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Adult neural stem/progenitor cells in response to their microenvironment : proliferation, differentiation, and migration

Basam Barkho

Basam Z Barkho

Candidate

Biomedical Science Graduate Program

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Xinyu Zhao, PhD , Chairperson

Lee Anna Cunningham, PhD

Paul McGuire, PhD

Michael Wilson, PhD

ADULT NEURAL STEM/PROGENITOR CELLS IN RESPONSE TO THEIR MICROENVIRONMENT:

PROLIFERATION, DIFFERENTIATION AND MIGRATION

BY

BASAM Z BARKHO

B.S., University of California, San Diego, 2002

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

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ABSTRACT

The plasticity of adult neural stem/progenitor cells allows a differential response to a variety of environmental cues. Over the past decade, significant research efforts have been devoted into understanding the regulation of neural stem/progenitor cells due to their promising potential for cell replacement therapies in adult neurological diseases. It has been demonstrated that after brain injury both endogenous and grafted neural stem/progenitor cells have the ability to proliferate to expand their number, migrate long distances to the lesioned site and differentiate into new specific neurons to replace the ones that have been lost. All these procedure are regulated by extrinsic cue found in the microenvironment surrounding the neural stem/progenitor cells. Several chemokines and growth factors have been identified that stimulate the proliferation, differentiation, and migration of endogenous or exogenous neural stem/progenitor cells. The first part of this dissertation work (Chapter 5) identifies the role of several extrinsic factors expressed and secreted by hippocampal astrocytes that regulate the neuronal differentiation of adult neural stem/progenitor cells in the neurogenic region of the dentate gyrus. While in non-

neurogenic regions, astrocytes secrete factors that inhibit the differentiation of adult neural stem/progenitor cells.

Cell migration is an essential component of neurogenesis in both embryonic and adult brains. Many critical signaling factors and molecules are involved in governing the dynamic process of cell migration, which includes chemotaxis, cytoskeleton restructuring, nuclear translocation, and extracellular matrix remodeling. Extracellular molecules regulate the interaction and communication of the cell with its microenvironment. Investigators have shown that extracellular matrix and matrix remodeling factors play a critical role in directing stem cell migration during development and in the response to brain injury. Identification the molecular pathways and mechanisms of these factors, involved in regulating stem cell fate choice and homing into the damaged areas is vital for new treatments in brain injury. The second part of this dissertaition (Chapter 6), I focus on demonstrating that several matrix metalloproteinases are demonstrated to play a role in both the migration and differentiation of adult neural stem cells/progenitor in response to stroke-induced chemokines. The role of matrix metalloproteinase in differentiation may be the first evidence of extracellular molecules effecting the intrinsic regulation of adult neural stem/progenitor fate choice.

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CHAPTER 1

Neural Progenitor/Stem Cells, Adult Neurogenesis, and the Environmental Niche

Stem cells are defined by their abilities to 1) self-renew and 2) differentiate into multiple cell types. The adult central nervous system (CNS) contains neural stem/progenitor cells (NSPCs) that can undergo either symmetric division that yields two identical daughter stem cells or asymmetric division that can give rise to one daughter stem cell and one committed precursor cell. Furthermore, a NSPC-derived precursor cell can become one of the three major cell types of the adult brain, a neuron, an oligodendrocyte, or an astrocyte. Multipotent NSPCs have been isolated from many regions throughout the adult mammalian brain (Gage, 2000). However, only two regions of CNS have been confirmed to have ongoing neurogenesis, a process defined as the production of new neurons. These neurogenic areas include the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subverntricular zone (SVZ) bordering the lateral ventricles. Recent publications suggest that slowly dividing NSPCs ($BLBP^+$, $Nestin^+$, $GFAP^+$, see Table 1.1 for definition of markers) of the SGZ give rise to $Nestin^+$, $GFAP^-$ transient amplifying cells and some of these cells differentiate into DCX^+ immature neurons (or neuroblasts) which migrate a short distance into the granule cell layer (Doetsch, 2003a). Newly matured $NeuN^+$ neurons can integrate into the hippocampal network by extending their axonal projections along mossy fiber pathways to form synapses with the CA3 pyramidal neurons. Whereas their dendrites extend in the opposite direction toward the molecular layer to form synapses with neurons from perforant pathway that orginate from neurons in the entorhinal cortex (see Figure 1.1)

(Ming and Song, 2005; Zhao et al., 2008). Adult neurogenesis in the hippocampus is suggested to play an important role in adult learning and memory (Kee et al., 2007; Ming and Song, 2005; Zhao et al., 2008); however, most experimental evidence have been correlational.

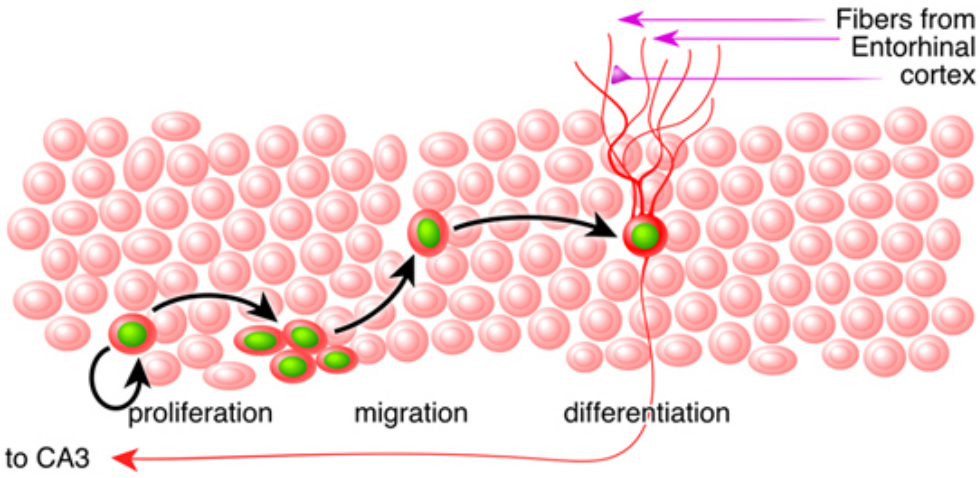

Figure 1.1: Neurogenesis in the adult hippocampal dentate gyrus. First, proliferation and fate determination: Stem cells in the subgranular zone of the dentate gyrus give rise to transit amplifying cells that differentiate into immature neurons. Next, migration: Immature neurons migrate into the granule cell layer of the dentate gyrus. Finally, integration: Immature neurons mature into new granule neurons, receive input from the entorhinal cortex and extend projections into CA3. (Adapted from Lie DC et al., Annu Rev Pharmacol Toxicol 2004)

In the SVZ, the cellular composition and architecture have been illustrated as a three-dimensional interconnected niche comprised of three major cell types (Doetsch et al., 1997). Neuroblasts (type A cells, DCX^+, doublecortin, see Table 1.1) organized as chains are ensheathed by the processes of slowly dividing SVZ cells with astrocytic properties (type B cells, $Nestin^+$, $GFAP^+$, see Table 1.1) and are adjacent to fast amplifying cells (type C cells, neuronal progenitor cell, $Nestin^+$, $GFAP^-$, Figure 1.2, left) (Alvarez-Buylla et al., 2001; Ma et al., 2009). Many of the neuroblast chains merge in the anterior and dorsal SVZ then migrate to the olfactory bulb in a chain configuration through a restricted path called the rostral migratory stream (RMS) formed by a tunnel of astrocytes

(Figure 1.2) (Doetsch and Alvarez-Buylla, 1996; Lois et al., 1996). These neuroblasts develop into functionally mature interneurons that are likely important for processing new information in the olfactory system.

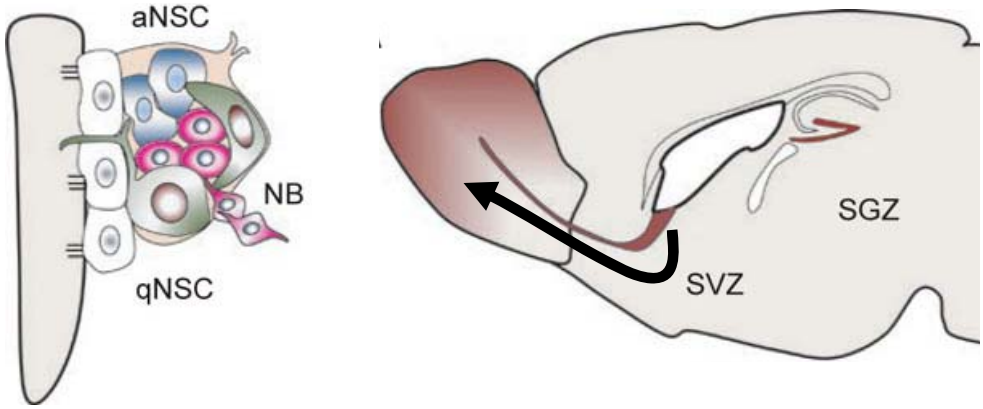

Figure 1.2: Neurogenesis in the subventricular zone (SVZ). Architecture in the adult subventricular zone (left). The adult SVZ consists of transit amplifiers (type C cells, aNSC, blue) that give rise to neuroblasts organized as chains (type A cells, NB, red) ensheathed by the processes of slowly dividing SVZ astrocytes (type B cells, qNSC, green). Ependymal cells (type E cells, white) line the ventricle and are sometimes displaced by type B cells that interlock between the ependyma to contact the ventricle. **Neuroblasts migrate out of the SVZ into the RMS and to the olfactory bulb (right).** Arrow represents route and direction of migration from the SVZ. (Adapted from Ma DK et al. Cell Research 2009)

NSPCs isolated from the rodent fetal brain or adult SVZ, DG, or forebrain, can be maintained as multipotent stem cells *in vitro* in serum-free media conditions with defined supplemental factors and the presence of mitogens -- basic fibroblast growth factor (bFGF) and epithelial growth factor (EGF) (Ming and Song, 2005). Clonal analyses have demonstrated that these NSPCs can be instructed to differentiate into all three major cell lineages of the brain (neurons, astrocytes, and oligodendrocytes) responding specifically to the exogenous signals administered to the culture (Kee et al., 2007). Therefore the *in vitro* culture of NSPCs has provided not only a good system for studying neurogenesis but also a source of cells for potential cell-based therapies (Horner and Gage, 2000).

Upon transplantation into normal or damaged CNS, these NSPCs can differentiate into neurons or glia based on the signals located within the local environment (Kolb et al., 2007; Magavi et al., 2000; Nakatomi et al., 2002). The regenerative capacity of NSPCs holds great potential for repairing damaged CNS resulting from stroke, trauma or neural degenerative disease such as Parkinson's disease, Alzheimer's, and amyotrophic lateral sclerosis.

TABLE 1.1 Cell Lineage Markers to Identify Phenotype Lineage

Lineage	**Antibody (Description)**
Neural Stem Cell	Nestin (intermediate filament protein), Prominin (membrane glycoprotein), BLBP (brain lipid binding protein)
Neuron	TujI (β-Tubulin), Doublecortin, DCX (microtubule cytoskeleton), NeuroD (transcription factor)
Astrocyte	GFAP (intermediate-filament protein), ABCA1(ATP-binding cassette transporter 1)
Oligodendrocytes	O4 (sulfatides, intermediate precursors), NG2 (Chondroitin Sulfate Proteoglycan, immature oligodendrocyte precursors)
Others	DCX (mirgratory cells), vascular cells (VE-CAM, PE-CAM-vascular adhesion molecules)

1.1 Neural Stem Cells *In vitro* for Studying Neurogenesis

It has been well-demonstrated that multipotent neural stem cells can be isolated from many regions of fetal and adult brains, including spinal cord and white matter (Gage, 2002; Sim and Goldman, 2005). These cells can be expanded *in vitro* using defined serum-free medium containing mitogenic growth factors such as basic fibroblast growth factor (FGF-2) and epithelial growth factor (EGF) (Temple and Alvarez-Buylla, 1999). In the absence of FGF-2 and EGF, adult NSPCs can spontaneously differentiate into all three major lineages of the CNS cells, neurons, astrocytes, and oligodendrocytes. The differentiation of adult NSPCs into cell each lineage can also be promoted by specific differentiating factors, such as retinoic acid (RA), forskolin, serum, leukemia inhibitory factor (LIF), bone morphogenetic protein 2 (BMP2), and insulin-like growth

factor 2 (IGF2) (Ming and Song, 2005). However, achieving directed differentiation into a homogenous population of CNS cell types is still a major challenge for adult NSPCs. When grafted into either embryonic or adult brains, adult NSPCs could differentiate into neurons in an area that supports neurogenesis, but only into glia in non-neurogenic regions (Shihabuddin et al., 2000), suggesting that these cells have the ability to respond to the extracellular signals present in the local brain environments (Gage, 2002). Therefore, isolated adult NSPCs, being self-renewing and multipotent, have proved to be a reliable *in vitro* model for understanding the molecular mechanism underlying their proliferation, differentiation, and migration.

1.2 Intrinsic Genetic and Epigenetic Program of Adult NSPCs

The self-renewal and fate specification of NSPCs are regulated by complex intrinsic mechanisms. It was found that mice of different genetic backgrounds exhibit distinct levels of adult neurogenesis both in standard housing conditions and during voluntary physical exercise (Kempermann and Gage, 2002; Kempermann et al., 1997), suggesting that heritable genetic and epigenetic programs are modulating adult neurogenesis. Many cell growth and differentiation genes have been identified to be directly involved in the regulation of NSPCs (Ming and Song, 2005). For example, cell growth and tumor suppressor phosphatase, PTEN, is critical in regulating the proliferation of NSPCs and a null mutation leads to increased NSPC self-renewal (Li et al., 2002) and cell migration (Marino et al., 2002) and subsequently an enlarged brain (Groszer et al., 2006). The basic helix-loop-helix (bHLH) proteins, such as Hes1 and Hes5, of the Hairy/Enhancer of split family protein, inhibit neuronal differentiation and promote astrocyte differentiation of NSPCs (Ishibashi et al., 1995; Wu et al., 2003). In

contrast, Hes6 enhances neuronal fate determination and represses astrocyte differentiation (Jhas et al., 2006). Cell cycle inhibitors, p21 and p27, inhibit the proliferation of NSPCs and are essential for normal CNS development (Doetsch et al., 2002; Siegenthaler and Miller, 2005). Mutation of Ataxia Telangiectasia Mutated, a DNA repair protein, leads to genomic instability and reduced neuronal differentiation of adult NSPCs (Allen et al., 2001). Orphan steroid receptor, TLX maintains adult NSPCs in a proliferative and undifferentiated state, and TLX-null NSPCs fail to proliferate (Zhang et al., 2008).

During recent years, epigenetic mechanisms have come to the center stage of neurogenic regulations. Epigenetic mechanisms refer to meiotically and mitotically heritable changes in gene expression that are not coded in the DNA sequence itself (Levenson and Sweatt, 2005). In mammals, epigenetic mechanisms include DNA methylation (Reik et al., 2001), histone modification (Jenuwein and Allis, 2001), and noncoding RNAs-mediated gene regulation (Bernstein and Allis, 2005). Several epigenetic factors have been shown to modulate adult neurogenesis. For example, deficiency in Methyl-CpG binding protein 1 (Li et al., 2008; Zhao et al., 2003) and polycomb protein Bmi-1 (Fasano et al., 2007; Zencak et al., 2005), as well as inhibitors to histone deacetylases (Siebzehnrubl et al., 2007) have been shown to affect adult neurogenesis. Recently, HMGA2, a chromatin-associated protein whose expression level declines with aging, has been shown to potentiate NSPC self-renewal through regulating the INK4a/ARF pathway (Nishino et al., 2008). In addition, several small noncoding RNAs, including miR-124, miR-9, let-7, etc, have been found to regulate stem cell differentiation. The particular, let-7b has been shown to regulate adult neurogenesis by

repressing HMGA2 (Foshay and Gallicano, 2007; Li and Zhao, 2008; Lindvall et al., 2004; Nishino et al., 2008). Many studies have shown that adult neurogenesis is highly sensitive to environment stimuli and experience (for a recent review, see (Li and Zhao, 2008)). A remarkable feature of epigenetic regulation is its ability to sense the extrinsic environmental changes and make inheritable modification in gene expression without modifying DNA transcription (Li and Zhao, 2008); therefore, epigenetic mechanisms could be the missing link that connects the genetic and environmental effects on adult neurogenesis. The molecular network formed by both genetic and epigenetic regulatory mechanisms likely determines the stem cell intrinsic properties that define the nature, responsiveness, and potentials of adult NSPCs. As described above in the previous section, this regulation of adult NSPCs can also be control by extrinisic signaling from the surrounding environmental niche.

1.3 Neural Stem Cell Niche

1.3.1 Extracellular Matrix

The extracellular matrix (ECM) contains a complex set of molecules that are tightly regulated. In the nervous system, the ECM has been shown to play an important role in neural development, including cell survival, migration, differentiation, axon growth, and synapse formation (Venstrom and Reichardt, 1993). Some of these molecules are transiently expressed at particular times during development and down-regulated during adulthood. The major ECM molecules of the CNS are fibrous matrix proteins (collagens, fibronectin and vitronectin), basement membrane proteins (laminin), tenascins, and proteoglycans. The interaction between the ECM molecules and cells is a dynamic process controlled through cellular receptors that promote cell adhesion, activate intracellular signaling pathway, and modulate the activity of several growth factors

(Venstrom and Reichardt, 1993). The cell surface receptors for the ECM are the integrins, a large family of α- and β- subunits, which can form >20 different receptors (Takada et al., 1997). These receptors can interact with many ligands and cell surface molecules, such as tyrosine kinases, G-protein coupled receptors (GPCRs), growth factor receptors, L1-CAM, or members of the tetraspanin family of proteins. When integrin receptors are directly or indirectly activated, they transduce signals through several pathways that include focal adhesion kinase (FAK), the Src family kinase fyn, MAP kinase, protein phosphatases, SH2-SH3 adaptors, Rho-family GTPases and phospholipid mediators. The activation of these signaling cascades ultimately results in a number of changes of integrin characteristics, such as plasma membrane localization, internalization, ligand affinity, signaling through intracellular proteins, interaction and the restructuring of the cytoskeleton, and transcriptional regulation (Schmid and Anton, 2003). These changes can directly affect the dynamics of cell-cell and cell-ECM interactions. For example, after ischemic injury, DCX^+ neuroblasts are in close proximity to the endothelial cells of vasculature (Ohab et al., 2006), suggesting a possible mechanism by which the neuroblasts migrate along the endothelial cells trail to reach the lesion area. Since the basal membrane of the vasculature consists of laminin, it is possible that matrix metalloproteases (MMPs) secreted by migrating neuroblasts degrade laminin during cell migration (to be discuss in full detail in Chapter 2). MMPs are a family of enzymes that collectively are able to degrade all the components of the ECM (Seiki, 2002; Stamenkovic, 2003). These proteases participate in a host of important physiological processes, including CNS development, embryological remodeling, wound healing, and angiogenesis, and their role in cancer cell metastasis has been studied extensively (Chang

and Werb, 2001; Mannello et al., 2006). To further support this notion, it has been shown that the migrating neuroblasts within the RMS express the laminin receptor, β1 integrin, and deficiency in this integrin inhibits the interaction among neighboring neuroblasts and abolishes their migration towards the olfactory bulb (Belvindrah et al., 2007). Our laboratory and others have found that laminin coating for *in vitro* migration assays is the most effective ECM component for mediating cell migration of NSPCs (Barkho et al., 2008; Kearns et al., 2003).

1.3.2 Cell-Cell Contact and Communication

The Gap junction proteins and cadherins have also been shown to play a role in NSPC proliferation and differentiation (Doetsch, 2003b; Li and Xie, 2005). However, the functions of these proteins have not been fully investigated in the neurogenic response to ischemic injury. Gap junctions play critical roles during embryogenesis, such as providing cell-cell communication for signaling pathways and intracellular transfer of ions, second messengers, and morphogens. Either the exchange or the decrease of such cytoplasmic factors through Gap junctions causes neural stem cells to exit the proliferative state and initiate differentiation (Dermietzel and Spray, 1993; Giaume and Venance, 1995). Connexin 43 (Cx43), a Gap junction protein, is expressed by embryonic neural stem cells to form contact between the stem cells and astrocytes. Also, Cx43 phosphorylation regulates the differentiation of NSPCs (Duval et al., 2002). In addition, a switch of Cx43 to Cx33 or Cx40 has been suggested to regulate hippocampal progenitor cells undergoing neuronal differentiation (Rozental et al., 1998). Furthermore, Gap junctions have been shown to play a role in cell migration during neural crest formation (Huang et al., 1998). The findings suggest further that Gap junction communication

mediates the expression of alpha1 connexins, resulting in the gain or loss of function in embryonic development, thereby illustrating their important role in cardiac neural crest migration.

Cadherins form cell-cell interaction through homotypic bonds across the extracellular gaps. These proteins have been shown to contribute to the molecular mechanism of cell differentiation through the activation of beta-catenin signaling pathway that is mediated by the cleavage of cadherin connecting cells (Huber and Weis, 2001; Shapiro, 2001). Furthermore, cadherins have been shown to be expressed by adult neurosphere cultures (Lobo et al., 2003), and it has been suggested that β-catenin signaling is involved in the adult neural stem cell niche (Doetsch, 2003b). A recent report shows that when proliferative neural precursor cells in the brain are clustered, they are associated with each other via cell adhesion molecules, such as N-cadherin/beta-catenin. As these cells migrate out of the clusters the cadherin/catenin signaling is reduced (Seki et al., 2007). These experiments suggest that the clustering cells migrating have a systematic cellular arrangement and intercellular communication, but further investigation of the role of Gap junctions in adult NSPC proliferation, migration and differentiation in response to injury is clearly needed. Regardless, the fact that Gap junctions and cadherins are crucial for the effects on adult NSPC proliferation, migration, and differentiation, this thesis does not focus on studying the role of these molecules in the effects of adult NSPC in response to their microenvironment of the stem cell niche.

CHAPTER 2

Neural Stem Cell Migration and Proteases

2.1 Cytokines and Chemokines in the Normal and Injured Brain

Cytokines are a group of signaling molecules that stimulate cellular function through autocrine, paracrine, or endocrine mechanisms. Although cytokines are best known for their functions in innate and adaptive immune responses, mounting evidence indicates their important roles in the adult brain (Das and Basu, 2008; Hagberg and Mallard, 2005; Vitkovic et al., 2000). Chemokines (chemotactic cytokines) are a family of small secreted cytokines, defined by the arrangement of the conserved cysteine residues, which are known to induce directed chemotaxis of responsive cells. The family of chemokines share common tertiary structures, and 20-50% sequence homology at both gene and protein levels (Laing and Secombes 2004). Some chemokines are considered pro-inflammatory and can be induced during an immune response, while others are expressed in the enviornment niche to establish homeostatic conditions. Current literature in cancer, immunology, and angiogenesis has demonstrated how cytokines and chemokines may mediate cellular responses to their environment. Chemokines can be released by many different cell types and serve to guide cells under various physiological conditions. Cells expressing chemokine receptors are attracted through increasing concentration of chemokines through chemokine receptor and activation of a variety of signaling pathways. Table 2.1 shows some of these chemokines, such as stromal cell-derived factor 1 (SDF-1α), have been shown to mediate the cell migration during normal angiogenesis, as well as cancer cell metastasis (see Table 2.1).

Table 2.1: Chemotactic Factors for Neural Stem Cell Migration

Chemokine and Cytokines	Receptor	Source in the Brain	NSPC effects
Stromal Derived Factor (SDF)-1α	(C-X-C motif) receptor (CXCR) 4	▪ Astrocytes ▪ Neurons	▪ Migration(Barkho et al., 2008) Differentiation(Barkho et al., 2008)
Stem Cell Factor (SCF)	c-kit receptor	▪ Neurons	▪ Migration(Sun et al., 2004)
Monocyte chemoattractant protein (MCP)-1	(C-C motif) receptor 2 (CCR2)	▪ Microglia ▪ Astrocytes	▪ Migration(Yan et al., 2007)
Tropic and Growth Factors			
Brain-Derived Neurotrophic Factor (BDNF)	TrkB	▪ Neurons	▪ Differentiation ▪ Proliferation ▪ Migration(Chiaramello et al., 2007; Zhang et al., 2005)
Glial cell-Derived Neurotrophic Factor (GDNF)	GDNF Receptor-α	▪ Astrocytes	▪ Differentiation ▪ Migration(Paratcha et al., 2006)
Vascular Endothelial Growth Factor (VEGF)	VEGF-R2 (migration)	▪ Endothelial Cells	▪ Migration(Barkho et al., 2008; Zhang et al., 2005) ▪ Differentiation(Barkho et al., 2008)
Transforming Growth Factor alpha (TGF-α)	TGF-α receptor	▪ Endothelial Cells	▪ Migration(Cooper and Isacson, 2004) ▪ Differentiation
basic Fibroblast Growth Factor (bFGF)	FGF receptor	▪ Endothelial Cells	▪ Migration(Zhang et al., 2005) ▪ Proliferation

(Adapted from Barkho BZ and Zhao X Book Chapter, *Adult Neurogenesis and Central Nervous System Diseases* 2010)

In the CNS, chemokines are typically known for a role in cell migration during brain development. For example, SDF-1α has been shown to have an obligate role in neuronal migration during the formation of the granule-cell layer of the cerebellum (Asensio and Campbell, 1999). However, upon neuroinflammatory injury, such as

multiple sclerosis and stroke, chemokines are produced by reactive cells, such as astrocytes and immune cells within the lesion area. These injury-induced chemokines have been reported to attract inflammatory cells and cause cell death in diseased or injured regions; however, it has also been shown that these chemokines can attract NSPCs and neuroblasts to the injured regions (Wiltrout et al., 2007).

2.2 Roles of Cytokines and Chemokines on NSPC Differentiation and Migration

Traditionally, cytokines and chemokines have been shown to be responsible for damaging neuroinflammation during diseases and CNS injuries (Wang et al., 2002a). For instance, transgenic mice that chronically overexpress interleukin (IL)-6 under the GFAP promoter exhibit CNS damage, with the severity of the damage correlating with levels of IL-6 expression (Wang et al., 2002a). To date, experimental evidence supports the hypothesis that high levels of cytokines play inhibitory roles in adult neurogenesis. For example, both IL-6 and LIF instruct embryonic NSPCs into the astrocyte lineage (Nakashima et al., 1999c). Moreover, GFAP promoter-IL-6 transgenic mice exhibit a 63% reduction in adult hippocampal neurogenesis and significantly reduced neuronal differentiation (Vallieres et al., 2002a). IL-6 inhibits RA-initiated neuronal differentiation of adult NSPCs and inflammation blockade restores hippocampal neurogenesis in irradiated adult brains (Monje et al., 2003). However, these cytokines and chemokines have been shown to have distinct biological effects when present at different concentrations and in various combinations (Ransohoff and Benveniste, 1996). Recent findings indicate cytokines can also have neuroprotective and regenerative effects (Liberto et al., 2004; Yoshida and Gage, 1992a). For example, using gene expression profiling, I have found that primary astrocytes derived from neurogenesis-supporting

embryonic CNS and adult hippocampus express many cytokines, chemokines, and inflammation-related proteins at relatively higher levels when compared to primary astrocytes derived from neurogenesis-inhibitory adult spinal cord and other non-neural cells (Barkho et al., 2006). Furthmore, as described in more detail in Chapter 5, I have also found that IL-1β and IL-6 promoted NSPC neuronal differentiation. These results contradict the current theory that inflammatory cytokines are only playing a role as inhibitors of adult neurogenesis and NSPC neuronal differentiation (Monje et al., 2003). To address this apparent inconsistency, I performed additional parallel experiments using both our condition and other published conditions, and found that in the presence of RA, high levels of IL-6 inhibited NSPC neuronal differentiation (Monje et al., 2003). However, in the absence of RA, relatively low levels of IL-6 promoted neuronal differentiation of adult NSPCs. Therefore it is apparent that the effects of cytokines on adult NSPCs are dependent on many factors, and are likely both context and concentration dependent.

CNS injuries drastically change the types and concentration of cytokines and chemokines in the brains, and therefore significantly alter the environment that NSPCs encounter. For instance, it has been shown that in the mouse ischemic model, astrocytes and endothelial cells express high levels of chemokines, such as stromal cell-derived factor-1, SDF-1α (Thored et al., 2006), and vascular endothelial growth factor, VEGF (Zhang et al., 2002). Both SDF-1α and VEGF have been well-characterized to attract NSPC-derived cells (Imitola et al., 2004; Miller et al., 2005; Zhang et al., 2003a). We and others have shown that NSPCs express CXCR4, the receptor for SDF-1α (Barkho et al., 2008; Thored et al., 2006; Tran et al., 2004). Imitola et al has demonstrated that exposure

to SDF-1α enhances proliferation and promotes migration of NSPCs, through the activation of the homing mechanism to direct NSPCs into the injured brain region(Imitola et al., 2004). I have found that, in the absence of mitogenic growth factors, SDF-1α and VEGF promote both neuronal and astrocyte differentiation of adult NSPCs (Barkho et al., 2008). Several other factors, such as monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1 (Kim, 1996; Otto et al., 2002) are also up-regulated in injured brains and have been suggested to play a role in stimulating neurogenesis after stroke (see Table 1 for summary of chemokines and growth factors role in cell fate) (Hallbergson et al., 2003).

Chemokine-induced cell migration requires the remodeling of the ECM. The chemotaxic functions of SDF-1 and VEGF are known to be mediated by the activation of MMPs (Pufe et al., 2004). Although MMPs have been investigated for their involvement in ischemic brain injuries, such as neuronal death and blood-brain barrier breakdown (Cunningham et al., 2005), their role in the neurogenic response of adult NSPCs after ischemic insults has only recently been considered. Neuroblast migration is known to require ECM remodeling (Bovetti et al., 2007), and MMP-9 immunoreactivity is colocalized with migrating neuroblasts (Lee et al., 2006). Furthermore, MMP-2 and MMP-9 expressed by endothelial cells promote neuroblast migration (Wang et al., 2006). As this will be discussed in more detail in Chapter 6, I have found that MMP-3 and MMP-9 not only play a role in the migration of the neuroblasts, but also are involved in NSPC differentiation. I knocked-down MMP-3 or MMP-9 using siRNAs in NSPCs, these cells have reduced neuronal differentiation in response to chemokines, such as SDF-1 and VEGF (Barkho et al., 2008). Our understanding of the functions of these endogenous

MMPs in regulating adult NSPC differentiation, proliferation, survival, and migration are, however still incomplete. Future studies in this area will significantly enhance our concept of the roles of ECM and proteases in brain injuries and repair.

2.3 Chemokine Signaling

It has been demonstrated that in embryonic stem cells, hematopoietic stem cells, immune cells, and cancer cells, SDF-1α specifically binds to CXCR4, a seven transmembrane domain receptor coupled to a $G\alpha_i$ protein. Upon SDF-1α binding, CXCR4 dimerizes and the tyrosine residues within its C-terminus domain become phosphorylated by receptor-specific kinases and/or Ser/Thr protein kinases, which in turn activates G proteins. This activation has been shown to induce several downstream signaling pathways, including protein kinase C (PKC), phospholipase C-γ and -ζ, FAK/paxillin, MAPK p42/44–ELK-1, and PI-3K–PKB–NF-κB, to name just a few (Petit, Goichberg et al. 2005). Activation of these pathways in CXCR4-positive cells regulates cellular locomotion, adhesion, and protein secretion, and cell migration. To date, the specific downstream signaling pathway that mediates SDF-1α–induced NSPC and neuroblast migration is not fully clear. In contrast, VEGF receptors (VEGFR1-3) have seven immunoglobulin-like domains in their extracellular portion, a single transmembrane spanning region, and an intracellular portion containing a split tyrosine-kinase domain. Of the three VEGF receptors, only VEGFR2 (also known as KDR/Flk-1) is known to mediate cell migration. VEGFR2 regulates the processes of cell locomotion, adhesion, and secretion through the p38/MAPK and FAK/paxillin pathways (Gerber et al., 1998). Even though the signaling pathway of both SDF-1α and VEGF are considered complex due to their interactions with multiple co-receptors, both factors have been well

documented to play important roles in cell migration (Cho et al., 2002; Duchek et al., 2001). Studies have shown that chemotactic function of SDF-1α and VEGF can be mediated by MMPs. For example, SDF-1α promotes the expression of MMP-2 to facilitate the migration of hematopoietic progenitor cells (Janowska-Wieczorek et al., 2000) and mesenchymal stem cells (Son et al., 2006) to their target regions. In Chapter 6, I have shown that SDF-1α and VEGF promote neuroblast migration through MMP activation (Barkho et al., 2008). The finding sets the stage for further studies that will provide a better understanding of the signaling mechanism underlying this regulation.

2.4 Cell Migration in the Brain

The patterns of chemokine and cytokine expression have been characterized throughout embryogenesis. Therefore, it is widely accepted that chemokine-mediated cell migration play an important role in the development of the nervous system (Gogat et al., 2004; McGrath et al., 1999). In recent years, the interest in understanding the physiological and pathological processes of stem cell migration has grown significantly, largely due to the increased evidence of neural progenitor cell migration in the adult brain under both normal and injured conditions. In the embryonic and the adult brains, neural cells demonstrate two unique ways of mobility, radial migration and tangential migration (Figure 2.1). Though the two different types of cell migration are not analyzed in this dissertation, it is important to discuss these processes because after brain injury in the adult, NSPCs convert from a similar process to radial migration, called chain migration, to migrating trangentially into the lesion area.

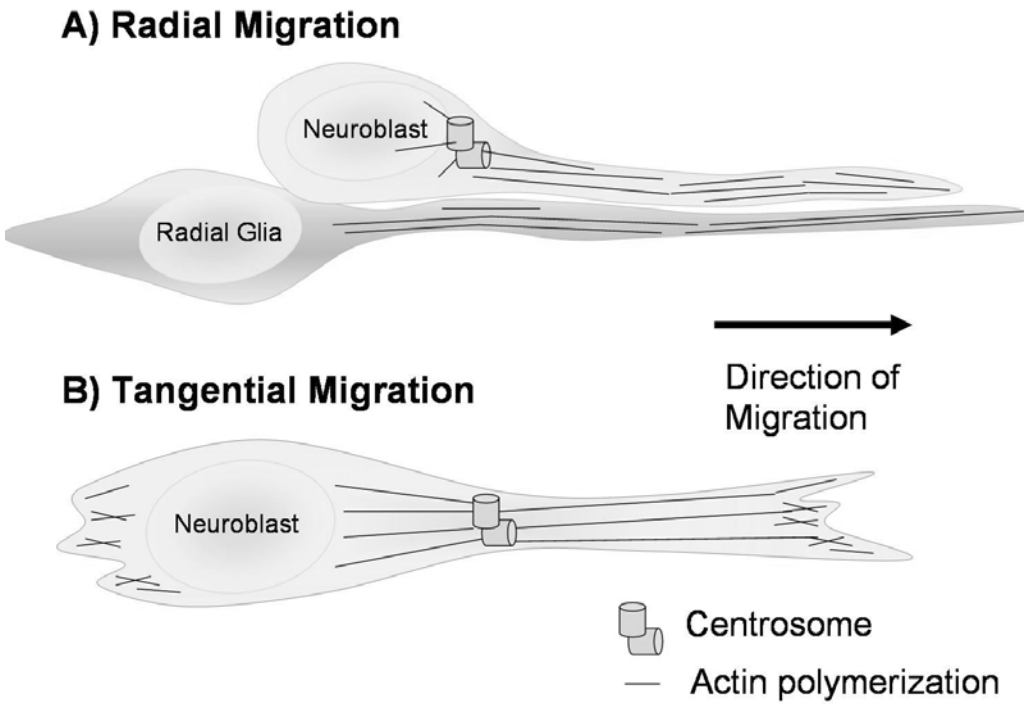

Figure 2.1: Two major types of cell migration in the embryonic and adult central nervous system. (A) Radial migration is found mostly during embryonic brain development. Neuroblasts migrate along radial glia processes to appropriate cortical zones and form the organized layers of the cortex. In migrating neurons, the centrosome, nuclear membrane, and the microtubule network form a complex, which allows the nucleus to move simultaneously with the centrosome. **(B)** Tangential migration is defined as non-radial neuronal translocation that does not require specific interactions with radial glia processes. In this migration, the centrosome and the nuclear membrane demonstrate a pronounced dissociation from each other. The movement of the nucleus follows the movement of the centrosome instead of the simultaneous movement of the nucleus-centrosome formation observed in radial migration. (Adapted from Barkho BZ and Zhao X Book Chapter, *Adult Neurogenesis and Central Nervous System Diseases* 2010)

In radial migration, newly born neurons migrate along radial glia processes (Figure 2.1A). This type of migration is mainly found during embryonic cortical development. In newly formed neuroepithelium, neuroepithelial cells (embryonic NSCs) divide by the basal ventricular zone and generate radial glia that extend long, scaffolding processes from the ventricular lumen to the pia matter of the developing brains (Noctor et al., 2007). Neuroblasts migrate along the processes of radial glia to appropriate cortical zones and form the organized layers of the cortex. Similar mechanisms of radial

migration also occurs during the development of the hippocampus and cerebellum (Maricich et al., 2001). The mechanism by which this migration occurs is unclear. However, it has been suggested that Gap junction signaling between the radial glia cells and the migrating neuroblasts are important in this migration and several chemotactic proteins expressed by Cajal-Retzius cells at the cortical marginal zone are critical for targeted migration (Noctor et al., 2001). Radial migration in the adult brains has only been shown to be in the cerebellum and the olfactory bulb (Hu et al., 1996; Rakic, 1972; Sotelo et al., 1994). In the adult cerebellum, Bergmann glia that guide Purkinje cells migration during development, continue to function as radial glia throughout adulthood and have been shown to guide the migration of neural cells after brain injury (Sotelo et al., 1994). Moreover, in the adult olfactory bulb, after neuroblasts exit the RMS, radial glia have been shown to guide newly differentiate interneurons to the appropriate regions of the olfactory bulb (Ghashghaei et al., 2007; Hu et al., 1996).

