGF specifically for patterning and for neurogenesis during cortical development. Nontheless the precises intracellular signalling and downstream effectors of FGF signalling during cortical development is still not well understood *(Iwata et al., 2009)*.

FGF-2 knock out experiments have shown viable and fertile mice but with various changes in neurogenesis, including midly thinner cortex, reduced number and density of neurons and glia, reduction in number of glutamatergic projection neurons but not GABAergic interneurons. Effects were more specific in the anterior regions of the cortex *(Vaccarino et al., 1999) (Chen et al., 2008)*.

Regulation of neurogenesis by FGF-2 signalling

Many factors are involved in the regulation of neural stem cells proliferation and maintenance which includes genetic aspects, growth factors, neurotransmitters, stress, environmental factors induced by ischemia/hypoxia and hormones. Neural stem cells are a rare population in the adult neurogenic SVZ and account for a minor fraction of the proliferative cells *(Morshead, 2004)*. Neural stem cells divide slowly with an average cell cycle time of over 15 days which has been proposed to allow them to retain their potential throughout life *(Morshead et al., 1994)*. Their progeny, the transit amplifying cells divide multiple times, with an estimated cell cycle of 12 hours and represent the majority of the dividing cells in the SVZ. The major progeny of the transit amplifying cells are the neuroblasts, which keep almost similar proliferative capacities but gradually become post-mitotic during their migration towards the olfactory bulb. Out of all growth factors, FGF-2 appears to be a key candidate that regulates the maintenance and proliferation of neural stem cells.

Different studies have demonstrated that FGF-2 signalling is a critical regulator of mammalian neurogenesis *(Ghosh et al., 1995; Temple et al., 1995; Ford-Perriss et al., 2001)*. FGF-2 has shown to have a major role for the maintenance of proliferative and undifferentiated population of NPCs *in vitro (Maric et al., 2003)*, and also *in vivo* within neurogenic zones of the SVZ *(Zheng et al., 2004)*. Self-renewal and differentiation of neural stem cells in the cortex is selectively regulated by FGF-2. NSCs exposed to bFGF *in vitro* can generate four stereotypical clonal expansion states: efficiently self-renewing, inefficiently self-renewing limited by apoptosis, exclusively neurogenic and multipotential. NSCs residing in the dorsal telencephalon co-express FGFR1 and FGFR3 and those residing in the ventral telencephalon also express

FGFR-2. Co-activation of FGFR-1 and FGFR-3 promoted symmetrical divisions of NSCs (self-renewal) whereas inactivation of either triggers asymmetrical division and neurogenesis from these cells *(Maric et al., 2007)*. After cerebral ischemia, FGF-2 can increase the pool of cells in neurogenic regions of the adult rat brain *(Ma et al., 2008; Oya et al., 2008)* and neonatal rat cortical brains *(Jin-qiao et al., 2009)*. Moreover, FGF-2 can reprogram native precursors of the adult brain rat which generate only glia, into neurons *in vitro (Palmer et al., 1999)*, and induce *in vitro* a neural stem cells phenotype into foetal forebrain progenitors *(Pollard et al., 2008)*. FGF-2 is also critical for the maintenance of pluripotency in human embryonic stem cells *(Amit et al., 2000) (Dvorak et al., 2006; Eiselleova et al., 2009)* and the reprogramming of primordial germ cells into pluripotent stem cells *(Durcova-Hills et al., 2006)*. It plays an important role in the preservation of neural stem/progenitor cells renewal *in vitro (Maric et al., 2003)* and the maintenance of an immature slow dividing stem cells population in the neurogenic niche of the SVZ *in vivo (Zheng et al., 2004)*.

FGF-2 signalling for brain repair

Increasing evidence suggests that FGF-2 is involved in neuroprotection and brain repair mechanisms. Due to its essential role as a niche factor, FGF-2 might be a determinant factor to provide structural repair after brain injury or in neurodegenerative disease. FGF-2 has been used already to stimulate the endogenous population of neural stem cells in the SVZ. It was demonstrated that two weeks after intracerbroventricular injection in rats, FGF-2 increased the number of newborn cells, most prominently newborn neurons in the olfactory bulb *(Kuhn et al., 1997)*. Furthermore chronic infusion of FGF-2 in the lateral ventricles has been shown to improve the regeneration of hippocampal neurons after global ischemia *(Nakatomi et al., 2002)*.

Recently, in a rat model of bilateral common carotid ligation it was shown that the administration of bFGF significantly increased the number of prolifertive cells in the SVZ and it was suggested also that bFGF promoted the differentiation of endogenous neural stem cells into neurons, oligodendrocytes and astrocytes *(Jin-qiao et al., 2009)*. Also in rats following traumatic brain injury, intraventricular infusion of bFGF significantly increased the number of proliferative cells in the SVZ and an increased number of newly generated cells survived 4 weeks after the injury, with a majority of them differentiating into neurons *(Sun et al., 2009)*. In a mouse model of Huntington disease it was shown that the sub-cutaneous administration of FGF-2 increased the number of proliferating cells by 30% in the subventricular zone of WT mice and by 150% in transgenic HD mice. It also induced the recruitment of neural progenitors to the striatum and cererbral cortex of HD mice. Interestingly FGF-2 showed also its neuroprotective effects since it prevent cell death in striatal cultures of HD mice *(Jin et al., 2005)*. It has been shown *in vitro* that binding of FGF-2 to ATP was essential for its neuroprotective effects *(Rose et al., 2009)*.

Whether FGF-2 is essential for endogenous brain repair may require further studies, which could aim at suppressing FGF-2 in animals subjected to cerebral ischemia. Nonetheless, the above studies suggest an appealing role for FGF-2 in brain repair and due to its critical involvement for the maintenance of neural stem cells it appears to be an interesting molecule to use for developing new brain repair strategies.

Goal of the project

In this work we wish to test the hypothesis that FGF-2 overexpression in transplanted neural progenitors improves the potential of these cells after transplantation and provides a strong population of progenitor cells available for tissue reconstruction after brain damage.

Consequently, we developed a novel model of neural progenitor cells transplantation in neonatal rat brain hypoxia-ischemia, using a lentiviral technology to overexpress FGF-2 in NPCs.

METHODS

Isolation, culture and differentiation of NPCs in vitro

Neural progenitor cells were isolated from the SVZ of newborn rat pups at P0. Mechanically, the SVZ was dissected from coronal slices of newborn rats and further trypsinized, as it was already described previously *(Lim et al., 2000; Zhang et al., 2003)*. NPCs were then purified by centrifugation using a Percoll gradient and further seeded onto matrigel dishes. Cells were allowed to expand in neurobasal medium supplemented with 20ng/ml of FGF-2. To induce differentiation, NPCs were trypsinized at DIV5, seeded onto matrigel coated dishes and allowed to expand during 48h in the presence of 20ng/ml of FGF-2. Differentiation was induced by removing FGF-2 and adding 20ng/ml of BDNF and 1% of fetal calf serum.

Lentiviral technology

To provide a population of neural progenitor cells with a robust overexpression of FGF-2 we used a lentiviral gene transfer system. Over the last decade, lentiviral vectors have opened new perspectives for a robust delivery of transgene to tissue *(Trono, 2000)*. The advantage of this system is that unlike retroviral technology, lentivirus can transduce all cells and not only dividing cells. Briefly, lentiviral particles carrying the gene of interest are generated by "producing cells" transfected with the vector of interest, a packaging system, and the envelope (Figure 5). For our experiments, the key issue was to construct a vector carrying the human form

of FGF-2 and the gene coding for a green fluorescent protein (GFP) to recognise all transduced cells after transplantation.

FGF-2 is normally not secreted *(Nugent et al., 2000; Dvorak et al., 2006; Chlebova et al., 2009)*. Therefore, to test the possibility of a paracrine effect of FGF-2, an immunoglobulin signal peptide was fused to the cDNA coding for the 18kDa form of the human FGF-2 (bFGF) to facilitate the secretion of FGF-2 *(Rinsch et al., 2001)*. The construct was cloned into the pWPI bicistronic lentiviral vector. Bicistronic vectors allows for simultaneous expression of a transgene and GFP marker together to facilitate tracking of transduced cells. In lentiviral technology, vectors contain a transgene, expressed form and elongation factor-1α promoter or its intron-less version, EF1α, a robust transcriptional element in most cell types. The pWPI bicistronic lentiviral vector is an HIV-1 derived SIN (self-inactivating) vector containing the EF1 α promoter and the EMCV-IRES-GFP cistron. FGF-2 is cloned in the PmeI site located between the EF1α promoter and the IRES_GFP sequences (Figure 5). We also produced control vectors carrying only GFP or red tomato markers which were produced as follows: pFUGW containing the ubiquitin promoter controlling the expression of GFP, pWPXL containing the EF1α promoter and controlling the expression of GFP and RIX-PGK-Tom-W vector 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. EF1α, ubiquitin and PGK promoters are ubiquitous and active in neural cells. The lentiviral technology allowed us to successfully transduce genes of interest into NPCs, which was done at DIV3. On average, 50-70’000 NPCs per 35mm culture dish were transduced using doses ranging from $5x10^4$ to $5x10^5$ TU of either control or FGF-2 lentiviral vectors.

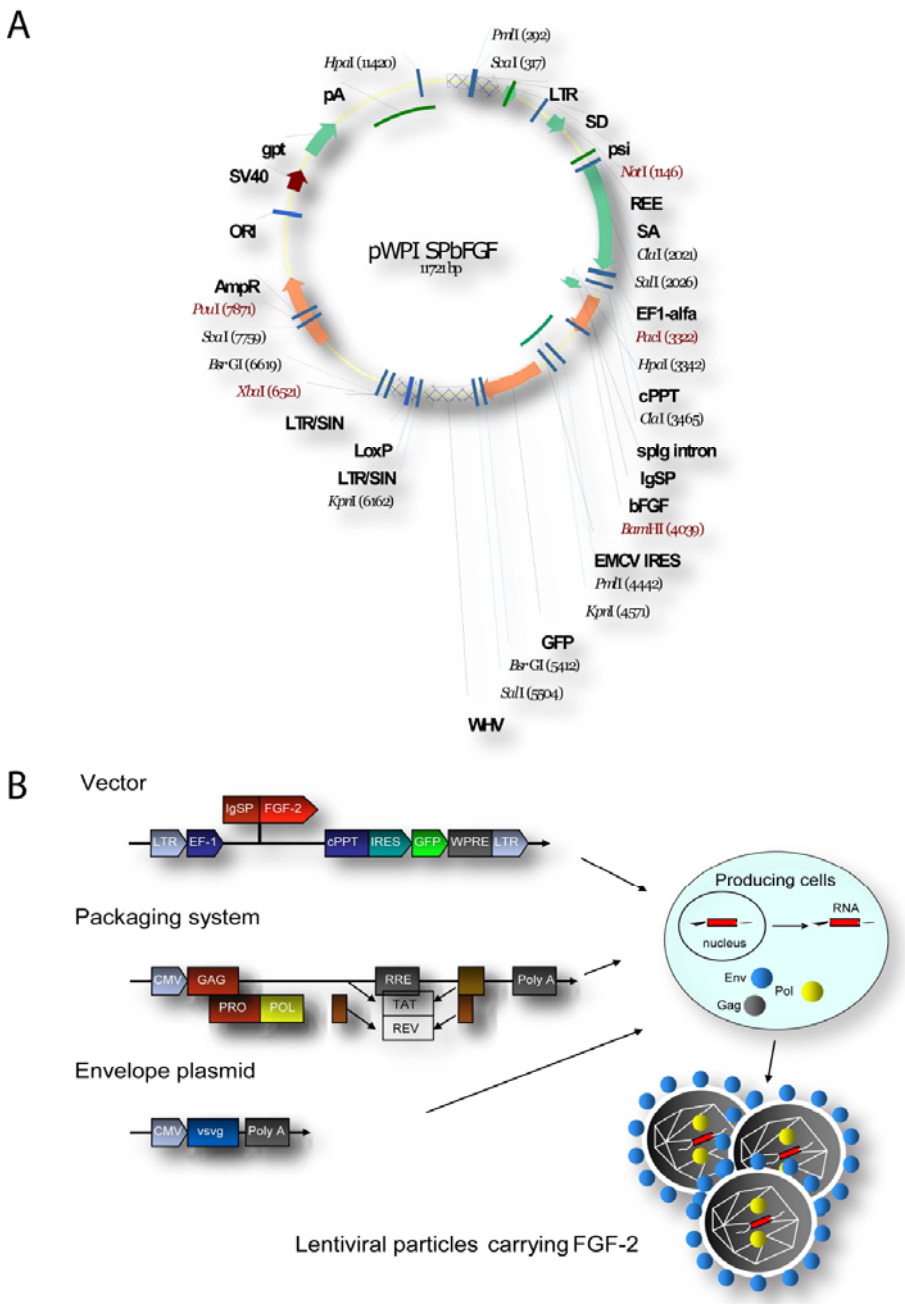

Figure 5: Lentiviral technology

(A) Map of the plasmid carrying the bFGF (FGF-2) vector, GFP and all relevant sequences necessary for its integration into the genome. (B) Schematical representation of the Vector carrying FGF-2 and GFP, the packaging system and the envelope plasmid that are required for the production of functional lentiviral particles from producing cells.

Neonatal rat model of NPCs transplantation after cerebral hypoxia-ischemia

To study the efficiency of FGF-2 overexpression in NPCs, in relation with the host tissue, we developed a neonatal rat model of transplantation in the intact cortex or after cerebral hypoxia/ischemia (Figure 6). The early postnatal phase was selected for our experimental approach since we believed that at that period the brain parenchyma is more permissive than during the adult period. The induction of cortical hypoxia/ischemia was performed by right common carotid electrocoagulation at P3 or P7 followed by 30 min of hypoxia at 6% of oxygen which was previously described by our colleagues in the laboratory *(Sizonenko et al., 2005)*. This model of cortical injury was selected because its distinctive alterations and neuronal loss in the deep layers of the somatosensory cortex, thus keeping supportive brain tissue for the migration of transplanted cells.

The effects of FGF-2 signalling in NPCs after transplantation in the cortex of neonatal rats and its relation with the host tissue was assessed using two approaches:

1) ***In vitro***, by developing an organotypic slice culture model to follow by time-lapse video recording, the migration of NPCs in a tissue context after *in vivo* cell transplantation.

2) ***In vivo***, by post hoc analysis of rat brains at different time points after grafting of FGF-2 transduced NPCs in the early postnatal rat cortex, this in normal conditions or after cerebral hypoxia/ischemia. These experiments provided evidence that FGF-2 overexpression not only enhanced the proliferative activity of transplanted NPCs but also significantly enhanced the migratory properties of grafted NPCs, making them more available for brain repair.

Transplantation techniques

For transplantation experiments, Wistar pups at postnatal day 3 (P3) were anesthetized with a mixture of Isofluran (Foren; 100%), O2 30%, and air 70%, and maintained in a stereotaxic frame. A small skin midline incision was performed at the surface of the skull and a small burr-hole was drilled on the right hemisphere. Approximately 20,000–50,000 (in 1 μl volume) of control or FGF-2-transduced NPCs were stereotactically injected in the cortex with a Hamilton syringe (coordinates from the bregma: 1.5-mm posterior, 2.5-mm lateral, 0.8-mm depth). After 1, 2 weeks or at later time points, rats were anesthetized with pentobarbital and sacrificed by intracardial perfusion of 0.9% saline followed by 4% PFA. Brains were extracted from the skull and postfixed in 4% PFA at 4°C and cryoprotected with sucrose 30% if cut on a cryostat or prepared for Vibratome section.

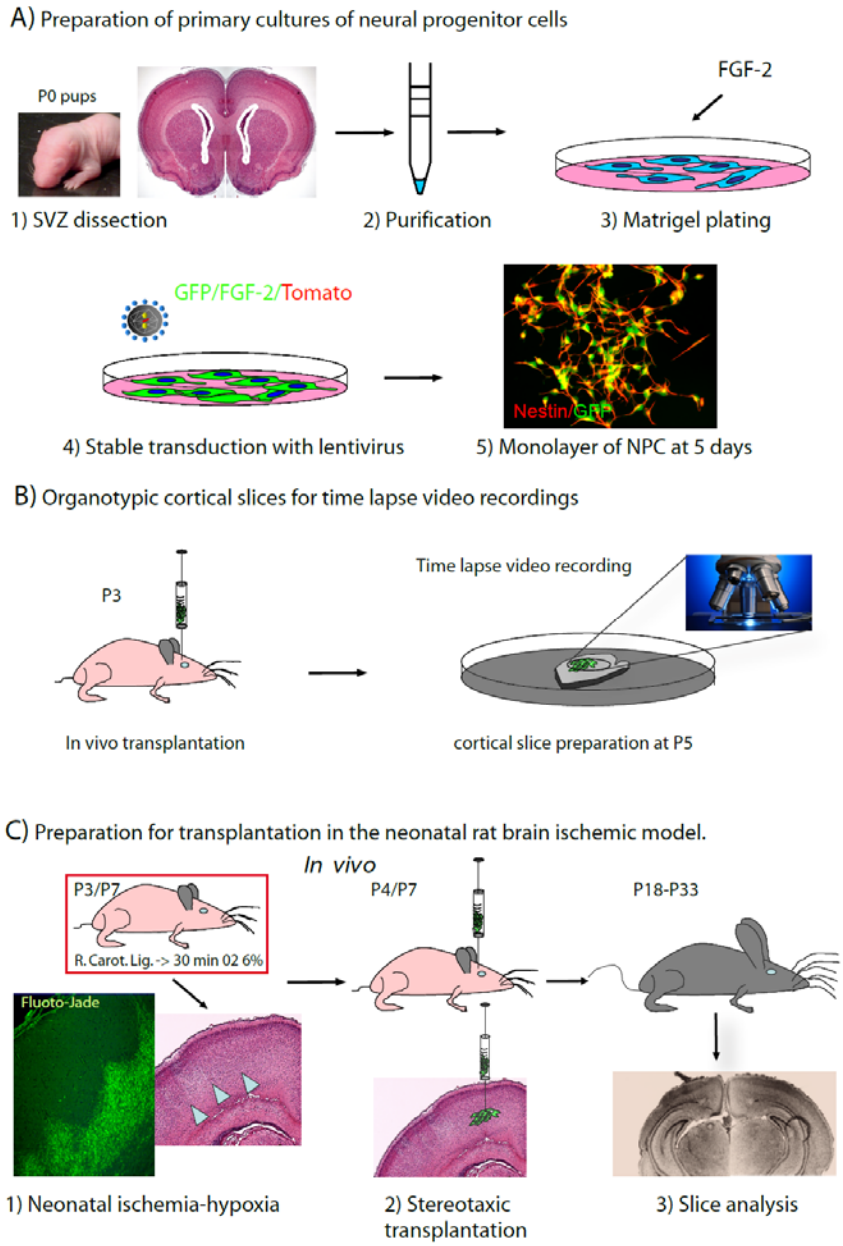

Figure 6: Elaboration of a transplantation model using a lentiviral technology

A) The SVZ of newborns pups at P0 was mechanically dissected; cells were then purified by centrifugation through a Percoll gradient, and then plated on a matrigel dish supplemented with FGF-2. Cells were then transduced with a lentiviral vector carrying the genes coding for GFP alone, FGF-2 and GFP or redTomato. A monolayer of NPCs was obtained at 5 days, with stable and robust GFP expression with positivity for the immature marker Nestin.

B) To study the migration of NPCs in a tissue context, using video time lapse recording organotypic slices were prepared from cortical sections 3 days after in vivo transplantation of NPCs.

C) The behaviour of transplanted NPCs was assessed after cerebral ischemia/hypoxia obtained by right common carotid electro-coagulation in early postnatal rat at P3 or P7 followed by 30 min of hypoxia at 6% Oxygen level.

MAIN OBJECTIVES OF THE THESIS

The identification of molecular signals that can enhance the capacity of grafted NPCs to invade an injured area in the brain is of key importance for neural cell replacement therapies. In this work we evaluate the possibility that the manipulation of neural progenitor cells prior to transplantation increases the efficiency of grafted cells to replace damaged or lost cells after brain injury, thus enhancing their potential for efficient brain repair strategies.

The first objective of our work was to establish a novel model of neural progenitor cell transplantation in the early postnatal rat cortex, using a lentiviral technology. FGF-2 is known to be a niche factor involved in the proliferation and maintenance of neural progenitor cells in the physiological germinative niches of the brain. We used a lentiviral technology to induce a robust over-expression of FGF-2 in neural progenitor cells prior to transplantation. We then grafted engineered neural progenitors in the intact cortex of early post natal rats, or after cerebral neonatal hypoxia/ischemia.

Our immediate goal with this strategy was to assess whether FGF-2 transduced NPCs showed an improved proliferation and migration potential after transplantation in the intact cortex. Two approaches were then used to assess the behaviour of transplanted NPCs:

In vitro, using an organotypic slice culture model for online time lapse video recording of NPCs;

In vivo, with post hoc analysis of brain cortical slice at different time points after transplantation.

