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Chenyu Liu April 12, 2016

# Promoting Effect of Wnt-3a and Stem Cell Factor Treatments on Cell Protection, Neurogenesis, Angiogenesis and Functional Recovery in Focal Ischemic Stroke Mice

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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By Chenyu Liu

Stroke is the third leading cause of death and a major cause of serious and long-term disability in the United States; however, very limited effective treatments are available for stroke patients. This study is aimed at finding a novel regenerative treatment for focal ischemic stroke by using stem cell factor (SCF) and Wnt-3a proteins. The Wnt-3a signaling pathway, through mediating gene expression and the β-catenin protein, is capable of inducing stem cell differentiation and migration, enabling it to be a potent neurogenesis agent. SCF, on the other hand, can promote angiogenesis by directly activating microvascular endothelial cells in the CNS. Because increasing vascularization of the ischemic penumbra region can help deliver more oxygen and nutrients, this should facilitate the process of neurogenesis and exert a synergistic therapeutic effect. Ultimately, we wanted to restore the neuro-vascular unit for functional recovery. We hypothesized that the combined treatment of Wnt-3a and SCF may work synergistically in treating stroke. Treatment was administered intranasally for 7 days, started on the day of stroke surgery. Immunohistochemical staining was used to quantify neurogenesis and angiogenesis. All treatment groups showed enhanced neurogenesis and angiogenesis, but no synergistic effect of the SCF/Wnt-3a combined group was observed. All treatment groups illustrated a cell protective effect as shown by Western blot analysis, and a synergistic effect was observed in the SCF/Wnt-3a combined group. Additionally, the sticky dots behavioral test was used to measure sensorimotor functional recovery. All treatment groups showed improved sensorimotor function, yet only a non-significant

trend of synergy was observed. Based on all our results, we concluded that SCF, Wnt-3a and SCF/Wnt-3a combined treatments all demonstrated a therapeutic effect on focal ischemia stroke. Our current data partially support the hypothesis. Considering that regeneration is a long-term process, further investigation at delayed time points will be necessary to test our hypothesis.

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TERMINOLOGY and ABBREVIATIONS

**AMPA receptor:** α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor, a type of glutamate receptor

**NMDA receptor:** N-methyl-D-aspartate receptor, a type of glutamate receptor

**BBB:** blood brain barrier

**Frz:** frizzled protein, the transmembrane protein receptor of Wnt protein

**Dsh:** Dishevelled protein, a critical regulator in the Wnt/β-catenin pathway

**GSK-3:** Glycogen synthase kinase 3, a critical regulator in the Wnt/β-catenin pathway

**β-catenin:** the effector of the Wnt/β-catenin pathway. Acting as a transcription factor and playing a role in initiating cell differentiation

**SVZ:** subventricular zone, stem cell niche of the lateral ventricle

**SGZ:** subgranular zone, stem cell niche of the hippocampus (the dentate gyrus)

**SCF:** stem cell factor, a type of hematopoietic growth factor

**EC:** endothelial cell, indicating brain microvascular endothelial cell in this thesis

**MCA:** middle cerebral artery

**CCA:** common carotid arteries

**NeuN:** neuronal nuclei, a marker that binds to neuronal DNA

**Glut-1:** Anti-glucose transporter-1, a marker for blood vessels

**BrdU:** Bromodeoxyuridine (5-bromo-2'-deoxyuridine), a marker for newly proliferated cells

**MMP-9/2:** Matrix metalloproteinase-9/2, an indicator of BBB disruption

**Cas-3:** Caspase-3, an indicator of cell apoptosis

## Abstract

Stroke is the third leading cause of death and a major cause of serious and long-term disability in the United States; however, very limited effective treatments are available for stroke patients. This study is aimed at finding a novel regenerative treatment for focal ischemic stroke by using stem cell factor (SCF) and Wnt-3a proteins. The Wnt-3a signaling pathway, through mediating gene expression and the β-catenin protein, is capable of inducing stem cell differentiation and migration, enabling it to be a potent neurogenesis agent. SCF, on the other hand, can promote angiogenesis by directly activating microvascular endothelial cells in the CNS. Because increasing vascularization of the ischemic penumbra region can help deliver more oxygen and nutrients, this should facilitate the process of neurogenesis and exert a synergistic therapeutic effect. Ultimately, we wanted to restore the neuro-vascular unit for functional recovery. We hypothesized that the combined treatment of Wnt-3a and SCF may work synergistically in treating stroke. Treatment was administered intranasally for 7 days, started on the day of stroke surgery. Immunohistochemical staining was used to quantify neurogenesis and angiogenesis. All treatment groups showed enhanced neurogenesis and angiogenesis, but no synergistic effect of the SCF/Wnt-3a combined group was observed. All treatment groups illustrated a cell protective effect as shown by Western blot analysis, and a synergistic effect was observed in the SCF/Wnt-3a combined group. Additionally, the sticky dots behavioral test was used to measure sensorimotor functional recovery. All treatment groups showed improved sensorimotor function, yet only a non-significant trend of synergy was observed. Based on all our results, we concluded that SCF, Wnt-3a and SCF/Wnt-3a combined treatments all demonstrated a therapeutic effect on focal

ischemia stroke. Our current data partially support the hypothesis. Considering that regeneration is a long-term process, further investigation at delayed time points will be necessary to test our hypothesis.

## Introduction

Stroke is the third leading cause of death and a major cause of serious and long-term disability in the United States. Every year, approximately 795,000 people suffer a stroke, and more than 140,000 people die of stroke attack (U.S. Centers for Disease Control and Prevention, 2015). For example, in 2006, stroke accounted for 1 of every 17 death in the United States (U.S. Centers for Disease Control and Prevention, 2006). On average, someone in the United States has a stroke every 40 seconds. Furthermore, stroke is a dominant cause of long-term disability in the United States. Seventy-five percent of stroke survivors had severe disability and decrease their employability (Coffey and Cummings, 2000). Stroke can also affect people emotionally. Some survivors are diagnosed with depression, anxiety disorders, panic attacks and flat affect (Braun et al., 2006), and also suffer from cognitive deficits, including perceptual disorders (Mercier et al., 2001), aphasia (Laska et al., 2005) and dementia (Thommessen et al., 2002). Stroke, also known as cerebrovascular accident (CVA), or "brain attack," is defined as occlusion of blood flow to the brain. This deprives a certain brain area of oxygen and nutrients supply, and can induce cell death within minutes (Hart and Kanter, 1990, Furlan et al., 1999). There are two main types of stroke: ischemic and hemorrhagic (National Institute of Neurological Disorders and Stroke, 2009). Ischemic stroke is caused by the decrease or interruption of blood supply to a certain brain area, and hemorrhagic stroke results from the rupture of a blood vessel, or intracranial bleeding induced by abnormal vascular structure. Both types of stroke result in a part of the brain not functioning properly. About 87% of strokes are ischemic, the rest being hemorrhagic (Kleindorfer et al., 2004). However, there is a condition known as "hemorrhagic transformation", wherein bleeding

develops inside areas of ischemia. Currently, it is unclear how many hemorrhagic strokes actually started as ischemic strokes (Donnan et al., 2008). Stroke costs the United States an estimated $34 billion each year. This total cost includes the health care services, medications treating stroke, and missed days of work by patients (Mozaffarian et al., 2016). However, there still are limited treatments for stroke victims -- both in terms of emergent treatment for stroke onset, controlling the cell death cascade after stroke, or treating the long-term physical, emotional and cognitive deficits.

