Review

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Regenerative Medicine

Understanding neural stem cell regulation *in vivo* and applying the insights to cell therapy for strokes

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The use of neural stem cell (NSC) therapy for the treatment of stroke patients is successfully paving its way into advanced phases of large-scale clinical trials. To understand how to optimize NSC therapeutic approaches, it is fundamental to decipher the crosstalk between NSC and other cells that comprise the NSC microenvironment (niche) and regulate their function, *in vivo*; namely, the endothelial cells of the microvasculature. In this mini review, we first provide a concise summary of preclinical findings describing the signaling mechanisms between NSC and vascular endothelial cells and *vice versa*. Second, we describe the progress made in the development of NSC therapy for the treatment of strokes.

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According to the WHO, neurological disorders are the leading cause of disability and the second leading cause of death worldwide. Neurological disorders of the CNS include neurodegenerative diseases, as well as CNS injury. The most commonly known neurodegenerative diseases include Alzheimer's disease, amyotrophic lateral sclerosis (ALS; also known as Lou Gehrig's disease and characterized by progressive death of motor neurons in the brain and spinal cord) and Parkinson's disease (characterized by progressive loss of dopaminergic neurons) [1]. As a result, these patients exhibit memory loss and physical disability.

Strokes (also known as cerebrovascular accidents), remain the most frequent CNS injury resulting from a vascular cause. There are two main types of stroke, ischemic and hemorrhagic [2]. In the USA ischemic strokes account for approximately 85% of all strokes, they are the third leading cause of death and remain a leading cause of long-term disability [3,4]. Ischemic strokes are caused by the interruption of the blood supply to the brain either via a blood clot (thrombus) or a dislodged clot (embolus). This arterio-occlusion caused by the clot prevents oxygen and nutrient perfusion to the brain, causing irreversible brain tissue damage. The two current treatments for strokes consist of removing the clot either by intra-arterial surgical intervention (known as thrombectomy) or intravenous delivery of the US FDA approved thrombolytic medication, a t-PA known as alteplase [5]. However, these treatments do not account for damaged vessels or lost neurons postischemic stroke; rather, only trigger reperfusion of the occluded vessel.

Unfortunately, in most cases, neurodegenerative diseases and CNS injury are reported when damage has already been done to the brain in terms of neuronal loss and vascular injury. Therefore, there is an urgent need to regenerate lost or damaged neurons by promoting neurogenesis, as well as promoting healthy revascularization via angiogenesis. Neural stem cell (NSC) therapy is a promising multi-action therapeutic candidate capable of secreting neuroprotective factors, while also promoting replacement of lost and damaged neural cell types via astrogliosis and neurogenesis and encouraging blood vessel regeneration via angiogenesis through the secretion of regenerative growth factors [6–13].

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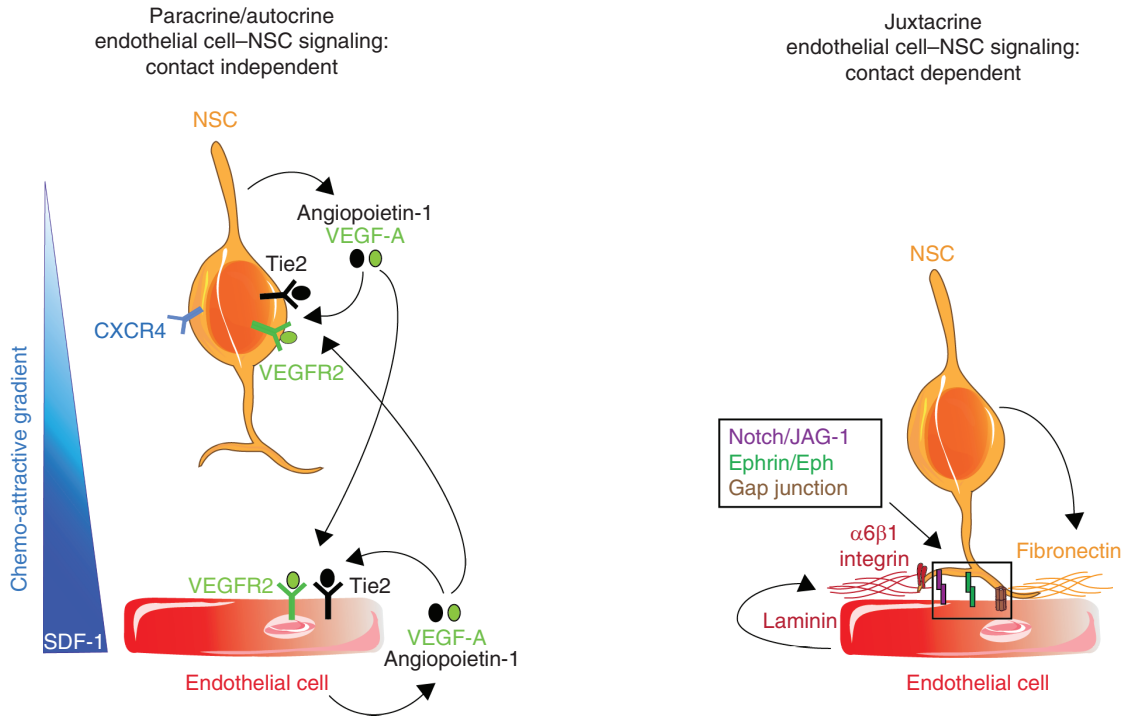