Furthermore, we wanted to assess if FGF-2 overexpression in NPCs could enhance the pool of grafted cells available for brain repair after transplantation in the ischemic cortex.

The second objective of our work was to test the possibility that ectopic neurovascular clusters could be generated in cortical regions of the rat brain and that these ectopic neurovascular clusters could be available for brain repair. In the germinative zone of the SVZ and the DG, neural stem cells are maintained within niches where microenvironmental cues play a fundamental role in their maintenance and proliferation. Within cortical regions, dormant resident stem cells could play a critical role in brain repair after cerebral ischemia, but the endogenous potential of these cells has been shown to be insufficient to adequately replace lost or damaged cortical cells.

One alternative would be to create in the cortex ectopic neurogenic niches that could be used to repair the injured brain. For this we tested the possibility that FGF-2 overexpression in neural progenitors prior to transplantation could enhance the interaction with their close host microenvironment, thus maintaining them in a more immature and proliferative state. We show that a particular characteristic of FGF-2 signalling in transplanted NPCs is their close association with host vessels, which maintains NPCs in a more immature and proliferative state. These neurovascular cortical clusters appeared to keep a neurogenic potential after being challenged by an ischemic injury.

RESULTS

SPECIFIC AIMS

1) (Publication 1)

In the initial part of the thesis we investigate how FGF-2 overexpression in NPCs enhances the potential of transplanted cells to repair the ischemic brain. We have for this purpose elaborated a novel model of NPCs transplantation in a rat model of neonatal cerebral ischemia/hypoxia using lentiviral gene transfer to overexpress FGF-2 in NPCs, and label them with GFP prior to their grafting. The efficiency of FGF-2 overexpression was assessed *in vitro* by analysing the amount of FGF-2 protein produced in the supernatant of FGF-2 transduced NPCs in culture.

The first objectives were to assess whether FGF-2 transduction in NPCs could enhance their proliferative and migratory behaviour thus keeping them in a more immature state after transplantation. These properties were tested on rat cortical slice using time lapse video recording after deposition of NPCs islet directly on the slice or by recording the progression of NPCs 2 days after *in vivo* transplantation and organotypic slice preparation. We further tested the behaviour of FGF-2 transduced NPCs *in vivo* after transplantation in the cortex of P3 rat, with a focus on the proliferation and immature state after engraftment. Moreover, the migration capacities from the site of injection was assessed, this compared to control NPCs. With this initial set of experiment we showed that effectively, after FGF-2 transduction NPCs can be kept more immature after transplantation as seen by the increased proliferation rate and the maintenance of nestin positivity. Moreover, a significant increase in the migration potential of FGF-2 transduced NPCs was seen on organotypic cortical slice but also after *in vivo* analysis.

Secondly, we investigate whether FGF-2 overexpression in NPCs could provide a significant advantage after cerebral hypoxia-ischemia. We transplanted FGF-2 transduced NPCs after cortical injury in the somatosensory cortex induced in the early postnatal period by common carotid ligation followed by 30 min of hypoxia at 6% oxygen. In this neonatal ischemic model we showed that the pool of NPCs available for brain repair was increased after FGF-transduction prior to transplantation, thus almost doubling the amount of immature neurons migrating in the infragranular cortex towards the site of injury. Interestingly one month after transplantation some FGF-2 transduced NPCs had differentiated in cells exhibiting mature neuronal characteristics.

With this first work we have demonstrated that the manipulation of NPCs with FGF-2 prior to transplantation, enhances their proliferative and migratory behaviour after transplantation by keeping NPCs in a more immature state, thus increasing the pool of migratory immature neurons available to replace lost cells in the sites of cortical injury.

2) (Publication 2)

In the second part of this thesis we investigate the close relation between grafted NPCs and the host microenvironment, as it has been strongly suggested that the intimate milieu surrounding NPCs in the germinative zones creates a supportive niche crucial for the maintenance of endogenous neural progenitor cells. A particular feature of FGF-2 transduced NPCs was their striking tendency to associate and form clusters with blood vessel after engraftment in the rat cerebral cortex. The objectives of this part of the work were to assess whether host blood vessels play a significant role for the supportive microenvironment of NPCs after transplantation and if this association maintains them in a more immature and proliferative rate.

To test this hypothesis, we investigated the proliferative and immature state of FGF-2 transduced NPCs associated with blood vessels, in comparison with FGF-2 transduced NPCs non-associated with blood vessels. FGF-2 transduced NPCs showed a distinct tendency to form clusters associated with blood vessels and this close interaction maintained them in a more immature and proliferative state at least two weeks after transplantation. Furthermore, to challenge the hypothesis that these vessels associated clusters of NPCs could keep a neurogenic potential, we applied cerebral hypoxia-ischemia injury, this after transplantation. As a proof of principle, this showed that compared to controls, injured areas of the cortex were able to recruit transplanted FGF-2 transduced NPCs and that some of them had differentiated into immature neurons migrating away from vessels in the ischemic zones.

In summary, in this second part of the work, we gave evidence that host vessels provide a supportive microenvironment for transplanted NPCs, keeping them in a more immature and proliferative state. FGF-2 signalling in NPCs appears to be critical to induce this association with the host vasculature. Moreover, the neurogenic potential of these ectopic neurovascular clusters was kept after transplantation making them available to migrate and differentiate in immature neurons after cerebral ischemia.

PUBLICATION 1

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

Alexandre G. Dayer, **Benoit Jenny**, Marc-Olivier Sauvain, Gael Potter, Patrick Salmon, Eloisa Zgraggen , Michiko Kanemitsu, Eduardo Gascon, Stephan Sizonenko, Didier Trono and Jozsef Z. Kiss.

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

Alexandre G. Dayer,[1,2,*] Benoit Jenny,[1,3,*] Marc-Olivier Sauvain,[4] Gael Potter,[1] Patrick Salmon,[1] Eloisa Zgraggen,[1] Michiko Kanemitsu,[1] Eduardo Gascon,[1] Stephane Sizonenko,[1] Didier Trono[4] and Jozsef Z. Kiss[1]

[1]Department of Neurosciences, University Medical Center, University of Geneva Medical School, Departments of [2]Adult Psychiatry and [3]Neurosurgery, University Hospital of Geneva, CH-1211 Geneva 4 and [4]School of Life Sciences, Ecole Polytechnique Federale de Lausanne (EPFL), CH-1015 Lausanne, Switzerland

*These authors contributed equally to this work.

Correspondence to: Jozsef Zoltan Kiss, Department of Neurosciences, University Medical Center (CMU), Rue Michel-Servet 1, 1211 Genève 4, Switzerland
E-mail: Jozsef.Kiss@medecine.unige.ch

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 anesthesized 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

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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

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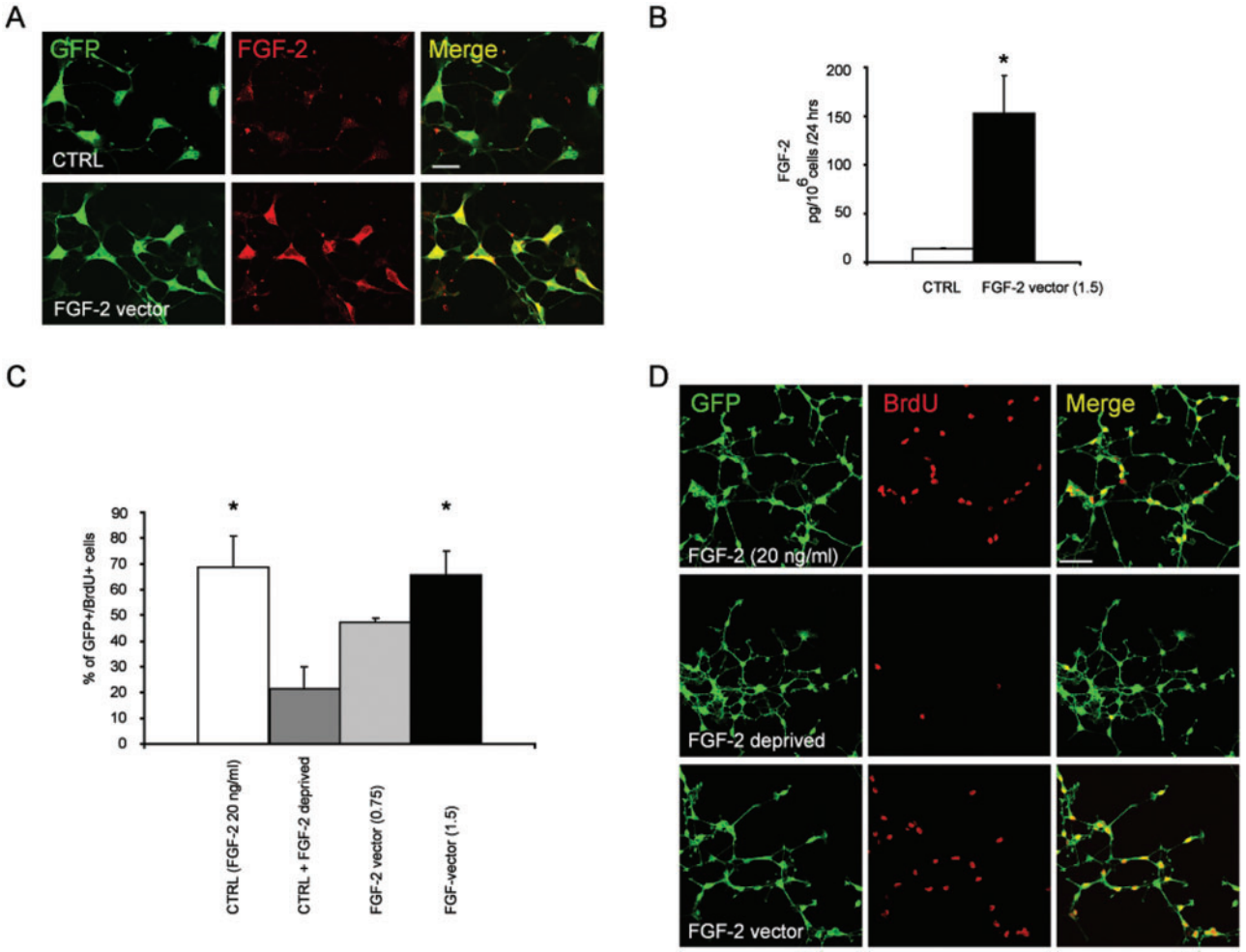

Fig. 1 FGF-2 transduction of neural progenitor cells (NPCs) maintains a pool of proliferative and immature cells *in vitro*. (**A**) Confocal images showing FGF-2-transduced NPCs displaying an intense FGF-2 cytoplasmic immunoreactivity compared to control NPCs. (**B**) Elisa quantification demonstrating 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

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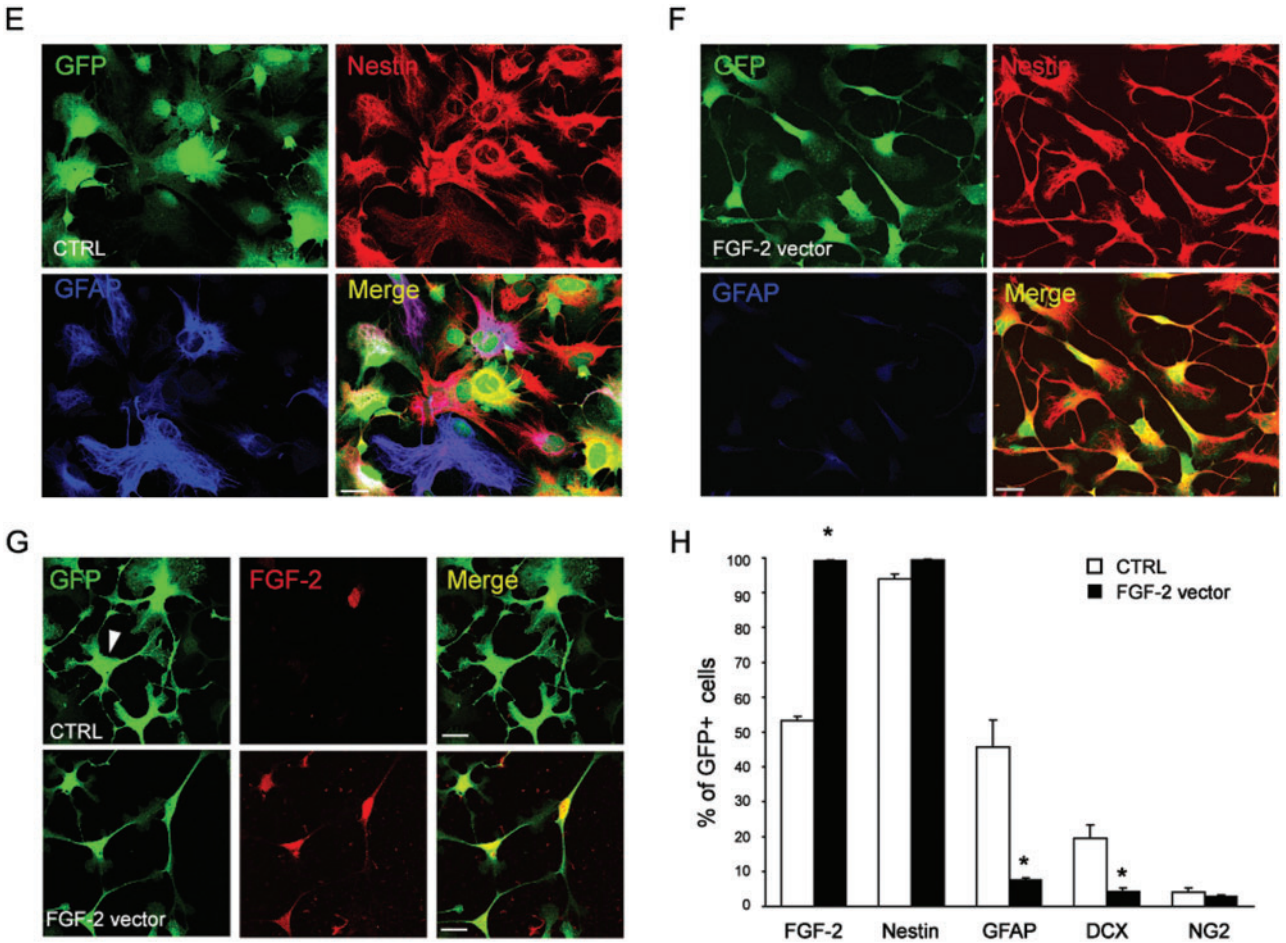

Fig. 1 Continued.

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

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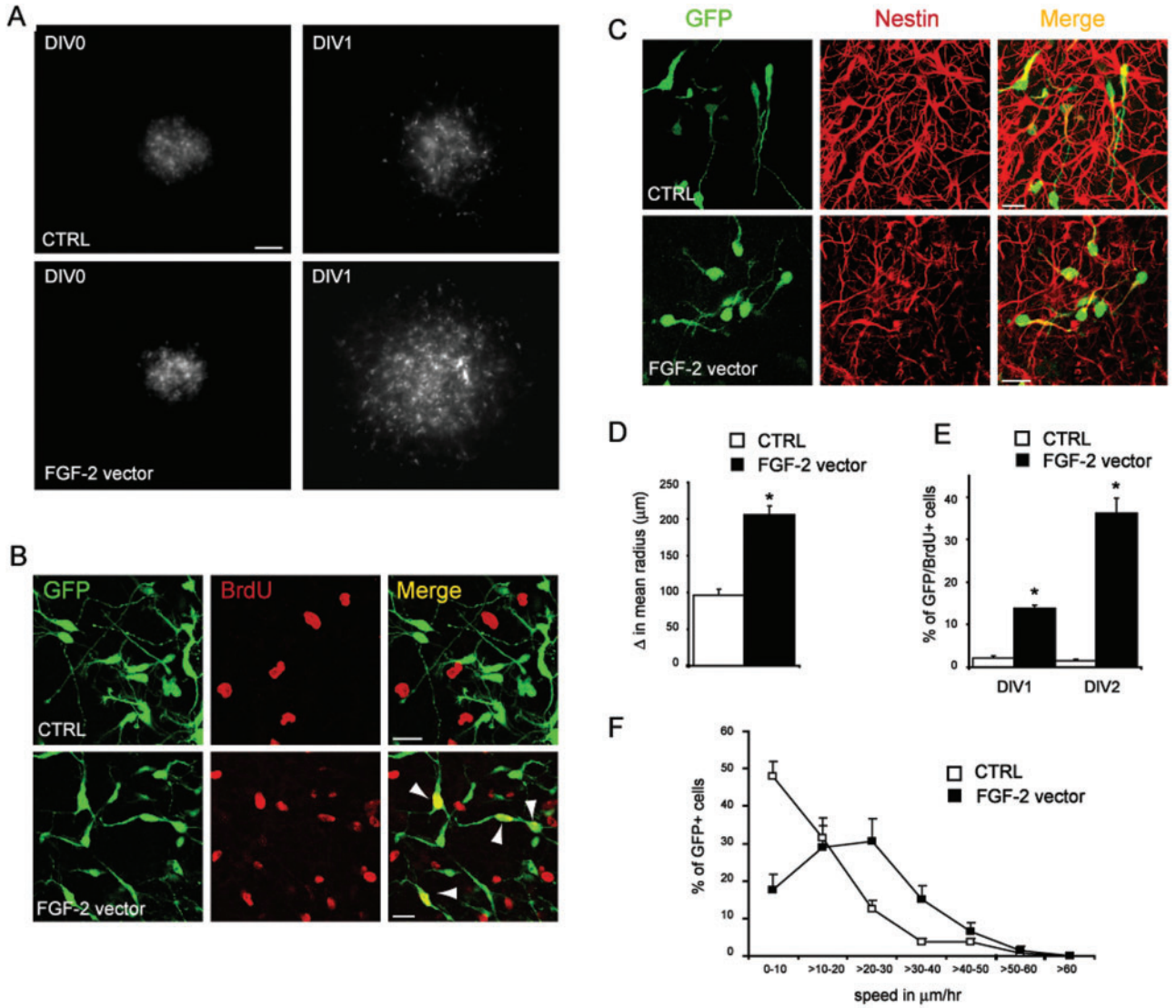

Fig. 2 FGF-2 transduction maintains a pool of proliferative and migrating NPCs after transplantation on cortical slices. (**A**) Epifluorescence images showing a larger increase in the size of a FGF-2-transduced GFP positive islet between DIV0 and DIVI compared to a control islet. (**B**) Confocal images showing that at DIVI 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 DIVI, 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 DIVI 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,

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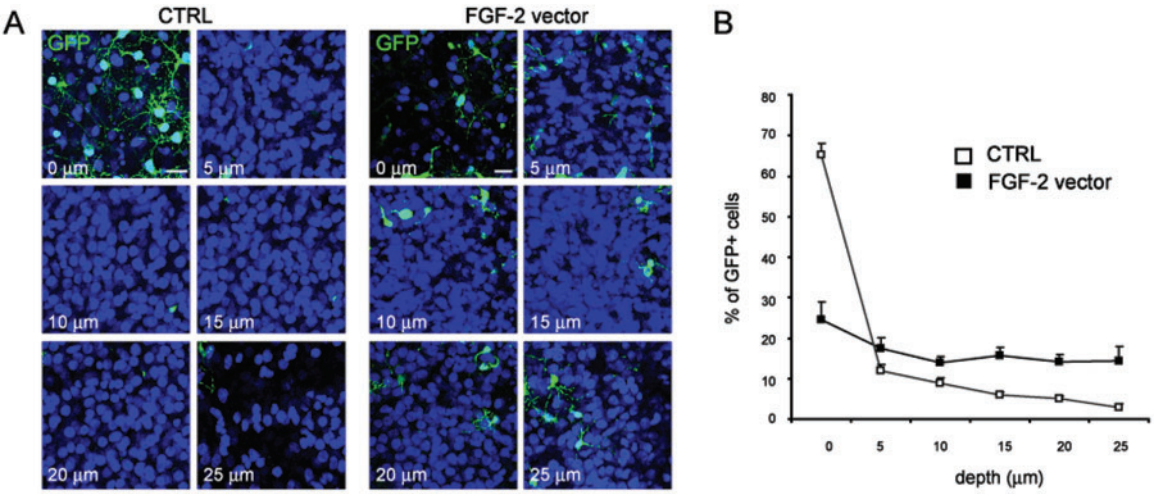