***Why study ischemic stroke: Pathophysiology of ischemic stroke***

There were two major pathophysiology pathways. First is the release of excitotoxic molecules. Neurotransmitter glutamate is released after stroke and it is excitotoxic (Kanekar et al., 2012). Cell death can be decreased by blocking the glutamate release or glutamatergic receptor (Shen et al., 2005) and over-expressing the glutamate transporter to increase the reuptake rate of glutamate. So why is glutamate excitotoxic? AMPA receptor, a well-studied glutamatergic receptor, will be activated by glutamate and depolarize the cell and then unblock the NMDA receptor by removing the magnesium block inside of the NMDA receptor. The NMDA receptor is permeable to $Na^+$, $K^+$ and $Ca^{2+}$ ions. A large influx of $Ca^{2+}$ ions is deadly for the cells. This will trigger intracellular signaling cascades and eventually lead to excitotoxic necrosis and apoptosis of the cells. This pathway is fast acting. Cell death starts within minutes after stroke and this accounts for the initial cell death. The second pathophysiology pathway is through obstruction of blood vessels. Neurons are not able to store glucose, so they need a constant supply of oxygen and nutrients by means of the cardiovascular system and astrocytes (Watters and

O'Connor, 2011). Obstruction of blood vessels in the brain by means of ischemic stroke induces a cellular cascade, and eventually leads to cell death. This will result in lack of oxygen supply (hypoxia), which disrupts the normal ATP synthesis (Martin et al., 1994, Harvey et al., 2011). Decreased cellular ATP level then disturbs the function of sodium-potassium pump ($Na^+/K^+$ pump). Normally, the $Na^+/K^+$ pump actively transports 3 $Na^+$ ions out and 2 $K^+$ ions into the cell. Failure of this active transportation against the concentration gradient results in increasing intracellular sodium concentration and cytotic edema, which leads to cell death cascade (Dirnagl et al., 1999, Durukan and Tatlisumak, 2007). However, because we have pre-stored ATP around the cells, so this patho-pathway will take hours to initiate cell death, which accounts for delayed cell death. Furthermore, oxygen radicals are produced during many enzymatic conversions as a consequence of ischemic cascade (Lo et al., 2003). Oxygen radicals can also interrupt the pentose-phosphate pathway, which leads to disruption of phospholipid bonds and nuclear DNA (Durukan and Tatlisumak, 2007). In addition, oxygen radicals are important signaling molecules that trigger inflammation and apoptosis, which accelerates the ischemic cell death cascade (Dirnagl et al., 1999).

Because the cell death cascade happens rapidly, researchers have been tried to develop stroke treatment from the aspect of increasing cell protection to attenuate cell death. However, over 200 clinical trials of cell protective treatments have failed. There were many treatments could work well on animals, but failed once moved on to human. Besides from complicated molecular differences between animal and human, the major

reason causes this failure was the short time window of treatment delivery. Therefore, researchers shifted focus from cell protection to regeneration therapy.

***Focal Ischemia Stroke: the Barrel Cortex***

Even though ischemic stroke can be fatal or cause long-term disability, some stroke victims indeed can slowly recover some functions. Therefore, understanding the mechanisms underlying the recovery of functions is critical to develop new therapeutic treatment. The study of mini-ischemic stroke -- that which is localized into a specific and well-known brain area -- helps promote understanding of the recovery mechanism. In our lab, we model stroke localized in a well-studied region of the sensorimotor cortex—the barrel cortex. Barrel cortex is the fourth layer of the somatosensory cortex, which receives inputs, particularly whisker sensory inputs for rodents, from the contralateral side of the body come in from the thalamus (Woolsey et al., 1975). In order to have a more comprehensive understanding of functional recovery including multiple behavioral aspects, modifications of the stroke surgery were designed. As describe in the Materials and Method part, middle cerebral artery occlusion (MCAO) surgery initial described by Wei et al. was permanent sutures of some branches of the MCA, which leads to specific barrel cortex stroke (Wei et al., 1995). The new surgery sutures the MCA in earlier parts of the branch that are farther away from the cortex, resulting in depleting blood supply to a larger cortex area, including the barrel cortex, sensory and motor cortex nearby (Wei et al., 2015). This enables researchers to measure and evaluate not only the recovery of whisker sensory function, but also sensorimotor function using diverse behavioral tests.

***Current therapy of Ischemic Stroke: Thrombolysis and Neuroprotection***

Considering the multifaceted pathophysiology of ischemic stroke, treatments have been developed from multiple approaches. Two major approaches are thrombolysis and neuroprotection. The most famous thrombolysis therapy -- and the only FDA-approved ischemic stroke treatment -- is the intravenous (IV) tissue-type plasminogen activator (tPA). tPA significantly improves outcome after an ischemic stroke, but the time-window is restricted within 3 hours of onset (Laatikainen and Mattila, 1995, Furlan et al., 2003). Consequently, due to this short window for administration, only 1-8.5% hospitalized patients are able to receive this treatment (Millan and Davalos, 2006). IA recombinant prourokinase (r-proUK) is another thrombolysis treatment for acute ischemic stroke under clinical trial. This intra-arterial thrombolysis treatment has a longer administration time window: up to six hours (Furlan et al., 1999). Recently, a plasminogen activator from the vampire bat saliva has been found effective and safe in DIAS and DEDAS trials, and scientists are currently investigating this further. (Furlan et al., 2006). Aspirin, clopidogrel or the combinations of the two, with other plasminogen activators, have also been confirmed as useful to treating ischemic stroke (Lee et al., 2002). The other approach to treating ischemic stroke is neuroprotection. Major investigations are focusing on citicoline (a cell-membrane stabilizer that maintains the ionic balance), and glutamate pathway modifiers (AMPA/kinate and NMDA receptors antagonist, glutamate recycle transporter, glutamate release blocker etc) (Shuaib, 2006, Harvey et al., 2011). Other subcategories, such as free-radical trapping agents and metal ion chelators, also aim at decreasing excitotoxic effect and thus can be considered neuroprotective (Shuaib, 2006).

Lastly, in addition to drugs helping with thrombolysis and neuroprotection, surgeries can also help with treating stroke.

### *Intranasal Delivery Of Treatment*

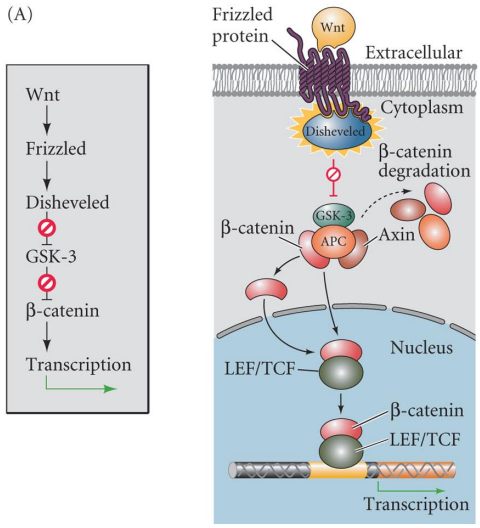
The blood brain barrier (BBB) is the greatest obstacle to treatment drug delivery into the central nervous system (CNS). The barrier monitors and restricts the exchange of hydrophilic compounds, small proteins and ions between plasma and the CNS. The BBB is critical and essential for preventing diseases and infections *in vivo*, however, it is a huge obstacle for delivering therapeutic agents into the CNS to treat pathobiology. Therefore, people who work with CNS diseases have long been trying to bypass the BBB to deliver treatments. Several attempts have been made. First, researchers have tried to design drugs that are permeable to the BBB, or deliver drugs with carriers or transporters (Lochhead and Thorne, 2012) that are able to cross the BBB. However, these methods are limited to small portion of the therapeutic agents. Different approaches such as intracerebral/intracerebroventricular injection enables precise deliver of the drug, yet is highly restricted in use due to its invasiveness and fatal side-effects. The most traditional treatment delivery is intravenous or intraperitoneal injection. This type of delivery is safe and well studied. However, drugs entering the blood circulatory system are easily degraded by the kidneys, thus lowering the dose that can reach the CNS. After all these attempts, a brand new approach—intranasal delivery – was developed. This method works due to the unique connection between the nasal mucosa and the brain. Drugs delivered intranasally do not require modifications to across the BBB. Drugs enter from the nasal mucosa, passing through the cribriform plate and are transported by bulk flow

through perineural channels and olfactory neural pathway, and then delivered directly to the brain parenchymal tissue and/or cerebral spinal fluid (CSF) (Hanson and Frey, 2008) (Talegaonkar, 2004). This is a non-invasive, fast and efficient method of drug delivery. Other researches have shown that intranasal delivery can deliver several other therapeutic agents including peptides, proteins, gene vectors and stem cells (Lochhead and Thorne, 2012). In our lab, we choose to deliver drugs and stem cells intranasally due to these advantages.

