Co-Transplantation of Endothelial Progenitor Cells and Neural Progenitor Cells for Treating Ischemic Stroke in a Mouse Model

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CO-TRANSPLANTATION OF ENDOTHELIAL PROGENITOR CELLS AND NEURAL PROGENITOR CELLS FOR TREATING ISCHEMIC STROKE IN A MOUSE MODEL

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Wang, Jinju, Ph.D., Biomedical Sciences Ph.D. program, Wright State University, 2016. Co-transplantation of endothelial progenitor cells and neural progenitor cells for treating ischemic stroke in a mouse model.

Ischemic stroke (IS) is a major cause of death and disability with limited and less effective therapeutics. Transplantation of stem cells, such as neuronal progenitor cells (NPCs) and endothelial progenitor cells (EPCs), is promising for treating IS. It is well known that neurogenesis and angiogenesis are critical for cerebral repair following ischemic injury. However, whether co-transplantation of EPCs and NPCs has synergistic effects remains unclarified. In addition, the PI3K/Akt pathway participates in modulating a widely range of cellular functions such as anti-apoptotic and anti-oxidant. The signal pathways responsible for the effects of grafted NPCs and EPCs are also waiting for in-depth investigation. The purpose of this dissertation was to determine whether co-transplantation of EPCs and NPCs has synergistic effects on IS via activation of the PI3K/Akt pathway. This project was divided into two parts.

Part 1: By using hypoxia/reoxygenation-induced ECs and neurons (SH-SY5Y cells) injury models in the transwell co-culture system, we found that co-culture with EPCs and NPCs synergistically 1) reduced reactive oxygen species (ROS) over-production and apoptosis in ECs and neurons; 2) up-regulated the protein
expressions of VEGF and VEGFR2 in ECs, and of BDNF and TrkB in neurons; 3) up-regulated the expression of p-Akt/Akt in ECs and neurons. These effects were abrogated by the PI3K inhibitor (LY294002).

Part 2: By employing a mouse model of IS, we found that co-transplantation of EPCs and NPCs synergistically 1) improved motor function; 2) alleviated the pathological indexes (infarct volume, cell apoptosis and ROS production) and improved cerebral microvascular density; 3) increased the levels of VEGF and BDNF, as well as p-Akt/Akt in the ipsilateral brain in both subacute and chronic phases; 4) increased IL-10 level, whereas decreased plasma TNF-α and IL-1α levels in subacute phase; 5) promoted neurogenesis and angiogenesis. All of these effects were abolished by LY294002.

Taken together, these data demonstrate that co-transplantation of EPCs and NPCs augments the therapeutic efficacies for IS via synergistically activating the PI3K/Akt pathway, which provides a novel therapeutic strategy for IS.
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CHAPTER I. Purpose & Specific Aims

According to the latest report from American Stroke Association, ischemic stroke (IS) is the fifth cause of death and the leading cause of long-term disability in the United States. IS leads to cellular and architectural damage to the brain by a plethora of complex mechanisms including deprivation of energy, increase in intracellular calcium, generation of free radicals, disruption of blood-brain barrier (BBB), inflammation (leukocyte and microglia activation), and apoptosis of neurons, endothelial cells (ECs) and other brain cells (1). Pathologically, cerebral damage consists of two major zones, core and penumbra. Of note, although the dead cells in the core area are irreversible, the injured cells in the ischemic penumbra are rescuable if proper treatments are timely administered (2). The theoretical strategies of treating IS include earlier reestablishing blood supply (reperfusion) with thrombolytic drug, preservation of blood flow through vascular protection and bilateral circulation, neuronal protection, and promotion of new vessel formation and neurogenesis.

To date, tissue plasminogen activator (tPA) is the only thrombolytic drug approved by the Food and Drug Administration (FDA) for acute IS (3). Only 2-5% of patients are eligible for tPA treatment, and there is a therapy window of 3-4.5 hours within symptom onset. Administration of tPA beyond this window increases the risk of intracerebral hemorrhage (4, 5). Mechanical thrombectomy is another
common treatment to remove blood clots, especially for patients with large-vessel occlusions who respond poorly to intravenous thrombolytics (6). Neuroprotective drugs have been intended to treat acute IS. The efficacies of several agents such as antioxidant agents (Edavarone, Uric acid) (7, 8), neurovascular protectors (Citicoline) (9), cytokine antagonists (IL-1ra) (10) have promising results in various animal stroke models. However, no single neuroprotective agent has been presently validated by randomized, placebo-controlled IS trials (11, 12). In addition, hypothermia is a potentially promising neuroprotective therapy, unfortunately, no major clinical study has demonstrated a role of it in the early treatment of IS (13). Therefore, there is a urgent need for developing new strategy for IS

Stem cell-based therapy has been emerging as a potential therapeutic approach for IS (14, 15). Embryonic stem cells (ESCs), mesenchymal stem cells (MSCs), and hematopoietic stem cells (HSCs) have been shown to be associated with some alleviated neurological deficits in experimental stroke models (15-21). Transplantation of ESC-derived neural progenitor cells (NPCs) has been shown to differentiate into neurons and glial cells and re-establish the connections with infarct areas (20) and lead to improved motor function (21) in the stroke mouse model. We have recently shown that early infusion of endothelial progenitor cells
(EPCs) can protect the cerebral vasculature and neurons from acute damage, as well as promote the recovery (angiogenesis and neurogenesis) of IS (22).

It is recognized that neurogenesis and angiogenesis play critical roles in the repair of cerebral injury (23). Numerous animal studies have demonstrated that ischemia could stimulate endogenous neurogenesis in the subventricular zone (SVZ) (24-28). Meanwhile, stroke-induced endogenous angiogenesis in the boundary zone could provide scaffolds to guide the neural NPCs in SVZ to migrate toward the infarct region (23, 25, 29-31). Unfortunately, endogenous neurogenesis and angiogenesis are insufficient to adequately restore the function of the damaged brain in IS (32), especially in disease conditions such as diabetes (22). Thus, transplantation of exogenous stem cells for promoting angiogenesis and neurogenesis should be a potential strategy for cerebral repair.

As we know, EPCs and NPCs are important components for angiogenesis and neurogenesis. Transplantation of NPCs (20, 33-35) or EPCs (22, 36-39) has been actively investigated for treating IS. NPCs have been shown to differentiate into neural cells, release neurotrophic factors and promote neurogenesis in the ischemic brain. Systemic administration of EPCs has been demonstrated to promote angiogenesis and enhance blood flow supply in the peri-infarct area/penumbra of IS (22, 36-39). All of these findings indicate the therapeutic
potential of NPCs and EPCs in IS, albeit the exact molecular mechanism needs more investigation. The phosphatidylinositol-3-kinase (PI3K) pathway is known to be involved in a wide range of cellular processes such as anti-apoptosis and anti-oxidant stress (40). *In vitro* studies have indicated that the PI3K pathway is associated with the beneficial effects of stem cells such as MSCs, EPCs on protecting ischemic ECs or neurons (41-43). Our lab previously determined that the PI3K/Akt pathway was responsible for the beneficial effects of EPCs in stroke mice (22), however, it is still unknown whether this pathway can modulate the function of NPCs.

Increasing evidence suggests that neurogenesis and angiogenesis are coupled in the brain (30, 31). The blockade of stroke-induced angiogenesis can reduce neurogenesis (30). By using the *in vitro* cell-culture system, Chopp and his colleagues showed that cerebral ECs isolated from IS mouse brain could enhance NPC proliferation and differentiation, while NPCs isolated from IS animal was able to promote the angiogenic ability of ECs (44). We have recently demonstrated that infusion of EPCs could promote both angiogenesis and neurogenesis in a stroke mouse model (22). Therefore, we speculate that co-transplantation of EPCs and NPCs could synergistically produce acute vasculature protection as well as augment neurogenesis and angiogenesis, and provide a superior approach over the single-cell type one.
The purpose of this dissertation is to investigate whether co-transplantation of NPCs and EPCs can augment the therapeutic efficacy for IS via activation of PI3K/Akt signal pathway. The findings of this proposal could provide a novel approach of stem cell-based therapy for IS. The following diagram (Fig 1) depicts the central hypothesis of this project.
I hypothesized that co-transplantation of EPCs and NPCs can synergistically promote EC and neuron protection, enhance angiogenesis and neurogenesis, and ameliorate neurological deficits of IS via activation of the PI3K/Akt pathway.
In order to test my hypothesis, I have proposed three Specific Aims:

Specific Aim 1: **To determine whether co-culture of EPCs and/or NPCs can protect ECs against hypoxia/reoxygenation (H/R)-induced apoptotic cell death, reactive oxygen species (ROS) overproduction and decreased tube formation ability through activating the PI3K/Akt signal pathway.** Human brain microvascular ECs were used to produce a H/R-induced injury model for the *in vitro* study. First, I evaluated the effects of EPCs and/or NPCs on H/R-injured EC apoptosis, ROS overproduction and tube formation by co-culture experiments. Second, I investigated whether the PI3K/Akt signal pathway was involved in these effects by performing signal pathway inhibition experiments.

Specific Aim 2: **To determine whether co-culture of EPCs and/or NPCs can protect neurons from H/R-induced apoptotic cell death and ROS overproduction via activating the PI3K/Akt signal pathway.** The SH-SY5Y cells were used to produce a H/R-induced injury model for *in vitro* study. First, I assessed the effects of EPCs and/or NPCs on H/R-induced SH-SY5Y cell apoptosis and ROS overproduction by co-culture experiments. Second, I evaluated whether the PI3K/Akt signal pathway was involved in these effects through performing signal pathway inhibition experiments.
Specific Aim 3: **To evaluate whether co-transplantation of EPC and NPC has synergistic effects on ameliorating acute damage and promoting neurological recovery via the activation of PI3K/Akt signal pathway in a mouse model of IS.** The therapeutic effects of EPCs and/or NPCs on IS were evaluated by measuring IS injury (infarct volume, neurological deficits) and tissue repair (cell death, microvascular density, angiogenesis, and neurogenesis). I studied whether PI3K/Akt signal pathway was involved in these effects by pathway inhibition experiments.
CHAPTER II. Introduction

IS occurs when there is a sudden loss of blood flow leading to interruption of blood supply to the brain. Normal cerebral blood flow (CBF) is approximately 50 - 60 ml/100g/min. The reduction in CBF below 20 ml/100g/min results in an electrical silence and less than 10 ml/100g/min causes irreversible neuronal injury (45). Lack of blood circulation to the brain deprives neurons of necessary glucose and oxygen, and leads to neuron death. The majority (~ 87%) of cerebral strokes are ischemic and mainly result from the occlusion of a major cerebral artery by a thrombus or an embolism. The remaining cases (~ 13%) are either intracerebral or subarachnoid hemorrhage stroke. Stroke is the No.5 cause of death and a leading cause of long-term disability in the United States. On average, a stroke occurs every 40 seconds and a patient dies of stroke every 4 minutes.

Pathophysiology of cerebral ischemia

The pathophysiology of stroke in the brain includes energy failure, loss of ion homeostasis, increased level of intracellular calcium, neuronal excitotoxicity, free radical overproduction, cytokine secretion, disruption of the BBB and activation of glial cells, etc., which are inter-related and tightly coordinated. Within a few minutes of cerebral ischemia, brain cells including neurons and ECs in the core area where the cerebral circulation is completely arrested are irreversible
damaged (46). A zone of less severely affected tissue which surrounds the necrotic core is called penumbra or peri-infarct area (Fig 2) (47). Notably, the cells in the ischemic peri-infarct area may undergo apoptosis after several hours or days. Therefore, cells within the ischemic peri-infarct area can be rescued if therapeutic interventions are applied in a timely manner.
**Fig 2. A diagram showing the ischemic brain.** The cerebral core is damaged and irreversible. The penumbra is functionally impaired but potentially rescuable in IS and is the target for therapy.
**Cerebral cell death following IS.** Necrosis and apoptosis are the principal mechanisms of cell death after ischemic injury (48, 49). The extent of cerebral cell death depends on duration and depth of the ischemic insult. Without proper intervention, infarcts initially developed in the core will progress to encompass both core and penumbral regions in the subacute and chronic phases, causing severe damage (2).

A large body of data has suggested that excitotoxicity, radical stress (oxidative stress and free radicals), and apoptotic-like pathways are implicated in ischemia-induced brain cell death (48, 50-52). When the brain fails to generate sufficient ATP by the reduction of blood flow supply, energy failure occurs and ion gradients lost, the cellular membrane depolarized, and glutamate reuptake process impaired. The accumulated glutamate binds to its postsynaptic receptors such as α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and N-methyl-d-aspartic acid (NMDA) (53, 54), and thereby promoting excessive influx of calcium. This calcium overload can trigger phospholipases and proteases that degrade cellular membranes as well as proteins, and in turn can damage the integrity of the endothelium and generate free radical species (48). In addition, excessive sodium and water influx result in cell swelling and edema (48). In parallel with ionic and free radical pathways, deleterious molecules such as caspases, calpains, cathepsin B, nitric oxide (NO), and poly-(ADP-ribose)
polymerase also promote cell death (55). Following ischemia, caspase activation occurs in response to pro-apoptotic signals such as downregulation of B-cell lymphoma-leukemia 2 (Bcl-2) and up-regulation of the Bcl2-associated X protein (Bax). Among the identified caspases, caspase 3 activation is recognized as one of the downstream events in the apoptotic cascade (56, 57). Caspases 1, 8, and 9 have also been shown to be involved in cerebral ischemia (58). The major mechanisms that causing cell death through necrosis and apoptosis are summarized in Fig 3.
Fig 3. Major mechanisms of cell death in IS. Interruption of blood flow leads to a series of ischemic cascades: excitotoxicity, free radicals production, mitochondria dysfunction, BBB impairment, inflammation, etc, which are the major causes of cell death.
Besides, autophagy has been found to be constitutively active in the central nervous system (CNS). In early cerebral ischemia, autophagy protects neuronal injury through degrading damaged organelles, eliminating ischemia/reperfusion-induced damaged components and toxic metabolites (59, 60). It can provide nutrient source for maintaining metabolism, ATP levels, cellular homeostasis and survival (61-63). However, it has also been noted that excessive activation of autophagy may promote autophagic cell death (64). The over-activation of autophagy might cause neuronal cells to self-digest their own necessary components or interact with the apoptotic cascade, thus promoting nerve cell death in the ischemic surrounding zones in cerebral ischemia (65-67). To date, the precise role of autophagy in the brain still remains controversial.

**Dynamic vascular changes following IS.** Cerebral ischemia results in dynamic vascular changes which could be broadly divided into three phases: acute (hours), subacute (hours to days) and chronic (days to months) (68, 69). Vascular pathophysiology in the acute phase causes disruption of the BBB and dysregulation between vasodilation and vasocontraction, which plays an important role in regulation of cerebrovascular blood flow in response to ischemia. There are a number of vasoactive factors that affect endothelial function in the acute phase. For example, plasma and brain tissue levels of endothelin-1 (ET-1), a potent vasoconstrictor, are increased in patients with IS as
well as in animal models (68). Local application of ET-1 has been shown to
induce neuronal damage (70), proving evidence for the involvement of ET-1 in
the pathophysiology of acute IS. NO is an important endothelial derived
vasodilator generated by endothelial NO synthase (eNOS). Vascular NO
production regulates cerebrovascular perfusion and protects against stroke by
increasing collateral flow to the ischemic area (71). Mice lacking eNOS exhibit
larger cerebral infarctions (72). In contrast, enhanced NO production by
administration of the eNOS substrate, L-arginine, or up-regulation of eNOS by
statins confer protection from stroke (73, 74). Another important factor is vascular
endothelial growth factor (VEGF), which promotes endothelial integrity by
stimulating NO production, is critical for angiogenesis. However, VEGF increases
BBB permeability in the acute phase after IS (75, 76). Angiopoietin-1 is another
growth factor involved in regulation of integrity of endothelium (77). The level of
angiopoietin-1 decreases immediately after ischemia which positively correlates
with increased BBB permeability.

In the subacute phase, a number of pro-inflammatory cytokines including
interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) are activated in response
to the hypoxia (78). These products increase the expressions of adhesion
proteins, intercellular adhesion molecule 1 (ICAM-1) and vascular adhesion
molecule 1 (VCAM-1), which interact with neutrophils and thereby allowing them to penetrate into the vasculature and brain tissue.

The potential mechanism of vascular changes in the chronic phase of IS involves induction of genes that participate in the regulation of apoptosis as well as stimulation of angiogenic factors in ECs. Programmed cell death is triggered by activation of cell surface receptors via several factors including TNF-α, superoxide, and IL-1β, all of which are stimulated in the acute and subacute phases. In response to these stimuli, a cascade of proteolytic enzymes known as caspases and other proteins such as Bax and transformation related protein 53 as well as anti-apoptotic proteins including Bcl2 are activated (79). Therefore, inhibition of apoptotic gene expression and stimulation of anti-apoptotic proteins may offer a vascular protection strategy in this phase. Additionally, angiogenesis is important for stroke recovery, since angiogenic stimulation generates new vessels, which could increase the collateral circulation, repair the BBB functionality (80). Taken together, maintenance the integrity of vascular structure is critical for brain tissue repair in ischemia.

**BBB impairment following IS.** Functional integrity of BBB elements is critical for protection of the CNS from harmful blood substances. The reported incidence of BBB disruption in acute IS varies considerably from 15% to 66%, depending on
stroke severity, the applied methodology and timing of the evaluation (81-85). Impairment of BBB leads to brain edema and further exacerbate brain damage (86). Brain edema can be classified as cytotoxic/cellular edema and vasogenic edema (87). Cytotoxic edema evolves within minutes to hours and is potentially reversible. It is characterized by swelling of all the cellular elements of the brain, including neurons and ECs, due to failure of ATP-dependent ion (sodium and calcium) transport, as well as the released free radicals. Vasogenic edema occurs over hours and days and is irreversible. It causes increased permeability of brain capillary ECs to macromolecular serum proteins (e.g., albumin), resulting in increase in extracellular fluid volume along with increased intracranial pressure. This may displace the brain hemisphere, or shift one compartment of the brain which in turn compresses neurons, nerve tracts, and cerebral arteries. A sustained increase in pressure will cause persistent ischemia and irreversible damage to brain cells. Additionally, extravasation of red blood cells might lead to hemorrhagic transformation of the infarcted area.

Apart from the deleterious effect on brain cells, hypoxia also causes loss of structural integrity of brain tissue and blood vessels, partly through the release of proteases like matrix metalloproteinases (MMP) which could aggravate BBB disruption and soften the tissue and eventually manifest into brain swelling (86). It is reported that MMP-9 released by neurons, glia and ECs results in BBB
damage through digestion of the endothelial basal lamina (88, 89). In addition, as discussed above, oxidative stress also triggers recruitment and migration of neutrophils and leukocytes to the cerebral vasculature, which release enzymes that increase vascular permeability. Finally, the leaky BBB facilitates transmigration of inflammatory cells, promoting the post-ischemic inflammatory response (90). To this end, reconstruction and maintainence of the integrity of BBB are critical procedures for treating IS.