Fig. 3 FGF-2 transduction increases the invasiveness of NPCs in cortical slices.(**A**) Confocal Z-stacks at 5 µm interval showing that FGF-2-transduced NPCs invade the whole thickness of the slice, whereas control NPCs are mainly located at the surface of the slice. Nuclear counterstain To-Pro-3 (blue). (**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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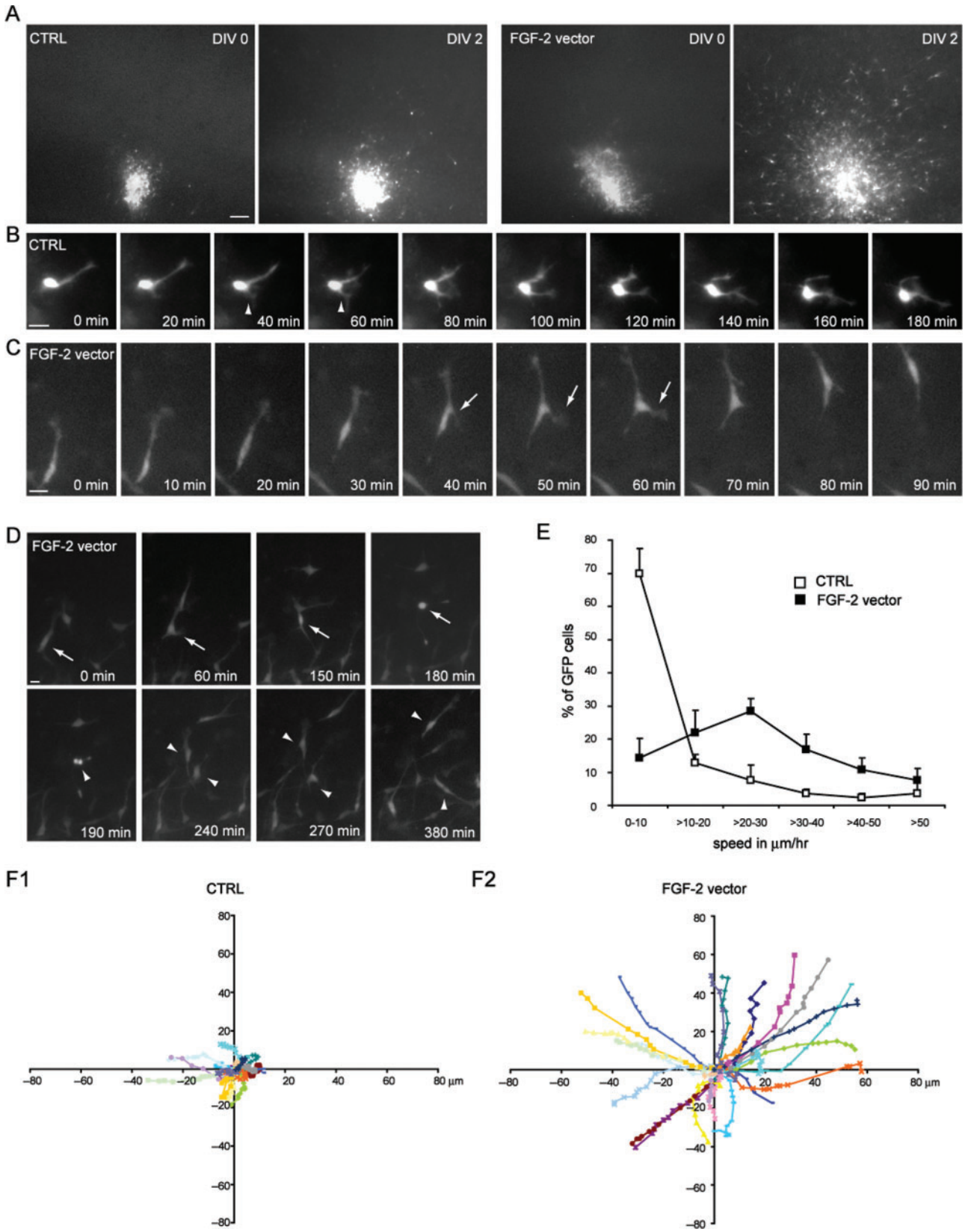

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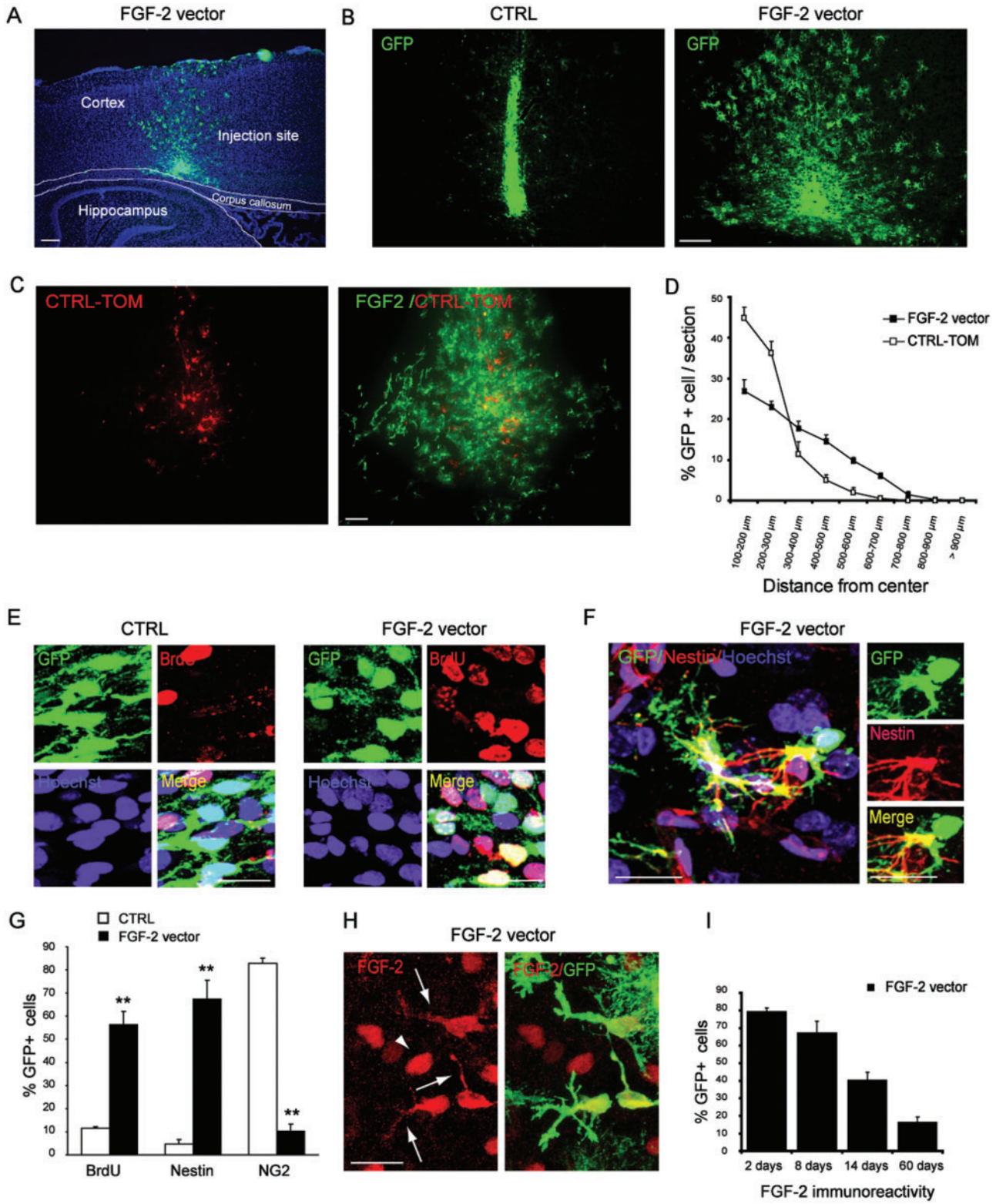

Fig. 5 FGF-2 transduction increases the dispersion of NPCs and maintains their proliferative immature phenotype after *in vivo* transplantation. (**A**) Epifluorescent image showing the site of transplantation in the cortex (left). (**B**) Dispersion of grafted control NPCs (middle) and FGF-2-transduced 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

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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.

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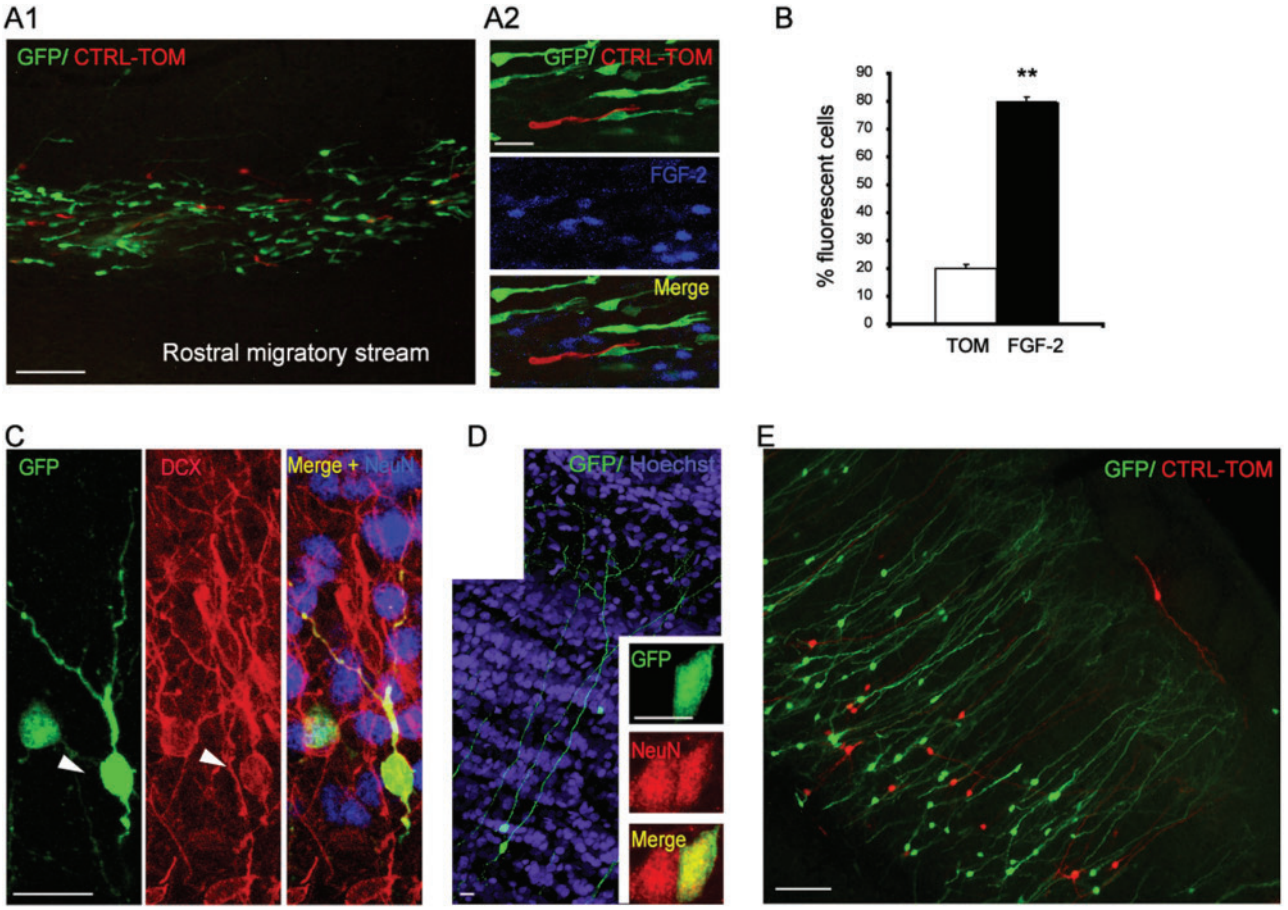

Fig. 6 FGF-2-transduction increases the pool of grafted neurons in the olfactory bulb. (A1) Epifluorescent image of a sagittal section showing FGF-2-transduced NPCs and tomato-labelled NPCs migrating along the rostral migratory stream 1 month after transplantation in the SVZ. (**A2**) Confocal images 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.

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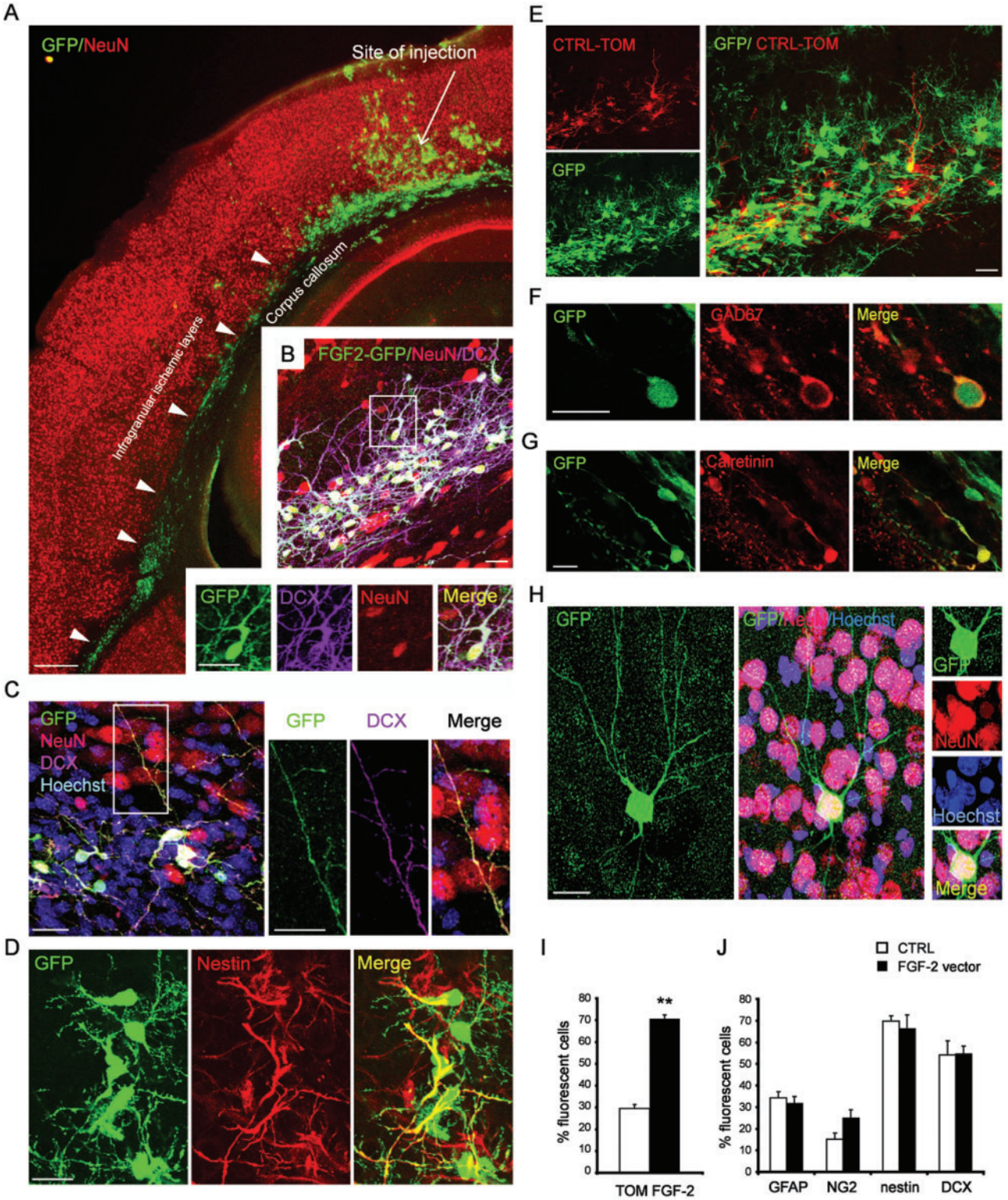

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and structural repair. Here we developed a novel system allowing the over-expression of FGF-2 in NPCs and studied its effects on several cellular functions after transplantation into the early postnatal rat cortex. We provide direct evidence for the 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

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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 hoc* 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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PUBLICATION 2

FGF-2 overexpression in transplanted neural progenitors promotes perivascular cluster formation with a neurogenic potential

Benoit Jenny, Michiko Kanemitsu, Oleg Tsupykov, Gael Potter, Patrick Salmon, Eloisa Zgraggen, Eduardo Gascon, Galina Skibo, Alexandre G. Dayer and Jozsef Z. Kiss

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Fibroblast Growth Factor-2 Overexpression in Transplanted Neural Progenitors Promotes Perivascular Cluster Formation with a Neurogenic Potential

BENOIT JENNY,[a,b] MICHIKO KANEMITSU,[a] OLEG TSUPYKOV,[c] GAEL POTTER,[a] PATRICK SALMON,[a] ELOISA ZGRAGGEN,[a] EDUARDO GASCON,[a] GALINA SKIBO,[c] ALEXANDRE G. DAYER,[a,d] JOZSEF Z. KISS[a]

[a]Department of Neurosciences, University Medical Center, University of Geneva Medical School, Geneva, Switzerland; [b]Department of Neurosurgery, University Hospital of Geneva, Geneva, Switzerland; [c]Department of Cytology, Bogomoletz Institute of Physiology, Kiev, Ukraine; [d]Department of Adult Psychiatry, University Hospital of Geneva, Geneva, Switzerland

Key Words. FGF-2 • Neural progenitors • Perivascular neurogenic cluster • Brain repair • Ischemia

ABSTRACT

Stem/progenitor cell-based therapies hold promises for repairing the damaged nervous system. However, the efficiency of these approaches for neuronal replacement remains very limited. A major challenge is to develop pretransplant cell manipulations that may promote the survival, engraftment, and differentiation of transplanted cells. Here, we investigated whether overexpression of fibroblast growth factor-2 (FGF-2) in grafted neural progenitors could improve their integration in the host tissue. We show that FGF-2-transduced progenitors grafted in the early postnatal rat cortex have the distinct tendency to associate with the vasculature and establish multiple proliferative clusters in the perivascular environment. The contact with vessels appears to be critical for maintaining progenitor cells in an undifferentiated and proliferative phenotype in the intact cortex. Strikingly, perivascular clusters of FGF-2 expressing cells seem to supply immature neurons in an ischemic environment. Our data provide evidence that engineering neural progenitors to overexpress FGF-2 may be a suitable strategy to improve the integration of grafted neural progenitor cells with the host vasculature thereby generating neurovascular clusters with a neurogenic potential for brain repair. STEM CELLS *2009;27:1309–1317*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The damaged mammalian brain, in particular the cerebral cortex, has a poor regenerative capacity. One of the many reasons accounting for this limited repair potential is the lack of a local population of neural progenitor cells (NPCs). Therefore, much focus has been placed on supplying a pool of NPCs by transplantation [1]. However, after engraftment in the cortex, NPCs rapidly lose their immature and proliferative phenotype and differentiate into glial cells, which severely limits their potential for efficient cell replacement [2–4]. In the adult nervous system, active neurogenesis is maintained in the subventricular zone of the lateral ventricle (SVZ) and the subgranular zone of dentate gyrus (SGZ) in the hippocampus [5]. In these regions, adult type stem cells and their progeny are localized in neural niches that provide a specific microenvironment where stem cells replenish themselves through self-renewal and give rise to different progenies through asymmetric divisions [6–8]. A particularly interesting feature of the neurogenic niche is the intimate association of NPCs with vessels and their extravascular basal lamina in both the SVZ and the SGZ [6, 9, 10]. Endothelial cells and the specialized basal lamina are believed to provide attachment for NPCs, generate signals to maintain stem cell properties and regulate proliferative activity [7, 11].

It may be possible to improve the brain repair capacities of transplanted NPCs by stimulating their interaction and ability to associate with the host vasculature. Fibroblast growth factor-2 (FGF-2) appears to play a key role in sustaining self-renewal of neural progenitor/stem cells in vitro [12] and maintaining immature proliferative populations in neurogenic

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Correspondence: Jozsef Z. Kiss, M.D., Department of Neurosciences, University Medical Center, University of Geneva Medical School, Rue Michel-Servet 1, 1,211 Genève 4, Switzerland. Telephone: +41223795202; Fax: +41223795402; e-mail: Jozsef.Kiss@medecine.unige.ch Received August 11, 2008; accepted for publication February 10, 2009; first published online in STEM CELLS *EXPRESS* February 26, 2009. © AlphaMed Press 1066-5099/2009/$30.00/0 doi: 10.1002/stem.46

niches in vivo [13]. In a previous study, we developed a novel system to overexpress FGF-2 in transplanted NPCs and showed that this manipulation significantly enhanced the capacity of these cells to generate a pool of progenitors and immature neurons [14]. In this work, we demonstrate that FGF-2 overexpression promotes the association of transplanted NPCs with vessels. The perivascular environment maintains grafted cells in an undifferentiated and proliferative state after transplantation in an intact cortex. Most importantly, these neurovascular clusters of progenitor cells can generate immature neurons in an ischemic environment, suggesting that they could function as ectopic neurovascular niches.

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

Primary cultures of NPCs were established from the subventricular zone of newborn Sprague Dawley rat brain as described previously [14]. The SVZ from coronal slices of newborn rat brains were dissected, mechanically dissociated and trypsinized. NPCs were purified using a 22% Percoll gradient centrifugated as previously described [15, 16], and seeded at 4×10^5 cells/dish concentration onto matrigel-coated dishes (1:500). Cells were allowed to expand in Neurobasal medium (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) supplemented with 20 ng/ml FGF-2 (human recombinant, R&D, Minneapolis, http://www.rndsystems.com), 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 or FGF-2 lentiviral vector. For transplantation, cells were then trypsinized at DIV5 centrifugated and re-suspended in Neurobasal medium.