***Wnt Signal Transduction Pathway and Neurogenesis Potential***

The Wnt protein family plays a crucial role in neural development and differentiation, and there are more than 20 subtypes in the Wnt protein family. (Wodarz and Nusse, 1998, Logan and Nusse, 2004, Davidson et al., 2007). Wnt proteins send important signals in early central nervous system development, contributing to brain and spinal cord differentiation and neural crest cell differentiation (Ciani and Salinas, 2005, Davidson et al., 2007). The specific Wnt protein used in this project is Wnt-3a, which is classified as canonical Wnt protein–a protein that is involved in the stabilization of β-catenin. This type of Wnt signaling pathway starts with the Wnt protein binding to the Frizzled protein, which is a trans-membrane protein receptor that couples with another protein named Disheveled (Dsh). In the cytoplasmic region, there are protein complexes that consist of GSK-3 and APC and other proteins. This protein complex can help stabilize and relocate β-catenin. The Dsh protein usually inhibits the GSK-3 protein complex: when GSK-3 is inhibited, β-catenin will not be stabilized, and will get degraded. However, when Frizzle activates Dsh, Dsh will disinhibit the GSK-3 complex and thus GSK-3 complex will start

stabilizing and then relocating β-catenin. Now, β-catenin is important because it is a signaling molecule that can cross nuclear membrane and bind to other transcription factors, thus inducing new transcriptions (Behrens et al., 1996, Willert et al., 2002, Zechner et al., 2003). These transcriptions (LEF/TCF family) include growth factors and other signaling molecules that will lead to cell developments and differentiations.



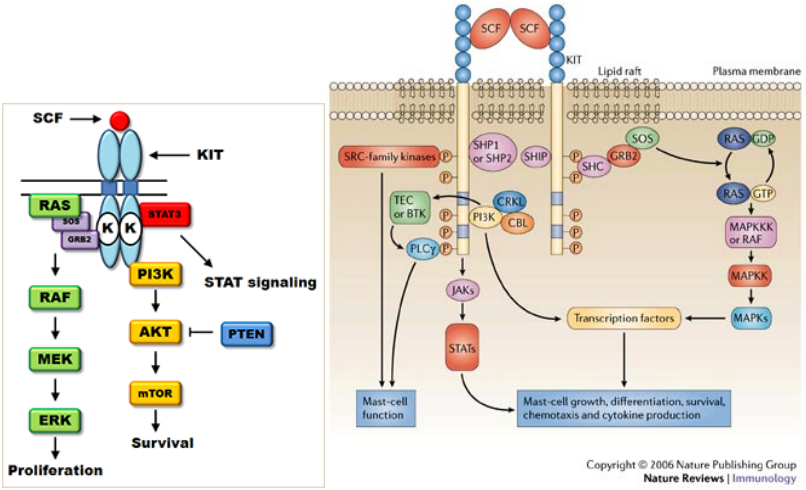

Developmental Biology, Seventh Edition, 2003 Sinauer Association, Inc.

So how can Wnt-3a help with treating stroke? The Wnt signaling pathway controls stem cell differentiation in multiple locations in our body. When a cell receives Wnt signal, β-catenin is stabilized and relocated into the nucleus where it turns on gene transcription, making proteins that tell the cells in the stem cell niche to maintain as adult stem cells. When the Wnt signal is absent, β-catenin is degraded and the repressor Groucho will inhibit gene transcription, which ceases the signal that tells the cells to maintain as adult stem cells. Therefore, stem cells will start differentiating. Furthermore, as stem cells initiate differentiation, Wnt-3a is also responsible for making these these progenitor cells migrate out of the stem cell niche (Zechner et al., 2003, Muroyama et al., 2004). In the

CNS, the subventricular zone (SVZ) and the subgranular zone (SGZ) are considered as two main stem cell niches in the brain (Varela-Nallar and Inestrosa, 2013). Wnt-3a also plays a role in proliferation and expansion of the neural progenitor cells, demonstrating mitogenic effects (Chesnutt et al., 2004). In fact, this mitogenic effect has been observed in many other cell types including mesenchymal stem cells (Boland et al., 2004, Davidson et al., 2007) and fibroblasts (Yun et al., 2005, Davidson et al., 2007).

***Stem Cell Factor Signaling Pathway and Angiogenesis Potential***

Stem cell factor (SCF) is a cytosine growth factor that plays an important role in hematopoiesis during embryonic development (Broudy, 1997). SCF binds to the tyrosine kinase c-Kit receptor. When the SCF ligand binds to the c-Kit receptor, it receptor homodimerizes and auto-phosphorylates at tyrosine residues (Zsebo et al., 1990). Activating the c-Kit receptor triggers many signal cascades, including the RAS/ERK, PI3-Kinase, and JAK/STAT pathways (Ronnstrand, 2004). SCF is essential for cells expressing c-Kit receptors, which include hematopoietic progenitor cells (HSC), mast cells, melanocytes, and germ cells. These cells are responsible for haemopoiesis, melanogenesis, and fertility (Ashman, 1999). According to previous literature, SCF can promote cell survival, proliferation, differentiation, adhesion, and migration (Majumder et al., 1988, Nocka et al., 1989, Simmons et al., 1994, Ashman, 1999, Sun et al., 2004).




https://www.mycancergenome.org/content/disease/acute-myeloid-leukemia/kit/276/
Nature Reviews|Immunology 2006

The RAS/ERK pathway promotes haemopoietic cell proliferation, and the mTOR pathway promotes the haemopoietic cell survival. Interestingly, when HSC matures, c-Kit receptors will be down regulated. Furthermore, SCF is also responsible for triggering HSC mobilization from its stem cell niche to peripheral blood (To et al., 1997). The other important signaling target of SCF is the mast cell. The JAK/STAT pathway is responsible for the growth, differentiation, survival, and migration of mast cells (Okayama and Kawakami, 2006). The mast cell, a type of white blood cell, derives from the myeloid stem cell and is part of the immune and neuroimmune systems (da Silva et al., 2014). Mast cells are crucial for cell protection by means of healing wounds, promoting angiogenesis, defending against inflammation, and securing BBB function (Polyzoidis et al., 2015a). Putting all this information together, SCF should be a potent agent of cell/neuroprotective effects, because it can promote stem cell and neuroimmune cell migration and haemopoietic/angiogenesis effects. In fact, as described by Sun et al, neuronal and glioma-derived SCF induced angiogenesis within the central nervous system by directly activating brain microvascular endothelial cell (ECs) (Sun et al., 2006).

The study addressed the possibility of SCF plays the pathological angiogenesis during tumor progression in CNS (Sun et al., 2006). Therefore, our lab thought it could also be a potent therapeutic agent in promoting angiogenesis around ischemic stroke area.

As mentioned before, neurons need constant oxygen and nutrients supply delivered by vascular system in the CNS. Ischemic stroke blocks these supplies from being delivered and triggers mass cell death. Therefore, proper re-establishment of the vascular system can benefit the neurogenesis process. Furthermore, we need reestablishment of the neuro-vascular units for functional recovery, which is our ultimate goal of helping stroke patients. Combining all these information, we hypothesized that Wnt-induced neurogenesis and SCF-induced angiogenesis will mutually facilitate each other, thus exerting a synergistic effect in treating stroke when they were combined.