Tangential migration is defined as non-radial glia-guided neuronal translocation that does not require specific interaction between migrating cells and radial glia processes (Ghashghaei et al., 2007) (Figure 2.1B). Another difference between radial and tangential migration is the mechanism by which the nucleus moves following the leading end in the direction of migration. In neurons undergoing radial migration, the centrosome, nuclear membrane, and the microtubule network form a complex, which allows the nucleus to move simultaneously with the centrosome, following the leading edge. In tangential migration, the centrosome and the nuclear membrane demonstrate a pronounced dissociation from each other. In this case, the nucleus moves after the centrosome instead of moving with the centrosome (Metin et al., 2008). Tangential migration occurs in many

locations of the developing brain. Most non-pyramidal inhibitory neurons are generated in ganglionic eminence rather than ventricular zone and these neurons migrate tangentially from the medial ganglionic eminence (MGE) to the dorsal telencephalon; from caudal ganglionic eminence (CGE) to dorsal telencephalon, from cortical-striatal boundary to the ventrolateral telencephalon (lateral cortical stream), and from lateral ganglionic eminence (LGE) to the olfactory bulb (through the RMS) (Corbin et al., 2001). The mechanism underlying this tangential migration is under further investigation. It has been suggested that the axonal guidance protein Slit can repel migrating neuronal precursors and facilitate the migration of these neurons from the anterior SVZ to the olfactory bulb (Wu et al., 1999).

In healthy adult brain, tangential migration continues in the RMS, in which migrating neuroblasts travel long distances through a glia tunnel formed by astrocytes from the SVZ to the olfactory bulb. In RMS, neighboring neuroblasts establish the scaffolding processes for other migrating neuroblasts to move along towards the direction of the olfactory bulb (Lois et al., 1996). The mechanism underlying this migration through the RMS is not completely clear. It has been suggested that several chemokines expressed in the olfactory bulb, such as glia-derived neurotrophic factor (GDNF) (Paratcha et al., 2006), brain-derived neurotrophic factor (BDNF) (Chiaramello et al., 2007), hepatocyte growth factor (HGF) (Garzotto et al., 2008), and netrin-1 (Murase and Horwitz, 2004), that are distributed through the RMS might play chemotactic roles in this process. However, removal of the olfactory bulb does not impede neuroblast migration through the RMS (Herz and Chen, 2006), suggesting a potentially important role of the glia tunnel, rather than chemokines, in instructing migration. In focal ischemic injuries of

striatum, neuroblasts and other SVZ-originated cells tangentially migrate towards the infarct region. This injury-induced migration is likely attracted by chemokines released by inflammatory and injured cells (Wiltrout et al., 2007). The migrating neuroblasts have been found to be next to blood vessels therefore laminin might serve as a substrate for this cell migration (Thored et al., 2007). We and others have found that laminin is required for SDF-1α-induced neuroblast migration, which supports this hypothesis (Barkho et al., 2008; Kearns et al., 2003). The molecular mechanism underlying tangential migrations in both healthy and injured adult brains remains to be further explored.

2.5 Molecular Mechanism of Cell Migration

Cell migration is a complex process, therefore requires the interplay of many genes that regulate a wide range of cellular functions, including chemoattraction/repulsion, cell adhesion, cell motility, and cytoskeletal dynamics (Figure 2.2). Mutations of the genes involved in cell migration have been linked to human neurological and developmental disorders, such as lissencephaly, cortical heterotopias, and microcephaly, etc (Gleeson and Walsh, 2000).

The initial cellular response to migratory cues is to polarize and extend the protrusion in the direction of migration. These protrusions are categorized into two types, large and broad lamellipodia or spike-like filopodia (Ridley et al., 2003). The extension of the leading edge of the cell is mediated by the polymerization of actin that is physically linked to transmembrane proteins, such as integrins. The leading edge further stabilizes the protrusion's structure by adhering to either the extracellular matrix or adjacent cells. These adhesions serve as grip for the cell body to contract toward the

protrusion, while a trailing edge is left attached. After the nucleus and cell body have moved forward, the trailing end of the cell disassembles allowing it to detach from the ECM contact. Using a variety of cell types as model systems, researchers have identified several critical factors that regulate cell migration. As I will discuss in the next few sections, some of these factors may also play important roles in NSPC and neuronal migration (Figure 2.2). However, these next section will not be discussed in detail in my dissertation, the all the factors are important to play a role in the global network of cell migration as seen in Figure 2.2. Therefore, I will discuss these sections briefly to emphasize their importance, and will be important to discuss in Future Directions (in Chapter 7) to discuss possible directions to continue the studies of this dissertation.

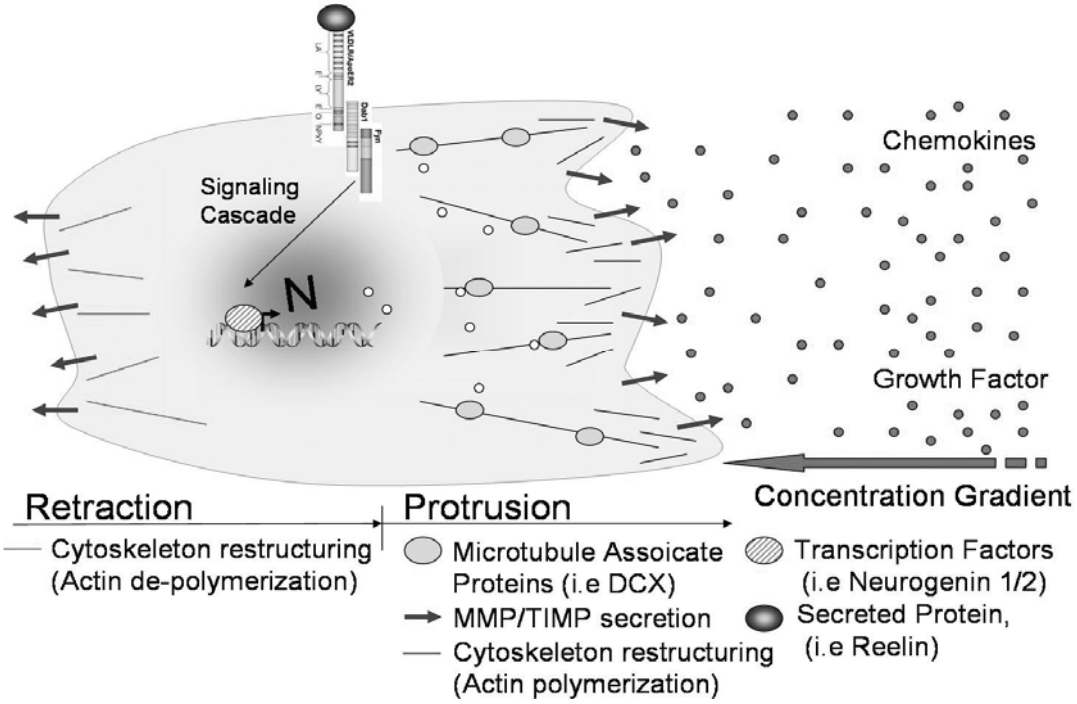

Figure 2.2: The molecular mechanism of NSPC migration induced by chemokines or growth factors. Cell migration is regulated by many intrinsic and extrinsic factors and their balanced action regulates both the extension of the leading edge and retraction of the trailing edge of the cells. Experimental investigation have identified some of these regulatory factors involved in cell migration, including transcription factors (Neurog1/2), cytoskeleton restructuring components (actin polymerization and de-polymerization), signaling pathways (GTPase and Rac), and secreted protein (reelin) and their binding

receptor/membrane proteins (e.g., VLDLR, ApoER2). (Adapted from Barkho BZ and Zhao X Book Chapter, *Adult Neurogenesis and Central Nervous System Diseases* 2010)

2.5.1 Extracellular Chemotactic Molecules

Several secreted proteins have been shown to be important for neuronal migration during development. Reelin is a large ECM-associated transmembrane glycoprotein that is secreted by Cajal-Retzius cells in the marginal zone of the developing cerebral cortex. Very low density lipoprotein receptor (Vldlr) and apolipoprotein E receptor type 2 (ApoER2) are canonical Reelin-binding receptors. Upon binding to these receptors, reelin activates intracellular effector protein Dab1, which is then phophorylated by non-receptor tyrosine kinases, Src and Fyn. How Reelin/Dab1 signaling regulate migratory process is still unknown, however it may influence the microtubule dynamics or cell adhesion. *Reeler* mice, with deficiency in Reelin, have abnormal motor function. During cortical development, migratory neurons in these mice apparently do not receive the critical cue that informs them of their position, therefore resulting in an inversion of the cortical layers. *Reeler* is characterized by an inverted lamination of the neocortex. In humans, Reelin (*RELN*) mutation result in lissencephaly and have also been linked to autism and other mental retardation disorders (Hong et al., 2000; Zaki et al., 2007). Reelin has been suggested to play a crucial role in synaptic plasticity in the adult brain (Herz and Chen, 2006). Whether Reelin plays a role in adult neurogenesis remains a question.

Neuregulins have been identified as neuronal ligands for tangential migration in the developing cerebral cortex and the hippocampus (Yau et al., 2003). The neuregulins (NRGs 1–4) are a family of proteins that have been identified as EGF-domain-containing molecules that serve as ligands for EGF-related receptors known as ErbB1-4. It has been shown that neuregulin-1-initiated ErbB2 and ErbB4 activation promotes the elongation of

radial glia and neuronal migration in the cerebellum and cerebral cortex during development (Anton et al., 1997; Rio et al., 1997). However, the question remains whether neuregulin-1 and ErbB receptors have a role in adult NSPC migration.

2.5.2 Microtubule and Cytoskeleton Proteins

During cell migration, centrosome is bound to the microtubule network and moves ahead pulling the nucleus forward (Metin et al., 2008) (Figures 2.1 and 2.2). This process requires the function of several cytoskeleton and microtubule-binding proteins involved in the rearrangement of centrosome and nuclear positioning. For example, LIS protein and its co-factor dynein, together with myosin II, are important for centrosome movement and nucleus translocation. Mutation in LIS1 gene results in lessencephaly or "smooth brains". Doublecortin (DCX) is a cytoplasmic protein which appears to direct neuronal migration by regulating the organization and stability of microtubules. As described in Chapter 1, DCX is highly expressed in migrating neuroblast and has been widely used as a marker for immature neurons. Migrating neuroblasts are bipolar as described above with a long leading protrusion and a short trailing edge extending from the soma. DCX has been shown to play a key role in cell polarity by recruiting tubulin polymerization and by stabilizing microtubules, which allows membrane trafficking through the leading edge (Feng and Walsh, 2001). DCX is critical for neuronal migration both during embryonic development and in adult brains. In DCX deficient mice migrating interneurons exhibit more branched processes, rather than a single pronounced leading edge, compared to their wild type littermates (Allen and Walsh, 1999; Friocourt et al., 2007). Mutation in DCX can also result in lissencephaly in humans. Since DCX gene is located on the X-chromosome, due to random X-chromosome inactivation, a

subpopulation of neurons in the heterozygote females with DCX mutation migrates approximately halfway to the correct cortical layer, therefore resulting in a "double cortex" phenotype (Gleeson et al., 1998). Cdk5 is a serine-threonine kinase that phosphorylates DCX at multiple site and activates it, allowing its binding to microtubules and other proteins (Graham et al., 2004). Cdk5 associates with a neuronal-specific activator, p35, and has been shown to play a variety of roles in both neuronal development and degeneration. Mutations of both Cdk5 and p35 result in serious neuronal migration defects in mice (Ohshima et al., 1996). DCX is highly expressed in migrating neuroblasts of both SGZ and RMS of adult brains, indicating a potential important role of DCX in cell migration in the adult rodent (Brown et al., 2003) and human (Curtis et al., 2007) brain.

2.5.3 Intracellular Signaling Proteins

Rho proteins generally cycle between an active GTP-bound conformation and an inactive GDP-bound conformation. Among 20 related Rho families of proteins, Rho A/B, Rac 1/2, and CDC42 have been shown to play a role in cell migration. Rac is required for lamellipodium extension induced by growth factors, cytokines and ECM components, and when Rac is inhibited or knocked out, cells cannot migrate (Roberts et al., 1999). CDC42 seems to function primarily through partitioning-defective-3 (PAR3), PAR6 and atypical protein kinase C (aPKC) isoforms to induce polarity. For example, CDC42 and the PAR6–PAR3–aPKC complex have been proposed to mediate the capture and stabilization of microtubules at the leading edge of the cell and to orientate the Golgi and microtubule-organizing centre during the establishment of migratory polarity (Heasman and Ridley, 2008). Finally, activation of Rho A/B has been shown to promote cell body

contraction and trailing tail detachment. On the other hand, cell adhesion to the ECM activates both Rac and Cdc42, for example, plating cells on fibronectin induces Rac and Cdc42 activation, which also is required for cell mobility (Ridley, 2001). Furthermore, activation of Rac activity also leads to increased expression of MMPs (Radisky et al., 2005; Zhuge and Xu, 2001), which are required for the degradation of the ECM for the cell to drive forward toward the direction of migration.

2.5.4 Transcription Factors

Homeobox gene distal-less homeobox 1 and 2 (Dlx1/2) are essential for tangential migration of GABAergic interneurons to neocortex by repressing p21-activated serine/threonine kinase PAK3 (Cobos et al., 2007). The Dlx1/2 double-mutant mouse exhibits a defect in tangential migration of neurons in the striatum and pallidum (Anderson et al., 1997). Dlx^+ cells initially differentiate into projection neurons and some of which migrate to the cerebral cortex and the olfactory bulb. Therefore, Dlx1/2 may play a role in both differentiation and migration that are prerequisite for neuronal migration (Eisenstat et al., 1999). More evidence for coupled differentiation and migration is seen with proneuronal bHLH transcription factors, Neurogenin 1 and 2 (Ngn1/2) (Ge et al., 2006). Transient expression of bHLH transcription factors initiates a cascade of other bHLH gene activation events that eventually lead to the expression of terminal neuronal differentiation genes. Ngn1/2 have been proposed to mediate the down-regulation of RhoA and up-regulation of DCX and p35 in cortical neuron migration (Heng et al., 2008). There has been no clear evidence that adult NSPCs use a similar mechanism to migrate, however this will be a good beneficial step for research to focus to understand the mechanism of brain-derived stem cell migration.

2.6 Matrix Metalloproteinase and Cell Migration

Chemokine-induced cell migration requires the remodeling of the ECM. The chemotactic functions of SDF-1α and VEGF are known to be mediated by the activation of MMPs (Pufe et al., 2004). MMPs are a family of enzymes that collectively are able to degrade all the components of the ECM (Seiki 2002; Stamenkovic 2003). MMPs participate in a host of important physiological processes, including CNS development, embryological remodeling, wound healing, and angiogenesis, and their role in cancer cell metastasis has been studied extensively (Chang and Werb, 2001; Mannello et al., 2006) (Table 2.2). The process of cell migration requires the coordinated regulation of cell-cell attachments, cell-matrix attachment, and matrix remodeling by the balance of MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs) (Woessner and Gunja-Smith, 1991). All MMPs have common structure domains, which include a pro-peptide domain, catalytic domain, hinge region and the haemopexin-like C-terminal domain. The pro-peptide inhibits activation of the catalytic domain until the appropriate signal releases another protease, typically another MMP, which binds the haemopexin-like C-terminal domain and cleaves the pro-peptide. With this event, the catalytic domain is able to cleave it target substrate, which include a wide range of extracellular components and proteins. Generally, MMPs are known to degrade the ECM surrounding the cells to allow the extension of its lamellipodium or filipodia. But to re-attach to the matrix, TIMPs are secreted immediately after MMPs to prevent the MMPs from degrading additional extracellular components and to allow the cell's extending end to adhere in the direction of the migration. However, the role of MMPs in chain migration or glia-guided migration of neuroblasts has not been well studied.

Table 2.2: Matrix Metalloproteinases (MMPs) in Cell Migration

MMPs	Other Names	Cells Regulated	Substrates
MMP-1	▪ Collagenase	▪ Cancer Metastasis	Collagens (I, II, III, VII, VIII and X); Gelatin; IL-1ß; MMP-2, & -9
MMP-2	▪ Gelatinase A ▪ Type IV Collagenase	▪ Cancer Metastasis ▪ Bone Marrow Homing ▪ Angiogenesis	Collagens (I, IV, V, VII, X, XI and XIV); Gelatin; elastin; fibronectin; laminin-1 and -5; decorin; IL-1ß; MMP-1, -9, & -13
MMP-3	▪ Stromelysin-1	▪ Cancer Metastasis ▪ Stroke-induced Migration	Collagens (III, IV, V, IX); Gelatin; decorin; fibronectin; laminin; elastin; IL-1ß; plasminogen; MMP-1; MMP-2/TIMP-2 complex; MMP-7, -8, -9, & -13
MMP-7	▪ Matrilysin	▪ Macrophage Migration	Collagen IV and X; Gelatin; decorin; fibronectin and laminin; insoluble fibronectin fibrils; ß4 intergrin; elastin; plasminogen; MMP-1, -2, & -9; MMP-9/TIMP-1 complex
MMP-9	▪ 92 kDa Gelatinase ▪ Gelatinase B	▪ Cancer Metastasis ▪ Development and Synaptic Plasticity ▪ Stroke-induced Migration	Collagens (IV, V, VII, X and XIV); Gelatin; elastin; fibronectin; IL-1ß; plasminogen
MMP-10	▪ Stromelysin-2	▪ Wound Healing	Collagens (III, IV and V); Gelatin; casein; elastin; MMP-1, & -8
MMP-12	▪ Macrophage Metalloelastase	▪ Macrophage Migration ▪ Cancer Metastasis	Collagen IV; Gelatin; elastin and k-elastin; fibronectin; vitronectin; laminin; fibrinogen; fibrin; plasminogen

***Modified from Matrix metalloproteinases Minireview from R&D Systems (www.rndsystems.com/mini_review_detail_objectname_MR99_MMPs.aspx)**.*

Although MMPs have been investigated for their involvement in ischemic brain injuries, such as neuronal death and blood-brain barrier breakdown (Cunningham et al., 2005), their role in the neurogenic response of adult NSPCs after ischemic insults has only recently been considered. Neuroblast migration is known to require ECM remodeling (Bovetti et al., 2007), and MMP-9 immunoreactivity is colocalized with migrating neuroblasts (Lee et al., 2006). Furthermore, MMP-2 and MMP-9 expressed by endothelial cells promote neuroblast migration (Wang et al., 2006). To elucidate the function of MMPs in NSPC migration, our lab has performed extensive molecular analyses to identify specific MMPs required for NSPC migration in response to two well known chemokines, SDF-1α and VEGF. As part of my dissertation in Chapter 6, I first confirmed that NSPC migration was sensitive to a pan-MMP inhibitor, GM6001. Then, to identify the specific MMPs may be required for this migration, we compared both mRNA and protein expression of MMPs in both migrated cells and stationary cells. Among all the potential MMPs analyzed (see Table 2.2)**,** I found that MMP-3 and MMP-9 play an important role in the migration of neuroblasts. I further defined the role and confirmed the specificity of these two MMPs using lentivirus-mediated siRNA knockdown. Surprisingly, I also found that these two MMPs were needed for NSPC differentiation to neurons (Figure 2.3E) (Barkho et al., 2008). It is apparent that chemokines-induced up-regulation of MMPs have dual function in both cell migration and differentiation. It will be interesting to explore whether these two MMPs are at the center of the regulatory pathways that link differentiation and migration and whether bHLH transcription factors are one of the key component in this process. The potential

functions of endogenous MMPs in adult NSPC differentiation, proliferation, survival, and migration remain to be explored for potential therapeutics of brain repair.

2.7 Methods for Analyzing Neural Stem Cell Migration

2.7.1 In vitro Migration Assays

In order to investigate the physiological and pathological processes of stem cell migration, researchers have developed a variety of in vitro methods to answer specific questions. The commonly used migrations assay is called filter or transwell assays, which have been developed based on a membrane migration assay, called the "Boyden Chamber assay" (Boyden, 1962). This assay was initially developed to measure a chemotactic response of cells by applying a chemoattractant source in the lower compartment of the chamber. More recently, transwells are used in normal tissue culture plates to simulate the same assay and are called "Modified Boyden Chambers" (Figure 2.3B). The pore size of the transwell membrane has to be small enough in relation to the size of the cells so that active migration, instead of passive gravity, is required for cells to pass through the pores. A pore size of 8 μm is the most commonly used for NSPCs. Since this method has been well characterized and widely used, I decided that this would be the best method to use for the experiment in the studies discussed in Chapter 6. Similar to the above described 2D surface assays, the membrane can be used as a bare artificial surface or it can be coated with a more physiologically relevant substance. It has been demonstrated by several other groups and as described in Chapter 6, NSPCs migrate more efficiently on laminin-coated membranes compared to membrane coated by other ECM proteins (Barkho et al., 2008; Kearns et al., 2003). However, it is evident that chemotaxis (directed movement) and chemokinesis (an undirected increase of migratory activity) cannot be well discriminated in this assay because, after a period of incubation time, an

equilibrium of the chemoattractant is reached between the top and bottom chambers. Therefore, a checkerboard assay was developed to address this problem, in which various concentrations of the chemoattractants are applied to the upper and lower compartment (Zigmond and Hirsch, 1973). Furthermore, Drell et al has designed a chemotactic chamber that allows constant replenishment of chemokine during the assay to achieve stabilized gradients of the chemoattractants (Drell et al., 2003; Entschladen et al., 2005). However such apparatus are not available commercially. Recently, several laboratories have published methods for culturing NSPCs in 3-dimensional matrix to understand the properties of cell migration (Brannvall et al., 2007; Watanabe et al., 2007; Widera et al., 2006). In vitro migration assays utilizing these 3-D culture system would likely yield results that is more comparable to in vivo system.

Another type of method, but less commonly used, is to investigate cell migration is 2D migration assays where the cells are plated on a glass or plastic surface coated with a protein matrix. Commonly used matrix are either brain ECM proteins, such as laminin, fibronectin, collagen type I or IV, hyaluronic acid, or ligands to the cells' surface receptors such as the intercellular adhesion molecule (ICAM)-1 (Porter and Hogg, 1997). Artificial substances or synthetic materials have also been used to mimic biological properties of the in vivo environment of the brain, such as cellular interactions and matrix elasticity (Saha et al., 2007). Since the cells often tightly adhere to these 2D substrates, they do not often translocate from one position to another. Therefore, cell migration is measured by the formation of polarity of the cells rather than the movement of the cells. It must be noted that not all cells that polarize will migrate, as shown in other migration assays. Since NSPCs are highly mobile in vitro, their migration cannot be assessed using

this assay. Scratching assay (wound healing model) is a modified 2D migration assay (Figure 2.3A). The "wound" is made by scratching through the cells, resulting in a gap between the remaining cells with a width of 0.1 to 0.5 mm. This "wound healing" is actually a combination of migration and proliferation since stem cells tend to grow to a confluent stage in culture dishes (Yarrow et al., 2004).

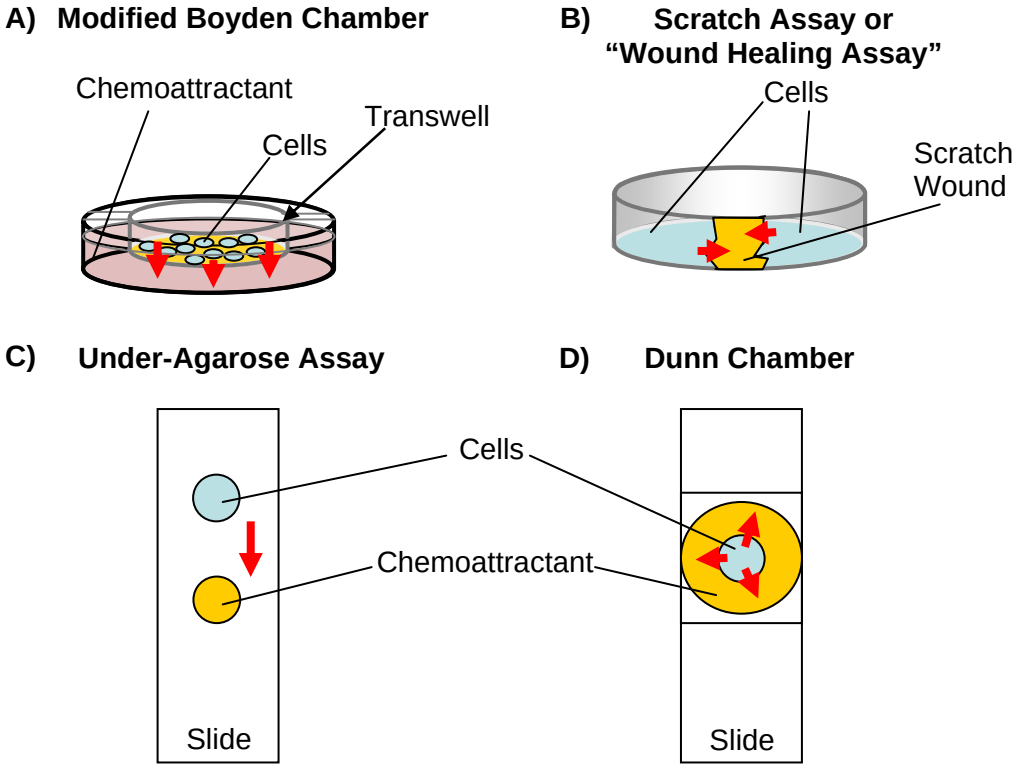

Figure 2.3: Schematic drawings of commonly used in vitro migration assays. (A) Modified Boyden chamber assay measures a chemotactic response of cells migrating through a membrane or filter, by applying a chemoattractant in the lower compartment of the chamber. Cells migrated to the bottom of the membrane are then counted. **(B)** The scratching or "wound" assay is made by scratching through cultured cells, resulting in a gap between the remaining cells with a width of 0.1 to 0.5 mm. Cell migration is measured by the distance and the speed of cells migrating to the "wounded" region. **(C)** In the under-agarose assay, two holes are cut out in the agarose with a certain distance. Cells are plated in one hole and chemokines are added in the other one. Migration is monitored by measuring the number of cells that have reached the hole containing chemoattractants. **(D)** Dunn chamber has concentric rings fixed on a slide, with cells plated in the center circle and chemokines applied to the outer ring. Cell migration is determined by their ability to reach the outer ring. (Adapted from Barkho BZ and Zhao X Book Chapter, *Adult Neurogenesis and Central Nervous System Diseases* 2010)

Other examples of migration assays include under-agarose assay, where cell culture dish are filled with solidified agarose. Then two holes are cut out in the agarose with a certain distance between the holes. Cells are plated in one hole and chemokines are added in the other one (Figure 2.3C) (Ridley et al., 2003). And Dunn chamber has concentric rings fixed on a glass slide, with cell plated in the center circle and chemokines applied to the outer ring (Figure 2.3D) (Zicha et al., 1991). In both of these methods, cell migration can then be monitored by fixing the cells and observing the distance traveled or a live-cell imaging system.

2.7.2 In vivo Migration Assays

In vivo tracing of migrating cells:

In both embryonic and postnatal brains, endogenous NSPCs can be marked by either nucleotide analog (e.g. BrdU) or cellular dyes (e.g. DiI) which are incoporated by cells through their plasma membrane. Their migratory route and final destination can be determined by histological methods at different time points (Entschladen et al., 2005). In the adult brain, these types of *in vivo* tracking of cell migration have been also used to study neuroblast migration within the DG of the hippocampus, and from the SVZ to the olfactory bulb (Ming and Song, 2005). Furthermore, the genetic marking can also be achieved by virus-based gene delivery (Ming and Song, 2005). Our laboratory is more familiar with the techniques of stereotaxic injections of virus in the adult brain (briefly describe below). However, to analyze gene expression in adult NSPC in response to brain injury, I used a mouse stroke model which limited my studies to using one surgical procedure. Therefore, rather than using virus injections to trace cells, in studies in Chapter 6, I labeled cells with phenotypic markers and ribroprobes to identify migrating

cells. In addition, migratory cells can also be genetically labeled in transgenic mice using promoters of the genes involved in cell migration, such as DCX, Dlx and Neurogenin1/2, to drive the expression of fluorescence proteins (e.g. GFP or YFP) or other marker proteins (e.g. LacZ). The advantage of genetically engineered mice is that these mice can be subjected to different physiological and environmental treatments to assess how external stimuli affect in vivo cell migration. These mice can also be used to cross with other mutant mice to study proteins that have interactive roles during cell migration (Barami, 2008).

Magnetic Resonance Imaging (MRI):

Both endogenous and grafted NSPCs have been shown to migrate long distances in response to injury. MRI provides the ability to non-invasively monitor the migration and fate of transplanted NSPCs in a longitudinal fashion, which allows more accurate interpretation of the functional improvement provided by the transplanted cells. Such non-invasive approaches are needed not only for clinical therapeutics utilizing exogenous cell replacement strategies, but provide a unique experimental opportunity to understand the biological factors that are important for NSPC migration and survival following transplantation. Modo et al used MRI to demonstrate long-distance migration of immortalized embryonic NSPCs following transplantation of these cells into the CNS (Modo et al., 2002). In this study, the embryonic NSPCs were transplanted into the contralateral hemisphere of rats at 3 months following middle cerebral artery occlusion (MCAO). The cells were found to migrate into the lesioned hemisphere within 7 days of transplantation, and repopulated the area of injury within 14 days, where some of the grafted cells gave rise to neuronal phenotypes. In order to detect transplanted cells,

cultured NSPCs have to be labeled by MRI contrast agents, such as paramagnetic iron particles or nanoparticles (reviewed by (Politi, 2007)). Several of these contrast agents are available as FDA approved agent for human clinical application. NSPCs can be labeled with these ion particles in vitro by using either DNA transfection method (Smrt and Zhao, unpublished data) or by direct incubation (Modo et al., 2002). When grated into rodent brains, these cells can be detected by non-invasive MRI. Several studies have used this method to show that grafted NSPCs migrate towards ischemic brain regions (Hoehn et al., 2002; Modo et al., 2004; Zhang et al., 2003b). However, the detection sensitivity is limited by the number of cells labeled and also by the resolution of MRI. In general, neither sensitivity nor resolution of the MRI is as good as histological methods.

Intravital microscopy:

The method of intravital microscopy allow scientists to analyze the process of cell migration in specific tissues in real time (Entschladen et al., 2005). In this method, the migrating cells are genetically marked with GFP and the migration of GFP^+ cells in brain slices can be monitored using live-cell confocal imaging coupled to specific software that could calculate the directionality, speed, and distance of cell migration (Platel et al., 2008; Yokota et al., 2007). This method is limited by the viability of brain slices, particularly adult brain tissues, maintained in an *in vitro* culture condition.

CHAPTER 3

Stroke and Stroke-Induced Neurogenesis

3.1 Stroke

Stroke is one of the leading cause of death and a major cause of adult disabilities. More than half of the stroke victims suffer some type of disabilities, ranging from different levels of minor weakness in a limb to a complete loss of mobility in one side of the body. Stroke may also cause a complete loss of ability to speak (MMWR Morbidity and Mortality Weekly ReportPrevention, 2001). Currently therapy for stroke requires a stringent rehabilitation program that includes both medicial and physical therapy. Nevertheless, two thirds of all the survivors will still have some type of disability in regular activities, include eating, walking, and using limbs.

Acute ischemic stroke is caused by cerebral artery occlusion and the loss or reduction of cerebral blood flow leading to an infarction of brain tissue. This event triggers two cascades of damage leading to cell death of neurons, astroyctes, and oligodendrocytes in the focal area (Dirnagl et al., 1999). First, the loss of oxygen or glucose to the brain region leads to the failure of cells in conducting normal physiological cellular functions, such as the depletion of ATP concentrations in the cell. One major cause of neuronal death is the glutamate excitotoxicity that result from the failure of ion pumps that control ion gradients and drive glutamate transporters to reuptake glutamate released in the synapse (Dirnagl et al., 1999). Furthermore, the ion pumps cause the neurotransmitter transporters in the membrane to transferred in the reverse direction and release glutamate into the synaptic cleft. Glutamate toxicity acts on receptors on the post-synaptic neuron, that cause a calcium influx, failure of the mitochondria, and eventually

deplete energy and neuronal death through apoptosis (Dirnagl et al., 1999). Second, the delayed brain insult includes the release of nitric oxide, oxygen free radicals, and other reactive oxygen species, which leads to further damage to neurons (Lo et al., 2003). In addition to the harmful effects on brain cells, ischemia can result in the loss of structural integrity of the protective blood-brain barrier (BBB), partly by triggering the release of MMPs and other proteases secreted by endothelial cells (Lo et al., 2003). The loss of vascular structural integrity results in a breakdown of the tight junctions between astrocytes of the brain and endothelial cells of the vascular system, which contributes to cerebral edema causing secondary progression of the brain injury. Even though the molecular targets that lead to cell death have been identified and current clinical therapies have attempted to block these targets, the efficiency of current therapeutics is unknown and no definitive treatment for stroke is available. Presently, recombinant tissue plasminogen activator, a drug used to break up blood clots during an ischemic stroke that also increases risk of bleeding, is the only approved agent used for stroke patients; however, success has varied in different patients. In addition, administration of this drug is time sensitive and other complications can occur such as increased infarct size and brain hemorrhage (Kilic et al., 2001).