**Inflammation following IS.** Increasing evidence demonstrate that inflammation plays a role in development and progression of stroke, especially in the context of reperfusion (91-95). Focal ischemia evokes a robust inflammatory response that begins within a few hours of onset and typifies the secondary or delayed response to ischemia. As noted above, interruption of cerebral blood flow leads to energy depletion and neuron death, which trigger immune responses and ultimately lead to inflammatory cell activation and infiltration. The recruitment of neutrophils to the ischemic brain begins with neutrophil rolling on the activated endothelial blood vessel walls which is mediated by selectins, followed by neutrophil activation and adherence which are mediated by integrin and immunoglobulin. When adhered to cerebral blood vessel walls, neutrophils transmigrate into the cerebral parenchyma, a process facilitated by BBB disruption. The recruitment of neutrophils can obstruct the microcirculation and
prevent complete restoration of cerebral blood flow after reperfusion. This blockage may cause further tissue damage after ischemia (94). In addition, the resident cells include ECs, neurons within brain tissue also secrete pro-inflammatory mediators such as IL-1, TNF-α, interleukin-6 (IL-6) and platelet-activating factor after an ischemic insult which contribute to the inflammatory response (96). These substances may produce an amplification of inflammatory signal cascades, and even disrupt of the BBB (86). What’s worse, these inflammatory mediators will ultimately appear in the ischemic penumbra and increase the cell death in this area. Among cytokines, IL-1, TNF-α, IL-6 and interleukin-10 (IL-10) are the most studied cytokines related to inflammation in stroke (97-99). IL-1 has been shown to cause up-regulation of E-selectin, ICAM-1 and VCAM-1 on cerebral ECs. The induction of these adhesion molecules may explain why the elevated level of IL-1 after ischemia increases neutrophil infiltration (94, 100). The detrimental effects of IL-1 is also demonstrated by another study showing that administration of recombinant IL-1 receptor antagonist reduces the severity of neurologic deficits and tissue necrosis in rats subjected to permanent middle cerebral artery occlusion (MCAO) (94, 101). IL-6 is largely thought of as a pro-inflammatory cytokine, and its biological activity overlaps with those of IL-1, but it is unclear whether the overall effect of IL-6 is beneficial or detrimental in the context of stroke (97). A clinical study revealed that a lower level of the anti-inflammatory IL-10 is related to larger infarctions and
poorer outcome in stroke patients (99). Like IL-1, TNF-α induces adhesion molecule expression in cerebral ECs and promotes neutrophil accumulation and transmigration (78).

In addition, IS is a powerful stimulus that triggers microglial activation. Once activated, microglia develop macrophage-like capabilities such as phagocytosis, cytokine (IL-6, TNF-α, etc.) production, ROS and NO production, antigen presentation and release of MMPs (MMP 9, MMP3) (102). As a result, peripheral leukocytes infiltrate into the brain that further exacerbate inflammation and brain damage. On the other hand, microglia release anti-inflammatory cytokines (IL-10, TGF-β, etc.) to restore tissue homeostasis by clearing pathogens or necrotic cells, and consequently attenuate the detrimental effects of inflammation and aid in tissue repair (95, 103). It has been shown that TGF-β released by microglia promotes an anti-inflammatory profile associated with increased proliferation and neuro-protection in the ischemic brain (104). Because of the critical roles in the immune response to stroke, microglia have become an important target for new therapeutic approaches to limit stroke damage, but therapeutic approaches that specifically target microglia are currently lacking.

**Current therapeutics for IS**
The most important step in alleviating ischemia injury is to quickly restore blood flow that occurs either naturally or with the aid of thrombolytic drugs. Yet, this might cause further damage the brain tissue because of the reperfusion injury. To date, administration of tPA to directly dissolve of thrombi in affected blood vessels is the gold therapy for acute IS. However, its application is often limited by a narrow window of 3-4.5 hours after stroke symptoms occur, and with deleterious side effects like hemorrhagic transformation which might exacerbate stroke injury (4, 5). Another treatment is an endovascular procedure (also called mechanical thrombectomy) which aims to remove the blood clot, especially for patients with large-vessel occlusions who respond poorly to intravenous thrombolytics (6). Other medications such as antiplatelet agent aspirin and anticoagulants warfarin are less effective once stroke occurs. In general, the current therapy for IS is limited, therefore, there is an urgent need for developing clinically effective treatments for IS.

**Stem cell-based therapy for IS: progress and prospects**

Accumulating studies have demonstrated that stem cell-based therapy is a promising approach for in IS (22, 38, 105). There are two major strategies for stem cell-based therapy for IS: one is mobilization of endogenous stem cells such as NPCs, EPCs, MSCs, the other one is transplantation of exogenous stem cells. Transplantation of stem cells has been shown to be directly beneficial to
the injured brain through stimulating angiogenesis and neurogenesis and thereby ameliorating neurological deficits (22, 38, 106).

**The promising of EPCs for treating IS.** EPCs are resident in the bone marrow (BM) blood sinusoids and circulate in the peripheral blood. Once hypoxia occurs, EPCs mobilize from BM to the injury site where they are differentiated into ECs to repair damaged endothelium and participate in new blood vessels formation (107). Therefore, EPCs are considered to play a very important role in vascular repair after vascular injury and in the maintenance of endothelial integrity. Over the last few years, EPCs have also been studied as biomarkers to assess the risk of cardiovascular diseases in human subjects. A low number of EPCs could predict severe functional impairment in several cardiovascular pathologies such as diabetes (108) and hypertension (109). Meanwhile, several studies have indicated that exogenous EPCs are one of the promising strategies for cell therapy after vascular ischemic injuries (110, 111). Indeed, EPCs can restore blood flow and promote vascular repair in the animal model of hindlimb ischemia (111). It is reported that peripheral blood-derived EPCs can incorporate into the foci of myocardial neovascularization (110). In addition, EPCs are considered to have a great potential for neurorepair following IS, based on their abilities to differentiate into ECs and to secrete protective cytokines and growth factors (112-114). Previous studies have demonstrated that transplantation of EPCs
provided a favorable environment for neuronal regeneration (15), reduced infarct volume accompanied with improved motor function in IS mice (113, 115). Our earlier animal studies showed that gene-modulated EPCs could protect vascular damage from ischemia (22), and exert better therapeutic efficacy in improving neurological deficit and enhancing angiogenesis than non-modulated EPCs in an IS mouse model (22, 38). Also, intravenous infusion of autologous EPCs after MCAO in rabbits showed functional improvement and increased microvessel density in the ischemic boundary area (116). Taken together, these findings suggest that EPCs possess therapeutic potential for IS.

**The promising of NPCs for treating IS.** Neurons are the core components of the brain that has a limited capacity for self-repair due to the inability of generating mature neurons to undergo cell division. In most cases, mature neurons are regenerated by adult NPCs, which are found mainly in 2 regions in brain: SVZ of the lateral ventricles (LV) and subgranular zone (SGZ) in the dentate gyrus (DG) of the hippocampus (117). The endogenous NPCs response to stroke has been broadly divided into three phases (118): 1) activation of the SVZ niche, 2) NPC migration and differentiation, 3) integration. Nevertheless, the endogenous neurogenic response after stroke is insufficient for self-repair of the ischemic brain. This is in part due to the fact that activated SVZ NPCs have to migrate long distances from the site of origin to the site of damage and where
they differentiate into neurons. Evidence have demonstrated that more than 80% of these immature neurons die before they reaching the damaged region, and only a small fraction (0.2%) of damaged neurons can be replaced (26), indicating that manipulation of endogenous NSCs alone could not be sufficient to adequately repopulate damaged brain cells. Transplantation of exogenous NPCs might be an alternative approach. Indeed, intravenous infusion of NPCs increases dendritic length and the number of branch points in host neurons (119). What’s more, the transplanted NPCs promote endogenous neurogenesis in the peri-infarct area (120) and ameliorate neurological behavioral deficits in IS animal models (121, 122). Embryo-derived NPCs can migrate toward the infarct region, survive and differentiate into mature neurons to some extent after transplantation (121). Collectively, these findings indicate that the great promising of NPCs for IS treatment.

**Other stem cells for treating IS.** There are several other types of stem cells such as ESCs, MSCs and HSCs have been used for treating IS in animal models (16-19). In general, the beneficial effects of these stem cells for treating IS are limited because of their application limitations. For instance, ESCs suffer from a series of constraints including ethical concerns, limited availability, potential of teratoma formation and immune rejection (16). HSCs have limited plasticity since they can only differentiate into blood and blood-related lineages. In addition,
there are only a small number of HSCs in BM and umbilical cord, the cells require \textit{ex vivo} expansion for clinical application (17). BM-derived MSCs may be superior to ESCs and HSCs. Previous studies have showed that BM-MSCs can enhance brain repair and ameliorate ischemic tissue damage (19), but they are unlikely to be an effective source for replacement of neural cells. The small quantity of MSCs in BM and the significant decrease of proliferation and differentiation potential with age limit the use of MSCs. Moreover, the invasive harvesting procedure of BM may lead to complications and morbidity (18). Besides, the extent to which MSC subpopulations can be directed to a neural cellular fate following transplantation remains a point of contention among researchers. More recently, inducible pluripotent stem (iPS) cells which are reprogrammed from somatic cells offer new prospects for regenerative medicine for stroke. The iPS cells behave in a manner similar to that of ESCs with high reproduction ability and pluripotency to differentiate into cells of any of the three primary germ layers like EPCs, NPCs, cardiomyocytes and smooth muscle cells (123-125). Some studies have demonstrated transplantation of iPS cells improved the sensorimotor functions (126, 127) and reduced infarct size (126) in the animal model of stroke. However, the use of iPS cells appears to have some ramifications. In fact, a high rate of tumorigenesis after transplantation of undifferentiated iPS cells is reported (128, 129).
Issues and perspectives of stem cell-based therapy for IS. To date, clinical trials of stem cell therapy have been conducted in patients with stroke including using MSCs (130, 131), bone marrow mononuclear cells (MNCs) (132, 133) and NPCs (134). Jiang and his colleagues delivered MSCs via catheter to the proximal end of the lesion artery in patients with stroke and observed improvement of the modified Rankin scale (130). However, the efficacy cannot be determined due to small number of enrolled patients. The transplanted teratocarcinoma-derived NPCs provided positive effects in some chronic stroke patients in phases I and II clinical trials, but no overall benefit was confirmed statistically (134). In general, cell transplantation therapy for stroke holds great promise, albeit many issues/questions remains to be clarified. For example, the type of cells and the number of cells should be used for obtaining greater therapeutic effects remain to be answered. How (route of delivery) and what time point should the exogenous cells be administered in order to improve survival and neural differentiation of transplanted cells remain unknown. The choice of cell to be transplanted following a stroke is likely to be dependent on the intended repair process. It seems most likely that stem cell-mediated neurological repair could be mediated by various trophins, which are either secreted from the cells, or are up-regulated in the host. In this regard, the transplanted cells function as trophic ‘machines’ secreting an diversity of factors, variably involved in angiogenesis, neurogenesis and host plasticity. Once the role of each trophin
has been characterized following a stroke, it may be possible to produce cells that home to the site of ischemia and over-express the desired factors. As mentioned above, the cell numbers and timing of delivery are further unresolved issues. There is some evidence of a dose-dependent mechanism of recovery in a study of a rodent model of stroke transplanted with MSCs (135), translation of this data to human studies however remains difficult. The timing of cell therapy is still unclear, which will need to take into account the mechanism of action. For example, a neuroprotective strategy would require acute delivery, whereas a neurorestorative strategy which may allow delivery from weeks to months after the event. Acute delivery of stem cells would also need to take into account many factors such as the clinical instability of stroke patients, the inflammatory response immediately post stroke (136), which may interfere in graft survival.

The optimum route of delivery for cells has not yet been established, though rodent models of stroke have showed functional improvement with intra-striatal, intravenous as well as intra-arterial delivery of stem cells (135, 137-140). It is suggested that intracerebral injection could precisely transplant cells into the striatum of ischemic brain. Other injection routes may lead to cells be trapped into systemic organs, with few cells reach the ischemic brain hemisphere (141). Also other groups state that the optimal route of cell transplantation might vary with different cell populations (139, 142). Additionally, how to enhance the restorative responses of the newly formed cells with the existing circuitry need to
be investigated. Longer-term studies are required to determine whether the cell-enhanced recovery is sustained and also to determine the tumorigenic potential of the cells. Tumors could possibly arise directly by transformation of the transplanted cells or indirectly by the induction of endogenous tumors through secretion of factors by the transplanted cells.

Taken together, the main objective of stem cell-based therapy is to repopulate the damaged tissue with functional cells, and these cells will integrate with the remaining functional native cells and contribute to the recuperation of the lost organ function. Due to the extensive overlap that exists with the mechanisms of ischemic damage activated by stroke, combinatorial therapy may represent the most promising direction for future stroke research.

**Signal pathways implicated in IS**

**The PI3K/Akt pathway.** The PI3K/Akt signal pathway is an important pro-survived pathway which widely participates in diverse cellular processes such as cell proliferation, apoptosis, and migration (40, 143, 144). It has been shown that phosphorylated Akt could promote cell survival by phosphorylating several substrates, including Bcl-2-associated death promoter (BAD), caspase-9, and Forkhead transcription factors (145). Activation of the PI3K/Akt pathway has been reported to reduce apoptosis of myocardial cells induced by ischemia (146,
The effect of brain derived neurotropic factor (BDNF) on promoting EC migration and enhancing the angiogenesis following myocardial infarction has been found to be modulated by the PI3K/Akt pathway (148). Likewise, activation of the PI3K pathway contributes to the protection of the brain from ischemic injury. It has been showed that VEGF protects the brain against focal cerebral ischemia through activation of the PI3K pathway (149), albeit it might increase the BBB permeability. BM-derived stem cells mediate cell survival and axonal outgrowth of neurons via activating the PI3K/Akt signal pathway (41, 42). More recently, we found that the PI3K/Akt/eNOS signal pathway was responsible for the therapeutic effects of modified EPCs (CXCR4 primed) in treating diabetic IS mice (22), and it mediated the protection effects of EPCs-derived microvesicles on ECs against H/R injury (43). All together, these findings indicate that appropriate activation of the PI3K pathway may be useful for promoting brain cell survival and thus reducing cell death after stroke.

The P38 MAPK pathway. Mitogen-activated protein kinases (MAPK) is an important family of enzymes transducing a wide range of extracellular signals like inflammation, growth factors, and toxic stimuli as well as integrating corresponding cellular responses (150). Among MAPKs, p38 is of particular importance since it transduces cellular inflammation (151). Recent reports have suggested that the p38 pathway is activated in neuronal cells (152-155).
Selective p38 MAPK inhibitors can promote the survival of neurons (152, 154, 156) and inhibit inflammatory mediators/proteins such as TNFα and IL-1b production (157, 158), inducible nitric oxide synthase (159), cyclooxygenase-2 (160), IL-6 (161), and IL-8 (162). Kawasaki et al showed that glutamate-induced apoptosis activates the p38 pathway through the activation of NMDA receptor in cerebellar granule cells, and that SB203580, a specific inhibitor for p38, inhibited glutamate-induced apoptosis (154). Ghatan and his colleagues suggested that p38 activity might play a critical role in NO-mediated neuronal cell death, by stimulating Bax translocation to the mitochondria, thereby activating the cell death pathway (163). In vivo studies have revealed that a significant increase of p38 activity was observed in the ischemic brain, whereas, no activation of p38 was detected in non-ischemic cortex or in the cortex following sham stroke surgery (164). But, astrocyte activation seems independent of the p38 MAPK because that delayed activation of p38 MAPK following ischemic injury corresponds with glial fibrillary acidic protein (GFAP) upregulation (indicator of astrocyte activation) in the penumbra region of astrocyte-specific p38 MAPK knockout mouse (165). Although there is a general consensus that the p38 pathway induce neuronal death, there are some reports that showed that the activation of the p38 pathway might be involved in neuronal cell survival (164, 166), suggesting that the roles of MAPK cascades on neuronal death and survival depend on the timing of expression in a given condition.
Altogether, targeting the p38 MAPK pathway might provide an opportunity for treating IS.
CHAPTER III. Co-culture of EPCs and/or NPCs protected ECs against H/R-induced apoptotic cell death, ROS overproduction and decreased tube formation ability through activating the PI3K/Akt signal pathway.

Rationale: Cell-cell interactions between neuronal and vascular compartments in the brain is important, because the change of vascular plays a critical role in homeostatic response mechanisms which balance oxygen supply to hypoxic stress-sensitive neurons (30, 167, 168). It is also known that brain ECs are critical components of the BBB. Increased BBB permeability leads to the development of tissue swelling, inflammatory cell infiltration and subsequently exaggerate injury in IS (169). Therefore, protection of ECs and maintenance of endothelium integrity could be an important strategy for reducing ischemic injury.

EPCs have been suggested to participate in EC protection, repair and angiogenesis (107). Transplantation of EPCs is a promising cell therapy for ischemic diseases such as acute myocardial infarction and stroke (22, 110, 170). Our previous studies have shown that EPC infusion promotes angiogenesis in mouse IS models (22, 38). EPCs released angiogenic growth factors, such as VEGF and insulin-like growth factor, have been suggested to be the beneficial effect of EPC conditioned medium on the viability of hydrogen peroxide-compromised human umbilical vein ECs (171, 172). Currently, we do not know whether EPCs can protect cerebral ECs against H/R-injury.
Transplantation of NPCs has also been shown to be effective for treating IS in animal models (20, 34). In addition to generating neurons, grafted NPCs could promote angiogenesis in a rodent stroke model (173). A recent report suggests that co-culture with NPCs decreases the passive permeability of brain ECs (174). Collectively, these studies indicate a crosstalk between NPCs and ECs. However, it is unclear whether NPCs and EPCs have synergistic effects on EC protection against hypoxia.

The PI3K/Akt signal pathway participates in various cellular processes such as cell survival and proliferation (40). Previous studies have shown that activation of the PI3K/Akt signal pathway promotes neuron survival (143, 144), cardiac microvascular EC migration (148), and axonal outgrowth compromised by oxygen-glucose deprivation (41, 42). It is unknown whether this pathway is involved in the mechanism of the benefits of NPCs and EPCs on IS.

The aims of this study were to determine whether co-culture of EPCs and/or NPCs can protect ECs against hypoxia/reoxygenation (H/R)-induced apoptotic cell death, reactive oxygen species (ROS) overproduction and decreased tube formation ability through activating the PI3K/Akt signal pathway.

Experimental design
The H/R injury model of ECs were induced by 6 hour hypoxic culture followed with 24 hour reoxygenation. During the reoxygenation period, ECs were divided into 10 co-culture groups (n=4/group): Vehicle, EPCs, EPCs + LY294002, EPCs + SU1498 + k252a, NPCs, NPCs + LY294002, NPCs + SU1498 + k252a, EPCs + NPCs, EPCs + NPCs + LY294002, EPCs + NPCs + SU1498 + k252a. At the end of experiment, the conditional culture medium from each group were used for enzyme-linked immunosorbent assay (ELISA) of VEGF and BDNF. Cells were collected for various analyses: 1) methyl thiazolyl tetrazolium (MTT) assay for cell viability, 2) flow cytometry for cell apoptosis and ROS analyses, 3) cell functional assays (paracellular permeability and tube formation ability), 4) western blot analysis of Akt, pAkt, eNOS, VEGFR2, etc.

Materials and methods

Cell culture of human iPS cells and human brain microvascular ECs

The vector-free viral-free human iPS cell line (iPS-DF-19-9/7T) was purchased from Wicell Research Institute (WI). The iPS cells were cultured with mTeSR1 medium (StemCell technologies, Vancouver, Canada) on matrigel-coated plates (0.5mg/well of a 6-well plate; BD Biosciences, CA), and expanded every 4-5 days according to the manufacturer’s instruction. Passage of 28 to 44 of human iPS cells were used for NPC or EPC induction.
Human cerebral microvascular EC cell line was purchased from Cell Systems (Kirkland, WA). ECs were seeded on regular tissue culture plates with attachment factor and cultured in CSC complete medium containing 10% fetal bovine serum (FBS), 2% culture Boost-R (human recombinant growth factors) and 0.2% Bac-off antibiotic solution under standard cell culture conditions (5% CO₂, 37°C). The CSC culture medium was replaced every 48 hours according to the manufacturer’s instruction. Passages 4 to 13 of ECs were used for experiments in this study.

