A neurovascular-unit-on-a-chip for the evaluation of the restorative potential of stem cell therapies for ischaemic stroke

Zhonglin Lyu¹,², Jon Park¹,², Kwang-Min Kim¹,²,³, Hye-Jin Jin¹, Haodi Wu², Jayakumar Rajadas², Deok-Ho Kim³,⁴, Gary K. Steinberg¹,⁵ and Wonjae Lee¹,²,⁶

The therapeutic efficacy of stem cells transplanted into an ischaemic brain depends primarily on the responses of the neurovascular unit. Here, we report the development and applicability of a functional neurovascular unit on a microfluidic chip as a microphysiological model of ischaemic stroke that recapitulates the function of the blood–brain barrier as well as interactions between therapeutic stem cells and host cells (human brain microvascular endothelial cells, pericytes, astrocytes, microglia and neurons). We used the model to track the infiltration of a number of candidate stem cells and to characterize the expression levels of genes associated with post-stroke pathologies. We observed that each type of stem cell showed unique neurorestorative effects, primarily by supporting endogenous recovery rather than through direct cell replacement, and that the recovery of synaptic activities is correlated with the recovery of the structural and functional integrity of the neurovascular unit rather than with the regeneration of neurons.

Ischaemic stroke is caused by an inadequate supply of blood to the brain and leads to deficits in neurologic functions¹. The stroke involves a series of spatial and temporal events, such as inflammatory and immune responses, cell death and differentiation, hypoxia, vascular damage and an altered cerebral microenvironment². Stem cell therapy has been highlighted as an emerging paradigm for stroke treatment, with support from experimental animal studies as well as clinical pilot studies¹. The clinical outcomes of stem cell therapies depend on a variety of factors, including the route, dosage and timing of administration, but the most critical factor seems to be the type of stem cells³. Each type of stem cell has unique traits and a distinct regenerative potential², and yet there is a lack of a reliable stroke model to systematically compare the efficacy across the broad range of candidate cell types and to investigate the underlying mechanism of stem cell therapeutics¹. This poses a major challenge in utilizing and advancing stem cell therapies.

The majority of research for developing and examining stem cell therapeutics is based on murine-based animal models and several approaches have shown some enhanced neuroprotective effects in animal studies¹. Although outcomes from early-phase cellular transplant clinical trials look promising, they do not always replicate the results from animal stroke models⁴. This might be due to the intrinsic differences between animal models and the human disease in terms of anatomy and physiology, the pathophysiological responses to injury or the injury mechanism⁵. The animal models also have the inherent limitations in time and cost efficiency to be an ideal testbed for characterizing the therapeutic potentials of multiple stem cell types on the entire cascade of pathological events induced by the stroke.

The neurovascular unit (NVU) has critical roles in stroke progression as well as the recovery process⁶. The NVU consists of brain microvascular endothelial cells (BMECs) and their neighbouring neural cells, that is, neurons and multiple types of glia cells⁷. These constituent cells in the NVU work in concert with one another and create a tight blood–brain barrier (BBB) that regulates the molecular transport into and out of the brain parenchyma to maintain the homeostasis of the microenvironment as well as the brain functions⁸. NVU not only mediates the drug delivery and infiltration of the transplanted stem cells into the ischaemically damaged brain parenchyma, but also engages in the fate determination of neural stem cells during post-stroke neuroregeneration⁹.

Owing to the clinical importance of the BBB in drug delivery to the brain parenchyma, there have been continuous efforts to develop in vitro BBB models. Major approaches in the past include BBB models with Transwell, a cone and plate viscometer, and hollow fibres⁸. More recently, there have been some notable advances in constructing a functional BBB on a microfluidic chip¹⁰–¹¹. However, most of these approaches have limitations to be directly applicable to establishing the complex three-dimensional (3D) tissue environment associated with the pathophysiological conditions of ischaemic stroke and the recovery process by transplanted stem cells.

In this Article, we developed a stroke microphysiological system to examine the neurorestorative capacity of stem cell therapy. Our stroke model uses human-derived cells and has an in vivo-like 3D microenvironment that recapitulates the natural interaction between the therapeutic stem cells and the host cells of the NVU. Our model serves as a reliable screening testbed to systematically analyse the neurorestorative behaviours of various stem cell types currently tested in clinics for stroke treatment.

Results

Chip design for the reconstruction of a functional BBB. The key advantage of an in vitro model is the ability to monitor cell
Fig. 1 | Characterization of the BBB reconstructed in a microfluidic chip. a. Physically intact barrier. Schematic of the chip design (i). The optimized protocol (ii). NEM/AM, mixture of neural expansion medium and astrocyte medium. NDM/AM, mixture of neural differential medium and astrocyte medium. ECM/PME, mixture of endothelial cell medium and pericyte medium. Neural cells include neurons, astrocytes and microglia at an expected final ratio of 8:4:1. Vascular cells include BMECs and pericytes at a ratio of 9:1. The spatial distribution of the NVU constituent cells in the chip (iii). A bright-field image showing the hydrogel (blue) deployed in the brain channel (iv). The well-defined boundaries (white arrows) between the blood-side (left) and brain (right) channels are shown in phase contrast (v) and fluorescence (vi) images. Endothelial cells and astrocytes were stained using anti-CD31 and anti-GFAP antibodies, respectively. The formed endothelium prevented free diffusion of the green fluorescent probes (FITC–dextran, 4 kDa) across it (vii). The image was taken 1 h after adding the probes. Single layers of confocal microscopy images of the formed endothelium on the side wall of the hydrogel (viii). The endothelial cells were stained for von Willebrand factor (vWF). The 3D reconstructed view of the confocal microscopy images showing the uniform endothelium (ix). Endothelial cells and astrocytes were stained for their specific markers, CD31 and GFAP, respectively. The log-transformed value of the apparent permeability coefficients, \( P_{\text{app}} \), of the endothelium in the chips (\( n = 5 \)) (x). Statistical analysis was performed using one-way analysis of variance (ANOVA) with Bonferroni–Holm post hoc test. The actual numbers are presented in Supplementary Table 1. DAPI, 4,6-diamidino-2-phenylindole. b, Biochemically intact barrier. Schematic of the hybrid culture condition with serum-containing medium in the blood-side channel and serum-free medium in the CSF-side channel (i). In the sample without endothelium (the white arrows indicate the hydrogel boundary), microglia (stained as green by IBA-1) showed upregulated expression of CD68 (red), a pro-inflammatory microglial marker, and appeared yellow (ii). In the sample with the reconstructed endothelium, most of the microglia did not express CD68 (iii). The CD68 expression between the samples with and without endothelium (\( n = 3 \)) (iv). All analyses were made 24 h after serum was added. Statistical analysis was performed using a one-tailed Student’s t-test (\( P = 0.0054 \)). c, Cell-selective barrier. Schematic of the incorporation of cancer cells (i). The prestained cells of two human breast cancer cell lines, MB-231 and MB-231Br, were injected into the blood-side channel. The fluorescent images for MB-231 (ii) and MB-231Br (iii), taken 3 d after the injection. The white arrows indicate the hydrogel barrier. Schematic of the incorporation of cancer cells (i). The prestained cells of two human breast cancer cell lines, MB-231 and MB-231Br, were injected into the blood-side channel. The fluorescent images for MB-231 (ii) and MB-231Br (iii), taken 3 d after the injection. The white arrows indicate the hydrogel barrier.
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channel was lowered to generate the surface tension between the top and the bottom surfaces (Fig. 1a (iii) and Supplementary Fig. 2) and to stably hold the liquid hydrogel prepolypearn in the brain channel (Fig. 1a (iv)). As expected, a well-defined boundary was formed between the blood-side and brain channels in the chip (Fig. 1a (v and vi)). The reconstructed endothelium prevented free diffusion of a fluorescent probe (FITC–dextran, 4 kDa) across itself (Fig. 1a (vii)). The probe size of 4 kDa is useful to evaluate the BBB functionality because most pathogens, such as viruses and bacteria, are larger than 4 kDa and the native BBB prevents these pathogens from entering the brain. Confocal microscopy images showed the continuous and physically intact endothelial barrier (Fig. 1a (viii and ix)) in contrast to the one in the previous chip design with micropoles (Supplementary Fig. 1). Our chip design is devoid of micrometre-scaled features, eliminating the need for the soft lithography process in chip production and enabling us to use a 3D printer (Supplementary Fig. 2).

Before the endothelium formation, there was a small population of astrocytes in the blood-side channel migrating from the brain channel (0.9 ± 0.3% (mean ± s.d.) of the totally incorporated astrocytes; n = 3; Supplementary Fig. 3). These migrated astrocytes, together with pericytes, supported BMECs to maintain the normal morphology of a smooth rounded shape throughout the blood-side channel (Supplementary Fig. 4), similar to the morphology of BMECs when co-cultured with both astrocytes and pericytes in 2D culture. The astrocytes and pericytes in the blood-side channel settled beneath the layer of the endothelial cells at the bottom, as the BMECs connected to each other, maturing to form an endothelium (Supplementary Fig. 3). This might be due to the angiogenic process whereby endothelial cell-to-cell junctions strengthen the connection between the neighbouring endothelial cells.

