Precise control of embolic stroke with magnetized red blood cells in mice

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Precise embolism control in immature brains can facilitate mechanistic studies of brain damage and repair after perinatal arterial ischemic stroke (PAIS), but it remains a technical challenge. Microhemorrhagic transformation is observed in one-third of infant patients who have suffered PAIS, but the underlying mechanism remains elusive. Building on an established approach that uses magnetic nanoparticles to induce PAIS, we develop a more advanced approach that utilizes magnetized erythrocytes to precisely manipulate de novo and in situ embolus formation and reperfusion in perinatal rodent brains. This approach grants spatiotemporal control of embolic stroke without any transarterial delivery of pre-formed emboli. Transmission electron microscopy revealed that erythrocytes rather than nanoparticles are the main material obstructing the vessels. Both approaches can induce microbleeds as an age-dependent complication; this complication can be prevented by microglia and macrophage depletion. Thus, this study provides an animal model mimicking perinatal embolic stroke and implies a potential therapeutic strategy for the treatment of perinatal stroke.
Perinatal stroke is a serious neurological disease that affects fetuses, preterm infants, and full-term infants; it is defined as cerebrovascular injury occurring between 20 weeks of pregnancy and 28 days after birth. A recent report revealed that the birth prevalence of perinatal stroke (1:1100) in full-term infants was higher than that in any of the previous reports and comparable to the incidence in the elderly population. The estimated risk for premature infants is believed to be 100 times higher than that of full-term infants. Perinatal arterial ischemic stroke (PAIS) is the most common type among the diverse subtypes of perinatal stroke. PAIS is clinically mostly asymptomatic, so its incidence has been underestimated, and its symptoms generally emerge after the neonatal period, such as cerebral palsy and cognitive dysfunction, which is also one of the reasons why PAIS seriously affects long-term disability and tetraplegia in neonates. Approximately 90% of PAIS cases occur in the middle cerebral artery (MCA). Unlike adult ischemic stroke, which is mainly caused by the progressive development of white thrombosis and the resultant artery stenosis, PAIS is dominantly caused by a red embolus, which is mainly made up of red blood cells and fibrins and distally originates from the placenta or the malfunctioning heart. To elucidate the fundamental molecular and cellular mechanisms of brain damage and repair after embolic stroke, diverse approaches have been produced, such as arterial delivery of pre-formed macro- or microemboli, local thrombin injection, rose bengal-based photothermalbosis or ultrashort laser pulse-induced thrombosis at the MCA. Although these models are closer to clinical stroke than mechanical occlusion approaches and are well documented in adult animals, they are not feasible in mice at the age of P0-7 because the arterial diameter is much smaller than that of adult mice. In addition, these traditional methods neither avoid arterial surgery nor produce reversible embolic occlusion in perinatal mice.

Hemorrhagic transformation can occur spontaneously in the infract tissue, complicate ischemia, and is associated with worse outcomes. Limited epidemiological studies have shown that pediatric patients have five times the incidence of hemorrhagic transformation than adult patients after focal ischemic stroke. During the subacute period, which is referred to as the time window from 1 to 5 days after ischemia onset, 30% of neonate patients have microbleeds as a complication, whereas less than 10% of adults do. There have been few reports comparing the hemorrhagic transformation frequency at the acute stage between neonates and adults. This merits further investigation in both epidemiological and basic research fields.

Microglia, the resident immune cells of the brain, are associated with cerebral vasculature and are among the first cells that respond to brain injury. The number of perivascular microglia increases gradually with stroke duration and activated microglia can phagocyte endothelial cells and promote blood vessel disintegration. In addition, blood-derived macrophages penetrate the blood-brain barrier after attaching to endothelial cells and infiltrate into the brain parenchyma, exacerbate inflammation and tissue damage, and break down the integrity of the blood-brain barrier. It has been reported that hemorrhagic incidence during the subacute period is decreased in adult mice by using minocycline, which inhibits microglia and macrophages. However, the cellular mechanism underlying hemorrhagic transformation after perinatal arterial ischemic stroke has yet to be determined.