Design and Production of Lentiviral Vectors

The description of the design and production of lentiviral vectors have been published previously [14]. Briefly, for the FGF-2 construct, a cDNA coding for the 18 kDa form of the human FGF-2 (bFGF) was fused to an immunoglobulin signal peptide to facilitate the secretion [17], and cloned into a pWPI bicistronic lentiviral vector. For control lentiviral vector, the expression of green fluorescent protein (GFP) was under the control of the pFUGW ubiquitin promoter (http://tronolab.epfl.ch). For control (GFP) and FGF-2 (GFP) lentiviral vectors we used titers ranging from 10^8 to 10^9 transducing units (TU)/ml. For transduction, doses ranging from 5×10^4 to 5×10^5 TU were used for either control or FGF-2 lentiviral vectors on NPCs dishes (~50,000–75,000 cells per 35-mm-culture dish).

NPCs Transplantation

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. A small skin midline incision was performed at the surface of the skull, and a small burr-hole was drilled on the right hemisphere. Approximately 20,000–50,000 (in 1 μl volume) control or FGF-2-transduced NPCs were stereotactically injected in the cortex with a Hamilton syringe (coordinates from the bregma: 1.5-mm posterior, 2.5-mm lateral, 0.8-mm depth). After 1 or 2 weeks, rats were anesthetized by pentobarbital and sacrificed by intracardial perfusion of 0.9% saline followed by 4% PFA. Brains were extracted from the skull and postfixed in 4% PFA at 4°C and cryoprotected with sucrose 30% if cut on a cryostat or prepared for Vibratome section.

Hypoxia-Ischemia Injury

Wistar pups that had previously received FGF-2-transduced NPCs transplantation in the somato-sensory cortex at P3 were subjected to hypoxia-ischemia injury at P7 following the same protocol as described previously [18]. Briefly, rats were anesthetized and a right common carotid permanent coagulation was performed by electrocoagulation with a bipolar instrument. This was followed by 30 minutes of hypoxia at 6% O_2 in a chamber kept at 37°C. Pups were then returned to the dam and breastfed ad libitum. After 7 days (P14), rats were anesthetized, sacrificed, and brains were extracted as described earlier.

Tissue Processing and Immunohistochemistry

Brain sections were cut with a cryostat (20-μm thick) (Leica CM 3050S) or on a Vibratome 1,500 (60-μm-thick sections) (Vibratome, St. Louis); sections were washed three times with 0.1 M phosphate buffer saline (PBS); incubated overnight at 4°C with a primary antibody diluted in PBS/0.5% bovine serum albumin (BSA)/0.3% Triton X-100; washed in PBS; incubated with the secondary antibodies against the appropriate species; nuclear counterstained with Hoechst 33,342 (Molecular Probes, Eugene, OR, http://probes.invitrogen.com; Invitrogen). The following primary antibodies were used: monoclonal mouse anti-Nestin clone rat-401 (1:1,000; Chemicon, Temecula, CA, http://www.chemicon.com), monoclonal mouse anti-RECA-1, clone HIS52 (1:250; Serotec), polyclonal rabbit anti-glial fibrillary acidic protein (GFAP) (1:500; Dakocytomation, Glostrup, Denmark, http://www.dakocytomation.com), polyclonal goat anti-doublecortin (DCX) (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com), polyclonal rabbit anti-GFP (1:1,000; Molecular Probes, Invitrogen), polyclonal goat anti-GFP (1:5,000; Novus Biologicals), monoclonal mouse anti-bromodeoxyuridine (BrdU), clone BMC 9,318 (1:100; Boehringer-Mannheim, Mannheim, Germany, http://www.boehringer.com), monoclonal rat anti-BrdU, clone BU1/75 (ICR1) (1:100; Oxford Biotech). The following secondary antibodies were used: anti-rabbit 568, 647, and 488, anti-mouse 568, 488, and 647, anti-goat 555 and 647, and anti-rat 555 (Molecular Probes, Invitrogen). For BrdU labeling, sections were incubated for 30 minutes at 37°C, in 2 N HCl for DNA denaturation followed by standard incubation.

BrdU Incorporation Experiments In Vivo

Intraperitoneal BrdU (50 mg/kg) was injected twice daily 3 days before sacrifice.

Image Processing and Quantification

A Zeiss LSM 510meta confocal microscope equipped with a 40× objective was used to obtain images of grafted GFP+ cells in sagittal or coronal sections. To image perivascular clusters, 1-μm-thick optical sections z-stacks were obtained and reconstructed in 3D. For all quantifications, the tract of injection was excluded and transplanted FGF-2-transduced NPCs were analyzed according to their association with cerebral vessels walls. NPCs were defined to be associated with vessels when their cell body was in direct contact with the wall of blood vasculature. For quantification of NPCs association with blood vessels, 1,182 FGF-2-transduced NPCs

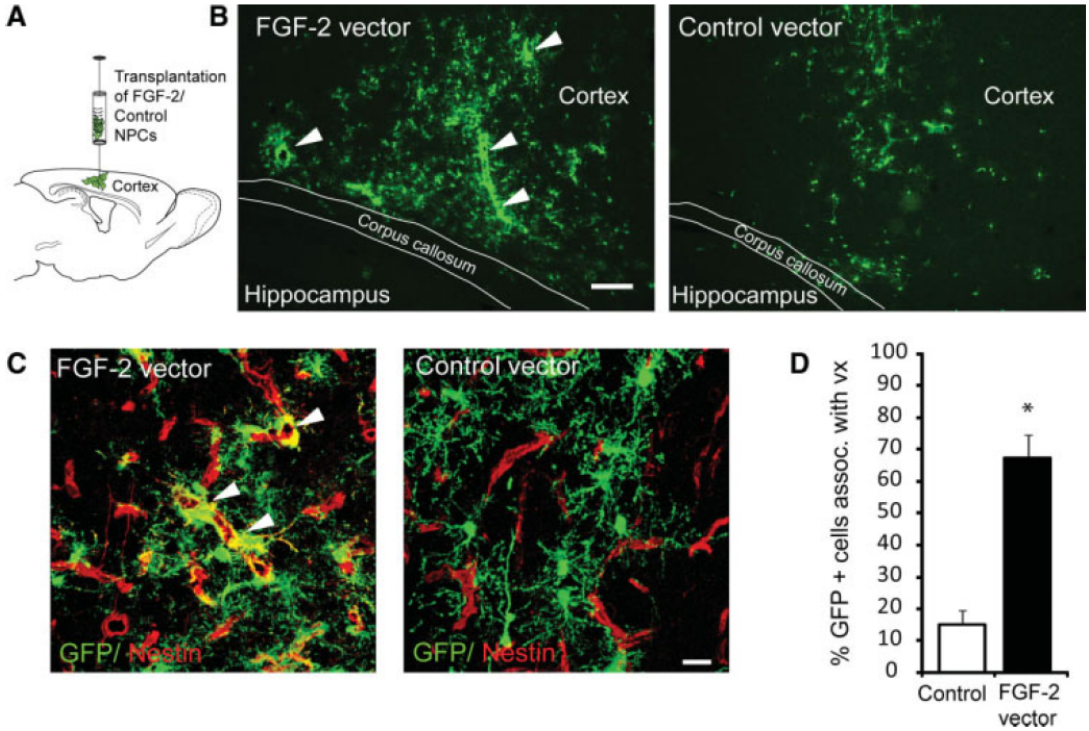

Figure 1. FGF-2 overexpression promotes the association of transplanted NPCs with blood vessels. **(A):** Schema of a rat brain sagittal section, showing the cortical localization of NPCs transplantation. **(B):** Epifluorescent images of sagittal brain sections showing the dispersion of FGF-2-transduced NPCs (left image) and control NPCs (right image), in the rat parietal cortex 2 weeks after transplantation. FGF-2 transduced NPCs have a clear tendency to accumulate around blood vessels (white arrow-heads, left image). **(C):** Confocal images of FGF-2-transduced NPCs (left image) and control NPCs (right image) 2 weeks after transplantation in the cerebral cortex, showing that FGF-2 transduced NPCs are in closed contact with blood vessels (white arrow-heads) compared with control NPCs. **(D):** Graph showing the percentage of FGF-2-transduced and control NPCs in association with blood vessels 2 weeks after transplantation. A total (100%) of 1,182 and 171 cells were counted in the FGF-2-transduced and control population, respectively. (Error bars indicate SEM, *, $p < .001$, t test). Abbreviations: control vector, GFP vector transduction in NPCs; FGF-2, fibroblast growth factor-2; FGF-2 vector, FGF-2 GFP vector transduction in NPCs; GFP, green fluorescent protein; NPCs, neural progenitor cells; vx, blood vessel. Scale bar = 200 μm for epifluorescence images and 20 μm for confocal images.

($n = 8$ transplanted animals) and 171 control NPCs ($n = 4$ transplanted animals) were counted. The surface area of blood vessels was measured on confocal images by using Zeiss LSM Image Browser, where transplanted cells were visible along with nestin-positive endothelial cells. The surface fraction of vessels was then determined by dividing the total surface area of vessels by the total sample area. The data was obtained from 4 animals transplanted with control NPCs and six animals transplanted with FGF-2 transduced NPCs. The total vessel surface measured in control group was 2.437 and 1.725 mm^2 in FGF-2 group. For BrdU and nestin quantification, 876 FGF2-transduced NPCs ($n = 5$ transplanted animals, 1-week survival) and 1,123 cells ($n = 6$ transplanted animals, 2-week survival) were analyzed, respectively. For BrdU quantification at 2 weeks, 1,008 FGF-2-transduced NPCs ($n = 5$ transplanted animals) were used. Cluster quantification was done by first counting the number of cells in direct contact to each other and then by determining whether the clusters were associated or not to blood vessels. Clusters were quantified on the basis of 898 cells ($n = 5$ transplanted animals) at 1 week and 632 cells ($n = 3$ transplanted animals) at 2 weeks. For each category composition: single cells, 2 cells, 3 cells, and more than 3 cells, we counted, respectively, 621, 86, 18, and 10 categories at 1 week and 431, 128, 17, and 22 categories at 2 weeks. For DCX quantification, FGF-2-transduced NPCs were quantified at P14 after a cerebral hypoxia-ischemia at P7 (722 cells, $n = 3$ transplanted animals) and at P14 in control brains (820 cells, $n = 3$ transplanted animals), also control NPCs were quantified at P14 after cerebral hypoxia-ischemia at P7 (285 cells, $n = 3$ transplanted animals).

Student t tests were performed using the software Minitab 13.32 and one-way analysis of variance using GraphPad Prism version 4.0.

Results

FGF-2-Transduced NPCs Associate with Blood Vessels

To explore the relationship of transplanted NPCs with the host vasculature, control (GFP lentiviral vector), and FGF-2-transduced (FGF-2-GFP lentiviral vector) NPCs were transplanted in the rat cortex at P3 (Fig. 1A), and brains were analyzed 1 and 2 weeks after engraftment. In agreement with our previous studies [14], we observed that FGF-2 overexpression increased the dispersion of grafted NPCs compared with controls (Fig. 1B). Moreover, we found that the majority of FGF-2-transduced NPCs consistently associated with the

vasculature whereas control cells did not show such a tendency (Fig. 1B, 1C). Confocal analysis confirmed that many FGF-2-transduced NPCs were in direct contact with vessels and often formed perivascular clusters (Figs. 2A, 2B, 1C). Cell counts indicated that 2 weeks after transplantation, the majority of FGF-2 transduced NPCs, 67.2% ± 7.3% (mean ± SEM) were in contact with nestin-positive vessels compared with only 15.2% ± 4.3% (mean ± SEM) of control NPCs ($p < .001$, *t* test) (Fig. 1D). As FGF-2 is known for its strong angiogenic potential [19], we examined the possibility that transplanted FGF-2 overexpressing cells could induce angiogenesis in the host tissue. For this purpose, we determined the surface fraction of blood vessels (total measured surface area of vessels divided with the total sample area of confocal images). We found that blood vessels occupied 6.1% ± 1.4% of the total area in the control group and 5.6% ± 1.3% (mean ± SEM) in the FGF-2 group suggesting that FGF-2-transduced NPCs may not induce local angiogenesis. Using a specific marker for endothelial cells, RECA-1, which recognizes a rat endothelial cell surface antigen, we further confirmed that a very high percentage 78.8% ± 4.7% (mean ± SEM) of transplanted FGF-2-transduced NPCs were associated to blood vessels. This value is slightly higher than that obtained with nestin staining of endothelial cells (67.2% ± 7.3%). We observed that in the case of nestin labeling, substantially less small-sized capillaries are seen and thus not considered for vessel association. Therefore, the proportion of vessels association could be slightly underestimated compared with the experiments in which RECA-1 was used to label endothelial cells. Together, these data indicate that FGF-2 overexpression promotes the association of NPCs with the vasculature.

The Perivascular Environment Maintains Progenitors in a Proliferative and Undifferentiated State

Focusing on FGF-2-transduced cells, we next sought to determine the influence of the perivascular environment on the proliferative activity and differentiation state of NPCs. First, we assessed the proliferation of NPCs by intraperitoneal injection of the S-phase marker BrdU at 7 and 14 days after transplantation, (six injections over 3 days and sacrifice of the animals after the last injection). We found a large number of BrdU-positive transplanted cells in the proximity of vessels at 1 week (Fig. 2A) and at 2 weeks (Fig. 2B). Quantitative analysis revealed that 1 week after transplantation 39.2% ± 4.3% (mean ± SEM) of FGF-2-transduced NPCs associated to vessels were BrdU positive compared with only 21.6% ± 3.7% (mean ± SEM) for grafted NPCs nonassociated with the vasculature ($p < .05$, *t* test), (Fig. 2C). Also 2 weeks after transplantation, 70.5% ± 3.6% (mean ± SEM) of FGF-2-transduced NPCs associated to vessels were BrdU-positive compared with only 38.5% ± 11.8% (mean ± SEM) for grafted NPCs nonassociated with the vasculature ($p < .05$, *t* test), (Fig. 2C). In comparison, control NPCs almost do not divide, ~10% at 1 week after transplantation, as described in our previous work Fig. 5 in [14]. These results provided evidence that FGF-2-transduced NPCs associated with vessels remained proliferative at least for 2 weeks after transplantation. Consistent with these results, FGF-2 transduced NPCs showed a clear tendency to form multicellular clusters around vessels (Fig. 2D). One week after transplantation the percentage of cells associated to vessels in the category of three cell-clusters was significantly increased compared with the percentage of cells associated to vessels in the category of single cells ($p < .05$, one-way analysis of variance, Tukey's multiple comparison test), (Fig. 2E). The same effect was found at 2 weeks after transplantation. These data indicate that in cellular clusters of 3 or more cells, the proportion of cells located in contact with vessels was more than 90%, suggesting that multiple cell clusters need the perivascular microenvironment. Moreover, the proportion of two cell clusters associated with vessels significantly increased 2 weeks after transplantation compared with that after 1-week survival (Fig. 2E) ($p < .05$, *t* test). Together, these results strongly suggest that the perivascular environment promotes the formation of proliferative clusters of FGF-2 overexpressing progenitors.

Normal neural progenitors as well as stem cells express the intermediate filament protein nestin [20]. To determine whether the proximity of vessels promotes the maintenance of an immature phenotype in transplanted NPCs, we examined nestin immunoreactivity in the grafted NPCs population. Quantitative analysis at 1 week after transplantation, revealed that the majority (>60%) of transplanted FGF-2 cells (Fig. 3A) expressed nestin independently of their association with the vasculature. Interestingly, 2 weeks after grafting, the proportion of nestin-positive cells was almost twofold higher in the vessel-associated population 68.9% ± 10.32% (mean ± SEM) than among nonvessel contacting FGF-2-transduced NPCs 38.2% ± 5.19% (mean ± SEM) ($p < .05$, *t* test). Moreover, NPCs nonassociated to vessels appeared to lose their nestin immunoreactivity after transplantation, as 63.4% ± 6.9% (mean ± SEM) were nestin-positive at 1 week compared with only 38.2% ± 5.19% (mean ± SEM) at 2 weeks ($p < .05$, *t* test) (Fig. 3A). Strikingly, clusters of FGF-2-transduced NPCs associated with vessels were strongly positive for nestin immunoreactivity (Fig. 3B, 3C). It appears therefore that at later survival time-points the proximity of blood vessels maintains grafted FGF-2 overexpressing in an undifferentiated state.

FGF-2-Transduced NPCs Associated to Vessels Supply a Pool of Immature Neurons After Cerebral Ischemia

A key issue is whether vessel-associated transplanted NPCs could provide immature neurons in response to brain injury. To reveal the neurogenic potential of NPCs associated to blood vessels, we transplanted FGF-2-transduced NPCs in the cortex of P3 rat and allowed NPCs to associate with blood vessels. We then exposed animals to cerebral hypoxia-ischemia at P7, by right carotid artery ligation followed by 30 minutes of hypoxia (6% O_2), a modified model of early rat postnatal hypoxia-ischemia we described earlier [18]. Rats were then sacrificed 7 days later at P14. Control animals received the same treatment but were not exposed to ischemia. In this ischemia model, areas of cortical injuries can be clearly identified by a distinct neuronal loss, (supporting information Fig. 1A, A1) and a strong glial reaction as revealed by GFAP immunoreactivity (supporting information Fig. 1B, B1) [18]. We found that in the ischemic cortex, 8.2% ± 0.7%, (mean ± SEM) of transplanted FGF-2-transduced NPCs became positive for DCX, a marker for immature neurons, compared with 0.5% ± 0.2%, (mean ± SEM) after transplantation into a nonischemic control cortex ($p < .01$, *t* test) (Fig. 4C). Moreover, after transplantation of control NPCs in the same ischemic model, significantly less transplanted NPCs, 1.4% ± 0.6% (mean ± SEM) showed an immature neuronal morphology with DCX positivity ($p < .01$, *t* test), and also showed no direct association with blood vessels (Fig. 4D). These immature neurons were found in areas of cortical injury (Fig. 4A) and the majority of DCX-positive NPCs were found in the vicinity of blood vessels 68.1% ± 3.5% (mean ± SEM) (Fig. 4B) compared with only 31.9% ±

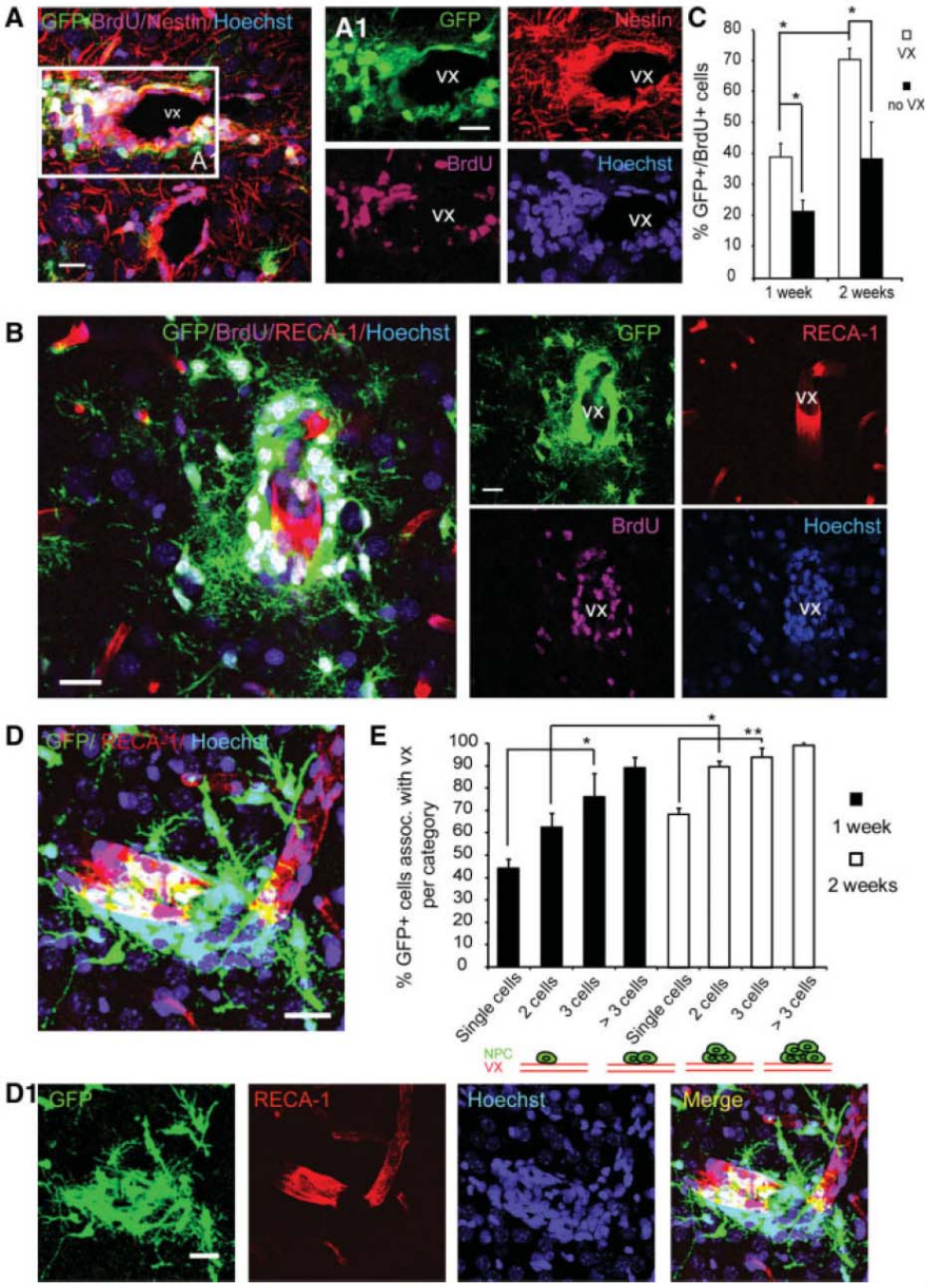

Figure 2. Grafted FGF-2-transduced neural progenitor cells (NPCs) form proliferative clusters of neural progenitors around vessels in the rat cerebral cortex. **(A):** Confocal images of FGF-2-transduced NPCs 1 week after transplantation in the cerebral cortex showing the formation of clusters associated with blood vessels. Many NPCs located in perivascular clusters are BrdU-positive as shown in the boxed area (A1). **(B):** Confocal images of FGF-2-transduced NPCs 2 weeks after transplantation in the cerebral cortex showing also the formation of clusters composed of many dividing BrdU-positive NPCs associated with blood vessels. **(C):** Graph showing the increased proportion of BrdU-positive cells in FGF-2-transduced cell clusters associated to vx compared with nonassociated to blood vessels (No vx), at 1 and 2 weeks after transplantation. A total (100%) of 582 and 294 cells were counted in the vx and no vx population, respectively at 1 week. At 2 weeks a total (100%) of 769 and 239 cells were counted in the vx and no vx population, respectively (Error bars indicate SEM, *, $p < .05$, t test). **(D):** Confocal images with color separation (D1) showing that grafted FGF-2-transduced NPCs can form clusters associated with blood vessels labeled with RECA-1. **(E):** Graph showing the proportion of single cells or clusters in contact with vessels, 1 week and 2 weeks after transplantation. At 1 and 2 week after engraftment the proportion of cells associated to vessels is greater for 3 cell-clusters compared with single cells (*, $p < .05$ and **, $p < .005$, respectively, One-way analysis of variance, Tukey's multiple comparison test). Also the tendency to associate with the vasculature increases at 2 weeks after transplantation for 2 cell-clusters (*, $p < .05$, t test). For clusters more than 3 cells the tendency to associate with vessels is very high at both 1 week and 2 weeks (error bar indicate SEM). Abbreviations: BrdU, bromodeoxyuridine; FGF-2, fibroblast growth factor-2; GFP, green fluorescent protein; vx, blood vessel. Scale bar = 20 μm.