All experimental procedures were approved by Wright State University Institutional Biosafety Committee and were in accordance with the approved guidelines.

**Induction of NPCs and EPCs from human iPS cells**

NPCs were produced from human iPS cells according to previous reports with slight modifications (143, 144). **Fig 4** shows the time flow of producing EPCs and NPCs from human iPS cells.
Fig 4. Time flow of induction of EPCs and NPCs from human iPS cells.

The generated NPCs were confirmed with neural progenitor markers nestin and pax6 using immunofluorescence, and differentiated into neurons which were verified by β-tubulin staining. The generated EPCs were purified using magnetic activated cell sorting (MACS) with EPC specific marker CD34 followed by flow cytometry analysis and functional analysis using the angiogenesis commercial kit. iPS cells: inducible pluripotent stem cells. NM medium: neurobasal medium; EBM2 medium: EPC medium; EPCs: endothelial progenitor cells; NPCs: endothelial progenitor cells; MACS: magnetic activated cell sorting.
In brief, the human iPS cell colonies were detached with dispase (2 mg/ml in DMEM/F12; StemCell Technologies, Vancouver, Canada) and used for embryonic body (EB) culture with EB medium (DMEM/F12+ 20% knock out serum + 1% nonessential amino acid + 0.1 mM 2-mercapethonal + 1% penicillin-streptomycin solution) in low-attachment dishes (Corning, NY) for 5-7 days. The DMEM medium and all supplement reagents were purchased from Gibco. For NPC generation, EBs were cultured in neural medium (NM: Neurobasal medium A + B27 minus Vitamin A (1x) + N2 (1x) + FGF (20 ng/ml) + EGF (20 ng/ml) + 1% penicillin-streptomycin solution) in low-attachment dishes for 7-9 days. The neurobasal medium A and all supplement reagents were purchased from Gibco. The generated NPCs were propagated in free-floating aggregates (neurospheres), and used for an in vitro differentiation assay to investigate neural differentiation ability as previously reported (175). The generated NPCs and neurons were confirmed by immunofluorescence analysis (176).

For EPC generation, EBs were cultured on gelatin (0.1%; Corning, NY) coated plates with EPC medium (EBM-2 medium + growth factor mixture + 5% FBS + VEGFA (50 ng/ml) + FGF (25 ng/ml) for 7-9 days (144). The EBM-2 medium and all supplement reagents were purchased from Gibco. The generated EPCs were purified by MACS with CD34-microbeads according to the manufacture’s protocol (Miltenyi Biotec Inc, CA), and assessed by flow cytometry and matrigel assay.
Characterization of generated NPCs

For immunofluorescence analysis, generated NPCs were cultured on matrigel-coated wells (0.25mg/well of a 12-well plate; BD Bioscience) with neural medium (NM: Neurobasal medium A + B27 minus Vitamin A (1x) + N2 (1x) + FGF (20 ng/ml) + EGF (20 ng/ml) + 1% penicillin-streptomycin solution) overnight. The next day, culture medium was removed. Cells were rinsed with phosphate buffer saline (PBS), and fixed with 4% paraformaldehyde (PFA) for 10 minutes at room temperature (RT), permeabilized and blocked with blocking buffer (PBS with 1% BSA and 1% Triton-100) for 30 minutes at RT. After being washed 3 times with PBS, cells were incubated with neural progenitor specific markers mouse anti-nestin (1:100; Pierce, CA), mouse anti-pax6 (1:50; Pierce, CA) and pluripotent specific marker rabbit anti-Oct3/4 (1:200; Abcam, MA) overnight at 4°C. Then, all samples were washed with PBS 3 times, 5 minutes each, and were incubated with secondary antibodies for 2 hours at RT. The secondary antibodies were Alexa flou 488-conjugated donkey anti-mouse, Cy3-conjugated donkey anti-mouse, or Cy3-conjugated donkey anti-rabbit (1:150; Jackson ImmunoResearch, PA). DAPI (0.5 ng/µl, Sigma, MO) was used to counterstain nuclei. All images were taken under an inverted fluorescence microscope (EVOS, Life Technologies, CA).
In order to determine the neuron differentiation capability of the produced NPCs, the generated NPCs were cultured on laminin-coated wells (5 µg/cm²; BD Biosciences, CA) with neuron differentiation medium (NPBM medium + 20 ng/ml BDNF + 20 ng/ml FGF + 20 ng/ml EGF + 1% penicillin-streptomycin solution) for 3 weeks. The NPBM medium and all supplement reagents were purchased from Gibco. The differentiated cells were rinsed with PBS, fixed with 4% PFA for 10 minutes at RT, permeabilized and blocked with blocking buffer (PBS with 1% BSA and 1% Triton-100) for 30 minutes at RT. After being washed 3 times with PBS, cells were incubated with neuron specific marker mouse anti-β-tubulin (1:100; Pierce, NY) overnight at 4°C and followed by 3 washes with PBS. Then, cells were incubated with Cy3-conjugated donkey anti-mouse secondary antibody (1:150; Jackson ImmunoResearch, PA) for 2 hours at RT. DAPI (0.5 ng/µl, Sigma, MO) was used to counterstain nuclei. All images were taken under an inverted fluorescence microscope (EVOS, Life Technologies, CA).

**Purification and characterization of generated EPCs**

In order to exclude the contamination of human iPS cells, the generated EPCs were purified by using MACS according to the manufacture’s instruction. In brief, the differentiated cells (1 x 10⁷ cells) were incubated with 10 µl CD34-conjugated microbeads (Miltenyi Biotec Inc, CA) for 20 minutes at 4°C, followed by 2 washes with PBS. The beads-binding cells were separated using an autoMACS.
separator (Miltenyi Biotec Inc, CA). All CD34+ cells were resuspended with EPC culture medium and cultured in a regular humidified incubator (5% CO₂, 37°C). For flow cytometry analysis, the purified EPCs were incubated with FITC-conjugated CD34, FITC-conjugated kinase insert domain receptor (KDR), FITC-conjugated Oct3/4 or FITC-conjugated IgG for 30 minutes (5 μl, eBioscience, CA) in 100 μl reaction volume in the dark. All antibodies were purchased from eBioscience. After incubation, all samples were analyzed under flow cytometry (Accuri C6 flow cytometer, BD Biosciences, CA). 10,000 events were collected for data analysis.

**H/R-injury models of ECs**

The H/R-injury model of ECs was produced as previously described (43). Briefly, ECs (4×10⁵/well) growing on 6-well plates were changed with fresh culture medium and cultured for 6 hours in a hypoxic chamber (1% O₂, 5% CO₂, and 94% N₂; Biospherix, NY), then re-oxygenated by incubation in a standard 5% CO₂ incubator for 24 hours. During the re-oxygenation period, ECs were co-cultured with EPCs and/or NPCs using the transwell co-culture system.

**Transwell co-culture of ECs with EPCs and/or NPCs**

The co-culture experiments were conducted by using the transwell co-culture system (Fig 5).
**Fig 5.** A diagram shows the transwell co-culture system used for EC, EPC and NPC co-culture. EPCs and NPCs were seeded into the transwell membrane inserts one day prior to co-culture. On the co-culture day, the insert containing EPCs and NPCs were placed into the wells containing ECs.
The protocol for co-culture was set up according to a previous report with minor modifications (44). In brief, the day before co-culture, NPCs (4×10^5/insert), or EPCs (4×10^5/insert), or a mixture suspension of NPCs (2×10^5) and EPCs (2×10^5) were plated into transwell membrane inserts (pore size, 0.4 μm; polycarbonate membrane, Greiner Bio-One, Germany) in NPC and/or EPC culture medium overnight (177). The ECs (4×10^5/well) subjected to hypoxic (1% O_2) were randomly divided into four groups and co-cultured with: 1) vehicle (EC culture medium only), 2) EPCs (co-cultured with EPCs), 3) NPCs (co-cultured with NPCs), 4) EPCs+NPCs (co-cultured with EPCs and NPCs), by placing the inserts containing EPCs and/or NPCs in the EC culture plates for 24 hours. For signal pathway study, LY294002 (PI3K inhibitor; 20 μM, Cayman Chemical Company, MI), and a combination of SU1498 (VEGFR inhibitor; 5 μM, BioVision Inc, CA) and K252a (TrkB inhibitor; 10 μg/ml, BioVision Inc, CA) were added to EC culture medium 2 hours prior to co-culture experiments in some groups (43, 44, 177, 178) and presented in the EC culture during the co-culture period. All inhibitors were dissolved with DMSO (Sigma, MO) and diluted with culture medium to yield desired concentrations. ECs cultured in normoxia (5% CO_2, 37ºC) were used as a control. After 24-hour co-culture period, ECs were used for apoptosis and viability analyses. The ROS production, tube formation and cellular permeability of ECs were also analyzed. The entire experimental design was showed in Fig 6.
Fig 6. Time flow for co-culturing H/R-injured ECs with EPCs and/or NPCs.

First, ECs were cultured in hypoxia condition for 4 hours, then pathway inhibitor or receptor blockers were added into the culture medium and ECs were continued to culture for another 2 hours at the same hypoxia condition. Lastly, the hypoxic ECs were co-cultured with EPCs and/or NPCs by transferring the
inserts containing EPCs and/or NPCs into the well of ECs in each group. After 24 hour co-culture, ECs were collected for several analysis.
Cell apoptosis, viability and ROS production analyses of ECs

The apoptosis assay of ECs was conducted using FITC-Annexin V/propidium iodide (PI) apoptosis detection kit (BD Biosciences, CA) as we previously described (43). In brief, after 24 hour co-culture with EPCs and/or NPCs, cell culture medium was removed and cells were rinsed twice with PBS, then detached with 0.25% trypsin/EDTA for 3-5 minutes, and centrifuged at 300 g for 6 minutes. The pelleted cells were resuspended with 100 µl 1x Annexin V-binding buffer and incubated with 5 µl FITC-conjugated Annexin V and 5 µl PI in the dark for 15 minutes at RT. FITC-conjugated IgG and PE-conjugated IgG served as isotype controls. All samples were analyzed by flow cytometry (Accuri C6 flow cytometer, BD Biosciences, CA). 10,000 events were collected for data analysis. The apoptotic cells were defined as Annexin V+/PI− cells. ECs cultured in normoxic condition served as controls. ECs in the vehicle group were cultured with EC culture medium only. The experiment was performed six times, and the relative cell apoptosis rate was expressed as fold relative to the cells in the vehicle group.

The viabilities of H/R-injured ECs in different groups were measured by using a methyl thiazolyl tetrazolium (MTT) kit (Invitrogen, CA) as we previously described with slight modification (43). Briefly, after 24 hour co-culture with EPCs and/or NPCs, the cell culture medium was replaced with 1 ml of fresh culture medium
with 100 µl of 12 mM MTT solution and incubated at 37 °C for 2 hours. Then 850 µl of medium was removed from each well and 500 µl of DMSO was added to each well and mixed thoroughly with the pipette, followed by incubation at 37 °C for 10 minutes. Finally, 100 µl of mixed solution from each well was transferred to a well of a 96-well plate. The 96-well plates were read by a plate reader (Bio-Tek, VT) at 570 nm. ECs cultured in normoxic condition served as controls. ECs in the vehicle group were cultured with EC culture medium only. The experiment was repeated six times, and the relative cell viability (%) was expressed as percentage relative to the cells in the vehicle group.

The intracellular ROS production in ECs was determined by dihydroethidium (DHE, Sigma, MO) (43). Briefly, after EPC and/or NPC co-culture, the EC culture medium was replaced with fresh cultured medium containing the DHE working solution (2 µM) and incubated at 37 °C for 2 hours. Then the cells were detached with trypsin and were analyzed by flow cytometer (Accuri C6 flow cytometer, BD Biosciences, CA), respectively. 10,000 events were collected for data analysis. The experiment was repeated six times. The relative level of ROS production in experimental groups was expressed as fold relative to the level of ROS in vehicle group.
For the above experiments, the theoretical additive effect of EPC plus NPC co-culture (Es) on ECs was calculated as the sum of the effect of the individual monotherapies. The synergistic effect of EPC plus NPC co-culture (Es) on ECs was calculated by using the formula: 
\[ Es = \frac{(E_{EPC+NPC} - E_{EPC} - E_{NPC})}{(E_{EPC} + E_{NPC})} \times 100\% \]

\( E_{EPC} \) represents the effect elicited by EPC co-culture. \( E_{NPC} \) represents the effect elicited by NPC co-culture. \( E_{EPC+NPC} \) represents the effect elicited by EPC and NPC co-culture.

**Tube Formation and endothelial permeability assays of ECs**

The tube formation ability of ECs was evaluated by using a tube formation assay kit (Millipore, MA) according to previously described with slight modification (43). In brief, ECMatrix solution (BD Biosciences, CA) was thawed on ice overnight, mixed with 10x ECMatrix diluents, and placed in a 24-well tissue culture plate at 37°C for 1 hour to allow the matrix solution to solidify. After co-culture with EPCs and/or NPCs, ECs were trypsinized with 0.25% trypsin/EDTA for 3-5 minutes, centrifuged at 300 g for 6 minutes, and resuspended with EC culture medium and counted by using a hemocytometer. Then, ECs were reseeded at a density of 5 x 10^3 - 1 x 10^4 onto the surface of the polymerized ECMatrix and incubated at 37°C in a CO₂ incubator for 12-16 hours. Tube formation was evaluated with an inverted light microscope. Tubes were quantified by counting sprouting microcapillary-like structures exhibiting lengths four times their width. Five
independent fields were assessed for each well, and the average number of tubes per field (magnification: 200x) was determined. The experiments were repeated six times, and data was expressed as the average number of tubes per field.

Change in macromolecular permeability of ECs was studied by using cell culture transwell insert method (179). In brief, ECs were seeded at a density of $1 \times 10^5$ cells/insert onto the upper compartment of the 24-well transwell inserts (pore size, 0.4 μm; polycarbonate membrane, Greiner Bio-One, Germany). On the same day, the EPCs or NPCs ($2 \times 10^4$ cells/well) were plated on the lower chamber of the transwell insert system. On the co-culture day, ECs growing on the 24-well transwell insert were cultured in a hypoxic condition (1% O$_2$) for 6 hours, followed by culturing in normoxic condition (21% O$_2$, 5% CO$_2$) for reoxygenation. During the reoxygenation period, the transwell inserts containing ECs were placed into the 24-well plates containing EPCs and/or NPCs, and ECs were co-cultured with EPCs and/or NPCs for 24 hours. FITC-conjugated dextran (1 mg/ml; 10k Dalton, Sigma, MO) was added to the upper compartment of the transwell inserts for 90 minutes before the end of the experiment. Then 100 μl medium was collected from the lower compartment and fluorescence was determined by using a fluorescent plate reader (Bio-Tek, VT). ECs cultured in normoxic condition served as control. ECs in the vehicle group were cultured with
EC culture medium only. The relative fluorescence intensity in experimental
groups was expressed as fold relative to the level of that of normoxia cultured
ECs. Likewise, Es was calculated as: $E_s = \frac{(E_{EPC + NPC} - E_{EPC} - E_{NPC})}{(E_{EPC} + E_{NPC})} \times 100\%$.

**Enzyme-linked immunosorbent assay of VEGF and BDNF**

The protein levels of VEGF and BDNF in the culture medium of EPCs and NPCs,
as well as in the culture medium of H/R-injured ECs co-cultured with EPCs
and/or NPCs, were determined using human VEGF and BDNF Enzyme-linked
immunosorbent assay (ELISA) kits according to the manufacturer's instruction
(R&D systems, MN), respectively. In brief, after 24-hour co-culture, the
supernatant of ECs in each group was collected and centrifuged at 300 g for 6
minutes to remove dead cells. Then the prepared supernatants were added (50
µl/well) to a 96-well plate coated with a primary antibody specific to human BDNF
or VEGF and incubated for 2 hours at RT. Then wells were washed for 4 times
with wash buffer, the HRP-conjugated BDNF or VEGF antibody was added and
incubated for 1 hour at RT. After 4 washes, substrate solution was added to each
well. After 30 minutes, the stop solution was added to each well and the optical
density of each well was measured at 450 nm. A standard curve was run for each
assay, and all standards or samples were run in duplicate.
Western blot analysis

H/R-injured ECs were harvested after co-cultured with EPCs and/or NPCs. Proteins were extracted with cell lysis buffer (Thermo Fisher Scientific, MA) supplemented with complete mini protease inhibitor tablet (Roche, Switzerland). Protein lysates were electrophoresed through SDS-PAGE gels and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 hour and incubated with primary antibodies rabbit anti-Akt (1:1000; Cell Signaling), rabbit anti-p-Akt (1:1000; Cell Signaling Technologies, MA), rabbit anti-VEGFR2 (1:1000; Cell Signaling Technologies, MA), rabbit anti-p-Flk1 (1:200; Santa Cruz Biotechnology, TX), and mouse anti-β-actin (1:4000; Sigma, MO) at 4°C overnight. After washing, membranes were incubated with horseradish-peroxidase-conjugated donkey anti-rabbit or donkey anti-mouse IgG (Jackson ImmunoResearch Lab, MI) for 1 hour at RT. Blots were developed with enhanced chemiluminescence developing solutions and images were quantified under ImageJ software. For detecting the protein expressions of Akt and p-Akt in all groups, two sets of gels were done. One set of gels was used to compare the difference between normoxia and hypoxia groups, and the other set of gels was used to compare the differences among different treatment groups in the hypoxia groups. All experiments were repeated for six times. Similarly, Es was calculated as: \( Es = \frac{(E_{EPC+NPC} - E_{EPC} - E_{NPC})}{(E_{EPC} + E_{NPC})} \times 100\% \).
**Statistical analysis**

All data are expressed as mean ± SEM. Comparisons for two groups were analyzed by Student’s t-test. Multiple comparisons were analyzed by one- or two-way ANOVA followed by Bonferroni’s post hoc analysis. The theoretical additive effect was calculated as the sum of the effect of the individual monotherapies. Two-way ANOVA was used to test levels of significance for the synergistic interaction of the EPCs and NPCs above an additive effect. A value of p < 0.05 was considered statistically significant.

**Results**

**NPCs and EPCs were successfully generated from human iPS cells**

As shown in Fig 7, human iPS cells grew as colonies staining positively for pluripotent markers, Sox2 and Oct3/4. The generated NPCs grew as neurospheres after 7-day neural induction, and expressed neural progenitor markers pax6 (98 ± 1%) and nestin (96 ± 1.5%), but not Oct3/4, indicating a high differentiation efficacy. The generated NPCs had ability of differentiating into neurons, which were evidenced by expressing neuron specific marker β-tublin.