We examined the endothelial tightness under different conditions, depending on the cell composition and the presence of flow, by calculating the apparent permeability coefficients (Fig. 1a (x) and Supplementary Table 1). In the presence of astrocytes and pericytes, the endothelium became significantly tighter to hinder the diffusion of the probe, FITC–dextran (Fig. 1a (x)). Further significant reduction in the permeability was observed after the flow of culture medium was introduced (Fig. 1a (x)). This tightening of the endothelium in the presence of flow is consistent with the reports of enhanced paracellular connectivity in the BBB by proper mechanical stimuli. The resulting permeability coefficients of our BBB model were ~6 × 10^−7 cm s^−1 and ~8 × 10^−9 cm s^−1 for 4 kDa and 70 kDa FITC–dextran, respectively, which are comparable to those of other in vitro and in vivo BBB models previously reported. The reconstructed BBB also showed the expected size-selective permeability as in functional BBB—the smaller the probe size, the better the diffusion across the BBB (Fig. 1a (x)). Another standard measure to assess the BBB tightness is transendothelial electrical resistance (TEER). TEER measurement is a simple, label-free and non-invasive method to quantify the barrier integrity. There is a broad range of TEER values reported for microfluidic BBB models, from a few hundreds to thousands of Ω cm^2, while their permeability coefficients are within a relatively narrow range of around 1 × 10^−6 cm s^−1 for 4 kDa FITC–dextran. This might be because the TEER values are largely dependent on the method of measurement and experimental procedures. Alternating currents (AC) are widely used for TEER measurement because direct currents (DC) can damage cells, and tetrapolar AC TEER measurement, using four electrodes, is more accurate than bipolar AC measurement as it is less influenced by the polarization impedance at the electrode-electrolyte interface. However, owing to the small surface area of the BBB in our chip, the resistance across the BBB was expected to reach several mega-ohms, beyond a measurable range of commercially available tetrapolar AC TEER meters. We therefore used a bipolar DC measurement, and the TEER value of the BBB in our chips was 370 ± 20.5 Ω cm^2 (mean ± s.d.) under the flow (Supplementary Fig. 5). The TEER value measured in our chip is lower than those reported in some of the microfluidic BBB models, but showed meaningful differences between conditions (Supplementary Fig. 5).

Once we confirmed the physical intactness of the endothelium, we examined the functional characteristics of the reconstructed endothelium as a biochemically intact barrier. One of the important functions of the cerebral endothelium in vivo is to isolate the neural cells in the brain parenchyma from any pro-inflammatory substances in the bloodstream. To maintain the original phenotype of the cells in each channel of our chip, we deployed two different types of medium: serum-containing endothelial medium in the blood-side channel and serum-free glial cell medium in the CSF-side channel (Fig. 1b (i)). The reason for this setup is that the endothelial cells require serum to maintain their original phenotype in vitro, whereas the glia cells show pro-inflammatory behaviours in the serum-containing culture medium. Serum, extracted from the whole blood, is an undefined mixture of proteins, hormones, minerals, growth factors and lipids. The reconstructed BBB therefore needs to prevent the entry of any pro-inflammatory substances from the serum in the blood-side channel. In the samples without the BBB, the microglia, a resident immune cell type in the brain, showed pro-inflammatory behaviours as expected (Fig. 1b (ii)) because they were directly exposed to the serum. By contrast, in the samples with the reconstructed BBB, the microglia did not show such pro-inflammatory behaviours (Fig. 1b (iii and iv)), confirming the BBB in our chip as a biochemically intact barrier, similar to the native BBB.

To be a clinically relevant model for stem cell therapy, the BBB on our chip should also exhibit distinct responses based on the traits of the invading cells. The neurorestorative efficacy of each stem cell type may depend on their ability to infiltrate across the tight BBB and reach the lesion site, and yet little is known about the native BBB responses to the candidate types of stem cells in therapy. Therefore, as a valid measure to show the cell-selective responsivity of the BBB, we propose to utilize the well-established metastatic behaviours of the two human breast cancer cell lines, MB-231 and its brain metastatic derivative population, MB-231Br (Fig. 1c). MB-231 infiltrates specifically across the BBB and exhibits much stronger metastatic tendency than MB-231 in an animal model. The reconstructed BBB in our model showed the expected cell-specific responses to these two types of invading cancer cells (Fig. 1c), confirming the in vivo-like functionality of our model and verifying its sensitivity to the traits of the invading cells.

Establishing the ischaemia. After confirming the formation of a functional BBB in our chip, we established an ischaemic condition. There are two major zones of ischaemic injury: the core infarct zone and the ischaemic penumbra, which is also called the peri-infarct rim. The core infarct zone is characterized by no blood supply and severe necrosis of neural cells, and is considered irreversibly injured. By contrast, the ischaemic penumbra, the rim surrounding the irreversibly damaged core, has just enough blood supply for the cells to survive but not enough to communicate and function properly. This peri-infarct rim has been considered to be a therapeutic target for post-stroke recovery. Thus, we targeted the establishment of an ischaemic condition that recapitulates this peri-infarct zone, sufficiently damaging cells and yet minimizing cell death.

The optimized ischaemic condition in our system was 2% O_2 with depletion of serum and glucose for 24 h in the absence of flow. Our ischaemic condition sustained the cell viability (Fig. 2a (i–iii)) while inducing detectable cytotoxicity (Fig. 2a (iv)), measured by the amount of the extracellular lactate dehydrogenase (LDH) released through the damaged cell membranes. We also observed that hypoxia-inducible factor-1a (HIF-1α), which is usually found in the cytoplasm of the cells under normoxic conditions
**Fig. 2 | Establishing ischaemia.** a, Live/dead assay: live cells were stained green by calcein AM, and dead cells were stained red by ethidium homodimer (EthD-1) in normoxia (i) and ischaemia (ii). Scale bars, 100 μm. Quantified viability measured using a live/dead assay (n = 3) (iii). Statistical analysis was performed using a one-tailed Student’s t-test (P = 0.16). Cytotoxicity measured by extracellular LDH level (n = 5) (iv). Statistical analysis was performed using a one-tailed Student’s t-test (P = 0.0087). In normoxia, HIF-1α is mainly located in the cytoplasm (v and vi). In our ischaemic condition, HIF-1α accumulated in the nucleus (vii and viii). All images were taken from the brain channel. n denotes the number of biological replicates, independent chips, used in each experimental condition. Data are mean ± s.d. Dots along the bar graphs represent individual data points. NS, P > 0.05; **0.001 < P < 0.01. b, Gene expression changes induced by ischaemia. log₂-transformed fold changes are plotted in the heat map. The genes are clustered into groups according to their functions. A more detailed description on the functions of each gene is presented in Supplementary Table 2.

(Fig. 2a (v and vii)), translocated to the nucleus (Fig. 2a (vii and vii)), as observed in the ischaemic brain in vivo[15]. According to the pattern of gene expression alteration in our chip (Fig. 2b), the ischaemic insult upregulated the genes in both the apoptotic and the antiapoptotic signalling cascades (apoptosis group), just as reported in animal ischaemic stroke model[16]. Oxidation–reduction reaction (redox group) was also upregulated, implying that the cells protected themselves against the increased intracellular levels of reactive oxygen species in ischaemia[17]. The upregulation of the neurotrophic and angiogenic factors (trophic factors group) suggests that the attempts of the ischaemically damaged cells to repair and remodel themselves[18]. The cells also exhibited typical neuro-inflammation responses against ischaemic stroke[19] as shown in the upregulated gene expressions of pro-inflammatory cytokines and integrin groups. The downregulated expression of extracellular matrix proteins (ECM proteins group), together with the enhanced activities of matrix metalloproteinases (MMP group) and the decreased interaction between the cells and ECM (cell adhesion group) imply that the ischaemic insult led to the impairment of tissue integrity as well as the subsequent tissue remodelling process. Overall, these gene expression patterns collectively indicate that our ischaemic condition induced inflammation and deterioration in tissue integrity as expected and also accompanied endogenous neuroprotection and tissue remodelling, as reported in many other in vivo stroke models[20]. More detailed information on individual genes is presented in Supplementary Table 2.

**Verifying the NVU behaviours.** To verify the functionality of the reconstructed NVU, we examined individual cell behaviours at various levels both under healthy and ischaemic conditions. At the gene level, we measured the expression alteration of the genes associated with a series of post-stroke pathological conditions and categorized them on the basis of their functional characteristics. As most of the genes are not cell specific and are involved in multiple cellular processes, this grouping is solely for the purpose of outlining the overall pattern of the responses across the cell population in our experiments. More detailed information on individual genes is presented in Supplementary Table 2.

Neurons are the primary component of the central nervous system and have critical roles in neurological functions. Considering the short lifespan and limited expansion capacity of the primary human neurons in vitro, we used the human induced pluripotent stem cell (hiPSC)-derived NPCs in our stroke model and optimized the culture conditions of the chip for their neuronal differentiation. The differentiated NPCs exhibited the neuronal morphology of a cell body and branches of axons and dendrites (Fig. 3a (i)), and expressed mature neuron markers such as microtubule-associated protein 2 (MAP-2) and synapsin I and II (SYN1/2), a family of proteins regulating neurotransmitter release at synapses (Fig. 3a (ii)). They also maintained proximity with the astrocytes in our chip (Fig. 3a (iii)). Under ischaemic conditions, they showed dendritic beading or fragmentation (Fig. 3a (iv)), which is a typical morphology of degenerating neurons[21], compared with the smooth and clear dendritic morphology observed in normoxia (Fig. 3a (ii)). They were also stained by the neuronal degeneration marker Fluoro-Jade (Fig. 3a (iv versus vii)), consistent with the reports from in vivo ischaemic stroke models[22].

The gene expression alteration by the ischaemia (Fig. 3a (vii)) shows that the endogenous repair (trophic factors group; Fig. 2b) led to the upregulation of the gene groups that are involved in neurite formation and synaptogenesis, but it was accompanied by the downregulation of genes related to synaptic plasticity. We also observed the excessive stimulation of an excitatory neurotransmitter, glutamate (glutamate group) and, at the same time, the decreased activity of an inhibitory neurotransmitter, gamma-aminobutyric acid (GABA) (GABA group; ABAT and GABRB1 encode an enzyme for GABA catabolism[23] and in one of the GABA receptors[24], respectively). These expression patterns imply the disrupted balance between neuronal excitation and inhibition in the ischaemic condition, potentially leading to the excitotoxicity typically observed in ischaemic stroke[25].

