Chapter 10
The Combination of Stem Cell Factor (SCF) and Granulocyte-Colony Stimulating Factor (G-CSF) in Repairing the Brain Post-acute Stroke

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Abstract Stroke represents the leading cause of long-term disability in adults worldwide. Most stroke survivors suffer from lifelong neurological deficits. Developing a pharmaceutical approach to enhance brain repair and improve functional outcomes post-acute stroke is a very important but less investigated area in stroke research. Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are the well-characterized vital hematopoietic growth factors for regulating hematopoiesis. Increasing evidence supports that SCF and G-CSF also play roles in the nervous system. Over the past decade, preclinical studies have demonstrated that SCF in combination with G-CSF synergistically enhances stroke recovery in the subacute or chronic phase. In this chapter, we have reviewed the biological function of SCF and G-CSF in hematopoiesis, neural plasticity, and neurogenesis, and summarized the preclinical studies illustrating the neurorestorative effects of SCF and G-CSF post-acute stroke.

Keywords Stem cell factor • Granulocyte-colony stimulating factor • Brain repair • Stroke • Subacute phase • Chronic phase

Abbreviations

AD	Alzheimer's disease
BBB	Blood-brain barrier
BDA	Biotinylated dextran amine

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BDNF	Brain-derived neurotrophic factor
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leucoencephalopathy
CNS	Central nervous system
CSF	Colony stimulating factor
CXCR4	C-X-C chemokine receptor type 4
DRGs	Dorsal root ganglia neurons
ECs	Endothelial cells
G-CSF	Granulocyte-colony stimulating factor
GCSFR	G-CSF receptor
GM-CSF	Granulocyte macrophage-colony stimulating factor
HPCs	Hematopoietic progenitor cells
HSCs	Hematopoietic stem cells
LTP	Long-term potential
NSCs/NPCs	Neural stem/progenitor cells
PPF	Paired-pulse facilitation
SCF	Stem cell factor
SDF-1	Stromal cell-derived factor 1
SGZ	Subgranular zone
SHRs	Spontaneous hypertensive rats
Sl	Steel gene
SVZ	Subventricular zone
tPA	Tissue plasminogen activator
U-type spines	Uncertain type spines
W	White-spotting gene
YFP	Yellow fluorescent protein

1 Introduction

Stroke remains the leading cause of long-term disability in adults worldwide [1, 2]. Stroke not only represents a serious medical condition but it also causes huge medical and financial burdens throughout the world [1–3].

A stroke has three clinical phases: the acute phase, subacute phase, and chronic phase. The exact time frame of these three phases varies among individuals as the duration of the three phases is dependent upon the size and location of the infarcts, the responsive capacity of cerebrovascular collateral circulation, the metabolic state of brain tissue, and patient's age and medical comorbidities. In general, the acute phase is the first 48 h after stroke symptom onset, the subacute phase represents the period from 48 h up to 3 or 6 months post-stroke, whereas the chronic phase starts 3 or 6 months after stroke [4–10].

Currently, there are only two therapeutic approaches available for stroke patients. The *first one* is the thrombolytic/thrombectomy treatment for ischemic stroke

patients in the acute phase. The therapeutic time windows for thrombolytic/thrombectomy approach are limited up to 4.5 h post-stroke for thrombolysis by tissue plasminogen activator (tPA) [11, 12] and within 6–8 h post-stroke onset for the thrombectomy [13–15]. The *other* treatment for stroke patients is physical therapy. The therapeutic window for physical therapy is restricted to the first 6 months post-stroke [16–18]. Due to the narrow time window and intracerebral hemorrhage risk of the thrombolytic/thrombectomy treatment [11, 13, 19], the majority of stroke patients are not able to receive this treatment in the acute phase [20]. In addition, many stroke survivors do not receive or complete the physical therapy post-acute stroke because of financial or family-related issues. Developing new therapeutic strategies, therefore, is highly important to reduce stroke-induced disability and enhance stroke recovery.

Over the past two decades, the vast majority of stroke research has targeted the neuroprotection in the acute phase, and little attention has been paid to enhancing stroke recovery in the subacute or chronic phase of stroke. In fact, the neuroprotective agents have all failed in clinical trials [21]. Searching for therapeutic approaches to improve stroke recovery post-acute phase becomes highly recognized in the stroke research field today.