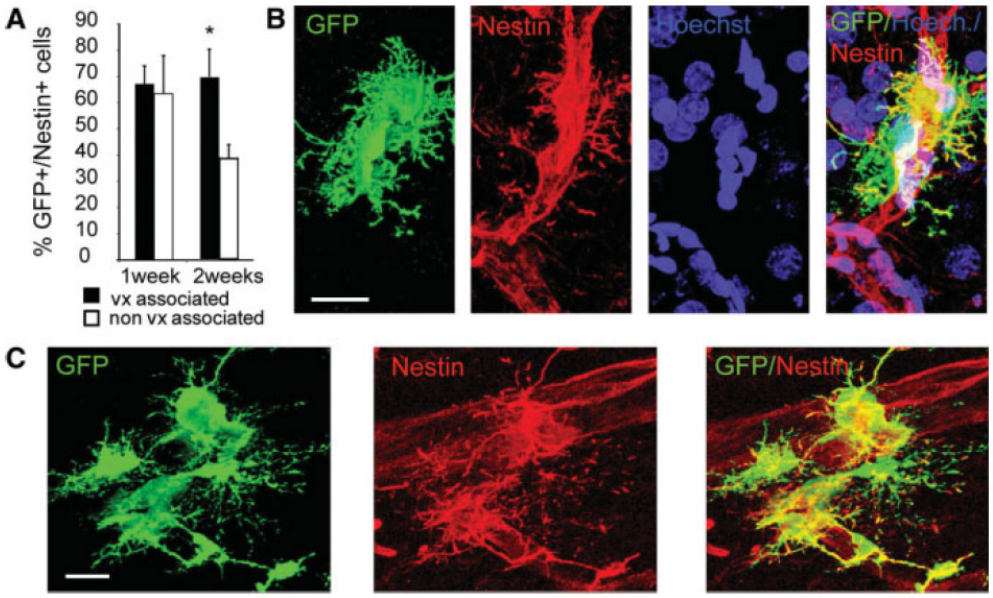

Figure 3. Neural progenitor cells (NPCs) in perivascular clusters stay immature at 2 weeks post-transplantation. **(A):** Graph showing that at 2 weeks a greater proportion of cells are nestin-positive in perivascular clusters compared with those nonassociated with blood vessels (*, $p < .05$, t test; error bar indicates SEM). **(B, C):** Confocal images showing two examples of nestin-positive cells in perivascular clusters of fibroblast growth factor-2-transduced NPCs 2 weeks after transplantation. Abbreviations: GFP, green fluorescent protein; vx, blood vessel. Scale bar = 20 μm.

3.5% (mean ± SEM) not associated with blood vessels ($p <$ .05) (Fig. 4F). Some DCX-positive NPCs appeared to migrate in "chain" along small vessels (Fig. 4D), and others seemed to migrate away from the vasculature (Fig. 4E). Together, these results suggest that vessel-associated FGF-2-transduced NPCs have a neurogenic potential when recruited by an ischemic environment.

Discussion

The major finding of this work is that overexpression of FGF-2 in transplanted neural progenitors increases their ability to associate with blood vessels and establish multiple cell clusters around the host vasculature. The perivascular environment appears to be critical in maintaining NPCs in an undifferentiated and proliferative phenotype. Moreover, we demonstrate that an ischemic environment can promote the generation of new neurons in the vicinity of blood vessels, suggesting that perivascular clusters of NPCs could function as neurovascular niches. These findings may open new possibilities to induce ectopic sites of neurogenesis in the damaged cortex and to generate a source of immature neurons for tissue repair.

The results of many studies indicate that cell death, restricted migration, and rapid differentiation into glial cells limit the use of transplanted NPCs for neuronal repair [2–4]. Recently, we demonstrated that FGF-2 expression in grafted neural progenitors cells could represent a useful strategy to overcome these limitations by maintaining NPCs in a migratory and proliferative state and by increasing the pool of immature neurons available for brain repair [14]. The data presented here suggest that the capacity of FGF-2-transduced cells to associate with the host vasculature could play an important role in this process. We showed that FGF-2 overexpression significantly increased the ability of NPCs to form multicellular clusters around vessels. Consistent with these results, we found a substantially higher proportion of proliferative and undifferentiated cells in the proximity of blood

Figure 4. Perivascular clusters of FGF-2-transduced neural progenitor cells (NPCs) generate immature neurons after cerebral ischemia. In these experiments, FGF-2-transduced NPCs were transplanted at P3, then cerebral hypoxia-ischemia was applied at P7 and brains were analyzed 7 days later. **(A):** Epifluorescent images of the ischemic cerebral cortex illustrating that FGF-2-transduced NPCs can be recruited toward the areas of cortical injuries (A1). The ischemic zone depicted by the dashed line is characterized by a high density cellular/glial reaction (increased Hoechst reaction). **(B):** Confocal images showing that after cerebral ischemia a fraction of FGF-2-transduced NPCs in contact to blood vessels is positive for the immature neuronal marker DCX (white arrow). **(C):** Graph showing the percentage of the total number of transplanted FGF-2-transduced cells (100% = 722) expressing DCX after ischemia versus intact cortex (100% = 820; *, $p < .01$, t test) and also the percentage of total number of control GFP-transplanted NPCs expressing DCX after ischemia (100% = 285) compared with FGF-2-transduced NPCs (*, $p <$ .01, t test). **(D):** Confocal images of control GFP-transplanted NPCs, showing that after cerebral ischemia, control NPCs show a more mature glial phenotype with no contact with blood vessels and also no DCX-positive immature neurons. **(E):** Confocal images showing a cluster of DCX-positive-grafted NPCs that appears to migrate along the vessel wall. **(F):** Confocal images showing DCX-positive-grafted NPCs that are in the proximity of blood vessels. Boxed area F1 and F2 showed in color separation the migration of GFP-positive DCX-positive NPCs migrating away from vessels (white arrow). **(G):** Graph showing that after cerebral ischemia, the majority of newly formed immature neurons are associated with blood vessels (*, $p < .05$). Error bars indicate SEM. Abbreviations: DCX, doublecortin; FGF-2, fibroblast growth factor-2; vx, blood vessel. Scale bar = 100 μm for epifluoresence images and 20 μm for confocal images.

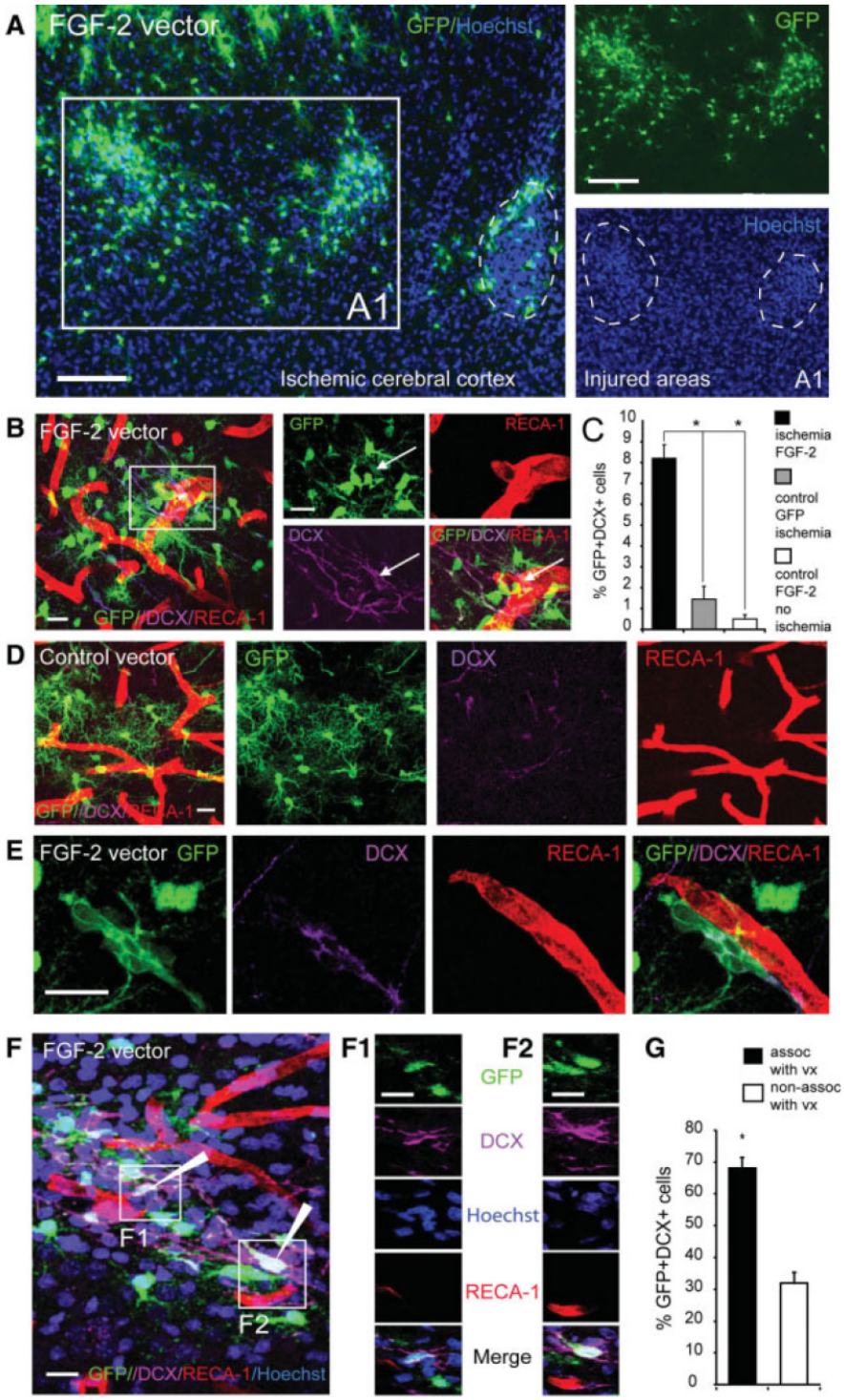
A
FGF-2 vector
GFP/Hoechst
A1
Ischemic cerebral cortex
GFP
Hoechst
Injured areas
A1
B
FGF-2 vector
GFP/DCX/RECA-1
GFP
RECA-1
DCX
GFP/DCX/RECA-1
C
% GFP+DCX+ cells
ischemia FGF-2
control GFP ischemia
control FGF-2 no ischemia
D
Control vector
GFP/DCX/RECA-1
GFP
DCX
RECA-1
E
FGF-2 vector GFP
DCX
RECA-1
GFP//DCX/RECA-1
F
FGF-2 vector
F1
F2
GFP//DCX/RECA-1/Hoechst
GFP
DCX
Hoechst
RECA-1
Merge
G
assoc with vx
non-assoc with vx
% GFP+DCX+ cells

Figure 4

vessels than in the nonvessel contacting population. These results confirm previous observations that 2 weeks after transplantation into the cortex, a significant proportion of FGF-2-transduced cells remains nestin-positive while very few control NPCs express this marker of immature neural progenitor cells [14]. In contrast, over 80% of control NPCs expressed NG2 a marker of oligodendrocyte progenitors [14]. Overall, these results suggest that FGF-2 transduction prolongs the maintenance of these cells in an immature and proliferative state. This conclusion is in agreement with the notion that FGF-2 is a major determinant in maintaining proliferative and undifferentiated populations of NPCs under in vitro conditions [12]. The data presented here further demonstrate that when grafted progenitors interact with the recipient host tissue, the effect of FGF-2 requires the perivascular environment. This is consistent with the hypothesis that maintaining a multipotential and proliferative phenotype of stem/progenitor cells in a tissue environment is not cell autonomous but depends on the external control from the environmental niche [21].

FGF-2 is known for its strong angiogenic potential [19] and has been shown to induce cerebral angiogenesis after intraventricular injection [22] and on organotypic cortical cultures of mice [23]. The possibility that transplanted NPCs secreting FGF-2 could induce local angiogenesis and recruit blood vessels for their micro-environmental supply is an interesting hypothesis. In our model, although we cannot formally exclude that FGF-2 expressing NPCs induce angiogenesis, our quantitative analysis of vessel densities after transplantation suggests that this is not the case.

The intimate contacts between neural stem/progenitor cells and vessels are believed to be essential for the maintenance of physiological neurogenic niches in the adult mammalian brain [6, 10]. Direct evidence for a functional relationship between endothelial and neural stem cells comes from coculture experiments, showing that endothelial cells release soluble factors that stimulate the self-renewal of neural stem cells, inhibit their differentiation, and enhance the production of neurons [11]. Our data provide strong support for this concept by demonstrating for the first time that the perivascular environment is critical in determining the fate of transplanted NPCs in vivo.

The precise cellular and molecular mechanisms that dictate the initiation and maintenance of vascular clusters after FGF-2 overexpressing NPCs transplantation remain unknown. FGF-2 overexpression in NPCs is clearly important for this process because control progenitors establish significantly less cellular clusters in the perivascular environment. In addition to controlling cell division and differentiation, FGF-2 could increase the incidence of vascular contacts indirectly through increasing the migratory and invasive potential of progenitors [14]. It is of interest that FGF-2 was shown to modify supportive niche cells for human embryonic stem (ES) through the secretion of paracrine factors influencing the self-renewal capacity of ES cells [24]. It is thus possible that reciprocal interactions between FGF-2 overexpressing NPCs and vascular cells such as endothelial cells (or pericytes, fibroblasts) establish a regulatory microenvironment that is favorable for the maintenance of an undifferentiated and dividing population of stem/progenitor cells. The "FGF-overexpression" strategy would offer a powerful model to explore interactions between NPCs and endothelial cells and to identify signals that mediate these interactions.

Summary

We demonstrate that in the intact cortex, perivascular clusters of FGF-2-transduced progenitors mainly comprise undifferentiated and dividing cells. Furthermore, an ischemic insult significantly increased the number of immature neurons that were found in the proximity of blood vessels with a significant additional benefit of FGF-2-transduced NPCs over control NPCs. In some cases, new neurons appeared to migrate in close contact to blood vessels suggesting that perivascular clusters could function as ectopic neurogenic vascular niches that can dynamically respond to environmental changes. Ectopic perivascular niches of neural progenitor cells were previously described under pathological conditions such as in the inflamed central nervous system [25, 26] and in brain tumors [27]. Whether the perivascular clusters identified in this study show further characteristics common to physiological neurogenic sites of the SVZ or dentate gyrus warrants additional studies. In conclusion, our results suggest that overexpression of FGF-2 in grafted neural progenitor cells is a reasonable strategy to improve tissue repair in a neonatal ischemic cortex and opens new therapeutic perspectives for brain regeneration using minimal genetic engineering of cells before transplantation.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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DISCUSSION

In this thesis we have developed a novel system allowing the robust over-expression of FGF-2 in NPCs and studied its effects on several cellular functions after transplantation into the early postnatal rat cortex, in particular the behaviour of FGF-2 transduced NCPs in the context of brain cortical ischemia. In addition to increasing the *in vitro* and *in vivo* proliferation and migration potential, an intriguing phenomenology was that FGF-2 overexpression in NPCs induced a strong association and cluster formation of transplanted cells with host rat cortical vessels. This association appeared to play a determinant role in maintaining neural progenitors in an immature state, thus keeping in the cortex a pool of transplanted NPCs ready to potentially repopulate areas of brain injuries.

The results provided here demonstrate that minimal genetic manipulation of NPCs with FGF-2 prior to transplantation gives them a distinct advantage over control NPCs and improves their repair potential by increasing the pool immature neurons migrating towards the sites of injury. Moreover, it promotes a special interaction with the host blood vessels similar to the interaction of neural stem cells and vessels in the germinative regions, where microenvironmental cues are determinant for the maintenance and self-renewal of neural stem cells. We believe that the pre-transplant engineering of neural progenitor cells using a lentiviral gene transfer system gives a new hope for brain repair strategies using neural stem cells and challenges critical issues seen after transplanting neural stem cells in the brain cortex.

In the light of further improving transplantation technologies for brain repair, we discuss here the advantages to use a lentiviral technology and the several mechanisms by which lentiviral manipulation of neural stem cells with FGF-2 significantly improves their potential to repair the injured brain cortex. Furthermore, we highlight why the generation of FGF-2 induced "ectopic cortical neurovascular clusters" could be an interesting concept to develop in cell based strategies for brain repair. However, the lentiviral manipulation of cells for therapeutic use has raised several concerns essentially with the risk of induced tumorigenesis and we highlight here some of these aspects.

Pre-transplant manipulations of neural stem cells with FGF-2: a new hope for brain repair strategies?

Lentiviral technology as a method of choice

Different gene transfer systems have been used to manipulate neural stem cells aiming to immortalize them, control their growth or differentiation. These different techniques include conventional transfection, liposome transfection, electroporation and most importantly viral mediated transfection *(Jandial et al., 2008)*. The advantage of viral mediated transfection is the excellent transfection rate and large transgene delivery capacity. Different types of viral technologies exist, using retroviruses, adenoviruses or lentiviruses. Retroviruses have been used for transduction of cells with high proliferative rate, leaving this method ineffective for mature tissue. The property of retroviruses is the reverse transcription of the RNA genome into a DNA copy which becomes integrated into the genome. The advantage with lentiviruses is the capacity

of infecting dividing and non-dividing cells, offering also a long term expression of the transgene and low toxicity *(Gallay et al., 1997)*. HIV and other lentiviruses can infect non-dividing cells because their preintegration complex carries karyophilic components recognized by the nuclear import machinery of the host cells. Retroviruses do not have this property and require the nuclear envelop to be disrupted which occurs only during mitosis *(Trono, 2003)*.

In neuroscience, the use of lentiviral technology is valuable, considering that most cells which compose the nervous system are in a post mitotic state and neural stem cells in the SVZ or the SGZ can have also slow dividing rates. We used in our model the lentiviral technology to label neural progenitor cells in green (insertion of GFP gene) or red (insertion of dtTomato gene), and to overexpress FGF-2. With this technology, we were able to follow the migration of cells through the brain tissue on cortical slice, and *in vivo*, up to two months after transplantation in the brain of rat pups.