Here, we report the development of an approach named embolic stroke induced by magnetic nanoparticle-coated red blood cells (SIMPLER). We were able to generate red thrombi de novo and in situ by aggregating magnetized red blood cells (mRBCs) at the distal MCA (dMCA) of mouse pups at the age of P0-7. We found that at the acute stage, perinatal mice had nine times the frequency of microhemorrhagic transformation as adults. The SIMPLER model is an advanced version of the SIMPLE model (stroke induced by magnetic nanoparticles) in the scenario of perinatal arterial embolic stroke in which most cases are caused by distally formed RBC-rich clots. Depletion of microglia and macrophages prevented microbleed complications.

**Results**

**Production of magnetized red blood cells (mRBCs).** To produce mRBCs by connecting magnetic nanoparticles (MNPs) to red blood cells, we utilized two distinct methods (Fig. 1a and Supplementary Fig. 1a). First, we used the principle of streptavidin-biotin interaction to attach MNPs to the RBC membrane (Fig. 1a and a-i). We linked biotin to the RBC membrane by using an anti-Ter119 antibody conjugated with biotin and specifically recognized the RBC membrane protein marker Ter119. Then, the resultant biotin-coated RBCs were incubated with streptavidin-modified MNPs (streptavidin-MNPs; see Fig. 1a-ii–a–ii and Methods). Thus, mRBCs were successfully produced via a strong interaction between streptavidin and biotin (Fig. 1b–f). Compared to the smooth surface of unmagnetized RBCs (Fig. 1b, d), scanning and transmission electron microscopy revealed that we successfully adhered MNPs (magenta arrows) to RBCs in vitro (Fig. 1c, e). MNPs did not detach from mRBCs after administration into the bloodstream (Fig. 1f, magenta arrows). We used these electron microscopic images to quantify the ratio between the free nanoparticles that did not bind to RBCs versus the total nanoparticles we used. This quantification revealed that most MNPs (92%) were tagged onto RBCs, and only 8% of them were free. Thus, the first method based on streptavidin-biotin binding can, with a high efficiency of 92%, tag MNPs onto the RBC membrane. In addition, the RBC quality was closely monitored by microscopic inspection at every single step (Supplementary Fig. 2). We ceased the mRBC preparation process if we found that under 90% of erythrocytes had a normal biconcave shape.

Alternatively, we aimed to load MNPs into RBC cytoplasm by using a classical DNA transfection reagent—Lipofectamine (Supplementary Fig. 1a). However, Lipofectamine failed to do so; instead, it tethered MNPs to the cell membranes of RBCs, as revealed by electron microscopy (Supplementary Fig. 1b–f). Compared to the smooth surface of unmagnetized RBCs (Supplementary Fig. 1b–i and 1e), MNPs were attached onto RBCs (yellow arrows, Supplementary Fig. 1c). High-magnification images show MNPs partially embedded into the dint of the RBC membrane (magenta arrow, Supplementary Fig. 1c–i). Some RBCs seemed structurally damaged (green arrows in Supplementary Fig. 1d), and the membrane of RBCs had holes (blue arrows in Supplementary Fig. 1d–i), implying that this method may harm RBCs. These results demonstrated that although mRBC was successfully produced as well, the second method based on Lipofectamine might have poorer quality than the first method. Both strategies were carried out throughout the entire study, unless otherwise indicated.

To assess how these mRBCs respond to the magnetic field gradient, we produced mRBCs using the two methods mentioned above and performed an mRBC enrichment assay that was achieved by providing a magnetic field to a drop of liquid that may contain MNPs alone, mRBCs or unmagnetized RBCs (see Methods). MNPs were rapidly pooled together and aggregated into a line (black arrows, Fig. 1g; Movie 1), as were mRBCs (green arrows, Fig. 1g–i; Movie 2). In contrast, unmagnetized RBCs remained steady state (Fig. 1g–ii; movie 3). The aggregation speed of mRBCs was slower than that of MNPs alone. mRBC took 90 s to accumulate into a line, whereas MNP took only 30 s (Fig. 1g–ii).
This result suggested that the weight and volume of RBCs may contribute to the slower motion of mRBCs.