We further examined how the ischaemic condition was reflected in the cytosolic calcium (Ca²⁺) oscillation pattern in the differentiated NPCs in our chip. The cytosolic Ca²⁺ imaging provides an indirect but accurate measure of the action potential generation in individual neurons[26], and represents various neuronal functions ranging from synaptic activity to cell–cell communication, adhesion, neurodegeneration and apoptosis[27]. The cytosolic Ca²⁺ images show that the differentiated NPCs exhibit the typical four patterns of cytosolic Ca²⁺ signals[28]: oscillatory (repeated brief increase in free Ca²⁺), transient (brief elevation due to Ca²⁺ influx through membrane calcium channels), sustained (sustained increase in Ca²⁺ level by both external and internal stores) or unnoticeable signals...
Fig. 3 | Behaviours of neurons, BMECs and pericytes. a, Behaviour of neurons. A phase contrast image of neurons in a normoxic sample (i). White arrows indicate the hydrogel boundary. The neurons derived from hiPSCs expressed mature neuronal markers, MAP-2 and synapsin I and II (SYN) (ii). NVU cellular components were immunostained for a neuronal marker, MAP-2, and an astrocyte marker, GFAP (iii). Fluoro-Jade C (FJ), a neuronal degeneration marker, barely stained the neurons in normoxia (iv). A phase contrast image of neurons in a hypoxic sample (v). Fluoro-Jade C stained the neurons in hypoxia (vi). Gene expression alteration by ischaemia (vii). Spontaneous oscillations of cytosolic calcium ions (Ca\(^{2+}\)) in differentiated NPCs (viii to xii). Representative calcium recording images (vii). NPCs were pre-stained with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiL) (red). Fluo-4 AM (green) was used to detect the cytosolic Ca\(^{2+}\) (green) was used to detect the cytosolic Ca\(^{2+}\). Representative calcium recording images (vii). NPCs were pre-stained with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiL) (red). Fluo-4 AM (green) was used to detect the cytosolic Ca\(^{2+}\) concentration. The DiL-expressing cells were randomly chosen from four independent samples, two each in normoxia (n = 34 cells) and ischaemia (n = 14 cells). The differentiated NPCs showed four distinct Ca\(^{2+}\) oscillation patterns for 10 min of recording (ix). The marked cells from 1 to 4 in (viii) showed oscillatory, transient, sustained and unnoticeable patterns, respectively. The ratio of cell numbers for each Ca\(^{2+}\) oscillation pattern (x) and the changes in the cell numbers due to ischaemia (xi). The amplitude and frequency of the calcium oscillation in the cells showing oscillatory Ca\(^{2+}\) signals (xii and xiii). The horizontal bars indicate the mean values and the dots represent individual data points. Statistical analysis was performed using one-tailed Student’s t-tests (P = 0.25 (xii) and P = 0.016 (xiii)). b, Behaviour of BMECs. Fluorescent (i versus iii) and phase contrast (ii versus iv) images show the BMEC morphology with or without flow. White arrows indicate the hydrogel boundary. The ZO-1 and VEGF expression spread all over the bodies of BMECs in ischaemia (v). The overall extent of ZO-1 expression in BMECs increased with the introduction of flow and reduced back in the ischaemic condition (n = 3) (vi). Statistical analysis was performed using one-way ANOVA with Bonferroni–Holm post hoc test (P = 0.0033 between without and with flow in normoxic samples; P = 0.0444 between normoxic samples with flow and ischaemic samples; and P = 0.02 between normoxic samples without flow and ischaemic samples). The spatial distribution of ZO-1 expression in an individual cell was quantified by counting the number of pixels (y axis) corresponding the fluorescence intensity (x axis) (vii). The ZO-1 expression in the ischaemic cells is dispersed throughout the entire cell body in contrast to the pattern of localized peaks shown in normoxic cells. The changes in log-transformed P\(_{\text{app}}\) by the presence of flow and ischaemic insult (n = 5) (viii). Statistical analysis was performed using one-way ANOVA with Bonferroni–Holm post hoc test (P = 0.000066 between without and with flow in normoxic samples; P = 0.0014 between normoxic samples with flow and ischaemic samples; and P = 0.022 between normoxic samples without flow and ischaemic samples). BMECs in ischaemia upregulated VEGF expression (n = 3) (ix). Statistical analysis was performed using one-way ANOVA with Bonferroni–Holm post hoc test (P = 0.050 between normoxic samples without and with flow; P = 0.0048 between normoxic samples with flow and ischaemic samples; and P = 0.0042 between normoxic samples without flow and ischaemic samples). Gene expression alteration by ischaemia (x). c, Behaviour of pericytes. A few pericytes are exposed between BMECs (i) and on the side wall of the brain channel (ii). The white arrows indicate pericytes. More detailed data on pericyte distribution are presented in Supplementary Fig. 3. Gene expression alteration by ischaemia (iii). More details on the functions of each gene are presented in Supplementary Table 2. Statistical significance is denoted by asterisks. n denotes the number of biological replicates, independent chips, used in each experimental condition. Data are mean ± s.d. Dots along the bar graphs represent individual data points. NS, P > 0.05; *P < 0.05, **P < 0.01, ***P < 0.001. For a–c, scale bars, 100 µm.
The ischaemic insult decreased the ratio of cells showing the unnoticeable Ca²⁺ signals, while increasing the ratio of cells showing both transient and sustained signals (Fig. 3a (x and xi)). The accumulated Ca²⁺ level in the cytoplasm is thought to lead to neuronal death in animal stroke models⁴⁶. Analysis of the alteration in oscillatory signal by ischaemia reveals insignificant changes in the amplitude but a significant increase in the frequency of the oscillation (Fig. 3a (xii and xiii)), indicating the increased Ca²⁺ influx into the cells⁴⁹. The excessive influx of Ca²⁺ together with the disrupted balance between neuronal excitation and inhibition (neurotransmitters group; Fig. 3a (vii)) show excitotoxic neurodegeneration⁵⁰ in our ischaemic samples.

BMECs are the primary cellular component of the cerebral vasculature, forming the BBB. Human primary BMECs were used throughout this study. BMECs have a high mitochondrial density, lack of fenestrations, low pinocytic activity and high density of adherent and tight junctions compared with the endothelial cells found in other tissues⁶⁰-⁶⁴. The tight junctions determine the paracellular tightness of the endothelial cells and the permeability across the BBB⁶¹. Zonula occludens-1 (ZO-1, also known as TJP1) is a dominant junctional adapter protein that regulates other junctional components, cell–cell tension, angiogenesis and BBB formation⁶². The flow through the blood-side channel increased the expression of ZO-1 and caused the shape of the cell body to elongate along the direction of the simulated bloodstream (Fig. 3b (i–iv)). The upregulated ZO-1 expression in the samples with flow led to the upregulated expression of other junctional proteins, vascular endothelial-cadherin and claudin-5 (Supplementary Fig. 6). The ZO-1 expression of the ischaemic samples was significantly decreased compared with the samples in normoxia with the flow, but was statistically comparable to the normoxic samples without the flow (Fig. 3b (v and vii)). Importantly, the expression of ZO-1, which is mainly localized on the cell membrane under normoxia conditions (Fig. 3b (iii and vii)), spread throughout the cell body under ischaemic conditions (Fig. 3b (v and vii)). This dispersed spatial distribution of ZO-1 in the ischaemic samples led to the increased permeability of fluorescence probe (4 kDa FITC–dextran; Fig. 3b (viii)), representing the reduced paracellular tightness under ischaemia. These results suggest that the paracellular tightness among the endothelial cells is affected more significantly by the extent of tight junction localization on the cell membrane, rather than the overall level of their expression. The BMECs in the ischaemic samples significantly increased the expression of the vascular endothelial growth factor (VEGF), one of the angiogenic factors (Fig. 3b (v and ix)), suggesting that the post-stroke vascular reorganization took place in our model, as observed in an animal stroke model⁶⁵. At the gene level (Fig. 3b (x)), the ischaemic insult decreased the endothelial paracellular connectivity (EC–EC junction group), but upregulated the genes involved in vasoconstriction and adhesion molecules for recruiting immune cells, as observed in animal stroke models⁶⁶-⁶⁸.

BMEC behaviours have been well documented in various experimental conditions. In a monoculture of human BMECs, the shear stress induced by the flow did not significantly affect the expression of the tight junction proteins or their morphology⁶⁹. By contrast, the flow condition in a monoculture of bovine BMECs led to the upregulation of tight junction proteins and the morphological alignment along the flow direction⁷⁰. In another in vitro study⁷¹, rat BMECs required the appropriate interactions with both the astrocytes and the pericytes to show their original pattern of tight junction localization around the cell membrane, as we observed in our model (Supplementary Fig. 4). Taken together, these results suggest that, for human BMECs to exhibit in vivo-like behaviours, they need some of the key components of the original BBB microenvironment: the mechanical stimuli by the blood flow and the heterocellular network in NVU. Our stroke model provides both of these essential microenvironment features, allowing for in vivo-like behaviours of human BMEC.

Pericytes are mural cells of the microvasculature, and regulate BBB permeability, angiogenesis, clearance, cerebral blood flow, neuroinflammation and stem cell activity⁷². We used human primary brain vascular pericytes throughout this study. Figure 3c (i and ii) shows that the pericytes in our chip expressed platelet-derived growth factor receptor beta (PDGFRβ), one of the pericyte-specific markers⁷³, and positioned themselves between the mature endothelium and the side wall of the brain channel. More detailed data on pericyte distribution are presented in Supplementary Fig. 3. The pericytes were activated in response to the ischaemic injury (pericyte markers groups in Fig. 3c (iii)), contributing to vascular inflammation⁷⁴. The interaction of pericytes with endothelial cells, which is crucial for the vascular stability under normal conditions⁷⁵, was downregulated (pericyte–endothelial cell interaction group).