A second characteristic of stroke injury is brain inflammation driven by the involvement of peripherally derived cytokines. The BBB is permeable to cells of the immune system, such as mononuclear phagocytes, T-lymphocytes, natural killer cells, and polymorphonuclear leukocytes (Giulian et al., 1989; Tsuchihashi et al., 1981). All these cells produce and secrete cytokines and therefore contribute to CNS inflammation and gliosis. In support of this notion, irradiation of the bone marrow or treatment with the

immunosuppressant colchicine attenuates gliosis, wound repair, neovascularization, and inflammation (Giulian et al., 1989). Ameboid microglia, a form of activated microglia, can be identified within several hours of ischemia (del Zoppo et al., 2007). Unlike healthy brain microvessels that are clear of inflammatory cells, brain microvessels in the ischemic regions are filled with leukocytes. This form of brain injury is linked with the expression of inflammatory factors, such as inflammatory cytokines (IL-1 and tumor necrosis factor (TNF)-α) and chemokines (IL-8 for neutrophils, MCP-1 for monocytes, CCL5, Leukotriene B4 (LTB4), CXCL10). The up-regulation of adhesion receptors (Intercellular CAM (ICAM)-1, selectins) support leukocyte adherence to the endothelium (Barone and Feuerstein, 1999). TNF-α and IL-1β predispose or "prime" endothelium for cellular adherence (Feuerstein et al., 1998). Additionally, adhesion molecules such as CD11/CD18 integrins are also thought to be pivotal in this inflammatory process (Pober and Cotran, 1990). The exact nature of the signaling mechanisms in brain inflammation still remains to be elucidated but undoubtedly involves TNF-α and IL-1β, chemotactic cytokines (e.g., chemokines such as IL-8), as well as the expression of adhesion molecules and proteinases that together promote cell adherence and infiltration and enhance permeability of brain endothelium (Barone and Feuerstein, 1999).

3.2 Stroke-Induce Neurogenesis

Recent experimental findings raise the possibility that functional improvement after stroke may be achieved through neural replacement by endogenous NSPCs residing in the adult brain, such as the SVZ (Figure 3.1a). Within the first week after focal ischemic insult and pronounced loss of striatal and cortical neurons, a large increase of SVZ NSPC proliferation is observed (Figure 3.1b). At two weeks after the injury, newly-

generated neuroblasts have been shown to re-route from the SVZ and RMS into the damaged area (distances up to 2mm in length), where some have been found to express mature neuronal markers at later time points (Figure 3.1c, d) (Kokaia and Lindvall, 2003; Lindvall et al., 2004). However, the number of neurons generated from endogenous NSPCs is extremely small (~0.2% of the striatal cells lost), and the survival of these new neurons in the lesion area is minimal. Therefore modulating endogenous NSPCs for brain repair is a critical issuce facing the development of effective stem cell therapy, but this will require more knowledge about the molecular mechanism that regulates NSPC proliferation, migration, and differentiation (this will be discussed further in the next section).

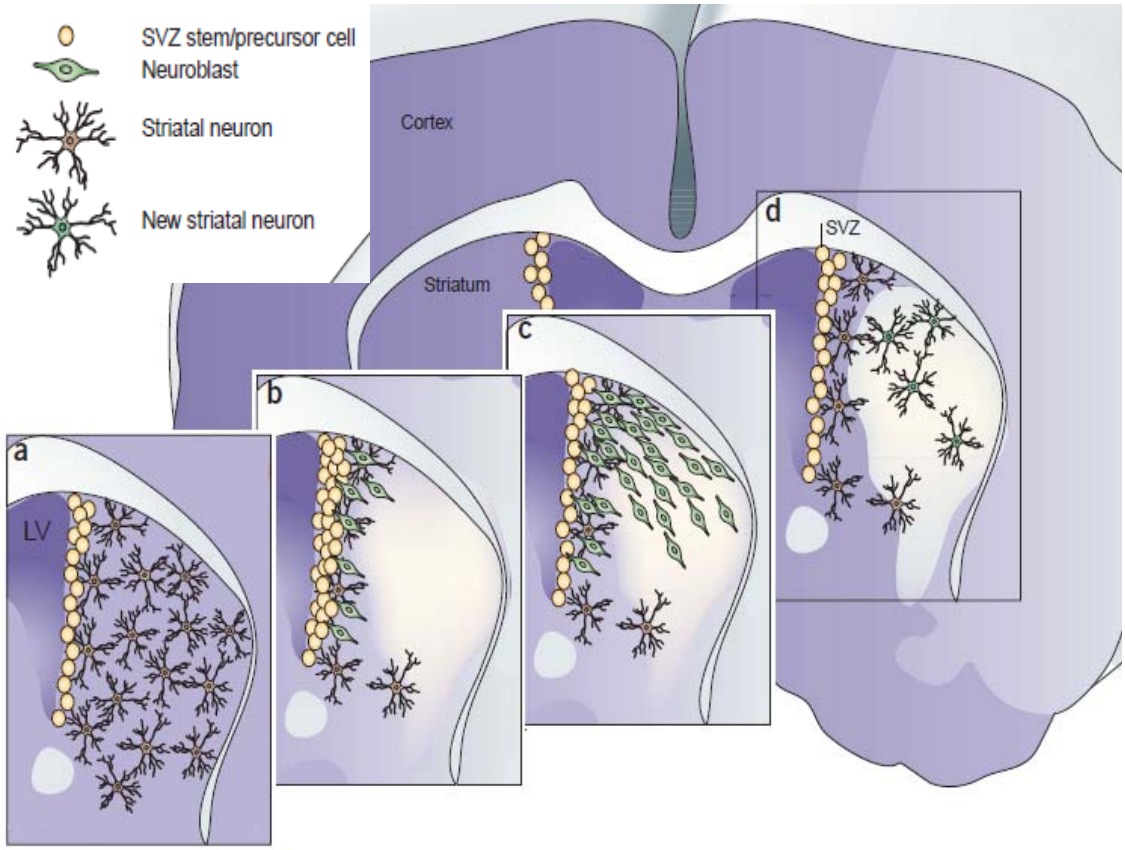

Figure 3.1: Adult NSPCs generate new striatal neurons after stroke. (**a**) NSPCs reside in the subventricular zone (SVZ). (**b**) Stroke induced by middle cerebral artery occlusion, which leads to death of striatal neurons (white area) and damage to overlying cortex, triggers increased proliferation of SVZ NSPC. (**c**) Newly formed neurons migrate

to the damaged part of the striatum. (**d**) New neurons express markers specific for striatal projection neurons or interneurons. LV, lateral ventricle. (Adapted from Lindvall et al. Nature Medicine 2004)

3.3 Therapeutic Application of Stem Cells

As short-term pharmaceutical treatments for brain injury have continued to have limited success, in recent years the potential for treatment using stem cells has become a subject of intense investigation. The discovery of injury-induced neurogenesis raises the possibility that functional improvement after stroke may be achieved through neuronal replacement by endogenous NSPCs residing in the adult brain (Thored et al., 2006). This is supported by the recent findings in brain injury rodent models, in which after one week following brain insult (e.g. epilepsy, stroke, and traumatic injuries), investigators have found an increased NSPC proliferation in the DG and the SVZ (Ming and Song, 2005). Although NSPC-based therapies for brain disorders, such as stroke, Parkinson's disease, Huntington's disease, multiple sclerosis and spinal cord injury, have been studied vigorously, most of these therapies have been performed only in experimental animal models. There are important issues that need to be resolved before any applications of such promising therapies in humans. Here I will discuss the optimal cell sources for transplantation (embryonic vs. adult; endogenous vs. exogenous cells), as well as the best route for cell administration (local vs. systemic transplantations). Early in stem cell therapy studies, clinicians and researchers have tried several experimental strategies for brain repair, such as promoting endogenous neural precursors to home into a damaged region and differentiate into desired cell types using growth factors or signaling molecules. They also have attempted to transplant neural progenitor cells that have been differentiated from adult NSPCs or embryonic stem (ES) cells in culture dishes (Rossi and Cattaneo, 2002). Valuable knowledge has been obtained from these experiments,

however, these attempts have had limited success in functional improvement and have provided insufficient evidence for a definitive procedure in NSPC-based therapy (Freed et al., 2001).

3.3.1 Endogenous Stem Cell Therapy

Endogenous adult NSPCs residing in the neurogenic niche have been suggested to be beneficial for brain repair due to their ability to support neurogenesis and gliogenesis during adulthood. However, after a variety of brain injuries (as described above), the microenvironment surrounding the NSPCs changes drastically due to the reactivity of neighboring cells such as astrocytes, endothelial cells and microglia. The release of cytokines and chemokines from these cells changes the microenvironment and therefore the niche for endogenous NSPCs. For example, as mentioned above, adult NSPCs in the SVZ initially experience a massive proliferative response within the first week of ischemic stroke in response to an increase of TNF-α (Katakowski et al., 2007). However, majority of these cells differentiate into glia cells stimulated by the high concentrations of inflammatory cytokines secreted by neighboring cells.

Even though endogenous NSPCs have been shown to have the capacity to replace lost neurons in animal models of cerebral ischemia (Arvidsson et al., 2002; Haas et al., 2005; Parent et al., 2002), the potential of functional recovery in humans is uncertain. In the rodent stroke model, the effect of endogenous NSPCs to generate new neurons that will survive and regenerate the damage area is currently insufficient (Kokaia and Lindvall, 2003). Therefore, current strategy of promoting NSPCs recruitment to the injured area has focused on the administration of high concentrations of cytokines, chemokines, and growth factors within the lesioned area. These factors

will attract endogenous NSPCs and provide a regenerative population of cells for the injured region. Initially, studies were focused on the administration of individual growth factors or cytokines such as BDNF (Benraiss et al., 2001) and VEGF (Jin et al., 2002). Though these results showed some promise of success, the functional recovery of animals was limited. More recently, it has been suggested that a combination of these factors might have a better outcome in improving brain function (Cairns and Finklestein, 2003; Toth et al., 2008). Results have shown that treatment with erythropoietin (EPO) in a rodent stroke model enhances neurogenesis and angiogenesis as well as improving neurological function after stroke (Wang et al., 2004). An administration of recombinant EPO leads to significant increase in VEGF and BDNF levels in the brain, suggesting that EPO can stimulate both angiogenesis and neurogenesis. Additional studies have shown that the coupling of these processes promote endogenous NSPC neurogenesis in brain injury, indicating that both angiogenesis and neurogenesis might be required to work simultaneously to support the integration of endogenous NSPCs. Thus, the most recent investigation pursued by researchers is to define the molecular mechanisms underlying the response of endogenous adult NSPCs to brain injury to obtain a better understanding of the basic biology of these cells' function in proliferation, migration, differentiation, and survival.

3.3.2 Exogenous Embryonic and Adult Neural Stem Cell Therapy

As described above, in brain injuries, either acute or chronic effects of cytokines and chemokine may modulate the NSPC niche, perturb stem cell properties, and interfere with the capability of endogenous NSPC in brain repair. Therefore, stem cell transplantation could produce an alternative therapeutic approach for brain injury. Both embryonic and adult NSPCs have the capability for brain repair because of their stem cell

properties to proliferate, migrate and differentiate. However, in this review we will briefly discuss all these cell types because of their significance to the field and to understand their advantages and disadvantages in therapeutics.

Embryonic Stem Cells

Embryonic stem cells are pluripotent cells because they can differentiate into all cell types found in the organs of the human body. In contrast, adult stem cells are generally limited to differentiating into cell types of their tissue of origin. Therefore, recently, fetal cells have been evaluated as a means to treat Parkinson's disease and have been transplanted into human patients. However, results using this cell type have not been optimistic since the results have not been as promising compared to pluripotent embryonic stem cells (Langston, 2005). The advantage of ES cells is that large numbers of specific cell types that can be easily grown in culture, while adult stem cells are rare in mature tissues and methods for expanding them in cell culture have not yet been optimized (Menendez et al., 2005; Shufaro and Reubinoff, 2004; Srivastava et al., 2008). This is an important distinction because a large number of cells are needed for stem cell replacement therapies. The use of embryonic stem cells in therapeutics has just been approved for the first clinical trial to be used in spinal cord injury. The delay in using these cell lines mainly arises from the ethical connotations, but there are still several key issues to resolve before applying these cells for a more complex therapy to the brain. Transplantation studies of embryonic stem cells have been performed in animal models and shown promising results; however, further knowledge is required before translating these studies to human therapy (Martino and Pluchino, 2006). The presumed disadvantage of exogenous stem cells is that introducing foreign cells into a patient could

cause transplant rejection. However, whether the recipient would reject donor embryonic stem cells has not been determined in human experiments.

Exogenous Adult Stem Cells

In this thesis, I focused on adult stem cells because currently these are the only types of cells (commonly hematopoietic stem cells, or HSCs) that have been beneficial in treating human diseases. The clinical potential of adult HSCs has been demonstrated in the treatment of several human diseases such as diabetes (Skyler, 2007), amyloidosis (Seldin et al., 2006) and some cancers including renal cell carcinoma (Childs et al., 2000). Also, more human clinical stem cells trials are currently in progress for treating other human diseases including CNS tumors, mastocytosis, and chronic granulomatous disease to name a few (clinicaltrails.gov). While studies as discussed above have generated considerable prospective for the regeneration of exogenous stem or progenitor cells as a therapeutic strategy, the successful development of stem cell recruitment therapy will also depend on our understanding of the proliferation, migration, differentiation, and functional integration of the grafted cells. This information will be critical for developing optimal procedures for using either autologous (stem cells from own self), or allogeneic (stem cells from donor) transplantation of immature stem cells, or differentiated cell types in the treatment of human diseases. Initial studies of exogenous adult stem cells transplanted into the injured brain have shown the regenerative capacity of rodent or human embryonic or fetal derived NSPCs to differentiate into neurons and astroglia in an injured brain (Aoki et al., 1993; Farber et al., 1988). One preclinical study investigated the effects of adult derived neural stem cell transplantation in the context of stroke in adult rats, suggesting an optimal point for

grafted cell survival is around 3 weeks after the stroke (Zhang et al., 2003b). In this experiment, adult neural progenitor cells that were transplanted intracisternally 48 hours following MCAO, survived and migrated toward the ischemic area as assessed by histological and radiological analysis. Some functional recovery was reported, but the underlying mechanisms need to be elucidated. Though these methods and other experiments have not been completely successful, studies such as these have brought us closer to understand the requirements of promoting NSPC transplantation for brain repair.

CHAPTER 4

Goal of Thesis

The goal of this thesis was designed to assess the role of extrinsic cues on adult NSPC properties. Experiments for this study were designed to examine the effects of these extrinsic cues in proliferation, migration, and differentiation of adult NSPCs, and to understand the mechanism which regulates stem cell properties in response to their microenvironment in a normal or injured niche.

The studies described in Chapter 5 (Barkho et al., 2006) aimed to identify the secreted chemokines, cytokines, and growth factors expressed by astrocytes that regulate neurogenesis in the adult brain. The hypothesis for this publication proposed that astrocytes in neurogenic regions expressed extrinsic factors that support adult NSPC neuronal differentiation, and that astrocytes derived from non-neurogenic regions would have the opposite effect. In this chapter, I first identified the differential gene expression profile between astrocytes derived from different CNS regions, by comparing astrocytes derived from neurogenic regions, such as the adult hippocampus and newborn hippcampus, with those derived from non-neurogenic regions, such as the adult spinal cord, and non-neural fibroblasts. Subsequent experiments were carrried out that mainly examined the effects of these secreted factors on the neuronal differentiation capacities of cultured adult NSPCs. Our results indicate that the factors found to be expressed by astrocytes derived from neurogenic regions can indeed support the neuronal differentiation of adult NSPCs, while the factors secreted by non-neurogenic astrocytes had opposite effect.

Experiments in Chapter 6 (Barkho et al., 2008) were designed to identify the extrinsic factors, and mechanisms that mediate the migration and differentiation of adult NSPCs in response to injury. These studies examined the hypothesis that in response to ischemia-induced chemokines, adult NSPCs differentiate into migratory cells to evoke their migration toward the site of injury through the expression of specific MMPs. Initial experiments of this project characterized the differentiation and migration of adult NSPCs in response to SDF-1 and VEGF that are two extrinsic factors expressed within the lesion area after brain injury. Next, this study focused to determine which chemokine-mediated MMPs have a role in the differentiation and migration of adult NSPCs. I have identified that MMP-3 and MMP-9 have a role not only in the migration of adult NSPC-differentiated migratory cells but also in the differentiation of these cells. Finally, experiments demonstrated that these MMPs are expressed in the migratory DCX^+ cells derived from SVZ NSPCs in a mouse MCAO model. This publication addresses a novel pathway and mechanism by which extrinsic cues mediate the differentiation and migration of adult NSPCs in response to brain injury.

Chapter 7 seeks to bring my thesis work together. It describes the finding detailed in the previous chapters and expands on the potential mechanisms by which MMPs may mediate adult NSPC migration and differentiation. This chapter addresses the next questions whether MMPs have a direct role in controlling NSPC differentiation towards a neuronal phenotype, and look at the possibility that a novel pathway may regulate the coupling of migration and differentiation of NSPC in response to their microenvironment either in a normal or injured brain.

Taken together, these publications should build on future scientific work to understand the basic mechanism by which extrinsic cues regulate the fate choice of adult NSPCs in the normal or injured adult brain. These findings will aid our understanding in the basic biology of stem cell, and consequently will help illustrate the general mechanisms regulating stem cell fate choice. Therefore, these studies may provide a small piece of the puzzle needed for future therapeutic applications of stem cells for repair after brain injury.

CHAPTER 5

Identification of Astrocyte-expressed Factors That Modulate Neural Stem/Progenitor Cell Differentiation

Basam Z. Barkho[1], Hongjun Song[2], James B. Aimone[3], Richard D. Smrt[1], Tomoko Kuwabara[3], Kinichi Nakashima[3], Fred H. Gage[3], and Xinyu Zhao[1]

1 Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, NM 87131

2 Departments of Neurology and Neuroscience, Institute for Cell Engineering, John Hopkins University School of Medicine, Baltimore, MD 21205

3 The Salk Institute for Biological Studies, Laboratory of Genetics, La Jolla, CA 92037

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ABSTRACT

Multipotent NSPCs can be isolated from many regions of the adult CNS, yet neurogenesis is restricted to the hippocampus and subventricular zone in vivo. Identification of the molecular cues that modulate NSPC fate choice is a prerequisite for their therapeutic applications. Previously, we demonstrated that primary astrocytes isolated from regions with higher neuroplasticity, such as newborn and adult hippocampus and newborn spinal cord, promoted neuronal differentiation of adult NSPCs, whereas astrocytes isolated from the nonneurogenic region of the adult spinal cord inhibited neural differentiation. To identify the factors expressed by these astrocytes that could modulate NSPC differentiation, we performed gene expression profiling analysis using Affymetrix rat genome arrays. Our results demonstrated that these astrocytes had distinct gene expression profiles. We further tested the functional effects of candidate factors that were differentially expressed in neurogenesis-promoting and –inhibiting astrocytes using in vitro NSPC differentiation assays. Our results indicated that two interleukins, IL-1β and IL-6, and a combination of factors that included these two interleukins could promote NSPC neuronal differentiation, whereas insulin-like growth factor binding protein 6 (IGFBP6) and decorin inhibited neuronal differentiation of adult NSPCs. Our results have provided further evidence to support the ongoing hypothesis that, in adult mammalian brains, astrocytes play critical roles in modulating NSPC differentiation. The finding that cytokines and chemokines expressed by astrocytes could promote NSPC neuronal differentiation may help us to understand how injuries induce neurogenesis in adult brains.

INTRODUCTION

During mammalian embryonic development, neurogenesis precedes gliogenesis, and neurons are generated from NSPCs with minimal influence from glia (Bayer et al., 1993). Upon birth, neurogenesis ceases in most brain regions; however, the dentate gyrus (DG) of the hippocampus and the subventricular zone of the lateral ventricles maintain neurogenic abilities throughout life (Gage, 2002). Unlike embryonic neurogenesis, NSPCs in adult CNS are in intimate contact with surrounding glia, which form a so-called "stem cell niche" that can influence NSPC proliferation and differentiation (Horner and Palmer 2003; Ming and Song 2005). Astrocytes may play critical roles in regulating neurogenesis in both intact adult brains and after injuries. Primary evidence of this is seen following neural injuries when reactive astrocytes secrete both growth factors and inflammatory proteins that can affect the survival of neurons and the regeneration of the neural network (Anderson et al., 2003; Chen and Swanson, 2003; Horner and Palmer, 2003; Liberto et al., 2004). Furthermore, astrocyte-expressed factors have been shown to promote NSPCs and embryonic stem (ES) cells to differentiate into neurons (Lim and Alvarez-Buylla, 1999; Nakayama et al., 2003; Song et al., 2002; Ueki et al., 2003). Therefore, determining how astrocytes regulate adult neurogenesis at the molecular level is an essential step toward understanding the regulation of adult neurogenesis. Our previous work indicated that both membrane-associated and secreted factors expressed by newborn hippocampal (NBH), adult hippocampal (ADH), and newborn spinal cord (NBS) astrocytes could significantly promote neuronal differentiation of co-cultured adult NSPCs (Song et al., 2002), whereas astrocytes isolated from nonneurogenic adult spinal cord (ADS) astrocytes and control adult skin fibroblasts (ASFs) exhibited inhibitory effects on NSPC neuronal differentiation (Song et al., 2002). Identification of

the genes that are responsible for such differential effects among these astrocytes will provide further understanding of the molecular cues that regulate adult neurogenesis. Here, we demonstrated that astrocytes isolated from different CNS regions had distinct gene expression profiles. By comparing neurogenesis-promoting astrocytes (NBH, ADH, NBS) and neurogenesis-inhibiting cells (ADH, ASFs), we have identified candidate genes that might modulate NSPC neuronal differentiation. Of the genes with significant differential expression, we have confirmed that insulin-like growth factor binding protein 6 (IGFBP6) and decorin, inhibitors of IGF and transforming growth factor- β (TGF-β2), respectively, and enkephalin, an opioid receptor agonist, inhibited neuronal differentiation of NSPCs that were cultured with NBH astrocytes. Unexpectedly, we found that two inflammatory cytokines, IL-1β and IL-6, could promote NSPC neuronal differentiation at relatively low concentrations, a finding that was contrary to the common assumption that inflammatory cytokines only inhibit neuronal differentiation of NSPCs (Monje et al., 2003; Vallieres et al., 2002b). Our work further supports the concept that, in mammalian brains, astrocytes can affect NSPC differentiation by expressing unique factors in different CNS regions and at different developmental stages, where astrocyte-derived factors such as pro-inflammatory cytokines may have contrasting effects depending on concentrations and components. Identification of these influences will facilitate our understanding of adult neurogenesis and the development of NSPC-based therapies.

MATERIALS AND METHODS

Cell culture

Isolation of astrocytes from different regions at different developmental stages was performed using established methods as described (Song et al., 2002). For NBH

astrocytes, hippocampus was dissected from brains of 3–6 rat pups (postnatal day 0), diced into small pieces in Hanks' balanced salt solution (HBSS) and 10 mM HEPES solution (Invitrogen), followed by enzymatic digestion by papain (Worthington #LS0003126, 100 μl of 40 μgP/ml) and DNase I (1 mg/ml) in HBSS/HEPES at 37°C for 20–30 min with occasional shaking. Digested tissues and cells were then allowed to settle to the bottom of the tube. Most of the supernatant was removed, and enzymatic digestion was stopped by adding 5 ml of Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Omega Scientific). Single cells were collected by passing the digested tissues and cells through a 70-μm cell strainer (BD Falcon), centrifuged, and resuspended in astrocyte medium [MEM (Invitrogen) supplemented with 10% FBS, 20 mM glucose, and N2 supplement (GIBCO)]. The isolated cells were plated in T25 flasks and cultured in a 37^{o}C, 5% CO2 incubator until cells reached confluence. The flasks were then shaken at 100 rotations/min for 3 days at room temperature to shake off proliferating cells and neurons. After changing to fresh astrocyte medium, the flake was incubated in 37°C incubator with 5% CO2 until the remaining astrocytes reached confluence. The astrocytes were then trypsinized and plated either for co-culture experiments or for enzyme-linked immunosorbent assay (ELISA). Nearly 100% of cells in these cultures were GFAP+ astrocytes.

Affymetrix genome array

RNA isolation and gene expression profiling were performed as described (Sandberg et al., 2000; Zhao et al., 2001). Briefly, confluent primary astrocytes grown on 10-cm dishes were harvested by using 1 ml of Trizol reagent (Gibco BRL, Gaithersburg, MD); then the total RNA was isolated according to manufacturer's protocol (Gibco). The

synthesis of cRNA targets and hybridization to Affymetrix U34A rat genome arrays were described in the Affymetrix manual (Affymetrix, Santa Clara, CA). Astrocytes isolated from individual animals were treated separately as independent replicates. Independent triplicates were used for each experimental condition. To assess the reproducibility of our data, the Affymetrix .CEL files were analyzed using MAS 4.1 (Affymetrix), and the target intensity was set at 200. The reproducibility of duplicate samples was analyzed by correlation coefficient of the average difference value and the number of falsely changed genes. The signal intensity representing expression levels of genes was used to determine correlation coefficients between replicates and experimental conditions to generate Figure 1A. To determine gene expression differences among these astrocytes, and between astrocytes and ASF, we used the "Present Calls" of MAS 4.1 software and obtained the lists of all the genes that have been identified as "Present" (P) in each astrocyte and ASF. We then compared these gene lists using the Venn Diagram function of GeneSpring 4.0.1 (Silicon Genetics, Redwood City, CA) to generate Figure 1, B and C.

Determination of candidate genes that affect NSPC differentiation

Affymetrix .CEL files from each of the five groups were analyzed using four different analytical tools: dChip1.2 (Li and Wong, 2001), MAS4.1 (Affymetrix), Felix Naef algorithms (Naef et al., 2002), and the drop method (Aimone and Gage, 2004). The use of multiple methods allowed us to minimize false negatives caused by analytical assumptions while providing a secondary measure of confidence when probe sets were returned by multiple methods (Aimone et al., 2004). The neurogenic astrocyte chips (NBH, ADH, and NBS) were compared to the nonneurogenic chips (ADS and ASF).

Pairwise fold changes were used in dChip, Felix Naef, and MAS, whereas Drop determined a statistical confidence (*c* value) between the two groups. The criteria used are as follows: dChip 1.2, pairwise fold change >1.3 and group *p* value < 0.05; Felix Naef, pairwise fold change > 1.3 and pairwise *p* value < 0.05; Affymetrix MAS4.1, pairwise fold change > 1.3; drop method, *c* value > 90%. We took all probe sets returned by multiple methods and/or by multiple probe sets as our candidate genes. These 86 probe sets representing 50 unique genes and 4 unannotated EST sequences are listed in Tables 1 and 2. Reference fold changes are from dChip and confidence values were from the drop method. Ranges in fold changes and confidence values are the maximum and minimum if multiple probe sets were returned for the same gene.

Real-time quantitative PCR

Analyses were performed using our previously established methods (Zhao et al., 2001). Briefly, total RNA was isolated from cells using Trizol (Gibco BRL, Gaithersburg, MD). The cDNA was synthesized using SuperscriptII reverse transcriptase (Invitrogen, CA). Each RNA sample and corresponding cDNA sample was generated from a single animal, and 3 or 4 independent samples were used for each experimental condition. PCR primers were designed using Primer Express software (Applied Biosystems) from Integrated DNA Technology (Coralville, IA). The primers and subsequent PCR products were first evaluated by gel electrophoresis to determine that a single PCR product of the predicted size was generated. The real-time PCR reactions were performed in an ABI 7700 Detection System (Applied Biosystems). Each cDNA sample was acquired in at least triplicate. Data analyses were performed according to the protocol provided by Applied Biosystems. Standard curves were generated using a

premade pool of rat CNS (a mixture of brain and spinal cord) total RNA. The amount of each mRNA for tested genes was calculated according to the standard curve for that particular primer set. Finally, the relative amount of the tested message was normalized to the level of an internal control message, either hypoxanthine phosphoribosyl transferase (HPRT) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results were statistically analyzed using a two-tailed, unpaired Student's *t*-test.

ELISA analysis

Confluent astrocyte cultures were changed from astrocyte medium (see above) into co-culture medium [MEM with 25 mM HEPES, 0.1% chicken egg albumin (Sigma A-5503), 0.2 M glucose, 1 mM sodium pyruvate, and N2 supplement (GIBCO)] (2 ml for 6-cm dish, and 6 ml for 10-cm dish). The medium was then collected after a 1-day or 4-day incubation period. The amounts of IL-1β and IL-6 protein secreted by astrocytes into the medium were determined by ELISA kits (R&D system; rat IL-1β/IL-1F2 kits, cat. #RLB00; rat IL-6 ELISA kit, cat. # R6000B). The medium was diluted to 1:2, 1:5, and 1:10 and assayed in duplicates for each concentration. The co-culture medium without astrocyte incubation was used as a baseline control. The amounts of IL-1β and IL-6 in each sample were calculated with standard curves generated by using the positive controls provided by the kits, according to manufacturer's protocols.

Recombinant proteins and other reagents

All recombinant proteins were purchased from R&D Systems, unless stated otherwise. The following proteins were used at 20 ng/ml for 4 days: rat IL-1β (cat. #501-RL), rat IL-6 (cat. #506-RL), human TGF-_2 (cat. #302-B2), human VCAM-1 (cat. #ADP5), rat IP-10 (cat. #1117-IP), and human cathepsin S (cat. #1183-CY). The

following proteins were used at 50 ng/ml for 4 days: human IGFBP-6 (cat. #876-B6), human decorin (cat. #143-DE), neuregulin/EGF domain peptide (NRG-ED, cat. #395-HB), neuregulin/extracellular domain peptide (NRG-ECD, cat. # 377-HB), and, Camarillo, CA). Blocking antibodies R&D Systems to inhibit IL-1β (# AF-501-NA) and IL-6 (#AF-506) were used at 1 μg/mL.

NSPC culture and in vitro differentiation analysis

Multipotent adult NSPCs and green fluorescent protein (GFP)-expressing adult NSPCs have been characterized previously using clonal analyses (Palmer et al., 1997; Takahashi et al., 1999). NSPCs were plated in polyornithine and laminin (P/L)-coated dishes using N2 media [DMEM/F12 (1:1) media with N2 supplement (Life Technologies, Gaithersburg, MD)] containing 20 ng/ml FGF2 (PeproTech, Inc., Rocky Hill, NJ) and cultured in a 5% CO_2 37°C incubator. For in vitro differentiation, cells were changed into N2 medium alone (negative control), N2 media containing factors to be tested, or N2 medium containing 1 μM *all-trans* retinoic acid (ATRA, positive control) for 4 days. Cells were then fixed by 4% paraformaldehyde, followed by immunocytochemical staining and stereological quantification as previously described (Zhao et al., 2003). Primary antibodies used were rabbit anti-type III β-tubulin (1:4,000, Babco, Richmond, CA) and guinea pig anti-GFAP (1:500; Advanced Immunochemical, Inc., Long Beach, CA). Secondary antibodies were all used at 1:250 dilution: donkey anti-rabbit Cy3 and donkey anti-guinea pig Cy5 (Jackson ImmunoResearch, West Grove, PA). Cell phenotypes were captured using an Olympus BX51 microscope equipped motorized stage. Cell counting was performed using a fractionator sampling design and formula (Gundersen et al., 1988) with the assistance of StereoInvestigator (MicroBright-Field)

using a 40X oil-submersion objective. Between 20 and 30 frames were sampled from each chamber slide well (~2-cm^2 area) and approximately 1,000 $DAPI^+$ cells were counted. The results were statistically analyzed using a two-tailed, unpaired Student's *t*-test. The image in Figure 5.5C was obtained using a Zeiss LSM510 laser scanning confocal microscope (Thornwood, NY) using split channel and *z*-stacking.

Co-culture assay

Co-culture of GFP-expressing NSPCs and astrocytes was performed as previously described (Song et al., 2002). Briefly, confluent primary NBH astrocytes grown in T25 flasks (see above for isolation method) were trypsinized and plated onto coated [10% Collagen (Becton Dickinson) 20 μg/ml poly-D-lysine (Becton Dickinson)] glass coverslips in 24-well plates (10,000 cells/coverslip). The cells were then incubated in astrocyte medium (see above) in a 5% CO2 incubator until confluence. One day before plating GFP^+ NSPCs onto the astrocyte feeder layer, the astrocyte medium was changed into co-culture medium (see above). GFP-expressing NSPCs were then plated at 7,500 cells/well onto an astrocyte feeder layer in co-culture media. Four hours after plating, recombinant factors or antibodies were added into the co-culture and incubated for 4 days in a 5% CO2 incubator. Cells were then fixed by 4% paraformaldehyde, followed by immunocytochemical analysis as described above.

Analysis of NeuroD1 promoter activity in NSPCs using luciferase assay

Murine NeuroD1 promoter (2 kb) was cloned by PCR from genomic DNA and inserted into a lentiviral vector, pCSCPW-Luci, at the site of cytomegalovirus (CMV) promoter by using the restriction enzyme sites of *Cla*I and *Bam*H1. The detailed method in constructing this plasmid has been submitted for a separate publication. The

production of lentivirus has been described elsewhere, and nearly 100% of the cells were infected [viral titers were >1.5 X 10^4 Tu/ng defined by the P24 assay (Kuwabara et al., 2004)]. NeuroD1 promoter driving luciferase was introduced into cultured NSPCs using a recombinant lentivirus [pCSCPW lentivector system (Naldini et al., 1996; Pfeifer et al., 2001)]. Because lentivirus is integrated into the cellular genome, all progenies of infected cells also contain the transgenes. NeuroD1-luciferase lentivirus-infected NSPCs were then plated onto 24-well plates (7.5 X 10^4 cells/well), and factors were added at 2 h post-plating. Cells were harvested 4 days later, using 100 μl/well 1X lysis buffer (Promega); 20 μl of each sample were used to analyze the luciferase activity using the Luciferase Assay System (cat. # E1500, Promega) and a luminometer (Bioscan, Washington, DC). The OD280 reading of each sample, representing the amount of protein and number of harvested cells, was measured after 1:100 dilution of the samples. The final NeuroD1 promoter activity of each sample was calculated as luciferase activity normalized by the OD280 reading. The results were statistically analyzed using a two-tailed, unpaired Student's *t*-test.

Analysis of GFAP promoter activity in NSPCs using luciferase reporter system

NSPCs were plated in P/L-coated 24-well plates (0.5 X10^5/well) as described above. The DNA plasmids carrying the 2.5-kb glial fibrillary acidic protein (GFAP) promoter firefly luciferase reporter gene (GF1L-pGL3) or its mutant version with STAT3 binding site mutated (GF1L-S-pGL3) and an internal control plasmid containing sea pansy luciferase driven by human elongation factor 1a promoter (EF1 α-Luc) (Nakashima et al., 1999a) were transfected into each well using FuGENE 6 Transfection Reagent (Roche cat. # 1815443) according to the manufacturer's instructions.

Immediately after transfection, NSPCs were treated with different factors and harvested 4 days later using 100 μl/well 1X lysis buffer (Promega); 20 μl of each sample was used to analyze the luciferase activity using the Dual Luciferase Assay System (Promega, #E1910a) in a luminometer (Bioscan). The final results of promoter activity were presented as firefly luciferase values normalized by EF1 α-Luc values. The results were statistically analyzed using a two-tailed, unpaired Student's *t*-test.

Cell proliferation assay

Analysis was performed as described (Lie et al., 2002) with modifications. Briefly, at 24 h post-plating (also factor treatment), BrdU was added to the NSPC culture at 2.5 μM final concentration for 8 h, followed by fixation using 4% PFA. Cells were then stained using rat anti-BrdU ascites (1:500; Accurate Chemicals, Westbury, NY), mouse Ki67 (1:1,000, NovoCastra Laboratories), and 1 μg/ml DAPI according to a published protocol (Lie et al., 2002). DAPI staining was weaker but still visible. The percentage of $BrdU^+$ cells or $Ki67^+$ cells over the total $DAPI^+$ cells was obtained using unbiased stereology (as described above). The results were statistically analyzed using a two-tailed, unpaired Student's *t*-test.