Figure 1. Endothelial cell and neural stem cell signaling in the adult subventricular zone. Paracrine/autocrine or contact-independent signaling involves soluble factors like VEGF-A, Ang-1 and SDF1. Juxtacrine or contact-dependent signaling involves membrane-bound receptors and their ligands, such as Notch/JAG1, Ephrin/Eph and junctional proteins including connexins that make up gap junction proteins.
NSC: Neural stem cell.

Preclinical studies of NSC therapy for strokes

NSC & neurogenesis

Adult NSC are multipotent neural cells that display long-term self renewal capacity and possess the ability to differentiate into astrocytes and oligodendrocytes (glial lineage), as well as granular and periglomerular neurons (neuronal lineage). In the adult mammalian brain, NSC reside in two germinal niches, the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) in the lateral ventricle. The adult SVZ is the largest niche for NSC that represent a small population of $GFAP^+$ astroglial cells. Within the SVZ, NSC exhibit an apico-basal polarity; that is, they protrude short apical processes on the ventricular side of the SVZ to contact the ependymal cell layer that lines the lateral ventricle. On the parenchymal side, NSC project long basal end-feet that contact microvascular endothelial cells within the SVZ. Neurogenesis is initiated when quiescent NSC are activated and give rise to fast dividing transit-amplifying progenitors. The transit-amplifying cells then assemble into small clusters and differentiate into chains of migrating neuroblasts that travel along the rostral migratory stream toward the olfactory bulb where they differentiate into granular and periglomerular neurons (mature interneurons) [14].

It is now well known that the SVZ microvasculature composed of endothelial cells is an integral component of the niche and is known to support NSC self renewal and maintenance, as well as the proliferation, differentiation and migration of neural progenitor cells [15,16]. Most importantly, the SVZ is comprised of a 'leaky' blood–brain barrier (BBB) where vascular endothelial cells lack astrocyte end feet and pericyte coverage. However, unlike the SVZ, the BBB comprising the SGZ is not 'leaky' under normal homeostasis conditions but shows leakage postischemic stroke [17,18]. This unique feature of the SVZ's BBB thus permits direct interactions between microvascular endothelial cells and NSC known as neurovascular coupling. Neuro–vascular interactions involve both juxtacrine (via membrane-bound proteins) and paracrine/autocrine (via secreted adhesion and systemic soluble factors) signaling, which are thought to regulate NSC self renewal and potential (depicted in Figure 1). Precise mechanisms that regulate neuro–vascular interactions in the SVZ are unclear [9,19,20]. However, over the years, there has been a substantial amount of work aimed at deciphering the role of contact-independent endothelial-cell NSC signaling in the regulation of NSC survival, proliferation and differentiation via either vascular endothelial cells secreted

soluble factors, namely PEDF [21] and betacelluline [22] or both endothelial cells and NSC expressed factors such as VEGF [23] and Ang-1 [24]. It was shown that: PEDF promotes NSC self renewal while betacelluline, VEGF and Ang-1 mainly induce NSC proliferation to promote SVZ neurogenesis [21,22,24]. Regarding contact dependent signaling, some studies identified Notch signaling as being an important molecular regulator of SVZ neurogenesis [25–27]. The role of Notch signaling is further discussed in section: vascular endothelial cell regulation of NSC.