Astrocytes are the dominant glial cell type in the brain and have many mediating roles in the heterocellular interactions in NVU⁷⁶. We used human primary astrocytes throughout this study. One of its roles is to sense neuronal metabolic activities and coordinate vasodilation and vasoconstriction to match the blood flow accordingly⁷⁷. Astrocytes perform these intermediary roles through direct contact-based interactions with the endothelial cells⁷⁸. In our chip, oxygen and nutrients are provided through only the blood-side and CSF-side channels such that the astrocytes in the brain channel would have to migrate and extend their endfoot towards the formed endothelial layer at the boundary to access the nutrients, therefore forming a physical contact with it. We indirectly confirmed this physical contact through the immunofluorescence staining of water-channel proteins encoded by aquaporin-4 (AQP4), the most abundant water channels in the brain⁷⁹. The water channels in astrocytes are localized around an astrocytic endfoot in direct contact with the blood vessel under normal conditions⁸⁰ (Fig. 4a (i and ii)). This polarized location reflects their mediating role in gaseous exchange including O₂, CO₂, and NO⁸¹. In inflammatory conditions such as ischaemia, the immunoreactivity of AQP4 in astrocytes bleeds away from the endfoot (Fig. 4a (iii and iv)), implying the disruption of the mediating role of AQP4 (ref. ⁸²). Furthermore, the astrocytes in the ischaemic samples showed reactive astrogliosis⁸³, characterized by abnormal hypertrophy (Fig. 4a (v–vii)), massive proliferation and upregulated expression levels of glial fibrillary acidic protein (GFAP) (Fig. 4a (viii)). Astrocytes failed to show these behaviours in the traditional 2D culture conditions (Supplementary Fig. 7). The gene expression pattern (Fig. 4a (ix)) reveals a heterogeneous population of astrocytes mixed with both A1 (inflammation-induced) and A2 (ischaemia-induced) phenotypes (astrocyte-reactive marker groups), as reported in vivo stroke models⁸⁵. The activated astrocytes in turn decreased their trophic support for the neurons in our ischaemic stroke model (astrocyte–neuron interaction group), consistent with the reports from other stroke models⁸⁶.

Microglia are the resident macrophages and the only immune cell type in the brain⁸⁷. Owing to the issues of reliable batch-to-batch reproducibility with human primary microglia, we used a transformed human microglial cell line (HMC3). Microglia in the brain show immediate pro-inflammatory responses to any injury or infection⁸⁸. Once activated, their pro-inflammatory changes in morphology are signified by the retraction and thickening of the processes and the hypertrophy of the cell body⁸⁹, which were reproduced in our ischaemic samples (Fig. 4b (i–iii)). They also promptly secrete interleukin-1β (IL-1β)—one of the pro-inflammatory (M1) phenotype markers—within a few hours of the onset of inflammation⁹⁰-⁹¹. However, this upregulation of IL-1β is only temporary and not sustained⁹²-⁹⁴. By contrast, the expression of the cluster of differentiation 68 (CD68) and ionized calcium-binding adapter molecule 1 (IBA-1) is upregulated during M1 phase and persists throughout the anti-inflammatory (M2) phase thereafter⁹⁵-⁹⁷. We observed...
Fig. 4 | Behaviours of astrocytes and microglia. a, Behaviour of astrocytes. AQP4 in the normoxic astrocytes is localized on the side wall of the hydrogel (i and ii), while AQP4 in the ischaemic astrocytes is spread over the cell bodies (iii and iv). GFAP expression of astrocytes under normoxic (v) and ischaemic (vi) conditions. The ratio between the longest and shortest axes of astrocytic cell bodies in normoxic and ischaemic samples (vii). $n=15$ cells from 3 independent chips were analysed. Statistical significance was determined using a one-tailed Student’s t-test ($P < 0.001$). Quantified GFAP expression levels of astrocytes in normoxic and ischaemic samples ($n=3$) (vii). Statistical significance was determined using a one-tailed Student’s t-test ($P = 0.030$). Gene expression alteration by ischaemia (viii). b, Behaviour of microglia. Microglia (stained with anti-IBA-1 antibodies) in ischaemia upregulated the expression of CD68 (i and ii). The ratio between the longest and shortest axes of microglial cell bodies in normoxic and ischaemic samples (iii). $n=15$ cells from 3 independent chips were analysed. Statistical significance was determined using a one-tailed Student’s t-test ($P < 0.001$). Microglial expression of IL-1β and CD68 in normoxic and ischaemic (for 3 h and 24 h) samples (iv to ix). Microglia upregulated the expression of IL-1β only during the first few hours of the onset of ischaemia ($n=3$) (x). Statistical significance was determined using one-way ANOVA with Bonferroni–Holm post hoc test ($P = 0.0019$ between samples of normoxia and ischaemia for 3 h; $P = 0.019$ between ischaemic samples for 3 h and 24 h; and $P = 0.113$ between samples of normoxia and ischaemia for 24 h). CD68 was expressed in microglia for the whole duration of ischaemia ($n=3$) (xi). Statistical significance was determined using one-way ANOVA with Bonferroni–Holm post hoc test ($P = 0.0019$ between samples of normoxia and ischaemia for 3 h; $P = 0.019$ between ischaemic samples for 3 h and 24 h; and $P = 0.012$ between samples of normoxia and ischaemia for 24 h). CD68 was expressed in microglia for the whole duration of ischaemia ($n=3$) (xi). Statistical significance was determined using one-way ANOVA with Bonferroni–Holm post hoc test ($P = 0.048$ between samples of normoxia and ischaemia for 3 h; $P = 0.43$ between ischaemic samples for 3 h and 24 h; and $P = 0.012$ between samples of normoxia and ischaemia for 24 h). Gene expression alteration by ischaemia (xii). More details on the functions of each gene are presented in Supplementary Table 2. The white arrows in a and b indicate the hydrogel boundary between the blood-side and the brain channels. $n$ denotes the number of biological replicates, independent chips, used in each experimental condition. Data are mean±s.d. Dots along the bar graphs represent individual data points. NS, $P > 0.05$; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. For a and b, scale bars, 100 μm.

These in vivo-like temporal patterns of the IL-1β and CD68 immunoreactivity in our stroke model (Fig. 4b (iv-xi)). By contrast, the traditional 2D culture conditions failed to induce these behaviour changes in microglia (Supplementary Fig. 8). The gene expression pattern of the microglia-reactive marker group in Fig. 4b (xii) indicates that the ischaemic onset led to the upregulation of both pro-inflammatory (M1 phenotype) and anti-inflammatory (M2a and M2b phenotypes) microglial markers, as observed in in vivo studies22. Both M2a and M2b are involved in phagocytosis and produce anti-inflammatory cytokines, although their activation signal pathways are distinct from each other23. By contrast, the M2c phenotype, which is usually regarded as a marker for the deactivating stage24, barely appeared during the 24 h time frame after the ischaemic onset in our stroke model, consistent with the report14 that M2c macrophages appeared only after the downregulation of the inflammation. Many other immune receptors and chemotactic markers also showed an upregulated expression level. The genes engaging both the innate and the adaptive immune responses were generally upregulated in our ischaemic condition (groups from toll-like receptor signal to major histocompatibility complex class-II molecule), as previously reported15. The gene expression of the purinergic receptors, which are involved in both immune cell regulation25 and neurogenesis26, was rather inconsistent (purinergic receptors group), although it was also clear that the overall immune responses were not well regulated right after ischaemia (immune regulation). These gene expression alterations indicate that the ischaemic insult triggered a broad spectrum of immune responses, from exacerbating the ischaemic injury to helping repair, as observed in other ischaemic stroke models27. Additional images of the NYU cells are presented in Supplementary Fig. 9.

Characterizing the neurorestorative potential of stem cells. A substantial number of studies has supported the neurorestorative potential of stem cells for stroke treatment, but there have also been a few reports contradicting some of these observations28–30. This could be partially because the experiments were all conducted under different conditions and/or focusing on different aspects of the complicated recovery processes. Being an in vitro system, our
stroke model enables the identical experimental conditions across large number of samples and over repetitions. It therefore serves as an effective testbed to systematically examine the neurorestorative ability of clinically relevant stem cells. The stem cells examined in our stroke model include hiPSC-derived NPCs (hNPCs), human embryonic stem cell derived neural stem cells (hNSCs), human haematopoietic stem cells (hHSCs), bone-marrow-derived mesenchymal stromal/stem cells (hBMSCs), adipose-derived mesenchymal stromal/stem cells (hAMSCs) and endothelial cell progenitor cells (hEPCs). We also examined the effect of reperfusion treatment alone, without stem cells, by reintroducing oxygen and glucose after ischaemic insult.

The neurorestoration after ischaemic stroke entails an expansive series of processes from neural cell regeneration and immune suppression, to the restoration of vascular structures and the recovery of heterocellular interactions in the NVU68. We selected 123 relevant genes that are involved in each of these aspects on the basis of the Human Neurogenesis PCR array as well as our own experimental data on the ischaemic responses in our chip (Fig. 5a (i)). The details on the relevant functions of the selected genes and the corresponding references are presented in Supplementary Tables 2 and 3. Figure 5a (i and ii) shows the gene expression alteration by stem cells and the related Gene Ontology (GO) terms based on the STRING database of protein–protein association networks79 (https://string-db.org/). The first three GO terms in Fig. 5a (ii) represent general functions (system development (GO:0048731), biological regulation (GO:0065007) and response to stimulus (GO:0050896)) and are common to most of the genes chosen for our study. The overall gene expression of the chosen set was generally upregulated by the incorporation of all types of stem cells as well as the reperfusion only (Fig. 5a (iii and iv)). When we considered the genes with changes in expression of greater than fourfold, hNPCs and hNSCs were mostly associated with strongly upregulated genes, whereas the opposite was true for hBMSCs (Fig. 5a (iv)). hEPCs turned up almost equal numbers of strongly up- and downregulated genes (Fig. 5a (iv)).

The stem cell incorporation as well as reperfusion generally invoked a positive influence on the generation of the cells in the nervous system (neurogenesis; GO:0022008), although with the exception of strongly upregulated genes, the extent of this influence varied across stem cell types (Fig. 5a (iii)). Furthermore, all of the groups enhanced the expression of genes involved in neuron migration (GO:0001764), neuron differentiation (GO:0030182), neuron fate commitment (GO:0048663), axonogenesis (GO:0007409) and gliogenesis (GO:0042063), although hBMSCs also showed an inhibiting influence on neuron differentiation and neuron fate commitment. Notably, the reperfusion upregulated the expression of all of the genes involved in neuronal migration, even though the extent was weak (Fig. 5a (iii)). As for the synapse responses, a similar pattern was revealed in synapse organization (GO:0050808) and regulation of synapse plasticity (GO:0048167), with a dominant positive influence from all experimental groups, except for the fact that hEPCs and reperfusion exhibited equally strong enhancing and inhibiting effects.