Stem cell factor (SCF) and granulocyte-colony stimulating factor (G-CSF) are the essential hematopoietic growth factors that critically regulate hematopoiesis [22–24]. Beside their roles in the hematopoietic systems, a large body of evidence shows that SCF and G-CSF also play roles in the nervous system. SCF and G-CSF do not only promote neural plasticity [25–30] and neurogenesis [31–33], but they can also enhance brain repair in both the subacute [34] and chronic phases [35–41].

In this chapter, we have reviewed the discovery of SCF and G-CSF in the hematopoietic system and current knowledge concerning the biological function of SCF and G-CSF in hematopoiesis, highlighted the studies demonstrating the effects of SCF and G-CSF in promoting neural plasticity and neurogenesis, and summarized up-to-date research progress regarding the effective and mechanistic determinations of SCF and G-CSF on brain repair in the subacute and chronic phases of experimental stroke.

2 The Discovery and Essential Role of SCF and G-CSF in the Hematopoietic System

SCF and G-CSF are the hematopoietic growth factors that are critically involved in regulation of blood cell production and mobilization of bone marrow stem cells. Since the discovery of SCF and G-CSF, great effort has been made to elucidate their biological function. Over the past six decades, there have been many breakthroughs in understanding the mechanisms underlying SCF- and G-CSF-regulated hematopoiesis and in developing potential therapies for using SCF and G-CSF in clinical trials. In this section, we have summarized the current understanding of the essential role of SCF and G-CSF in the hematopoietic system.

The discovery of SCF (also known as kit ligand, steel factors and mast cell growth factor) and its receptor c-kit took place in 1990s [42]. Observations of white spots on a few mice among thousands of laboratory mice lead to the identification for the loci of steel (Sl) and white-spotting (W), which encode SCF and c-kit receptor, respectively [43]. Mutations at either of these two loci result in similar phenotypes with coat color alterations, anemia, and lack of mast cells in the tissue and neonatal mortality [44]. These findings offer critical information concerning the *in vivo* function of SCF and c-kit, and highlighting its important roles in hematopoiesis, melanogenesis and fertility. In addition, it has been demonstrated that the W mutation-induced c-kit dysfunction affects hematopoietic stem cells and hematopoietic progenitor cells (HSCs/HPCs), while the Sl mutation impairs stromal cell function. These findings are in accordance with the *in vitro* study that was reported in 1977 [45]. In this *in vitro* study, Dexter and Moore demonstrated the stromal-dependent hematopoietic cells culture, and proposed that Sl and W encoded a ligand-receptor pair.

Many studies have revealed that there are two forms of natural SCF due to alternative splicing of the DNA transcripts. A shorter form consists of 220 amino acids and produces a membrane-bound form of SCF. The soluble SCF, which consists of 165 amino acids, is derived from a full length 248 amino acids cleaved in the extracellular domain [46]. Both the soluble and membrane-bound forms of SCF are biologically active. However, the two forms of SCF have distinct but overlapping roles [47]. Membrane-bound SCF is expressed on stromal cells, endothelial cells (ECs) and fibroblasts in the bone marrow and induces more persistent tyrosine kinase activation than soluble SCF [48]. In 1991, Brannan and colleagues reported that Steel-Dickie mice exhibited anemia, pigmentation and germ cell defects as these mice only produced soluble SCF due to genome deletion affecting the transmembrane and cytoplasmic domain [49]. These research findings suggest that membrane-bound SCF plays a unique biological role in the stromal cells, ECs and fibroblasts in the bone marrow. C-kit is expressed on normal hematopoietic cells and several other cell types, including mast cells [50], melanocytes [51] and a wide range of non-hematopoietic cell types as ECs [52], interstitial cells [53] and astrocytes [43]. Interaction between SCF and c-kit is the initial and key step for triggering the downstream signaling. It has been shown that the SCF/c-kit system has an important function not only in mouse but also in humans and other primates due to its pleiotropic effects on hematopoietic cell survival, proliferation, differentiation and mobilization [54]. SCF acts directly on HSCs/HPCs, promotes HSC/HPC entry to the cell cycle, and facilitates HSC/HPC proliferation [55]. SCF enhances the primitive HSC survival by suppression of apoptosis [56, 57]. In 1992, Valent and colleagues reported that SCF induced mast cell development from immature hematopoietic cells in human bone marrow [58], suggesting the effect of SCF on cell differentiation. This study was also confirmed by Irani and colleagues, who observed similar results in human fetal liver [59]. In addition, SCF is also a potent agent for mobilization of murine and human HSCs from bone marrow to peripheral blood [43], which shares a similar role as G-CSF.