In our experience, the use of the lentiviral technology has been a non-toxic tool to over-express FGF-2 in NPCs. In the culture medium FGF-2 protein, was increased ~10 fold compared to primary cultures of NPCs transduced only with a control vector, this shown by Elisa analysis. Also FACS analysis allowed us to calculate that in our system, 2 to 3 copies of FGF-2 lentiviral vectors were incorporated in the genome. Although lentiviral technology offers a robust and long lasting effect, we observed, by analyzing the level of FGF-2 expression at different survival points after transplantation, a gradual down regulation of FGF-2 production over a period of two months *in vivo*. This was correlated with a progressive loss of migratory and proliferative capacities of FGF-2 over-expressing NPCs after *in vivo* transplantation. Different reasons could explain this phenomenology including decreased transcription levels, increased mRNA instability, decreased mRNA translation and increased protein degradation. Surprisingly, FGF-2

transplanted in the anterior SVZ continued to express FGF-2 one month after transplantation, suggesting that FGF-2 down-regulation might be region specific. Most importantly, lentivral FGF-2 transduction showed no toxicity as transplanted NPCs kept a similar morphology *in vitro* compared to non-transduced NPCs.

Drawbacks of the lentiviral approach

Despite that in our model the use of lentiviral technology has been an efficient and safe technology to permanently over-express our gene of interest in cells prior to transplantation many concerns have been raised over the past two decades with the use of HIV derived lentivirus in clinical approaches. The main risk is the fact that tumours could develop from the permanent overexpression of genes, especially growth factor like FGF-2. In our model, we have not observed any tumour-like formation, up to 40 days after *in vivo* transplantation.

Risk of tumorigenesis induced by lentivirus

In retroviral technologies, the integration event from retroviruses has been historically believed to be random, and the chances to accidently disrupt or activate a gene, a phenomen called insertional mutagenesis very remote. Initially the use HIV-1 derived vectors (lentivirus) originated from the natural history of HIV, a retrovirus able to infect even non-dividing cells with a high efficient and non-oncongenic potential *(Cohen, 1996) (Naldini et al., 1996)*. Unfortunately it was discovered a decade ago, that a few children treated for a blood disease with a murine leukaemia virus (MLV)-based gene therapy vector developed leukaemia *(Check, 2002; Kaiser, 2003)*. This raised serious concerns about the safety of these approaches and the hypothesis of a random integration was uncertain.

The main risks of retroviral based therapy to induce cancer is a consequence of either of the following events: 1) integration of the gene into the host DNA leading to neoplasia due to insertional mutagenesis by over expressing tumorigenic or growth factor genes 2) the possible unintended generation of replication competent vector-derived viruses: replicant competent retrovirus (RCR) or replicant competent lentivirus (RCL) *(Wilson et al., 2009)*.

1) A characteristic of retroviral replication is the reverse transcription of viral RNA genome into a DNA copy that becomes integrated into the host cell genome. Unfortunately when transcriptional elements contained into the retrovirus activate a nearby growth stimulating gene through effects on either the gene promoter or enhancer, this can link to uncontrolled proliferation and cancer *(Trono, 2003)*. The virally encoded integrase mediates the integration between the viral DNA and the host genome, which integration is supposed to be random. HIV and other lentiviruses can infect non-dividing cells because their pre-integration complex carries karyophlic determinants recognized by the nuclear import machinery of the host cells. In contrast, MLV and most other retroviruses lack this property and require the disruption of the nuclear envelope that occurs during mitosis to come into contact with host cells chromosomes *(Trono, 2003)*. It was demonstrated that integration sites in the human genome from the lentivirus HIV-1 and from the oncoretrovirus murine leukaemia virus (MLV) are different and that MLV prefers to integrate in 5' flanking regions of genes near the start of transcriptional units, in contrast to HIV-1 which preferred to integrate anywhere across the transcriptional unit but not upstream from the transcriptional start *(Wu et al., 2003)*. This suggested some advantages of lentiviral based approaches and highlighted the need for *in vivo* biosafety models to assess the

risks of adverse events from retroviral and lentiviral vectors *(Bauer et al., 2008; Bokhoven et al., 2009).*

2) Another risk from the use of lentivirus is the generation and propagation of replication competent lentiviruses (RCLs) during vector production due to recombination of vector plasmids or *in vivo* due to the mobilization of vector proviral DNA by infectious retrovirus such as HIV *(Connolly, 2002).* The generation of RCL occurs most likely form homologous recombination between overlapping sequences *(Pauwels et al., 2009).*

How can we reduce the oncogenic risk?

If lentivirus are supposed to be less tumorigenic than other retrovirus like gammaretroviral vectors, further improvement needs still to be made in lentiviral design to reduce the risk of triggering cancer by upregulating cellular protoconcogenes *(Modlich et al., 2009).* The lentiviral integration pattern plays and important role in cancer induction. It was already known that transcriptional active long terminal repeats (LTR) play an important role in oncogenesis especially in gamma retrovirus induced tumours *(Lenz et al., 1984).* The major role for transcriptional active LTR in genotoxicity was further demonstrated by Montini et al. who showed that, in a Cdkn2a knockout mouse model (a model for insertional mutagenesis), vectors with a SIN (self inactivating) carry the enhancer promoter in an internal position, before the gene of interest, so that it is not introduced into an LTR. This reduces therefore the risk of neoplasia by a factor 10. Overall, it is suggested that the safest design for retroviral vectors to reduce the risk of insertional mutagenesis is the combination of a SIN LTR with a moderate active internal promoter *(Modlich et al., 2009; Montini et al., 2009).* In our model we have used an HIV-1 derived SIN vector containing the EF1 α promoter and the EMCV-IRES-GFP cistron. After 40

days follow-up after transplantation we have not detected any GFP positive cell agglomerates suggesting the formation of a tumour *in vivo*.

Another approach to diminish the tumorigenic risk is to develop integrase defective lentiviral vectors. After vector internalization in the cell and reverse transcription in the cytoplasm, the vector-double strand DNA is incorporated into the pre-integration complex. A critical component of the pre-integration complex is the viral integrase (IN) which catalyzes viral integration DNA into the genome. Once the integration complex reaches the nucleus, IN mediates the integration between viral and host DNA *(Banasik et al.)*. Although integration in the genome is generally the gold standard of lentiviral gene transfer, DNA episomes can be generated by lentivirus. Episomes can still generate protein but they do not persist in dividing cells because they are note integrated and lack an origin of replication. An attractive approach to avoid the risk of detrimental insertional mutagenesis is the generation of lentivirus lacking an integrase, which is an appropriate option when permanent expression of the transgene in the genome is not necessary. Still the efficiencies of such construct are controversial and some early HIV-integrase defective lentiviruses have been reported to be non-functioning. In general the duration of gene expression varies according to the rapidity of cell turnover. In the brain some studies have shown expression of transgenes up to 4 months *(Rahim et al., 2009)*. Nontheless, integrase defective LV could represent and interesting approach to reduce the risk of insertional mutagenesis *(Banasik et al.)*.

Although many concerns have been raised since the use of retroviral vectors for experimental and or clinical therapies, considerable improvements have been made to assess biosafety issues

for lentiviral vectors, leading to the development of new vector design, combining improved safety and increased transduction efficiencies.

Neural stem/progenitor cells engineered to express FGF-2

Roles of FGF-2 in the maintenance of immature and proliferative state

The maintenance of an immature and proliferative state after transplantation is a critical issue for neural stem/progenitor cells grafted in the cortex. In our preliminary data control NPCs grafted in the cortex of neonatal rats rapidly differentiate into glia or oligodendrocytes. This occured as rapidly as one week after transplantation, thus making transplanted cells unavailable for brain repair purpose. Studies have demonstrated that by losing their immature state neural stem cells lose their migration and invasive potential *(Soares et al., 2004)*.

In our model we showed that lentiviral FGF-2 overexpression in grafted NPCs was sufficient to maintain their proliferative and immature phenotype as well as their migratory properties *in vitro* in an organotypic tissue complex as well as *in vivo* (Figure 7). Islets of FGF-2 transduced NPC deposited on organotypic cortical slices showed a significant increase in their proliferation rate compared to control NPCs. At DIV2, 35% of FGF-2 transduced NPCs divided compared to less then 3% of control NPCs. After *in vivo* transplantation into the cortex of neonatal rats, more than 50% of FGF-2 transduced NPCs were still dividing compared to only 10% of control NPCs. FGF-2 transduced NPCs kept their immature phenotype as more than 65% of the cells were positive for nestin a marker of immaturity, as compared to less than 5% of control NPCs. An

interesting observation was that after grafting, nuclear and cytoplasmic expression of FGF-2 gradually decreased over a period of 60 days, concurrently with NPCs losing their immature state and differentiating into glial cells. FGF-2 considered as a niche factor for the maintenance of neural stem cells in the germinative niches of the SVZ and the SGZ significantly improved in our model the maintenance of a pool of NPCs after engraftment into cerebral cortex.

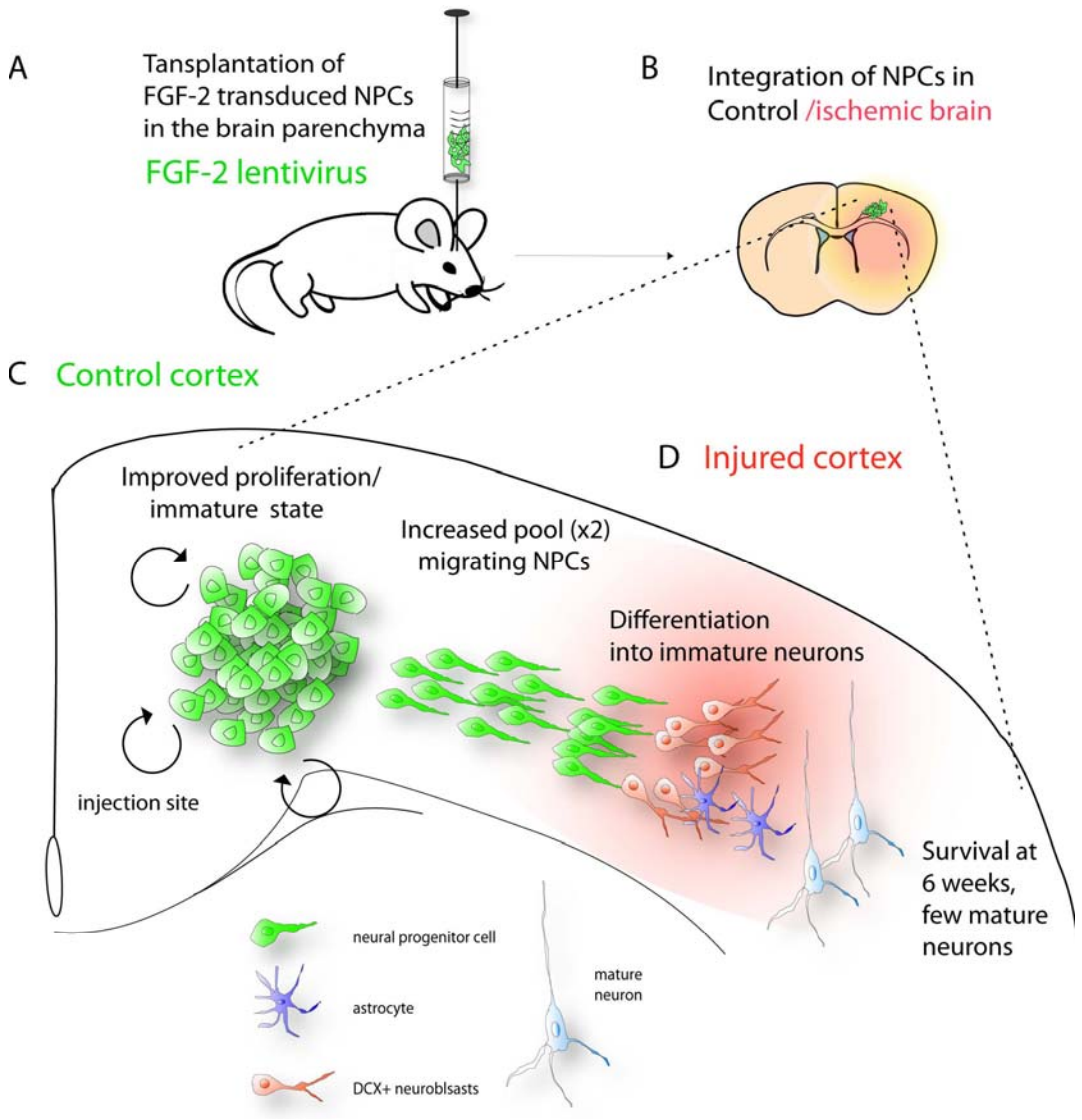

Figure 7: FGF-2 overexpression in transplanted NPCs significantly improves their potential for brain repair

Schematical representation of the different steps were FGF-2 overexpression in transplanted NPCs improves their potential for brain repair strategies. (A) NPCs are manipulated prior to transplantation and transduced with a lentiviral vector carrying FGF-2 and/or GFP. (B) The effects of FGF-2 overexpression in NPCs are assessed *in vivo* in two different conditions: a normal cortex or an ischemic cortex. (C) FGF-2 overexpression in NPCs significantly increases the pool of cells after transplantation, by maintaining their proliferation rate, immature state and also improves their migration capacities. (D) In the ischemic cortex, FGF-2 overexpression significantly increased the pool of migrating cells available for brain repair. These cells were still responsive to

local cues induced by the ischemic environment and differentiate into immature neurons and later on into a few more mature neurons.

Impact of FGF-2 overexpression on migration and invasive potential

To reach the sites of cortical brain injuries and travel through the brain parenchyma, transplanted neural stem cells need to keep strong migratory properties when interacting with the host tissue. FGF-2 signalling in NPCs increased their migration rate and invasive capacities on organotypic slice culture, as 30% of FGF-2 transduced NPCs travelled at a speed of 20-30µm/h compared to only ~10% of control NPCs. This was also related to a significant increase in the distance travelled by NPCs through cortical organotypic culture. For comparison, an *in vitro* study assessed the speed of SVZ neuroblasts migrating out of neurospheres and showed that post ischemic SVZ neuroblasts migrated at 7.07 µm/h on average compared to 5.53 µm/h for non stroke SVZ neuroblasts which represents the migration speed of 70% of control NPCs on our organotypic slices *(Zhang et al., 2007)*. In fact FGF-2 significantly improved the invasive capacities of NPCs as their migration radius assessed by single cell track analysis increased dramatically, from less then 20µm in control NPCs to mostly 40-60µm in FGF-2 transduced NPCs.

Understanding the mechanisms involved in cell migration is important to improve their capacity in achieving efficient cellular replacement for brain repair. Cell migration is a highly integrated multistep process and the migrating cell is polarized with complex regulatory pathways which determine its forward movement and interaction with the extracellular matrix (ECM). In general cell migration can be conceptualized as a cyclic process. The initial response of a cell to a migrating-promoting agent is to polarize and extend protrusions in the direction of migration. These protrusions can be large, broad lamellipodia or spike-like filopodia, are usually driven by

actin polymerization and are stabilized by adhering to the ECM or adjacent cells via transmembrane receptors linked to the actin cytoskeleton. These adhesions serve as traction sites for migration as the cell moves forward over them and they are dissembled at the cell rear allowing detaching *(Ridley et al., 2003)*.

Neuroblasts migrating towards the sites of brain injury need to fulfil these tasks and some studies are emerging showing their migratory characteristics. Cells migrating outside the SVZ towards a striatal injury seem to divide and migrate, with cells induced by stroke migrating and dividing much more rapidly that non stroke induced SVZ cells. These migrating cells show a leading process extending from the soma in the direction migration and tailing process much shorter *(Zhang et al., 2007)*. In our study we have demonstrated on cortical slices that migrating FGF-2 transduced NPCs can switch form a bipolar to a multipolar morphology and that migrating FGF-2 transduced NPCs can also divide "en route" while the two daughter cells will continue their migration.

After *in vivo* transplantation, FGF-2 signalling in NPCs was critical to improve the migration and invasive capacities of NPCs through the brain parenchyme. Strikingly, control NPCs stayed constantly trapped within the needle track injection whereas FGF-2 transduced NPCs dispersed homogeneously away from the injection site creating a diffuse area in the brain cortex infiltrated with proliferative and immature, nestin positive neural progenitor cells.

Different studies have shown that neural progenitor cells after transplantation in a non-ischemic cortex or striatum remain within the needle track injection. After transplantation of neural precursors cells obtained from mouse embryo into the mouse striatum, most of these cells remained at the injection sites and died within one week *(Jin et al., 2005)*. In a study assessing

the effects of stroke after neural progenitor cell transplantation, it was obvious that neural progenitors transplanted in control cortex did not migrate compared to the migration induced by cerebral ischemia *(Guzman et al., 2008)*. Another study showed that hNSCs transplanted in non-ischemic cortex remained significantly within the needle track injection whereas they show some degree of migration after cortical ischemia *(Kelly et al., 2004)*. Together this emphasizes that the capacity for grafted neural stem cells to invade the surrounding brain parenchyme is seriously impaired and that the pre-transplantation manipulation of NPCs with FGF-2 offers a reasonable approach to enhance the migratory capacities of NPCs after transplantation.

Mechanisms of FGF-2 signalling in transplanted NPCs

As FGF-2 overexpressed in NPCs is secreted in our model we hypothesized that paracrine signalling to the neighbour cells could account for the increased proliferation and migration. To test this hypothesis we co-transplanted control red tomato NPCs and GFP-FGF-2 transduced NPCs, and showed that in fact FGF-2 transduced NPCs had dispersed in the cortex whereas control cell stayed in the injection sites. This strongly suggested that a crosstalk through paracrine signalling between FGF-2 NPCs and control NPCs was not sufficient to increase the migratory properties of transplanted cells. We speculated therefore that an autocrine signalling loop accounted for the effects observed with FGF-2 transduced NPCs. In our experiments, both FGF-2 receptors FGFR-1 and FGFR-2 were expressed at the mRNA and protein level on NPCs, further supporting the evidence that over-expression of FGF-2 in the extracellular compartment could function through specific receptors and maintain the biological properties of FGF-2 transduced NPCs. It may also be possible that the 18 kDa isoform of FGF-2 after binding to its

cognate receptors could be internalized and reach the nucleus thus functioning as an intracrine manner *(Sorensen et al., 2006)*.

Integration of FGF-2 transduced NPCs in the olfactory bulb

Whether FGF-2 overexpressing NPCs could still respond to local signalling cues and integrate into the host tissue as neurons was a critical issue of this project. Co-transplantion of FGF-2 transduced NPCs and control tomato-labelled NPCs in the physiological neurogenic environment of the SVZ, showed that at different time points after transplantation both FGF-2 transduced NPCs and control NPCs were found in the SVZ, along the rostral migratory stream and in the olphactory bulb where they had differentiated into immature neurons expressing doublecortin. Six weeks after transplantation, NPCs in the olfactory bulb showed well developed processes, extending typical dendrites and expressing more mature neuronal marker, like NeuN, displaying the characteristics morphology of granular interneurons. At 4 weeks after transplantation, neurons derived from FGF-2 transduced NPCs were 4 times more abundant than control tomato labelled neurons. This loss of immature phenotype was strongly correlated with a down regulation of FGF-2 expression, since less than 5% of transplanted cells were expressing FGF-2. The demonstration that FGF-2 transduced NPCs could still respond to the physiological environment and integrate in the host tissue was a critical step in this project. It showed that the pre-transplant manipulation of NPCs with FGF-2 would not alter their capacities to function as normal progenitor cells, and would significantly increase the pool of neural progenitors available for brain repair.

Integration of FGF-2 transduced NPCs after cortical hypoxia- ischemia

One of the goals in restorative medicine is to replace lost or damaged cells. It was crucial to demonstrate that a significant amount of transplanted cells efficiently reached and integrated the areas of damaged cortex. In our study, NPCs over-expressing FGF-2, transplanted in the ischemic cortex of neonatal rats showed improved proliferative properties compared to control cells, doubling the amount of cells available for brain repair 2 weeks after transplantation. At this time point, FGF-2 was expressed in only a third of the transplanted cells indicating that the downregulation of FGF-2 also occurred in the ischemic environment. A large fraction of the transplanted cells remained nestin positive but most importantly, more than 50% of the cells expressed the immature neural marker DCX. This provided evidence that the ischemic environment also had a neurogenic influence on NPCs, in contrast with the control non ischemic environment where only a few neurons/ immature neurons could be observed. At later time point a few cells showed a more complex neuronal phenotype, indicating that the lentiviral over-expression of FGF-2 does not prevent neuronal differentiation, this probably taken into account the FGF-2 downregulation in transplanted cells. These results supported the evidence that in a hostile diseased environment, FGF-2 signalling contributed to the maintenance of proliferative cells and increased significantly the pool of neural progenitors available to replace lost cells in the regions of damaged cortex.