In the post-stroke recovery process, it is also important to suppress the inflammation initiated by the ischaemia80. The hNPCs and hNSCs most strongly upregulated inflammation-related genes (inflammation response, GO:0006954). While hAMSCs also slightly upregulated the genes in the inflammation response group, hEPCs, hBMSCs, hHSCs and perfusion slightly suppressed the genes in this group. To examine more specific inflammatory responses, we measured the expression of glial phenotype markers (coloured letters in Fig. 5a (i); a magnified heat map is presented in Supplementary Fig. 10) and examined the influence of the translated stem cells on their inflammatory behaviours. As for the effects on BMECs (red letters in Fig. 5a (i) and Supplementary Fig. 10), hNPCs, hNSCs and hEPCs upregulated the expression of the tight junction protein 1 (TJP1), but the expression of Claudin 5 (CLDN5) was downregulated in all groups, despite the fact that TJP1 and CLDN5 interact closely to form the BBB81. The expression of platelet and endothelial cell adhesion molecule 1 (PECAM1)—one of the endothelial adhesion molecules that is responsible for immune cell recruiting after brain injury82—was all effectively downregulated. For the effects on pericytes (yellow letters in Fig. 5a (i) and Supplementary Fig. 10), only hAMSCs suppressed the expression of a reactive pericyte marker, chondroitin sulfate proteoglycan 4 (CSPG4). The expression of CD248, which is involved in the role of pericytes in mediating angiogenesis83, was upregulated by hNSCs and hAMSCs only, and generally suppressed by the rest. Regarding the influence on the microglial activities (green letters in Fig. 5a (i) and Supplementary Fig. 10), all of the groups failed to suppress the expression of the microglial-reactive marker CD68, which is usually upregulated throughout the whole inflammatory phase84–86. By contrast, CD86, a proinflammatory M1 phenotype marker, was suppressed by all groups, except for hAMSCs and hHSCs. hHSCs enhanced the expression of CD206, which is an anti-inflammatory M2a phenotype marker, while hEPCs and hBMSCs promoted the expression of CD32a, which is an anti-inflammatory M2b phenotype marker. hBMSCs, hAMSCs and reperfusion upregulated the expression of CD163, a microglia-deactivating phenotype marker. As for the effects on astrocytes (purple letters in Fig. 5a (i) and Supplementary Fig. 10), there was no group suppressing the expression of VIM, a pan-reactive astrocyte marker. hNPCs were the only ones that suppressed the expression of C3, an A1-reactive (inflammation) astrocyte marker. In all of the groups, the expression of CD109, an A2-reactive (ischaemia) astrocyte marker, was downregulated. Taken together, the expression pattern of these astrocyte-reactive markers suggests that, 7 d after the ischaemic insult, there was little influence from the ischaemia itself, and yet the astrocytes still retained their inflammatory behaviours. The expression of interferon induced transmembrane protein 3 (IFITM3), involved in neurotrophic support of astrocytes, was upregulated in all groups, while the expression of fatty-acid-binding protein 7 (FABP7), another gene with a similar function, was upregulated only by hNPCs, hEPCs and hBMSCs. The complexity in the overall gene

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Fig. 5 | Characterization of the neurorestorative potential of stem cells. a. Gene expression alterations 7 d after incorporating stem cells in ischaemic samples (i). Detailed descriptions of the functions of each gene are provided in Supplementary Tables 2 and 3. The gene names written in red, yellow, green and purple are the phenotype markers of endothelial cells, pericytes, microglia and astrocytes, respectively. A magnified heat map is presented in Supplementary Fig. 10. GO terms associated with each gene are highlighted in dark or light yellow (ii). The percentage of genes associated with each GO term according to their fold changes in expression (increase in red and decrease in blue) (iii). The gene count of the entire population of genes according to their fold changes in expression (iv). The genes with changes in expression of greater than fourfold are shown on the right. Statistical significance in the up- and downregulated gene counts is denoted by asterisks. b. The genes (red) expressed differentially by the stem cell types with the NDC (i). The cut-off thresholds are P = 0.05 (red dashed line) and expression changes of greater than twofold (blue dashed line). The five most significant GO terms that emerged from the GO enrichment analysis of the genes identified in (i) (ii). GO terms associated with each gene are highlighted in yellow. c. The GO term enrichment networks, built with only the genes with changes in expression of greater than fourfold, show the dominant restorative pathways for each stem cell type. The clustered circles around the GO-term labelled circle indicate its significant subtypes in GO hierarchy. The size of a circle is inversely proportional to the false-discovery rate (FDR) (bottom right). Detailed GO terms are shown in Supplementary Fig. 11.
expression pattern suggests that all six types of stem cells as well as reperfusion have their own pathways to suppress the neuroinflammation induced by the ischaemia.

When the gene expression pattern was hierarchically clustered (agglomerative hierarchical clustering on the basis of Euclidian distances using Addinsoft XLSTAT), hNPCs and hNSCs, with more
restricted fate commitment to neural cells, stood out as a separate group from the rest (dendrogram in Fig. 5a (i)). The differential expression analysis between the two groups (Fig. 5b (i)), the group with neural differential capacity (NDC) versus the group without NDC) identified 27 genes. We performed the GO enrichment analysis on the identified genes based on the STRING database9 and found that the stem cells with NDC were beneficial in neurogenesis (GO:00022008) and other closely related GO terms (Fig. 5b (ii)). The stem cells with NDC also had positive effects on regulating the signal cascade of mitogen-activated protein kinase (GO:0043408), which is an important regulator of ischaemic and haemorrhagic cerebral vascular disease).

To better distinguish the neurorestorative characteristics of each type of the stem cells, we performed the GO enrichment analysis focusing on the genes with expression changes of greater than fourfold after stem cell incorporation to identify the dominant therapeutic pathways for each stem cell type (Fig. 5c). The detailed GO terms are shown in Supplementary Fig. 11. Each GO term, represented by a circle, is placed according to the functional hierarchy, with sub-trees grouped together under the parent GO term circle. The size of a circle indicates the significance of the stem cell influence on the given GO term. Positive influence/upregulation is denoted in red while the negative influence/down-regulation is denoted in blue. hNPCs showed the greatest potential for neurogenesis (GO:0022008), especially regarding the generation of neurons (GO:0048699; Fig. 5c (ii)), although it had stimulating effects on other restorative functions as well. The influence of hNSCs is more evenly spread out across diverse aspects, such as forming and maturing tissue structures (anatomical structure development, GO:0048856) and developing multicellular organism (multicellular organism development, GO:0007275; Fig. 5c (ii)). hNSCs also showed a strong capacity in developing blood vessels (GO:0001568), a fundamental environment for restoring NVU function, as well as promoting the movement of cells (locomotion, GO:0048011), an important feature for reorganizing the ischaemically damaged structure (Fig. 5c (ii)). Other relative advantages of hNSCs included enhanced adaptation to environmental changes (response to stimulus, GO:0050896), and better regulation of immune responses (regulation of immune system process and immune response, GO:0002682 and GO:0050776, respectively; Fig. 5c (ii)). Notably, hNSCs strongly suppressed the acute inflammatory responses (acute-phase response, GO:0006953; Fig. 5c (ii)). hNSCs were also least associated with the pathways in cancer (hsa05200 in Supplementary Fig. 11) based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database22. Across the whole range of the GO terms, hEPGs exhibited the tendency of simultaneous promotion and inhibition (Fig. 5c (iii)), while hBM-SCs consistently inhibited most of them (Fig. 5c (iv)).

Compared with the top three influential stem cell types (hNPCs, hNSCs and hEPGs), the rest of the experimental groups (hBM-SCs, hAMSCs, hHSCs and reperfusion therapy) induced relatively smaller changes in gene expression. We therefore performed the GO enrichment analysis on the genes with expression changes of greater than twofold for these groups (Supplementary Fig. 12). In contrast to the distinct characteristics that emerged from the previous analysis for the three most influential stem cell types (Fig. 5c and Supplementary Fig. 11), these groups exhibited relatively inconsistent influences over the GO terms grouped together for related functions (Supplementary Fig. 12). A few consistent trends spotted were neurogenesis promotion by hHSCs and vasculature development (GO:0001944) inhibition by hAMSCs and reperfusion.

We further examined the synaptic activities as an important and reliable parameter to estimate the extent of the post-stroke recovery (Supplementary Fig. 13). On the basis of the GO enrichment analysis of the genes with expression changes of at least fourfold or twofold, we compared the significance of the GO terms related to the synapse and neurotransmitters, and examined how much each stem cell type promoted the synaptic activities. This analysis also revealed a streak of strong positive influences of hNSCs on many aspects of the neurorestoration at synapse level, including synapse organization (GO:0050808) and synaptic transmission (GO:0007268), as well as the regulation of the transmission pathways of the relevant neurotransmitters such as glutamate (GO:0035249), acetylcholine (GO:0007271) and GABA (GO:00051932). Given the molecular and functional complexity of the synapses and the importance of their coordination in neurological functions, hNSCs stand out as the highly promising therapeutic agent among all of the stem cells evaluated in our system.

**Tracking the therapeutic stem cells.** Although the exact mechanism underlining the neurorestorative effects of the therapeutic stem cells for stroke is still unknown, there is accumulating evidence that the therapeutic effects of stem cell therapies are mediated by indirect mechanisms, such as releasing trophic factors and immune regulatory cytokines, promoting endogenous stem cell migration and enhancing endogenous neural plasticity and function recovery. However, albeit rarely, there have also been reports that the transplanted stem cells directly replace the host cells, reconstituting the damaged neural circuitry. The primary factor attracting the stem cells towards the infarcted brain parenchyma seems to be the inflammatory responses of the NVU, such as the upregulation of cytokines, cell adhesion molecule (CAM) and MMP, which is observed in our NVU model as well.