The discovery of G-CSF occurred much earlier than SCF. The identification of G-CSF happened in the 1960s by *in vitro* assays measuring the ability of the growth factors to stimulate colony formation of bone marrow cells (see review by [60]). During the 1970s, a detailed category of colony stimulating factor (CSF) had been published, and G-CSF had been defined as a stimulator specific for colonies containing predominantly neutrophils [61]. G-CSF is produced by a variety of cells, of which, monocyte/macrophage lineage cells are the most prominent source [60]. G-CSF is also produced by normal mesothelial cells [62], fibroblasts [63] and ECs [64]. G-CSF, like other growth factors, exerts its biological functions by binding to the G-CSF specific receptor, G-CSFR. G-CSFR expression has been found on a variety of hematopoietic cells, including myeloid progenitors, mature neutrophils, monocytes, myeloid cells, lymphoid leukemia cells, and normal B and T cells [65].

Generally, G-CSF is known to have multiple functions in regulation of HSC/HPC proliferation, differentiation and mobilization, neutrophil production and mobilization from the bone marrow, neutrophil progenitor cell proliferation and differentiation, and the state of functional activation of neutrophils. In 1987, Tamura and colleagues reported that G-CSF mobilized large numbers of hematopoietic cells from the bone marrow into the circulation [66]. In addition, increased progenitor cells of all lineages were detected in the spleen of G-CSF-treated mice. These results were further confirmed by Duhrsen and colleagues in cancer patients with G-CSF treatment [67]. The administration of G-CSF to the patients showed significant increases of circulating HSCs, followed by a slight reduction in the frequency of bone marrow progenitor cells. However, the absolute number of the progenitor cells in the bone marrow was still increased. Together, all these data support the efficacy of G-CSF on HSC/HPC mobilization and proliferation. As peripheral blood is one of the important sources for stem cell transplantation, the biological effects of G-CSF in HSC/HPC mobilization and proliferation therefore allow autologous and allogeneic HSC transplantation in the clinical setting [68]. G-CSF is also a strong stimulator for neutrophil activation. Masja and colleagues reported that G-CSF increased the release of inflammatory granules [69]. In addition, G-CSF stimulates the survival and primitive proliferation of progenitor cells *in vitro* by combination with other factors. McNiece and colleagues observed more numerous and larger colonies of progenitor cells after combination treatment of G-CSF and GM-CSF as compared to either single factor [70].

G-CSF also shows a synergistic effect with SCF in regulating many important biological responses. As stated earlier, both SCF and G-CSF have effects on regulating survival, proliferation, differentiation, and mobilization of HSCs/HPCs and hematopoietic lineage; the combination of SCF and G-CSF shows an enhanced effect. SCF in combination with G-CSF increases more progenitor cell mobilization in peripheral blood than SCF or G-CSF alone [71]. Many clinical trials have further confirmed this finding. Combined treatment of SCF and G-CSF show beneficial effects on peripheral blood progenitor cell mobilization with an increased number of CD34+ cells/kg in circulating system in patients who have received high dose chemotherapy for lymphoma [72, 73], breast cancer [74] and multiple myeloma

[75]. The synergistic effect of SCF + G-CSF on HSC/HPC proliferation has also been illustrated. The synergistic effect of combined treatment of SCF and G-CSF in HSC/HPC proliferation is not only because of the enhanced ligand/receptor interaction [76], but it also due to the marked shortening of the duration of G0/G1 phase [76]. A direct effect of SCF and G-CSF on cell cycle distribution has been identified, and this effect is mainly induced by the regulation of cyclin-dependent kinase inhibitor p27kip1 [77]. Besides, the combination of SCF and G-CSF also shows a synergistic enhancement of STAT3 and MAPK signaling [76], which is involved in promoting the cell proliferation.