After 7-day EPC induction, approximately 48 ± 2.1% of cells positively expressed endothelial progenitor marker CD34. In order to get a pure population of EPCs, we used CD34-conjugated microbeads to purify the generated EPCs which were
further analyzed by flow cytometry. Results showed that the CD34-conjugated microbeads purified cells positively expressed CD34 (96 ± 2.1%) and KDR (95 ± 1.8%). As expected, the purified EPCs did not express Oct3/4. In addition, the generated EPCs had tube formation ability as revealed by matrigel assay.
Co-culture with EPCs and NPCs synergistically protected ECs from H/R-induced apoptosis and compromised viability via activating the PI3K pathway

After exposed to the hypoxic condition for 6 hours, ECs were co-cultured with EPCs and/or NPCs for 24 hours, followed with apoptotic and MTT assays. Results (Fig 8) showed that co-culture with EPCs and NPCs exerted a greater effect on decreasing H/R-injured EC apoptosis than that co-culture with EPCs or NPCs alone did (vehicle: 32.1 ± 1.5%, EPCs: 18.8 ± 1.4%, NPCs: 26.1 ± 1.3%, EPCs+NPCs: 10.3 ± 2%; p<0.05, vs. vehicle, or EPCs, or NPCs). Similarly, as shown in Fig 9, the EC viability was also synergistically increased by co-culture with EPCs and NPCs (vehicle: 62 ± 5%, EPCs: 80 ± 6%, NPCs: 74 ± 4%, EPCs+NPCs: 94 ± 5%; p<0.05, vs. vehicle, or EPCs, or NPCs).

Moreover, our data showed that the PI3K inhibitor (LY294002) pre-treatment could completely block the abovementioned effects of EPCs and/or NPCs, suggesting that the beneficial effects of EPCs and NPCs are mediated by the PI3K pathway. To define the contribution of VEGFR2 and TrkB (PI3K upstream molecules) in these effects, their respective inhibitors SU1498 and K252a were pre-added in the co-culture system as described. Interestingly, the results revealed that blockade of the VEGF/VEGFR2 and BDNF/TrkB signals only partially reduced the effects of EPCs and NPCs, indicating that the VEGF/VEGFR2 and BDNF/TrkB signals partially mediate the anti-apoptotic of
EPCs and NPCs on H/R-injured ECs. Other molecules might also be trigger for the activation of PI3K in this co-culture system and deserves further exploration.
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Fig 8. EPCs and NPCs reduced apoptosis of H/R-injured ECs via activating the PI3K pathway. PI/FITC-Annexin V apoptosis assay was conducted on H/R-injured ECs co-cultured with EPCs and/or NPCs for 24 hours as described in Material and Methods. A1, representative flow plots of EC apoptotic rate. A2,
summarized data of the apoptotic rate of ECs, showing that the combination of EPCs and NPCs offered better anti-apoptotic effect than EPCs or NPCs alone. Blocking the PI3K pathway diminished the effect of EPCs and/or NPCs. Also the PI3K pathway upstream blockers, SU1498 and K252a, reduced the effects of EPCs and NPCs. *p< 0.05, vs. Normoxia; #p< 0.05, vs. vehicle. Data are expressed as mean ± SEM, n=6/group/measurement. LY294002: PI3K inhibitor; SU1498: VEGFR2 inhibitor; K252a: TrkB inhibitor. (Wang et al, Molecular Brain, 2016)
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Fig 9. EPCs and NPCs promoted the survival of H/R-injured ECs via activating the PI3K pathway. MTT assay was conducted on H/R-injured ECs co-cultured with EPCs and/or NPCs for 24 hours as described in Material and Methods. A1, representative morphology images showing the viability of ECs. A2, summarized data showing EC viability which was synergistically increased
when co-cultured with the combination of EPCs and NPCs than that co-cultured with EPCs or NPCs alone. Blocking the PI3K pathway diminished the beneficial effect of EPCs and/or NPCs. Additionally, the PI3K pathway upstream blockers, SU1498 and K252a, reduced the effect of EPCs and NPCs. *p< 0.05, vs. Normoxia; #p< 0.05, vs. vehicle. Data are expressed as mean ± SEM, n=6/group/measurement. LY294002: PI3K inhibitor; SU1498: VEGFR2 inhibitor; K252a: TrkB inhibitor. (Wang et al, Molecular Brain, 2016)
Co-culture with EPCs and NPCs synergistically decreased the oxidative stress of H/R-injured ECs via activating the PI3K pathway

As shown in Fig 10, ROS production was decreased in H/R-injured ECs co-cultured with EPCs or NPCs (vehicle: 1.6 ± 0.1, EPCs: 1.3 ± 0.08, NPCs: 1.4 ± 0.07; p<0.05, vs. vehicle). Moreover, co-culture with EPCs and NPCs decreased ROS production to a larger extent than that with EPCs or NPCs alone did (EPCs+NPCs: 1.08 ± 0.09; p<0.05, vs. EPCs, or NPCs).

As expected, pre-treatment with PI3K inhibitor, LY294002, completely blocked the anti-oxidative effect of EPCs and/or NPCs on H/R-injured ECs. Pre-treatment with a combination of the PI3K upstream blockers SU1498 and K252a only partially reduced the anti-oxidative effects of EPCs and NPCs on H/R-injured ECs. All of these data indicate that the anti-oxidative effect of EPCs and NPCs is mediated by the PI3K signal pathway.
Fig 10. EPCs and NPCs decreased ROS production via activating the PI3K pathway. The histogram showing that ROS over-production was much decreased in H/R-injured ECs co-cultured with EPCs and NPCs than that co-cultured with EPCs or NPCs alone. LY294002 entirely abrogated the anti-oxidative effect of EPCs and/or NPCs, and the combination of SU1498 and
K252a partially reduced such effect. *p< 0.05, vs. Normoxia; #p< 0.05, vs. Vehicle. Data are expressed as mean ± SEM, n=6/group/measurement.

LY294002: PI3K inhibitor; SU1498: VEGFR2 inhibitor; K252a: TrkB inhibitor.

(Wang et al, Molecular Brain, 2016).
H/R-compromised tube formation ability of ECs was synergistically improved by co-culturing with EPCs and NPCs via activating the PI3K signal pathway

We further assessed whether co-culture with EPCs and/or NPCs altered the tube formation function of ECs exposed to H/R. The results (Fig 11) showed that EPCs or NPCs alone increased the tube formation ability of H/R-injured ECs (vehicle: 16 ± 3, EPCs: 27± 2, NPCs: 25 ± 3; p<0.05, vs. vehicle). Moreover, co-culture with EPCs and NPCs exhibited a synergistic effect on improving the tube formation ability of ECs compromised by H/R (EPCs+NPCs: 34± 2, p<0.05, vs. EPCs, or NPCs).

In order to elucidate the possible role of the PI3K pathway in the effect of EPCs and/or NPCs on EC tube formation, the pathway specific inhibitor LY294002 was used in the co-culture study. Our results showed that PI3K inhibition entirely abolished this effect of EPCs and NPCs. Similarly, to further explore whether VEGF/VEGFR2 and BDNF/TrkB signals could be responsible to trigger the activation of the PI3K pathway, we pre-added their respective inhibitors SU1498 and K252a into the co-culture system. As we expected from the data of apoptotic and MTT assays, blockade of the VEGF/VEGFR2 and BDNF/TrkB signals only partially reduced the effect on tube formation. Therefore, we speculate that additional mechanism could exist in the activation of PI3K signal pathway by EPCs and NPCs.
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Fig 11. EPCs and NPCs improved the angiogenic function of H/R-injured ECs via activating the PI3K pathway. A1, representative plots of tube formation. Scale bar: 200 μm. A2, summarized data of EC tube formation, showing that EPC and NPC co-culture offered synergistic effects on improving EC function compared to EPCs or NPCs alone. And such synergistic effect could
be blocked by LY294002, or partially abolished by the combination of SU1498 and K252a. *p< 0.05, vs. Normoxia; #p< 0.05, vs. Vehicle. Data are expressed as mean ± SEM, n=6/group/measurement. LY294002: PI3K inhibitor; SU1498: VEGFR2 inhibitor; K252a: TrkB inhibitor. (Wang et al, Molecular Brain, 2016).
The endothelial permeability was improved by co-culturing with EPCs and NPCs

Under physiological conditions, the endothelial membrane is impermeable to macromolecules (mass weight around 70 k Dalton) (180). We performed permeability assay to evaluate whether co-culture of EPCs and/or NPCs could improve the barrier function of ECs compromised by H/R. As expected, H/R injury increased trans-endothelial permeability to FITC-conjugated dextran (mass weight around 10 k Dalton). Co-culture of EPCs or NPCs decreased the flux of FITC-dextran (p<0.05, vs. vehicle), and EPCs combined with NPCs was more effective in decreasing the FITC-dextran flux through the EC monolayer (p<0.05, vs. vehicle, or EPCs, or NPCs; Fig 12).
Fig 12. EPCs and NPCs modulated the permeability of H/R-injured ECs. A, fold change of FITC-dextran flux, showing that the combination of EPC and NPCs had better effects than EPCs or NPCs alone on improving the endothelial barrier function of H/R-injured ECs. *p< 0.05, vs. Normoxia, #p< 0.05, vs. vehicle. Data are expressed as mean ± SEM, n=6/group/measurement. (Wang et al, Molecular Brain, 2016).
Co-culture with EPCs and NPCs elevated the levels of VEGF and BDNF in the conditioned medium of ECs exposed to H/R

In order to explore the mechanisms underlying the protective benefits of EPCs and NPCs, we performed an ELISA assay to determine the levels of VEGF and BDNF. As shown in Fig 13A, the baseline VEGF level was detected in the culture medium of ECs, EPCs, and NPCs prior to the co-culture experiments. After 24 hour co-culture, a higher level of VEGF was detected in ECs co-cultured with EPCs alone than that in vehicle, and there was no significant difference of the VEGF level in the vehicle and NPC co-culture groups. The level of VEGF was synergistically elevated in the medium of ECs co-cultured with EPCs and NPCs (p<0.05, vs. vehicle, or NPCs, or EPCs). In Fig 13B, we showed the baseline level of BDNF in the culture medium of EC, EPCs, and NPCs prior to the co-culture experiments. After 24 hour co-culture, the BDNF level was increased in the culture medium collected from ECs co-cultured with NPCs, whereas, no significant difference of BDNF level was observed between the vehicle and EPC treatment groups. Moreover, the BDNF level was remarkably increased in the ECs co-cultured with EPCs and NPCs.
**A**

![VEGF Bar Graph](image)

- **Normoxia**
  - ECs
  - EPCs
  - NPCs
  - Vehicle
  - Co-cultured with EPCs
  - Co-cultured with NPCs
  - Co-cultured with EPCs + NPCs

- **Hypoxic ECs**
  - Co-cultured with EPCs
  - Co-cultured with NPCs
  - Co-cultured with EPCs + NPCs

**B**

![BDNF Bar Graph](image)

- **Normoxia**
  - ECs
  - EPCs
  - NPCs
  - Vehicle
  - Co-cultured with EPCs
  - Co-cultured with NPCs
  - Co-cultured with EPCs + NPCs

- **Hypoxic ECs**
  - Co-cultured with EPCs
  - Co-cultured with NPCs
  - Co-cultured with EPCs + NPCs

**Vegetable**

P<0.05

**Potato**

# #

**Mango**

P<0.05

**Tomato**

# #
Fig 13. EPCs and NPCs modulated VEGF and BDNF secretion of H/R-injured ECs. A-B, summarized data showing the levels of VEGF and BDNF in culture medium of ECs, EPCs, NPCs and H/R-injured ECs co-cultured with EPCs and/or NPCs. *p< 0.05, vs. Normoxia, #p< 0.05, vs. vehicle. Data are expressed as mean ± SEM, n=6/group/measurement. (Wang et al, Molecular Brain, 2016).
The level of VEGFR2 was upregulated and ratios of p-Flk1/VEGFR2 and p-Akt/Akt were increased in H/R-injured ECs co-cultured with EPCs and NPCs. As shown in Fig 14A, co-culture of EPCs and NPCs increased the expression level of VEGFR2 in H/R-injured ECs (p<0.05, vs. vehicle, or EPCs). Whereas the VEGFR2 level was not changed in ECs co-cultured with EPCs alone (p>0.05, vs. vehicle), suggesting EPCs and NPCs protect ECs against H/R injury through up-regulating the expression of VEGFR2.

Western blot results demonstrated that the expression ratios of p-Flk1/VEGFR2 (Fig 14B) and p-Akt/Akt (Fig 14C) in H/R-injured ECs were increased by co-culture with EPCs or NPCs alone, with a greater increase in those ECs co-cultured with a combination of EPCs and NPCs. As expected, the PI3K inhibitor LY294002 completely abolished the phosphorylation of Akt, suggesting that the PI3K/Akt signal pathway is activated in ECs co-cultured with EPCs and NPCs. A combination of SU1498 and K252a decreased the phosphorylation of Akt, reflecting that it at least partially depends on the upstream molecules VEGFR2 and TrkB.
A

VEGFR2/β-actin of ECs

Normoxic ECs

Hypoxic ECs

VEGFR2/β-actin

# #
P<0.05

Co-cultured cultured with EPCs

Co-cultured cultured with NPCs

Co-cultured cultured with EPCs+ NPCs

B

p-Flik1 / VEGFR2 of ECs

Normoxic ECs

Hypoxic ECs

p-Flik1 / VEGFR2

# #
P<0.05
Fig 14. Co-culture with EPCs and NPCs activated the PI3K/Akt signal pathway on H/R-injured ECs. A, VEGFR2 expression was significantly up-regulated in H/R-injured ECs co-cultured with NPCs or the combination of EPCs.
and NPCs. B, the protein expression ratio of p-Flk1/VEGFR2 was significantly increased in H/R-injured ECs co-cultured with EPCs or NPCs, with a higher ratio in ECs co-cultured with the combination of EPCs and NPCs. C, the protein expression ratio of p-Akt/Akt was increased in H/R-injured ECs co-cultured with EPCs and NPCs, and this effect was blocked or reduced when ECs were pre-treated with PI3K inhibitor LY294002 or VEGFR2 inhibitor SU1498 or TrkB inhibitor K252a. *p< 0.05, vs. Normoxia; #p< 0.05, vs. vehicle; Data are expressed as mean ± SEM, n=6/group/measurement. (Wang et al, Molecular Brain, 2016).
Discussion

In the present study, we showed that EPCs and NPCs produced from human iPS cells had synergistic beneficial effects on H/R-injured brain ECs. The major findings include: i) Co-culture with EPCs and NPCs synergistically protected ECs from H/R-induced apoptosis and dysfunction; ii) The levels of VEGF and BDNF in the medium of ECs co-cultured with EPCs and NPCs were increased; iii) Co-culture with NPCs up-regulated VEGFR2 expression and its phosphorylation on ECs; iv) Blockade of the VEGFR2 and Trkb or PI3K/Akt pathway inhibited or abolished the protective effects of EPCs and NPCs (Fig 15).
Fig 15. Proposed molecular mechanism for the protective effect of EPCs and NPCs on H/R-injured brain ECs. Co-culture with EPCs and NPCs synergistically increased the survival ability, decreased the oxidative stress and improved the angiogenic and barrier functions of H/R-injured ECs, via activating the PI3K/Akt signal pathway that mainly depended on the progenitor paracrine (VEGF and BDNF) mediated signals. (Wang et al, Molecular Brain, 2016).
ECs are unique and critical in maintaining normal BBB function (181). Impairment of BBB occurs in the early stage of ischemic brain injury, leading to subsequent brain swelling and inflammatory responses (86). Therefore, protecting brain ECs from H/R-induced injury will theoretically alleviate brain tissue damage in ischemic stroke. Nevertheless, there is no clinically effective strategy to protect ECs against H/R-induced injury in acute IS. Transplantation of stem cells has been shown to accelerate the functional recovery of IS by promoting angiogenesis and neurogenesis (106). Indeed, others and our studies have demonstrated that engrafted EPCs or NPCs can alleviate acute ischemic injury and promote angiogenesis and neurogenesis in an IS mouse model (20, 22, 34, 38). However, it is unknown whether there are synergistic effects if EPCs and NPCs are combined to treat ischemic-reperfusion stroke. In this study, we examined the effects of EPCs and NPCs on H/R-injured brain ECs in vitro. It is well known that iPS cells have unlimited self-renewal ability and are able to differentiate to various types of cells with less ethical issues for clinical applications (125, 127). We successfully differentiated human iPS cells into EPCs and NPCs. To mimic the status of ECs in acute IS, we produced an in vitro model of brain EC H/R injury, characterized with decreased viability, increased apoptosis and cellular permeability, increased ROS production, as well as compromised tube formation ability (43). By using this model, we found that co-culture with EPCs or NPCs alone had beneficial effects on protecting ECs from
H/R-induced injury, including increase in apoptosis, ROS production and intercellular permeability, and decrease in viability and capillary formation. Moreover, co-culture with both EPCs and NPCs achieved synergistic effects on those measurements by 18-28% increase.

Numerous studies have shown that VEGF and BDNF are respectively responsible for the beneficial effects of EPCs and NPCs (182, 183). In order to determine whether EPC-derived VEGF and NPC-derived BDNF are the major factors involved in the observed effects of EPCs and NPCs in this study, we have analyzed the levels of VEGF and BDNF in the culture medium of ECs. Our results showed that EPC co-culture increased VEGF, but not BDNF level in the EC medium, whereas, NPC co-culture increased BDNF, but not VEGF level in the EC medium. More importantly, the data revealed that EPCs and NPCs complementarily increased the VEGF and BDNF levels in the co-culture medium of ECs. In the present study, we did not study the dose-dependent effects of VEGF and BDNF on ECs did not compare if co-application of VEGF and BDNF is more significant than simply increasing the dose of VEGF or BDNF alone. However, our results revealed that the combination of EPCs and NPCs have synergistic effects on ECs. For exploration of the underlying mechanism, we analyzed the expression of VEGFR2 and its phosphorylation. The results showed that co-culture with both EPCs and NPCs synergistically increased the
expression of p-Flk1/VEGFR2 in ECs. Of note, we found that co-culture with NPCs, but not EPCs, up-regulated the expression of VEGFR2 on ECs. This suggests that NPCs can mediate the synergistic effects by its secreted BDNF and provides the basis of the synergistic effects observed in the co-culture group combining EPCs and NPCs. Furthermore, we examined the role of VEGF/VEGFR2 and BDNF/TrkB signal pathways in the beneficial effects of EPC/NPC co-culture. Our data showed that blockade of both signals largely decreased the abovementioned effects of EPCs and NPCs, suggesting that these effects are mainly dependent on the VEGF/VEGFR2 and BDNF/TrkB signals. These data are in consistent with the notion that VEGFR2 and TrkB are the major modulators of endothelial survival (184). Collectively, VEGF and BDNF are the major factors for responsible of the synergistic effects of EPCs and NPCs in the co-cultures, although there are unidentified factors contributing a minor part.

The PI3K is a downstream pathway molecule of VEGFR2 and TrkB, which mediates various cell activities includes cell survival, cell proliferation (185-187). We found that both EPCs and NPCs increased the level of p-Akt/Akt in ECs. And there was a synergistic effect on the level of p-Akt/Akt when EPCs and NPCs were simultaneously applied. The synergistic effect of EPC plus NPC co-culture was 30% on up-regulating the protein expression ratio of p-Akt/Akt. Moreover,
the protective effects elicited by EPCs and/or NPCs were abolished by blockade of PI3K with LY294002. Taken together, our data indicate that the PI3K pathway is responsible for the beneficial effects of EPCs and NPCs.

In conclusion, our data demonstrate that EPCs and NPCs can offer synergistic benefits in protecting brain ECs against H/R injury by VEGF and BDNF paracrine-mediated activation of the PI3K/Akt signal pathway. These findings provided fundamental rationale for us to develop cell-based therapy for IS.
CHAPTER IV. Co-culture of EPCs and/or NPCs protected neurons from H/R-induced apoptotic cell death and ROS overproduction via activating the PI3K/Akt signal pathway.