Migration of FGF-2 transduced NPCs towards the sites of cortical injury

The migration potential of transplanted NPCs towards the sites of injuries is an important aspect for brain repair strategies and many studies have reported that transplanted neural stem cells could migrate towards the damaged regions *(Jin et al., 2003; Kelly et al., 2004; Kim et al., 2004;*

Guzman et al., 2008). In our experiments we observed a clear tendency for transplanted cells to migrate from the site of injection towards the areas of ischemic cortex. These migrating cells had the morphological appearance of "migrating neuroblasts" with a leading edge and rear tail and the majority of them were positive for doublecortin marker.

Different models of brain injuries have shown increased migration of endogenous immature DCX positive neurons from the SVZ into the striatum *(Gordon et al., 2007; Masuda et al., 2007)*. After cortical ischemia induced by middle cerebral artery occlusion immature neuroblast migrate mostly into the striatum and at the lateral boarder of the striatum *(Arvidsson et al., 2002)* and it has been shown that migration in the RMS was increased concomitantly *(Gotts et al., 2005)*. Also, after induced cerebral haemorrhage near the internal capsula of rats, increased neuroblastic migration is seen in the peri-hemmorrhage areas *(Masuda et al., 2007)*. In a model of quinolinic acid-induced striatal cell loss, a population of dividing cells originated form the SVZ to populate the injured striatum *(Gordon et al., 2007)*, but the migration of neuroblasts into the injured cortical areas remained very limited. After cerebral ischemia induced by middle cerebral artery occlusion, doublecortin-immunoreactive cells in the rostral subventricular zone, but not the dentate gyrus, migrated into the ischemic penumbra of the adjacent striatum and, some via the rostral migratory stream and lateral cortical stream, into the penumbra of ischemic cortex *(Jin et al., 2003; Hayashi et al., 2005)*.

Interestingly, after stroke neuroblasts adopt specific migratory routes and the scaffold provided by the ECM is determinant for migration. Cells migrating outside the SVZ after stroke seemed to preferentially migrate in association with blood vessels *(Ohab et al., 2008)*, forming elongated aggregates of chain like structures associated with blood vessels *(Yamashita et al., 2006; Shin et*

al., 2008). This resembled the chain migration of neuroblasts in the RMS which is also in relation with blood vessels *(Bovetti et al., 2007).* In our work we observed also that FGF-2 transduced NPCs had the striking tendency to migrate as clusters of cells around blood vessels. Migration of cells outside the SVZ depends on local cues and chemoattractant factor which play a determinant role in the process. After cerebral ischemia different factors are upregulated during brain ischemia and might play a determinant role in the migration of neural progenitors towards the site of brain damage.

The ischemic cortex induces a local inflammatory response were specific molecules could direct the migration of grafted cells. Interestingly, in our model, the ischemic environment had a strong impact on the fate of transplanted FGF-2 transduced NPCs since about 50% of the population differentiated into DCX positive immature neurons. Within the graft core, a significant proportion of the cells were also positive for the neuronal marker NeuN. Transplanted NPCs appeared to migrate in the direction of the damaged regions of the brain but it was not demonstrated that FGF-2 confers a significant advantage for the directed migration of NPCs towards the zones brain injuries.

Different factors overexpressed in cerebral ischemia have been highlighted has key molecules for the directed migration of NPCs. One of them is the vascular endothelial growth factor (VEGF), a strong angiogenic factors that promotes the migration of endothelial cells has, been shown to be chemoattractant for FGF-2 stimulated NPCs *in vitro (Zhang et al., 2003).* Migrating NPCs *in vitro* are also responsive to stem cell factor (SCF), and monocyte chemoattractant protein-1 (MCP-1) *(Xu et al., 2007). In vivo,* NPCs transplanted in rat brains have shown to migrate towards source of infused MCP-1 *(Magge et al., 2009).* Recently it was observed that sonic

hedgehog a well known developmental morphogen could regulate the migration of neuroblasts in the SVZ, and the rostral migratory stream to the olphactory bulb mainly by functioning as a cheomattractant factor *(Hor et al., 2009)*. The stromal derived factor-1/ chemokine receptor 4 (SDF-1/CXCR4) pathway seems to have an important role in the process since it was shown that stroke could enhance the migration of neural progenitor cells which was attenuated by neutralizing antibody against CXCR4 *(Robin et al., 2006)*. After brain injury, local astrocytes and endothelial cells upregulate SDF-1α which is chemoattractant for neural progenitor cells expressing the receptor CXCR4 *(Imitola et al., 2004)*. MCP-1 also plays an important role *in vivo* for the migration of neural progenitors cells towards the sites of injury after transient middle cerebral artery occlusion *(Yan et al., 2007)*.

The inflammatory response after cerebral ischemia provides local cues that could act as strong chemoattractant factors for transplanted neural progenitor cells. Whether FGF-2 signalling in NPCs improved the directed migration of NPCs towards molecules secreted by the sites of injury remains to be determined.

Survival of neural progenitors in the cortex

Once neural progenitor cells have reached the site of injury they need to differentiate, integrate into functional circuits and survive for long period. In our model, long term survival rate of transplanted NPCs were carried out up to 60 days after transplantation. At this time point and in the non-ischemic cortex, FGF-2 was significantly down regulated with less than 20% of the cells expressing FGF-2 compared to 80% 2 days after transplantation. Nonetheless, at this time point a reasonable amount of NPCs had survived and could be visualised in the rat cortex. By losing their immature properties, NPCs had essentially differentiated into glial cells, astrocytes and

oligodendrocytes. Interestingly, in the olfactory bulb, one month after engraftment, transplanted cells had also significantly down-regulated FGF-2, and differentiated in more mature cells demonstrating the typical morphological features of more mature neurons. Moreover, in the ischemic context also, one month after transplantation, transplanted cells were still visible in the cortex and a small fraction of the surviving cells had differentiated into cells presenting the morphological features of mature neurons with extensive dendrites and were positive for the neuronal marker NeuN.

The long term survival and fate of neural cells available for brain repair is a major issue, either for endogenous repair, but mostly after transplantation. After transient middle cerebral artery occlusion the new born cells migrating out of the SVZ towards the injured striatal area of the brain express doublecortin (DXC) in the first week after focal ischemia indicating an immature and neuronal phenotype *(Jin et al., 2003)*. Migrating DCX cells can also reach the ischemic cerebral cortex, travelling in a chain along the interface between the cortex and the corpus callosum and appear in cortical regions surrounding the infarct 24 hours after the ischemic event. Some of theses cells are observed in the cortex as long as two weeks *(Jin et al., 2003)* or three weeks after the injury *(Zhang et al., 2007)*. The differentiation of cells migrating out of the SVZ might depend on the ischemic model used and the location where these cells reach their final destination. A fraction of these cells will differentiate into glial cells, expressing GFAP or NG2 with various proportion in the striatal or cortical regions near the ischemic core *(Hayashi et al., 2005)*. Overall the fraction of mature cortical neurons surviving in the boarder of injured regions is very low *(Arvidsson et al., 2002)*.

For these reasons, transplantation has opened new hopes to increase the pool of cells available for long term brain repair. Most studies show the survival rate of transplanted neural progenitors

within one month *(Kelly et al., 2004)*, or two months *(Webber et al., 2007)* sometimes up to three months *(Imitola et al., 2004; Srivastava et al., 2009)*. A few studies showed that more than one year after transplantation, transplanted neural progenitors could survive through the injured areas of mice brain *(Shear et al., 2004)*. In our model survival rates were carried out up to 40 days after transplantation, and at this time point, FGF-2 transduced NPCs were still visible and showed more dispersion than control NPCs.

Overall, scientific evidence strongly suggests that endogenous cortical neurogenesis after cerebral ischemia is not sufficient to promote adequate brain repair, and that transplantation of neural progenitors is a reasonable choice to increase the pool of neural cells available for brain repair. In our experience, the pre-transplant manipulation of NPCs with FGF-2 using the lentiviral technology as shown to be in our model a robust approach to improve maintenance and survival of transplanted NPCs.

FGF-2 overexpression in transplanted NPCs: perspectives.

In our experience, the pre-transplant modulation of NPCs with FGF-2 has offered a significant improvement of the brain repair capacities of grafted neural progenitor cells. Thus further development of this strategy could be achieved: 1) The modulation of FGF-2 expression after transplantation would offer the possibility to induce the differentiation of cells towards a specific phenotype. 2) The generation of artificial ectopic cortical neurovascular niches dispersed in the cortex and available as a launch pad to replace lost cells after cerebral injury would be an

interesting development of the "ectopic neurovascular clusters of NPCs" induced by the pre-transplant manipulation of NPCs with FGF-2.

Modulation of gene expression

The permanent expression of FGF-2 in NPCs raises issues with tumour development but also the possibility that the constitutive expression of FGF-2 in NPCs would prevent their differentiation in mature neurons and push towards an immature interneuronal phenotype. This is not the ultimate goal for cellular replacement therapies, since motor function for example needs pyramidal neurons restoration or replacement. Would the modulation of FGF-2 expression in NPCs or sequential expression of lineage specific genes be beneficial in our model?

Inductive constructs or sequential expression of genes as an innovative solution

The modulation of gene expression by lentiviral vectors has been the focus of many groups, using lentiviral approach and different constructs have already been developed. Initially constructs to switch the gene delivered on or off (Tet on/off) have been developed, this after the administration of doxycycline in the system. Transduction of human 293T cells with a tet regulated vector carrying the GFP reporter gene showed that withdraw of doxycycline from the milieu induced a 500-fold increase in GFP expression *(Kafri et al., 2000)*. For our model, the regulation of FGF-2 under a tetracycline transactivator could be used to stop the expression of FGF-2, once NPCs have migrated and established in the cerebral cortex, therefore allowing NPCs to differentiate in more mature neurons, thus this system depends on the use of constant administration of antibiotics.

A novel interesting system, developed to modulate the expression of genes using lentiviral technology is the use of interference RNA (iRNA) which interferes with RNA traduction at a posttranscriptional level based on sequence homology in the cells transfected by the lentivirus *(Corbeau, 2008)*. Using such systems allows to downregulate a gene of interest. Even more, the further development of drug inducing system for conditional RNAi allows the modulation of any endogenous gene in the cell.

To obtain the desired phenotype, the modulation of more than one gene expression is sometimes required. An interesting concept is the sequential expression of gene, which would allow the expression of a gene later after transplantation pushing transplanted NPCs towards a neuronal lineage. In this field dual-promoter lentiviral vectors have been developed, for constitutive or regulated gene expression. A tetracycline-dependant transactivator can drive the inducible expression of a transgene delivered on the same lentiviral vector than the gene of interest *(Gascon et al., 2008)*.

The alternative for co-expression of different protein is the use of bicistronic or multicistronic mRNAs containing an internal ribosomal entry site (IRES). One of the drawbacks of these constructs is the significant low expression of the two cDNA placed downstream from the IRES *(Yu et al., 2003)*, although this might depend directly on the transgene itself, position within the construct, the total number of transgenes expressed and the copies of proviral insertion *(Chinnasamy et al., 2009)*. We used in our work, a pWPI bicistronic lentiviral vector (HIV-1 derived SIN vector) containing the EF1-α promoter and the EMCV-IRES-GFP cistron. Expression of both GFP and FGF-2 have remained satisfactory in our experiments, thus allowing us to detect the presence of fluorescent cells in rat cortical preparation directly under the microscope without any immunostaining, this up to 40 days after transplantation. Also although

the expression of FGF-2 was shown to be significantly downregulated in transplanted cells after 60 days, almost 80% of the cells showed cytoplasmic FGF-2 expression 2 days after transplantation.

The possibility of sequential expression of genes becomes an interesting concept when we suspect in our present work that the overexpression of FGF-2 pushes NPCs towards interneuronal phenotypes, preventing therefore transplanted NPCs to acquire full neuronal mature characteristics. In fact in our long term survival after 2 months, only a very few transplanted FGF-2 transduced NPCs had differentiated into more mature neuronal phenotypes. In the ischemic cortex after one month, grafted cells could differentiate into neurons expressing the γ-aminobutyric acid (GABA) and glutamic acid decarboxylase (GAD67). At later survival time point, some cells expressed the calcium-binding protein, calretinin. Only very few cells had acquired a more neuronal morphology and expressed the neuronal marker NeuN. This could be due to the fact that FGF-2 overexpression, although down regulated at later time points, might prevent NPCs to differentiate into mature neurons and also drive the differentiation into a more interneuronal phenotype. The development and use of sequential gene expression in this setting would be an interesting approach to overcome these limitations and allow the damaged brain to be repopulated with eventually efficient primary sensory or motor pyramidal neurons, this from transplanted NPCs.

Generating artificial/ectopic cortical neurovascular clusters: a key for modern brain repair strategies?

A fascinating characteristic of FGF-2 over-expression in NPCs was the generation of perivascular clusters with the host vasculature as early as one week after transplantation. Compared to control NPCs, the large majority of FGF-2 transduced NPCs associated with the host vasculature. Interestingly, this dynamic relation created between NPCs and the vasculature had a significant impact on the maintenance and proliferation of transplanted cells. We observed that compared to FGF-2 transduced NPCs non-associated to blood vessels, clusters of FGF-2 transduced NPCs associated with vessels, showed a significant increase in their proliferation rate and maintenance of their immature state, revealed by nestin positivity. This observation highlights the intriguing interaction between transplanted NPCs and the host vasculature as an important mechanism for neural stem cells maintenance and proliferation after transplantation. Moreover, it showed that FGF-2 signalling in NPCs was determinant to promote this interaction.

The generation of ectopic neurovascular clusters might be an interesting concept in brain repair strategies. It recalls the interaction between neural stem cells and endothelial cells which occurs in the physiological niches of the SVZ of the lateral ventricle and the SGZ of the dentate gyrus in the hippocampus. This interaction seems to be crucial for the maintenance and self-renewal of endogenous neural stem cells *(Alvarez-Buylla et al., 2002; Doetsch, 2003)*. Within the physiological neurogenic niches neural stem cells reside in close contact with the vascular component and its extravascular basal laminae *(Mercier et al., 2002)*. The concept of vascular niches has been hypothesized for many organs during development and during the adult life

(Nikolova et al., 2007) (Moore et al., 2006), and also for brain tumour stem cells *(Calabrese et al., 2007)*. Recently the concept of the "neurovascular niche" has emerged, where endothelial and neural stem cells closely interact with each other.

For the perspective of future therapeutic brain repair approaches the possibility of generating "ectopic neurogenic niches" in the diseased cortex is appealing. Therefore, understanding the mechanisms and molecular pathways involved in this particular neural stem/endothelial cell relation is of key importance. Moreover, novel development in transplantation technologies could involve the preconditioning of neural stem cells in artificial matrix such as hydrogel which would provide all the structural and molecular support for neural stem cells *(Liu et al., 2003; Bible et al., 2009; Uemura et al.)*. These new approaches require the development of the most appropriate structural and molecular support for neural stem cells maintenance and activation after an ischemic insult. It raises several relevant questions which would help developing technologies to enhance neural stem cells maintenance in "artificial neurovascular niches":

1) How is the endogenous neurovascular niche activated after an ischemic insult?

2) How is it possible to induce such an interaction with transplanted neural stem cells?

Activation of the neurovascular niche after cerebral ischemia

A relevant question for efficient brain repair strategies is how neurovascular niches are activated after an ischemic insult and how they can increase their neuronal production. In our model, we designed an experiment where FGF-2 transduced NPCs were transplanted in the postnatal rat cortex, allowing for neurovascular cluster formation. Ischemia-hypoxia was applied at a later

time point to test the possibility that these neurovascular clusters might respond to an ischemic event and produce new immature neurons which could be recruited by the sites of injury. Interestingly, we observed that after cerebral ischemia-hypoxia, the zones of cortical loss of neurons were recruiting transplanted FGF-2 transduced NPCs and that within these regions some cells had acquired the phenotype of migrating neuroblasts, some of them travelling in close association or away from vessels.

Together, it suggested that these ectopic cortical neurovascular clusters could sense some degree of cerebral injury and respond by increasing their production of immature neurons. In fact it has been already suggested that endogenous neurogenic niches might function as a "sensor" able to identify an hypoxic event and respond with an increase in neuronal production *(Liu et al., 1998; Kee et al., 2001; Jin et al., 2003)* or simply after a physical exercise *(Aberg et al., 2008; Lou et al., 2008; Wu et al., 2008)*, but also after stimulation via exposure to an enriched environment *(Leal-Galicia et al., 2007)*.

Hypoxia influences the endothelial/neural stem cell relationship *in vitro* and can also directly affect *in vivo* the angiogenic/neurogenic response within the neurogenic niches of the SVZ or SGZ. *In vivo,* neurovascular coupling is important outside the SVZ for the migration of neuroblasts to the sites of injury (Figure 8).

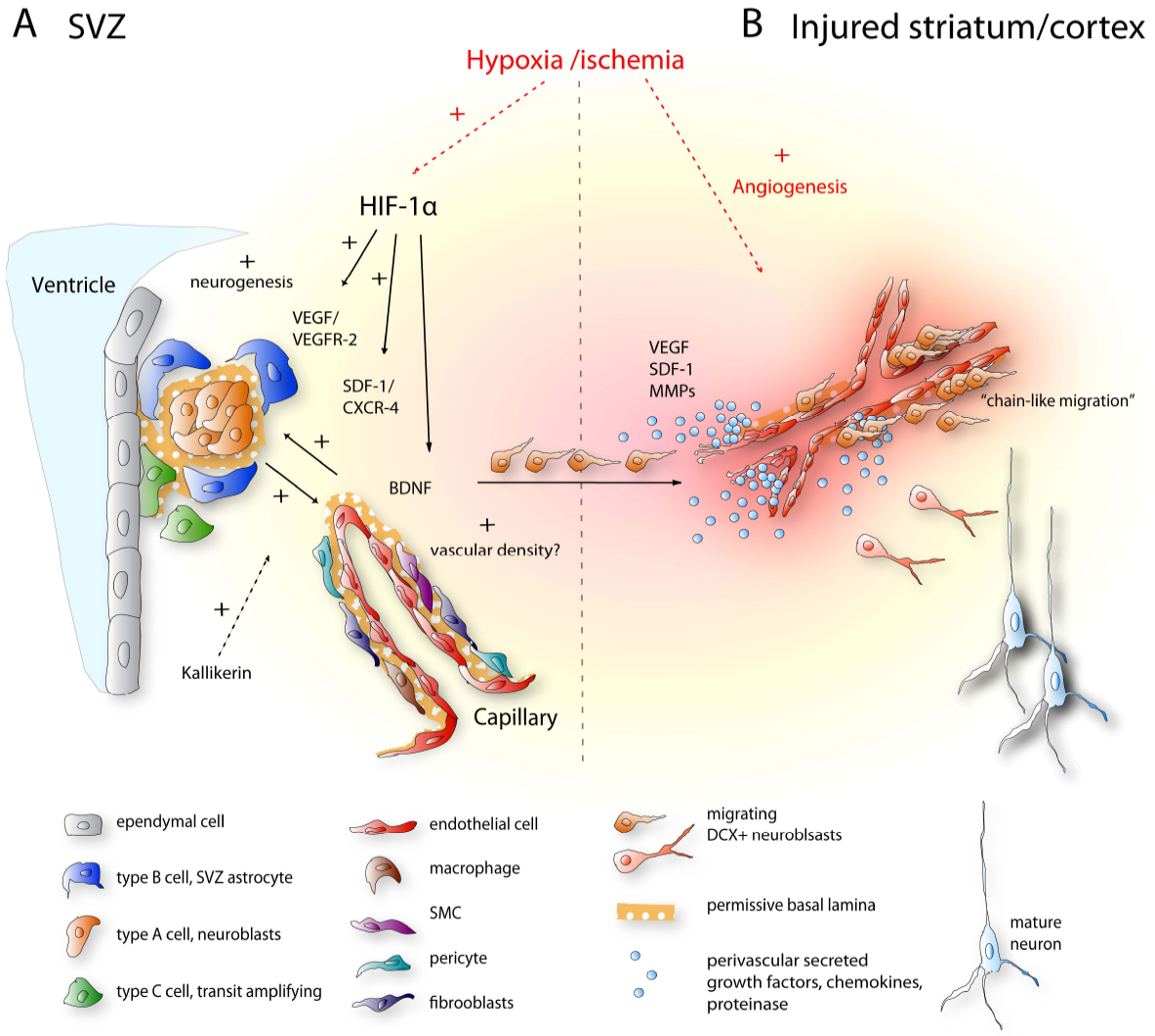

Figure 8 : Neurovascular niche "activation" by cerebral hypoxia/ischemia

The endogenous brain repair mechanisms are activated by cerebral ischemia/hypoxia. The neurovascular niches of the SVZ will provide new immature neurons which migrate towards the sites of brain injury with a distinct tendency to associate and migrate as chain-like migration along blood vessels. (A) The hypoxia inducible factor HIF-1alpha and its many down stream effectors (VEGF, SDF-1, BDNF) seems to be an important player in the SVZ that will stimulate neurogenesis, concomitantly with a low grade angiogenesis. (B) Hypoxia/ ischemia stimulates the vasculature to release in the perivascular microenvironment factors (VEGF, SDF-1, MMPs) which will be determinant for the survival and navigation of newly formed immature neurons through the brain parenchyma.