RESULTS

Neurogenesis-Promoting and –Inhibiting Astrocytes Exhibited Different Gene Expression Patterns

We have previously shown that primary type I astrocytes isolated from the spinal cord and the hippocampus of both newborn and adult rats had distinct effects on the neurogenesis of co-cultured adult NSPCs (Song et al., 2002). To identify the genes that were responsible for such different effects, we performed gene expression profiling on astrocytes isolated from NBH, NBS, ADS, and ADH. We used ASF samples as negative

controls. For each condition, astrocytes were isolated from at least 3 rats, cultured, and then processed independently as replicates. The total RNA isolated from these astrocytes and from ASF was labeled and hybridized to Affymetrix rat genome U34A high-density oligonucleotide microarrays using established methods (see Materials and Methods). We first assessed the reproducibility of our data by analyzing the signal intensity of each gene (generated by Affymetrix MAS4.1 software) between replicates and between experimental conditions. Our data sets had 96–99% correlation coefficients (CCs) between replicates, whereas the CCs between experimental conditions were 83–96% (examples of correlation coefficient plots are shown in Figure 5.1a). To determine whether these astrocytes indeed expressed different sets of genes, we used MAS4.1 software to identify genes that were defined as "Present" (the expression levels of these genes were above the threshold in at least two of three replicates) in each type of astrocyte. Using the Venn Diagram function of GeneSpring software (Silicon Genetics, CA), we compared genes that were common or unique to each type of astrocytes. We found that among 5,190 genes that were "Present" in at least one astrocyte sample, 3,394 (65.4%) genes were shared by all four types of astrocytes, 914 (17.6%) genes were shared by three types of astrocytes, 537 (10.3%) genes were shared by two types of astrocytes, and 527 (10.2%) genes were only "Present" in one type of astrocyte (Figure 5.1b). In addition, our data indicated that the newborn (NBH and NBS) astrocytes shared more common genes with each other (4,271 genes, a sum of genes shared by NBH and NBS as displayed in Figure 5.1b) than the adult samples (ADH and ADS, 3,706 genes), indicating that more molecular divergence has developed in the adult CNS. Furthermore, the hippocampal samples (NBH and ADH) shared more common genes (4,161) than

spinal cord astrocytes did (NBS and ADS: 3,631), indicating that the adult hippocampal astrocytes might be more similar to their newborn counterparts at gene expression levels than the adult spinal cord astrocytes to their newborn counterparts. Among the 3,394 common genes shared by astrocytes, 3,231 genes (95.2%) were also shared with ASF, whereas 163 genes (4.8%) were astrocyte- specific (Figure 5.1c).

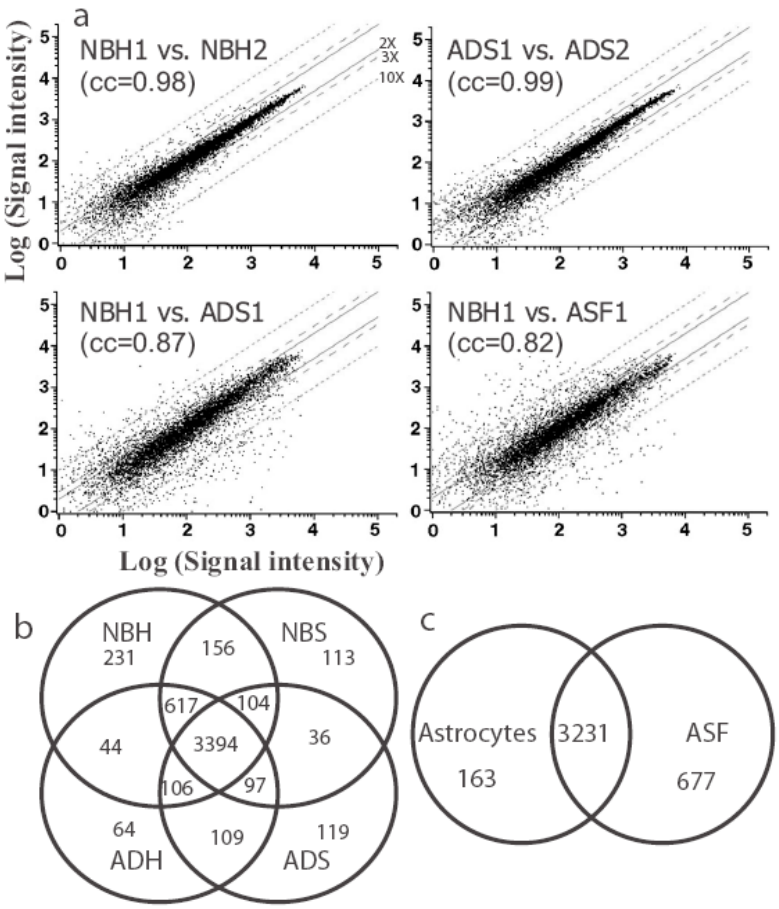

Figure 5.1: Astrocytes derived from adult and newborn hippocampus and spinal cord express different sets of genes. (a) Sample correlation coefficient (cc) plots of signal intensity (representing gene expression levels) obtained by using MAS4.1 software (Affymetrix); Note that replicates (NBH1 vs. NBH2, ADS1 vs. ADS2) have similar gene expression profiles (high cc values), whereas samples from different tissues (NBH vs. ADS, NBH vs. ASF) have distinct gene expression profiles (low cc values); **(b)** a modified Venn Diagram showing numbers of genes that are unique or common among these astrocytes derived from different tissues; Among 5,190 genes that are "Present" in at least one astrocyte sample, 3,394 (65.4%) genes were shared by all four types of astrocytes, 914 (17.6%) genes were shared by 3 types of astrocytes, 537 (10.3%) genes were shared by two types of astrocytes, and 527 (10.2%) genes were only "Present" in

one type of astrocyte; **(c)** among the 3,394 common genes shared by the astrocytes, 3,231 genes are also shared with ASF.

To obtain genes that were differentially expressed by neurogenesis-promoting and -inhibiting astrocytes, we grouped NBH, NBS, and ADH together as the neurogenesis-promoting group, and ADS and ASF as the neurogenesis-inhibiting group. Our initial data analysis using MAS4.1 software indicated that even though these astrocytes expressed different sets of genes, many of these genes were expressed at low levels and/or did not exhibit large fold changes among astrocytes (see online data set). We thought that many important functional genes might be expressed at low levels and their mild fold changes could have significant impact on cellular functions. To include genes with low expression levels and genes with mild fold changes, while minimizing the number of false positives, we performed data analysis of Affymetrix .CEL files using four different analytical software programs: dChip1.2 (Li and Wong, 2001), MAS4.1 (Affymetrix), Felix Naef algorithms (Naef et al., 2002), and the drop method (Aimone and Gage, 2004) using relaxed data analysis criteria (see Materials and Methods for details). The use of multiple methods allowed us to minimize false negatives caused by analytical assumptions while providing a secondary measure of confidence when probe sets were returned by multiple methods. Such a data analysis approach has previously been used to analyze Affymetrix results successfully (Aimone et al., 2004). A total of 178 probe sets were found by at least one method using these criteria, and 48 of them were returned by multiple methods. Interestingly, of the 130 single-method probe sets, 38 were identified by at least one other probe set for the same gene. We included 50 unique genes and 4 unannotated expressed sequence tag (EST) sequences identified either by multiple methods and/or multiple probe sets in our candidate lists (Tables A.5.1 and A.5.2, see

Appendix A). We obtained the reference fold changes and confidence values using dChip and drop analyses, respectively. The complete data files are published online as supplemental data and the original data set (.CEL files) will be available for further data mining. To identify the genes expressed by astrocytes that affect NSPC fate, we focused on genes encoding secreted and cell-surface proteins and confirmed expression patterns of these genes of interest using real-time quantitative PCR (examples are shown in Figure 5.2). For all genes analyzed, the PCR results strongly correlated with Affymetrix gene array data.

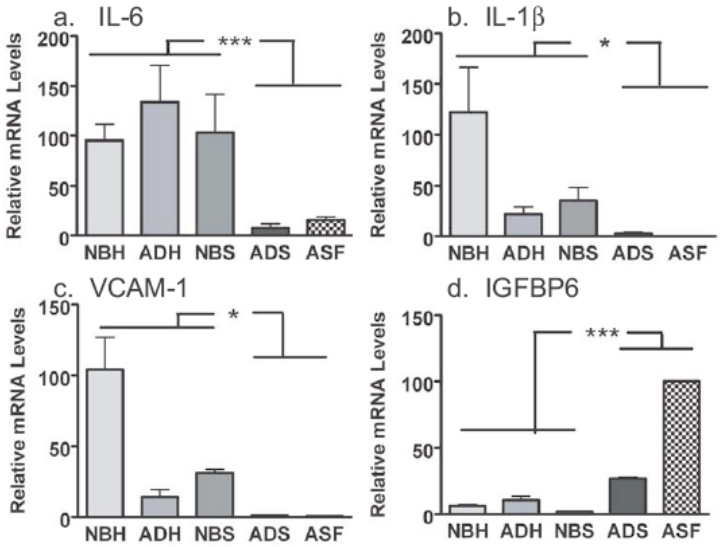

Figure 5.2: Gene expression profiles of sample candidate genes were confirmed using real time quantitative PCR. (a) IL-6; **(b)** IL-1ß; **(c)** VCAM-1; and **(d)** IGFBP6. The relative mRNA levels of each candidate genes were obtained by normalizing to an internal control, GAPDH. PCR results were consistent with the results obtained by microarray analysis.*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$ (unpaired t-test).

Proteins Expressed at Higher Levels in Neurogenesis-Inhibiting Astrocytes Inhibited Neuronal Differentiation of Adult NSPCs

Our goal was to identify the factors that affected NSPC differentiation; therefore, we compared results from this study with genes that we have previously found to be differentially expressed between neurogenic regions (DG of hippocampus tissue) and nonneurogenic regions (CA1 of hippocampus and spinal cord tissue) of adult CNS (Zhao

et al., 2001). Because both membrane and secreted factors expressed by astrocytes were shown to affect NSPC differentiation (Song et al., 2002), we focused on these groups of factors, with emphasis on the factors known to share the same pathways, for further functional tests. We selected insulin-like growth factor-6, IGFBP6, a negative regulator of IGF2 signaling (Jones and Clemmons, 1995) that was expressed at higher levels by ADS astrocytes, because we previously found that IGFBP5, a positive regulator of IGF signaling, had a higher expression level in neurogenic tissue (Bondy and Lee, 1993; Zhao et al., 2001). Furthermore, we found that TGF-β2 was expressed at higher levels in neurogenic tissue (Zhao et al., 2001), whereas decorin, a small proteoglycan that can inhibit TGF-β2 (Hausser et al., 1994), was expressed at higher levels in ADS astrocytes. We also selected enkephalin, an agonist for delta opioid receptor, in our study because we found it expressed higher levels in nonneurogenic regions in our previous study (Zhao et al., 2001). Activation of the delta opioid system has been shown to inhibit adult hippocampal neurogenesis (Eisch et al., 2000; Persson et al., 2003), and we hypothesized that enkephalin may directly inhibit NSPC neuronal differentiation. To analyze the functional effect of neurogenesis inhibiting proteins, we used GFP-expressing NSPCs co-cultured with NBH astrocytes. NBH astrocytes promoted neuronal differentiation of co-cultured NSPCs (Figure 5.3a, b, d, Control) (Song et al., 2002). We treated co-cultured NSPCs and astrocytes with recombinant IGFBP6, decorin, or enkephalin at 50 ng/ml for 4 days and analyzed differentiation of NSPCs using immunofluorescent staining for an early neuronal marker, (III-tubulin (TuJI). The results showed that IGFBP6 (41.3% inhibition, $p < 0.05$, $n = 4$), decorin (Dcn, 44.1% inhibition, $p < 0.01$, $n = 4$), and enkephalin (enk, 53.5% inhibition, $p < 0.05$, $n = 4$) all significantly reduced neuronal

differentiation of NSPCs co-cultured with NBH astrocytes, compared to untreated co-cultures (Figure 5.3c, d), whereas neurogenesis-promoting candidate factors, such as IL-1β, had no inhibitory effect on NSPC neuronal differentiation in this co-culture assay (data not shown). We further performed the dose–response analysis of IGFBP6 and found that IGFBP6 could significantly inhibit neuronal differentiation of NSPCs at concentrations as low as 10 ng/ml (40.0% inhibition, $p < 0.05$, $n = 4$); an increased concentration of IGFBP6 had a more profound inhibitory effect (Figure 5.3e). Therefore, IGFBP6, decorin and enkephalin, which were expressed at higher levels by astrocytes in nonneurogenic regions, indeed had inhibitory effects on NSPC neuronal differentiation.

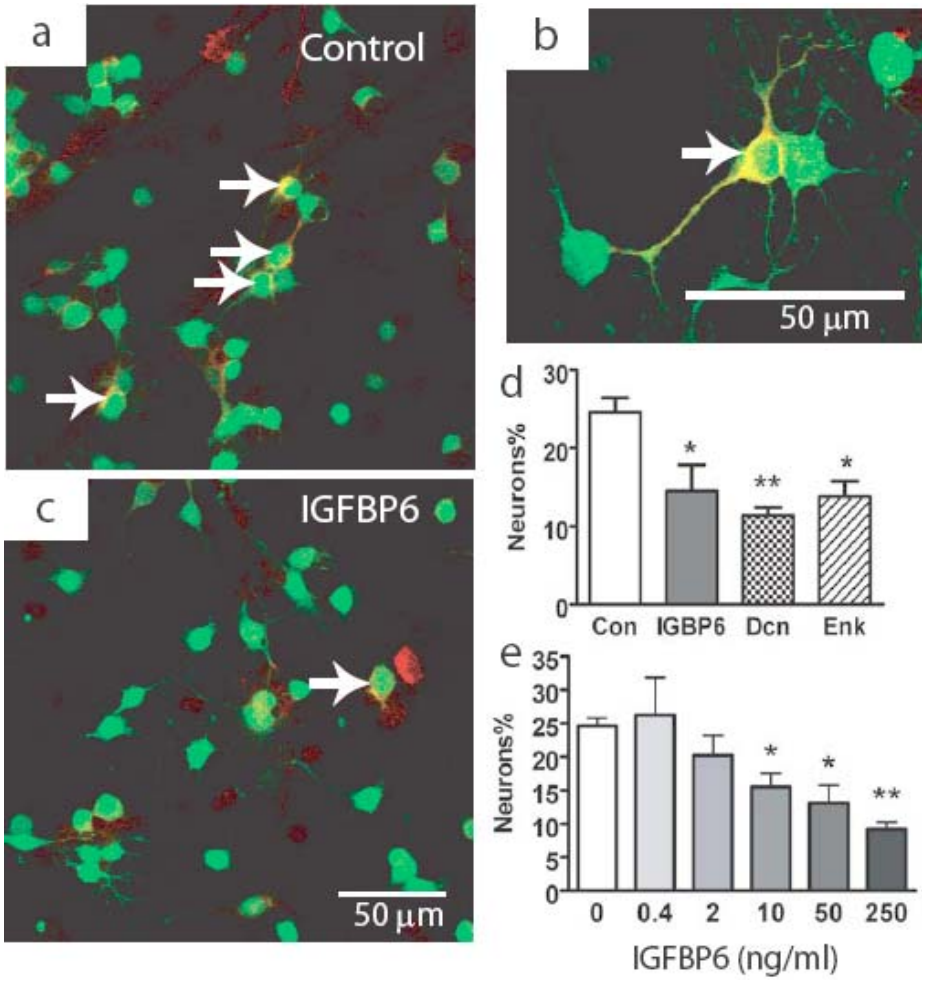

Figure 5.3: Factors expressed at higher levels in neurogenesis-inhibiting astrocytes and non-neurogenic regions inhibited NSPC neuronal differentiation promoted by NBH astrocytes. (a, b, c) GFP-expressing NSPCs (green) differentiated into TuJI+ neurons (red) when co-cultured with NBH astrocytes for 4 days, in the absence (a, b) or presence of 50ng/ml IGFBP6 (c). **(d)** Quantification of immunofluorescent staining indicated that IGFBP6, decorin (Dcn) and enkephalin (Enk) significantly reduced neuronal differentiation (represented by the percentage of TuJI+ neurons) of NSPCs that were co-cultured with NBH astrocyte; **(e)** Dose-dependent inhibition of NSPC neuronal differentiation by IGFBP-6. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ (unpaired, 2-tailed student's t-test).

Proteins Expressed at Higher Levels by Neurogenesis-Promoting Astrocytes Enhanced Adult NSPC Neuronal Differentiation

To determine the functional effect of neurogenesis promoting factors, we used adult NSPCs cultured on P/L coated plates that exhibited a very limited effect on neuronal differentiation of NSPCs even in the absence of the mitogen, FGF-2 (Song et al., 2002). To confirm the neurogenesis-promoting effect of the large number of candidate factors, we used luciferase reporter systems that allowed us to analyze the functional effects of many factors in a timely manner. To detect neuronal differentiation of NSPCs, we used promoter activity analysis of NeuroD1, a basic helix–loop–helix transcriptional factor that is expressed when NSPCs initiate their neuronal differentiation (Hsieh et al., 2004; Miyata et al., 1999). NSPCs were infected with a recombinant lentivirus carrying NeuroD1 promoter-luciferase reporter, and nearly 100% of the cells were infected as described previously (Kuwabara et al., 2004). To analyze the effect of these factors on astrocyte differentiation of NSPCs, we transfected NSPCs cultured on P/L-coated plates with GFAP promoter-luciferase construct and a control EF1α, sea pansy luciferase construct, and about 20–30% of the cells were transfected (Nakashima et al., 1999b). NSPCs infected or transfected with luciferase constructs were treated with individual factors at 20 ng/ml, in the absence of FGF-2, for 4 days, followed by luciferase activity assays. To simplify our initial analysis, we used 20 ng/ml for all candidate

factors, because most growth factors for adult NSPCs, such as FGF-2, were used at this concentration (Palmer et al., 2001; Zhao et al., 2003). Examples of promoter activity results are shown in Figure 5.4, A and B. Both the EGF domain of neuregulin (NRG/ED) and IL-6 increased the NeuroD1 promoter activity in NSPCs (NRG/ED, 63% increase, $p < 0.05$, $n = 8$, *t*-test; IL-6, 19.3% increase, $p < 0.05$, $n = 14$), but the effects of both were less than the positive control, RA treatment (Figure 5.4a, RA, 112.5% increase, $p < 0.001$, $n = 14$). Using BrdU labeling, we subsequently found that NRG/ED also significantly increased NSPC proliferation (data not shown), which may account for the proliferation effect of NBH astrocytes on NSPCs (Song et al., 2002). We decided to focus on the factors that mainly modulated NSPC differentiation in this study; the functional analyses of NRG will be published elsewhere. Among tested factors, only IL-6 significantly increased astrocyte differentiation of NSPCs, shown by increased GFAP promoter activity (Figure 5.4b, 6.7-fold increase, $p < 0.05$, $n = 6$), which was consistent with published literature (Nakashima and Taga, 2002). Its effect was also smaller than that of LIF (Figure 5.4b, 49.8-fold increase, $p < 0.05$, $n = 6$). Neither IL-6 nor LIF increased activity of mutant GFAP promoter with mutation in the STAT3 binding site, indicating that both IL-6 and LIF act through the JAK/STAT pathway to instruct adult NSPCs into the astrocyte lineage, similar to what has been shown in embryonic NSPCs (Nakashima and Taga, 2002). Because both the NeuroD1 and GFAP promoters that we used were partial promoters and displayed relatively high basal activities in undifferentiated NSPCs, the functional effects of certain factors on NSPC differentiation might not be detected by this method. Therefore, we further tested the neuronal differentiation effects of a subset of candidate factors using more accurate

immunofluorescent staining with antibodies against early neuronal marker (TuJI) and astrocyte marker (GFAP). The phenotypes of treated cells were quantified using unbiased stereology methods. We found that most factors tested did not significantly increase neuronal differentiation of NSPCs, but two cytokines, IL-1β and IL-6, could promote neuronal differentiation of treated NSPCs when used at 20 ng/ml (Figure 5.4c and Figure 5.5b, c; IL-6, 6.3-fold increase, $p < 0.01$, $n = 7$; IL-1β, 5.0-fold increase, $p < 0.01$, $n = 7$). To confirm that the concentrations we used were within physiological ranges, we analyzed the amounts of IL-1β and IL-6 proteins released into the culture media by NBH astrocytes using ELISA assays and found that NBH astrocytes secreted 7.9 ng/ml of IL-6 and 1.5 ng/ml IL-1β into their culture medium (total of 6 ml of medium collected from one 10-cm plate of confluent NBH astrocytes). We collected medium after either a 1-day or 4-day incubation periods and found no significant difference in the amounts of IL-1β and IL-6 in the culture medium secreted by astrocytes, indicating that the amounts of IL-1β and IL-6 proteins expressed by astrocytes reached their stable levels after 1 day of incubation. Because the local concentration of IL-1β and IL-6 secreted by astrocytes adjacent to co-cultured NSPCs was likely higher, we decided to use IL-1β and IL-6 at the 20 ng/ml concentration for the rest of this study. To further confirm the effects of IL-1β and IL-6 on the neuronal differentiation of NSPCs, we used specific blocking antibodies to these two cytokines and found that these antibodies specifically blocked the neuronal differentiation effects of the IL-1β and IL-6 (Figure 5.4c, $p < 0.05$, $n = 3$ for both, independent experiments from Figure 5.4e). To investigate whether IL-1β and IL-6 indeed mediated the neurogenesis-promoting effect of NBH astrocytes, we further added the specific blocking antibodies to the NSPCs and primary astrocyte co-culture, and the

results showed that the blocking antibodies against IL-6 and IL-1β partially blocked the neural differentiation of NSPCs promoted by NBH astrocytes (Figure 5.4d, $p < 0.5$, $n = 3$ for all conditions). Consistent with the literature and our luciferase assay results, IL-6 also increased astrocyte differentiation of NSPCs (Figure 5.4e, $GFAP^+$%, 2.8-fold increase, $p < 0.01$, $n = 4$), whereas IL-1β had no such effect ($p < 0.05$, $n = 3$). To determine whether IL-1β and IL-6 also affected NSPC proliferation, we incubated cytokines-treated NSPCs with BrdU for 8 h to label proliferating cells, followed by immunofluorescent staining and quantification. We found that in the absence of FGF-2, neither IL-1β nor IL-6 significantly altered the cell proliferation (indicated by percentage $BrdU^+$ cells) and cell survival (indicated by number of $DAPI^+$, non-apoptotic cells/well) of treated NSPCs compared to untreated NSPCs (data not shown), indicating that, at the concentration we used in our assays, both IL-1β and IL-6 promoted NSPC differentiation without significantly affecting cell proliferation and cell death. Using standard RT-PCR, we also confirmed that adult NSPCs expressed mRNA of the receptors for IL-1β (IL-1R1 and IL-1RII) and IL-6 (IL-6R and gp130) (data not shown). IL-6 has been shown to inhibit the neuronal differentiation of cultured NSPCs that were treated with RA (Monje et al., 2003). To understand the difference between our results and the published literature, we treated cultured NSPCs with both 50 ng/ml [published concentration in (Monje, Toda et al. 2003)] and 20 ng/ml (our condition) IL-6 in the absence and presence of RA for 60 h [published condition (Monje et al., 2003)] and 4 days (our condition). Consistent with published work, we found that 50 ng/ml IL-6 inhibited NSPC neuronal differentiation that was initiated by RA (data not shown). However, in the absence of RA, 20 ng/ml IL-6 increased the neuronal differentiation of NSPCs (Figure 5.4c) and did not

inhibit RA-triggered neuronal differentiation (Figure 5.5c, RA vs. IL-6 + RA: $p = 0.4$, $n = 4$; RA vs. IL-1β + RA: $p = 0.2$, $n = 4$), suggesting that IL-6 could have distinct effects on NSPC neuronal differentiation at different concentrations and in different context.

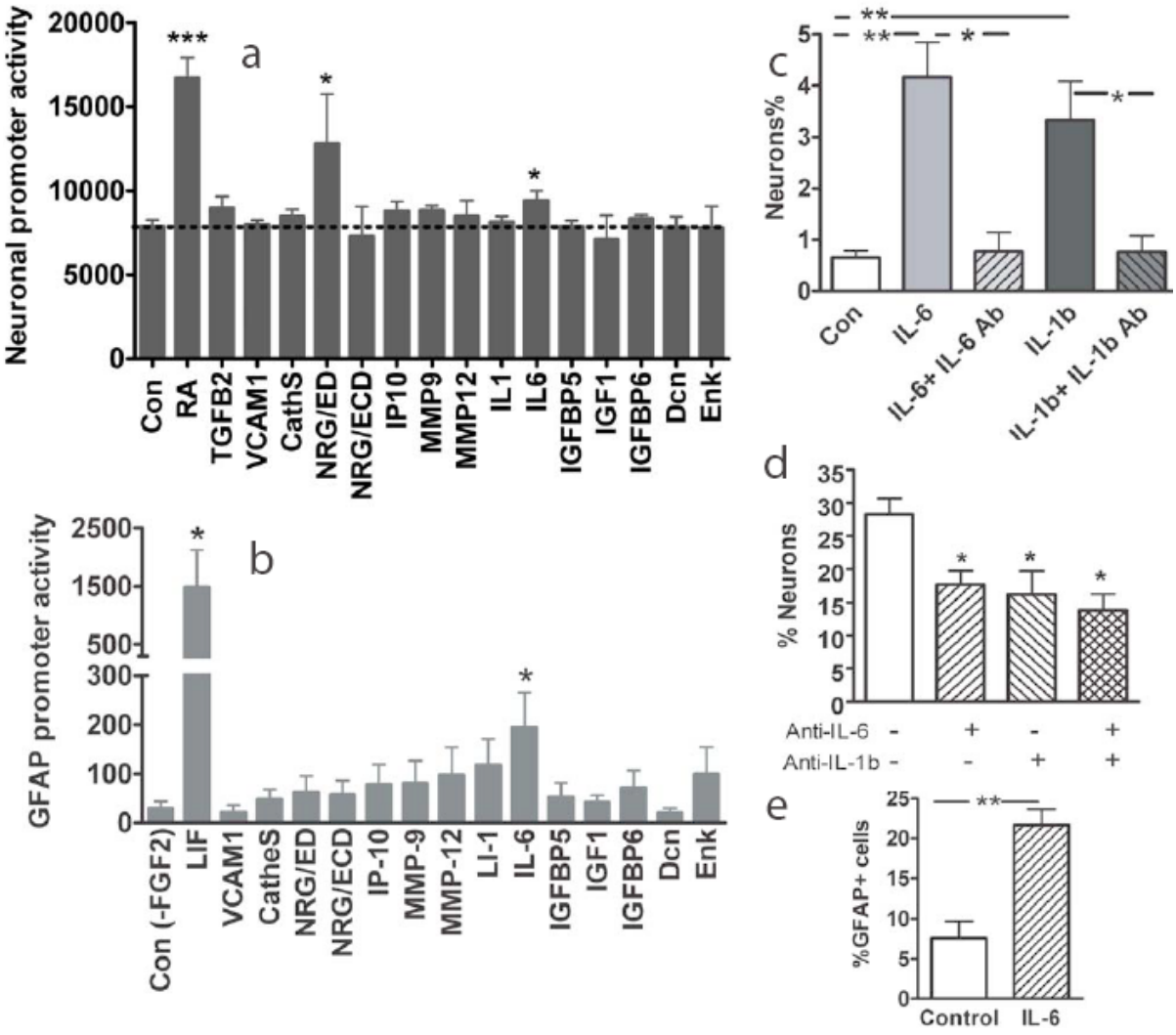

Figure 5.4: Inflammatory cytokines, IL-1 and IL-6, could modulate affect NSPC differentiation. (a) Both NRG/ED ($p<0.05$, n=8) and IL-6 ($P<0.05$, n=14) increased NeuroD1 promoter activity in NSPCs; **(b)** IL-6 treatment increased GFAP promoter activity in NSPCs ($p<0.05$, n=6); **(c)** Both IL-1 and IL-6 promote the neuronal differentiation of NSPCs ($p<0.01$, n=7 for both) and such effect could be blocked by their specific blocking antibodies ($p<0.05$, n=3 for both); **(d)** Antibodies to IL-6 and IL- blocking the neurogenic effects of NBH astrocytes on co-cultured NSPCs; (Control un-treated NSPCs co-cultured with NBH astrocytes). **(e)** IL-6, but not IL-1 also increased GFAP+ astrocyte differentiation of NSPCs ($p<0.05$). *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ (unpaired, 2-tailed, student's t-test).

Combination of Factors Had More Profound on Neuronal Differentiation of Adult NSPCs

Because the majority of the candidate factors that we tested showed no effect on NSPC neuronal differentiation in either luciferase assays or immunofluorescent staining, we considered two explanations: first, even though some of the factors were expressed at higher levels by neurogenesis-promoting astrocytes, they might not have any functional effect on neuronal differentiation of NSPCs; second, because neurogenesis-promoting astrocytes expressed these factors together, a combination of these factors might be necessary to have a significant effect on NSPC differentiation. Therefore, we treated cultured NSPCs with different combinations of the neurogenic candidate factors and analyzed their effect on NeuroD1 promoter activity in NSPCs using the luciferase assay. After a series of experiments with different combinations, we found that a combination of IL-1β, IL-6, vascular cell adhesion molecule-1 (VCAM-1), interferon-induced protein 10 (IP-10, also known as CXCL10), cathepsin S, and TGF-β2 had the strongest effect on NSPC neuronal differentiation (Figure 5.5a, Combo, 1.6-fold increase $p < 0.01$, $n = 14$). In addition, the effect of this combination was significantly higher than IL- 1β or IL-6 alone (Figure 5.5a, d). We further confirmed the effect of these combined factors on NSPC neuronal differentiation using immunofluorescent staining and quantification (Figure 5.5b, d; Combo, 12.0-fold increase, $p < 0.001$, $n = 4$; Combo vs. LI-6, 1.1-fold higher, $p < 0.05$, $n = 4$; Combo vs. IL-1β, 1.4-fold higher, $p < 0.01$, $n = 4$). Using BrdU labeling, we confirmed that the combination of these factors also did not alter the proliferation of treated NSPCs, compared to untreated NSPCs. It has been shown that RA can initiate NSPCs to differentiation down the neuronal lineage (Kuwabara et al., 2004; Palmer et al., 1999). However, the downstream signaling mechanism of RA is not fully

understood. To understand whether IL-1β, IL-6, or the combination of factors was acting through the same pathways as RA, we treated NSPCs with RA and these factors at the same time. We found that neither IL-1β nor IL-6 had an additional effect on the neuronal differentiation of RA-treated NSPCs (Figure 5.5d; IL-1β $p < 0.2$; IL-6, $p < 0.4$, $n = 4$). The combination of IL-1β, IL-6, VCAM1, IP-10, cathepsin S, and TGF-β2 (Combo) also did not significantly increase the neuronal differentiation of RA-treated NSPCs (Combo vs. Combo + RA, $p < 0.1$, $n = 4$). These results suggested that these factors did not have a synergistic effect with RA, and therefore these factors might activate through the same pathway as RA to induce NSPC neuronal differentiation.

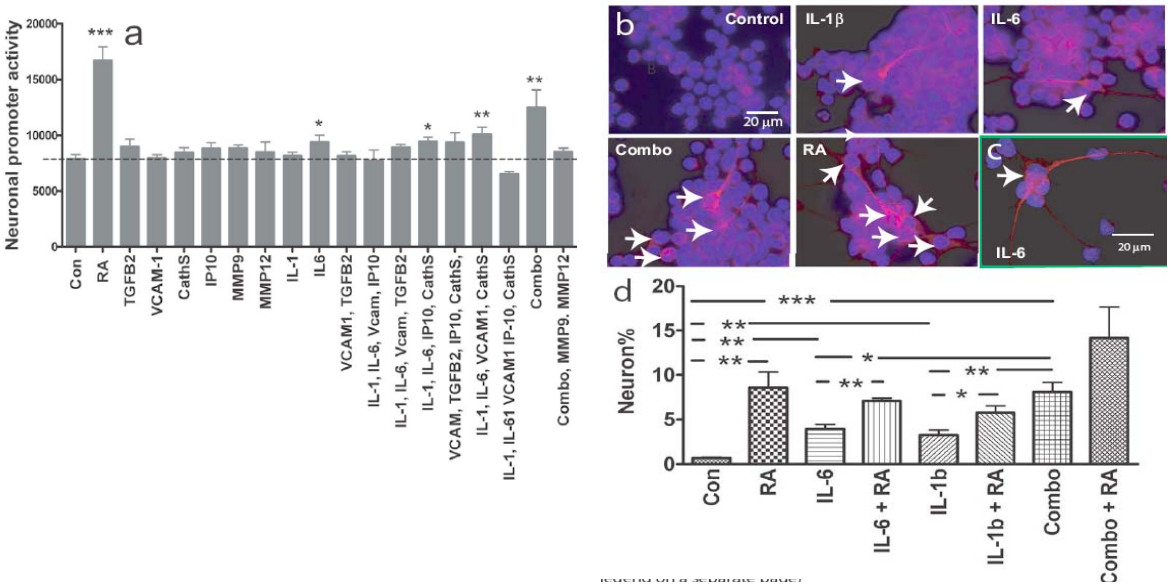

Figure 5.5: A combination of factors expressed at higher levels by neurogenesis-promoting astrocytes and tissues promoted neuronal differentiation of NSPCs. (a) The combination of IL-1β, IL-6, VCAM1, IP-10, Cathepsin S, and TGFβ2 (Combo) enhanced NeuroD1 promoter activity in NSPCs, analyzed by luciferase activity assays($p<0.01$, $n=14$); **(b)** Immunofluorescent images showing TuJI+ neurons (red) differentiated from NSPCs that were treated by IL-1β, IL-6, Combo and RA in the absence of FGF-2. Such fluorescent staining results were quantified using unbiased stereology quantification to generate data shown in Fig 4c and Fig 5d. **(c)** A Z-stack confocal image of a TuJI+ neurons differentiated from IL-6 treated NSPCs; **(d)** IL-1β, IL-6 and Combo could promote neuronal differentiation of NSPCs indicated by the percentage of TuJI+ neurons. Combo was significantly more potent than IL-1β and IL-6 alone. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$ (unpaired, 2-tailed, student's t-test).