Rationale: Neurons are the most vulnerable cells in the brain in response to ischemia/reperfusion insult. Neurons in the core area will be irreversibly damaged within a few minutes after ischemia occurs, and the cells in the ischemic penumbra will undergo apoptosis in several hours or days following IS (46). The core region will enlarge with the cells in penumbral region undergoing death (2). Therefore, strategies intended to prevent neuronal death in the penumbra should be critical for treating IS.

It is well documented that ROS generation in normal cells is under tight homeostatic control. Chronic and/or abrupt increases in ROS level above a physiological threshold may destroy cellular components (lipids, protein, and DNA) and ultimately trigger cell death via apoptosis or necrosis (188). Accumulating studies have shown that oxidative stress participates in neural death in IS (189-192). In clinical studies, low plasma concentrations of antioxidants are found to be associated with high degree of neurological impairment in IS (193, 194). The application of antioxidant compounds like dehydroascorbic acid, Ebselen, Edaravone have shown to be effective in combating oxidative stress in the animal study (195, 196). Albeit most of
antioxidant agents have shown neuroprotective effects in the animal model of brain ischemia, only a few antioxidants have been or are currently tested in clinical trials (196). Edaravone delayed infarcts and edema evolution and decreased mortality in the acute stage of patients. However, it did not significantly improve functional outcome among the surviving patients (197).

Previous studies have demonstrated that recombinant BDNF and VEGF can decrease neuron apoptosis in animal stroke models (198-201). Other neurotrophins like glial cell-derived neurotrophic factor and insulin-like growth factor I, also exert protective actions by inhibiting death mechanisms of neurons (202, 203). As we know, NPCs and EPCs can release neurotrophic/growth factors such as BDNF and VEGF (182, 183). Whether EPCs and NPCs could protect neurons against ischemic-induced cell apoptosis and oxidative stress have not been studied, and the corresponding molecular mechanism remains unclear.

A previous report showed that insulin-like growth factor I protects against neuron death via activating the PI3K/Akt pathway which in turn inactivating the cell death transcription factor FOXO family (204). The PI3K/Akt signal pathway has also been found to be responsible for BM-derived stromal cells on mediating cell survival and axonal outgrowth of neurons (41, 42). More recently, we found that
EPCs-derived microvesicles protected ECs against H/R injury through activating PI3K/Akt/eNOS signal pathway (43). Based on these observations, we speculate that the PI3K/Akt pathway is a mediator for EPCs and NPCs on protecting neurons against ischemia.

Our main goal with this study was to determine the anti-apoptotic and anti-oxidant effects of co-culture of EPCs and NPCs on neurons following H/R-injury, and to identify whether the PI3K/Akt pathway is involved in.

Experimental design
The H/R injury model of SH-SY5Y cells were induced by 6 hour hypoxic culture followed with 24 hour reoxygenation. During the reoxygenation period, SH-SY5Y cells were divided into 10 co-culture groups (n=4/group): Vehicle, EPCs, EPCs + LY294002, EPCs + SU1498 + k252a, NPCs, NPCs + LY294002, NPCs + SU1498 + k252a, EPCs + NPCs, EPCs + NPCs + LY294002, EPCs + NPCs + SU1498 + k252a. At the end of experiment, the conditional culture medium from each group were used for enzyme-linked immunosorbent assay (ELISA) of VEGF and BDNF. Cells were collected for various analyses: 1) methyl thiazolyl tetrazolium (MTT) assay for cell viability, 2) flow cytometry for cell apoptosis and ROS analyses, 3) western blot analysis of Akt, pAkt, p-VEGFR2, VEGFR2, etc.
Materials and methods

Cell culture of human neurons

Human neuron cell line (SH-SY5Y cells) was purchased from ATCC. The SH-SY5Y cells were cultured with Eagle’s minimum essential medium and F12 medium (1:1 mixture) supplemented with 10% FBS in regular tissue culture plates. The culture medium was replaced every 4-5 days.

H/R-injury model of neurons

The H/R-injury model of neurons was produced as previously described (43). Briefly, SH-SY5Y cells (4×10^5/well) growing on 6-well plates were changed with fresh culture medium and cultured for 6 hours in a hypoxic chamber (1% O_2, 5% CO_2, and 94% N_2; Biospherix, NY), then re-oxygenated by incubation in a standard 5% CO_2 incubator for 24 hours. During the re-oxygenation period, SH-SY5Y cells were co-cultured with EPCs and/or NPCs using the transwell co-culture system as described in Fig 16.
Fig 16. A diagram shows the transwell co-culture system used for SH-SY5Y cells, EPC and NPC co-culture. EPCs and NPCs were seeded into the transwell membrane inserts one day prior to co-culture. On the co-culture day, the insert containing EPCs and/or NPCs was placed into the well containing SH-SY5Y cells.
Co-culture SH-SY5Y cells with EPCs and/or NPCs

The day before co-culture, NPCs (4×10^5/insert), or EPCs (4×10^5/insert), or a combination of NPCs (2×10^5) and EPCs (2×10^5) were plated into transwell membrane inserts (pore size, 0.4 μm; polycarbonate membrane, Greiner Bio-One, Germany) in NPC and/or EPC culture medium overnight (44). The SH-SY5Y cells (4×10^5 /well) subjected to hypoxia (1% O_2) were randomly divided into four groups and co-cultured with: 1) vehicle (SH-SY5Y cell culture medium only), 2) EPCs (co-cultured with EPCs), 3) NPCs (co-cultured with NPCs), 4) EPCs+NPCs (co-cultured with EPCs and NPCs), by placing the inserts containing EPCs and/or NPCs in the SH-SY5Y cell culture plates for 24 hours. For signal pathway study, LY294002 (PI3K inhibitor; 20 μM, Cayman Chemical Company, MI), and a combination of K252a (TrkB inhibitor; 10 μg/ml, BioVision Inc, CA) and SU1498 (VEGFR inhibitor; 5 μM, BioVision Inc, CA) were added to cell culture medium 2 hours prior to co-culture experiments (43, 44, 177, 178) and presented in the SH-SY5Y culture during the co-culture period. All inhibitors were dissolved with DMSO (Sigma, MO) and diluted with culture medium to yield desired concentrations. SH-SY5Y cells cultured in normoxia (5% CO_2, 37° C) were used as a control. The experimental design was showed in Fig 17.
Fig 17. Time flow of co-culturing SH-SY5Y cells with EPCs and/or NPCs.

First, SH-SY5Y cells were cultured in hypoxia condition for 4 hours, then pathway inhibitor or receptor blockers were added into the culture medium and SH-SY5Y cells were continued to culture for another 2 hours at the same hypoxia condition. Lastly, the hypoxic SH-SY5Y cells were co-cultured with EPCs and/or NPCs by
transferring the inserts containing EPCs and/or NPCs. After 24 hour co-culture, SH-SY5Y cells were collected for several analysis.
**Cell viability, apoptosis and ROS production analyses of SH-SY5Y cells**

The viabilities of H/R-injured SH-SY5Y cells were measured by using a MTT kit (Invitrogen, CA) as we previously described with slight modification (43, 205). Briefly, after 24-hour co-culture with EPCs and/or NPCs, the cell culture medium was replaced with 1 ml of fresh culture medium with 100 µl of 12 mM MTT solution and cells were incubated at 37 ºC for 2 hours. 850 µl of medium was removed and 500 µl of DMSO was added to each well, mixed thoroughly with the pipette, incubated at 37 ºC for 10 minutes. Finally, 100 µl of mixed solution from each well was transferred to a well of a 96-well plate. The 96-well plates were read by a plate reader (Bio-Tek, VT) at 570 nm. SH-SY5Y cells cultured in normoxic condition served as a control. SH-SY5Y cells in the vehicle group were cultured with SH-SY5Y cell culture medium only. All experiments were performed six times, and the relative cell viability (%) was expressed as percentage relative to the cells in the vehicle group.

The apoptosis assay of SH-SY5Y cells was conducted using FITC/PI-Annexin V apoptosis detection kit (BD Biosciences, CA) as we previously described (43, 205). In brief, after 24-hour co-culture with EPCs and/or NPCs, the cell culture medium was removed and rinsed twice with PBS, then cells were de-attached with 0.25% trypsin/EDTA for 3-5 minutes, centrifuged at 300 g for 6 minutes. The cell pellet was resuspended with 100 µl 1x Annexin V-binding buffer and
incubated with 5 µl FITC-conjugated Annexin V and 5 µl PI in the dark for 15 minutes at RT. FITC-conjugated IgG and PE-conjugated IgG served as isotype controls. All samples were analyzed by flow cytometry (Accuri C6 flow cytometer, BD Biosciences, CA), respectively. 10,000 events were collected for data analysis. The apoptotic cells were defined as Annexin V+/PI− cells. SH-SY5Y cells cultured in normoxic condition served as controls. SH-SY5Y cells in the vehicle group were cultured with SH-SY5Y cell culture medium only. All experiments were performed six times, and the relative cell apoptosis rate was expressed as fold relative to the cells in the vehicle group.

The intracellular ROS production in SH-SY5Y cells was determined by DHE (Sigma, MO) (205). Briefly, after EPC and/or NPC co-culture, the SH-SY5Y cell culture medium was replaced with fresh cultured medium containing the DHE working solution (2 µM) and incubated at 37 ºC for 2 hours. Then the cells were detached with trypsin and were analyzed by flow cytometer (Accuri C6 flow cytometer, BD Biosciences, CA), respectively. 10,000 events were collected for data analysis. The experiment was repeated six times. The relative level of ROS production in experimental groups was expressed as fold relative to the level of ROS in vehicle group.
For all experiments, the theoretical additive effect of EPC plus NPC co-culture (Es) on SH-SY5Y cells was calculated as the sum of the effect of the individual monotherapies. The synergistic effect of EPC plus NPC co-culture (Es) on SH-SY5Y cells was calculated by using the formula: 

\[ \text{Es} = \frac{(E_{EPC+NPC} - E_{EPC} - E_{NPC})}{(E_{EPC} + E_{NPC})} \times 100\% \]

\( E_{EPC} \) represents the effect elicited by EPC co-culture. \( E_{NPC} \) represents the effect elicited by NPC co-culture. \( E_{EPC+NPC} \) represents the effect elicited by EPC and NPC co-culture.

**ELISA of VEGF and BDNF**

The protein levels of VEGF and BDNF in the culture medium of H/R-injured SH-SY5Y cells co-cultured with EPCs and/or NPCs were determined using human VEGF and BDNF ELISA kits according to the manufacturer’s instruction (R&D systems, MN), respectively. In brief, after 24-hour co-culture, the supernatant of SH-SY5Y cells in each group was collected and centrifuged at 300 g for 6 minutes to remove dead cells. The prepared supernatants were added (50 µl/well) to a 96-well plate coated with a primary antibody specific to human BDNF or VEGF and incubated for 2 hours at RT. Then wells were washed 4 times with wash buffer, the HRP-conjugated BDNF or VEGF antibody was added and incubated for 1 hour at RT. After 4 washes, substrate solution was added to each well. Then, after 30-minute incubation, the stop solution was added to each well.
and the optical density of each well was measured at 450 nm. A standard curve was run for each assay, and all standards or samples were run in duplicate.

**Western blot analysis**

H/R-injured SH-SY5Y cells were harvested after co-cultured with EPCs and/or NPCs. Proteins were extracted with cell lysis buffer (Thermo Fisher Scientific, MA) supplemented with complete mini protease inhibitor tablet (Roche, Switzerland). Protein lysates were electrophoresed through SDS-PAGE gels and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 hour and incubated with primary antibodies rabbit anti-Akt (1:1000; Cell Signaling Technology, MA), rabbit anti-p-Akt (1:1000; Cell Signaling Technology, MA), rabbit anti-TrkB (1:1000; Cell Signaling Technology, MA), rabbit anti-p-TrkB (1:200; Santa Cruz Biotechnology, TX), and mouse anti-β-actin (1:4000; Sigma, MO) at 4°C overnight. After washing, membranes were incubated with horseradish-peroxidase-conjugated donkey anti-rabbit or donkey anti-mouse IgG (Jackson ImmunoResearch Lab, MI) for 1 hour at RT. Blots were developed with enhanced chemiluminescence developing solutions and images were quantified under ImageJ software. All experiments were repeated at least six times. Similarly, Es was calculated as: $\text{Es} = \frac{(E_{EPC+NPC} - E_{EPC} - E_{NPC})}{(E_{EPC} + E_{NPC})} \times 100\%$. 
Statistical analysis

All data are expressed as mean ± SEM. Comparisons for two groups were analyzed by Student’s t-test. Multiple comparisons were analyzed by one- or two-way ANOVA followed by Bonferroni’s post hoc analysis. The theoretical additive effect was calculated as the sum of the effect of the individual monotherapies. Two-way ANOVA was used to test levels of significance for the synergistic interaction of the EPCs and NPCs above an additive effect. A value of p < 0.05 was considered statistically significant.

Results

Co-culture with EPCs and NPCs synergistically protected SH-SY5Y cells from H/R-induced apoptosis and compromised viability via activating the PI3K pathway

According to the results obtained from flow cytometry analysis (Fig 18), the apoptotic rates of SH-SY5Y cells were significantly decreased in both the EPC co-culture alone and NPC co-culture alone groups as compared to that in the vehicle group (culture medium only). Also, a higher percentage of early apoptotic SH-SY5Y cells were rescued by the combination culture of EPCs and NPCs than that rescued by EPC or NPC alone. In the set of signal pathway study, we found that the PI3K inhibitor LY294002 completely blocked the anti-apoptotic effect of EPCs and NPCs, suggesting that the beneficial effects of EPCs and NPCs are
mediated by the PI3K pathway. To define the contribution of VEGFR2 and TrkB (PI3K upstream molecules) in this effect, the respective inhibitors SU1498 and K252a were pre-added in the co-culture system as described. The results showed that blockade of the VEGF/VEGFR2 and BDNF/TrkB signals only partially reduced the effect of EPCs and NPCs, indicating that the VEGF/VEGFR2 and BDNF/TrkB signals partially mediate the anti-apoptotic of EPCs and NPCs on H/R-injured SH-SY5Y cells.
Fig 18. EPCs and NPCs reduced apoptosis of H/R-injured SH-SY5Y cells via activating the PI3K pathway. FITC/PI-Annexin V apoptosis assay was conducted on H/R-injured SH-SY5Y cells co-cultured with EPCs and/or NPCs for 24 hours as described in Material and Methods. A, summarized data of
the apoptotic rate of SH-SY5Y cells, showing that the combination of EPCs and NPCs offered better anti-apoptotic effect than EPCs or NPCs alone did. Blockade of the PI3K pathway abrogated this effect. And the PI3K pathway upstream blockers, SU1498 and K252a, remarkably reduced the effects of EPCs and NPCs. *p< 0.05, vs. Normoxia; #p< 0.05, vs. vehicle. Data are expressed as mean ± SEM, n=6/group/measurement. LY294002: PI3K inhibitor; SU1498: VEGFR2 inhibitor; K252a: TrkB inhibitor.
Upon the results of MTT assay (Fig 19), SH-SY5Y cell viability was increased in those co-cultured with EPCs alone or NPCs alone as compared to that in the vehicle group (culture medium only). The combination of EPC and NPC co-culture significantly improved the H/R-compromised viability of SH-SY5Y cells. Likewise, for signal pathway study, we pretreated the cells with PI3K inhibitor LY294002, as well as its upstream molecule inhibitors SU1498 and k252a, respectively. We found that LY294002 completely, and a combination of SU1498 and k252a only partially inhibited the pro-survival effect of EPCs and NPCs on SH-SY5Y cells.
A1

<table>
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<tr>
<th>Condition</th>
<th>Image Description</th>
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<tr>
<td>Normoxia Vehicle</td>
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<tr>
<td>Co-cultured with EPCs</td>
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<td>Pre-treated with LY294002</td>
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<tr>
<td>Co-cultured with NPCs</td>
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<tr>
<td>Pre-treated with SU1498+K252a</td>
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<td>Co-cultured with EPCs + NPCs</td>
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Fig 19. EPCs and NPCs improved the viability of H/R-injured SH-SY5Y cells via activating the PI3K pathway. A1, representative morphology images showing the viability of SH-SY5Y cells. A2, summarized data showing SH-SY5Y cell viability which was synergistically increased when co-cultured with the combination of EPCs and NPCs than that co-cultured with EPCs or NPCs alone.
Blocking the PI3K pathway diminished the beneficial effect of EPCs and/or NPCs. And SU1498 and K252a reduced the effect of EPCs and NPCs. *p< 0.05, vs. Normoxia; #p< 0.05, vs. vehicle. Data are expressed as mean ± SEM, n=6/group/measurement. LY294002: PI3K inhibitor; SU1498: VEGFR2 inhibitor; K252a: TrkB inhibitor.
EPCs and NPCs synergistically decreased H/R-induced ROS production in SH-SY5Y cells via activating the PI3K pathway

As shown in Fig 20, the level of ROS production in SH-SY5Y cells was determined using DHE staining and the intensity was analyzed by flow cytometry. Results showed that the ROS production was significantly decreased in H/R-injured SH-SY5Y cells co-cultured with EPCs or NPCs as compared to that in the vehicle group (vehicle: 22 ± 1.1%, EPCs: 17.6 ± 1.2%, NPCs: 17.4 ± 1.4%, p<0.05, vs. vehicle). Also, it was much decreased in SH-SY5Y cells co-cultured with combination of EPCs and NPCs (EPCs+NPCs: 12.3 ± 1.1%, p<0.05, vs. EPCs, or NPCs) than that co-cultured with EPCs or NPCs alone. Likewise, pre-treatment with PI3K inhibitor, LY294002, completely diminished the anti-oxidative effect of EPCs and/or NPCs on H/R-injured SH-SY5Y cells, and a combination of the PI3K upstream blockers SU1498 and K252a only partially reduced this effect.
Fig 20. EPCs and NPCs decreased ROS production in SH-SY5Y cells via 
activating the PI3K pathway. A1, representative flow cytometry plots showing 
the fluorescence intensity of DHE staining of SH-SY5Y cells. A2, the quantitative
data showing that ROS level was much decreased in H/R-injured SH-SY5Y cells co-cultured with EPCs and NPCs than that co-cultured with EPCs or NPCs alone. LY294002 could entirely diminish the anti-oxidative effect of EPCs and/or NPCs, and the combination of SU1498 and K252a partially reduced such effect. *p< 0.05, vs. Normoxia; #p< 0.05, vs. Vehicle. Data are expressed as mean ± SEM, n=6/group/measurement. LY294002: PI3K inhibitor; SU1498: VEGFR2 inhibitor; K252a: TrkB inhibitor.
Co-culture with EPCs and NPCs elevated the levels of BDNF and VEGF in the culture medium of H/R-injured SH-SY5Y cells

In order to explore the mechanisms underlying the beneficial effects of EPCs and NPCs on SH-SY5Y cells, we performed ELISA assay to determine the levels of the two cytokines in the SH-SY5Y cell culture medium. As shown in Fig 21A, after 24-hour co-culture, a higher level of VEGF was detected in SH-SY5Y cells co-cultured with EPCs alone than that in vehicle (p<0.05, vs. vehicle), whereas, no significant difference was observed between the vehicle and NPC co-culture groups (p>0.05). The level of VEGF was also elevated in the medium of SH-SY5Y cells co-cultured with EPCs and NPCs (p<0.05, vs. vehicle, or NPCs). In Fig 21B, we found that the BDNF level was increased in the culture medium collected from SH-SY5Y cells co-cultured with NPCs (p<0.05, vs. vehicle), but no significant difference was observed between the vehicle and EPC treatment groups (p>0.05). And it was remarkably increased in the SH-SY5Y cells co-cultured with EPCs and NPCs.
Fig 21. EPCs and NPCs modulated the levels of VEGF and BDNF in the culture medium of H/R-injured SH-SY5Y cells. A-B, summarized data showing the levels of VEGF and BDNF in culture medium of H/R-injured SH-SY5Y cells co-cultured with EPCs and/or NPCs. *p< 0.05, vs. Normoxia, #p< 0.05, vs. vehicle. Data are expressed as mean ± SEM, n=6/group/measurement.
The expression of TrkB was up-regulated and ratios of p-TrkB/TrkB and p-Akt/Akt were increased in H/R-injured SH-SY5Y cells co-cultured with EPCs and NPCs