With our stroke model, we were able to track the therapeutic stem cells and assess the extent of the direct cell replacement. We examined each of the major indicators of the cell replacement: the extent of adhesion to the BBB, the number of surviving cells, the extent of infiltration into the ‘brain’ channel and differentiation into various neural cell types in NVU. We first prepared green fluorescent protein (GFP)-expressing stem cells using lentiviral factors. The transfection efficiency of this process is presented in Supplementary Fig. 14. The number of stem cells initially adhering
to the BBB was less than 5% of the total cell number in the chip in most cases (Fig. 6a (i) and 6b (i) and Supplementary Fig. 15). After 7 d of stem cell injection, the cell viability counts for those attached to the BBB were in general either decreased (hNPCs, hNSCs and hHSCs) or only slightly increased (hBMSCs and hAMSCs) (Fig. 6a (i) and 6b (ii) and Supplementary Fig. 15). By contrast, hEPCs vigorously proliferated and infiltrated into the brain channel (Fig. 6a (i) and 6b (ii and iii)). At the same time point, 7 d after transplantation,
all of these therapeutic stem cells barely expressed their stem cell markers (Fig. 6a (ii)) that they originally expressed in 2D cultures (Supplementary Fig. 16). This does not mean that they completed differentiation by that time, because only a very limited number of the cells were mature enough to express the markers of their predicted lineages (Fig. 6a (iii) and 6b (iv)). Only hNPCs and hNSCs showed detectable neural differentiation and even those were less than 0.01% of the total cell number in the chip. The extremely limited stem cell differentiation suggests that the direct cell replacement is not a major mechanism underlying the neurorestorative effects of stem cell therapy, adding to the recent growing evidence against it.86

Discussion

Here we present a microphysiological stroke model to systematically evaluate the efficacy of stem cell therapy. Our data revealed three key aspects of NVU microenvironments that are required for in vivo-like behaviours of the constituent cells: the formation of the intact BBB, the heterocellular network and the proper mechanical stimuli by blood flow (Fig. 1). The brain-like microenvironment ensures that the cells in our model retain their native behaviours and show clinically relevant responses to ischaemic insult (Figs. 2, 3 and 4). Our model served as an efficient screening testbed to examine the neurorestorative potential of the stem cells used in preclinical trials. We systematically analysed how each type of stem cell influenced the gene activities during the complicated disease progression and recovery processes (Fig. 5). We also utilized our stroke model to track the stem cell behaviours therapeutic in the ischaemically damaged NVU (Fig. 6).

The design of our microfluidic chip well suited the need of establishing a functional BBB and at the same time enabled the real-time monitoring of the therapeutic stem cells moving across the BBB. Similar chip designs have been proposed to build a functional BBB: positioning cells side-by-side by using micropoles (AIM Biotech)90 or a flow-guiding structure (PhaseGuide technology, Mimetas)91. The design of such a chip is useful for observing the behaviour of drugs or cells passing through the BBB in a 3D environment. Another mainstream method in BBB chip design is to vertically stack hierarchical tissue structures between porous membranes.12,47,37,87. The chips with this design are also commercially available (Emulate), and have been used to reconstruct a functional BBB29–39. A 3D NVU structure to examine the metabolic consequences of BBB inflammation was reconstructed on the basis of this design47. The advantage of this chip design is that the BBB is formed on a 2D membrane so that the dynamics of the endothelium can be monitored in the same focal plane of optical microscopes. This design poses challenges to visualize the heterocellular interactions across vertical layers, necessitating confocal imaging. Moreover, the cellular interactions are inevitably interfered by the porous membranes. What differentiates our design is the absence of physical structures between two neighbouring channels, which enables the cellular interactions free from any potential interference due to physical structures. However, we acknowledge a couple of limitations of our chip design. First, the new design involves different heights and therefore reduces the possible contact surface for the cells in the neighbouring channels to interact with. Second, the use of a generic hydrogel of which the components are not fully defined made it difficult to characterize the dynamics in the extracellular matrix, especially the basement membrane in the BBB that actively participates in BBB regulation47. Third, our current model uses polydimethyl siloxane (PDMS) and therefore retains the PDMS-inherent pitfalls, such as cytotoxicity, non-specific adsorption of bioactive molecules and gas passage65,92. As an alternative, microfluidic chips prepared with plastic injection moulding have been proposed to overcome these limitations of PDMS, as well as for large-scale production purposes89. Our chip design without microstructures should be compatible with the injection moulding and the combined methodology would be ideal for industrial-scale mass production.

Our stroke model delineated the neurorestorative behaviours of each candidate stem cell type for stroke treatment (Fig. 5). The benefits of hNPCs and hNSCs, the stem cells with the capacity to differentiate into neural cells, consistently stood out in many aspects related to the post-stroke recovery processes. Our hNPCs were tested by the manufacturer to ensure that more than 80% of their progeny differentiate into neuronal cells. The hNSCs used in this research were initially isolated from fetal cortical brain tissue at 13.5 weeks gestation (M031 clone) and classified as neural stem cells due to their ability to self-renew and produce progeny cells that differentiate into neural cells90. On the basis of our GO analysis, hNPCs showed the strongest ability to generate neurons (GO:0048699) and hNSCs exhibited compelling positive effects on the overall structural and functional integrity in NVU. Notably, the recovery of NVU functionality, such as gliogenesis (GO:0042063), blood vessel development (GO:0001568) and immune system process (GO:0002376), was also linked to the enhanced synaptic activities, both mediated by hNSCs. Given the importance of the synaptic activities in rewiring the neuronal network and neurological functions, this result suggests that restoring the overall NVU functionality may be more critical for stroke treatment than replenishing neurons themselves. It is important to take into account the limitations of our approach as well when interpreting these results. First, our efficacy evaluation focused only on the gene level, as represented by GO functional analysis, and did not cover the entire range of interactions across different levels associated with post-stroke recovery. The transcriptionomics was also performed on the whole-cell population, which has limitations in showing cell- or tissue-specific changes, and there is always the risk of overinterpreting the GO analysis results. Second, the contribution from the peripheral immune cells crossing the BBB was not addressed in our model and they could also have important roles in the post-ischaemic inflammation95 and, as we incorporated neurons differentiated from NPCs rather than mature neurons, our chip could contain subsets of neurons with heterogeneous maturity. Third, as the flow in our chip was bidirectional, generated by a rocking shaker, the endothelial cells would activate different signal pathways of mechanotransduction compared with the unidirectional blood flow in vivo100–102.

The results from tracking the stem cells (Fig. 6) suggest that the therapeutic effects of the stem cells arise mainly through the indirect mechanism of supporting the endogenous recovery, rather than direct cell replacement. At the time of gene expression alteration analysis, the number of stem cells left in our samples was mostly less than 1% of the whole cell population. The presence of such a small population itself could not possibly be the major driving force to induce the observed magnitude-fold changes in the gene expression for the whole cell population. This implies that the presence of the remaining stem cells themselves have had a minor influence on efficacy evaluation. Similar observations were reported in both animal models and clinics that the transplanted stem cells barely reached the ischaemic region, but still induced meaningful therapeutic effects92. On the basis of these observations and implications, the preclinical evaluation of the candidate stem cells for cell therapy would be more effective and relevant if focusing on their ability to restore the damaged NVU both structurally and functionally, rather than tracing the fate of the transplanted stem cells themselves in vivo.

Many of the previous studies have presented conflicting results, not only on the neurorestorative potential of each stem cell type in varying conditions, but also on the mechanism by which stem cells exert their therapeutic effects84. The possible reason for these controversies could be the fact that the efficacy evaluation was focused only on a few aspects, lacking comprehensive analysis on the overall recovery process. Another reason could be the comorbidities that often accompany stroke, such as hypertension, high cholesterol and
diabetes, that complicate the disease progression and treatment. As such, stem cell therapy would be most effective using a personalized approach based on the comprehensive health condition of individual patients. We expect that in vitro stroke models, such as the ones presented in this study, would serve as an ideal testbed to develop personalized stem cell therapies, by using patient-derived cells and simulating the unique pathophysiological condition of individual patients. The personalized stroke model could in turn serve as an efficient testbed to screen many different candidate stem cells and identify the optimal stem cell regimen for the given patient. The multiomics approach, presented in some of the recent studies, could further expand our understanding of the post-stroke neurorestoration process and our in vitro stroke model is readily applicable for that purpose as well.

Taken together, our approach recapitulated the NVU behaviours in the normal and ischaemic conditions in vitro and enabled efficient and systematic evaluation of the stem cell therapy, overcoming the limitations of both the animal models and the currently available in vitro models. The findings from this study, especially the characterization of the neurorestorative potential of various stem cells, can steer the direction of the stem cell therapeutics in research as well as in clinics. Our approach presented in this work is also immediately applicable to a wide range of other diseases associated with the vasculature, opening up new possibilities in the field of precision medicine.

Methods

Cell culture. NVU constituent cells. Human primary astrocytes (ScienCell, 1800) were cultured on T75 precoated flasks with 2% poly-l-lysine solution (Sigma-Aldrich) in an astroculture medium (LAM, ScienCell) before use. Human microglial cell-line (HMC3, ATCC, CRL-3304) was maintained in Eagle’s minimum essential medium (EMEM, ATCC) containing 10% fetal bovine serum and 1% penicillin-streptomycin. hNPCs (Millipore, SC0035) were maintained on T75 precoated flasks with 1% Matrigel (BD Matrigel Matrix High Concentration) in NEM. Human primary BMECs (ScienCell, 1000) were cultured on T75 precoated flasks with 2% collagen solution (Sigma-Aldrich) in an endothelial cell medium (ECM, ScienCell, 1001). Human brain vascular pericytes (ScienCell, 1200) were grown on T75 precoated flasks with 2% poly-l-lysine solution (Sigma-Aldrich) in a pericyte medium (PM, ScienCell, 1201). T75 flask coated with Matrigel and those coated with poly-l-lysine solution were prepared by incubation at 37 °C for 1 h and overnight, respectively. T75 flask coated with collagen were prepared by incubation at 4 °C overnight.