DISCUSSION

In this study, we performed gene expression profiling of primary astrocytes that exhibited differential effects on the neuronal differentiation of adult NSPCs (Song et al., 2002). In conjunction with our previous study analyzing genes differentially expressed in neurogenic and nonneurogenic regions (Zhao et al., 2001), we identified candidate genes that potentially affect NSPC lineage determination. Using luciferase reporter assays, we tested the functional effects of a large number of the candidate genes encoding secreted and membrane proteins on adult NSPC differentiation, followed by confirmation using more precise immunofluorescent staining and quantification. We demonstrated that two interleukins, IL-1β and IL-6, and a combination of a group of factors that included these two interleukins could promote NSPC neuronal differentiation, whereas IGFBP6, decorin, and enkephalin inhibited neuronal differentiation of cultured adult NSPCs. These results provide molecular evidence that astrocytes play critical roles in modulating adult NSPC fate choices. The finding that cytokines and chemokines could promote adult NSPC neuronal differentiation might help us to understand how injuries induce neurogenesis in adult brains. The lack of neurogenesis in adult spinal cord could be due to both the lack of positive regulators and the presence of inhibitory factors. Our findings indicate that at least three molecules that were expressed at higher levels in ADS astrocytes or spinal cord tissue had negative effects on the neuronal differentiation of NSPCs: IGFBP-6, decorin, and enkephalin. IGF1 has been shown to promote neurogenesis both in vivo and in cultured NSPCs (Aberg et al., 2000; Aberg et al., 2003; Bondy and Cheng, 2004). IGFBPs are a family of proteins that can regulate IGF function both positively and negatively (Jones and Clemmons, 1995). Although IGFBP5, a

positive regulator of IGFs, was highly expressed in the DG as opposed to nonneurogenic tissues (Zhao et al., 2001), IGFBP6 is a negative regulator of IGF signaling with highest specificity to IGF2 (Bienvenu et al., 2004). Over-expression of IGFBP6 in astrocytes of transgenic mice (GFAP promoter- IGFBP6 transgenic mice) leads to decreased IGF levels and brain size (Bienvenu et al., 2004). The function of IGFBP6 in adult neurogenesis has not been shown previously. In our functional assays, neither IGFBP5 nor IGFs had significant effects on NSPC neuronal differentiation, probably due to the fact that our NSPC culture medium contained a high concentration of insulin. By contrast, IGFBP6 did inhibit neuronal differentiation of adult NSPCs co-cultured with NBH astrocytes in our study, possibly by inhibiting the effect of insulin expressed by NBH astrocytes. Our finding suggests that the high levels of IGFBP6 expressed by ADS astrocytes may contribute, at least partially, to the inhibitory effect of ADS astrocytes on NSPC neuronal differentiation in the adult spinal cord. It will be interesting to know whether GFAP promoter-IGFBP6 transgenic animals have reduced adult hippocampal neurogenesis. In addition, TGF-βs have been shown to inhibit cell proliferation but promote differentiation (Bottner et al., 2000). Previously, we found that TGF-β2 was expressed at higher levels in neurogenic tissues than in nonneurogenic tissues (Zhao et al., 2001). However, when we applied TGF-β2 alone to NSPCs, we did not observe any effect on either NSPC proliferation or differentiation, but TGF-β2 was a necessary component of the combined factors (Combo) that exhibited the strongest effects on promoting NSPC neuronal differentiation in our assay. Interestingly, in this study we found that decorin, an extracellular proteoglycan that can bind and inhibit several cytokines including TGF-β2 (Hausser et al., 1994), was expressed at higher levels in

ADS astrocytes. We then confirmed that decorin could significantly inhibit NSPC neuronal differentiation promoted by NBH astrocytes (Figure 5.3a). Our data suggest that IGF/IGFBPs and TGF- β2/decorin pathways may be involved in regulating NSPC neuronal differentiation in the adult stem cell niche. Additional studies at molecular and cell signaling levels and further in vivo assays will unveil how these two pathways regulate adult neurogenesis. Traditionally, cytokines and chemokines have been shown to be responsible for damaging neuroinflammation during diseases and CNS injuries (Wang et al., 2002a). Transgenic mice that chronically overexpress IL-6 under the GFAP promoter (GFAP promoter-IL-6 mice) exhibit CNS damage, with the severity of the damage correlating with levels of IL-6 expression (Wang et al., 2002a). Cytokines and chemokines can have distinct biological effects when present at different concentrations and in various combinations. On the other hand, recent findings indicate cytokines can also have neuroprotective and regenerative effects (Liberto et al., 2004; Yoshida and Gage, 1992b). The effects of cytokines on adult neurogenesis have recently triggered great attention because of their potential roles in regenerating damaged adult CNS. To date, experimental evidence supports the hypothesis that cytokines play inhibitory roles in adult neurogenesis. For example, both IL-6 and LIF instruct embryonic NSPCs into the astrocyte lineage (Nakashima et al., 1999a). GFAP promoter-IL-6 transgenic mice exhibit a 63% reduction in adult hippocampal neurogenesis and significantly reduced neuronal differentiation of NSPCs in vivo (Vallieres et al., 2002b). IL-6 inhibits RA-initiated neuronal differentiation of adult NSPCs and inflammation blockade restores hippocampal neurogenesis in irradiated adult brains (Monje et al., 2003). The role of cytokines in promoting neuronal differentiation of adult CNS stem cells or progenitor cells have not

been shown previously. The fact that cultured adult NSPCs express receptors for both IL-1 and IL-6 indicates that these cells are ready to respond to these stimulations. To our surprise, our gene expression analysis indicated that neurogenesis-promoting astrocytes express many cytokines, chemokines, and inflammation related proteins at higher levels than did neurogenesis inhibitory cells. Specifically, we found that IL-1β and IL-6 promoted NSPC neuronal differentiation, contradicting the current theory that inflammatory cytokines, such as IL-6, inhibit adult neurogenesis and NSPC neuronal differentiation (Monje et al., 2003). To address this apparent inconsistency, we performed additional parallel experiments using both our condition and published conditions, and found that in the presence of RA, high levels of IL-6 [50 ng/ml used in (Monje et al., 2003)] inhibited NSPC neuronal differentiation. However, in the absence of RA, relatively low levels of IL-6 (20 ng/ml, used in this study) promoted neuronal differentiation of adult NSPCs (Figures 5.4 and 5.5). Because IL-6, like many cytokines, can have distinct physiological effects at different concentrations and in different biological contexts, the differences between our results and published work could be due to the differences in the amounts and conditions that we used in the experiments. It is also possible that IL-6 modulates adult NSPC fate differently, depending on the context. When other neurogenic cues (such as RA) are present, IL-6 inhibits neuronal differentiation of adult NSPCs (Monje et al., 2003), but in the absence of other neurogenic cues, IL-6 promotes neuronal differentiation of adult NSPCs (this study). The results of our present study suggest that the effects of cytokines on adult NSPCs are complex and are likely context and concentration dependent. Interestingly, we found that even though TGF- β2, IP- 10, cathepsin S, and VCAM1 did not display any effect on

NSPC neuronal differentiation when applied alone, the combination of these factors and IL-1β and IL-6 could significantly promote neuronal differentiation of NSPCs. This is consistent with our finding that IL-1β and IL-6 antibodies partially blocked the effects of co-cultured NBH astrocytes on NSPCs. IP-10 is a chemokine induced by several cytokines and has chemoattractant effects on immune cells such as monocytes and lymphocytes (Huang et al., 2000). VCAM-1 is an extracellular matrix adhesion protein that regulates cell–cell interaction and can be induced by both IL-1β and IL-6 during neural inflammation (Huang et al., 2000). Cathepsin S belongs to a group of cysteine proteases of the papain family and is involved in major histocompatibility complex (MHC) presentation during immune responses. Cathepsin S degrades proteins in the extracellular matrix (Nakanishi, 2003) and may modulate cell-surface receptors and hence cellular functions. The role of these factors that are normally involved in inflammatory or immune responses, to promote neuronal differentiation of NSPCs is unclear. Extensive studies have demonstrated that injuries can induce neurogenesis in adult brain regions that are normally nonneurogenic; however, the underlying mechanism is not clear. Our current findings provide a new perspective on understanding injury-induced neurogenesis by identifying the molecular cues, which will facilitate the understanding of adult neurogenesis and the development of NSPC-based therapies. The fact that we did not observe a synergistic effect between these factors and RA suggests that these factors may act through the same pathways as RA to promote NSPC neuronal differentiation. The signaling mechanisms underlying the neuronal differentiation effect of these factors are currently under investigation. In summary, we have performed the initial identification and functional analysis of a group of positive and negative regulators

expressed by astrocytes that can modulate adult NSPC differentiation, and we have provided molecular evidence that astrocytes play critical roles in modulating adult NSPC fate determination. Further mechanistic studies using antibodies and signaling pathway inhibitors will unveil the molecular mechanisms underlying the cytokines regulation of NSPC proliferation and fate choice both in normal brains and after injuries.

CHAPTER 6

Endogenous Matrix Metalloproteinase (MMP)-3 and MMP-9 Promote the Differentiation and Migration of Adult Neural Progenitor Cells in Response to Chemokines

Basam Z. Barkho[1], Ari E. Munoz[1], Xuekun Li[1], Lu Li[1], Lee Anna Cunningham[1] and Xinyu Zhao[1]

1 Department of Neurosciences, University of New Mexico School of Medicine, Albuquerque, NM 87131

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ABSTRACT

Adult neurogenesis is regulated by both intrinsic programs and extrinsic stimuli. The enhanced proliferation of adult NSPCs in the subventricular zone and the migration of neuroblasts toward the ischemic region in adult brains present a unique challenge as well as an opportunity to understand the molecular mechanisms underlying the extrinsic cue-induced neurogenic responses. Matrix metalloproteinases (MMPs) are a family of proteinases known to play a role in extracellular matrix remodeling and cell migration. However, their presence in adult NSPCs and their potential function in injury-induced adult NSPC migration remain largely unexplored. Here we demonstrate that in response to two injury-induced chemokines, stromal cell derived factor 1 (SDF-1) and vascular endothelial growth factor, adult NSPCs differentiated into migratory cells that expressed increased levels of MMP-3 and MMP-9. Whereas differentiated neuroblasts and a subpopulation of astrocytes migrated toward the chemokines, undifferentiated progenitors did not migrate. Blocking the expression of MMP-3 or MMP-9 in adult NSPCs interfered with both the differentiation of adult NSPCs and chemokine-induced cell migration. Thus, endogenous MMPs expressed by adult NSPCs are important for mediating their neurogenic response to extrinsic signals.

INTRODUCTION

Adult NSPCs participate in active neurogenesis in two locations of the adult brain: the subgranular zone of the dentate gyrus in the hippocampus and the subventricular zone (SVZ) bordering the lateral ventricles. In response to focal ischemic injury, the proliferation of adult NSPCs residing in the SVZ increases. Subpopulations of these newborn adult NSPCs differentiate into neuroblasts and migrate away from the SVZ and the rostral migratory stream into the damaged region (Kokaia and Lindvall,

2003). Several injury-induced factors produced by reactive cells, such as astrocytes, endothelial cells, and immune cells, are known to modify the niche for adult NSPCs (Thored et al., 2006; Zhang et al., 2002). Among these factors, stromal cell-derived factor 1 (SDF-1) and vascular endothelial growth factor (VEGF), acting through their cell surface receptors chemokine (C-X-C motif) receptor 4 (CXCR4) and vascular endothelial growth factor receptor 2 (VEGF-R2), respectively, are found to be chemoattractive to new cells generated in the SVZ (Imitola et al., 2004; Robin et al., 2006; Zhang et al., 2003a). Nevertheless, the exact mechanism by which these chemokines promote the ischemia-induced migration of adult NSPCs is unclear. Chemokine-induced cell migration requires the remodeling of the extracellular matrix (ECM). The chemotaxic functions of SDF-1 and VEGF are known to be mediated by the activation of matrix metalloproteinases (MMPs) (Janowska-Wieczorek et al., 2000; Pufe et al., 2004). MMPs are a family of enzymes that collectively are able to degrade all the components of the ECM (Seiki 2002; Stamenkovic 2003). MMPs participate in a host of important physiological processes, including CNS development, embryological remodeling, wound healing, and angiogenesis, and their role in cancer cell metastasis has been studied extensively (Chang and Werb, 2001; Mannello et al., 2006). Although MMPs have been investigated for their involvement in ischemic brain injuries, such as neuronal death and blood-brain barrier breakdown (Cunningham et al., 2005), their role in the neurogenic response of adult NSPCs after ischemic insults has only recently been considered. Neuroblast migration is known to require ECM remodeling (Ohab et al., 2006), and MMP-9 immunoreactivity is colocalized with migrating neuroblasts (Lee et al., 2006). Furthermore, MMP-2 and MMP-9 expressed by endothelial cells promote neuroblast

migration (Wang et al., 2006). However, whether adult NSPCs express MMPs and what the potential functions of these endogenous MMPs are in adult NSPC differentiation, proliferation, survival, and migration remain unknown. In this study we demonstrate that MMP activity promotes the adult NSPC neurogenic response to stroke-induced chemokines. In response to SDF-1 and VEGF, adult NSPCs differentiate into migratory cells that express higher levels of MMP-3 and MMP-9 compared with non-migratory cells. Blocking the expression of endogenous MMP-3 and MMP-9 in adult NSPCs using specific small interfering RNAs (siRNAs) inhibited both adult NSPC differentiation into the neuronal lineage and subsequent cell migration in response to SDF-1 and VEGF. Furthermore, we show that migrating neuroblasts in a rodent focal ischemia model express endogenous MMP-3 and MMP-9 mRNAs. This study presents the first evidence for the function of endogenous MMPs expressed by adult NSPCs in the regulation of their neurogenic response to ischemic insult.

MATERIALS AND METHODS

Neural Stem/Progenitor Cell Culture

Isolation procedure and culture conditions for multipotent mouse adult NSPCs were as described (Zhao et al., 2003). Briefly, adult NSPCs were kept under proliferating conditions using N2 medium (Dulbecco's modified Eagle's medium/Ham's F-12 medium [1:1] [DM-25; Omega Scientific, Tarzana, CA, http://www.omegascientific.com] supplemented with N2 [Life Technologies, Gaithersburg, MD, http://www.lifetech.com], L-glutamine [25030; Invitrogen, Carlsbad, CA, http://www.invitrogen.com], and 1X Antibiotic-Antimycotic [15240; Invitrogen]), 20 ng/ml fibroblast growth factor-2 (FGF-2; Peprotech, Rocky Hill, NJ, http://www.peprotech.com), and 20ng/ml epidermal growth factor (EGF; 100-15; Peprotech) and incubated at 5% CO_2 at 37°C.

Recombinant Proteins and Reagents

Human recombinant SDF-1α/PBSF (350-NS/CF; R&D Systems Inc., Minneapolis, http://www.rndsystems.com) and human recombinant VEGF (293-VE/CF; R&D Systems) were used at 100 ng/ml. GM6001 MMP inhibitor (CC1000; Chemicon, Temecula, CA, http://www.chemicon.com) was used at a final concentration of 10 μM.

Cell Migration Assay

Cell migration assays were performed on the basis of published methods with modifications (Zigmond and Hirsch, 1973). Briefly, Transwells with 8.0-μm Pore Polycarbonate Membrane Inserts (#353097; BD Biosciences, San Jose, CA, http://www.bdbiosciences.com) were coated with polyornithine and laminin as described (Barkho et al., 2006). Approximately 1 X 10^5 mouse adult NSPCs were plated onto the upper chamber of an insert that fit into 1 well of a 24-well plate in N2 medium without growth factors or chemokines. After 60 minutes to allow the cells to attach to the surface of the membrane, SDF-1 or VEGF was added at 100 ng/ml into the lower chamber of the Transwell. For the MMP inhibitor studies, GM6001 (dissolved in dimethyl sulfoxide [DMSO]) was diluted in phosphate-buffered saline and administered to both top and bottom chambers at a final concentration of 10 μM, whereas the controls received an equal volume of DMSO in both chambers. The cells were then incubated for 16 hours in a cell culture incubator. For quantification of migrated cells, the lower chamber was fixed with 4% paraformaldehyde (PFA) and stained with 4, 6-diamidino-2-phenylindole, and the cells remaining in the upper chamber were scraped off with a cotton swab. The membrane was cut out from the insert and mounted onto a glass slide with DAVCO-PVA and coverslipped. For lentivirus-infected cells, the membrane was immunostained using

an anti-green fluorescent protein (anti-GFP) antibody (described below) to amplify the GFP signal. To determine whether lentivirus itself has deleterious effect on adult NSPCs, cells infected by either control lentiviruses or siRNA lentiviruses, as well as uninfected cells, were plated onto the Transwell membranes for 16 hours without inducing migration, followed by GFP signal amplification as described above. Cell numbers were estimated using unbiased stereology (MicroBrightField Inc., Williston, VT, http://www.mbfbioscience.com) and a 20X objective lens on an Olympus BX51 epifluorescent microscope (Olympus, Tokyo, http://www.olympus-global.com). The results were statistically analyzed using a two-tailed, unpaired Student's *t* test.

Immunohistochemistry

Primary antibodies used were mouse anti-CXCR4 (1:1000, eBioscience, #14-6009-81), mouse anti-VEGFR2 (1:1000, eBioscience, #14-5821-81), mouse immunoglobulin G (IgG) (1:1000, Sigma, #15381), mouse anti-Nestin (1:1000, BD Pharmingen, #556309), rabbit anti-Sox2 (1:1000, Cell Signaling, #2748), goat anti-DCX (1:1000, Santa Cruz, #sc-8066), rabbit anti-type III β-tubulin (1:4000, Babco, Richmond, #PRB-435P), guinea pig anti-GFAP (1:5000, Advanced Immunochemical, Inc., Long Beach, CA), rabbit anti-MMP-3 (1:1000, Chemicon, #AB810), rabbit anti-MMP-9 (1:500, Chemicon, # AB19106), mouse anti-GFP (1:500, Molecular Probes, #A11120), mouse anti-NG2 (1:1000, Chemicon, #AB5320), and rabbit immunoglobulin G (IgG) (1:1000, Sigma, #15006). Secondary antibodies were all used at 1:250 dilutions: donkey anti-rabbit Cy3, donkey anti-mouse Alexa 488 (Invitrogen), and donkey anti-guinea pig Cy5 (Jackson ImmunoResearch, West Grove, PA). Cell quantification was performed as described above.

Cell Proliferation Assay

Cell proliferation was performed as described (Barkho et al., 2006) with modifications. Briefly, at 8 hour post-plating of NSPCs in proliferating medium, BrdU was added to the NSPC culture at 2.5 μM final concentration and for an additional 8 h, followed by fixation using 4% PFA. Cells were then stained using rat anti-BrdU ascites (1:500; Accurate Chemicals, Westbury, NY), and 1 μg/ml DAPI according. The percentage of $BrdU^+$ cells over the total $DAPI^+$ cells was quantified using unbiased stereology method as described above. The results were statistically analyzed using a two-tailed, unpaired Student's *t*-test.

Real-Time Quantitative Polymerase Chain Reaction

Real time PCR was performed using established methods (Barkho et al., 2006; Zhao et al., 2003). Briefly, total RNA was isolated from cells using either TRIzol (Gibco BRL, Gaithersburg, MD) for cells grown on culture dishes or the Ambion RNAqueous Kit (Ambion, Austin, TX, #AM1912) for cells plated on transwells. The cDNA was synthesized using a Superscript II kit (Invitrogen, CA). Each RNA sample was generated from a single dish or well and at least 3 independent samples were analyzed for each experimental condition. PCR primers were designed using Primer Express software (Applied Biosystems) and ordered from Integrated DNA Technology (Coralville, IA). The primers and subsequent PCR products were first evaluated by gel electrophoresis to determine that a single PCR product of the predicted size was generated. The real-time PCR reactions were performed in an ABI 7700 Detection System (Applied Biosystems). Each sample was acquired in at least triplicate. Data analyses were performed according to the protocol provided by Applied Biosystems. Standard curves were generated using

total RNA isolated from mouse CNS (a mixture of brain and spinal cord). The amount of each mRNA for tested genes was calculated according to the standard curve for that particular primer set. Finally, the relative amount of the tested message was normalized to the level of an internal control message, glyceraldehyde-3-phosphate dehydrogenase (Gapdh). The results were statistically analyzed using a two-tailed, unpaired Student's *t*-test. PCR primers used were as follows:

Gene (RefSeq Accession)	Forward	Reverse
mouse CXCR4 (NM_009911)	ACGCCATGGCTGACTGGTAC	CCAGGATAAGGATCACCGTA
mouse VEGF-R2 (NM_010612)	TCCGGAGCCATCCACTTCAA	TTGGACAGCATCACCAGCAGT
mouse MMP-3 (NM_010809)	AGTCTACAAGTCCTCCACAG	TTGGTGATGTCTCAGGTTCC
mouse MMP-2 (NM_011594)	GATGTCCAGCAAGTAGATGC	TGAAGTCACCAGGTGAAGGA
mouse MMP-7 (NM_010810)	GAGATCATGGAGACAGCTTC	TGTTGATGTCTCGCAACTTA
mouse MMP-9 (NM_013599)	TGAGTCCGGCAGACAATCCT	TCTTGGTCTGCGGATCCTCA
mouse MMP-10 (NM_019471)	CTTAGATGCTGCCTATGAGG	CATGATGATCAGCACAGCAG

Western Blotting

Western blotting was performed based on standard protocol (Zhao et al., 2003). Briefly, NSPCs were collected using homogenization buffer. After centrifugation to eliminate insoluble membranes, the protein concentration was determined using Bradford reagent (Bio-Rad, CA). For each sample, protein structure dissociated by boiling at 90^{o}C for 10 min, protein size was resolved through a 4-12% Bis-Tris gel (Invitrogen, #NB0341BOX) electrophoresis and transferred to a nitrocellulose membrane (BioRad, TransBlot Transfer Medium, #162-0115). Nonspecific sites of the membranes were saturated by 1-hour incubation in TBST (150 mM NaCl, 20 mM Tris, pH 7.5, 1% Tween 20) containing 5% nonfat milk. The membrane was incubated overnight at 4°C with appropriate primary antibody: either goat anti MMP-3 (1:1000, AbCam, #AB18898), rabbit anti MMP-9 (1:1000, Chemicon, #AB19106), rabbit anti MMP-12 (1:1000, ABcam, #AB39876) in TBST containing 1% nonfat milk. Membrane was then rinsed and incubated for 1 hour at room temperature with the corresponding horseradish peroxidase-conjugated rabbit or goat anti rabbit secondary antibody (Pierce, #1858415) diluted 1:5000 in 1% milk solution. Peroxidase activity was revealed using the enhanced chemiluminescence detection system (ECL and SuperSignal, Pierce) and exposed for 1 to 5 min on X-ray films (FUJI photo Film Co. LTD). The membrane was then stripped using Restore Western Blot Stripping Buffer (Pierce, #21059) for 60 min at 37°C, followed by incubation with a mouse antibody against β-actin (1:2000, Sigma, #A5441) as protein loading control.

Recombinant Lentiviruses and In Vitro Gene Knockdown

The vectors expressing MMP-siRNA driven by a U1 promoter were purchased from SuperArray (SureSilencing siRNA plasmid: MMP-3, KM03673G; MMP-9,

KM03661G; SuperArray Bioscience Corp., Frederick, MD, http://www.superarray.com). The U1-siRNA cassettes were subsequently cloned into a third-generation lentiviral vector (Verma and Gage, 2000). Lentivirus production was performed as described previously (Barkho et al., 2006). A detailed description is provided in supplemental data. All recombinant DNA research was performed on the basis of NIH guidelines.

Mouse Middle Cerebral Artery Occlusion

All animal procedures used in this study were approved by institutional animal care and use committees. Middle cerebral artery occlusion (MCAO) followed by reperfusion was conducted using the intraluminal method as described (Doetsch, 2003b; Kokovay and Cunningham, 2005). Adult male C57BL/6 mice (3 months of age; weight, ~25 g) were used for a 60-minute MCAO followed by reperfusion. At 14 days post-MCAO, mice were sacrificed, and brains were rapidly removed, quick-frozen in isopentane equilibrated in dry ice-ethanol slurry, and stored at -80°C until further processing. Coronal brain sections (20 μm) were prepared using a cryostat and arranged on slides (Superfrost Plus; VWR, West Chester, PA, http://www.vwr.com). A detailed description of MCAO is provided in supplemental data.

Fluorescence In Situ Hybridization

Fluorescence in situ hybridization (FISH) was performed as described (Guzowski et al., 1999). Briefly, the DNA templates for riboprobe synthesis were cloned using PCR on the basis of GenBank sequences (supplemental data). PCR products were ligated to TOPO II vector (#45-0640; Invitrogen) and sequenced before they were linearized to generate templates for riboprobe synthesis. Digoxigeninlabeled antisense and sense cRNA riboprobes were made using MAXIscript T7/SP6 (Ambion #1322 or #1326;) and

digoxigenin RNA labeling mix (Roche Diagnostics, #11277073; Basel, Switzerland, http://www.roche-applied-science.com). For FISH, the riboprobes were hybridized on fixed brain sections at 56°C overnight and detected with the use of a commercial tyramide signal amplification cyanine-3 or fluorescein tyramide signal amplification kit (PerkinElmer Life and Analytical Sciences, #SAT704A001EA or SAT701001EA; Boston, http://www.perkinelmer.com). *z*-Stack images were captured using a Zeiss laser scanning confocal microscope (LSM 510-META; X40 oil; 1.2 numerical aperture) at 1-μm intervals. For quantification of DCX and MMP double-labeled cells, we took three confocal images spanning from the SVZ to the border of infarct area on the ipsilateral side (Figure 6.5B, red squares). In each confocal image, we first selected 10–15 DCX^+ cells and determined whether they were also MMP-3^+ or MMP-9^+. Then we selected 10–15 MMP^+ cells and determined whether they were DCX. Three independent MCAO animals with confirmed stroke lesions were used in this quantification.

RESULTS

SDF-1 and VEGF Induce the Differentiation of Multipotent Adult NSPCs

To define the molecular mechanism by which adult NSPCs migrate in response to chemokines, we first isolated adult NSPCs from adult mouse brains and determined the effects of SDF-1 and VEGF on these cells. These primary adult NSPCs cultured under proliferating conditions express both immature progenitor cell markers, sex determining region Y-box 2 (Sox2) and Nestin (Figure 6.1A), indicating relative phenotypic homogeneity. When cultured under proliferating conditions, adult NSPCs incorporated the thymidine analog bromodeoxyuridine (BrdU; Figure 6.1B). Upon growth factor withdrawal, adult NSPCs differentiated into all major brain cell types, including neurons (neuronal class III β-tubulin [Tuj1]), astrocytes (glial fibrillary acidic protein [GFAP];

Figure 6.1C), and oligodendrocytes (detected by NG2, a chondroitin sulfate proteoglycan expressed by oligodendrocyte progenitor cells, supplemental Figure 6.1E). To examine adult NSPC response to SDF-1 and VEGF, we used immunocytochemistry and reverse transcriptase-PCR and confirmed that these adult NSPCs indeed expressed both CXCR4, the receptor for SDF-1 (Figure 6.1D; supplemental Figure 6.1C), and VEGF-R2, the receptor that mediates VEGF-induced cell migration (Figure 6.1E; supplemental Figure 6.1C). Using real-time quantitative polymerase chain reaction (qPCR), we further demonstrated that the mRNA levels of CXCR4 and VEGF-R2 in adult NSPCs did not change either after growth factor withdrawal or upon stimulation by SDF-1 or VEGF (supplemental Figure 6.1A, 6.1B). Thus, cultured adult NSPCs express both receptors, CXCR4 and VEGF-R2, indicating the potential responsiveness of these cells to exogenous SDF-1 and VEGF. We and other laboratories have previously shown that chemokines and cytokines promote the proliferation and differentiation of adult NSPCs (Barkho et al., 2006; Monje et al., 2003). Therefore, we first examined how SDF-1 and VEGF affect stem cell properties of primary adult NSPCs in the absence of the mitogens FGF-2 and EGF. Compared with control conditions (growth factor withdrawal), allowing for spontaneous differentiation, both SDF-1 and VEGF treatment led to a 51.6% increase in neuronal differentiation ($Tuj1^+$ cells; $p < .001$; Figure 6.1G, 6.1H) and 60.0% and 69.2% increases, respectively, in astrocyte differentiation ($GFAP^+$ cells; $p < .001$; Figure 6.1I, 6.1J). The number of oligodendrocytes ($NG2^+$) showed no significant difference after treatment with SDF-1 or VEGF compared with the control (supplemental Figure 6.1D, 6.1E).

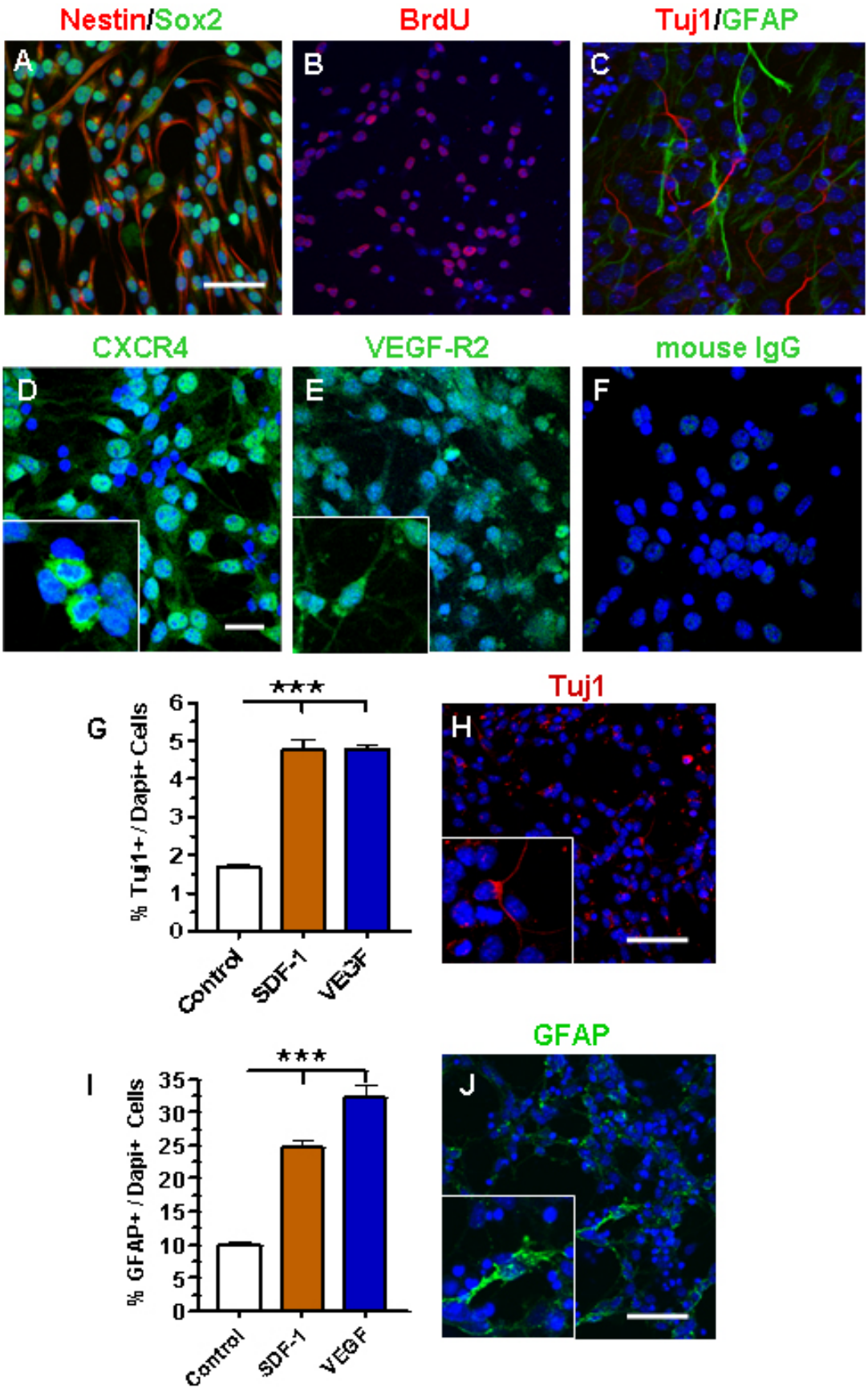

Nestin/Sox2
BrdU
Tuj1/GFAP
A
B
C
CXCR4
VEGF-R2
mouse IgG
D
E
F
G
***
% Tuj1+ / Dapi+ Cells
0
1
2
3
4
5
6
Control
SDF-1
VEGF
Tuj1
H
I
***
% GFAP+ / Dapi+ Cells
0
5
10
15
20
25
30
35
Control
SDF-1
VEGF
GFAP
J

Figure 6.1: Multipotent adult NSPCs differentiate in response to SDF-1 and VEGF. **(A)** Cultured adult NSPCs expressed immature stem cell markers, Sox2 (green) and Nestin (red). **(B)** Under proliferating conditions, cultured adult NSPCs incorporated BrdU (red). Blue, Dapi. **(C)** In the absence of growth factors, adult NSPCs differentiated into neurons (Tuj1, red) and astroctyes (GFAP, green). **(D-F)** Proliferating adult NSPCs expressed the receptors for SDF-1 and VEGF. **(D)** CXCR4 (green) and **(E)** VEGFR2 (green). **(F)** Control immunostaining using mouse IgG. Scale bar = 50 μm**.** Blue, DAPI. Insets in D and E show higher-magnification images. **(G)** Both SDF-1 and VEGF promoted neuronal differentiation of adult NSPCs after 16 hours of treatment (*** $p < 0.001$, $n = 3$). **(H)** Example of $Tuj1^+$ neurons quantified in (G). Scale bar = 50 μm **(I)** Both SDF-1 and VEGF led to increased astrocyte differentiation of adult NSPCs after 16 hours of treatment (*** $p < 0.001$, $n = 3$). **(J)** Example of $GFAP^+$ astrocytes quantified in (I). Scale bar = 50 μm.

We used 8-hour BrdU pulse labeling to assess the effects of SDF-1 and VEGF on adult NSPC proliferation in the absence of mitogen. There was no evident difference in the number of dividing cells under either the SDF-1- or VEGF-treated conditions compared with control cultures (supplemental Figure 6.1F). Therefore, neither SDF-1 nor VEGF functions as a mitogen for adult NSPCs, but both promote the differentiation of adult NSPCs. Finally, to ensure that the effect of these chemokines on adult NSPC differentiation was not due to their effects on cell survival, we performed a propidium iodide permeability assay and found that neither SDF-1 nor VEGF altered the cell death of adult NSPCs compared with control growth factor withdrawal conditions (supplemental Figure 6.1G). Hence, cultured adult NSPCs express the receptors for SDF-1 and VEGF, and these chemokines promote both neuronal and astrocyte differentiation of adult NSPCs without affecting proliferation or survival.

SDF-1 and VEGF Induce the Migration of Adult NSPC-Differentiated Neurons and Astrocytes

To dissect the molecular mechanisms underlying adult NSPC migration in response to SDF-1 and VEGF, we established an in vitro migration assay based on a modified Boyden chamber assay (Imitola et al., 2004; Thored et al., 2006). Adult NSPCs

were plated in the top chamber of the cell culture insert (Transwell), and then either SDF-1 or VEGF was added to the bottom chamber. At different time points, we fixed, stained, and quantified the cells that migrated to the bottom chamber (Figure 6.2A). We discovered that most cells plated onto noncoated cell culture inserts failed to migrate toward either chemokine. Since the ECM is known to be important for cell mobility (Kearns et al., 2003; Tate et al., 2004), we coated tissue culture inserts with several different ECM components native to the brain and compared their ability to promote cell migration in response to SDF-1 and VEGF. Consistent with previous reports (Kearns et al., 2003), we found that laminin is the most effective ECM coating for mediating cell migration (approximately 1.75-fold more effective than fibronectin; Figure 6.2B). We therefore decided to use laminin for further migration studies. An example of cells that migrated to the bottom chamber on a laminin-coated insert is shown (Figure 6.2C, control vs. SDF-1). Next we determined the time course of migration and found that migrating cells began to appear in the bottom chamber at 10 hours and reached a plateau at 16 hours post-chemokine addition, with approximately one-third of the total plated cells having migrated (Figure 6.2D). When fresh SDF-1 or VEGF was administered to the bottom chamber at 16 hours, significantly more cells migrated, suggesting that the plateau at 16 hours did not represent a limited potential of plated adult NSPCs but rather equilibrium of the chemokine gradient (supplemental Figure 6.2A). These data indicate that isolated adult NSPCs migrated through a laminin coated matrix toward SDF-1 and VEGF, which allows us to distinguish the molecular mechanisms underlying chemokine-induced adult NSPC migration using this assay. There is ample evidence demonstrating the migration of neuroblasts to ischemic regions (Gotts and Chesselet, 2005; Jin et al., 2003; Kokaia

and Lindvall, 2003). However, few studies have characterized other migratory cell types derived from proliferating adult NSPCs residing in the SVZ. Since we observed an effect of SDF-1 and VEGF on the differentiation of adult NSPCs (Figure 6.1), we next characterized the phenotypes of those cells derived from adult NSPCs that either migrated (in the bottom chamber) or remained stationary (in the top chamber) after 16 hours of chemokine-induced migration. We observed that $Nestin^+$ immature cells and $GFAP^+$ astrocytes constituted the majority of the stationary cells, along with some other cells of unknown identity (Figure 6.2E). The majority of migratory cells were DCX^+ and $Tuj1^+$ neuroblasts, plus some $GFAP^+$ astrocytes (Figure 6.2F). Thus, upon SDF-1 or VEGF stimulation, most of the $Nestin^+$ cells (92%–94%) and the majority of $GFAP^+$ astrocytes (76%–83%) failed to migrate (Figure 6.2G), whereas ~98% of the DCX^+ and 80%–87% of the $Tuj1^+$ neuroblasts migrated (Figure 6.2H). Not all of the DCX^+ cells were positive for $Tuj1^+$ in either the migration assay (Figure 6.2I) or the differentiation assay (supplemental Figure 6.2C–6.2E), possibly because these two markers label different stages of neuronal differentiation. These data suggest that the differentiation and migration of adult NSPCs are integral components of the SDF-1- and VEGF-induced phenotypic response. Since the expression levels of the chemokine receptors did not change upon chemokine treatment (Figure 6.1), this suggests that cellular differentiation is likely a required process for the migration of neuronal and astrocyte cell lineages.