To determine the RBC magnetization efficiency, we used the mRBC enrichment assay to calculate the percentage of unmagnetized RBCs. At an MNP dose of 0.5 pg per RBC for the magnetization reaction, we found that ~70% of RBCs were not magnetic. In other words, >30% of RBCs were magnetized (Fig. 1h, light blue dots). This result was consistent with the results from electron microscopy imaging by counting mRBCs versus total RBCs (scanning electron microscopy, 31.3%, 148 of 427 cells, transmission electron microscopy, 30.1%, 196 of 654 cells, Fig. 1h, dark and light brown dots). These results indicated that the mRBC enrichment assay can indeed reflect the magnetization efficiency of RBCs. Therefore, we further assessed
the dose-dependency of RBC magnetization. We found that mRBC yield was augmented when MNP dosage gradually increased from 0.2 to 1.5 pg per RBC and turned to a decline at the higher dosages (Supplementary Fig. 1g). The highest dose of 13.5 pg per RBC killed RBCs directly (Supplementary Fig. 1g). Notably, at the 1.5 pg per RBC reaction dosage, we found that only 16% of RBCs were not magnetic, indicating that more than 80% of total RBCs were magnetized (Fig. 1h, darker blue dots; Supplementary Fig. 1g). Hereafter, a dosage of 1.5 pg per RBC was used throughout the following study. These results together indicated that the RBC magnetization efficiency was dose dependent and that an overdose of MNPs (13.5 pg per RBC) damaged RBCs.

We video-recorded the entire process of an mRBC floating toward a magnet (Fig. 1i; Movie 4). The mRBCs used in this experiment were produced by the Lipofectamine-based method. All RBCs were stained green by using the lipophilic dye DiO. Immediately after adding the mixture of unmagnetized RBCs and mRBCs that were both DiO positive into a chamber with a cylindrical magnet (1.5-mm diameter) glued at the center, all the RBCs initially floated in stochastic directions in the unsettled buffer. However, 10 s later, we observed that many DiO-mRBCs were trapped onto the surface of the magnet (Fig. 1i, green dots), and other RBCs were still floating around (Fig. 1i, white stars, Movie 4). We particularly focused on the region indicated by the magenta circle inside the yellow boxed area. The floating path of one DiO-mRBC is traced by a long-curved magenta dashed arrow (Fig. 1j). We noticed that it took only 5 s for this DiO-mRBC to be attracted onto the magnet (magenta arrow, Fig. 1j, Movie 4). The possibility that gravity caused the RBCs to settle was ruled out because the erythrocyte sedimentation rate is on the time scale of minutes to hours, which is far behind the mRBC’s motion. Taken together, these results demonstrated that mRBCs were successfully produced and responded to the magnetic field gradient rapidly and effectively in vitro.

mRBCs aggregated in vivo in response to magnetic force. Next, we aimed to aggregate the mRBCs in vivo (Fig. 2a). We first examined whether DiO-mRBCs (Lipofectamine-based method) remained magnetic after their entry into the adult mouse circulation. DiO-mRBCs that were drawn back from the bloodstream were aggregated by a magnetic field again, whereas unmagnetized DiO-RBCs were not (Fig. 2b, c). This result demonstrated that MNPs did not entirely dissociate from RBCs after circulating in the endogenous bloodstream environment.

Therefore, we directly monitored DiO-mRBC behavior in vivo by taking advantage of transparency of the skull of perinatal mouse pups (postnatal day 3, P3). DiO-mRBCs were delivered through the superficial temporal vein (Fig. 2a). Hereafter, all mRBCs were generated by using the first method based on the principle of streptavidin-biotin interaction. The injected mRBCs less than 1 million per gram of mouse body weight failed to aggregate in vivo (Table 1). Injection of 1-2 million DiO-mRBCs g⁻¹ gradually aggregated in the microvessels around and under the magnet (Fig. 2d, e, magenta arrows and Table 1). It took only 3 s to form two additional DiO-mRBC clots (○ vs. ◊ in Fig. 2d). Twelve seconds later, these 3 initially separated clots were aggregated together (○ vs. ◊ in Fig. 2d). These data demonstrated that a magnetic field was able to trap the flowing DiO-mRBCs at targeted vessels in a few seconds.