In order to elucidate the possible mechanism for the synergistic effects of EPCs and NPCs on H/R-injured SH-SY5Y cells, we examined whether EPCs could affect the expression of growth factor receptor in SH-SY5Y cells. As shown in Fig 22A, we found that the TrkB expression level was elevated in SH-SY5Y cells co-cultured with EPCs as well as co-cultured with a combination of EPCs and NPCs. No difference was observed between the NPCs and vehicle groups. Meanwhile, western blotting analysis indicated that the expressions of p-TrkB/TrkB (Fig 22B) and p-Akt/Akt (Fig 22C) were remarkably up-regulated in SH-SY5Y cells co-cultured with a combination of EPCs and NPCs when compared to that of cells co-cultured with EPCs or NPCs alone. LY294002 reversed the phosphorylation of Akt, and a combination of TrkB inhibitor and VEGFR2 inhibitor partially blocked it, reflecting that the phosphorylation of Akt at least partially depended on the upstream molecules VEGFR2 and TrkB. Collectively, all of these data indicate that EPCs and NPCs protect SH-SY5Y cells against H/R injury in a PI3K/Akt-dependent way.
A

TrkB

β-actin

\[ \begin{align*}
\text{Vehicle} & \quad \text{Co-cultured with EPCs} & \quad \text{Co-cultured with NPCs} & \quad \text{Co-cultured with EPCs + NPCs} \\
\end{align*} \]

Hypoxic SH-SY5Y cells

B

p-TrkB

TrkB

β-actin

\[ \begin{align*}
\text{Vehicle} & \quad \text{Co-cultured with EPCs} & \quad \text{Co-cultured with NPCs} & \quad \text{Co-cultured with EPCs + NPCs} \\
\end{align*} \]

Hypoxic SH-SY5Y cells
C

- Normoxic SH-SY5Y cells
- Co-cultured with EPCs
- Vehicle
- Co-cultured with NPCs
- Co-cultured with EPCs+NPCs

**p-Akt/Akt of SH-SY5Y cells**

Hypoxia  
-  
+  
+  
+  
+  
+  
+  
+  
P<0.05

LY294002  
-  
-  
-  
+  
-  
-  
-  
+  
P<0.05

SU1498+K252a  
-  
-  
-  
-  
+  
-  
-  
+  
P<0.05

p-Akt  
Akt  
β-actin
Fig 22. Co-culture with EPCs and NPCs upregulated the levels of TrkB and p-TrkB/TrkB, and activated the PI3K/Akt signal pathway on H/R-injured SH-SY5Y cells. A, TrkB expression was significantly up-regulated in H/R-injured SH-SY5Y cells co-cultured with EPCs or the combination of EPCs and NPCs. B, the ratio of p-TrkB/TrkB was significantly increased in H/R-injured SH-SY5Y cells co-cultured with EPCs or NPCs, with an even higher ratio in SH-SY5Y cells co-cultured with the combination of EPCs and NPCs. C, the ratio of p-Akt/Akt was increased in H/R-injured SH-SY5Y cells co-cultured with EPCs and NPCs, and this effect was blocked or reduced when SH-SY5Y cells were pre-treated with PI3K inhibitor LY294002 or VEGFR2 inhibitor SU1498 or TrkB inhibitor K252a. *p< 0.05, vs. Normoxia; #p< 0.05, vs. vehicle; Data are expressed as mean ± SEM, n=6/group/measurement.
**Discussion**

In this study, we demonstrated that co-culture of EPCs and NPCs confers neuroprotection against H/R by decreasing apoptosis and oxidative stress. We further revealed that the anti-apoptotic and anti-oxidative effects of EPCs and NPCs are mediated through activation of the PI3K/Akt pathway. More interestingly, we discovered that EPCs and NPCs can produce synergistic benefits on H/R-induced injury in our experimental model (**Fig 23**).
Fig 23. Proposed molecular mechanism for the protective effects of EPCs and NPCs on H/R-injured neurons (SH-SY5Y cells). Both EPCs and NPCs increased the cell viability, decreased the apoptosis and oxidative stress of H/R-injured SH-SY5Y cells, via activating the PI3K/Akt signal pathway that mainly depends on the progenitor paracrine (VEGF and BDNF) mediated signals.
Combination of EPCs and NPCs offered synergistic benefits on H/R-injured SH-SY5Y cells.

IS can trigger complex cellular events that lead to neuronal cell death in a progressive manner (48, 51, 52). As we mentioned above, the penumbra has been recognized as an area at risk and should be the battle ground for identifying therapeutic targets for stroke therapy (206). The brain is relatively deficient in antioxidant species, with lower activity of glutathione peroxidase (207). And the brain cells are considered highly susceptible to oxidative stress which can lead to a number of cell death paradigms. Therefore, developing safe and effective therapy to prevent neuron death in the penumbra is important for cerebral tissue repair. In this study, we used transwell co-culture system for the first time to elucidate the protection effects of EPCs and NPCs on neurons subjected to H/R-injury. Our data clearly demonstrated that co-culture with EPCs or NPCs alone increased the neuron viability compromised by H/R injury, decreased H/R-induced neural apoptosis and ROS over-production. What’s more, co-culture with the combination of EPCs and NPCs elicited synergistic effects by 26-30% increase as compared to their additive effects. The data indicate that combination of EPCs and NPCs provide better therapeutic efficacy in protecting neurons against H/R injury.
For determining the possible mechanisms underlying the effects of EPCs and NPCs, we measured the levels of BDNF and VEGF in the co-culture medium. BDNF, predominately synthesized and secreted from neurons, is one of the neurotrophic factors for neuron survival (208). Intravenously administration of BDNF prior to focal ischemia injury (209) or intracerebral infusion of BDNF following ischemia (210) have been shown to result in a significant reduction of the infarct volume and/or improvement of behavioral recovery. VEGF, a potent angiogenic factor, has been reported to have diverse actions beyond its proangiogenic effect. For instance, VEGF has been shown to have neuroprotective effects (increase cell viability) in vitro by rescuing neurons subjected to ischemia or serum deprivation (211, 212). All of these findings suggest that BDNF and VEGF could protect neurons from acute injury and promote brain regenerative recovery in IS. In this study, our data showed that NPC co-culture mainly increased the level of BDNF in the co-culture medium, but not for VEGF. Similarly, EPC co-culture raised the level of VEGF but not BDNF in the co-culture medium. Not surprisingly, we found that the levels of both VEGF and BDNF were increased in the medium when co-cultured with EPCs and NPCs together, indicating this approach could offer at least additive beneficial effects since both factors are involved.
In order to assess whether the increased VEGF and BDNF were involved in the beneficial effects of EPCs and NPCs on H/R-injured neuron, we analyzed the receptor expression and downstream pathways. TrkA, TrkB and TrkC are the most common types of Trk receptors. Each of the receptors has different binding affinity to a certain type of neurotrophin. TrkB receptor has high affinity for BDNF and been shown to be essential for the survival-promoting action of BDNF (213). It is reported that BDNF binds to TrkB receptor and thereby affecting the function of the nervous system (214). In the present study, we found that NPC co-culture remarkably up-regulated the phosphorylation of TrkB which could be attributed to the increased expression of BDNF in the neuron culture medium, indicating that NPCs activate the BDNF/TrkB pathway in H/R-injured neurons. On the other hand, we found that EPC co-culture significantly raised the expressions of TrkB and the phosphorylation of TrkB in H/R-injured neurons. These data further explain the synergistic protection effects of EPCs and NPCs on H/R-injured neurons.

Previous studies have demonstrated that VEGF and BDNF can prevent neuronal apoptosis via activation of the PI3K/Akt signaling cascade (211, 212, 215-218). In this study, inhibition of the PI3K upstream BDNF/TrkB and VEGF/VEGFR2 signal pathways largely reduced the synergistic effects of EPCs and NPCs on H/R-injured neurons. These data suggest that the beneficial effects of EPCs and
NPCs are mainly dependent on the VEGF/VEGFR2 and BDNF/TrkB signals (143, 212, 217, 218). Meanwhile, we found that both EPCs and NPCs increased the phosphorylation of Akt, a direct downstream molecule of the PI3K, whereas, blockade of the BDNF/TrkB and VEGF/VEGFR2 signal pathways significantly decreased its expression, reflecting that the activation of the PI3K/Akt is the downstream of the BDNF/TrkB and VEGF/VEGFR2 pathways. All of these data indicate that the PI3K/Akt pathway activated by the VEGF/VEGFR2 and BDNF/TrkB signals is responsible for the beneficial effects of EPCs and NPCs on neurons.

In conclusion, our data demonstrate that co-culture of EPCs and NPCs offer synergistic benefits in protecting SH-SY5Y cells against H/R injury through VEGF and BDNF paracrine-mediated activation of the PI3K/Akt signal pathway, which provides the basis for co-transplantation of EPCs and NPCs for IS.
CHAPTER V. EPC and NPC co-transplantation had synergistic effects on ameliorating acute damage and promoting neurological recovery via the activation of PI3K/Akt signal pathway in a mouse model of IS.

Rationale: IS is one of the most severe health problems in the world. On average, a stroke occurs every 40 seconds and a patient dies of stroke every 4 minutes. Unfortunately, non treatment can restore the brain function of IS is currently available (3). Administration of tPA is effective for some patients, but it has a narrow therapeutic window and a risk of hemorrhagic transformation (4, 5). Mechanical thrombectomy is a promising approach for patients with large-vessel occlusions who respond poorly to intravenous thrombolytics (6). Other treatment options like neuroprotection agents are appear to be less effective (11-13).

In response to stroke insult, NPCs that reside in the SVZ and DG in the brain are activated. They migrate towards the injury site and differentiate into neural cells to replace the damaging cells (118). However, more than 80% of these immature neurons die before them reaching the damaged region and only a small fraction of damaged neurons can be replaced (26), indicating that endogenous NPCs alone are insufficient to adequately repopulate the damaged neurons. Besides activating NPCs, EPCs could be mobilized by ischemia from BM under chemo-
attraction to the injury site where they are differentiated into ECs to repair
damaged endothelium and participate in angiogenesis (107). Unfortunately, the
function of endogenous EPCs is often compromised in cardiovascular diseases
such as hypertension, diabetes, and coronary artery diseases which are high risk
factors for stroke (108, 109, 219). Thus, endogenous EPCs are insufficient for
vascular repair and angiogenesis in response to ischemia. Therefore,
transplantation of exogenous NPCs and EPCs could be a potential strategy for
treating IS.

Indeed, an increasing number of animal studies have suggested that stem-cell
based therapy holds great promising for IS (14, 15, 26, 34, 135). The
transplanted NPCs have been shown to release neurotrophic factors (220, 221),
ameliorate neurological behavioral deficits in IS (121, 122), as well as promote
endogenous neurogenesis in peri-infarct area (120). Meanwhile, it is reported
that transplanted EPCs could reconstitute the integrity and function of the BBB,
decrease the pro-inflammatory response and enhance the repair of damaged
tissue in IS (222). Previously, our lab have demonstrated that infusion of EPCs
promotes vascular protection (22) and angiogenesis (22, 38). All of these findings
suggest that NPCs and EPCs are promising cells for treating IS.
It is known that neurogenesis couples to angiogenesis in the ischemic brain. Stroke-induced new blood vessels in the penumbra region provide scaffolds to guide NPCs to the infarct region (23, 25, 29-31). Cerebral ECs isolated from stroke mice could secrete cytokines to attract NPCs (44). Moreover, it is reported that blockage of stroke-induced angiogenesis can reduce neurogenesis (30). In our in vitro studies, we have demonstrated that co-culture of EPCs and NPCs could protect ECs and neurons against H/R injury via activating the PI3K/Akt pathway. Nevertheless, whether co-transplantation of EPCs and NPCs can synergistically promote vascular protection and enhance angiogenesis and neurogenesis on IS has not been studied.

The objective of this study was to investigate whether co-transplantation of NPCs and EPCs could initiate a synergistic beneficial effect on treating IS via activating the PI3K/Akt pathway. The findings of this study could provide a novel strategy for treating IS.

**Experimental design**

Adult C57BL/6 male mice (8-10 week old) were used for this study. All mice were subjected to MCAO followed by reperfusion surgery, and randomly assigned into five groups for cell transplantation (n=12/group): vehicle (PBS), NPCs, EPCs, NPCs+ EPCs, and NPCs+EPCs+LY294002. LY294002 (1 µl, 1 mM) were
administrated after the MCAO-reperfusion surgery via brain microinjection. The $3 \times 10^5$ NPCs or $3 \times 10^5$ EPCs were resuspended in $3 \mu l$ PBS and injected into two sites of the right striatum after the surgery. The microinjection positions and cell numbers were determined based on a previous study (140). The Rotarod behavior test of experimental mice were conducted on 1 day prior to surgery day (day -1), surgery day (day 0), after middle cerebral artery occlusion (MCAO) surgery, and day 10 after MCAO surgery. To label the new generated cells, mice were injected with bromodeoxyuridine (BrdU, 65 mg/g/day, i.p.) for sequential 7 days before sacrifice. All mice were sacrificed at either day 2 or day 10 after cell transplantation. Brains were collected for different measurements (n=6/group/measurement): 1) For Fluoro-Jade staining to determine the infarct volume; 2) IHC analysis for microvascular density, neurogenesis and angiogenesis; 3) Terminal deoxy-xynucleotidyl transferase dUTP nick end-labeling (TUNEL) staining to assess brain injury; 4) ROS measurement for oxidative stress; 5) Western blot analysis to identify pathway related gene protein expression (VEGF, BDNF, TrkB, p-TrkB, VEGFR2, p-Flk1). All mice received cyclosporine A (10 mg/kg/day, s.c) from the day of surgery.

For all experiments, the theoretical additive effect of EPC and NPC co-transplantation (Es) on animals was calculated as the sum of the effect of the individual monotherapies. The synergistic effect of EPC and NPC co-
transplantation (Es) on animals was calculated by using the formula: \( Es = \frac{(E_{EPC+NPC} - E_{EPC} - E_{NPC})}{(E_{EPC} + E_{NPC})} \times 100\% \). \( E_{EPC} \) represents the effect elicited by EPC co-culture. \( E_{NPC} \) represents the effect elicited by NPC co-culture. \( E_{EPC+NPC} \) represents the effect elicited by EPC and NPC co-culture.

**Materials and methods**

**Experimental animals**

Male C57BL6/J mice (8-10 weeks of age; weight ranges from 22-25 g) purchased from Harlan Laboratories (Indianapolis, Indiana) were used in this study. Mice were maintained in a 22°C room with a 12-hour light/dark cycle and fed with standard chow and drinking water ad libitum. All experimental procedures were approved by the Wright State University Laboratory Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Institutes of Health (NIH). **Fig 24** shows my experiment design.
Fig 24. Experimental timeline delineates the *in vivo* study to determine the therapeutic effects of EPCs and/or NPCs on IS mice.

Cyclosporine A was administrated daily from day 0 to day 10. LY292004 was injected with cell suspensions.
MCAO surgery

The MCAO surgery was performed as we previously described (22, 38). Briefly, mice were anesthetized by inhaling 2.5% isoflurane. The body temperature of mice was maintained using a water-jacketed heating pad. The left common carotid artery (CCA) was exposed and ligated distal to the bifurcation. Then the left external carotid artery (ECA) was ligated and cut for exposure of the left internal carotid artery (ICA). A suture was placed under the ICA and lightly lifted to prevent blood backflow from the head. Then, a small incision was made on the CCA between the ligation and carotid bifurcation. A 7-0 nylon monofilament suture with a rounded head coated with poly-L-lysine was inserted through the small incision and advanced into the ICA until resistance was detected (about 9-10 mm distal to the bifurcation). The suture was left in place by ligation for transient MCAO for 90 minutes. To restore the middle cerebral artery blood flow (reperfusion), a second anesthesia (2.5% isoflurane) was given, the knot on the left ICA was momentary loosed and the suture was withdrawn. The remaining suture was shortened. The incision was cleaned and closed with a surgical suture. After a 30-minute reperfusion, mice were received cell transplantation by using brain microinjection.

Rotarod test
To determine whether transplantation of EPCs and/or NPCs can promote functional recovery in the IS mice, we performed rotarod test which provides an index of forelimb and hindlimb motor coordination and balance (223). All animals were trained for 3 days (3 trials/day) prior to the surgery day. The rotarod test was performed 1 day prior to MCAO surgery, after MCAO surgery (before cell transplantation), and at day 2 and 10 after MCAO according to the previously described protocol (135). In brief, mice were placed on an accelerating rotarod rungs. The speed was increased from 5 to 35 rpm every 30 seconds. The time of each mouse remained on the rungs was recorded. The mean duration (in seconds) on the rungs was averaged from 3 trials. A trial ended if the animal fell off the rungs or gripped the device and spun around for 2 consecutive revolutions without attempting to walk on the rungs. Data is presented as the average duration normalized to that of vehicle mice prior to MCAO.

**Cell transplantation via brain microinjection**

EPCs (3 x 10^5 cells), NPCs (3 x 10^5 cells), and a mixture of EPCs and NPCs (1.5 x 10^5 EPCs and 1.5 x 10^5 NPCs) were prepared in 3 μl sterile PBS immediately prior to use (140). Brain microinjections was performed after 2 hours of the MCAO surgery by using a 10 μl syringe and UMP3 micro syringe pump combined with the Micro4 controller (World Precision Instruments, FL) as previously reported (224). Briefly, mice were anesthetized using a ketamine:
xylazine mixture (100:8 mg/kg) and placed on the stereotaxic frame. An incision was made on the back of the head (around 1 cm). The injection guide was lowered closely to the brainstem surface pointing the injection site (coordinate 1: 0.5 mm anterior, 2.2 mm lateral to bregma, and 2.5 mm from brain surface), then a small hole was drilled in the skull for injection. The micropipette of the syringe filled with cell suspension was lowered through the guide cannula into the injection site (coordinate 1: 0.5 mm anterior, 2.2 mm lateral to bregma, and 2.5 mm from brain surface). After injection, the micropipette was lowered into another coordinate of the left striatum (coordinate 1: 0.5 mm posterior, 2.2 mm lateral to bregma, and 2.5 mm from brain surface). Each injection was 1.5 μl over 45 seconds and was left in place for 5 minutes to minimize upward flow of cell solution after raising the micropipettes. Mice in the vehicle group received 2 injections of PBS, 1.5 μl for each site. For pathway study, LY294002 was dissolved in 3% dimethyl sulfoxide (DMSO, Thermo Fisher Scientific, MA) and diluted with sterile PBS to a working concentration of 10 mM (225). The diluted LY294002 (1μl, 10 mM) was mixed with the mixed EPC and NPC suspension and injected into some mice. Each mouse received cyclosporine A (10 mg/kg, subcutaneously) daily after the cell transplantation (226).

Functional evaluation of neurological deficits
The evaluation of neurological deficits was carried out on day 2 and 10 after cell transplantation according to a five-point scale (22, 38): 0, normal motor function; 1, flexion of contralateral torso and forelimb upon lifting the whole animal by the tail; 2, circling to the contralateral side but normal posture at rest; 3, leaning to the contralateral side at rest; 4, no spontaneous motor activity.