In mice, the SVZ primarily allows the genesis of new interneurons destined for the olfactory bulb and this process persists into adulthood [28]. However, in the human brain, SVZ production of new neurons is highly reduced by 2 years of age and little to no neurogenesis is observed after childhood [29,30]. Regarding SGZ hippocampal neurogenesis, contradicting studies from different groups contribute to the continued debate about whether human hippocampal neurogenesis persists throughout adulthood and aging [31,32]. Nonetheless, it is well accepted that there is no detectable olfactory bulb neurogenesis in adult humans [33]. The absence of endogenous SVZ NSC to engage in neurogenesis in the adult human brain is a challenge for promoting neuroregeneration post injury. To devise strategies to circumvent this roadblock, there is extensive ongoing preclinical research using animal models of NSC implantation poststroke to promote endogenous repair and behavioral recovery. However, to achieve effective functional therapeutic effects of NSC implantation, it is essential to understand how NSC are regulated within their *in vivo* microenvironment. Insights gained from such studies will ultimately enable the transplantation of NSC alongside cues required for their proper engraftment and desired differentiation in the affected region, as well as for the promotion of neovascularization.

Vascular endothelial cell regulation of NSC

It is now well established that angiogenesis is a key factor allowing effective behavioral recovery post-NSC intracerebral implantation [34]. When the clinical grade human NSC, CTX0E03, were transplanted in a rat model of a stroke, induced via transient middle cerebral artery occlusion, neovascularization was observed near the injected cells [35]. To decipher how implanted NSC induce angiogenesis in response to a stroke, researchers established an *in vitro* model mimicking SVZ neuro–vascular interactions. They cocultured human cerebral microvascular endothelial cells and human NSC (STR0C05 and CTX0E03 cell lines derived from legally aborted fetal striatum and cortex, respectively) to dissect the signaling interactions between these cell types. They analyzed the secretome in the supernatant from NSC–endothelial cell cocultures, compared with monoculture supernatant and observed a dramatic increase in the pro-angiogenic factor VEGF-A in coculture conditions. VEFG-A was previously implicated in the behavioral efficacy of NSC transplanted in stroke-injured tissues [9,34]. Additionally, in NSC–endothelial cell cocultures, genes associated with angiogenesis and vessel maturation (i.e., Ang-1 and PDGF-AB) are increased [9]. In a follow-up study, NSC and vascular endothelial cells were isolated via fluorescent-activated cell sorting (FACS) after coculture to evaluate the differential expression of genes associated with angiogenesis, cell survival and migration. Interestingly, their results showed that both NSC and endothelial cells upregulate the pro-angiogenic factor VEGF-A [19]. These results suggest that interdependent signaling between endothelial cells and NSC promotes angiogenesis, which is required for optimal NSC therapeutic effects.

Another study of implanted NSC employed GFP-tagged adherent neural stem cell type 4 (ANS4) cells, which are derived from an area encompassing the SVZ surrounding the lateral wall of the forebrain ventricle of 2-month-old (adult) CD1 mice. These cells were confirmed to be representative of SVZ neural cells by expression of genes, such as *SOX2, OLIG2* and *MASH-1*, and were found to be capable of differentiating into neurons and astrocytes when subjected to a permissive differentiation environment [36,37]. When the ANS4-GFP cells were encapsulated together with mouse brain endothelial cells (bEnd.3) in polyethylene glycol (PEG)-based microbeads, the endothelial cells maintained NSC quiescence and accelerated the degradation of PEG microbeads through proteolytic metalloproteinase activity, facilitating the exclusion of NSC from the microbeads post-transplantation [38]. Although this study was performed under normal brain homeostatic conditions, it demonstrated the importance of vascular endothelial cells for proper NSC delivery within engineered PEG-based microbeads [36–38]. These investigators also discussed the absence of microglia and pericytes in their model and suggested that more complex engineered models of the 3D neurovascular niche may be desirable to fully optimize NSC therapeutic effects [9,19,38].

In earlier studies, SVZ neurosphere-expanded cells were transplanted into the murine SVZ under normal brain homeostasis. The transplanted NSC were found to migrate toward the vasculature in response to the chemokine CXCL12, also termed as SDF1, which is secreted by endothelial cells, creating a chemoattractive gradient (depicted in Figure 1). It was shown that SDF1-mediated recruitment of NSC to the vasculature was mediated through the CXCR4, which is highly expressed by SVZ progenitor cells [39]. Furthermore, when cultured NSC were treated

with SDF1, they exhibited increased expression of the mitogen factor EGFR, which is highly enriched in activated NSC, suggesting that endothelial cell secretion of SDF1 contributes to the activation and proliferation of NSC near the vasculature [39,40]. Unfortunately, injecting the SDF1 chemokine *in vivo* in an injured brain is not a clinically viable strategy because of the undesirable inflammation promoted by SDF1-CXCR4 signaling. Interestingly, to counter this side effect, one preclinical study implanted human NSC derived from human induced pluripotent stem cells (iPSC)-NSC (discussed in section: iPSC-NSC) in mouse brains pretreated with a mutated form of the SDF1 ligand (termed SDV1a) prior to implantation. Interestingly, the SDV1a pretreatment promoted the migration of transplanted NSC to the site of injury without promoting inflammation [41].