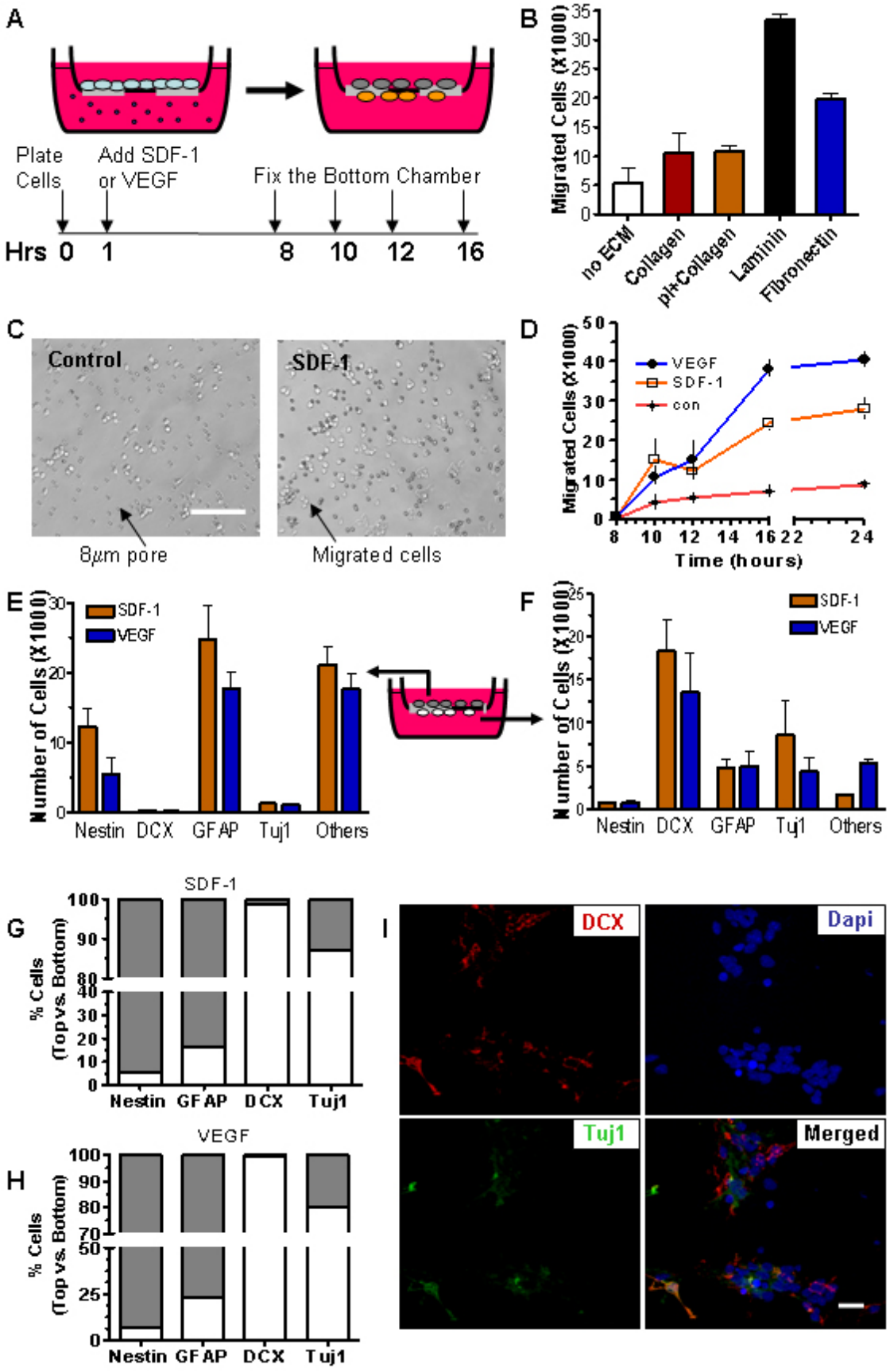

A
Plate Cells
Add SDF-1 or VEGF
Fix the Bottom Chamber
Hrs 0 1 8 10 12 16
B
Migrated Cells (X1000)
no ECM
Collagen
pl+Collagen
Laminin
Fibronectin
C
Control
SDF-1
8μm pore
Migrated cells
D
Migrated Cells (X1000)
VEGF
SDF-1
con
Time (hours)
E
Number of Cells (X1000)
SDF-1
VEGF
Nestin DCX GFAP Tuj1 Others
F
Number of Cells (X1000)
SDF-1
VEGF
Nestin DCX GFAP Tuj1 Others
G
SDF-1
% Cells (Top vs. Bottom)
Nestin GFAP DCX Tuj1
H
VEGF
% Cells (Top vs. Bottom)
Nestin GFAP DCX Tuj1
I
DCX
Dapi
Tuj1
Merged

Figure 6.2: Adult NSPCs differentiate and migrate in response to SDF-1 and VEGF. **(A)** Experimental schematics showing in vitro migration assay. Tissue culture inserts with an 8.0-μm pore were used to separate the top and bottom chambers. Adult NSPCs were plated onto the membrane of the top chamber, and either SDF-1 or VEGF was administered to the bottom chamber. At specific time points, the migratory and stationary cells were analyzed. **(B)** Quantification of cell migration on different ECM substrates indicated that laminin was the most effective ECM at promoting cell migration (n = 4). **(C)** Bright-field image of cells migrated to the bottom of chambers on laminin-coated inserts. Control: no SDF-1 was added. Scale bar = 100 μm. **(D)** Time course of adult aNSPC migration toward SDF-1 and VEGF. The cell migration was initially observed at 10 hours and plateaus at 16 hours (n = 3). **(E)** Majority of stationary cells on the top chamber were $Nestin^+$ immature cells and $GFAP^+$ astrocytes (n = 3). **(F)** Majority of migratory cells at the bottom were DCX^+ and $Tuj1^+$ neuroblasts and some $GFAP^+$ astrocytes (n = 3). **(G)** The percentage distribution of different lineages of cells between the top chamber and the bottom chamber after SDF-1 treatment. **(H)** The percentage distribution of different lineages of cells between the top chamber and the bottom chamber after VEGF treatment. **(I)** Example of migratory DCX^+ (red) and $Tuj1^+$ (green) cells on the bottom chamber. Merged image showing the colocalization of DCX- and Tuj1-expressing cells. Blue, Dapi. Scale = 20 μm.

Endogenous MMP-3 and MMP-9 Are Involved in Chemokine-Induced Adult NSPC Migration

With the establishment of an effective in vitro migration assay, we next determined whether endogenous MMPs play a role in the migration of adult NSPCs. We found that a broad-spectrum MMP inhibitor, GM6001, significantly reduced adult NSPC migration in response to either SDF-1 or VEGF (Figure 6.3A), suggesting that MMPs sensitive to GM6001 are involved in the migration of adult NSPCs. To identify specific MMPs involved in adult NSPC migration, we selected candidate MMPs already known to be involved in other migratory processes, such as cancer metastasis, angiogenesis, and stem cell homing (Kucia et al., 2005). We first compared the mRNA expression levels of the candidate *MMPs* in migratory cells collected from the bottom chamber versus stationary cells from the top chamber after exposure to SDF-1 and VEGF. Using qPCR, we found that *MMP-3* mRNA levels were 43.8% and 37.5% higher in migratory cells compared with stationary cells in SDF-1- and VEGF-induced migration, respectively ($p <$

.05; Figure 6.3B). Moreover, *MMP-9* mRNA levels were 53.5% and 75.0% higher in migratory cells compared with stationary cells in SDF-1 and VEGF treatment, respectively ($p < .05$; Figure 6.3C). The expression of *MMP-2, -7*, and *-10* mRNA transcripts, however, did not differ between migratory and stationary cells (supplemental Figure 6.3A–6.3C). We then confirmed that MMP-3 and MMP-9 protein levels were also higher in both the medium (Figure 6.3D; quantification is shown in supplemental Figure 6.3F, 6.3G) and cell lysate collected from the top or bottom chambers (Figure 6.3E; quantification is shown in supplemental Figure 6.3H, 6.3I). Furthermore, we confirmed that the DCX^+ neurons that migrated to the bottom chambers of Transwells are positive for MMP-3 and MMP-9 immunoreactivity (Figure 6.3F–6.3H). Since MMP-2 is known to be regulated at the protein expression and activity levels, rather than the transcriptional level, we also analyzed the protein expression levels of MMP-2, but we did not detect differential expression of this MMP in the migratory cells (supplemental Figure 6.3D). Therefore, mi- grated cells express higher levels of MMP-3 and MMP-9 at both the mRNA and protein levels. Although pharmacological MMP inhibitors have been widely used to study the roles of MMPs in cellular functions, these inhibitors lack specificity, which confounds data interpretation. We therefore decided to use siRNAs that specifically knockdown endogenous MMP-3 and MMP-9 expression to investigate whether increased expression of MMP-3 and MMP-9 in migrating cells is critical for the migration of these cells. To this end we used lentiviral vectors that expressed MMP-3-siRNA, MMP-9-siRNA, or control nonsilencing (NC)-siRNAs under a U1 RNA polymerase III promoter and enhanced green fluorescent protein under a cytomegalovirus promoter, so that the cells that underwent acute knockdown of MMPs could be tracked

by GFP fluorescence (supplemental Figure 6.3E). To determine whether lentivirus itself had a deleterious effect on adult NSPCs, we plated cells infected by control viruses (lenti-GFP and lenti-NC-siRNA), as well as by MMP-3- and MMP-9-siRNA lentiviruses, onto the Transwell membranes for 16 hours without inducing migration (no chemokine added). We found that all virus-infected cells had a similar percentage of GFP^+ cells (32.8% $\pm$ 2.4% infection efficiency), suggesting that neither lentivirus nor MMP-siRNAs had a negative effect on cell survival. To assess the nonspecific effect of the virus on cell migration, we then determined that the number of migrated cells in control lentivirus-infected conditions was comparable to that of uninfected cells (supplemental Figure 6.3L), suggesting that the lentivirus itself had no significant negative effect on our in vitro assay. We then confirmed that these siRNAs could indeed reduce the protein-expression levels of endogenous MMP-3 (Figure 6.3I) and MMP-9 (Figure 6.3J) compared with the NC-siRNA, lenti-GFP, and uninfected control cells (quantification is given in supplemental Figure 6.3J, 6.3K). To determine the effect of MMP-3 and MMP-9 knockdown on cell migration, we infected proliferating adult NSPCs with siRNA-lentivirus for 24 hours, allowing the siRNA sequences to be incorporated into the genome. Then the cells were plated onto migration chambers and subjected to cell migration assays. We found that acute knockdown of either MMP-3 or MMP-9 led to a significant reduction in cell migration in response to either SDF-1 (Figure 6.3K) or VEGF (Figure 6.3L) compared with the lentivirus-NC-siRNA infected or lentivirus-GFP-infected cells. Specifically, we observed that MMP-3-siRNA-infected cells had a 33.8% reduction in SDF-1-stimulated migration ($p < .05$) and a 38.6% reduction in VEGF-stimulated migration ($p < .01$; Figure 6.3K) compared with the NC-siRNA-infected cells.

Knockdown of endogenous MMP-9 led to a 42.3% reduction in SDF-1-stimulated cell migration ($p < .05$) and a 53.3% reduction in VEGF-stimulated cell migration compared with the NC-siRNA-infected cells ($p < .01$; Figure 6.3L). Therefore, both endogenous MMP-3 and MMP-9 expressed by adult NSPCs are involved in their migration toward stroke-induced chemokines. Together these data indicate that in response to SDF-1 and VEGF, adult NSPCs differentiate and express higher levels of MMP-3 and MMP-9, which are required for their migratory response.

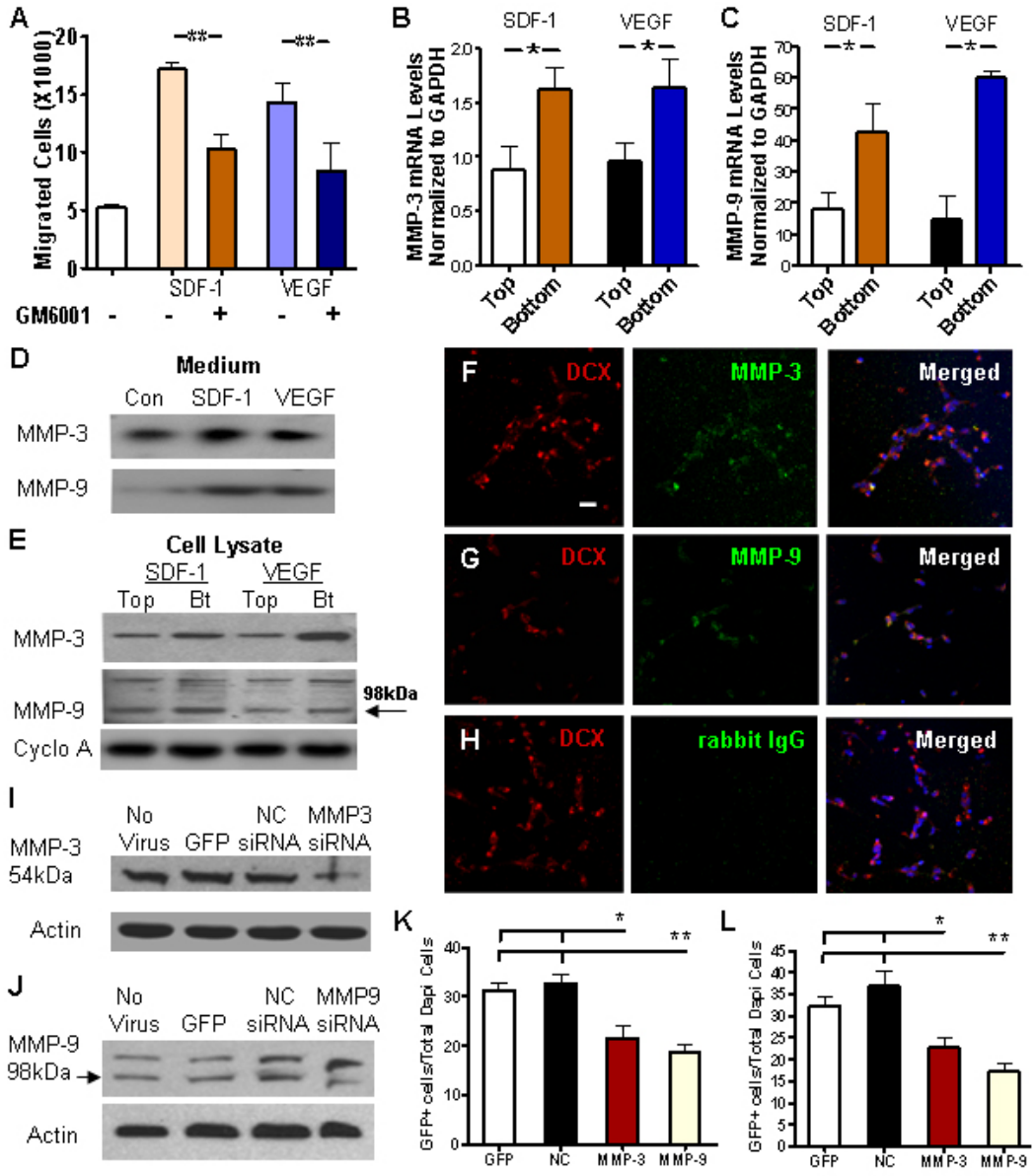


Figure 6.3: MMP-3 and MMP-9 expressed by adult NSPCs are important for their migration response to SDF-1 and VEGF. (A) A broad-spectrum MMP inhibitor, GM6001, inhibited SDF-1– or VEGF-induced cell migration (** $p < 0.01$, $n = 4$). **(B, C)** qPCR analysis of migratory and stationary cells demonstrated that mRNA levels of **(B)** MMP-3 and **(C)** MMP-9 were significantly higher in migratory cells (bottom chamber) compared with stationary cells (top chamber) ($n = 4$). **(D, E)** The protein levels of MMP-3 and MMP-9 in the culture medium **(D)** and cell lysate **(E)** of the bottom chamber (Bt) were higher than those in the top chamber (Top). **(F-H)** Migrated DCX+ neuroblasts (red) express MMP-3 (F green) and MMP-9 (G, green). Mouse IgG, instead of MMP antibodies, was used as negative control (H, green). Scale bar = 20 μm. **(I)** Western blot analysis showing lentivirus-MMP-3-siRNA could efficiently knockdown endogenous MMP-3 (54 kDa) in adult NSPCs compared with the control lentivirus (lentivirus-NC-siRNA and lentivirus-GFP)-infected adult NSPCs and uninfected adult NSPCs (β-actin antibody used as a loading control). **(J)** Western blot analysis showing lentivirus-MMP-9-siRNA could efficiently knockdown endogenous MMP-9 (98Kda) in adult NSPCs compared with control lentivirus-infected adult NSPCs and uninfected adult NSPCs. **(K, L)** The knockdown of MMP-3 and MMP-9 led to reduced cell migration in response to either SDF-1 (K) or VEGF (L) (** $p < 0.01$, $n = 3$).

MMP-3 and MMP-9 Are Involved in Adult NSPC Proliferation and Differentiation

Since we demonstrated that SDF-1 and VEGF led to both the differentiation of adult NSPCs and increased MMP-3 and MMP-9 expression in migrating cells, we next investigated whether MMP-3 and MMP-9 were involved in SDF-1- and VEGF induced adult NSPC differentiation. MMP-3- or MMP-9-siRNA lentivirus-infected adult NSPCs were treated with SDF-1 or VEGF in the absence of stem cell mitogens, and we used Tuj1 expression as an index of neuronal differentiation and GFAP expression for glial differentiation (NC-siRNA vs. MMP-9-siRNA; Figure 6.4A, 6.4B). Consistent with what we found in uninfected cells (Figure 6.1G, 6.1J; supplemental Figure 6.4A, 6.4B), SDF-1 and VEGF treatment led to increased neuronal and astrocyte differentiation in both NC-siRNA-infected and lentivirus-GFP-infected adult NSPCs (Figure 6.4C, 6.4E). However, acute knockdown of either MMP-3 or MMP-9 abolished SDF-1- or VEGF-induced neuronal differentiation (Figure 6.4C). Such inhibitory effects became more apparent when we compared the fold induction of neuronal differentiation by SDF-1 and VEGF in

siRNA-infected adult NSPCs with that of controls (Figure 6.4D, dotted line). Interestingly, acute knockdown of MMP-9 inhibited the neuronal differentiation induced by VEGF more than that induced by SDF-1 (Figure 6.4D). On the other hand, acute knockdown of MMP-3, but not MMP-9, significantly potentiated VEGF-induced astrocyte differentiation (Figure 6.4E, 6.4F). Using BrdU pulse labeling, we determined that MMP-3- siRNA and MMP-9-siRNA had no effect on the proliferation of SDF-1- or VEGF-treated adult NSPCs in the absence of mitogens (supplemental Figure 6.4C–6.4E), which is consistent with our observation that neither SDF-1 nor VEGF affected adult NSPC proliferation in the absence of mitogens (supplemental Figure 6.1G). On the other hand, under proliferating conditions (in the presence of FGF-2 and EGF), cell division was reduced in MMP-3-siRNA- or MMP-9-siRNA-infected cells compared with NC-siRNA-infected cells (supplemental Figure 6.4F), suggesting that intrinsic MMP-3 and MMP-9 are required for mitogen-dependent maintenance of adult NSPCs. Together, these data indicate that both MMP-3 and MMP-9 play important roles in the fate choice of adult NSPCs into the neuronal and astrocyte lineages in response to SDF-1 or VEGF.

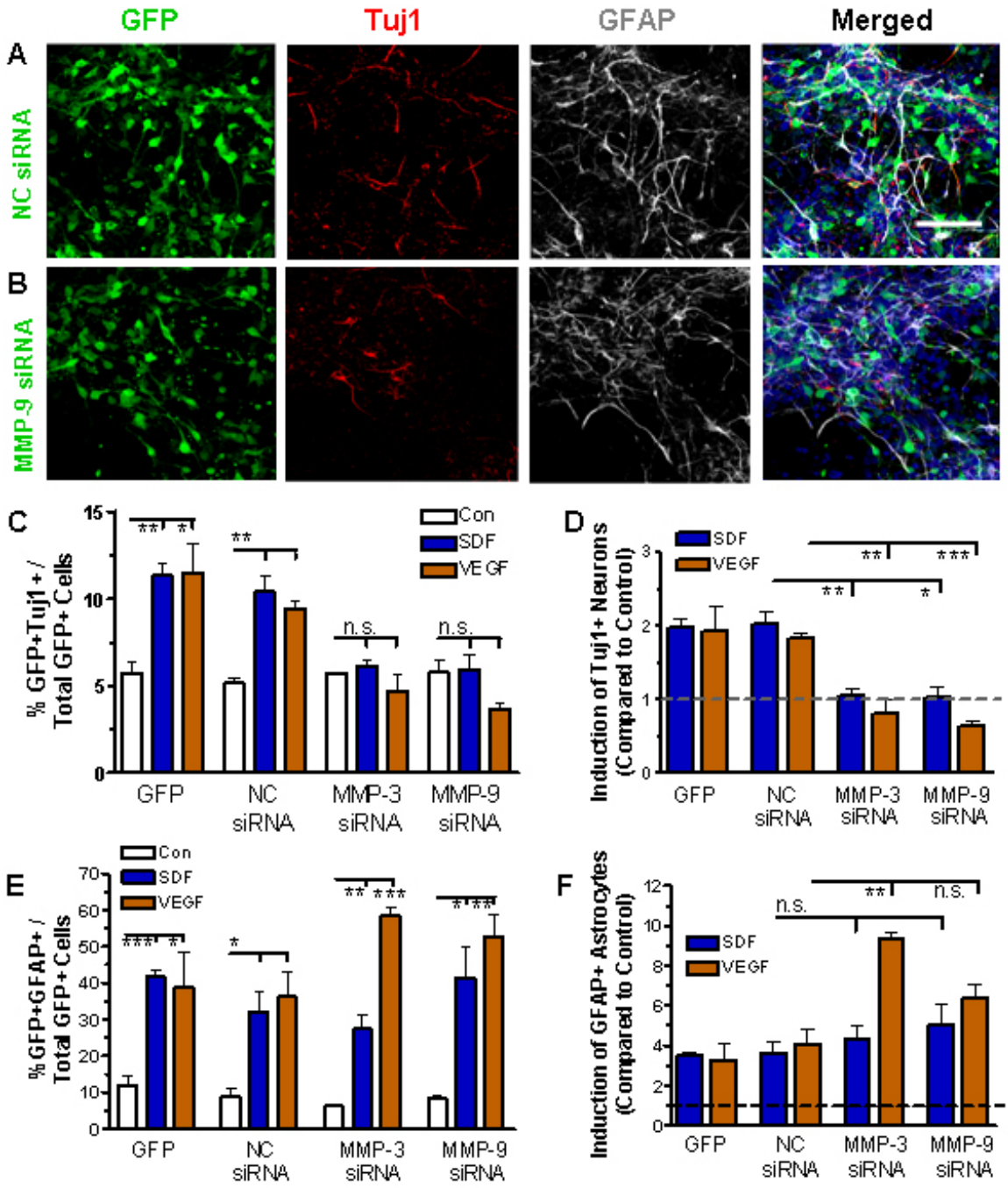


Figure 6.4: Knockdown of MMP-3 and MMP-9 interferes with SDF-1– and VEGF-induced adult NSPC differentiation. (A-B) Sample images showing that lentivirus-NC-siRNA (A) and Lentivirus-MMP-9-siRNA (B) -infected cells differentiated into Tuj1+ neurons (red) or GFAP+ astrocytes (white) in the presence of SDF-1. Blue, Dapi. Green, GFP; Scale bar = 50 μm. **(C)** Acute knockdown of MMP-3 or MMP-9 using lentivirus-siRNA abolished SDF-1– and VEGF-induced neuronal differentiation compared with lentivirus-NC-siRNA– and lentivirus-GFP–infected adult NSPCs. **(D)** The neuronal induction by SDF-1 or VEGF was abolished by acute knockdown of MMP-3 or MMP-9. The same data shown in E was normalized to a no-chemokine control condition (shown in dotted line). **(E)** Acute knockdown of MMP-3 (** $p < 0.01$) using lentivirus-siRNA

potentiated VEGF- but not SDF-1–induced astrocyte differentiation compared with controls. MMP-9-siRNA did not have a similar effect ($p = 0.1$) **(F)** Same data shown in F was normalized to a no-chemokine condition (shown in dotted line). *, $p < 0.05$; $p < 0.01$; ***, $p < 0.001$; ns, non-significant.

Neuroblasts in a Rodent Stroke Model Express Endogenous MMP-3 and MMP-9

To verify that migrating neuroblasts in stroke brains indeed express endogenous MMP-3 and MMP-9, we decided to use a mouse model of MCAO. Recent studies have shown that in this rodent stroke model, DCX^+ migrating neuroblasts are double labeled for MMP-9 immunoreactivity (Lee et al., 2004); however, since most MMPs are secreted into the ECM, it is difficult to assess the origin of this metalloproteinase on the basis of the immunoreactivity of its antibody. We therefore decided to use multicolor FISH to examine the colocalization of MMP mRNA and DCX mRNA expressed by migrating neuroblast at 2 weeks post-MCAO (Figure 6.5A). Mice received a 60-minute MCAO to one hemisphere (ipsilateral; Figure 6.5B) and were analyzed at 2 weeks post reperfusion, when ischemia-induced cell migration was at its peak (Kokaia and Lindvall, 2003). We observed that there was an increased FISH positive hybridization signal for both MMP-3 and MMP-9 mRNAs in the ipsilateral striatum compared with the contralateral side (Figure 6.5C–6.5N); notably, normal adult brain tissues express undetectable levels of MMP-3 or MMP-9 (Seiki, 2002; Stamenkovic, 2003). In addition, high-resolution confocal analysis indicated that both MMP-3 (Figure 6.5C, red) and MMP-9 (Figure 6.5I, red) riboprobes were colocalized with DCX riboprobe (migrating neuroblast; Figure 6.5D, 6.5J, green). Neither antisense probes on the contralateral side (Figures 6.5H, 6.5N; supplemental Figures 6.5A–6.5C, 6.5G–6.5H) nor sense riboprobes on the ipsilateral side (supplemental Figure 6.5D–6.5F, 6.5J–6.5L) showed positive signal ($^+$). To determine the extent to which DCX^+ cells in the ischemic region expressed MMP-3 and MMP-9, we

selected consecutive regions between the SVZ and the infarct core and quantified the colocalization of DCX^+ to MMP^+ cells (Figure 6.5B, red boxes). We found that all of the DCX^+ cells expressed mRNA of MMP-3 and MMP-9 and that ~90% of $MMP\text{-}3^+$ or $MMP\text{-}9^+$ cells were also DCX^+. Therefore, migratory neuroblasts in ischemic brains express endogenous MMP-3 and MMP-9, supporting a role for these MMPs in stroke-induce neurogenesis.

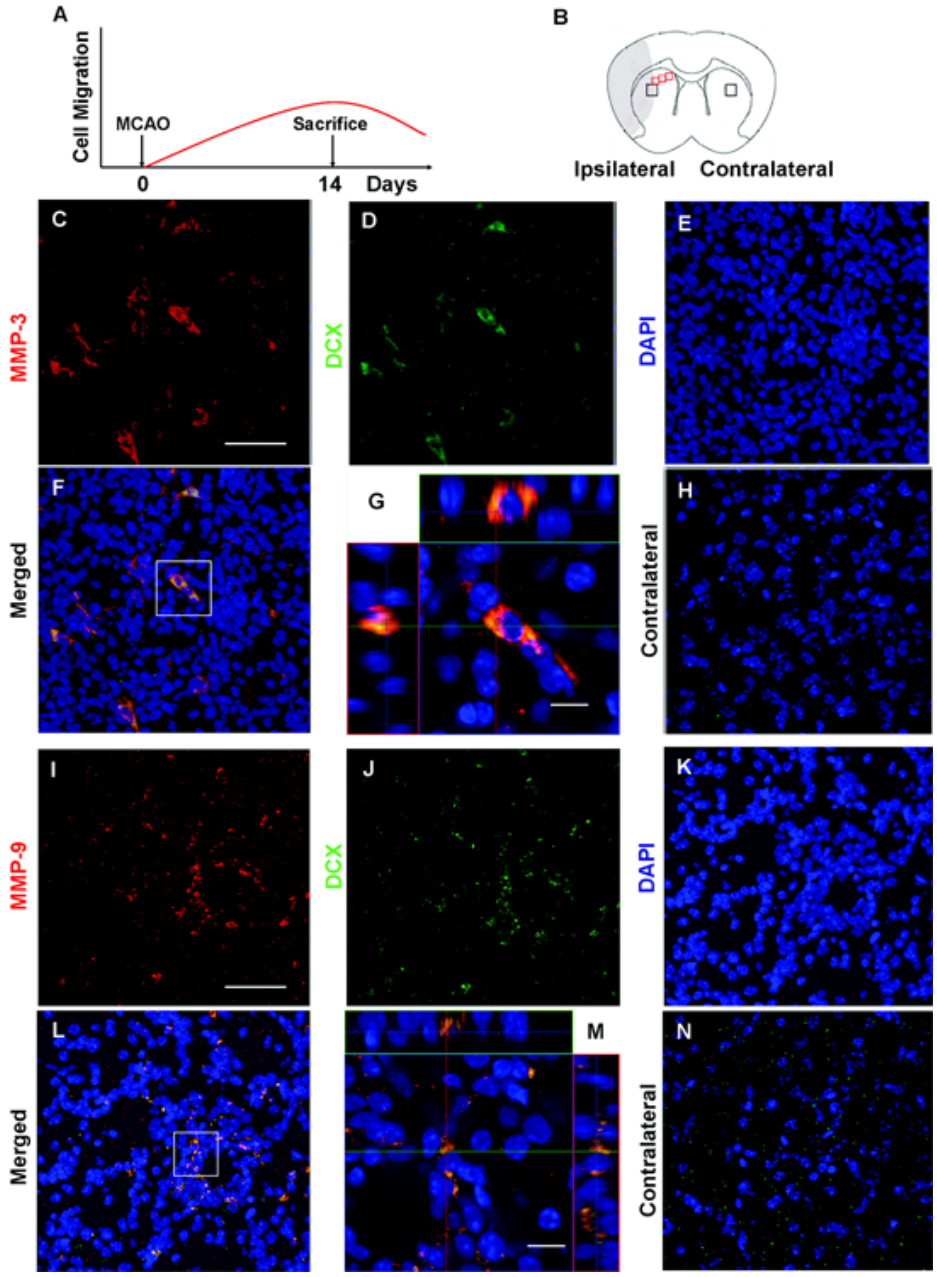


Figure 6.5: Migrating DCX$^+$ cells in an MCAO mouse model express MMP-3 and MMP-9 mRNA. (A) Mice were analyzed at 14 days post-MCAO, corresponding to the peak of neuroblast migration (based on literature (Kokaia and Lindvall, 2003; Lindvall et al., 2004). **(B)** Schematic diagram showing mouse brains subjected to unilateral MCAO. Both ipisilateral and contralateral sides were analyzed. The grey boxes indicate the brain regions where images were taken and shown in (C-M). The red boxes indicate the regions subjected to quantification of DCX$^+$ and MMP$^+$ cells. **(C-G)** Ribroprobes detected increased MMP-3 mRNA (C, red) in DCX mRNA expressing neuroblast (D) in the ipsilateral side compared with the contralateral side (H). Scale bar = 50 μm. **(G)** Image of a single cell from F (white box) showing that MMP-3 and DCX mRNA are expressed in the same cells. Scale bar = 20 μm. **(I-M)** Ribroprobes detected increased MMP-9 mRNA (I, red) in DCX mRNA expressing neuroblast (J) in the ipsilateral side but not the contralateral side (N). Scale bar = 50 μm. **(N)** Image of a single cell from L (white box) showing that MMP-9 and DCX mRNA are expressed in the same cells. Scale bar = 20 μm.

DISCUSSION

The discovery of a neurogenic response subsequent to an ischemic injury has generated vast interest, largely because of the desire to understand more about the functions and plasticity of the adult human brain. However, despite the extensive efforts and substantial progress made to date, the molecular mechanisms underlying adult NSPC differentiation, proliferation, survival, and migration remain unclear (Gotts and Chesselet, 2005). One obstacle has been the complexity of the in vivo environment that adult progenitors encounter after a brain injury (Kokaia and Lindvall, 2003; Parent, 2003). Injured brain tissue and surrounding regions contain many dynamically changing cell types, ranging from astrocytes to endothelial and immune cells (Thored et al., 2006; Zhang et al., 2002). The power behind our study is a well-defined in vitro assay that allowed us to identify the molecular characteristics of migrating cells in response to two well-studied stroke-induced chemokines, namely SDF-1 and VEGF. By using these two distinct chemokines that act via independent receptors, we can also derive a relatively common mechanism for injury induced adult NSPC migration. Using this assay system, we found that adult NSPCs differentiated and that differentiated cells, as opposed to

undifferentiated cells, migrated in response to chemokines. Our most intriguing finding is that such differentiation and migration are mediated, at least partially, by endogenous MMP-3 and MMP-9 expression by adult NSPCs. This represents the first evidence to account for the way extrinsic cues trigger the endogenous MMP expression that leads to downstream adult NSPC phenotypic changes and cell migration.

SDF-1 and VEGF Direct Adult NSPC Differentiation and Migration

Enormous efforts have been devoted to the identification of chemokines that attract adult NSPCs and newborn cells in injured brains; among these, the most studied are SDF-1 and VEGF (Hill et al., 2004; Kolodziej et al., 2008; Zhang et al., 2003a). It has been shown that in response to ischemic injuries, adult SVZ NSPCs differentiate into DCX^+ neuroblasts that migrate into the injured regions (Belmadani et al., 2006; Kokaia and Lindvall, 2003; Yamashita et al., 2006); however, the repertoire of the migratory cell types derived from adult SVZ NSPCs has not been fully characterized. We found that in response to both of these chemokines, the majority of DCX^+ and $Tuj1^+$ neuroblasts migrate, whereas nearly all $Nestin^+$ immature cells remain stationary, despite the fact that these cells may express comparable levels of receptors for these chemokines. Nevertheless, some researchers have found that $Nestin^+$ undifferentiated cells did migrate in response to ischemia (Imitola et al., 2004; Yamashita et al., 2006). It is possible, however, that the migration of these cells is triggered by other chemokines, such as stem cell factor (Yoshida et al., 2001) and monocyte chemoattractant protein-1 (Yan et al., 2007) in stroke brains. Such possibilities await further investigation. We also found that approximately 20%–25% of $GFAP^+$ astrocytes migrated toward SDF-1 and VEGF, but we could not distinguish the morphological differences between migratory and stationary

astrocytes. Whether these astrocytes belong to a distinct type of astrocyte (e.g., reactive astrocytes) is unclear. Because of the complex and dynamic nature of the in vivo post-ischemia environment, more research using a well-defined assay system is needed to determine the functional effect of other extrinsic cues on adult NSPCs.