**Quantification of infarct volume**

On day 2 and 10 following cell transplantation, the brains were immediately collected after transcardially perfusion with PBS followed by 4% PFA, fixed in 4% PFA overnight followed in 4% PFA plus 30% sucrose for 3 days. Then the brains were dried with wipes (Kimberly-Clark, TX), wrapped with aluminum foil, and stored at -80°C. Then, the brains were cut into coronal sections (15 μm thickness) and sequentially put into six separate wells of 6-well plates containing 2 ml of PBS. The cerebral ischemic damage was revealed by Fluoro-Jade (Histochem, Jefferson, AR) staining as we previously reported (38). In brief, six brain sections from rostral to caudal were selected for representing a whole brain. The selected brain sections were mounted on microscope slides and air-dried at 30°C overnight. Slides were then sequentially placed in decreasing concentrations (100, 95, 85, and 75%) of ethanol for 3 minutes each, in distilled deionized water for 1 minute, and in 0.06% KMNO₄ for 15 minutes. Then the slices were stained with 0.001% Fluoro-Jade in 0.1% acetic acid for 30 minutes
at RT in the dark, rinsed with ddH₂O four times for 1 minute each, dried for 20
minutes, cleared in xylene, and coverslipped with DPX mounting medium (BDH
Laboratories, Poole, UK). All images were taken under an invertible fluorescence
microscope (EVOS, Life technologies, CA), and analyzed by using the Image J
software (NIH). We calculated the infarct volume as a percentage of the whole
hemisphere using the following formula: [(area of contralateral hemisphere)-(area
of remaining ipsilateral hemisphere)] / (area of contralateral hemisphere) x 2
x100% (227).

**Measurement of cerebral microvascular density**

On day 2 and 10 after cell transplantation, the brains were collected and
sectioned using a cryostat into 15-µm thickness coronal sections as described
above. The cerebral microvascular density (cMVD) in the peri-infarct area was
revealed by CD31 staining as previously reported (22, 38). Briefly, the free-
floating brain slices were blocked for 1 hour with 1% donkey serum in Tris-
buffered saline, reacted with rat anti-mouse CD31 (1:50 in 1% donkey serum; BD
Biosciences, CA) primary antibody or PBS (for negative control) at 4°C overnight,
washed, incubated with Alexa Fluor 594-conjuated donkey anti-rat IgG (1:200,
Molecular Probes; Invitrogen, CA) secondary antibody for 1 hour at RT. After 3
washes, sections were coverslipped with fluorescence mounting medium.
Images were captured with a confocal microscope (Olympus FV1000, Japan).
Six brain slices from rostral to caudal were selected for representing a whole brain. Five random areas in the peri-infarct regions, determined by staining the adjacent sections with Fluoro-Jade as described above, were chosen from each slice. Quantification of vessel density was analyzed using Image J software (NIH). The mean cMVD from six sequential brain slices of individual mouse was calculated and expressed as numbers/mm².

**Immunohistochemistry analysis**

On day 10 after cell transplantation, the brains were collected and sectioned into 15-μm thickness coronal sections as above described. The selected sections were incubated with mouse anti-HuNu (1:50; Millipore, MA), or mouse anti-BrdU (1:50; Abcam, MA), or rabbit anti-NeuN (1:50, Millipore, MA), or rat anti-CD31 (1:50; BD Biosciences, CA) primary antibody for overnight at 4°C. Next, brain sections were reacted with Cy3-conjugated donkey anti-rat, or Cy3-conjugated donkey anti-rabbit, or FITC-conjugated donkey anti-mouse secondary antibody (1:250; Invitrogen, CA) for 2 hours at RT in the dark. The positive cells in the peri-infarct area of each section were visualized using confocal microscopy (Olympus FV1000, Japan). HuNu⁺CD31⁺ cells represented transplanted EPCs incorporated with microvessels. Angiogenesis was determined as BrdU⁺CD31⁺ cells and neurogenesis was determined as BrdU⁺NeuN⁺ cells according to previous reports (22, 38). Six brain slices from rostral to caudal were selected for
representing a whole brain. Five random microscopic fields (magnification: 200×) in the peri-infarct regions, determined by staining the adjacent sections with Fluoro-Jade as described above, were chosen from each slice. The percentage of neurogenesis or angiogenesis, and the percentage of transplanted EPCs incorporated with microvessels, was analyzed using Image J software (NIH), respectively. The percentage of HuNu+CD31+ cells from six sequential brain slices of individual mouse was calculated as: HuNu+CD31+ cells/HuNu+ cells x 100%. The neurogenesis and angiogenesis from six sequential brain slices of individual mouse was calculated and expressed as numbers/mm².

Measurement of ROS production in brain tissue

On day 2 and 10, mice were deeply anesthetized by intraperitoneal injection of pentobarbital, perfused through the heart with PBS. The brains were collected and immediately frozen in dry ice. For DHE staining, the brains were sectioned into 15-µm thickness coronal sections, mounted onto pre-chilled gelatin-coated slides and air-dried for 30 minutes. Then, brain sections were incubated with DHE (2 µmol/L) in dark for 10 minutes at RT (228, 229). After incubation, sections were washed 3 times with PBS. Following the final wash, sections were coversliped and imaged using a fluorescence microscope (EVOS, Life Technologies, CA). The DHE fluorescence of five randomly chosen microscopy fields located in the peri-infarct areas was quantified using Image J. Six slices
from rostral to caudal of each brain were averaged to present the data of each mouse. The data were expressed as percentage of fluorescence level relative to the level of ROS in vehicle (PBS-treated) group.

**In situ apoptosis detection by TUNEL staining**

In situ apoptosis was analyzed by TUNEL assay kit (Roche, Switzerland) according to the manufacturer’s instructions. In brief, brain slices (10-µm) mounted on gelatin-coated slides were dried overnight at 30°C. After being washed 3 times with PBS, the tissue was permeabilized with 0.1% TritonX-100/0.1% sodium citrate for 2 minutes on ice, washed, and incubated with freshly prepared TUNEL reaction mixture in a humidified atmosphere for 60 minutes at 37°C in the dark. Brain slices incubated with label solution served as negative controls. Cell nuclei were stained with DAPI. Tissue samples were examined under a fluorescence microscope (Leica, Germany). Apoptotic cells were counted at five arbitrarily selected microscopic fields. Six slices from rostral to caudal of each brain were averaged to present the data of each mouse. Apoptotic rate were calculated as: TUNEL+ cells / DAPI+ cells x 100%.

**ELISA of IL-10, TNF-α, IL-1α in plasma**

The levels of anti-inflammatory factor IL-10 and pro-inflammatory factors TNF-α and IL-1α in mouse plasma were measured by ELISA kits (R&D Systems, MN)
according to the manufacture’s instruction. In brief, after 2 days of cell transplantation, mice were deeply anesthetized by intraperitoneal injection of pentobarbital. A volume of 0.5–1 ml of peripheral blood was taken from the left ventricle of heart into a syringe containing 1% volume of heparin (1,000 U/ml; Sigma, MO). Then the whole blood was centrifuged at 2,000g for 20 minutes, the supernatant was considered as plasma, aliquoted and stored at -20°C. For analysis, plasma was thawed and diluted with Calibrator Diluent RD5T (1:1 volume; R&D Systems, MN). Then the diluted plasma was added (50 µl/well) to a 96-well plate coated with a primary antibody specific to mouse IL-10, TNF-α or IL-1α, and incubated for 2 hours at RT. All wells were washed with wash buffer, and added with HRP-conjugated IL-10, TNF-α or IL-1α antibody was and incubated for 1 hour at RT. After 4 washes, substrate solution was added to each well. 30 minutes later, stop solution was added and the optical density of each well was measured at 450 nm. A standard curve was run for each assay, and all standards or samples were run in duplicate.

**Western blot analysis**

H/R-injured ECs were harvested after co-cultured with EPCs and/or NPCs. Proteins were extracted with cell lysis buffer (Thermo Fisher Scientific, MA) supplemented with complete mini protease inhibitor tablet (Roche, Switzerland). Protein lysates were electrophoresed through SDS-PAGE gels and transferred
onto PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 hour and incubated with primary antibody: rabbit anti-Akt (1:1000; Cell Signaling Technologies, MA), rabbit anti-p-Akt (1:1000; Cell Signaling Technologies, MA), rabbit anti-VEGF (1:1000; Cell Signaling Technologies, MA), rabbit anti-VEGFR2 (1:1000; Cell Signaling Technologies, MA), rabbit anti-p-Flk1 (1:200; Santa Cruz Biotechnology, TX), rabbit anti-BDNF (1:200; Santa Cruz Biotechnology, TX), rabbit anti-TrkB (1:1000; Cell Signaling Technologies, MA), rabbit anti-p-TrkB (1:200; Santa Cruz Biotechnology, TX), and mouse anti-β-actin (1:4000; Sigma, MO) at 4°C for overnight. After washing, membranes were incubated with HRP-conjugated donkey anti-rabbit or donkey anti-mouse IgG (Jackson Immuno Research Lab, MI) for 1 hour at RT. Blots were developed with enhanced chemiluminescence developing solutions and quantified under ImageJ software. All experiments were repeated at least six times.

**Statistical analysis**

All data are expressed as mean ± SEM. Comparisons for two groups were analyzed by Student’s t-test. Multiple comparisons were analyzed by one- or two-way ANOVA followed by Bonferroni’s post hoc analysis. The theoretical additive effect was calculated as the sum of the effect of the individual monotherapies. Two-way ANOVA was used to test levels of significance for the synergistic
interaction of the EPCs and NPCs above an additive effect. A value of $p < 0.05$ was considered statistically significant.

**Results**

**Co-transplantation of EPCs and NPCs synergistically improved motor function.**

We conducted the accelerating rotarod test and used the classic neurological deficit score scale to assess the motor function of IS mice that received cell transplantation. According to the results of the rotarod test (**Fig 25A**), after MCAO-reperfusion surgery, mice displayed a significantly shorter duration of remaining on the rotarod rungs compared with before stroke. At day 2 and 10, the mice received EPCs or NPCs exhibited a better performance than those received PBS only, and the mice co-transplanted with EPCs and NPCs had the best performance. Meanwhile, the results of neurologic deficit score assessment (**Fig 25B**) showed that transplantation of EPCs or NPCs alone decreased the neurologic deficit score, and co-transplantation with EPCs and NPCs further decreased it on both day 2 and 10. Whereas, administration of the PI3K inhibitor LY294002 completely blocked the improved motor function elicited by the combination therapy on the IS mice.
Rotarod (% of baseline)

Days after MCAO

EPCs
NPCs
EPCs+NPCs
PBS
EPCs+NPCs
+LY294002

A
Fig 25. Co-transplantation of EPCs and NPCs effectively improved the motor function and reduced neurologic deficit on IS mice. A, the averaged rotarod test performance of mice in different treatment groups at different time points. B, summarized data showing the neurologic deficit score assessed on day 2 and/or day 10 after cell transplantation in all groups. * p<0.05, vs. PBS. N=6-12/group.
Co-transplantation of EPCs and NPCs synergistically decreased the infarct volume.

We used Fluoro-Jade staining to evaluate the infarct size. As shown in Fig 26 and Table 1, EPCs alone or NPCs alone reduced the infarct size. What’s more, co-transplantation with EPCs and NPCs remarkably decreased the infarct size as compared to that EPCs alone, or NPCs alone, or PBS did on both subacute (day 2) and chronic phases (day 10). However, there was no significant difference of the infarct volume between the EPCs+NPCs+LY294002 group and PBS group on both time points.
Fig 26. Co-transplantation of EPCs and NPCs synergistically reduced the infarct volume on both day 2 and day 10. A, representative brain slices (15-µm thickness) stained with the Fluoro-Jade in all treatment groups. N=6 mice/group.
<table>
<thead>
<tr>
<th>Group</th>
<th>Infarct volume (%)</th>
<th></th>
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<tr>
<td></td>
<td></td>
<td>D2</td>
<td>D10</td>
</tr>
<tr>
<td>PBS</td>
<td>23 ± 1</td>
<td>23.5 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>EPCs</td>
<td>19.5 ± 0.9 *</td>
<td>17 ± 1 *</td>
<td></td>
</tr>
<tr>
<td>NPCs</td>
<td>20 ± 0.8 *</td>
<td>18 ± 0.8 *</td>
<td></td>
</tr>
<tr>
<td>EPCs+NPCs</td>
<td>14 ± 1 * #</td>
<td>6 ± 0.6 * #</td>
<td></td>
</tr>
<tr>
<td>EPCs+NPCs+LY294002</td>
<td>20 ± 1.3</td>
<td>21 ± 1.5</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. The percentage of infarct volume to the whole hemisphere of mice in each group. *p <0.05, vs. PBS; #p <0.05, vs. EPCs or NPCs at the same time point. Data are expressed as mean ± SEM. N=6 mice/group.
Co-transplantation of EPCs and NPCs synergistically reduced cell apoptosis in the peri-infarct area.

**Fig 27A** shows representative images of TUNEL staining for identifying apoptotic cells in the ipsilateral and contralateral brain on 2 and 10 days after cell transplantation, respectively. There were barely detectable TUNEL$^+$ cells in the contralateral brain. The percentage of TUNEL$^+$ cells (TUNEL$^+$/DAPI$^+$ x 100%) was decreased in the ipsilateral brain of the mice transplanted with EPCs or NPCs alone. Also the percentage of TUNEL$^+$ cells was further decreased in mice co-transplanted with EPCs and NPCs (**Fig 27B**). Likewise, we found that PI3K inhibitor LY294002 can abrogate the anti-apoptotic effect of EPCs and NPCs on IS mice.
<table>
<thead>
<tr>
<th></th>
<th>D2</th>
<th>D10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contralateral</td>
<td>Ipsilateral</td>
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<tr>
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<td><img src="image" alt="PBS" /></td>
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<td>NPCs</td>
<td><img src="image" alt="NPCs" /></td>
<td><img src="image" alt="NPCs" /></td>
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<tr>
<td>EPCs+NPCs</td>
<td><img src="image" alt="EPCs+NPCs" /></td>
<td><img src="image" alt="EPCs+NPCs" /></td>
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<tr>
<td>EPCs+NPCs +LY294002</td>
<td><img src="image" alt="EPCs+NPCs +LY294002" /></td>
<td><img src="image" alt="EPCs+NPCs +LY294002" /></td>
</tr>
</tbody>
</table>
Fig 27. Co-transplantation of EPCs and NPCs synergistically reduced cell apoptosis in the peri-infarct area on both day 2 and day 10. A, representative images showing TUNEL staining in the ipsilateral and contralateral hemispheres of the brain. Green: TUNEL; Blue: DAPI. Scale bar: 100 µm. B, quantitative data showing that EPC or NPC transplantation alone decreased cell apoptosis in the peri-infarct area, with a greater effect elicited by the combination of EPCs and NPCs. Data are expressed as mean ± SEM. N=6 mice/group.
Co-transplantation of EPCs and NPCs synergistically reduced ROS production in the peri-infarct area.

To assess whether co-transplantation of EPCs and NPCs could reduce ROS production, we conducted DHE staining in the brain tissue. Fig 28A shows representative images of DHE staining in contralateral and ipsilateral of the brain. As shown in Fig 28B, transplantation of EPCs alone or NPCs alone decreased ROS production in the peri-infarct area as compared with PBS. Also we found that co-transplantation of EPCs and NPCs elicited a synergistic anti-oxidative effect on both day 2 and day 10 when compared to the effect of EPCs or NPCs alone. It was also found that the anti-oxidative effect of the EPCs and NPCs could be completely inhibited by LY294002.
Co-transplantation of EPCs and NPCs synergistically decreased ROS production in the peri-infarct area on both day 2 and day 10. A, representative images showing DHE staining in contralateral and ipsilateral hemisphere of the brain on both time points. Red: DHE staining. B, quantitative data showing that EPC or NPC transplantation alone displayed anti-oxidative effect in the peri-infarct area, with a greater effect elicited by the combination of
EPCs and NPCs. *p <0.05, vs. PBS. Data are expressed as mean ± SEM. N=6 mice/group
Co-transplantation of EPCs and NPCs synergistically increased cMVD in the peri-infarct area.

In addition, we measured the cerebral microvascular density in the peri-infarct area using immunofluorescence staining with anti-CD31. As shown in Fig 29, on day 2, the cMVD (CD31+ cells) was increased in the mice transplanted with EPCs or NPCs alone. A greater increase of cMVD was noted in mice grafted with the combination of EPCs and NPCs. On day 10, a synergistic increase of cMVD was observed in the co-transplantation group compared with the mice transplanted with EPCs or NPCs alone. Again, there was no significant difference between the mice treated with PBS and the ones treated with EPCs and NPCs plus LY294002 at both time points.
A

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Fig 29. Co-transplantation of EPCs and NPCs synergistically increased cMVD in the peri-infarct area on both day 2 and 10. A, representative images showing cMVD level in the ipsilateral hemisphere of the brain on both time points. Red: CD31 staining. B, quantitative data showing that EPC or NPC transplantation alone increased cMVD in the peri-infarct area, with a greater effect elicited by the combination of EPCs and NPCs. *p <0.05, vs. PBS. Data are expressed as mean ± SEM. N=6 mice/group. Scale bar: 50 µm.
Co-transplantation of EPCs and NPCs suppressed the ischemia-triggered inflammatory response on day 2.

Post-stroke inflammation plays an important role in brain recovery. We analyzed the level of pro-inflammatory and anti-inflammatory factors in the mouse plasma on day 2 using ELISA. Our results (Fig 30) showed that the level of IL-10 was increased in mice that received EPCs or NPCs alone as compared with that in PBS-treated mice. Moreover, there was a higher level of IL-10 in the plasma of mice treated with the combination of EPCs and NPCs than that in the EPCs or NPCs treatment group. In contrast, the levels of pro-inflammatory factors TNF-1α and IL-1α were decreased in the plasma of mice transplanted with EPCs or NPCs alone, and an even lower level was observed in the plasma of those co-transplanted EPCs and NPCs. There was no significant difference of the levels of IL-10, TNF-1α and IL-1α in the plasma of mice between EPCs+NPCs+LY294002 and PBS groups.
Co-transplantation of EPCs and NPCs synergistically increased anti-inflammatory factor protein level, and decreased pro-inflammatory factor protein level in mouse plasma on day 2. A, IL-10 protein expression level. B-C, the protein levels of TNF-1α and IL-1α. *p <0.05, vs. PBS. Data are expressed as mean ± SEM. N=6 mice/group.
Co-transplantation of EPCs and NPCs synergistically promoted neurogenesis and angiogenesis in the peri-infarct area.

Fig 31A shows the representative images of neurogenesis (BrdU+NeuN+) in the brain of IS mice. No significant difference was observed between the EPC transplanted and PBS-treated mice. Data showed that NPC transplantation significantly increased neurogenesis on day 10, and co-transplantation of EPCs and NPCs synergistically increased neurogenesis in the peri-infarct area (Fig 31B). This increased neurogenesis was abrogated by LY294002.

Upon the results of double staining of BrdU (label newly generated cells) and CD31 (EC marker), we found that the angiogenesis (BrdU+CD31+) was significantly promoted in mice transplanted with EPCs, and co-transplantation with EPCs and NPCs synergistically increased the angiogenesis in the peri-infarct area on day 10 (Fig 32). There was no significant difference between the NPC transplantation and PBS treatment groups. In addition, our data showed that the synergistic effects of EPCs and NPCs on angiogenesis was abolished by LY294002.
Fig 31. Co-transplantation of EPCs and NPCs synergistically promoted neurogenesis on day 10. A, representative images showing the newly generated neurons. Bottom panel is an enlarged view of the white box region in the respective upper panel. White arrows point the merged cells (BrdU$^+$NeuN$^+$). Red: NeuN; Green: BrdU. Scale bar: 50 μm (upper panel); 25 μm (bottom panel).