In terms of juxtacrine signaling, studies conducted under normal brain homeostatic conditions reported that canonical Notch signaling via EphrinB2 (Ephrin signaling) and JAG-1 maintains NSC quiescence [26]. In contrast, the noncanonical Notch ligand, EGFL7 known as an inhibitor of JAG-1-induced Notch signaling, acts as a neurovascular regulator of SVZ-NSC controlling olfactory perception and behavior [25]. Another study conducted in middle cerebral artery occlusion stroke-induced mice, reported that stroke enhances the SVZ's BBB 'leakiness' to facilitate direct access to stroke-induced increased levels of VEGF-A_{165} to niche cells [41]. Additionally, these authors reported that the increase in VEGF-A levels post stroke activates Notch signaling by a transient induction of the Notch ligand DLL4 mRNA levels to enhance SVZ neurogenesis post stroke [27].

It has also been reported that Cx43 is the main protein comprising gap junctions of SVZ endothelial cells and NSC; however, the role of Cx43 signaling in endothelial cell and NSC regulation is still unclear [20]. Furthermore, NSC upregulate fibronectin which is known to improve NSC survival and migration *in vivo*, while laminin, a basement membrane protein which promotes cell adhesion through α6β1 integrin, is selectively upregulated in endothelial cells [19]. Paracrine/autocrine and juxtacrine signaling between endothelial cells and NSC are summarized in Figure 1.

Collectively, these studies suggest that, to optimize the therapeutic effects of transplanted NSC (discussed in section: PISCES clinical study of NSC therapy for stroke), it is important to think about engineering 3D biomimetic niches that include the different cell types and factors required to maintain NSC *in vivo*, as well as to promote axonal outgrowth from transplanted NSC-derived neurons for functional integration and synaptic connections into host brain circuitry. These might include: SVZ niche cells (vascular endothelial cells and pericytes/smooth muscle cells and microglia); vascular growth factors that promote neovascularization (i.e., VEGF-A); neurotrophic factors; and synthetic chemo-attractants (i.e., SDV1a) to direct NSC migration to the site of injury for proper engraftment. Engineering biomaterials for clinical therapies is further reviewed in [42] and [43].

iPSC-NSC

The STR0C05 and CTX0E03 human NSC lines and animal models are useful tools to advance the understanding of NSC regulation at the bench level [44]. Unfortunately, the fetal human NSC lines are not easily available to researchers and studies in mouse models are expensive, time consuming and not always representative of human biology.

Hence, the ability to reprogram adult human somatic cells (usually fibroblasts) into iPSC-NSC represents an alternate avenue to explore NSC regulation and therapy for stroke [45]. Moreover, iPSC-NSC, unlike fetal NSC that cannot be used as an autologous cell source, can be generated from a patient's own somatic cells, preventing the risk of immune rejection associated with allogeneic transplants. Preclinical data on rodent brains post-stroke transplanted with iPSC-NSC showed: increased neural plasticity that was mediated by increased expression of VEGF-A secreted by the transplanted cells themselves [46]; differentiation into site specific neuronal cells that functionally integrated into host circuitry and offered protection from stroke-associated neurological deficits [47]; and enhanced proliferation of cells in the SVZ and migration of cells expressing markers of immature neurons to the site of stroke damage [8,48].

To date, unlike the CTX0E03 human fetal NSC line (further discussed in section: PISCES clinical study of NSC therapy for stroke), there are no clinical trials for stroke using autologous iPSC-NSC transplantation [49]. One explanation would be that animal studies in the field have seen effective results from iPSC-NSC transplanted directly after a stroke (acute) or 24 h–1 week (subacute) after the onset of the injury. However, it takes at least 7 weeks to generate and differentiate a sufficient number of iPSC-NSC from a patient's fibroblasts to a desirable neuronal phenotype, and this time frame of implantation post stroke might compromise the efficacy of iPSC-NSC therapy [50]. Nonetheless, an appealing use of iPSC-NSC remains in the high-throughput 3D microfluidic brain-

on-a-chip device allowing the study of human cell–cell interactions, as well as drug screening for neurodegenerative diseases such as Alzheimer's and Parkinson's disease [51,52].