Endogenous MMPs Promote Adult NSPC Migration in Response to Injury

A collection of cancer studies has linked chemokine-induced MMP expression to the migration of the cells expressing them (reviewed by (Chang and Werb, 2001)). However, the roles of MMPs expressed by adult NSPCs in cell migration have gone largely unexplained. Moreover, earlier studies into the roles MMPs play in cell migration were carried out using MMP inhibitors that generally lack specificity. Our study made use of MMP-3- and MMP-9-specific siRNAs, providing clear evidence for the involvement of endogenous MMP-3 and MMP-9 expressed by adult NSPCs in chemokine-induced migration. A major challenge to identifying the sources of MMPs is that most MMPs are secreted proteins; therefore their immunoreactivity is localized on both target and source cells, making it difficult to discern which exact cells express these MMPs. For example, MMP-9 immunoreactivity is colocalized with DCX^+ cells (Lee et al., 2004), but we also know that endothelial cell-secreted MMP-2 and MMP-9 are important for neuroblast migration (Wang et al., 2006). To demonstrate the presence of endogenous MMPs in adult NSPC-derived neuroblasts and support our in vitro finding, we used multicolor FISH and confocal microscopy to colocalize the MMP mRNAs with DCX mRNAs in the same cells, which yielded definitive evidence for the expression of MMPs by migrating neuroblasts. We anticipate that the methodology we developed in this manuscript (i.e., qPCR of migrated cells, siRNAs for MMPs, and multicolor FISH)

will advance the current standard for the study of MMP functions in the brain. By using both SDF-1 and VEGF, we have found that MMP-3 and MMP-9 are required for the migration that occurs in response to chemokines, suggesting that the signaling pathways initiated by these two chemokines converge at MMP-3 and MMP-9 expression in mediating cell migration. Both SDF-1 and VEGF are known to activate MMP transcription, which leads to cell migration (Chiu et al., 2007; Janowska-Wieczorek et al., 2000). Cell migration has been studied extensively in other cell systems, and the process is appreciated as a balanced action involving MMPs and their natural inhibitors, TIMPs, and a dynamic interaction between the cells and the ECM. These ECM factors include laminin (Ohab et al., 2006), integrins (Belvindrah et al., 2007), cadherins (Monea et al., 2006), and growth factor receptors (Thored et al., 2006). The activation of MMPs and subsequent cleavage of these factors may trigger intracellular signaling that leads to changes in NSPC functional properties. Our data provide the first evidence that MMP-3 and MMP-9 expressed by adult NSPCs contribute to the balance shift that is important for cell migration.

Endogenously Expressed MMP-3 and MMP-9 Promote Adult NSPC Differentiation

Despite the intense interest in the role of MMPs in cancer cell migration and in brain development and tissue injuries, their functions in adult NSPC proliferation and differentiation are poorly understood (Chang and Werb, 2001; Mannello et al., 2006). We demonstrated that the acute reduction of endogenous MMP-3 and MMP-9 in adult NSPCs abolished the neuronal differentiation induced by SDF-1 and VEGF but promoted the astrocyte differentiation induced by VEGF. In addition, we showed that MMP-3 and MMP-9 expressed by proliferating adult NSPCs are critical for their proliferation in the

presence of mitogen. These data provide an intriguing model in which MMPs, although conventionally thought of as extracellular protease, could modulate the basic properties of adult NSPCs: namely, proliferation and fate determination. Although MMP-3- and MMP-9-null mice have been generated by other laboratories (Coussens et al., 2000; Mudgett et al., 1998), the advantage of using MMP-specific siRNAs is that we were able to avoid compensatory effects from other mechanisms during mouse development, which can confound data interpretation. Future studies using NSPC-specific inducible Cre mice (Lagace et al., 2007) together with floxed MMP-3 and MMP-9 condition knockout mutant mice (these mice are yet to be made) will make a valuable in vivo extension of our current study. Recent studies have suggested that ECM remodeling and changes in cell-cell interactions, such as the breakdown of laminin and integrins, have an effect on the differentiation of embryonic and adult stem cells (Chen et al., 2007; Flanagan et al., 2006). Therefore, the breakdown of MMP substrates may activate secondary mechanisms that stimulate the differentiation of adult NSPCs. To our surprise, as chemokines promote the differentiation of adult NSPCs, we also observed a distinctive migratory response on the part of differentiated cells compared with immature undifferentiated cells, suggesting that differentiation and migration are integral parts of the adult NSPC response to chemokines. Since a lack of MMP-3 or MMP-9 hindered the capability of adult NSPCs to differentiate into the neuronal lineage, our data suggest that chemokines may promote a neurogenic response, at least in part, by inducing the expression of endogenous MMPs in adult NSPCs.

CHAPTER 7

Summary, Future Directions and Scientific Impact

Summary

The plasticity of adult NSPCs allows these cell types to control their cell fate in response to a variety of environmental cues. Major efforts in stem cell research have been devoted to understanding the regulation of NSPC, because of the potential of these cells as replacement therapies in adult neurological diseases. In the developing brain and in specific regions of adult brains, these cells are found to proliferate, migrate certain distances into specific regions, integrate into existing networks, and ultimately differentiate into specific neurons. Furthermore, it has been demonstrated that after brain injury both endogenous and grafted NSPCs also have the adaptive ability to proliferate, migrate long distances to the lesioned site, and differentiate into new neurons to replace the ones that have been lost. This course of regeneration is regulated by extrinsic cues found in the microenvironment surrounding the NSPCs, such as chemokines, cytokines, and growth factors. *The goal of my thesis is to identify these extrinsic cues that could stimulate the processes of proliferation, differentiation, and migration on endogenous or exogenous NSPCs*. In the first part of this thesis, I identified several key cytokines, such as IL-1β and IL-6 that are expressed and secreted at higher levels by astrocytes isolated from brain regions that support normal neurogenesis compared to those derived from non-neurogenic CNS regions. I showed that these cytokines individually can regulate the neuronal differentiation of NSPCs derived from adult forebrains. While in non-neurogenic regions, astrocytes secrete factors that inhibit the neuronal differentiation of adult NSPCs. To no surprise, factors secreted by astrocytes in neurogenic promoted

neurogensis whereas, factors expressed in the non-neurogenic region have the reverse effects. In the second part of this thesis, I investigated the role of extrinsic cues on regulating fate choice of adult NSPCs after stroke-induced brain injury. The goal of these studies was to understand a mechanism by which stroke-induced chemokines, such as SDF-1 and VEGF, regulate the differentiation and migration of NSPCs in response to injury. I was able to identify MMP-3 and MMP-9 as crucial players in regulating the process of NSPC neuronal differentiation and migration in response to the stimulation by SDF and VEGF. In summary, the results of my thesis work has helped in identifying potential roles of extrinsic cues in regulating the differentiation and migration of adult NSPC in both normal and injured brain. Therefore, we anticipate that we will have a better understanding of the basic mechanism of NSPC fate choice of proliferation, migration, or differentiation in response to their surrounding microenvironment.

A thorough understanding in the mechanisms that regulate NSPCs is needed for efficacious therapeutic approaches for brain diseases. Further knowledge of cytokines, chemokines, and MMPs in mediating NSC functions might provide novel molecular targets for treating neurological disorders or disease. My work has pointed to the next steps of this project that will provide better understanding in how MMPs might function in the complex network of stem cell homeostasis and differentiation. Therefore my findings, together with future extension of this work, will provide the potential foundation for controlling stem cell fate in response to extrinsic cues by manipulating MMP expression. This information will be important not only for knowing how endogenous adult NSPCs respond to the microenvironment in the normal brain, but also for understanding how NSCPs adjust to the changing environment in the damaged brain.

Furthermore, this information will help to determine the proper environment and conditions needed for culturing different stem cell types in *in vitro* before they can be properly studied outside the brain and serve as appropriate model systems for understanding the molecular mechanisms controlling stem cell properties.

Critique of Work

Due to time constrain, I did not have time to finish some of the analyses needed for a more complete understanding in the mechanism by which MMP regulate the adult NSPC differentiation and migration. However, I have tried to address some of the remaining questions as discussed in the Future Direction.

One direction that I would have liked to take this research was to understand how adult NSPCs respond to their microenvironment *in vivo* in the normal brain or after injury. First, cultured NSPCs are a heterogeneous population of cells. Therefore, it may be argued that only a subpopulation of NSPCs has the capability to respond to chemokine treatment. One weakness of this current research is the lack of a true comparison to the actual phenomenon that occurs in stroke-induce neurogenesis. Since it is impossible to truly replicate the *in vivo* settings using cultured cells; animal experiments would resolve some of these problems (see Future Direction). However, because techniques to perform *in vivo* injections were not available at the time, therefore, the focus of the current dissertation work was limited to several chemokines that are known to be expressed in the injured area and to cultured adult NSPCs in an *in vitro* setting (see Chapter 6). When time permitted to study the role of MMPs *in vivo*, due to technical difficulty, I was only able to optimize a simple method of electroporation of MMP siRNA into the brains as shown in the Appendix C.3. We can use this technique to answer the simple question of

whether the same *in vitro* effects of MMP siRNA knockdown, as we showed in Chapter 6, can be observed in the migration and differentiation of adult NSPCs in healthy or injured brains.

My dissertation work also lack a detailed analysis of migratory cells in animal MCAO stroke model, such as studying more time points post MCAO and utilizing other methods to identify migrating cells. For example, a possible pitfall is that the time point I have selected for analyzing MMP expression after MCAO may have missed the crucial time point or specific stages to observe significant expression of the MMPs. Therefore, experiments with more time points, such as at the start and during the peak of migration (Day 7 and 14, respectively), would provide more information. In addition, to determine whether MMP-3 or MMP-9 are involved in the proliferation of adult NSPC in response to stroke, analysis at an earlier time point, in this case Day 3 post-MCAO will be necessary. This additional information would help to determine whether using MMP siRNA knockdown would be an appropriate technique to analyze the effects of MMP in adult NSPCs in response to MCAO (as proposed in the Future Direction, see below). Furthermore, in the dual color FISH study described in Chapter 5, I only analyzed DCX^+ cells, which does not identify other types of adult NSPCs-derived cells that are also capable of expressing MMP3 and 9 in response to injury. Consequently, I have developed FISH probe templates to analyze expression of other genetic markers of different cell phenotypes, which can be used to answer this question in the future. To expand the advantage of dual color FISH further to determine the origin of MMPs expression in cells, it would be useful to combine MMP FISH with immunohistochemistry with cell lineage markers. However, using FISH and immunohistochemistry simultaneously is a

novel technique and has not been optimized in our laboratory yet. Similar consideration applies to the *in vitro* study shown in Chapter 6. It would be informative if I performed dual color FISH to analyze migratory versus stationary cells the *in vitro* migration assay to identify which cell phenotypes express MMP mRNA in response to SDF-1 and VEGF. These experiments would help identify which NSPC-derived cells express MMP-3 and/or MMP-9 in response to stroke-induced chemokines, SDF-1 or VEGF. However, this technique would need to be optimized to perform FISH on membranes of migration chambers.

Additional *in vitro* experiments are also needed to address the role of MMP in regulating adult NSPC properties. For example, I will need to determine whether SDF-1 and VEGF have a direct role on NSPC migration and differentiation. To answer this question, I considered treating NSPCs with pathways inhibitors of SDF-1 and VEGF signaling, either by blocking the appropriate receptors or by inhibiting their downstream signaling. Although these experiments have been performed by other laboratories, these studies would have given us further evidence and confirmation of my hypothesis and model. Second, it is unclear whether there are any synergistic effects between MMP-3 and MMP-9 on NSPC migration and differentiation. To resolve this I have planned treating cells with both MMP-3 and MMP-9 siRNA simultaneously followed by determining the effects of chemokines on adult NSPC differentiation, proliferation, and migration in the absence of both MMPs. However, the problem of the approach is that it would be difficult to distinguish the effect of each MMP because both migration and differentiation are affected by inhibiting individual MMPs in the adult NSPCs. However, due to the interest of publishing the novel finding described in Chapter 6, this work has

not been performed, but is being considered by other members in the laboratory as a new project.

Future Directions

In this thesis, I demonstrated the effects of the molecular environment surrounding NSPCs on their proliferation, migration, and differentiation. Also, I identified a potential novel pathway in which MMPs may have a direct role in determining NSPC fate. This initial observation has provided important leads for future studies. Based on my work and literature, as summarized in Figure 7.1, MMP-3 and -9 up-regulation in response to SDF1 could have multiple effects on NSPCs.

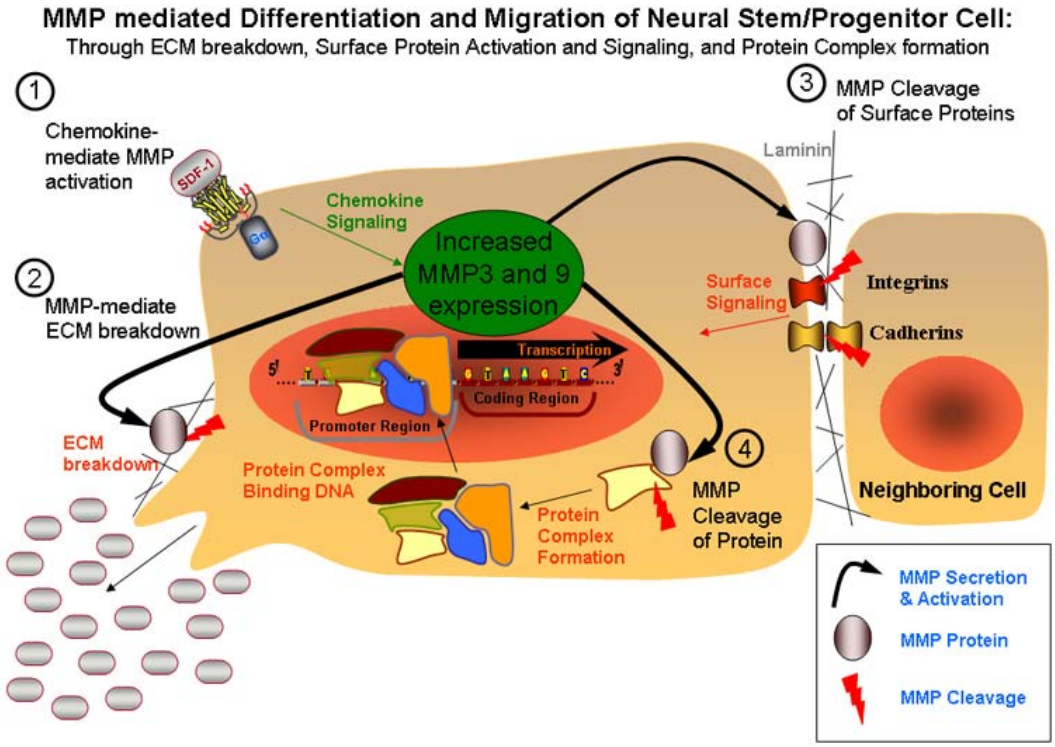


Figure 7.1: Model of the role of MMP in adult NSPC migration and differentiation. **1)** Extrac1, signal the activation of MMP-3 and -9. **2)** MMP is secreted locally to promote the breakdown of the ECM and drive the migration and differentiation of the NSPC toward the concentration gradient of chemokines. **3)** MMP cleaves surface proteins, such as integrins and cadherins, to simulate the migration and differentiation of NSPCs. **4)** A possible novel role of MMP3 and MMP9 in the cleavage of intracellular proteins to promote the formation of transcription complexes and driving the expression of specific genes necessary for the migration and differentiation of NSPCs.

To expand this work, it will be important to investigate the novel pathway underlying how MMPs regulate NSPC neural differentiation in response to SDF1 and VEGF. Aiming for this goal, I began to investigate the potential downstream targets affected by the acute knockdown of MMP-3 and -9 in NSPCs during neuronal differentiation (data shown in Figure 6.4). I used quantitative RT-PCR gene arrays to investigate which of the specific pathways related to NSPC differentiation are altered in the absence of MMP3 and MMP9, in response to SDF-1 (Appendix C.1). The data suggest that several genes involved in neuronal differentiation and neuronal phenotype are expressed at lower levels when either MMP-3 or -9 were knocked-down through siRNA silencing compared to the control non-silencing siRNA condition. This suggests that MMP-3 and -9 could directly or indirectly regulate the expression of neuronal genes, such as Neuregulin 1 (Nrg1), Paired Box gene 6 (Pax 6), Sonic Hedgehog (SHH), and Neurogenic differentiation 1 (NeuroD1) in the response of chemokine treatment (Appendix C.1). The next step is to identify the indirect or direct pathway by which MMP activity regulates the expression of these genes. One known pathway by which MMP activation can regulate NSPC neuronal differentiation is the cleavage of extracellular cell-cell contact molecules, such as cadherins that in turn signal the release of β-catenin into the nucleus for gene transcription (Figure 7.1.3). However, this pathway is an indirect mechanism that MMPs can regulate neuronal gene expression through the cleavage of extracellular molecules to activate intercellular signaling. MMPs could act through a more direct but unknown pathway that can regulate the expression of these genes. For example, a protease Cathepsin L has been shown to regulate gene expression by degradation of histone

proteins, which revealed a new mechanism through which protease could be involved in cellular gene regulation (Duncan et al., 2008). Thus, MMPs may have a similar mechanism in regulating pathways of neuronal differentiation. Recently, more evidence has demonstrated that MMPs are expressed in the cytoplasm of cells (Choi et al., 2008; Golubkov et al., 2005; Luo et al., 2002; Yamaguchi et al., 2005). Although there is no definitive evidence to date that demonstrates intracellular MMPs have protease activity in the brain or within stem cells, intercellular MMP activities have been found to account for acute myocardial dysfunction (Wang et al., 2002b) or after ischemia injury (Deschamps et al., 2005). Recent studies have shown that MMPs are also present in the nucleus of cardiac myocytes and MMP-3 could function as a DNA binding proteins and regulates gene transcription (Eguchi et al., 2008; Kwan et al., 2004). Thus, experiments described here may suggest a potential role of MMPs in the cleavage of proteins intracellular to regulate the expression of genes of neuronal differentiation in response to their environment. A possible mechanism is that MMP-3 or MMP-9 may cleave proteins, such as transcription factors, and change the configuration of these proteins allowing them to interact with protein complexes or histone proteins to activate DNA transcription (Figure 7.1.4). However, since this is a new area of stem cell research, further experiments are needed to determine both direct and indirect roles of MMPs in regulating the neuronal differentiation of adult NSPCs in the response to SDF-1 and VEGF. Consequently, this question is currently being addressed by a postdoctoral fellow in the laboratory to pursue this area of research.

This thesis has also guided a potential project that focuses on the role of MMPs in regulating differentiation and migration in vivo within normal and injured brains. As

described above, a major question I planned to address is whether the differentiation and migration of adult NSPCs are coupled in response to SDF-1 stimulation. Based on pervious literature, it has been demonstrated that genes for neuroblast migration are turned on simultaneously with genes of differentiation (Ge et al., 2006). Evidence from the gene pathway array results suggest that after knockdown of MMP-3 and -9 in adult NSPCs exposed to SDF-1 fail to express both genes involved in neural differentiation and genes associated with cell migration, such as Fez1, Nrp2, and Rac1 (Appendix C.1). To address this question, I have planned experiments to use siRNA knockdown of MMP-3 and -9 in the SVZ to monitor neuroblast migration and differentiation *in vivo* within the RMS and into the olfactory bulb. My preliminary data has indicated that knockdown of either MMP-3 or MMP-9 impairs neuroblast migration into the RMS (see Appendix C.3). However, there was a lack of consistency between several experiments because variation of DNA transfection. In addition, the harshness of the method (electroporation in the adult SVZ brain) used in the procedure led to high mortality of animals after surgery. Therefore, I moved on to a more familiar method to our laboratory which is to use viral delivery of siRNA into the brain (lenti-virus injections to flood the lateral ventricle or directly into the SVZ). Both experiments are feasible, however the number of lentivirus infected adult NSPCs was very small (data not shown) and the lentivirus also lacked specificity for targeting proliferating cells. Therefore, to target specifically for adult NSPCS, the next plan was to deliver retrovirus (infects only dividing cells) that carries the MMP siRNA into the lateral ventricle or the SVZ. This should provide the specificity and number of cells that we seek to target in the brain. I anticipate that both

differentiation and migration will be drastically impaired upon acute MMP-3 or MMP-9 knockdown in both normal and injured brains.

Scientific Impact

The identity of adult NSPCs is distinguished not only by its gene expression profile and differentiation patterns, but also by the ability of their progeny to incorporate into the existing network. Therefore, neuronal migration is a critical step that the brain needs to precisely regulate both in the embryo and in adult, and in healthy and in injured brains. The knowledge in NSPCs migration is largely derived from the study of embryonic neuronal migration. Some of the factors that regulate new neurons in development are shared by the adult brain in regulating NSPC migration, such as the SDF-1α and DCX, as described above. A direct comparison between embryonic and adult NSPC migration might significantly advance our knowledge in identifying the molecular mechanism of NSPC migration during normal and stroke-induced neurogenesis in adult brains. The roles of extracellular protease in injury-induced neuronal migration have only recently caught attention. Questions remain how these proteases mediate migration and differentiation, and whether differentiation and migration are mechanistically coupled. New advances in both knowledge and technologies will certainly provide more insight in this important and exciting process.

As we have described above, the ability of both transplanted embryonic stem cells and adult endogenous progenitor cells to differentiate into major neural cell types of the CNS, has provided a promising outlook for using stem cells to repair injured adult brains. Consequently, understanding the molecular basis of stem cell plasticity will lead to more effective therapeutic treatment for injuries such as ischemic stroke. However, several

critical questions remain concerning cell-based therapies before stem cell treatment can be explored for human patients. Past research in stroke-induced neurogenesis has left us with new challenges that must be answered to understand adult NSPCs potential in brain repair, such as how to precisely control the proliferation, differentiation and migration of transplanted or endogenous stem cells. In addition, several important issues remain to be resolved, such as the source of stem cells, the administration route of stem cells, the combination of trophic factors, the immunosuppression method to prevent graft rejection, and the prevention of tumor formation. To advance our knowledge in these areas, it is critical that a wide range of cell types, from embryonic stem cells to adult-derived cells, are studied. Furthermore, translating studies performed in animals to human patients is a critical step in therapeutic development. Investigators are currently studying many methods to determine the possibilities and limitations of pluripotent stem cells, such as cell injections, viral methods, micropumps, or even manipulating stem cells to deliver transgenes. Other methods are needed to investigate whether regional or global transplantation of cells is more suitable, and to assess the proper selection or cocktail of transgenes to manipulate the differentiation, migration and survival of NSPCs. Knowledge of the molecular mechanisms regulating all stem cell types are required to optimize their responses to the environment into which they will be introduced, and therefore the best possible method could be administered to repair damaged CNS.

Understanding the molecular basis of stem cell plasticity may result in more efficacious therapeutic techniques to promote CNS repair after an ischemic stroke. The work completed here has provided knowledge of the fundamental aspects of endogenous MMP in the role of NSPC differentiation and migration in response to the extrinsic

injury-induced chemokines. For example, the expression of MMPs in different types of stem cells, such as hematopoietic, neural, and embryonic stem cells may represent a stem cell signature. Recognition of the variations in these cells may lead stem cell research to understand how to manipulate adult stem cells that are limited in expanding in culture to increase the amount of cells for transplantation or to control endogenous cell for a more timely and quantitative response to repair. Furthermore, this might lead to more efficient utility of other sources of somatic stem cells for CNS repair. For example, mesenchymal stem cells, which are readily available from patients themselves, currently have been found to have a very low potential for trans-differentiation into neural cells. In summary, this dissertation research can help understand a part of the basic mechanisms that regulate stem cells for maintenance in tissue homeostesis, during development or adulthood, and for adapting to an environment after brain insult.

APPENDIX A

Chapter 5 Supplementary Tables

Table A.5.1: Genes that are expressed at higher levels in neurogenesis-promoting astrocytes

Gene Symbol	Gene Description	dChip Fold Change* (Range)	Drop Confidence# (Range)	Detected by Affymetrix Probe Sets &
Fabp7	fatty acid binding protein 7, brain	16.62	98%	U02096_at
MMP-9	matrix metalloproteinase 9 (gelatinase B)	16.27	100%	U24441_at
Tf	Transferrin	12.34 - 18.98	99.8% - 100.0%	U31866_g_at, *D38380_g_at*
Cxcl10	chemokine (CXC motif) ligand 10	15.3	100%	U17035_s_at
Best5	Best5 protein	9.97 - 17.19	98.3% - 100.0%	*Y07704_g_at, Y07704_at*
Cd74	CD74 antigen	7.79 - 17.08	98.3% - 99.0%	*X13044_at, X13044_g_at*
GFAP	glial fibrillary acidic protein	7.75 - 15.49	99.6% - 99.6%	AF028784mRNA#1_s_at, *AF028784cds#1_s_at*
MMP-12	matrix metalloproteinase 12	9.49	99%	X98517_at
Ccl3	chemokine (CC motif) ligand 3	8.55	100%	U22414_at
Cxcl2	chemokine (CXC motif) ligand 2	8.29	100%	U45965_at
IL-1β	interleukin 1 beta	5.31 - 10.36	99.6% - 100.0%	M98820_at, M98820_g_at, E01884cds_s_at
Ass	arginosuccinate synthetase	7.15	100%	X12459_at
VCAM-1	vascular cell adhesion molecule 1	6.11 - 7.14	99.9% - 100.0%	X63722cds_s_at, *M84488_at*
Serpine1	serine (or cysteine) proteinase inhibitor, member 1	5.62	100%	M24067_at
Gch	GTP cyclohydrolase 1	3.78 - 7.16	71.5% - 99.5%	M58364_at, *E03424cds_s_at*
Enpp2	ectonucleotide pyrophosphatase/ phosphodiesterase 2	5.06 - 5.79	99.6% - 99.6%	*D28560_at, D28560_g_at*
Lyz	lysozyme	5.28	87%	rc_AA892775_at
IRF-7	interferon regulatory factor 7	4.76 - 5.54	97.2% - 100.0%	*rc_AA799861_at, rc_AA799861_g_at*
CatheS	cathepsin S	4.84	94%	L03201_at
RT1.S3	MHC class Ib RT1.S3	4.81 - 4.83	99.7% - 99.9%	*rc_AI235890_s_at, AF029240_at*
Olr1	oxidised low density lipoprotein (lectin-like) receptor 1	1.41 - 7.76	98.1% - 99.2%	AB005900_at, rc_AI071531_s_at
Lgals5	lectin, galactose binding, soluble 5	3.88	100%	L21711_s_at
Ckb	creatine kinase, brain	3.5 - 4.16	96.9% - 97.2%	M57664_at, M57664_g_at
Cdh2	cadherin 2	1.92 - 5.2	71.5% - 98.9%	AF097593_g_at, *AF097593_at*
IL-6	interleukin 6	1.66 - 5.01	90.2% - 100.0%	M26745cds_s_at, *M26744_at*
Mx2	myxovirus (influenza virus) resistance 2	3.1	100%	X52713_at
RT1-M3	RT1 class Ib, locus M3	3.07	97%	U16025_g_at
Lgals9	lectin, galactose binding, soluble 9	3.03	98%	U72741_g_at
Ndrg2	N-myc downstream-regulated gene 2	2.89	97%	rc_AA799560_at
Itga7	integrin alpha 7	1.69 - 3.77	96.9% - 99.6%	X74293_s_at, X65036_at, X65036_g_at
Argbp2	Arg/Abl-interacting protein 2	2.3 - 2.98	21.9% - 99.2%	rc_AA891194_s_at, *AF026505_at*
Atp1b1	ATPase, Na+/K+ transporting, beta 1 polypeptide	1.64 - 3.04	99.3% - 99.9%	rc_AI230614_s_at, rc_AI112173_at
Ler3	immediate early response 3	2.11	92%	X96437mRNA_at
Rtn1	reticulon 1	1.21 - 2.95	19.0% - 62.2%	*U17604_at, X52817cds_s_at*
---	Unknown EST	1.67	100%	rc_AA891797_at
C1qb	complement component 1, q subcomponent, beta polypeptide	1.2 - 1.28	2.2% - 2.8%	*X71127_g_at, X71127_at*
Nrg1	neuregulin 1	1.86 - -1.57	0.6% - 60.2%	*U02320_s_at, U02315_g_at, U02322_s_at*

Reference fold changes were obtained by dChip analysis; #, Confidence values were obtained by Drop analysis were indicated; the ranges are the maximum and minimum if multiple probe sets were returned. &, probe sets in italic were identified by only one data analysis software.

Table A.5.2: Genes that are expressed at lower levels in neurogenesis promoting astrocytes

Gene Symbol	Gene Description	dChip Fold Change* (Range)	Drop Confidence# (Range)	Detected by Probe Sets&
Sepp1	selenoprotein P, plasma, 1	-5.77	100%	rc_AI230247_s_at
Fibl3	EGF-containing fibulin-like extracellular matrix protein 1 precursor (Fibulin-3) (T16 protein)	-4.78 - -5.01	99.9% - 99.9%	*D89730_g_at, D89730_at*
Dcn	decorin	-3.54 - -5.42	99.8% - 99.9%	*Z12298cds_s_at, X59859_i_at, X59859_r_at*
Sfrp4	secreted frizzled-related protein 4	-4.21	100%	AF012891_at
Anpep	alanyl (membrane) aminopeptidase	-1.84 - -5.26	25.1% - 97.2%	*AF039890mRNA_s_at, M25073_at*
IGFBP-6	insulin-like growth factor binding protein 6	-2.83	97%	M69055_at
LOC191574	3-alpha-hydroxysteroid dehydrogenase	-1.92 - -3.27	97.8% - 98.3%	D17310_s_at, S35751_f_at
Bst1	bone marrow stromal cell antigen 1	-2.41	100%	D49955_at
Col5a1	Collagen, type V, alpha 1	-1.94 - -2.56	97.5% - 99.6%	*rc_AA859757_g_at, AJ005394_at, rc_AA859757_at*
Maf	v-maf musculoaponeurotic fibrosarcoma (avian) oncogene homolog (c-maf)	-2.17	100%	U56242_at
Sc65	SC65 synaptonemal complex protein	-2.02 - -2.16	97.2% - 100.0%	*X65454_at, X65454_g_at*
---	Unknown EST	-2.02	97%	rc_AA893180_at
FGFR1	Fibroblast growth factor receptor1	-1.96 - -2.03	91.1% - 99.9%	*D12498_s_at, S54008_i_at*
---	Similar to cDNA sequence BC019776	-1.84	99%	rc_AI639012_at
---	Unknown EST	-1.83	100%	rc_AA893082_at
Npr2	natriuretic peptide receptor 2	-1.46	-95%	M26896_at
---	Unknown EST	-1.38	93%	rc_AA799299_at

Reference fold changes were obtained by dChip analysis; #, Confidence values were obtained by Drop analysis were indicated; the ranges are the maximum and minimum if multiple probe sets were returned. &, probe sets in italic were identified by only one data analysis software.

APPENDIX B

Chapter 6 Supplementary Figures

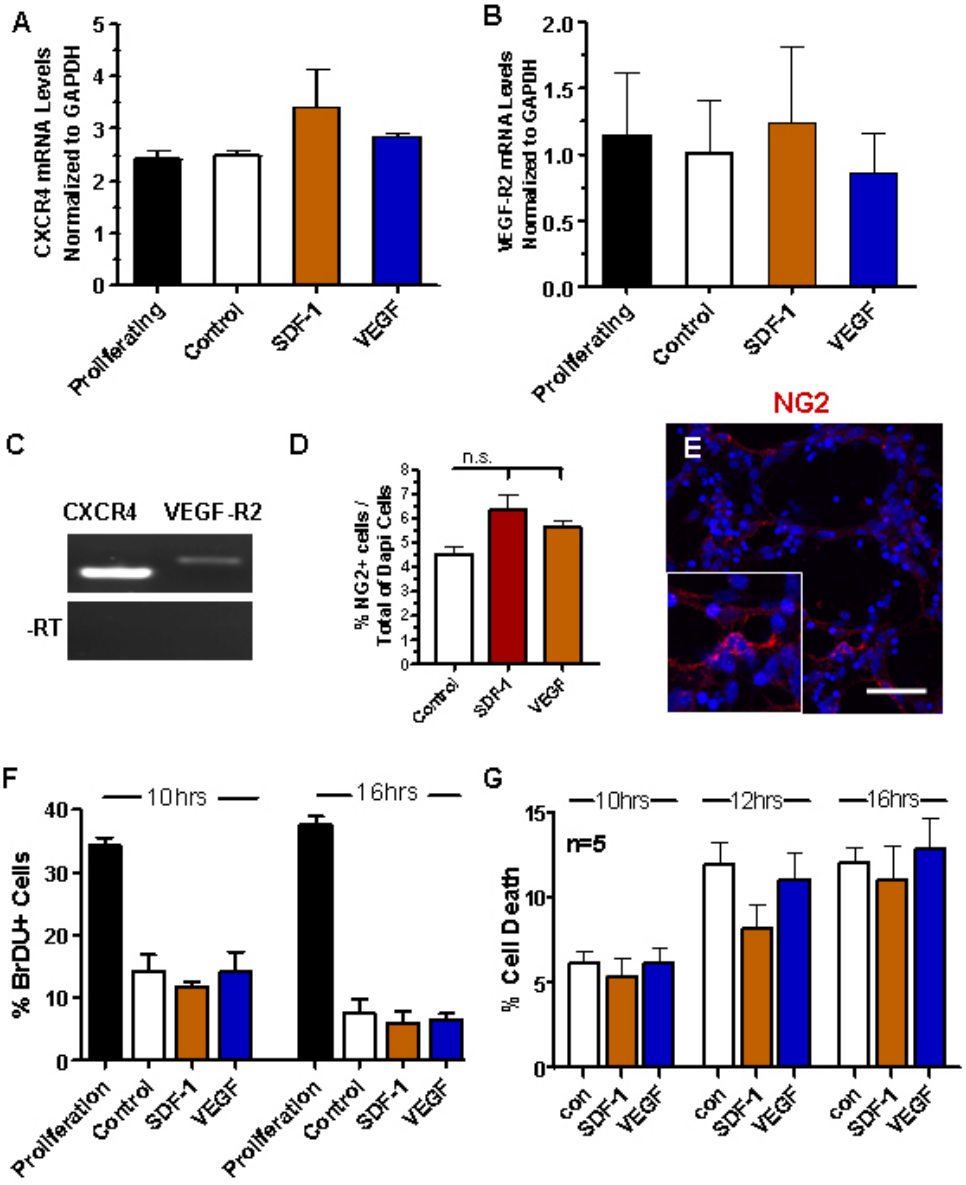


Figure B.6.1: SDF-1 and VEGF do not affect adult NSPC proliferation or survival and do not affect oligodendrocyte differentiation. (A, B) qPCR analysis of cultured NSPCs demonstrated that **(A)** CXCR4 or **(B)** VEGF-R2 did not show differential gene expression across experimental conditions (n = 3). **(C)** Standard PCR representing expression of CXCR4 and VEGF-R2 in adult NSPCs cultured under proliferating conditions. Negative control, no reverse transcriptase (-RT) was added in cDNA synthesis. **(D)** Neither SDF-1 nor VEGF has a significant effect on oligodendrocyte differentiation of adult NSPCs after 16 hours of treatment (n = 3). **(E)** A representative image of $NG2^{+}$ oligodendrocytes quantified in (D). Scale bar = 50 μm. **(F)** Proliferation

analysis using BrdU pulse labeling showed no change in cell division when adult NSPCs were treated with either SDF-1 or VEGF compared with the negative control (growth factor withdrawal alone) at 10 or 16 hours. Adult NSPCs grown in the presence of the growth factors FGF-2 and EGF were used as a positive control for BrdU incorporation (n = 3). **(G)** Cell death analysis using propidium iodide permeability showed no change in the percentage of dead cells after either SDF-1 or VEGF treatment compared with the negative control (growth factor withdrawal alone) at 10, 12, and 16 hours (n = 3).