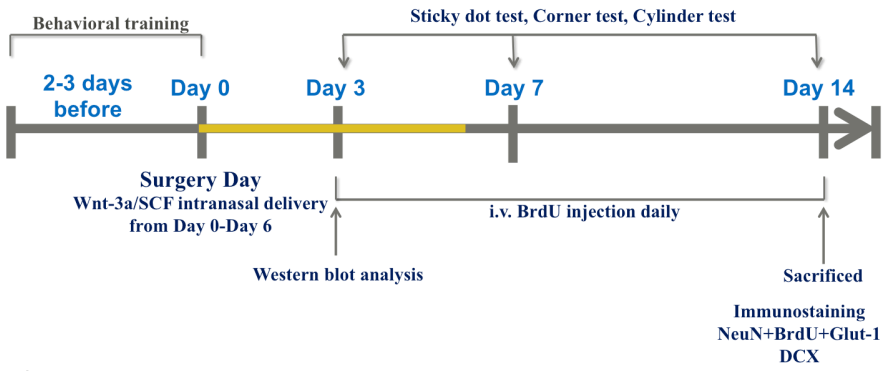
## Materials and Methods

### *Ischemia Animal Model:*

Ten to 12-16 week-old male mice (Charles River, Wilmington, MA, USA) weighing 25-35 grams were used. Animals were kept in groups of five before and after surgery on sawdust bedding, with free access to water and a normal diet. The animal protocols were approved by institutional Animal Care and Use Committee (IACUC) at Emory University. The animal local ischemia stroke model was produced by a modified middle cerebral artery occlusion (MCAO) surgery as described by Wei et al. (Wei et al., 1995). Animals were anesthetized with 4% chloralhydrate. Multiple branches of the middle cerebral artery (MCA) were permanently ligated (surgically sutured). Common carotid arteries (CCA) occlusion was used to reduce the barrel cortex blood flow to less than 20%, as measured by laser Doppler scanning.

### *Experimental Groups and Drug Administrations:*

Mice were randomly divided into four groups: 1) stroke-saline group as control, 2) stroke-Wnt-3a group, 3) stroke+SCF, and 4) stroke+Wnt-3a/SCF as experimental groups. Daily intranasal injection of Wnt-3a/SCF (50ng/25μL) started on the third day after surgery until sacrifice (day 14).



***BrdU Administrations***

Five-bromo-2-deoxyuridine (BrdU) was administered intraperitoneally (i.p) on the third day after surgery to all animals to label the proliferation of new cells until sacrifice (day 14). The volume of BrdU injected was based on animal weights (50mg/kg).

***Coronal Sectioning and Tissue preparations***

Mice were perfused on day 14 followed by the protocol described by Gage et al. (Gage et al., 2012) for tissue fixation. Brains were sectioned into 10μm coronal brain sections, each separated by 90μm per slide. Each brain was cut into a total of 80 sections to collect the subventricular zone (SVZ) and stored in -80ºC.

***Immunohistochemical Staining***

Brain sections were fixed in 10% formalin for 10 minutes, then washed by phosphate buffered saline (PBS) for three times (3*5min, 15 min for perfused slides). Slides were then incubated by -20 ºC methanol for 14 minutes (2*7min) and were completely air-dried for at least 10 minutes. After rehydrating with PBS, slides were incubated in 0.2% TritonX-100 for 45 minutes and then washed by PBS for 3 times. Brain sections were blocked in 1% fish gel (Sigma, St Louis, MO) for 1 hour. Primary antibodies including neuronal nuclei (1:300, NeuN, Millipore; Billerica, MA), newly proliferated cells (1:200, BrdU, Serotec; Raleigh, NC) and collateral vessel epithelial cells (1:400, Glut-1, Millipore; Billerica, MA) were incubated at 4ºC overnight. Brain sections were washed by PBS for 3 times and incubated with secondary antibodies including donkey anti-mouse 488mm (1: 400, Invitrogen; Grand Island, NY), Donkey anti-rat Cy-3 (1:500,

Jackson Immunoresearch; West Grove, PA), goat anti-rabbit Cy-5 (1:200 Jackson Immunoresearch; West Grove, PA) for 2-4 hours. Neuroblast cell migration was measure by DCX staining. Brain sections were fixed in 10% formalin and methanol as described before. Samples were completely air-dried and incubated in 0.2% TritonX-100 for 5 minutes and were blocked in 1% fish gel for 1 hour. Primary antibodies DCX anti-goat and BrdU anti-rat were incubated at 4ºC overnight. Secondary antibodies donkey anti-goat 488mm and donkey anti-rat Cy-3 were incubated for 2-4 hours. After secondary antibody incubation, all slides were washed in PBS for three times and were sealed and stored at 4ºC (dark) for imaging.

***Cell Counting***

Florescent pictures of the sample were taken under 40x magnification. Six images of the para-infract/penumbra region, defined as within 500 μm from the edge of the infarct area (Fisher, 2004), were taken for each brain. Total neuronal cells (NeuN), blood vessels (Glut-1) and newly proliferated cells (BrdU) were counted. Newly grown neuronal cells and blood vessels were identified by co-labeled with BrdU. All cells were counted by ImageJ and processed blindly.

***Western Blot Analysis***

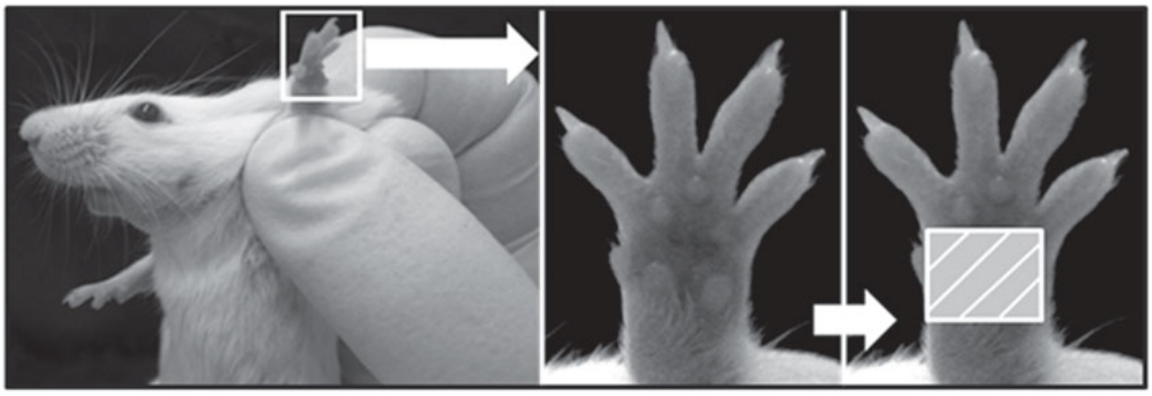
Peri-infarct/penumbra region in fresh brain tissue was prepared for western blot analysis. Tissues were homogenized in lysis buffer (25mM Tris-HCl (pH 7.5), 5mM EDTA, 0.1%SDS, 100mM NaF. 150mM NaCl, 1% TritonX-100, leupeptin, aprotinin and pepstatin). Samples were centrifuged at 15,000 rpm for 15 minutes and the resulting

supernatant was collected. All samples were assayed by Bicinchoninic Acid Assay to identify the protein concentrations. Proteins (30 μg) from each sample with dye reagent were loaded into the wells of a 12-18% SDS-PAGE gradient gel, along with molecular weight markers. Samples were run at 150V about 1 hour until protein markers had adequately separated. Samples were then transferred onto polyvinyl difluoride (PVDF) membrane. The membrane was blocked and incubated with primary antibodies including matrix metalloproteinase-9/2 (MMP9/2; 1:500; Santa Cruz, Dallas, TX); cleaved caspase-3 (1:500; Cell signaling; Sigma, St Louis, MO). Bax (1:100, ThermoFisher, Waltham, MA) and mouse β-actin antibody (1:6000; Sigma) were applied overnight at 4°C as described in Choi et al. (Choi et al., 2012). After washing with Tris-Buffered saline (TBS), alkaline phosphatase-conjugated secondary antibodies were applied for 1 to 2 hr at room temperature. After incubation, the membrane was washed by nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3'-indolyphosphate solution (BCIP). The intensity of each band was visualized and measured and β-actin was used to normalize the expression ratio of each target protein.