Alternatively, the magnet was kept on top of the vein sinus for 9 s and then removed. We found that DiO-mRBCs were trapped in the venous sinus, which has a large luminal space (○ vs. ◊ in Fig. 2e, magenta arrow). It took more than 30 s for those previously aggregated DiO-mRBCs to undergo gradual dispersal (○ vs. ◊ in Fig. 2e, magenta arrow), indicating that we can manipulate mRBC motions. Apparently, these accumulated mRBCs did not fully obstruct the venous sinus. Thus, we increased the mRBC number up to 12 million g⁻¹ and resulted in full occlusion (Movie 5). In addition, we used Alexa Fluor 488-labeled mRBCs (Alexa Fluor 488-mRBCs) instead of DiO-mRBC in Fig. 2d). We intended to aggregate Alexa Fluor 488-mRBCs to induce a blood clot in the dMCA, which is the most affected vessel in ischemic stroke. Notably, Alexa Fluor 488-mRBCs were selectively trapped at the targeted artery (magenta arrows in Fig. 2f, g), whereas barely any Alexa Fluor 488 signals were detected in the neighboring vessels (green arrows in Fig. 2f, g). This result demonstrated, again, that we were able to magnetize RBCs with high efficiency in vitro; otherwise, we would have observed unmagnetized Alexa Fluor 488-RBCs in neighboring regions. The occlusion caused by the red thrombus in the distal middle cerebral artery of a P3 mouse was reversible (Movie 6). These experimental results strongly demonstrated that we can spatiotemporally control red thrombus formation and reperfusion at the distal middle cerebral artery of perinatal mouse pups by manipulating mRBCs.

Perinatal arterial embolic stroke was induced by occluding the dMCA using SIMPLEr. Given that 90% of PAIS cases occur at the MCA, we next aimed to test the capability of SIMPLEr to induce hypoperfusion in the territory of the dMCA. Assessed by laser speckle contrast imaging, SIMPLEr, using 6 mg kg⁻¹ MNPs, successfully achieved a regional reduction in blood supply by more than 50% in the brain region outlined by the magenta dashed line (Fig. 3a). Notably, SIMPLE, using the same low dose, failed (Fig. 3b). Only when the MNP dosage increased to 80 mg kg⁻¹ did SIMPLE achieve a similar degree of hypoperfusion as SIMPLEr (Fig. 3c, d). It took 30 min for the blood flow to reach the low perfusion plateau that lasted up to 10 h (Fig. 3d). This indicates that both SIMPLEr and SIMPLE could result in permanent occlusion if magnets were not removed (Fig. 3d). Furthermore, we used two-photon live imaging and revealed that the blood flow direction, as in adult mice, was disrupted in the arteriolar branches of the MCA following occlusion (Movie 7). The deposition of MNPs in SIMPLEr mouse livers, kidneys and spleens was negligible compared to that in SIMPLE

**Fig. 1 Produce magnetized RBC (mRBC) in vitro. a-a-i** Schematic illustration of the principle of streptavidin-biotin-based mRBC generation. a-ii TEM image and particle diameter analysis a-iii of streptavidin-MNPs synthesized by our collaborators. Representative SEM (b, c) and TEM (d, e) images of unmagnetized RBCs (b, d) and mRBCs (c, e), respectively. Magenta arrows indicate MNPs. f TEM image of ultrathin sections from a segment of the middle cerebral artery, in which mRBCs accumulated. Endothelia and vascular smooth muscle cells are highlighted in magenta and green. Magenta arrows indicate MNPs. Time-lapse pictures of MNP (g), mRBC (g-i) and unmagnetized RBC (g-ii) enrichment upon magnetic gradient. Black arrows indicate aggregated MNPs, and green arrows show accumulated mRBCs. h Quantitative analysis of the RBC magnetization efficiency (SEM, 31.3%, 148 of 427 cells; TEM, 30.1%, 196 of 654 cells, three independent experiments were performed for each group of mRBC enrichment). The effect size between two groups at doses of 0.5 and 1.5 pg per RBC is 18.2. i Snapshot of trapped DiO-mRBCs on a cylindrical magnet with a 1.5-mm diameter. The dashed circle indicates the edge of the magnet, and white stars indicate the floating unmagnetized DiO-RBCs and DiO-mRBCs. j Still images of the trajectory of one mRBC movement indicated with dashed magenta arrow.
mice (Supplementary Fig. 3 and Movies 8–11). Thus, SIMPLeR is superior to SIMPLE by using a much lower amount of MNPs.