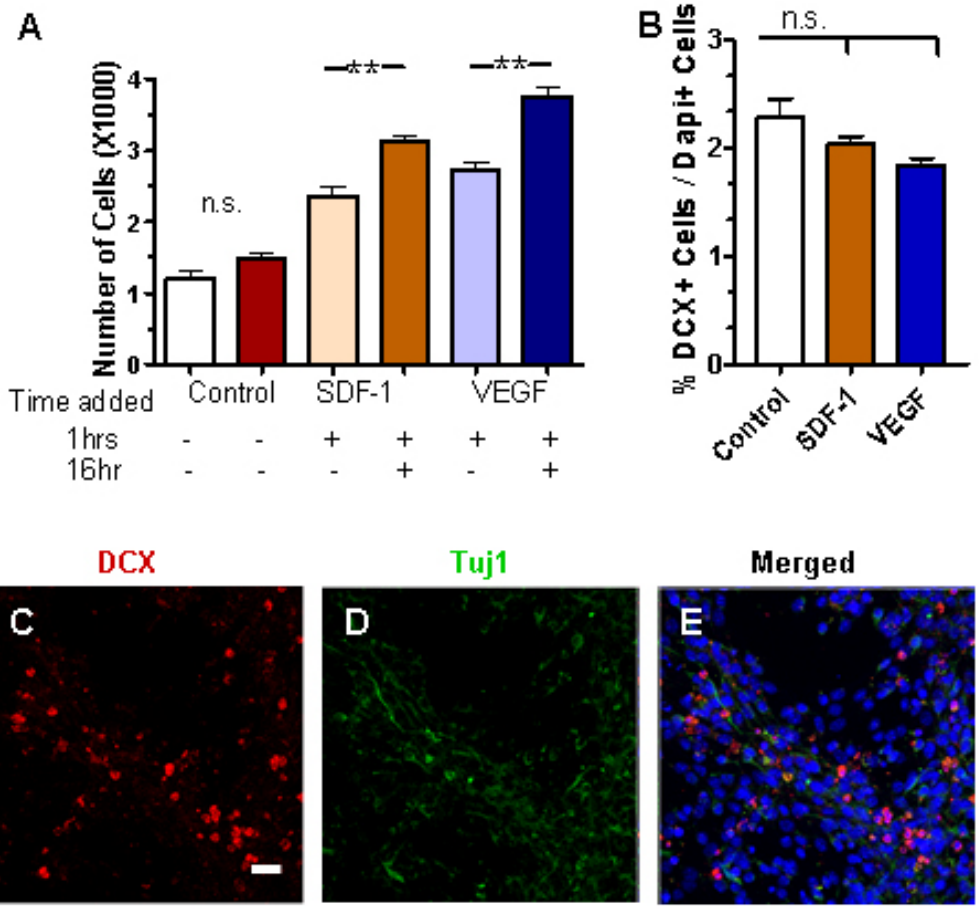


Figure B.6.2: The plateau of cell migration at 16 hours is partially due to chemokine equilibrium. (A) In the cell migration assay, administering second doses of SDF-1 or VEGF at 16-hours post-plating resulted in significantly more cells migrated to the bottom chamber, compared with only one application of chemokines at 1 hour post-plating. ** $p < 0.01$ **(B)** When adult NSPCs were plated onto laminin-coated coverslips, rather than a migration chamber, neither SDF-1 nor VEGF had any effect on the differentiation of adult NSPCs into DCX^+ after 16 hours of treatment (n = 3). **(C-E)** In the same assay as **(B)**, no colocalization of **(C)** DCX^+ and **(D)** $Tuj1^+$ cells was observed after 16 hours of treatment with SDF-1. Scale bar = 20 μm. **(E)** Merged image showing the DCX- and Tuj1-expressing cells.

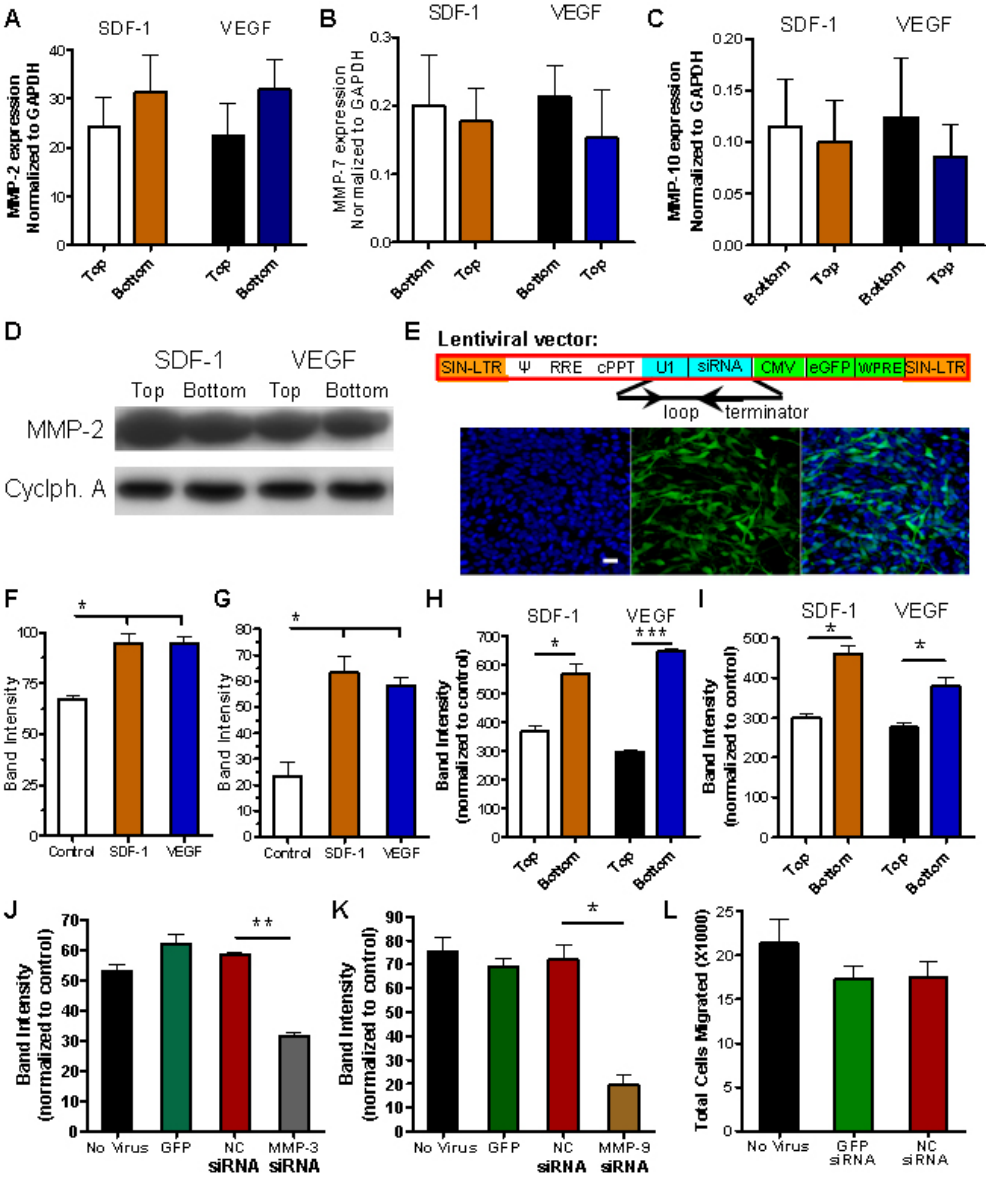


Figure B.6.3: MMP-3 and MMP-9, but not MMP-2, -7, or -10, were differentially expressed in migratory versus stationary cells. (A-C) qPCR analysis demonstrated that mRNA levels of **(A)** MMP-2, **(B)** MMP-7, and **(C)** MMP-10 exhibited no significant difference between migratory and stationary cells (n = 4). **(D)** The protein levels of MMP-2 in the cell lysate of migrating cells (Bottom) were not significantly different compared with that in stationary cells (Top). Cyclophilin A antibody was used for loading control. **(E)** Lentivirus expressing both siRNA and GFP infected (see methods for details) adult NSPCs with good efficiency without apparent adverse effect. Structure of lentiviral vector used to express siRNAs is shown: SIN-LTR, self-inactivating long terminal repeat; Φ, HIV packaging signal; cPPT, central polypurine track; CMV, cytomegalovirus promoter; WRE, woodchuck hepatitis virus response element. Loop, the loop of the siRNA hairpin; terminator, the terminator sequence of siRNA. Representative images of lentiviral NC-siRNA–infected adult NSPCs at 24 hours post-infection. Blue, Dapi; Green, GFP. **(F, G)** Quantification of Western blot band intensities (for Figure 3D)

demonstrates higher protein levels of **(F)** MMP-3 and **(G)** MMP-9 in the culture media of migratory cells compared with stationary cells responding to SDF-1 or VEGF. Equal volume of cell culture medium was loaded onto each lane. **(H, I)** Quantification of Western blot band intensities (for Figure 3E) demonstrates higher protein levels of MMP-3 and MMP-9 in migratory cells compared with stationary cells responding to SDF-1 or VEGF (compared with loading control Cyclophilin A). **(J, K)**. Quantification of Western blot band intensities (for Figure 3I, J) demonstrates gene knockdown efficiency of siRNAs for MMP-3 (J) and MMP-9 (K) (β-actin was used as a loading control). **(L)** The total number of migrated cells was not significantly affected by infection of two different control lentiviruses compared with uninfected adult NSPCs. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

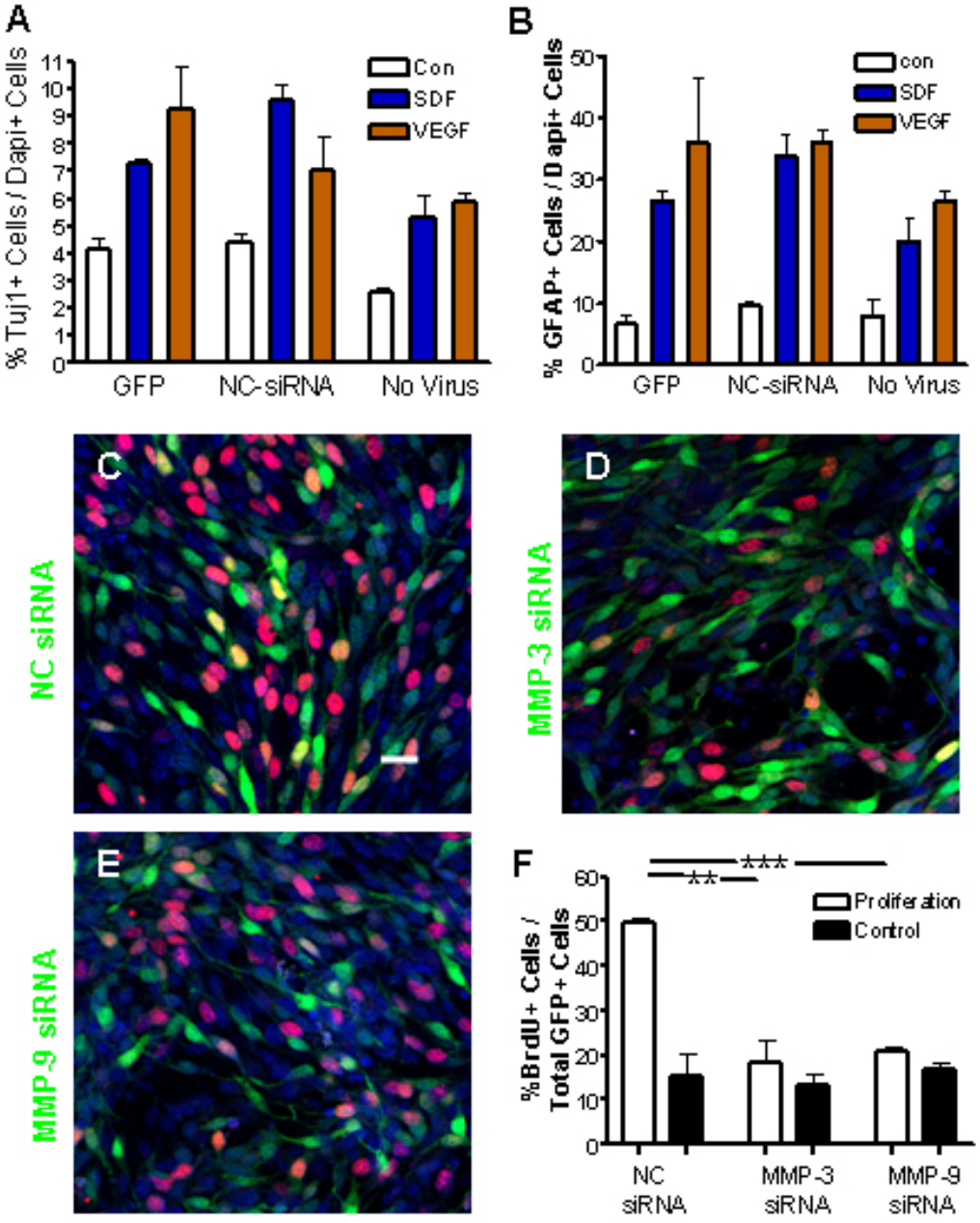


Figure B.6.4: MMP-3 and MMP-9 are important for mitogen-dependent adult NSPC proliferation. (A) Two control lentiviruses, Lenti-GFP and Lenti-NC-siRNA, had a mild effect on the neuronal differentiation of NSPCs. However, the induction of neuronal differentiation by SDF-1 and VEGF was not affected by viral infection compared with uninfected cells, suggesting that this lentivirus system could be used for analyzing the effect of gene knockdown on SDF-1– and VEGF-induced neuronal differentiation. **(B)** Neither control virus had a significant effect on SDF-1– and VEGF-induced astrocyte differentiation. **(C-F)** Acute knockdown of MMP-3 or MMP-9 using siRNAs affect adult NSPC proliferation. **(C)** Lentivirus-infected adult NSPCs expressing NC-siRNA (green) incorporated BrdU (red) under proliferating conditions. Scale bar = 20 μm **(D, E)** Lentivirus-infected adult NSPCs expressing **(D)** MMP-3 or **(E)** MMP-9–siRNA (green) incorporated BrdU (red) under proliferating conditions. **(F)** Quantification of BrdU+ cells among total GFP^+ cells indicated that MMP-3– or MMP9-siRNA–infected adult NSPCs had reduced proliferation compared with NC-siRNA–infected cells under proliferating conditions (white bars); however, neither siRNA had an effect on cell division in the absence of mitogens (black bars). ** $p < 0.01$ and *** $p < 0.001$.

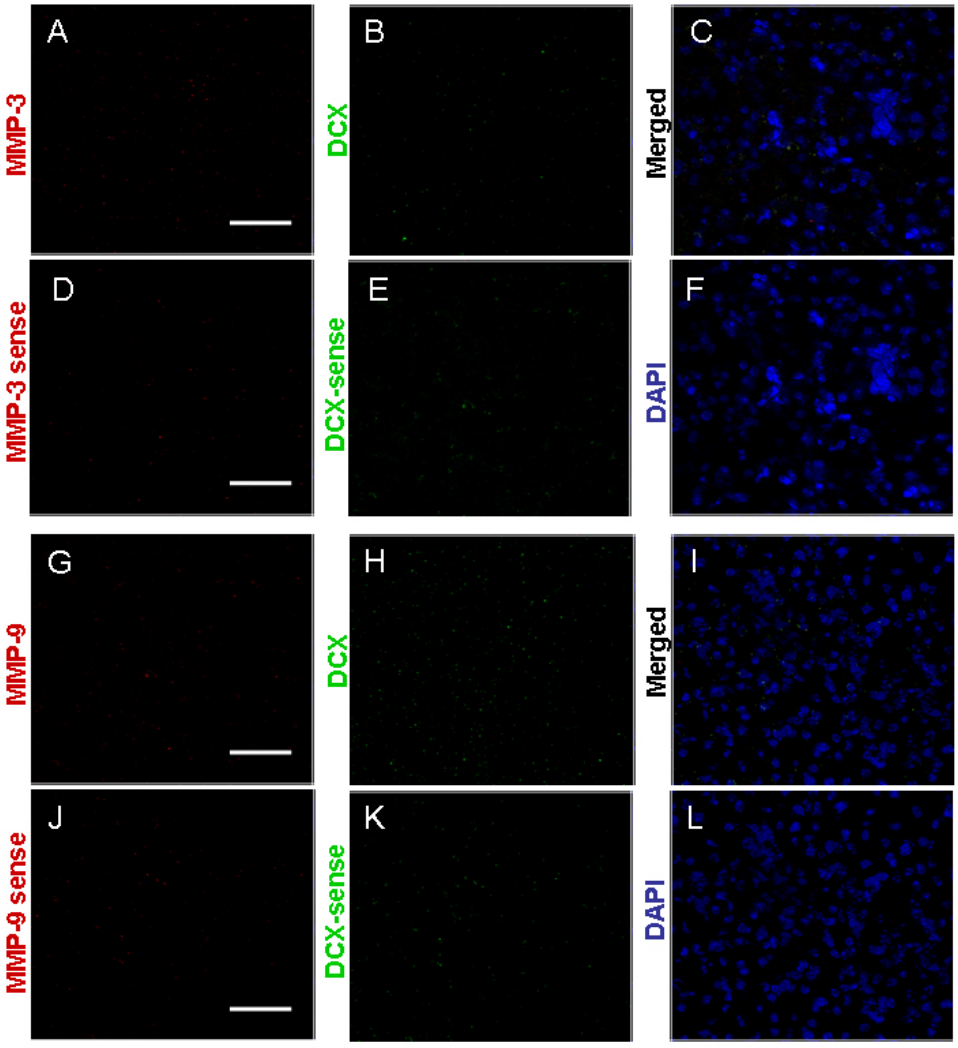


Figure S5: Sense riboprobes did not show detectable signals in either the ipsilateral or contralateral side of the brain. (A-C) Ribroprobes detected no **(A)** MMP-3 mRNA (red) and no **(B)** DCX mRNA (green) expression in the contralateral side after MCAO. **(C)** Merged image (A) and (B). Scale bar = 50 μm. **(D-F)** Sense ribroprobes detected no **(D)** MMP-3 mRNA (red) and no **(E)** DCX mRNA expression in the contralateral side after MCAO. **(F)** Dapi image. Scale bar = 50 μm. **(G-I)** Ribroprobes detected no **(G)** MMP-9 mRNA (red) and no **(H)** DCX mRNA (green) expression in the contralateral side after MCAO. **(I)** Merged image (G) and (H). **(J-L)** Sense ribroprobes detected no **(J)** MMP-9 mRNA (red) and no **(K)** DCX mRNA expression in the contralateral side after MCAO. **(L)** Dapi image. Scale bar = 50 μm.

APPENDIX C

Thesis Supplementary Data

C.1 Quantitative PCR Gene Expression Array

Goal of the experiment: To determine gene expression changes in NSPCs with acute MMP3 or MMP9 siRNA knockdown upon SDF-1 treatment compared to NSPCs infected with lentivirus expressing a non-silencing control siRNA.

Cells: NSPCs (independent culture as triplicates in each condition) were cultured in 6cm petri-dishes, infected with lentivirus twice for 24 hours (to obtain ~100% infection efficiency) in the proliferation conditions (FGF2/EGF). The cells were then changed to medium with SDF-1 (100 ng/ml) in the absence of FGF2/EGF. After 16hours, the cells were collected with trizol and RNA was isolated.

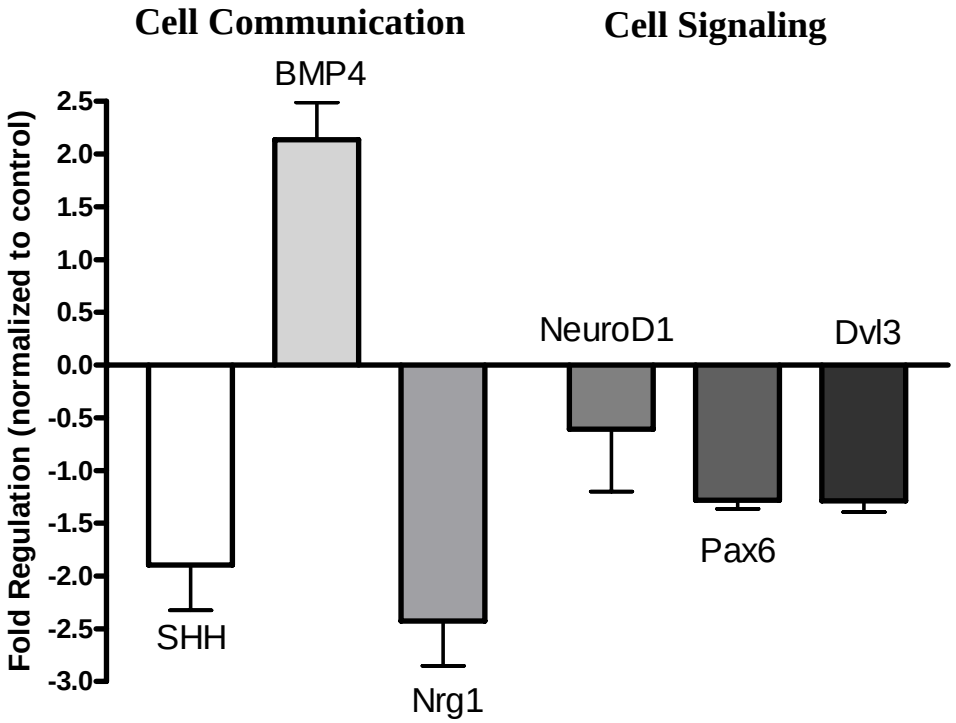
Array: The RNA was isolated by Trizol and chloroform isolation (see methods above). Real-time qPCR was run with 0.5ug of RNAs for each condition (see Table C.1) and loaded in SABioscience 96-well array (Mouse Superarray GEArray Neural Stem Cell and Neurogenesis, 9 total plates) for each condition (repeated three times). Plates were run in the ABI 7300 machine with 40 cycles of 95 degrees for 30 seconds and 60 degrees for one minute. C_t values from the machine were exported to be analyzed with PCR data analysis software.

Table C.1: Mouse Superarray GEArray Neural Stem Cell and Neurogenesis Setup

Sample #	Condition	NSPC Treatment	# of Experiments
1	NC-siRNA	SDF-1	3
2	MMP3-siRNA	SDF-1	3
3	MMP9-siRNA	SDF-1	3

Data analysis: Data was analyzed using the Superarray PCR Array Data Analysis Software (http://www.sabiosciences.com/pcrarraydataanalysis.php).

Figure C.1: Mouse Superarray GEArray Expression Analysis-Fold Regulation compared to control (NC-siRNA).



Others Down-regulated
Mobility and Extracellular molecules

Fez1	Fasciculation and elongation protein zeta 1	Interaction w/ DISC1
Nrp2	Neuropilin 2	Cell migration and axon guidance
Tnr	Tenascin R (restrictin, janusin)	Neuronal differentiation and outgrowth

C.2 Mouse WG-6 Illumina Whole Genome Microarray

Goal of the experiment: To determine gene expression changes in NSPCs with acute MMP3 or MMP9 knockdown upon SDF-1 and VEGF treatment.

Cells: NSPCs (n=1) were cultured in 10cm petri-dishes, infected with lentivirus for 24 hours (to obtain ~100% infection efficiency) in the proliferation conditions (FGF2/EGF). The cells were then changed to medium with no growth factor, SDF-1 or VEGF (at 100ng/ml) in the absence of FGF2/EGF. After 16hours, the cells were collected with trizol.

Array: Illumina microarrays were run with 1ug RNA and were analyzed by Mouse WG-6 Illumina Whole Genome Microarray.

Data analysis: Data was analyzed using BeadStudio Gene Expression Module V3.2 software.

Table C.2: Mouse WG-6 Illumina Whole Genome Microarray Setup

Sample #	Condition	Adult NSPC Treatment
1	Control	NC-siRNA
2	Control	MMP-3 siRNA
3	Control	MMP-9 siRNA
4	SDF-1	NC-siRNA
5	SDF-1	MMP-3 siRNA
6	SDF-1	MMP-9 siRNA
7	VEGF	NC-siRNA
8	VEGF	MMP-3 siRNA
9	VEGF	MMP-9 siRNA

Example of Differential Gene Expression (Relative Expression)-Figures C.2 (n=1)

Figure C.2.1 Nrg1

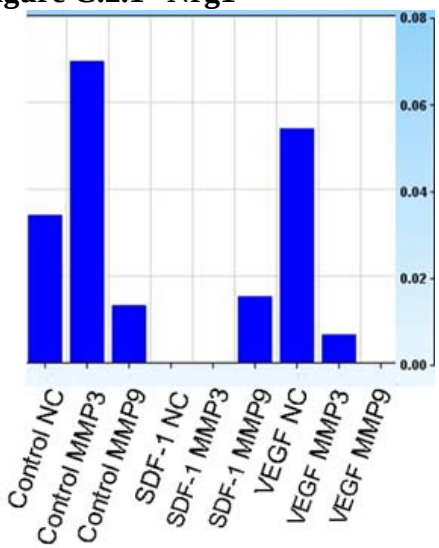


Figure C.2.2 SHH

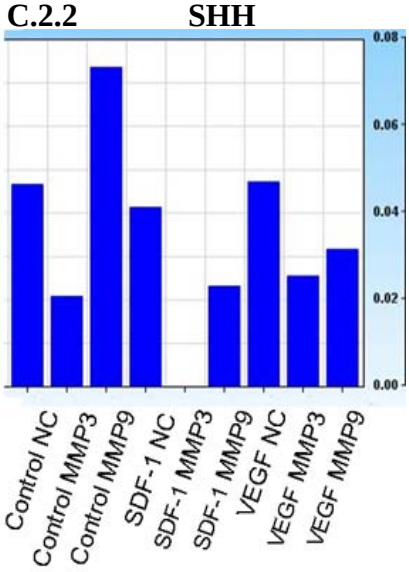


Figure C.2.3 NeuroD1

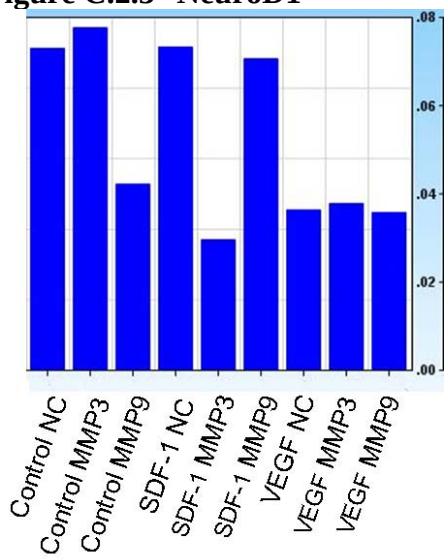
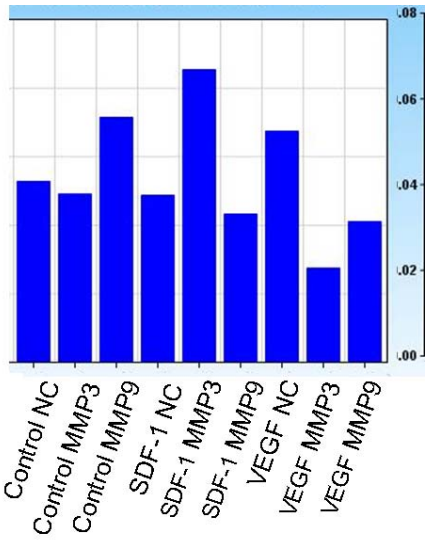


Figure C.2.4 Pax6



C.3 In Vivo Gene Delivery

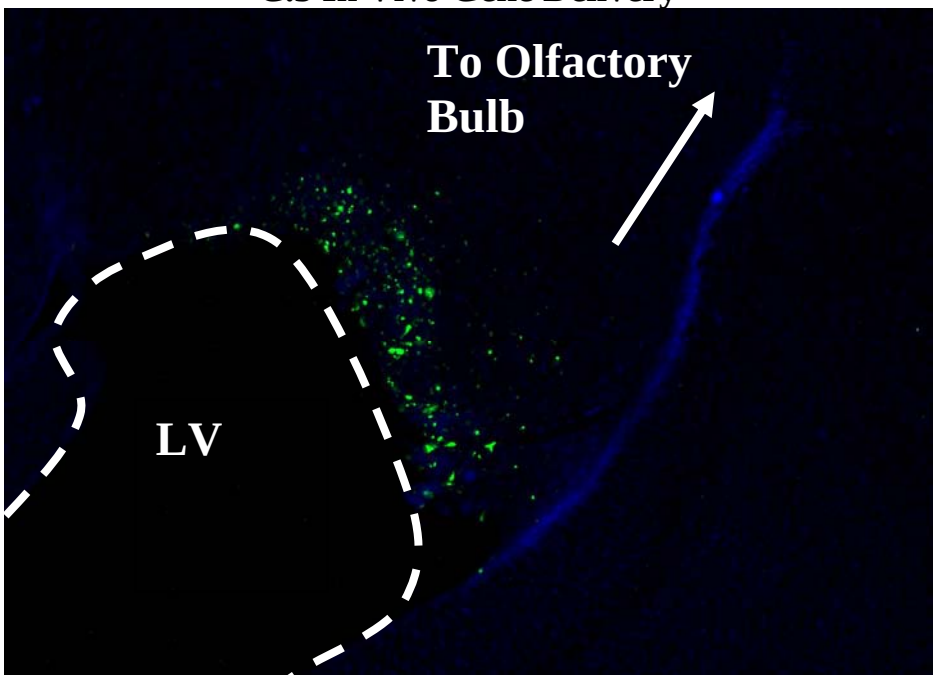


Figure C.3.1: NC-siRNA plasmid injected into the Lateral Ventricle (LV) of adult mice and electroporated in the direction of the SVZ.

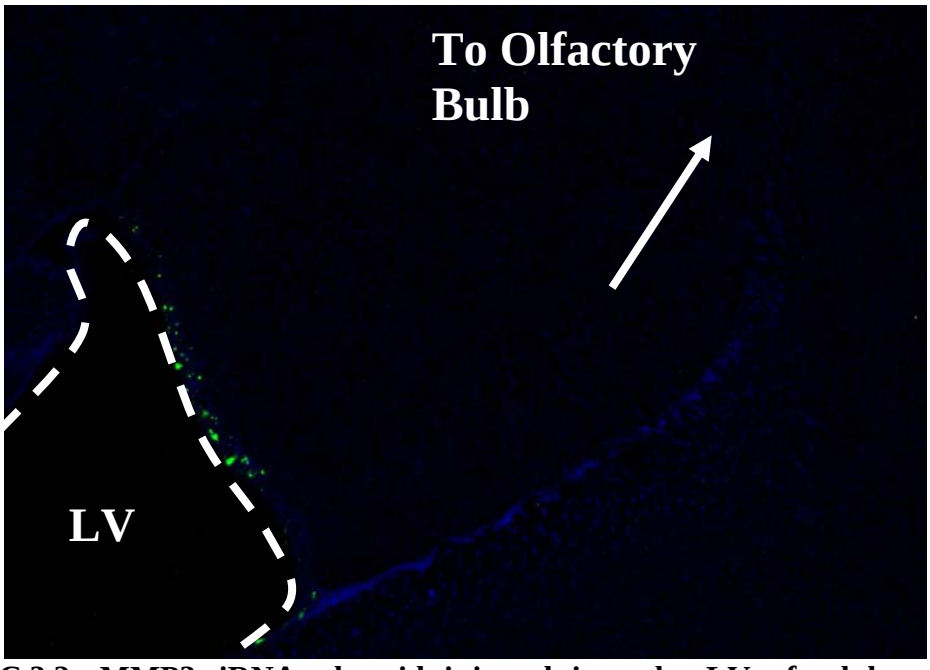


Figure C.3.2: MMP3-siRNA plasmid injected into the LV of adult mice and electroporated in the direction of the SVZ.

Images show saggital sections with electroporated plasmid expressing GFP (green) and all cells stained with Dapi (blue). 20ug of DNA was injected in the LV. Electroporated with the Nugene CUY21EDIT Square Wave Electroporator, with 5 pulses at 200V for 50 milliseconds. Animals were sacrificed one week after electroporation.

LIST OF ABBERVATIONS

ApoER – Apolipoprotein E Receptor

bFGF – basic Fibroblast Growth Factor

bHLH – basic Helix-Loop-Helix

BBB – Blood-Brain Barrier

BDNF – Brain-Derived Neurtrophic Factor

BMP – Bone Morphogenetic Protein

BLBP – Brain Lipid Binding Protein

CAM – Cadherin Adhesion Molecule

CC R(L) – C-C motif Receptor (Ligand)

CNS – Central Nervous System

Cx – Connexin

CXC R(L) – C-X-C motif Receptor (Ligand)

DCX – Doublecortin

DG –Dentate Gyrus

Dlx – Distal-Less homeobox

ECM – Extracellular Matrix

EGF – Epithelial Growth Factor

ELK – Ets Like Kinase

EPO – Erythropoietin

ES cells – Embryonic Stem cells

FISH – Fluorescence in situ hybridization

FAK – Focal Adhesion Kinase

GDNF – Glial cell-Derived Neurotrophic Factor

GFAP – Glial Fibrillary Acidic Protein

GPCR – G-Protein Coupled Receptor

GTP – Guanine Triphosphate

Hes – Hairy/Enhancer of Split

HMGA – High Mobility Group AT-hook

HSC – Hematopoietic Stem Cells

IGF – Insulin-like Growth Factor

IL – Interleukin

LIF – Leukemia Inhibitory Factor

LTB4 – Leukotriene B4

MAPK – Mitogen-Activated Protein Kinase

MCAO – Middle Cerebral Artery Occlusion

MCP – Monocyte Chemoattractant Protein

MIP – Macrophage Inflammatory Protein

miR – microRNA

MMP – Matrix Metalloproteinase

MRI – Magnetic Resonance Imaging

NeuN – Neuronal Nuclei

NF-κB – nuclear factor kappa B

Ngn – Neurogenin

NSPC – Neural Stem/Progenitor Cells

PAK – p21-Activated Protein Kinases

PKB (C) – Protein Kinase B (C)

RA – Rectinoic Acid

Rac1 – Ras-related C3 botulinum toxin substrate 1

Rho – Ras homolog gene family

RMS – Rostral Migratory Stream

RT-PCR – Real-Time Quantitative PCR

SDF – Stromal Derived Factor

SCF – Stem Cell Factor

SGZ – Subgranular Zone

siRNA – small interfering RNA

SVZ – Subvernticular Zone

TIMP – Tissue Inhibitors of Matrix Matalloproteinase

TGF – Transforming Growth Factor

TNF – Tumor Necrosis Factor

VEGF (R) – Vascular Endothelial Growth Factor (Receptor)

Vldlr – Very Low Density Lipoprotein Receptor

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