PISCES clinical study of NSC therapy for stroke

One of the most advanced clinical trials using NSC in human patients with chronic ischemic stroke is the Pilot Investigation of Stem Cells in Stroke (PISCES) study. PISCES Phase I (PISCES-I) was the first-in-man study that consisted of intracerebral stereotactic implantation of increasing doses (2, 5, 10 and 20 million cells) of the allogeneic human NSC line CTX0E03 in 11 men who had an ischemic stroke 30 months prior to implantation. The CTX0E03 human NSC are clonally derived from human fetal cortical neuroepithelial cells and transformed by retroviral insertion of a conditional immortalization transgene, c-MycER^{TAM}. The injection of CTX0E03 human NSC did not induce the formation of tumors, no seizures were observed post surgery and modest neurological and functional improvements were observed in some patients. The take home message from PISCES-I was feasibility and tolerability since no cell-related safety issues were observed up to 24 months post stereotactic intracerebral injection of CTX0E03 human NSC in patients with chronic ischemic strokes. However, the main limitation of the study was the small sample size, which limited the number of patients exposed to the highest dose [53,54].

On the other hand, the PISCES Phase II trial (PISCES-II) was a multicenter study of 23 participants, including both male and female participants. Patients were recruited at earlier time points than PISCES-I following a stroke, specifically between 2 and 13 months post stroke, referred to as subacute stages. The therapy consisted of injecting 20 million CTX0E03 cells, the highest dose tested in PISCES-I and the study confirmed few cell-related adverse effects up to 1 year post-transplantation. Functional outcomes were set for evaluation at 30, 90, 180 and 360 days after treatment. The primary outcome measure used to evaluate functional recovery was a minimum two-point improvement in the Action Research Arm Test number 2 score, which involved grasping a 2.5 cm^3 block and moving it from starting point to the target position [55]. The study primarily found functionally relevant improved arm movement during subacute stages of stroke recovery in patients presenting with residual upper limb motor function at baseline [56].

PISCES-I (number NCT01151124) and PISCES-II (number NCT02117635) are registered with ClinicalTrials.gov. Fortunately, PISCES-II results were sufficient to justify further clinical investigation and; therefore, the FDA approved the commencement of a randomized, placebo controlled PISCES-III (NCT03629275) clinical trial in the USA in patients living with chronic stroke disabilities. PISCES-III will be a multicenter trial across up to 40 centers in the USA with plans to enroll approximately 130 patients (a bigger group than PISCES-II to adequately represent the heterogeneity of stroke). Unfortunately, the start of the trial is currently on hold due to COVID-19-related restrictions.

Nonetheless, to continuously and rapidly advance the use of NSC therapy, there is an urgent need to escalate preclinical research of cell therapies for stroke without sacrificing scientific rigor. The Stem Cell Therapy as an Emerging Paradigm in Stroke meetings promote bridging between preclinical and clinical research by bringing together academic and industry leaders, as well as experts from regulatory authorities, to discuss the latest development in cell therapies for stroke. Importantly, they continue to publish updated recommendations for preclinical and clinical research [43].

Even though the field of NSC therapy post stroke has approached a promising state because of clinical applications, we must bear in mind that only a low percentage (~0.2%) of these cells can reach the damaged area to fully mature and integrate in the infarcted region. To date, evidence that transplanted NSC-derived neurons can establish functional synapses to allow host brain control of neuronal activity, is still lacking. However, in preclinical stroke models and human stroke patients, NSC treatment enhances: the formation of neurovascular units comprised of endothelial cells, pericytes, basal lamina, astrocytes, pericapillary microglia and neurons, and promotes brain tissue repair through secretion of neurotrophic and regenerative growth factors [57]. Furthermore, transplanted NSC can differentiate into astrocytes forming the glial scar, protecting peri-infarct neurons from glutamate-induced excitotoxicity [58]. In conclusion, despite absence of evidence for functional synapses, the neuroprotective effects induced by the transplanted NSC might be contributing to the modest motor skills recovery observed in PISCES-II patients.