***Behavior Assessment***

Behavioral tests were conducted to measure functional recovery. The adhesive removal test was done to measure the recovery of sensorimotor function as previously described (Bouet et al., 2009, Freret et al., 2009). As shown in the picture, a small sticky dot is taped on forepaws of animals, one side at a time, and latency time (the time it takes for the animal to notice the dot) and removal time (the time it takes for the animal to take the dot off) were measured. Animals had training sessions for three days before the stroke

surgery, and the basal level of performance was recorded. Behavior tests were done on days 3, 7 and 14 after stroke surgery. There were 3 trials separated by at least 15 minutes for each animal.



The adhesive removal test; Nature Protocols 4, 1560 - 1564 (2009) (Bouet et al., 2009)

***Statistical Analyses***

All statistical analyses were performed using Graphpad Prism 5.0 software. Multiple comparisons were performed by one-way ANOVA followed by Tukey's post hoc analysis to identify significant difference. Changes were identified as with significant with p-value less than 0.05, labeled with *. Mean values were reported with standard error mean (SEM).

## Results

### 1. SCF, Wnt and SCF/Wnt combined treatments all demonstrated enhanced neurogenesis

Fluorescent pictures of immunohistochemical staining were used to quantify the neurogenesis. As green represents neuronal nuclei (NeuN) and red represents newly proliferated cells (BrdU), co-labeled NeuN and BrdU were considered as newly synthesized neurons. Only complete overlaps were considered as BrdU positive cells, as indicated by white arrows in figure 2D. All three treatment groups showed significant difference compared to stroke animals treated with saline controls. Wnt alone showed significantly higher neurogenesis than SCF alone or combined. (N number of saline, SCF, Wnt, SCF/Wnt are 10, 10, 8, 12)

### 2. SCF, Wnt and SCF/Wnt combined treatments all demonstrated enhanced Angiogenesis

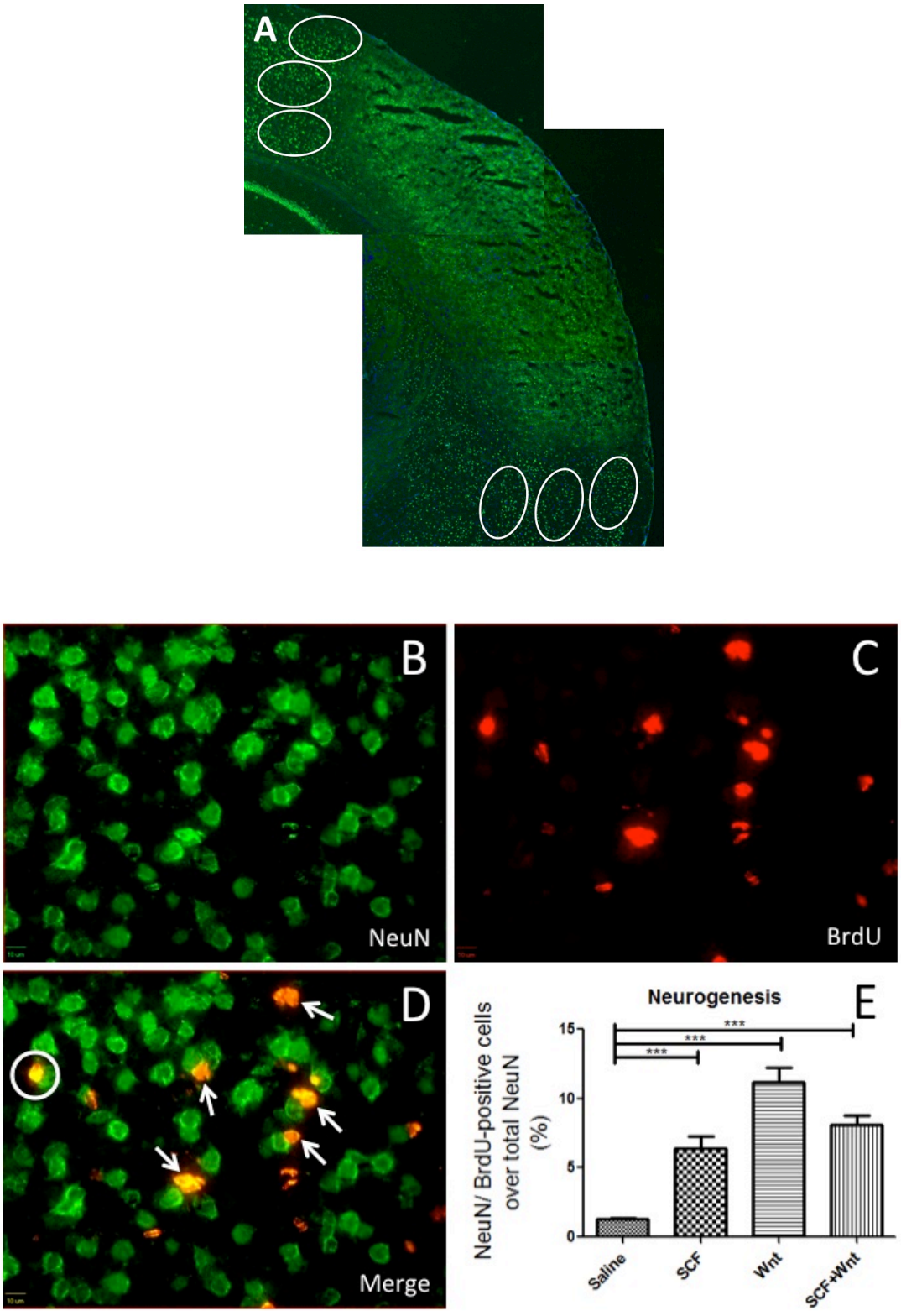
Fluorescent pictures of immunohistochemical staining were used to quantify the angiogenesis. As blue represents blood vessels (Glut-1) and red represents newly proliferated cells (BrdU), co-labeled Glut-1 and BrdU were considered as newly synthesized neurons. New-grown blood vessels were indicated by white arrows in Figure 3C. All three treatment groups showed significant difference compared to stroke animals treated with saline controls. SCF alone showed significantly higher neurogenesis than Wnt alone or combined. Not surprisingly, Wnt showed the lowest angiogenesis effect, and there was no significant difference between SCF and SCF+Wnt. (N number of saline, SCF, Wnt, SCF/Wnt are 10, 10, 8, 12)

**3. All treatment groups demonstrated cell protection effect. Combined group showed trend of synergistic effect**

Western blot analysis was used to measure the neuroprotective effect of SCF and Wnt treatments. MMP-9 and MMP-2 were used to measure BBB disruption. All treatment groups showed significantly less BBB disruption. The combined treatment had significantly lower BBB disruption level than SCF or Wnt alone. Cas-3 was used to measure cell apoptosis. Treatment groups demonstrated a trend of decreasing cell apoptosis, although not showing significant differences. (N number of sham, saline, SCF, Wnt, SCF/Wnt are 2, 4, 3, 2, 2)