3 The Role of SCF and G-CSF in the Central Nervous System: Neural Plasticity and Neurogenesis

In addition to the effects of SCF and G-CSF in the hematopoietic system, increasing evidence shows that SCF and G-CSF also play a role in the central nervous system (CNS). Receptors for SCF and G-CSF have been found to express in the brain [78, 79], particularly in the neural stem cells/neural progenitor cells (NSCs/NPCs) [31–33, 80], and in cerebral neurons [32, 80] of adult mice and rats. It has been demonstrated that both SCF and G-CSF can pass through the blood-brain barrier [32, 81]. These findings suggest that hematopoietic growth factors, SCF and G-CSF, may have biological function in the CNS.

Numerous *in vitro* and *in vivo* studies have examined the contribution of SCF and G-CSF in the neuronal plasticity. SCF and G-CSF have been shown to play a key role in regulation of the neural plasticity in both the developing and adult brains. *In vitro* studies have shown that SCF supports the survival of c-kit-positive dorsal root ganglia neurons (DRGs) and promotes the neurite outgrowth of mouse embryonic DRGs through the c-kit receptor tyrosine kinase activity [25]. In addition, SCF has also shown to increase the neurite outgrowth of cultured cortical neurons [30]. In cultured brain slices, SCF selectively promotes outgrowth of commissural axons, which highly express SCF receptor [82]. *In vivo* studies, commissural axons fail to exit the floor plate in SCF and c-kit mutant mice [82]. In addition, c-kit conditional knockout mice show delayed extension of callosal fibers within the contralateral cortex and fail to innervate their target area [83]. At the functional level, SCF mutant mice exhibit a reduction of baseline synaptic transmission between dentate gyrus and hippocampal CA3 pathway and show deficits in spatial learning and memory [27]. C-kit mutant rats and mice both display impairments of paired-pulse facilitation (PPF) and long-term potential (LTP) in the hippocampal mossy fiber-CA3 pathway and a deficit in performance in Morris water maze task [28, 84]. In mouse brain slices, SCF binding to c-kit receptor activates PI3K/PLA_2 intracellular pathway, modulates PPF and LTP, and regulates synaptic transmission in the hippocampus [84]. These studies suggest that SCF/c-kit signaling is involved in the structural and functional regulation of synaptic plasticity. In addition to SCF, G-CSF also participates in neural plasticity.

G-CSF knockout mice show impaired LTP, reduced densities of NMDA receptors and dendritic complexity of hippocampal neurons in the dentate gyrus and the CA1 region, and deficiency in spatial learning and memory [29]. G-CSF treatment restores impaired long-term depression (LTD) in a mouse model of Alzheimer's disease (AD) [85]. In addition, subcutaneous injection of G-CSF increases the dendritic length and complexity of pyramidal neurons in the peri-infarct cortex in the cerebral ischemia rats [86]. Furthermore, it has been demonstrated that G-CSF in combination with SCF synergistically promotes neurite outgrowth and network formation of cultured cortical neurons through the PI3K/AKT/NF-kB/BDNF pathway [30]. Collectively, these research data suggest that SCF and G-CSF, the two hematopoietic growth factors, act as neurotrophic factors to regulate the neural plasticity during development and maturity.