Conclusion

This mini review summarizes the current promising advances made in the use of transplanted human NSC to promote endogenous repair and functional recovery in stroke-injured patients. The PISCES clinical studies showed

that human NSC therapy is feasible and tolerable without cell-related adverse effects and produced improved motor skills in patients in subacute stages of stroke recovery. Although the task of treating stroke is scientifically (preclinical) and clinically challenging due to lack of perfect *in vitro* and *in vivo* models, budgetary constraints and the huge amount of time required to address all the relevant factors, FDA approval of the PISCES-III clinical trial brings hope to the scientific and medical communities for continued improvement in the treatment of strokes.

Importantly, mechanistic studies at the bench level have greatly contributed to the understanding of the various parameters that influence stroke therapies. Indeed, based on recent preclinical findings, we highlighted the importance of vascular endothelial cells in NSC regulation. Endothelial cell regulation of NSC is an important factor to take into account when considering the fabrication of biomimetic NSC niches that could be transplanted *in vivo* for clinical therapies. In addition, to thoroughly understand how vascular endothelial cells regulate NSC and *vice versa*, it is crucial to continue bench research focused on elucidating the role of paracrine/autocrine and juxtacrine signaling between the two cell types. Further mechanistic insights will ultimately serve to improve stroke therapies.

Future perspective

The role of NSC, endothelial cells and their associated paracrine/autocrine, as well as juxtacrine, niche signaling explored in animal studies are very challenging to translate into clinical studies of stroke patients. Diversity of patients, variabilities in injury location and extent of brain damage make it difficult to integrate preclinical knowledge into clinical strategies for stroke patients. The next phase of study will consist of learning from human stroke patient trial data and applying that to future preclinical work to move the field forward.

In the coming years, we can envision the injection of genetically engineered and ethically justified 3D biomimetic NSC niches comprised of all the *in vivo* required cues that maintain NSC survival, migration and neuronal differentiation. These would include vascular endothelial cells, NSC, pericytes, smooth muscle cells, microglia, laminin α6β1 derived-peptide (YIGSR), vascular growth/neurotrophic factors, chemo-attractants and axonal outgrowth-promoting factors. Ultimately, improved functional recovery of stroke patients should be clinically viable because of the extensive preclinical work currently being conducted.

Executive summary

Preclinical studies of neural stem cell therapy for strokes

- The 'leakiness' of the blood–brain barrier of the adult subventricular zone allows for direct interactions between vascular endothelial cells and neural stem cell (NSC) via either contact-independent (soluble factors) or contact-dependent mechanisms (membrane-bound proteins).
- Studying the NSC niche using *in vivo* preclinical models and *in vitro* culture systems, under both homeostasis and stroke conditions, allows to decipher the different cues required for proper NSC therapy.
- Angiogenesis, as well as neurogenesis, are critical to achieve effective and functional recovery post-NSC transplantation.
- Preclinical studies identified neuro–vascular interactions as key factors regulating NSC behavior, maintenance, survival and migration.
- Crosstalk intercellular signaling between vascular endothelial cells and NSC can promote both angiogenesis and neurogenesis for optimal NSC therapeutic effects.

Clinical studies of NSC therapy for strokes

- There are no clinical trials for stroke using autologous induced pluripotent stem cell-NSC transplantation, but they can be used *in vitro* for drug screening for neurodegenerative diseases.
- The Pilot Investigation of Stem Cells in Stroke (PISCES) clinical trials are registered with ClinicalTrials.gov.
- PISCES-I showed tolerability and feasibility while PISCES-II showed modest motor skill recovery in patients between 2 and 13 months post stroke.
- PISCES-III is currently on hold due to COVID-19-related restrictions.
- To date, there is no evidence showing that new neurons post-NSC therapy integrate into host brain circuitry for the establishment of functional synapses.
- Motor skill improvement post-NSC transplantation is mainly attributed to neuroprotection and neurotrophic rescue of peri-infarct tissues.
- Continuous learning from preclinical studies and clinal trial data will allow to better understand of the complex challenges associated with NSC therapy.
- Successful fabrication of transplantable 3D biomimetic NSC niches will allow for enhanced improvement in functional recovery of stroke patients.

Financial & competing interests disclosure

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• **Performed coculture of the clinical grade neural stem cells (NSC; CTX0E03) with vascular endothelial cells and showed that the pro-agiogenic factor VEGF-A was dramatically increased.**

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• **Demonstrates the importance of vascular endothelial cells for proper NSC delivery within engineered microbeads.**

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