Stem cells. hEPCs were purchased from Celsprogen (San Pedro, 37089-01) and were expanded on T75 flask precoated with the extracellular matrix for hEPC expansion (Celsprogen, E36053-65-775) in complete hEPC growth medium (Celsprogen, M36053-1370). hBMECs (Gibco, A15632) and hAMSCs (Gibco, PCS-500-011) were maintained in a mesenchymal stem cell medium (ScienCell, 7501). hNSCs (NR1), which were initially isolated from fetal cortical brain tissue at 13.5 weeks gestation (M031 clone) and derived from the embryonic stem cell line H9, were cultured under the same conditions as the hiPSC-derived NSCs. hHSCs were purchased from ATCC (PCS-800-012) and were used directly for experiments without subculturing. The medium was changed every 2–3 d. Cells were passaged when the confluency reached approximately 80%. We used 0.25% trypsin-EDTA to split astrocytes, BMECs, and pericytes when the confluency reached approximately 80%. We used 0.05% trypsin-EDTA to split hiPSC-derived NSCs, astrocytes and microglia were embedded in a basement membrane extract (BME) hydrogel (Cultrex reduced growth factor basement membrane matrix type R1, Trevigen, 3433-001-R1) and then injected into the brain channel of the chips. hNPCs were suspended in a neural expansion medium (NEM, Millipore, SCM004) suspended with 2 mM glutamine and 0.02 μg/ml fibroblast growth factors (FGF)-2. To obtain astrocytes and microglia in their resting state, they were sustained in aM without serum and astrocyte-conditioned medium (ACM, ScienCell, 1811), respectively, for 1 d before injection. The density of the suspension for each cell type was ~8 × 10^6 cells per ml. We prepared cell mixture by mixing hNPCs, astrocytes and microglia at the ratio of 8:4:1 (n/n/n) and then with BME type R1 hydrogel prepolymer (gelcell, 4:1 (v/v)). According to the vendor, more than 80% of the hNPCs commit to mature neurons, making the final cell ratio for neurons, astrocytes and microglia fall in the range of 5–6:4–5:1 (n/n/n), similar to the naive brain. The gel–cell mixture was injected into the brain channel of a chip that was placed onto a cold pack. The total number of the incorporated neural cells in the brain channel was around 4 × 10^7.

After injection, chips were transferred into incubator four-well cell culture plates (Thermo Fisher Scientific, 267061) and incubated at 37 °C in a cell culture incubator for 30 min for gelation. After gelation, the serum-free mixed medium of NEM, serum-free AM and ACM (8:4:1, v/v/v), referred to as NEM/AM, was injected into both the blood-side and the CSF-side channels and then changed every day. From day 3 after the injection, NEM was replaced with a neural differentiation medium (NDM) (Millipore, SCAM 11), referred to as NDM/AM in Fig. 1a (ii). The culture medium was changed every other day for the next 2 d until BBB reconstruction.

BBB reconstruction. BMECs and human pericytes were suspended in ECM and PM, respectively, at a density of ~1 × 10^6 cells per ml. BMECs and pericytes were mixed at a 9:1 (n/n) ratio on the basis of the literature and 10μl of the cell suspension was injected into the blood-side channel of a chip after the neural cells were co-cultured for 4 d in the brain channel. The total cell number in the blood-side channel was around 1 × 10^6. We tilted the chip a little bit for BMECs and pericytes to adhere to the side wall of the hydrogel in the brain channel and incubated it for 3 h. Then we removed old medium and injected fresh mixed medium (ECM/PM; 9:1 (v/v), final serum content of 4.7% (v/v), referred to as ECM/PM in Fig. 1a (ii)) into the blood-side channel to remove any unattached cells and debris. We changed the mixed medium of ECM and PM in the blood-side channel and the mixed medium of NDM, AM and ACM in the CSF-side channel every other day. Chips were cultured for a further 3 d for BBB formation.

Shear stress on the BBB. To apply the shear stress of flow in the physiological range as in the brain microvasculature (0.01–10 dyne cm^−2), we generated a pulsatile bidirectional flow by placing the samples on a rocking see-saw shaker (Mimetas, Organoflow L). We adopted the flow condition of a previous study in which a functional BBB was established. We modulated the design parameters of our model on the basis of the equation below:

\[
\tau = \frac{6 \times Q \times \mu}{b \times b^2}
\]

where \( r \) is the shear stress (dyne cm^−2), \( Q \) is the flow rate (cc s^−1), \( r \) is the viscosity of culture medium, \( b \) is the channel width and \( h \) is the channel height. On the basis of the Poiseuille's law,

\[
Q = \frac{D^P \times \pi \times D^p}{128 \times \mu \times L}
\]

\[
D = \frac{2 \times h \times b}{h + b}
\]

where \( D^P = r \times \pi \times \sin \theta \) (the pressure difference between the inlet and outlet), \( \theta \) is the tilt angle of a shaker, \( L \) is the channel length and \( r \) is the liquid density.

The mean shear stress during a given time period is proportional to the following parameters:

\[
\tau \propto \frac{h^3 \times b^3 \times \sin \theta}{h + b^3} \times \sin \theta
\]

In previous studies, we used the experimental set-up with \( h \approx 220 \mu m, b = 400 \mu m, \theta = 7^\circ \) (Organoplate, Mimetas) and a tilting frequency of 16 min, the maximum shear stress was estimated to be 1.7 dyne cm^−2 on the basis of a
Numerical model implemented with Python software. Our set-up (h = 400 μm, b = 1 mm, θ = 4° and a tilting frequency of 1 min) was expected to generate 3.4 dyne cm⁻² of the maximum shear stress at a higher frequency.

**Induction of in vitro ischemic conditions.** To induce ischaemia, the chips were placed in the incubation chamber of an EVOS fl auto imaging system with 2% O₂ and 5% CO₂ for 24 h (ref. 4). Before the chips were incubated in the hypoxic chamber, the culture medium was replaced with serum- and glucose-free DMEM (Gibco, 11966025) that had been flushed with nitrogen gas for 1 min before use. There was no flow during the ischemic period. The samples under normoxic conditions were cultured an incubator with 5% CO₂ and atmospheric O₂ concentration (~20%).

**Functional characterization of the reconstructed BBB.** Evaluation of BBB as a physically intact barrier. To evaluate the physical intactness of the formed BBB in a microfluidic chip, we fitted FITC-conjugated dextran (70kDa and 4kDa) into the blood-side channel and monitored the diffusion of the dextran across the BBB. We took fluorescence images (at 488 nm) at different time points during 1 h after the probe injection and the fluorescence intensities were measured with ImageJ (NIH). The permeability coefficients were calculated using the equation below:

\[
P_{app} = \frac{1}{A} \times \frac{dQ}{dt} = \frac{A \times (\bar{I}_{brain} - \bar{I}_{blood})}{\Delta t} \times \frac{I_{brain}}{A_{brain}}
\]

where \( A \) is the surface area of the membrane, \( C_{in} \) is the initial concentration on the donor side, \( dQ/dt \) is the transport rate, \( I_{brain} \) is the hydrogel volume in the brain channel, \( I_{brain} \) is the mean fluorescence intensity in the brain channel, \( I_{blood} \) is the mean fluorescence intensity in the blood-side channel and \( I_{t} \) is the initial fluorescence intensity.

This equation assumes that flux across the imaging boundary is negligible, and transcellular diffusion is constant. In our chip, these assumptions were safely met for the time intervals (\( \Delta t \)) of shorter than 15 min; that is, there was no significant difference between \( P_{app} \) calculated with 5, 10 and 15 min of \( \Delta t \) (n = 5, P > 0.05). \( \Delta t \) was set to 10 min. We calculated the \( P_{app} \) of the BBB (\( P_{brain} \)) based on the \( P_{app} \) of the whole barrier (\( P_{barrier} \)) and the endothelium itself (\( P_{endo} \)) using the following equation.

\[
\frac{P_{barrier}}{P_{app}} = \frac{1}{P_{app}} + \frac{1}{P_{endo}}
\]

**Evaluation of BBB as a biochemically intact barrier.** We examined whether the formed endothelium could isolate the neural cells in the brain channel from the serum in the blood-side channel. First, we added the serum-containing medium of ECM and PM (9:1, v/v) containing 10% fetal bovine serum into the blood-side channel and monitored the diffusion of the dextran across the BBB. We took fluorescence images (at 488 nm) at different time points during 1 h after the probe injection and the fluorescence intensities were measured with ImageJ (NIH). The permeability coefficients were calculated using the equation below:

\[
P_{app} = \frac{1}{A} \times \frac{dQ}{dt} = \frac{A \times (\bar{I}_{brain} - \bar{I}_{blood})}{\Delta t} \times \frac{I_{brain}}{A_{brain}}
\]

where \( A \) is the surface area of the membrane, \( C_{in} \) is the initial concentration on the donor side, \( dQ/dt \) is the transport rate, \( I_{brain} \) is the hydrogel volume in the brain channel, \( I_{brain} \) is the mean fluorescence intensity in the brain channel, \( I_{blood} \) is the mean fluorescence intensity in the blood-side channel and \( I_{t} \) is the initial fluorescence intensity.

This equation assumes that flux across the imaging boundary is negligible, and transcellular diffusion is constant. In our chip, these assumptions were safely met for the time intervals (\( \Delta t \)) of shorter than 15 min; that is, there was no significant difference between \( P_{app} \) calculated with 5, 10 and 15 min of \( \Delta t \) (n = 5, P > 0.05). \( \Delta t \) was set to 10 min. We calculated the \( P_{app} \) of the BBB (\( P_{brain} \)) based on the \( P_{app} \) of the whole barrier (\( P_{barrier} \)) and the endothelium itself (\( P_{endo} \)) using the following equation.

\[
\frac{P_{barrier}}{P_{app}} = \frac{1}{P_{app}} + \frac{1}{P_{endo}}
\]

**Calcium imaging and analysis.** To record the cytosolic calcium oscillation in the neurons differentiated from hNPCs, we preincubated the hNPCs with Dil (1.0 μM) and DiD (5 μM) before culturing the cells for 3 h. After culturing, the cells were fixed with 4% PFA in PBS and mounted with ProLong Diamond antifade reagent (Thermo Fisher Scientific). The fluorescence images were acquired at different focal planes ranging from z = 0 to 100 μm in 10-μm intervals. For 3D reconstruction of the images, the 3D Viewer plugin of ImageJ was used.