The role of SCF and G-CSF in promoting neurogenesis and directing NSCs/NPCs to give rise to neurons has been illustrated in both *in vitro* and *in vivo* studies. There are two neurogenic regions in the adult mammalian brain, including the subventricular zone (SVZ) surrounding the anterior part of lateral ventricles and subgranular zone (SGZ) of the hippocampal dentate gyrus. NSCs/NPCs in these regions have regenerative potential, which has been postulated as a likely source for neural repair. Infusing SCF into the cerebrolateral ventricle has been shown to increase the number of newborn neurons in the SVZ [31]. Injection of anti-c-kit antibody into the cisterna magnum increases the number of cell death and results in thinning of the cerebral cortex, suggesting essential role of SCF/c-kit for cortical progenitor cell survival [87]. In cultured NSCs/NPCs, G-CSF is shown to promote the differentiation of NSCs/NPCs into neurons in a dose dependent manner [32, 88]. In G-CSF knockout mice, hippocampal neurogenesis is strongly diminished, and the mice show deficits in behavioral plasticity [29]. Peripheral or intraventricular administration of G-CSF has been demonstrated to increase the neurogenesis and promote the proliferation and differentiation of NSCs/NPCs, not only in the intact mice and rats [32, 88], but also in the animal models of neurological disorders, including the cerebral ischemia, perinatal hypoxia, irradiation-induced brain injury, traumatic brain injury, AD, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and bacterial meningitis [32, 34, 89–98]. G-CSF-induced neurogenesis is probably associated with G-CSF receptor-mediated phosphorylation of transcription factor STAT3/5 [88]. Besides, combination of SCF and G-CSF has been reported to have a synergistic effect in facilitating the proliferation of intrinsic NSCs/NPCs in a mouse model of cerebral ischemia [34]. When adding SCF and G-CSF into the culture medium during the proliferating stage of NSCs/NPCs, SCF in combination with G-CSF (SCF + G-CSF) shows a dual function in directing cell cycle arrest and promoting neuronal fate commitment through the regulation of neurogenin 1 [33]. Together, these studies reveal that SCF and G-CSF are involved in the regulation of NSC/NPC proliferation and neurogenesis.

In addition to promoting the proliferation and differentiation of intrinsic NSCs/NPCs, the combination of SCF and G-CSF also mobilizes bone marrow-derived cells, causing them to migrate into the brain and differentiate into various types of

cells, including neurogenesis. The fate of bone marrow-derived cells in the brain is dependent upon the microenvironment of the brain. In the subacute and chronic stroke brain, SCF + G-CSF treatment augments bone marrow-derived endothelial cells and neurons [34, 36]. In the brains of CADASIL mice, SCF + G-CSF selectively directs bone marrow-derived cells toward neuronal fate commitment [99]. In the APP/PS1 transgenic mice, bone marrow-derived microglial cells are significantly increased in the brain following SCF + G-CSF treatment, suggesting that SCF + G-CSF treatment leads to an enhancement in microglial fate commitment of bone marrow-derived cells in the brain with β-amyloid deposits [92, 100, 101]. G-CSF treatment has also been shown to mobilize bone marrow-derived mesenchymal stem cells, promote the migration and differentiation of mesenchymal stem cells into the neurons, and contribute to neurogenesis in the brains of AD mice [102]. The C-X-C chemokine receptor type 4 (CXCR4)/stromal cell-derived factor 1 (SDF-1) has been shown to be a key mediator in G-CSF-based recruitment of bone marrow-derived cells [102]. Together, these studies suggest that although bone marrow-derived cells possess different phenotypes in various brain conditions, these cells may participate in the neurogenesis and brain repair.

4 SCF and G-CSF Combination in Brain Repair Post-acute Stroke: Effective and Mechanistic Determinations

As stated in the previous section, substantial evidence has revealed the capacity of SCF and G-CSF in promoting neural plasticity and neurogenesis, and the permeability of the BBB to SCF and G-CSF. These discoveries provide a scientific base for seeking to determine the therapeutic effectiveness of SCF and G-CSF in enhancing brain repair and stroke recovery in the subacute phase and/or the chronic phase of stroke.

Here we highlight the preclinical studies demonstrating the efficacy and possible mechanisms of SCF and G-CSF in brain repair during subacute or chronic phases of experimental stroke.

4.1 The Effects of SCF and G-CSF on Brain Repair in the Subacute Phase of Stroke

There are a few preclinical studies demonstrating the therapeutic efficacy of SCF and G-CSF in the subacute phase of stroke. Using a transient focal ischemia model in Sprague-Dawley rats, Lee and co-workers [103] reported that intraperitoneal injections of G-CSF for 3 days beginning at 4 or 7 days post-ischemia led to motor function improvement, infarction size reduction, and increased angiogenesis. Kawada and colleagues [34] injected SCF and G-CSF subcutaneously during the period of 11–20 days after induction of focal cerebral ischemia in C57BL mice, and