How does hypoxia influence the endothelial/neural stem cells in vitro?

To test the hypothesis that cerebral ischemia might influence the direct interaction between endothelial and neural stem cells *in vitro*, Teng et al used a co-culture system of cerebral endothelial cells activated by ischemia and neural progenitors isolated from the ischemic SVZ. Endothelial cells activated by ischemia, isolated from the stroke boundary of the rat brain were co-cultured with neural progenitor cells isolated from the SVZ of adult normal rat which increased neural progenitor cell proliferation and neural differentiation while reducing astrocytic differentiation *(Teng et al., 2008).*

How does hypoxia/ischemia influence the neurovascular niche of the SVZ in vivo?

Many studies have shown how cerebral ischemia stimulates neurogenesis in the germinative regions of the adult brain *(Liu et al., 1998; Kee et al., 2001; Yagita et al., 2001; Iwai et al., 2003; Jin et al., 2003)*, and some of the molecular mechanisms that induces neurogenesis and gene profiling of the SVZ after ischemia have been explored *(Liu et al., 2007; Scholzke et al., 2007).* Intermittent hypoxia stimulates the proliferation and differentiation of neural stem/progenitor cells in the SVZ and DG *(Zhu et al., 2005)* and neurogenesis after stroke can continue for months *(Thored et al., 2006).*

Although peri-infarct cortical angiogenesis and its link to VEGF have been well documented *(Greenberg, 1998; Szpak et al., 1999; Marti et al., 2000; Wei et al., 2001; del Zoppo et al., 2003)*, the role of ischemia/hypoxia on the behaviour of endothelial cells or vascular structures in the germinative zone of the SVZ and their relation to neurogenesis is less well understood, and it is debated whether and increased vascularity is observed in the SVZ after cerebral ischemia *(Thored et al., 2006) (Thored et al., 2007) (Gotts et al., 2005).*

After stroke, many genes in the SVZ are up-regulated, some of them participating in neurogenesis, others in angiogenesis. Gene profiles have been analyzed of adult SVZ cells in non-stroke and stroke mice. Non-stroke SVZ cells express sets of genes that are important for neural progenitor cell proliferation, differentiation and migration. In addition, stroke SVZ cells express many genes that are involved in neurogenesis during embryonic development, indicating that adult SVZ cells recapture embryonic molecular signal after stroke. Many of these genes are associated with angiogenesis, like angiopoietin 2, FGF-1, VEGF-B. This suggests that stroke induces gene profile changes associated with neurogenesis and angiogenesis in the adult SVZ cells *(Liu et al., 2007; Liu et al., 2007; Tonchev et al., 2007)*.

Potential role of the "master switch" HIF in the neurovascular niche activation

Molecular pathways might orchestrate the activation of neurovascular niches in response to cerebral ischemia and upregulated down stream effectors involved in a coordinate induction of angiogenesis/neurogenesis. The angiogenic response after hypoxia is mainly coordinated by HIF-1α known as the "master angiogenic switch" and many of its downstream effectors like VEGF. Whether HIF-1α plays a role in the neurovascular niche of the SVZ for the induction of neurogenesis after stroke is relevant for the mechanisms which could be involved in the activation of ectopic neurogenic niches.

In a model of chronic neonatal sublethal hypoxia, CD-1 mice were much more resistant than C57BL/6 mice as they lived at least for 30 days compared to 13 days for the C57BL/6 mice. Interestingly it was seen that although the microvascular density of the SVZ decreased in both strain under hypoxic condition, CD-1 mice kept an increased density of microvessels in the SVZ compared to the other mice. This resistance to hypoxia was related to an increased expression of

HIF-1α protein in the whole brain of CD-1 pups compared to C57BL/6 pups and to an increased level of the factors modulated by HIF-1 α such as VEGF, SDF-1, BDNF and their cognate receptors. Moreover, cultured NPCs form the SVZ of CD-1 pups showed increased expression of HIF1 α than C57BL/6 pups under hypoxic condition, with concomitant induction of VEGF-A165, Nrp-1, CXCR-4, SDF-1, thus highlighting that HIF-1α, modulates the combined angiogenic/neurogenic response from the neurogenic niches of the SVZ *(Li et al., 2008)*.

The induction of neurogenesis from the endogenous neurovascular niche after cerebral ischemia is a fine regulated process where the coupling between hypoxia inducible angiogenic factors and neurogenic factors might be a determinant step to initiate the production of new immature neurons ready to be recruited by the sites of brain injury (Figure 8A).

Association of NPCs with the vasculature outside germinative niches after cerebral ischemia

Efficient endogenous cortical repair after brain injury depends on neuroblasts migration from the SVZ and resident neural stem/progenitor cells to be recruited to the sites of brain damage *(Bjorklund et al., 2000)*. In our model, after hypoxia-ischemia, FGF-2 transduced NPCs seemed to migrate along blood vessels as DCX positive cells. During this process, the importance of neurovascular coupling might be critical for efficient brain repair which raises relevant questions: 1) Does the microvascular environment provide support for the proliferation and survival of immature neurons? 2) Do vessels and their basal membrane provide attachment for neuroblasts towards the sites of brain injury?

A few studies showed that immature neurons induced by the ischemic insult establish themselves in a perivascular environment of the injured brain. In a model of focal stroke in the mouse somatosensory barrel field cortex, labelled endogenous neural progenitor cells migrate out of the

subventricular zone into a unique neurovascular niche in peri-infarct cortex, mediated by SDF-1 and Ang-1 *(Ohab et al., 2006)*. Furthermore, the migration of neuroblasts associated associated vessel after stroke appears to share similar features with normal neuroblasts migration in rostral migratory stream *(Ohab et al., 2008)*. This feature was also observed after MCA occlusion in the adult mouse, where migrating neuroblasts from the SVZ to the striatum formed elongated aggregates of chain like structures associated with blood vessels *(Yamashita et al., 2006)*. Also in the cerebral cortex of rat after focal ischemia Nestin and BrdU-colabelled cells were observed near endothelial cells lining cerebral vessels *(Shin et al., 2008)*.

The directed migration of newly generated immature neurons towards the site of injury appears be related in part by the microenvironmental factors secreted by the vasculature in the areas of brain injury. Many of these factors or chemokine like VEGF, SDF-1 have been shown to be chemoattractant for neural progenitors in different experimental models. SDF-1 is known to be chemoattractant for neural progenitors cells after cerebral ischemia *(Imitola et al., 2004)*, and up-regulated by endothelial cells *(Hill et al., 2004; Li et al., 2008)* and in the close perivascular environment *(Miller et al., 2005)* after focal cerebral ischemia. VEGF is also a key factor for angiogenesis and the directed migration of endothelial cells. It is a chemoattractant in vitro for FGF-2 stimulated neural progenitor cells *(Zhang et al., 2003)*. Whether the over-expression of VEGF by the vasculature or microvascular environment in the areas of cortical injury is relevant for the directed migration of endogenous neural progenitors cells, is not clearly identified *(Schanzer et al., 2004)*. However, its contribution to neurogenesis and neuromigration in a VEGF over-expression mice model of cortical stroke is relevant *(Wang et al., 2007)*.

The migration of neuroblasts through the brain parenchyma might also require the support of the ECM and subsequent degradation to create a migration channel. MMPs are strongly involved in the migration of endothelial cells *(Rundhaug, 2005)* and secreted by the ischemic cerebral microvasculature. They represent potential candidates helping the progression of immature neural cells through the brain parenchyma. Support for this comes from *in vitro* experiments where endothelial cells activated by erythropoietin (EPO), over-express MMP2 and MMP9. This significantly increases the migration of neuroblasts *(Wang et al., 2006)*. In vivo observations show that MMPs are expressed by the ischemic vasculature and migrating neuroblasts are associated to blood vessels *(Yamashita et al., 2006) (Lee et al., 2004; Lee et al., 2006)*.

The basal lamina also contributes for an important part to the interaction between vessels and migrating neuroblasts towards the sites of brain injury *(Mueller et al., 2006)*. Its alteration and damage following ischemia increases the microvascular permeability and change the microvascular environment configuration releasing factors *(Wang et al., 2007)*.

The brain vasculature around the injured brain plays an important role for the migration and survival of immature neurons towards the sites of lesions. Evidence shows that factors and chemokines secreted in the vessel microenvironment may help this process and would be of much benefit to improve the efficiency in generating functional ectopic neurogenic niches (Figure 8B).

How to generate ectopic cortical neurovascular clusters?

Interestingly, the generation of ectopic neurovascular clusters can be obtained by different transplantation techniques or by the pre-transplant manipulation of neural progenitor cells that

enhances this relation, as shown in our work with FGF-2 over-expression in transplanted neural progenitors (Figure 9).

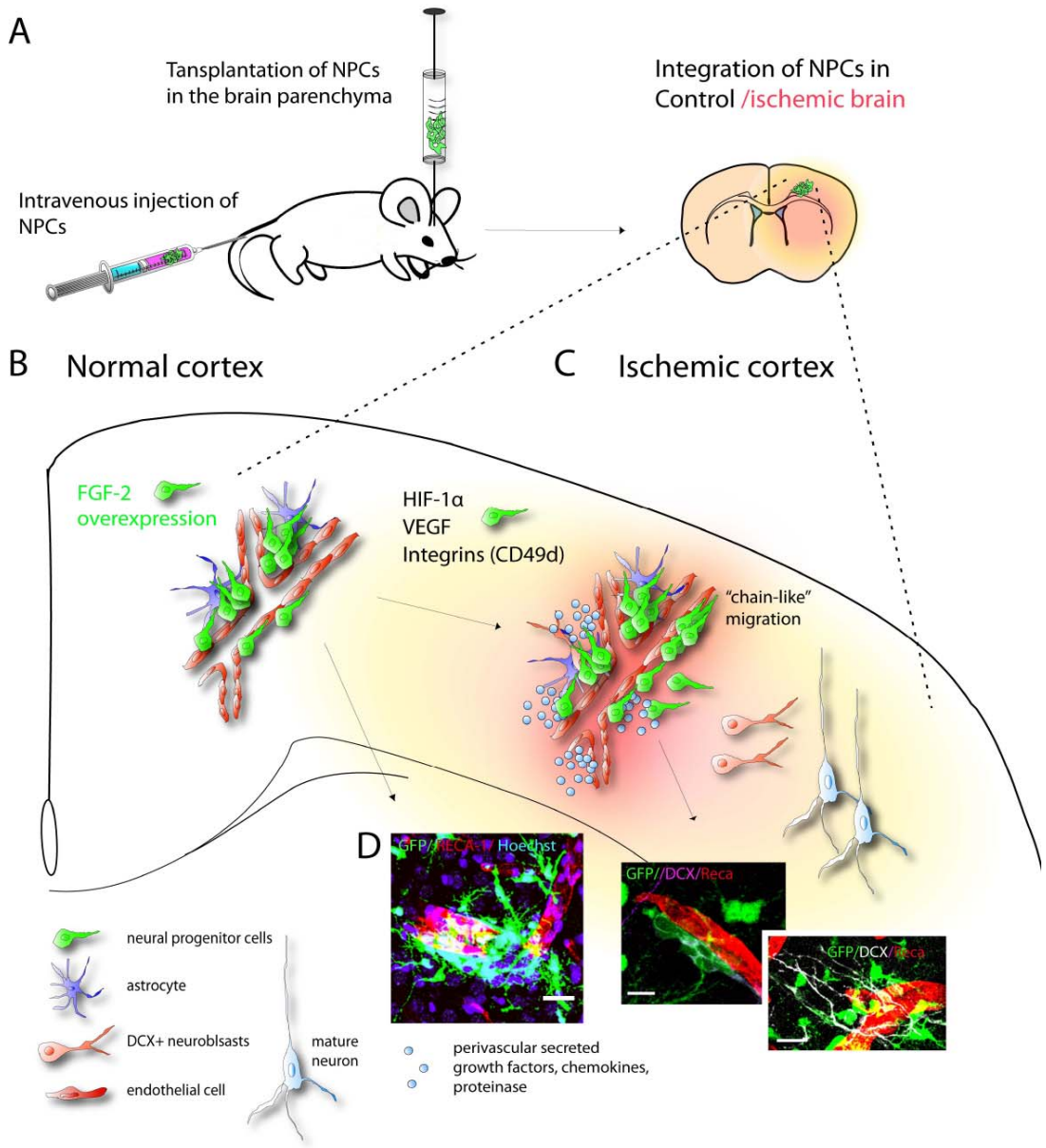

Figure 9: Ectopic perivascular niches after transplantation of neural progenitors.

An interesting strategy to improve brain repair would be to create in the cerebral cortex ectopic neurogenic niches that would provide sufficient new immature neurons which could be recruited by the sites of injury. (A) Representation of two different approaches of transplantation: one via the vascular system, the other by direct intra-cerebral injection. (B) Both of these techniques can provide the formation of ectopic neurovascular clusters to a various degree. FGF-2 overexpression has been shown to increase the formation of perivascular clusters. (C) After cerebral ischemia the activated vasculature seems to secrete factors that are important for the maintenance of these perivascular clusters that are able to provide immature neurons to ischemic

sites. The activated or inflamed vasculature with alteration of the basement membrane plays an important role for the "homing" of neural progenitor cells through the vascular system to the site of cerebral injury. Angiogenic factors (HIF-1alpha, VEGF) have been shown to play a role in the perivascular cluster formation. The physical attachment to the vasculature is a key player and intergrins like CD49d expressed on neural progenitor cells are major determinants for the vascular attachment of transplanted neural stem cells. (D) Confocal images showing that FGF-2 overexpressing neural progenitors preferentially associate with blood vessel after transplantation (left panel). After cerebral ischemia, these perivascular clusters seem to provide new immature neurons which migrate along blood vessels like a "chain migration" (middle panel) and are found in close relation to vessels near the sites of injury.

(Confocal photographs taken from Jenny et al, Stem cells, 2009)

Injection of neural stem cells in the vascular system

A few studies describe a close relation between grafted neural progenitors and host vessels by the injection of neural progenitor cells directly in the vascular system *(Chu et al., 2003; Pluchino et al., 2003; Fujiwara et al., 2004; Takeuchi et al., 2007)*. For example, in a model of rat transient forebrain ischemia, intravenously transplanted human neural stem cells were able to migrate towards the damaged hippocampus, proliferate and differentiate in mature neurons and astrocytes. Many of the cells were seen in the perivascular areas of the dentate gyrus, and some of them were found, on and around cerebral microvessels *(Chu et al., 2003)*.

In a model of chronic CNS inflammation, intravenously injected adult neural progenitors constitutively activated integrins and functional chemokine receptors to selectively enter the inflamed CNS. These undifferentiated cells survived repeated episode of CNS inflammation where they stayed in the perivascular area where reactive astrocytes, inflamed endothelial cells and T cells produced neurogenic and gliogenic regulators, VEGF Noggin, Notch1 and Notch3 *(Pluchino et al., 2005)*. It was suggested that this intravenous systemic transplantation protocol promotes the formation a new anatomical and functional entity. The atypical ectopic

(perivascular) niche would be functionally similar to endogenous germinal niches but differs in terms of cellular components and regional tropism *(Martino et al., 2006)*.

The ECM and especially adhesion molecules to the basal membrane may play an important role in the interaction between neural stem cells and the vasculature. A fraction of NSCs express the integrin CD49d. In a recent study, these cells were sorted by fax analysis and were transplanted via intracarotid injection in a stroke model of mouse. Significantly more NSCs were found in the cortex, the hippocampus the subventricular zone in the ischemic hemisphere in animals receiving CD49d+ NSCs compared to animals receiving CD49d- NSCs *(Guzman et al., 2008)*.

Direct transplantation of neural stem cells in the brain parenchyma

The association of neural progenitor cells and the host vasculature is also described after direct transplantation in the brain parenchyma. The niche microenvironment provided by endothelial cells in vitro, but also the stabilization and recruitment of the vasculature by neural progenitor cells seems to be valid also in the *in vivo* setting. Recently it was shown that neural progenitor cells *in vitro* were able to express constitutively HIF-1α and VEGF, this with an upregulation after oxygen and glucose deprivation. Also they have been shown to promote endothelial cell morphogenesis and prevent endothelial cell death under hypoxic condition. These cells transplanted in the striatum 3 days before the induction of transient focal ischemia via 30 min of MCA occlusion, were found as perivascular densities around microvessels *(Roitbak et al., 2008)*.

Moreover, it was shown that co-transplantation of endothelial cells and neural stem/progenitor cells increased survival and proliferation of ischemia-induced neural stem/progenitor cells and also accelerated neuronal differentiation compared to the transplantation of neural precursors

alone *(Nakagomi et al., 2009)*, thus suggesting that the preconditioning of neural progenitors cells with niche factors improves the maintenance of neural stem cells.

Finding molecules that improve the interaction between transplanted neural stem cells and the vasculature would be beneficial for brain repair strategies. In our work, we highlight that FGF-2 is an attractive candidate, since transplanted FGF-2 transduced NPCs were able to specifically associate with the vasculature of the cerebral cortex. The close interaction of NPCs with the host vasculature was a clear advantage, thus maintaining them in a more immature and proliferative state compared to cells non-associated with vessels. To show that these perivascular clusters of FGF-2 transplanted neural progenitors were capable of activation after an ischemic insult, we transplanted FGF-2 transduced NPCs in the cortex of neonatal rats allowing them to form neurovascular clusters and then applied a hypoxic-ischemic insult. One week later, we could clearly observe that FGF-2 transduced neural progenitors were recruited inside the zones of cortical injuries where neurons had disappeared and were able to produce new immature neurons, migrating along or away from the vasculature *(Jenny et al., 2009)*.

Together, these studies suggest that the intimate relation between transplanted neural progenitors cells and the host vasculature might be key to create an “ectopic niche-like microenvironment” where secreted factors would play a role for the maintenance and self renewal of neural progenitor cells. Within these “neurovascular ectopic niches”, neural progenitor cells could be more receptive and ready to replace lost or damaged cells after cortical injury.

GENERAL CONCLUSIONS

In this work we have demonstrated that the lentiviral manipulation of NPCs with FGF-2 prior to transplantation significantly improves the potential of NPCs for brain repair. FGF-2 transduced NPCs maintained after transplantation into the cortex of the neonatal rat an immature and proliferative phenotype, thus preserving significant migratory properties. After hypoxia-ischemia the pool of NPCs invading the zones of cortical damage significantly increased with lentiviral FGF-2 transduction prior to transplantation. More than 50% of FGF-2 transduced NPCs differentiate into immature neurons after cerebral ischemia.

An attractive effect of lentiviral FGF-2 manipulation of NPCs was the striking tendency of NPCs to associate with the host vasculature, thus generating "cortical ectopic neurovascular cluster". For further development in transplantation strategies we believe that a key issue for the maintenance of neural stem cells would be to supply a suitable surrounding microenvironment which could act as a niche providing all the factors and cellular interaction essential for their self-renewal and proliferation. In our model the interaction with the host vasculature was significantly favourised by FGF-2 signalling, maintaining NPCs in a more immature and proliferative state after transplantation. Moreover, these "ectopic neurovascular clusters" preserved a neurogenic potential since after cortical hypoxia-ischemia, immature neurons could be found migrating away from the vasculature, towards the regions of neuronal loss. The concept that the manipulation of neural stem cells prior to transplantation could enhance the formation of neurovascular ectopic niches in the brain cortex is, from our perspective, a significant step towards the establishment of efficient biotechnological strategies in brain repair.

Generating "cortical ectopic niches" after transplantation is an attractive concept for brain repair strategies. Future progress in transplantation technologies could involve the preconditioning of neural stem cells in an artificial matrix such as hydrogel which would provide all the structural and molecular support essential for neural stem cells maintenance, thus reproducing an "artificial niche for neural stem cells". This matrix could be injected into areas of brain damage and function as "artificial ectopic niches", thus functioning as a launch pad available for cell replacement. Developing such technologies could significantly improve the delivery of neural stem cells in the different zones of brain injury. Moreover, it would increase the chances for one single cell to reach its final destination and integrate into a functional network, thus improving the efficiencies of brain repair strategies.

Abbreviations

bFGF/FGF-2	: basic fibroblast growth factor/ fibroblast growth factor-2
NPCs	: neural progenitor cells
NSCs	: neural stem cells
ESCs	: embryonic stem cells
CNS	: central nervous system
SVZ	: subventricular zone
SGZ	: subgranular zone
DG	: dentate gyrus
ECM	: extracellular matrix
EGF	: epidermal growth factor
VEGF	: vascular endothelial growth factor
HIF-1α	: hypoxia induicible factor-1α
SDF-1	: stromal derived factor-1
MMP	: matrixmetalloproteinase
DCX	: doublecortin

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