**Calcium imaging and analysis.** To record the cytosolic calcium oscillation in the neurons differentiated from hNPCs, we preincubated the hNPCs with Dil (1.0 μM) and DiD (5 μM) before culturing the cells for 3 h. After culturing, the cells were fixed with 4% PFA in PBS and mounted with ProLong Diamond antifade reagent (Thermo Fisher Scientific). The fluorescence images were acquired at different focal planes ranging from z = 0 to 100 μm in 10-μm intervals. For 3D reconstruction of the images, the 3D Viewer plugin of ImageJ was used.
were determined using the Agilent 210 Bioanalyzer. The RNA amount obtained from each chip was as follows for each experimental condition: a chip with 10 μg under normoxia generated about 250 ng; the same condition followed by 24 h of ischaemia generated about 130 ng; a chip with 1 μg under normoxia, used as the control group in neurorestorative efficacy evaluation, generated about 400 ng; a chip with reperfusion only, generated about 200 ng; a chip with therapeutically stem cells, generated about 350–750 ng depending on the stem cell type. Total RNA was reverse-transcribed to cDNA using a high-capacity cDNA reverse transcription kit (Applied Biosystems, 4368814). Quantitative PCR (qPCR) was performed in a StepOnePlus real-time PCR system (Applied Biosystems) using TaqAdvanced Universal SYBR Green Supermix (Bio-Rad, 1725272 A) to quantify the expression levels of the genes of interest. qPCR amplification was achieved with 40 cycles of 30 s at 95 °C, 15 s at 95 °C and 30 s at 65 °C. We distinguished the signal from noise using the StepOnePlus real-time PCR software and further checked manually to ensure that the obtained Cq values had indeed captured real signals. Customized qPCR plates were designed and fabricated by ScienCell. Neurogenesis qPCR plates were purchased from Qiagen. We first chose 12 genes with well-known ischaemic behaviours and confirmed the reproducibility of their expression in triplicate, and we measured the expression of the final 123 genes with more than six independent chips for each experimental condition.

Evaluation of the neurorestorative potential of stem cells. We examined the neurorestorative potential of various types of stem cells. Twenty-four hours after the ischaemic insult, the serum- and glucose-free DMEM medium was replaced with NDM/AM at a ratio of 8:4 (v/v). One ml of medium of NDM, serum-free AM and hAMSCs (8:4:1 (v/v/v)) in the CSF-side channel. The stem cells were then replaced with NDM/AM (the mixed medium of NDM, serum-free AM and hAMSCs, hBMSCs and hEPCs. We used a prestaining method for hHSCs because the virus transfection efficiency of hHSCs was not sufficient. We purchased ready-to-use GFP lentiviral particles from GenTarget and used them according to the manufacturer’s protocol with some modifications. More specifically, cells were cultured in a 48-well plate until the confluency reached 50–75%. Cell culture medium was removed before transduction and 0.25 ml of fresh medium and 15 μl of virus solution were added to each well. Cells were cultured in a cell culture incubator for 2–3 d without medium change in between to achieve a desirable transduction efficiency. hHSCs were prestained with Vybrant DiO cell-labelling solution (Invitrogen, V22886). hHSCs at 1 × 10^6 cells per ml were incubated with staining medium (10 μl labelling solution per 1 ml culture medium) in a 96-well plate for 20 min in a cell culture incubator and washed three times with sterile PBS (pH 7.4) for use. After injecting each stem cell into the blood-side channel of a chip after ischaemic insult, images were taken every other day for up to 7 d to track stem cell infiltration. At the end of the seven days, stem cells were immunostained for neurogenesis, GFAP for gliogenesis and VWF for vasculogenesis). The extravasation extent of both cancer and stem cells was quantified by image scoring (ImageJ, NIH).

Statistical analyses. Every independent experiment was repeated at least three times, and the results are presented as the mean ± s.d. For the quantitative analysis of fluorescence images, we obtained at least three images from different samples and used ImageJ (NIH) image analysis software to quantitatively analyse the aspects of interest. Statistical significance was evaluated using one-sided Student’s t-tests for two-group comparisons and one-way ANOVA with Bonferroni–Holm post hoc test for multiple-group comparison (Daniel’s XL Toolbox). P < 0.05 was considered to be significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. The main data supporting the results in this study are available within the paper and its Supplementary Information. The raw and analysed datasets generated during the study are too large to be publicly shared, yet they are available for research purposes from the corresponding author on reasonable request.

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**Author contributions**

Z.L. and K.-M.K. designed and performed the experiments, and analysed and interpreted the data; J.P. designed experiment; H.-J.J. and H.W. performed the experiments; W.L. conceived and supervised the project, designed and performed experiments, and analysed and interpreted the data. The manuscript was mainly written by W.L. with contributions from all of the authors.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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**Correspondence and requests for materials** should be addressed to W.L.

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Software and code

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Data collection

Optical images were obtained with an EVOS fl auto imaging system (Life Technologies) or an LSM 880 confocal microscope (Zeiss). Real-time qPCR was performed in a StepOnePlus real-time PCR system (Applied Biosystems). We observed spontaneous calcium oscillations in Dil labeled cells using a confocal microscope (Carl Zeiss, LSM 710, Göttingen, Germany) under 37 deg and 5 % CO2. Time-lapse video recordings of calcium signals were made with an LSM 710 confocal microscope (Zeiss).

Data analysis

Image analysis was performed using ImageJ 1.53a (NIH). Statistical analyses were performed using Excel (Version 2103, Microsoft) and Daniel's XL Toolbox (version 2.0). Hierarchical clustering analysis was performed using XLSTAT program (Version. 2018.5, Addinsoft).

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| Sample size | For imaging analysis, we used more than 3 distinct samples.
| For qPCR analysis, we first chose 12 genes with well-known ischaemic behaviours and triplicated using different batches of the cells. After confirming the reproducibility, we measured the expression of the final 123 genes reported in this study. We collected 4–6 independent samples for each experimental group.
| For WB analysis, we collected 4 independent samples for each experimental group.

| Data exclusions | No data were excluded from analysis.

| Replication | All experimental measurements were done at least with three biological replicates.

| Randomization | No randomization was used.

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| MRI-based neuroimaging | X

Antibodies

| Antibodies used | Sheep polyclonal anti-human CD31/PECAM-1 (R&D Systems, Cat. No.: AF806, 1:20), rabbit polyclonal anti-human GFAP (Sigma, Cat. No.: G9269, 1:100), chicken polyclonal anti-human GFAP (Synaptic Systems, Cat. No.: 173006, 1:500), rabbit polyclonal anti-human AQP4 (Novus Biologicals, Cat. No.: NBP1-87679, 1:2000), mouse monoclonal anti-human ZO-1 (Invitrogen, Cat. No.: 339100, 1:100), rabbit polyclonal anti-human von Willebrand Factor (vWF, Sigma, Cat. No.: F3520, 1:200), mouse monoclonal anti-human vWF (Sigma, Cat. No.: AMAB90931, 1:500), mouse monoclonal anti-human podoplanin (PDPPN) (E-1) (Santa Cruz Biotechnology, Cat. No.: SC376695, 1:100), rabbit polyclonal anti-human Synapsin 1/2 (Synaptic System, Cat. No.: 106003, 1:1,000), chicken polyclonal anti-human MAP2 (Abcam, Cat. No.: ab5392, 1:10,000), goat polyclonal anti-human IBA-1 (Abcam, Cat. No.: ab5076, 1:200), rabbit polyclonal anti-human IL-1β (Abcam, Cat. No.: ab9722, 1:100), mouse monoclonal anti-human CD68 (Bio-Rad, Cat. No.: MAC5709, 1:100), rabbit monoclonal anti-human CD44 (Invitrogen, Cat. No.: 19H8I4, 1:500), mouse monoclonal anti-human CD34 (Life technology, Cat. No.: BI-3CS, 1:250), mouse monoclonal anti-human Nestin (ThermoFisher, Cat. No.: MA1-5840, 1:250), rabbit monoclonal anti-human PDGFR β (Cell Signaling, Cat. No.: 3169, 1:100), mouse monoclonal anti-human HIF-1α (Abcam, Cat. No.: ab6066, 1:200), Claudin-5 (Invitrogen, Cat. No.: 34-1600, 1:150), VE-cadherin (Invitrogen, Cat. No.: 14-1449-82, 1:200), ZO-1 (Invitrogen, Cat. No.: 339100, 1:250), and β-actin (Sigma, Cat. No.: A1978, 1:20,000).

| Validation | All antibodies were chosen on the basis of the validation statements of the manufacturers' product information and the corresponding literature verifying their applications. No new application for primary antibodies was used in this work.
### Eukaryotic cell lines

**Policy information about cell lines**

| Cell line source(s) | Human induced pluripotent stem cell (hiPSC)-derived neural progenitor cells (hNPCs) (Millipore, Cat. No.: SCC035). Human brain microvascular endothelial cells (hBMECs) (ScienCell, Cat. No.: 1000). Human brain vascular pericytes (ScienCell, Cat. No.: 1200). Human astrocytes (ScienCell, Cat. No.: 1800). Transformed human microglia (HMC3, ATCC, Cat. No.: CRL-3304). Human endothelial progenitor cells (hEPC) (Cat. No.: 37089-01, San Pedro, CA, USA). Human bone marrow-derived mesenchymal stem cells (hBMSC, Gibco, Cat. No.: A15652). Human adipose-derived mesenchymal stem cells (hAMSC, Gibco, Cat. No.: PCS-500-011). Human hematopoietic stem cells (hHSC) ATCC (Cat. No.: PCS-800-012). |
| Authentication | We purchased all cell lines (except MB231br, provided by Joan Massagué at Sloan Kettering Institute via a materials transfer agreement; and NR1, generated by co-author Steinberg) from manufacturers, which provided the authentication information. For MB231br and NR1, we reference appropriate literature providing the detailed authentication process. |
| Mycoplasma contamination | All cells were provided with results from mycoplasma-contamination tests. |
| Commonly misidentified lines (See ICLAC register) | No commonly misidentified cell lines were used. |