observed that the SCF and G-CSF treatment increased the number of bone marrow-derived neuronal cells in the ipsilesional hemisphere and promoted the proliferation of intrinsic NSCs/NPCs in the SVZ. In addition, they also found that the SCF + G-CSF synergistically enhanced NSC/NPC proliferation in the SVZ when compared with treatment of SCF or G-CSF alone [34]. How SCF + G-CSF optimally repairs the brain in the subacute phase of stroke has not been clarified. Using the same treatment paradigm as reported by Kawada and colleagues [34], SCF + G-CSF treatment was found to upregulate IL-10, an anti-inflammatory cytokine, and to reduce infiltration of microglial/macrophages in the infarcted brain [104]. Although inhibiting inflammation by SCF + G-CSF may provide a favorable microenvironment for neurogenesis in the subacute phase of stroke, the causal link among the SCF + G-CSF-induced neurogenesis, anti-inflammation, and motor function improvement remains to be elucidated.

4.2 The Effects of SCF and G-CSF on Brain Repair in the Chronic Phase of Stroke

Most stroke patients still carry different degrees of disability when they enter into the chronic phase of stroke although many of them have received thrombolytic therapy in the acute phase [19] and physical therapy during the subacute phase [16]. However, in the chronic phase, there has been no therapy available for enhancing stroke recovery as it has been believed that the opportunity for obtaining recovery is largely ended by the time stroke patients enter the chronic phase [17, 105].

Brain plasticity is an intrinsic ability of the brain to reorganize its function and modify its structure in response to stimuli and injuries from both internal and external sources. Accumulating evidence supports that brain plasticity exists throughout a person's lifespan [106–111]. Accordingly, there is a possibility that a stroke-damaged brain may still be reparable during the chronic phase.

Over the past decade, our research team has demonstrated the safety, efficacy and possible mechanisms of SCF and G-CSF on stroke recovery in the chronic phase of stroke using rat and mouse models of cerebral cortical ischemia.

4.2.1 The Efficacy, Safety and Effective Dosage of SCF and G-CSF on Stroke Recovery in the Chronic Phase of Stroke

Systemic administration of SCF and G-CSF during the period of 3.5–6 months after cerebral cortical ischemia has been demonstrated and validated to be effective for brain repair in spontaneously hypertensive rats (SHRs), C57BL mice, or transgenic mice with C57BL genetic background [35–39, 41]. SHRs are used for making a stroke model because hypertension is the most important risk factor for stroke in humans [112]. Chronic hypertension leads to extensive pathological changes in the cerebrovasculature [113, 114]. Numerous studies have illustrated that the cerebral

cortical ischemia model in SHRs shows a more consistent and larger infarction in the cortex than in normotensive rats due to poor collateral circulation [35, 80, 114–121]. This model also causes permanent deficits in somatosensorimotor function that last up to the chronic phase of stroke [35, 80, 118–122]. In addition to the cortical infarct model in SHRs, we also use C57BL mice or transgenic mice with C57BL genetic background to make the cerebral cortical infarct model for exploring the mechanisms behind the SCF and G-CSF-enhanced brain repair in chronic stroke.

First of all, the therapeutic efficacy of SCF and G-CSF on stroke recovery in the chronic phase has been examined using the cerebral cortical ischemia model in SHRs. SCF (200 μg/kg), G-CSF (50 μg/kg), or SCF + G-CSF was subcutaneously injected daily for 7 days beginning at 3.5 months post-ischemic stroke. Among the treatment groups, only the SCF + G-CSF treatment led to a stable and long-term (17 weeks) improvement in somatosensory motor function. SCF alone treatment improved functional outcomes but the improvement did not present as stable as the SCF + G-CSF combination treatment. G-CSF alone treatment, however, did not result in functional benefits. The research data of field-evoked potentials lent further support to the neurobehavioral findings and revealed a reestablished normal pattern of somatosensory pathways by SCF + G-CSF treatment [35]. These findings provide first evidence that SCF + G-CSF combination treatment in the chronic phase of stroke can enhance stroke recovery.