Extensive hypoperfusion leads to neuronal death. At 6 h after dMCA occlusion, SIMPLeR (6 mg kg\(^{-1}\)) induced massive neurodegeneration, as revealed by Fluoro-Jade C staining (Fig. 3e). SIMPLE did not cause any neuronal death when administered at 6 mg kg\(^{-1}\) (Fig. 3f), but it did when administered at 80 mg kg\(^{-1}\) (Fig. 3g–h).

The infarction volume was far beyond the aggregation site of mRBCs, as evidenced by the sections in Fig. 3e–g being at least 2 mm remotely posterior to the occlusion site in the dMCA. Neurodegeneration took place in the somatosensory cortex region with a sharp boundary separating the nonischemic area (Fig. 3e and g). Hoechst, NeuN, and GFAP staining revealed condensed nuclei, neuronal loss, and damaged radial glial processes in the ischemic area.
or heart atrium are most likely the major causes. Moreover, there is emerging evidence suggests that emboli generated in the placenta vessels, including platelets and white blood cells. (Fig. 3s-i). This red embolus-like structure mimics the emboli generated in the human heart atrium and deep vein thrombus. In contrast, SIMPLE used MNPs as the central component of the obstruction (green outline Fig. 3p and s-ii panel, Supplementary Fig. 6). These results suggested that SIMPLER more closely simulated clinical emboli than SIMPLE.

### Table 1 Relationship between the injected amount of mRBCs and vascular occlusion formation.

| Age | Weight (g) | mRBCs (million g⁻¹) | Occlusion formation |
|-----|------------|----------------------|---------------------|
| P8  | 3.23       | 2.14                 | Yes                 |
| P8  | 3.32       | 1.53                 | Yes                 |
| P8  | 3.12       | 1.41                 | Yes                 |
| P8  | 3.07       | 1.33                 | Yes                 |
| P8  | 3.44       | 0.89                 | No                  |
| P8  | 3.55       | 0.86                 | No                  |
| P8  | 3.36       | 0.61                 | No                  |

(Supplementary Fig. 4a–c). Neuronal density decreased by 25% to 33% in both the SIMPLEr and SIMPLE models (Supplementary Fig. 4d). Low-dose SIMPLE (6 mg kg⁻¹) did not cause any detectable defects (Supplementary Fig. 4b, d). Approximately 15% of the whole brain volume of the P5 mouse pup was infarcted, as indicated by TTC staining (Fig. 3i), while T2-weighted MRI scanning revealed a smaller infarction size, 0.85% following a 7-hour occlusion (Fig. 3g) and 3.18% following a 14.5-hour occlusion (Fig. 3j and l). A second MRI scan of the same mouse from Fig. 3j in young adulthood (P50) revealed that the infarction evolved to a thinner somatosensory cortex (yellow arrows) compared to the healthy contralateral side (white arrows, Fig. 3m). The success rate of red thrombus formation in the dMCA induced by SIMPLER was 100%, but the success rate of stroke induction was 71% because it was not necessary for all occlusions resulting in ischemia. The survival rate was 100%, as all the pups that were subjected to the occlusion lasted for 7–10 h. Not only the hemorrhagic transformation probability but also the severity of microbleeds became more obvious (Fig. 4e, f). A European cooperative acute stroke study subdivided hemorrhagic transformation into the hemorrhagic infarction (HI) type (with punctate foci of petechial hemorrhage) and the parenchymal hematoma (PH) type (with more confluent foci)²⁰. The microbleed pattern shifted from a micro-scale punctate shape (Fig. 4e-2h) to a petechial shape (gray arrows in Fig. 4e-h) and progressed further to confluent bleeding foci (black arrow in Fig. 4e-6h). A short ischemia duration (2–5 h) caused only minor hemorrhagic infarction (HI)-type transformation, whereas parenchymal hematoma (PH)-type transformation more than 24 h after SIMPLER, the occlusive material evolved, indicated by the fact that fibrin emerged to enwrap RBCs (Fig. 3o and s-i). The component ratio of fibrin-like structures accounted for up to 39%, RBCs occupied 23% and 38% of the total area left, including platelets and white blood cells. (Fig. 3a-i). This red embolus-like structure mimics the emboli generated in the human heart atrium and deep vein thrombus. In contrast, SIMPLE used MNPs as the central component of the obstruction (green outline Fig. 3p and s-ii panel, Supplementary Fig. 6). These results suggested that SIMPLER more closely simulated clinical emboli than SIMPLE.