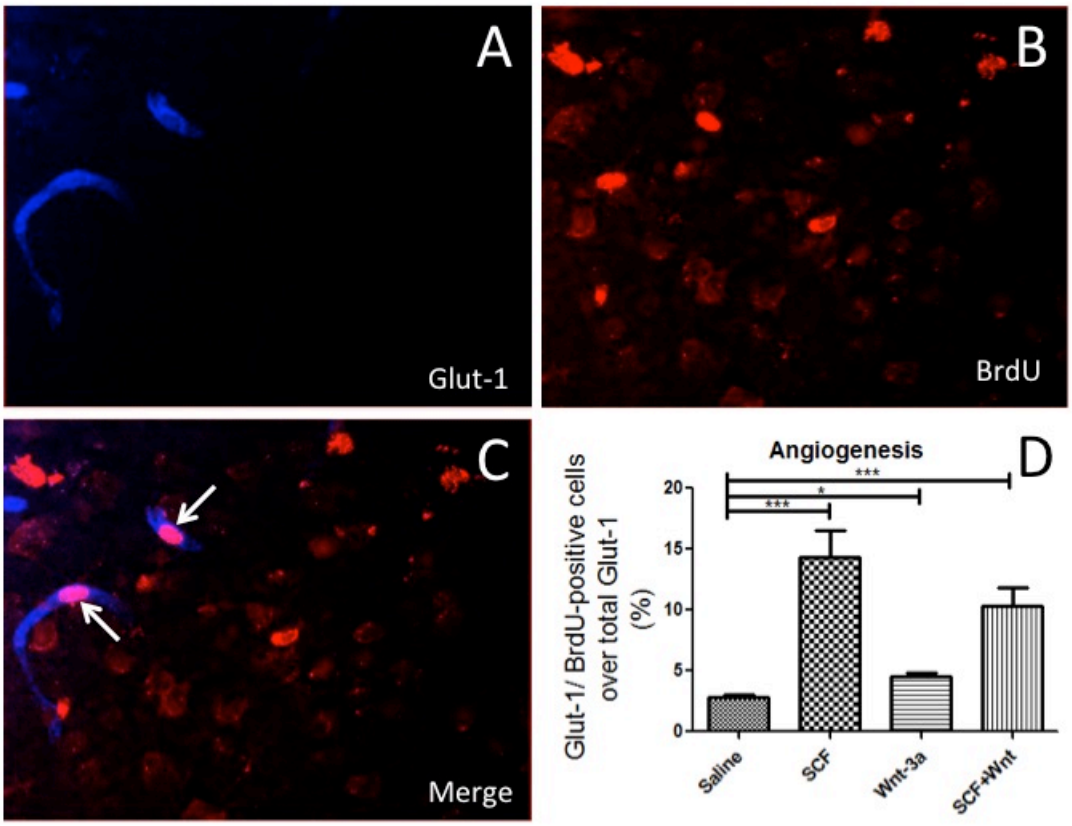
**4. All treatment groups showed sensorimotor functional recovery**

The sticky dot behavioral test was used to assess functional recovery. Animals had stroke in their barrel cortex, affecting their sensorimotor functions. The results corresponded with the predictions. An ANOVA test across latency and removal time of all five groups of animals on their right paws showed no significant difference. However, SCF and SCF+Wnt treatment groups demonstrated less latency time, and all three treatment groups showed decreased removal time, suggesting the functional recovery of sensorimotor ability. There was no significant different across three treatment groups in removal time, yet the trend showed the combined treatment decreased the removal time the most. (N number of sham, saline, SCF, Wnt, SCF/Wnt are 5, 3, 10, 8, 12)


A
B
NeuN
C
BrdU
D
Merge
E
Neurogenesis
NeuN/ BrdU-positive cells over total NeuN (%)
15
10
5
0
***
***
***
Saline
SCF
Wnt
SCF+Wnt

(n=10, 10, 8, 12)

**Figure 1. Both SCF and Wnt treatments enhanced neurogenesis.** (A) White circles indicate the locations of pictures taken. (B) and (C) NeuN were represented by green and BrdU were represented by red. Pictures were taken at 40x magnification. (D) Merged NeuN and BrdU channels. Arrows indicated co-labeled cells, which were considered as NeuN/BrdU positive cells, interpreted as newly proliferated neuronal cells. Only complete-overlap can be counted as BrdU positive cells. White circle indicates a non-complete-overlap NeuN, which was not considered as BrdU positive cell. (E) Percentage of neurogenesis. All three treatment groups had significantly higher neurogenesis rate than saline control. Wnt alone is significantly higher than SCF but the difference between Wnt and SCF+Wnt was not significant. Mean values were reported with standard error mean (SEM).




(n=10, 10, 8, 12)

**Figure 2. SCF and SCF+Wnt combined treatment groups showed enhanced angiogenesis.** (A) and (B) blood vessels were indicated by green and newly proliferated cells were indicated by red. (C) Merged Glut-1 and BrdU channels. Arrows indicated co-labeled cells, which were considered as Glut-1/BrdU positive cells, interpreted as newly proliferated blood vessel epithelial cells. (D) Percentage of angiogenesis. All three treatment groups had significantly higher angiogenesis rate than saline control. SCF alone is significantly higher than the other two treatment groups. Mean values were reported with standard error mean (SEM).