Given the fact that stroke has the highest incidence in the elderly [2], the safety, efficacy, and optimal dosage of SCF + G-CSF combination treatment on chronic stroke recovery have been assessed in experimental stroke using aged SHRs and C57BL mice [37]. Six dosages of SCF + G-CSF ranging from 5 μg/kg (SCF) and 2.5 μg/kg (G-CSF) to 200 μg/kg (SCF) and 50 μg/kg (G-CSF) have been examined [37]. The treatment was initiated at 3–4 months post-experimental stroke. All the tested dosages did not show either acute or chronic toxicity to the livers and kidneys, demonstrating the safety of SCF + G-CSF treatment for chronic stroke in the aged population. The higher dosages (SCF/G-CSF: 200/50,100/25, and 50/25 μg/kg) showed the most effective outcomes in mobilizing circulating stem cells and in stably improving functional recovery. The intermediate dose of SCF + G-CSF (20/10 μg/kg) displayed a short-term improvement, whereas the dosages less than 20/10 μg/kg did not lead to functional improvement in chronic stroke in aged SHRs. These findings demonstrate that SCF + G-CSF treatment for chronic stroke recovery is a safe and effective therapeutic approach for the aged population and acts in a dose dependent manner.

4.2.2 The Possible Mechanisms Underlying the SCF + G-CSF-Enhanced Recovery in the Chronic Phase of Stroke

We have employed the approaches of bone marrow-derived cell tracking, molecular manipulation, live brain imaging, whole brain imaging, axon tracking, immunohistochemistry, confocal imaging, and neurobehavioral testing to determine how SCF + G-CSF repairs a stroke-damaged brain in the chronic phase.

By tracking bone marrow-derived cells through bone marrow transplantation, our study has revealed that increased bone marrow-derived endothelial cells and bone marrow-derived neurons are involved in SCF + G-CSF-enhanced angiogenesis and neurogenesis in the brain of chronic stroke [36].

Previous studies have shown that the receptors for SCF and G-CSF are expressed in cerebral neurons [32, 80] and cerebral endothelial cells [81] of adult mice and rats, and that both the SCF and G-CSF can pass through the blood-brain barrier of the adult rodent brain [32, 81]. Can SCF + G-CSF treatment in chronic stroke remodel the neural networks in an aged brain? To address this question, we used 2-photon microscopy to scan the brain area adjacent to the infarct cavity before and after SCF + G-CSF treatment in aged Thy-1-YFPH mice (C57BL background) [38]. In the brains of Thy-1-YFPH mice, the yellow fluorescent protein (YFP) is exclusively expressed in the layer V pyramidal neurons [123]. The mushroom spines with large heads on the dendrites are unique spines forming functioning synapses [124, 125]. Before SCF + G-CSF treatment, the mushroom spines of layer V pyramidal neurons were decreased, and the uncertain type (U-type) spines, which cannot build synapses with other neurons, were increased in the chronic stroke brain. This observation indicates that reduced synaptic circuits occur in the peri-infarct cavity cortex in the chronic stroke brain. However, 6 weeks after treatment, increased mushroom spines with decreased U-type spines were found in the brains of SCF + G-CSF-treated stroke mice. In addition, the densities of dendrites and PSD-95 were also increased in the ipsilesional cortex by SCF + G-CSF treatment. These findings demonstrate that SCF + G-CSF intervention in the chronic phase of stroke enhances synaptic network regeneration in the ipsilesional cortex of aged brains.

To distinguish whether SCF + G-CSF can directly modulate neural network formation, we carried out an *in vitro* study by determining neurite outgrowth of primary cortical neurons [30]. We found the expression of SCF and G-CSF receptors on the neurite growth cones. SCF + G-CSF showed synergistic effects in promoting neurite extension, activating NF-kB, and upregulating brain-derived neurotrophic factor (BDNF). Blockage of NF-kB activation eliminated the SCF + G-CSF-increased neurite outgrowth and BDNF production [30]. These data demonstrate the direct and synergistic efficacy of SCF + G-CSF in promoting neurite outgrowth, which is the initial step for generating neural networks. SCF + G-CSF enhances neurite extension through the NF-kB signaling.