B, summarized data showing the average number of BrdU$^+$NeuN$^+$ cells in per mm$^2$ in the ipsilateral of mice grafted with EPCs and/r NPCs. *p<0.05, vs. PBS. Data are expressed as mean ± SEM. N=6 mice/group.
Figure A shows the effect of different treatments on cell proliferation and differentiation. Cells were stained with BrdU/CD31 to visualize proliferation and differentiation. The treatments included PBS, EPC, NPC, EPC+NPC, and EPC+NPC+LY294002. The images demonstrate the presence of BrdU-positive cells (green) and CD31-positive cells (red), indicating cell proliferation and differentiation.
Fig 32. Co-transplantation of EPCs and NPCs synergistically promoted angiogenesis on day 10. A, representative images showing the angiogenesis in the peri-infarct area. Bottom panel is an enlarged view of the white box region in the respective upper panel. White arrows point the merged cells (BrdU<sup>+</sup>CD31<sup>+</sup>). Red: CD31; Green: BrdU. Scale bar: 50 μm (upper panel); 25 μm (bottom panel). B, summarized data showing the average number of BrdU<sup>+</sup>CD31<sup>+</sup> cells in per mm<sup>2</sup> in the ipsilateral of mice treated with EPCs and/or NPCs. *p <0.05, vs. PBS. Data are expressed as mean ± SEM. N=6 mice/group.
Co-transplantation of EPCs and NPCs synergistically promoted EPC incorporation with microvessels on day 10.

As shown in Fig 33, we found that a higher percentage of transplanted EPCs were merged with microvessels in the co-graft group than that in the EPC transplantation alone group. No difference was found in the NPC transplantation and PBS treatment groups.
Fig 33. Co-transplantation of EPCs and NPCs synergistically promoted EPC incorporation with microvessels on day 10. A, representative images showing the grafted EPCs merging with the microvessels. HuNu: grafted cells; CD31: microvessels. A2, Scale bar: 50 µm. A2, summarized data showing the percentage of HuNu−CD31+ cells over the total number of HuNu+ cells in the two groups.
Co-transplantation of EPCs and NPCs synergistically increased the level of VEGF and activated the VEGF/VEGFR2 pathway in the ipsilateral brain

In order to determine the molecular basis underlying the beneficial effects of co-transplantation of EPCs and NPCs, we examined the protein expression level of VEGF and the activation of its receptor VEGFR2. Western blot results revealed that VEGF level was increased in the ipsilateral brain of mice treated with EPCs as well as the combination of EPCs and NPCs, and there was no significant difference of VEGF expression in between NPC alone treated and PBS treated mice on both day 2 and 10 (Fig 34A1 & B1). Also, the expression of p-Flk1/VEGFR2 was up-regulated in the ipsilateral brain of mice treated with EPCs as well as with the combination of EPCs and NPCs on both time points (Fig 34A1 & B2). These data suggest that the VEGF/VEGFR2 signal pathway is activated in the ipsilateral brain of mice that treated with EPCs alone and with the combination of EPCs and NPCs.
**A1**

VEGF

β-actin

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**A2**

p-Flk1

VEGFR2

β-actin

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* P<0.05
**Fig 34.** Co-transplantation of EPCs and NPCs synergistically up-regulated the levels of VEGF and its receptor phosphorylation in the ipsilateral brain.

A1-A2, VEGF and p-Flk1/VEGFR2 protein expression levels on ipsilateral brain on day 2; B1-B2: VEGF and p-Flk1/VEGFR2 protein expression levels on ipsilateral brain on day 10. Upper: representative western blot bands; bottom: quantitative data. *p <0.05, vs. PBS. Data are expressed as mean ± SEM. N=6 mice/group.
Co-graft of EPCs and NPCs synergistically increased the level of BDNF and activated the BDNF/TrkB pathway in the ipsilateral brain

As shown in Fig 35, we found that the levels of BDNF and p-TrkB/TrkB were increased in the ipsilateral brain tissue of mice transplanted with NPCs on both day 2 and 10 as compared to that in the mice treated with PBS. No difference was observed between the mice received EPCs and PBS. Co-transplantation of EPCs and NPCs synergistically increased the protein expression levels of BDNF and p-Flk-1/VEGFR2 in the ipsilateral brain of mice at both time points. These results indicate that the BDNF/TrkB pathway was activated in the ipsilateral brain of mice received NPCs and the combination of EPCs and NPCs.
Fig 35. Co-transplantation of EPCs and NPCs synergistically up-regulated the protein expression levels of BDNF and its receptor phosphorylation in the ipsilateral brain. A1-A2, BDNF and p-TrkB/TrkB protein expression levels on the ipsilateral brain on day 2; B1-B2: BDNF and p-TrkB/TrkB protein expression levels on the ipsilateral brain on day 10. Upper: representative western blot bands; bottom: quantitative data. *p <0.05, vs. PBS. Data are expressed as mean ± SEM. N=6 mice/group.
Co-transplantation of EPCs and NPCs increased the ratio of p-Akt/Akt.

To determine whether the downstream PI3k/Akt pathway is involved in the therapeutic effects of EPCs and NPCs, we measured the protein expression of p-Akt/Akt. The results (Fig 36) revealed that the ratio of p-Akt/Akt was higher in EPC or NPC transplantation alone group than that in the PBS group. And EPC and NPC co-transplantation synergistically up-regulated its expression on both day 2 and day 10 after cell graft. LY294002 blocked the increased expression of p-Akt/Akt in the ipsilateral brain tissue.
Fig 36. Co-transplantation of EPCs and NPCs synergistically up-regulated the level of p-Akt/Akt in the ipsilateral brain. A, p-Akt/Akt protein expression on the ipsilateral brain on day 2; B: p-Akt/Akt protein expression on the ipsilateral brain on day 10. Upper: representative western blot bands; bottom: quantitative data. *p <0.05, vs. PBS. Data are expressed as mean ± SEM. N=6 mice/group.
Discussion

In the present study, we have demonstrated that co-transplantation of EPCs and NPCs synergistically alleviates inflammation response, reduces cell death, decreases infarct volume, improves motor function, enhances neurogenesis and angiogenesis accompanied with up-regulation of neurotrophic and angiogenic factors (BDNF and VEGF) in the ischemic hemisphere brain. Moreover, we revealed that the therapeutic effects of EPCs and NPCs are mediated by the PI3K/Akt pathway.

With the development of regenerative medicine, stem cells have been considered to be of great potential for treating IS (14, 15). Up to date, various stem cell delivery routes such as intravenous, intracerebral, intra-artery, and intraventricular have been applied in treating stroke (230). Intracerebral injection was chosen in our study because the cells could be precisely transplanted into the striatum of ischemic brain without cell loss. Other injection routes could lead to stem cells trapped in systemic organs, with few stem cells reaching the ischemic brain hemisphere (141). Previous studies showed that both early and delayed cell transplantation had beneficial effects on IS (140, 231). Previously, our group has reported that infusion of EPCs could maintain cerebral vascular density during acute ischemia (22), reduce the neurological deficit score and promote angiogenesis in IS mice (22, 38). In the present study, EPCs and/or
NPCs were stereotactically injected into the brain at 2 hours post IS surgery, in order to assess whether early transplantation could confer neurovascular protection.

In this study, our data showed that NPCs improved the rotarod performance and decreased the neurological deficit score. As expected, we found that EPC transplantation improved the motor function, which is consistent with our previous study (22). Also we found that co-transplantation of EPCs and NPCs elicited synergistic effect on improving the motor function. The gradual motor function improvement over the 10 days indicates that the combination of EPCs and NPCs has a recovery-synergistic effect. From the pathological analysis, we found that transplantation of EPCs or NPCs slightly decreased infarct volume (~ 3% reduction in EPC or NPC transplanted mice), and this reduction was enhanced in the co-transplantation group (~ 9% reduction) on day 2 after cell transplantation, and even a higher reduction (~17%) on day 10 after cell transplantation. These data suggest that co-transplantation of EPCs and NPCs could synergistically ameliorate tissue damage in the brain. Here, we also found that the effects of co-transplantation of EPCs and NPCs on behavior performance and infarct size were entirely blocked by LY294002, reflecting that the PI3K pathway mediates the beneficial effects elicited by EPCs and NPCs. These data are supported by
our previous study showing the PI3K/Akt pathway is responsible for the beneficial effects of EPCs on treating IS on diabetic mice (22).

It is known that cell apoptosis occurs in brain ischemia (232). In the present study, we found a much lower percentage of TUNEL+ cells in the mice co-transplanted EPCs and NPCs than that treated with EPCs or NPCs, reflecting the synergistic effect of EPCs and NPCs on reducing cell death. This data is in agree with previous reports showing that the transplanted NPCs have anti-apoptotic effect in treating hemorrhagic stroke (233, 234). Besides, this result further supports the observation of improved motor function in mice co-transplanted with EPCs and NPCs. Blockade of the PI3K pathway diminished this anti-apoptotic effect, reflecting that this pathway is responsible for the anti-apoptotic effect of EPCs and NPCs. Taken together, these data indicate the synergistic effects of EPCs and NPCs on reducing cell apoptosis in the peri-infarct area in the brain.

Oxidative stress has been shown to play an important role in tissue damage during ischemia/reperfusion, being involved in the gradual expansion of a cerebral infarct (93, 235). Numerous experimental and clinical observations have showed the increased free radical formation during stroke injury (190, 236). In our study, EPC or NPC transplantation notably reduced the increased ROS production. What’s more, co-transplantation of EPCs and NPCs synergistically
decreased ROS production in the peri-infarct areas at 2 and 10 days after cell transplantation. This data could help to explain the synergistic effects of EPCs and NPCs on reducing the infarct size. It also is supported by a previous study showing that amelioration of tissue damage in the peri-infarct area contributed to the reduction of infarct volume (237). Further work is needed to analyze whether the majority of ROS production detected was located in neurons.

Besides ROS, inflammation is also implicated in the pathogenesis of IS (1, 93). It is known that with the onset of ischemia, ECs and neurons release a number of cytokines like TNF-1α and IL-1α (93, 95) and stimulate the post-stroke inflammation (238). On the other hand, inflammation has beneficial effects such as removal of damaged tissue by phagocytic cells. The positive versus negative effects of inflammation following stroke still remains controversial (91), but it is generally considered that inflammation does more harm than good after stroke, especially in the early stage of ischemic injury. Here, we found that co-transplantation of EPCs and NPCs synergistically suppressed ischemia-triggered inflammation in the subacute phase, indicating that co-transplantation of EPCs and NPCs has vascular protective effects in the subacute phase of IS. Meanwhile, this protective effect was reflected by the synergistically increased microvascular density in the peri-infarct tissue.
It is known that functional repair requires the recovery of both neural and vascular components in the damaged area after brain ischemia (80, 239). Re-establishing the vascular network in the infarct areas might have a neuroprotective effect and is important for treating IS. EPCs are believed to play an important role in maintaining endothelial/vascular integrity and angiogenesis (114, 240). Increasing evidence shows that injection of ex vivo expanded EPCs significantly improved cardiac function in the animal model of myocardial infarction (241, 242), and increases the neovascularization in the hindlimb ischemia model (241, 243, 244). In IS, endogenous angiogenesis is poor in the infarct area (245). Measurement of newly generated ECs (BrdU+ CD31+ cells) for the index of angiogenesis is commonly used by others and us (38, 221, 246). In this study, we obtained the evidence that EPC transplantation increased angiogenesis, and co-transplantation of EPCs and NPCs synergistically elicited a higher level of angiogenesis in the peri-infarct area, providing the basis for the overall synergistic effects elicited by EPCs and NPCs. To date, whether transplanted EPCs can merge with blood vessels remains unknown. Here, we first reported that the grafted EPCs incorporated with microvessels (~12% HuNu+ cells co-labeled with CD31+ cells) on day 10 after cell transplantation. Furthermore, more grafted EPCs (~25% HuNu+ cells co-labeled with CD31+ cells) merged with microvessels in the co-transplantation group. Taken together, these data indicate that co-transplantation of EPCs and NPCs synergistically
protected cerebral vasculature against ischemia and promoted tissue repair following IS. In our study, we observed that NPCs increased neurogenesis (BrdU+ NeuN+ cells) in the peri-infarct region, which is consistent with previous reports (121, 122). Also this effect was synergistically enhanced by the combination of EPCs and NPCs, suggesting that EPCs have the recovery-enhancing effects on treating IS.

In the present study, we also assessed the contribution of BDNF and VEGF on the therapeutic effects of EPCs and NPCs. VEGF has been shown to be important for suppression of inflammation and angiogenesis in the peri-infarct region (247). In line with such a role of VEGF, it was reported that increase of VEGF expression could lead to significant increase of long-term potentiation in the hippocampus through modulation of synaptic plasticity in mature neurons (248). BDNF is known to be neuroprotective through influence the generation and survival of neurons (198, 208, 249, 250). In our study, we found that the levels of BDNF and VEGF in the ipsilateral brain tissue were increased in mice treated with EPCs or NPCs alone, and synergistically up-regulated in mice co-transplanted with EPCs and NPCs. This finding supports the synergistic efficacy of EPCs and NPCs in vivo, because the increased BDNF and VEGF may provide mutually supportive trophic mechanisms of neural protection and vascular protection in the microenvironment and prevent the brain from injury. Meanwhile,
the synergistically increased angiogenesis might lead to the increase of blood flow which in turn facilitating the neurotrophic and angiogenic factor delivery, resulting in an overall synergistic therapeutic effects.

Besides the increased level of VEGF and BDNF, we also found that EPC transplantation up-regulated p-Flk1/VEGFR2 level, and NPC transplantation up-regulated the level of p-TrkB/TrkB which was supported by a previous study (251). The ratio of p-Akt/Akt was found to be up-regulated in EPC or NPC alone treated mice, and to be synergistically raised in the combination treatment group. These results indicated VEGF/VEGFR2/PI3K/Akt and BNDF/TrkB/PI3K/Akt pathways were activated in the mouse brain. Collectively, all of data suggest that increased VEGF and BDNF signaling are responsible for the beneficial effects exhibited by the co-transplantation of EPCs and NPCs, albeit there might be other mechanisms not explored in this study.
CHAPTER V. CONCLUSIONS

In summary, the work presented in this dissertation focuses on investigating the synergistic effects of co-transplantation of EPCs and NPCs on treating IS and the underlying mechanism by using *in vitro* transwell co-culture system and *in vivo* IS mouse model. Our data demonstrated that co-culture with EPCs and NPCs synergistically inhibited H/R-induced oxidative stress and apoptosis in both ECs and SH-SY5Y cells via activating the VEGF/VEGFR2/PI3K/Akt and BDNF/TrkB/PI3K/Akt pathways. Co-transplantation of EPCs and NPCs synergistically improved motor function and alleviated the pathological indexes in IS mice (a lower infarct volume, a lower portion of cell apoptosis, decreased ROS production, improved cMVD, suppressed inflammatory response, as well as increased angiogenesis and neurogenesis). All of these findings will help us to develop a novel treatment strategy for IS.
CHAPTER VI. FUTURE DIRECTIONS

In the future, a long-term study will be needed to determine whether the vascular protection and tissue recovery elicited by co-transplantation of EPCs and NPCs are sustained. Whether the transplanted cells can functionally integrate in host tissue including synaptic connectivity and reorganized neuronal network in the infarct area need to be investigated. Also whether co-transplantation of EPCs and NPCs can promote endogenous neurogenesis and angiogenesis in IS need to be evaluated. Further evaluations (such as dose-response, tumor-like neoplasm) are needed before considering the clinical application of EPCs and NPCs. In addition, we did not investigate whether the extracellular vesicles released from EPCs and NPCs participated in the observed synergistic effects in the present study. Extracellular vesicles are cellular membrane vesicles which are released into the extracellular space. They carry a cargo of proteins and genetic materials (mRNAs, miRNAs, etc.). It is well known that extracellular vesicles participate in intercellular communication. In our previous study, we have shown that BM EPC-derived extracellular vesicles could merge with ECs, and delivery the cargo to ECs thereby modulating EC functions {131}. Increasing evidence have demonstrated the potential efficacy of stem cell-derived extracellular vesicles in treating stroke and other neurodegenerative diseases {450, 451}. It is crucial to determine whether the progenitor-derived extracellular
vesicles could replace their parent cells, serving as cell-free strategy for treating IS. The advantages of cell-free strategy include capable of transferring a wide range of cargoes, and easily passing through the BBB, etc.
CHAPTER VII. BIBLIOGRAPHY


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Appendix A. LIST OF ABBREVIATIONS

Akt: protein kinase B
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)
BBB: blood brain barrier
BM: bone marrow
BDNF: brain-derived neurotrophic factor
BrdU: bromodeoxyuridine
Bcl-2: B-cell lymphoma-leukemia 2
Bax: Bcl-2-associated X protein
BAD: Bcl-2 associated death promoter
CNS: central nerve system
CBF: cerebral blood flow
CXCR4: chemokine (C-X-C motif) receptor 4
cMVD: cerebral microvascular density
CCA: common carotid artery
DG: dentate gyrus
DAPI: 4',6-diamidino-2-phenylindole
DMSO: dimethyl sulfoxide
DHE: dihydroethidium
EPCs: endothelial progenitor cells
ECs: endothelial cells
ESCs: embryonic stem cells
EB: embryonic body
EGF: endothelial growth factor
EDTA: ethylenediaminetetraacetic acid
ET-1: endothelin-1
ELISA: enzyme-linked immunosorbent assay
eNOS: endothelial NO synthase
ECA: external carotid artery
FBS: fetal bovine serum
FDA: Food and Drug Administration
FGF: fibroblast growth factor
FITC: fluorescein isothiocyanate
Flk-1: fetal liver kinase 1
hiPS cells: human inducible pluripotent stem cells
H/R: hypoxia/reoxygenation
HSCs: hematopoietic stem cells
HRP: horseradish peroxidase
HuNu: human nuclei
IS: ischemic stroke
IL-1α: interleukin-1 α
IL-10: interleukin-10
ICAM: intercellular adhesion molecule 1
ICA: internal carotid artery
KDR: kinase insert domain receptor
LV: lateral ventricular
MSCs: mesenchymal stem cells
MTT: methyl thiazolyl tetrazolium
MCAO: middle cerebral artery occlusion
MMP: matrix metalloproteinases
MACS: magnetic activated cell sorting
NPCs: neural progenitor cells
NPBM: neural progenitor basal medium
NMDA: n-methyl-d-aspartic acid
NO: nitric oxide
PI3K: phosphatidylinositol-3-kinase
PBS: phosphate buffer saline
PFA: paraformaldehyde
PI: propidium iodide
p-Akt: phosphorylated protein kinase B
p-TrkB: phosphorylated tyrosine kinase receptor B
p-Flk1: phosphorylated fetal liver kinase 1
ROS: reactive oxygen species
RT: room temperature
tPA: tissue plasminogen activator
TrkB: tyrosine kinase receptor B
TNF-α: tumor necrosis factor α
TUNEL: terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling
VEGF: vascular endothelial growth factor
VEGFR2: vascular endothelial growth factor receptor 2
VCAM-1: vascular adhesion molecule 1
SVZ: subventricular zone
SGZ: subgranular zone