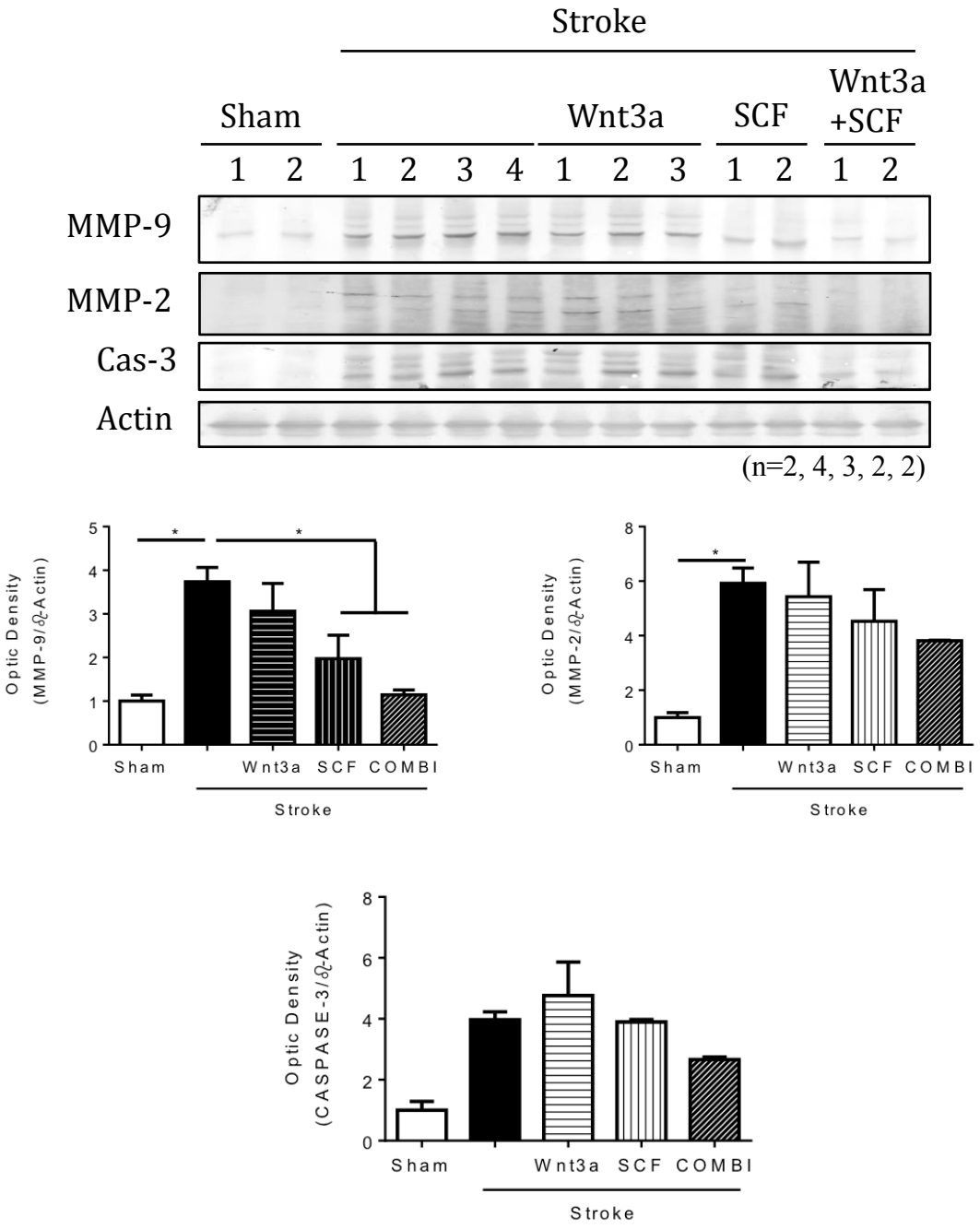



**Figure 3. Wnt3a, SCF, and the co-treatment after stroke in mice showed cell protective effect.** (A) Expression of MMP-9, MMP-2, Cas-3 and β-Actin. (B) Quantification of protein expression normalized by β-Actin expression. All treatment groups showed the trend of reducing cell apoptosis, illustrating cell protection effect.

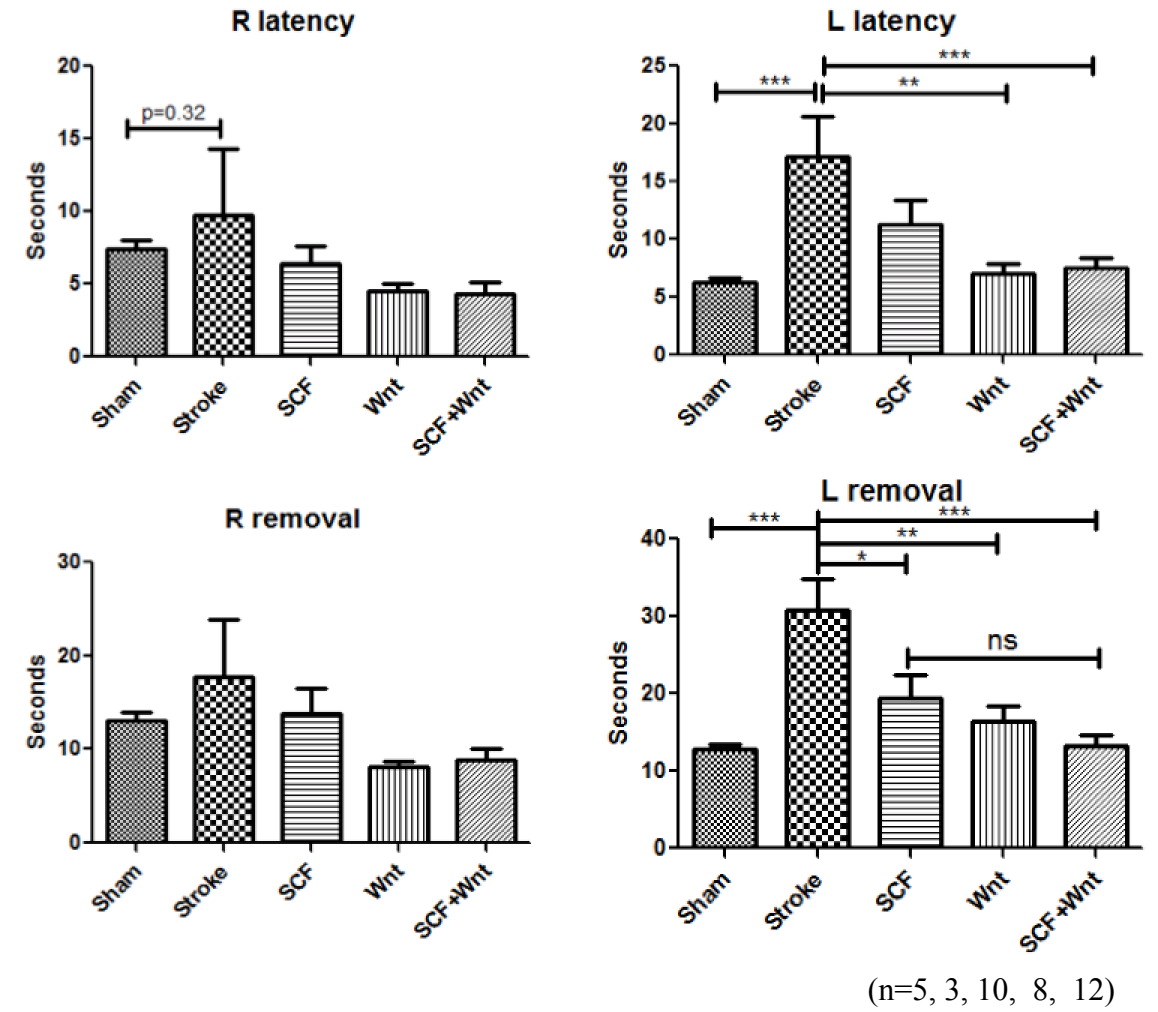



**Figure 4 SCF, Wnt and SCF/Wnt combined treatment significantly improved sensorimotor function recovery.** (A)(B) The latency and removal time on the right did not show any significant difference across all experimental group, which corresponded with our prediction. Mice had unilateral stroke on the right sensory cortex. Therefore, they should be intact on their sensorimotor function on the right side. (C) and (D) Saline control group displayed significant deficit in both latency and removal time on their left side. All treatment groups showed the trend of lowering the latency and removal time, suggesting improvements of sensorimotor functions.

**Discussion:**

In this investigation, we reported the therapeutic effects of SCF and Wnt treatments in cell protection, neurogenesis, angiogenesis, and functional recovery in treating focal ischemic stroke. Our current data partially support the hypothesis. Considering that regeneration is the long-term process, further investigation at delayed time point will be necessary to test our hypothesis.

***Histology data of Neurogenesis and Angiogenesis***

Neurogenesis was not considered possible until late 90s when researchers identified the two main stem cell niches in the brain: the subventricular zone (SVZ) of the lateral ventricle, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Doetsch and Scharff, 2001). Previous literature had shown the relationship between the Wnt signaling pathway and neurogenesis in the CNS – more specifically, in the hippocampus (Lie et al., 2005). Recently, many researchers, including our lab, have started to use Wnt as a therapeutic agent for stroke and other brain injuries. Wnt expression is up-regulated in the SVZ and it can enhance neurogenesis after stroke (Morris et al., 2007, Shruster et al., 2012). Our previous data have illustrated that Wnt-3a increases neurogenesis 14 days after ischemic stroke. Because of our unique barrel cortex focal ischemic stroke, we were able to observe Wnt-3a-induced neuronal stem cell migration from the SVZ to cortex 14 days after stroke. In this investigation, Wnt-3a demonstrated consistent neurogenesis ability. As mentioned in the introduction, studies have demonstrated that SCF is capable of neurogenesis *in vitro* and *in vivo* by means of haemopoietic stem cell mediation (Jin et al., 2002). The results of our project were thus consistent with these previous results. SCF