Based on the *in vitro* findings, we then sought to use NF-kB inhibitor for blocking SCF + G-CSF-promoted neural network regeneration and to elucidate whether there is a dependent link between the SCF + G-CSF-enhanced neural network remodeling in the ipsilesional cortex and the SCF + G-CSF-improved motor function in chronic stroke. In an *in vivo* study [39], the NF-kB inhibitor was infused into the lateral ventricle through an osmotic pump for 7 days beginning at 1 h before a 7 day treatment (s.c.) of SCF + G-CSF, which was initiated 4 months after cortical ischemia. To track axons projecting from the contralesional hemisphere, an anterograde neuronal tracer, biotinylated dextran amine (BDA), was injected into the somatosensorimotor cortex in the contralesional hemisphere. After motor function

testing 2 and 6 weeks after treatment, mice were sacrificed at 10 weeks post-treatment. Our findings have revealed that SCF + G-CSF-increased BDA-labeled axons, PSD-95 accumulation, and blood vessel density in the peri-infarct cavity is eliminated by NF-kB inhibitor. In addition, the SCF + G-CSF-induced motor functional improvement is also prevented by NF-kB inhibitor. These data suggest that the SCF + G-CSF-improved functional outcome in chronic stroke may depend on the regeneration of neural networks and vasculature in the peri-infarct cavity cortex. However, this terminal determination study is limited to clarify the dynamically causal link between the SCF + G-CSF-promoted neural network rewiring and functional improvement in chronic stroke.

To over come this limitation, we conducted a unique study combining live brain imaging and motor function evaluation to simultaneously examine the dependent relationship between the SCF + G-CSF-enhanced synaptic network remodeling and motor function improvement in the chronic phase of experimental stroke [40]. To prevent the influence of behavioral testing-induced neural network remodeling, the following two sets of experiments were carried out simultaneously: (1) Thy1-YFPH mice with cortical infarction for live brain imaging at 2 and 6 weeks post-SCF + G-CSF treatment, and (2) Thy1-YFPH mice with cortical infarction for motor function assessment at 2 and 6 weeks post-SCF + G-CSF treatment. The SCF + G-CSF treatment was initiated at 6 months post-experimental stroke. We observed that once the SCF + G-CSF-increased mushroom spines in the ipsilesional motor cortex were eliminated by NF-kB inhibitor, the SCF + G-CSF-improved motor function was simultaneously prevented. This observation provides solid evidence validating a clear causal link between SCF + G-CSF-promoted neural network remodeling and motor functional improvement and strongly supporting that the SCF + G-CSF-enhanced neural network rewiring in the ipsilesional motor cortex is required for SCF + G-CSF-improved motor function in the chronic stroke.

It is worth noting that the SCF + G-CSF-increased dendritic spine head size, PSD-95 accumulation, and blood vessel density in the peri-infarct cortex are much greater than in the contralesional hemisphere of chronic stroke brain and in the intact control mouse brain [39, 40]. Blocking the SCF + G-CSF-induced “over growth” of synaptic networks and vasculature in the peri-infarct cortex by NF-kB inhibitor leads to abolition of the SCF + G-CSF-improved motor function in chronic stroke [39, 40]. These findings reveal that SCF + G-CSF-strengthened synaptic function in the peri-infarct motor cortex plays a vital role in motor functional improvement in chronic stroke.

5 Concluding Remarks

SCF and G-CSF were initially discovered as critical hematopoietic growth factors to regulate hematopoiesis. SCF in combination with G-CSF has been demonstrated to have synergistic effects in promoting the proliferation, differentiation and survival of HSCs/HPCs, and in mobilization of HSCs/HPCs into the blood.

Numerous studies have demonstrated that both SCF and G-CSF are crucially involved in neural plasticity and neurogenesis. These findings significantly advance our knowledge of these two hematopoietic growth factors: the biological function of SCF and G-CSF is not only limited in the hematopoietic system but it also acts in the CNS. In addition to the synergistic effects of SCF + G-CSF in the hematopoietic system, our research team has, for the first time, illustrated that the combination of SCF and G-CSF also synergistically promote neurite outgrowth of primary cortical neurons.

Over the past decade, the contribution of SCF and G-CSF in brain repair post-acute stroke has been determined. Importantly, it has been demonstrated that SCF in combination with G-CSF synergistically enhances brain repair in the subacute phase (by Kawada's group) and chronic phase (by our research team) of experimental stroke. These findings extend current understanding concerning the neurorestorative efficacy of SCF + G-CSF in brain repair post-acute stroke and provide a new approach for enhancing stroke recovery.

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