alone showed a neurogenesis effect, although the percentage was lower than those of the Wnt and Wnt/SCF combined groups. Therefore, we concluded that Wnt alone is enough to promote neurogenesis. In addition, all treatment groups showed an angiogenesis effect. According to Polyzoidis et al., the SCF signal is critical for activating mast cells, which are responsible for wound healing, angiogenesis, and BBB protection (Polyzoidis et al., 2015b). Therefore, we hypothesized that SCF will promote angiogenesis in the brain. Our results illustrated that SCF enhanced angiogenesis and the enhancement was significantly stronger than Wnt alone and Wnt/SCF combined group. Our data showed that Wnt alone is not capable of angiogenesis. Interestingly, the histology data did not demonstrate the predicted synergistic effect of combined group. First of all, by observing the data, we found that combine group had lower neurogenesis than Wnt alone and had lower angiogenesis than SCF alone. The interpretation could be the sub-cascade of two singling pathway might disrupt each other because SCF attenuated Wnt-induced neurogenesis, while Wnt attenuated SCF-induced angiogenesis. As described by Tartaglia et al., depending on specific signals, a protein tyrosine phosphatase can act either positive or negative regulator of the ERK, JAK/STAT and some other signaling cascades (Shi et al., 1998, Tartaglia et al., 2001). Therefore, it is possible that a molecule activated by the Wnt pathway shuts down one cascade of the SCF pathway and vise versa. However, the interaction between these two pathways is not clear. More basic studies need to be done in order to understand the interactions. Also it is possible that SCF and Wnt are activating multiple pathways and only some of them are synergistic while others are not. For example, we observed synergistic effect in western blot analysis of cell protection but did not observe synergy in the histology data. We speculated that the cell protection

pathways are interacting synergistically but not the regeneration pathways. Another possible explanation is that regeneration is a long-term process (Rama et al., 2010) and it could take up to few months to show therapeutic effects. Therefore, further investigation at delayed time points will be necessary.

***Western Blot Analysis Of Cell Protection Effects***

In the western blot analysis, all treatment group demonstrated cell protection effect. The Wnt/SCF combined group showed a significant synergistic effect in lowering BBB disruption level and, although not significant, a trend of synergistically lowering cell apoptosis rate. Antibodies to matrix metalloproteinase (MMP) have been shown to be associated with BBB opening and brain edema (Shigemori et al., 2006), and in a rat cortical contusion model, researchers found that MMP-9 was up-regulated after the injury (Aoki et al., 2002, Shigemori et al., 2006). MMP-2 is another enzyme that plays a critical role in regulating BBB permeability during ischemic conditions (Jin et al., 2010). Thus, MMP-9 and MMP-2 are often used as indicators of BBB disruption. In our results, all treatment groups showed lower MMP-9/2 expressions. Although only the decreases in MMP-9 expression were significant, MMP-2 showed similar trend. We interpret this difference in light of the molecular and functional differences between MMP-9 and MMP-2. According to a recent clinical research, MMP-2 upregulation was only observed in patients with mild stroke in a short time window (<12 hrs). However, MMP-9 upregulation was observed much later (days after stroke) and was related to more severe stroke (Lucivero et al., 2007). Earlier experimental studies found evidence that MMP-2 was responsible for the initial opening of the BBB after ischemic stroke, and that it was

blocked by a MMP inhibitor (BB-1101) within three hours (Rosenberg et al., 1998). However, MMP-9 mediates the second, delayed opening of BBB after cerebral ischemia, suggesting further damage of the tight junction and basal lamina protein of the BBB (Lee et al., 2007, Rosenberg and Yang, 2007, Yang et al., 2007). Animals for this western blot analysis were sacrificed on day 3 after stroke. Therefore, MMP-9 expression would be a more precise indication of the BBB disruption at this time point. It is possible that MMP-2 expression was already suppressed by the MMP inhibitor (BB-1101) in the brain because MMP-2 indicates earlier (within hours) BBB disruption level after ischemic stroke. Therefore, we cannot detect a significant difference between the decrease of BBB disruption level of saline control and treatment groups by MMP-2. However, the trend aligned with our prediction that the combined treatment group showed highest decrease of BBB disruption, suggesting a cell protection effect. In summary, we concluded from the western blot analysis that SCF, Wnt and SCF/Wnt combined treatments all demonstrated cell protective effect, and there is a possibility that SCF/Wnt combined treatment works synergistically. Also this data was highly consistent with our behavioral data, which I will explain later. Our interpretation of the synergy in western blot analysis was SCF and Wnt both triggered multiple pathways that played roles in different results. For example, the pathways of neurogenesis and angiogenesis might be conflicting with each other while cell protection pathways are mutually facilitating.

***Behavioral Assessment And Functional Recovery***

In order to measure the beneficial effect in promoting functional and motor recovery, we also performed behavioral tests. The adhesive removal test (sticky dots test) is an acute

measurement of the recovery of sensory motor function (Bouet et al., 2009, Freret et al., 2009). Since animals suffered a stroke in the barrel cortex, which controls their sensorimotor functions, stroke animals were expected to have longer latency and removal time because they would not be as sensitive as before (longer latency) and their motor coordination would also decrease (longer removal). In addition, since the stroke is unilateral on the right hemisphere, the animals should have deficits in sensing and removing the sticky dots on their left paws, yet be relatively unaffected on their right paws. Studies have reported that Wnt and SCF lead to functional recovery by using different behavior tests, including the limb placement test (Komitova et al., 2005), foot fault test (Hernandez and Schallert, 1988, Zhao et al., 2007), corner test, and cylinder test (Li et al., 2004) as described previously. However, these tests often are limited in testing one aspect of sensorimotor function recovery. For example, the limb placement test and foot fault test only indicates motor function recovery, while the corner and cylinder tests only indicate the sensory function recovery. In this investigation, the sticky dots test was used because it can indicate both sensory and motor functional recovery (Bouet et al., 2009). Our results fit with our predictions. At day 3, ANOVA test across all groups on the data of their right side did not show any significant difference, suggesting relatively intact neurological function on the right side. However, all treatment groups had significant decrease in both latency and removal time on their left side, suggesting recovery of the sensorimotor functions. Although there was no significant differences among three treatment groups (SCF, Wnt, SCF/Wnt combined), we speculate it was due to insufficient N numbers. However, the tread was consistent with our prediction. A poweranalysis should be ran to see how many more animals we need to add in order to

generate significant data. Interestingly, this data was consistent with the western blot data because they were both performances of the day 3 animals. We interpreted the functional improvements as a result of cell protection effect of Wnt-3a and SCF. To further confirm the therapeutic effects, including cell protection and regeneration, more comprehensive and sophisticated behavioral tests should be done. In addition, considering that regeneration is a long-term process, behavioral tests at short and delayed time points should be included as well.

## Limitations

In this study, we concluded that SCF, Wnt and SCF/Wnt combined treatments all demonstrated therapeutic effect on focal ischemia stroke. However, because the SCF/Wnt combined group only showed synergistic effect in some of the tests, we could only concluded it is possible that combined treatment will have synergistic effect, yet needs further investigations.

There are many limitations and improvements can be made in this study. First, we know that Wnt pathway induces neurogenesis by promoting neural stem cell to migrate out of the stem cell niche, but sole histology data of newly proliferated neurons were not able to illustrate this. Protein DCX localization overlaps microtubules in cultured neurons, therefore often used as an indicator of neuronal migration (Gleeson et al., 1999). A doublecortin (DCX) and BrdU immunohistochemical staining can make the study more comprehensive because we can visualize the migration pathway of the newly proliferated neurons. Secondly, a day 14 western blot analysis of neurotrophic factors, for example, brain-derived neurotrophic factor (BDNF), vascular endothelial growth factor (VEGF) etc., will support the observation of neurogenesis and angiogenesis molecularly. Third, a long-term behavioral assessment should be included to demonstrate the overall trend of behavioral improvements. For example, a fold-change (post-stroke latency and removal time/baseline removal time) of day 3, day7 and day 14 data can illustrate the sensorimotor recovery more clearly.

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