**SIMPLEr and SIMPLE utilized distinct materials to obstruct vessels.** Although the etiology of PAIS is not yet thoroughly clear, emerging evidence suggests that emboli generated in the placenta or heart atrium are most likely the major causes. Moreover, there is no atherosclerosis that is often related to white thromb in such early developing brains. It would be important to take a close look at the nature of the obstructions generated by these two models. At 30 min after SIMPLEr, transmission electron microscopy revealed that many RBCs, including both mRBCs and unmagnetized RBCs, were stuck at the dMCA segment (Fig. 3n and Supplementary Fig. 5), which was from the same segment boxed in Fig. 2f-g (see Supplementary Fig. 5). The large volume of RBCs made the major contribution (92.6%) to obstructing the vascular lumen, while the MNPs accounted for very little volume (1.75%, Fig. 3n, s). This shows the underlying reason why SIMPLER utilizes only a small amount of MNPs can cause cerebral infarction, which usually requires a higher dose of SIMPLE.

**Depletion of CSF1R+ macrophages prevented cerebral ischemia-induced microbleeds.** Hemorrhagic transformation occurs spontaneously in the infarcted tissue, complicates PAIS and is associated with worse outcomes. However, the underlying cellular mechanisms are largely unknown. Consistent with our previous report, both SIMPLEr (6 mg kg⁻¹) and SIMPLE (80 mg kg⁻¹) resulted in petechial microbleeds in P3 mouse pups (Fig. 4a, c), but a low dose of SIMPLE (6 mg kg⁻¹) did not (Fig. 4b). Notably, this study recorded the dynamic process of bleeding by using two-photon cranial imaging of mouse pups that underwent focal cerebral ischemia (Movie 12). These petechial hemorrhages were usually remote from the infarcted area with a rim of restricted diffusion (Fig. 4a and c), similar to clinical observations. When subjected to in utero photothrombosis (see Methods), gestational day 19 mouse embryos exhibited microhemorrhage as well (Fig. 4d). However, it was not certain whether this hemorrhage resulted directly from endothelial damage or indirectly from photothrombosis-induced ischemia. Nonetheless, our model successfully mimicked microhemorrhagic transformation following PAIS.

We further ascertained the relationship between hemorrhagic transformation probability and ‘occlusion duration’ by using P3 to P5 mouse pups. We found that 11 of 17 pups did not have hemorrhagic transformation at 2 h after ischemia (Fig. 4e left panel), and 6 of 17 pups (35%) had a very mild hemorrhagic transformation, observed as punctate microbleeds (white arrows in Fig. 4e-2h). The hemorrhagic transformation rate gradually increased and reached 92% pups (38 out of 41) when dMCA occlusion lasted for 7–10 h. Not only the hemorrhagic transformation rate but also the severity of microbleeds became more obvious (Fig. 4e, f). A European cooperative acute stroke study subdivided hemorrhagic transformation into the hemorrhagic infarction (HI) type (with punctate foci of petechial hemorrhage) and the parenchymal hematoma (PH) type (with more confluent foci). The microbleed pattern shifted from a micro-scale punctate shape (Fig. 4e-2h) to a petechial shape (gray arrows in Fig. 4e-h) and progressed further to confluent bleeding foci (black arrow in Fig. 4e-6h). A short ischemia duration (2–5 h) caused only minor hemorrhagic infarction (HI)-type transformation, whereas parenchymal hematoma (PH)-type transformation...
emerged and accounted for one-sixth at 6–10 h following occlusion (Fig. 4f). Thus, there was a positive relationship between the hemorrhagic transformation rate and ischemia duration within hours (Fig. 4f). In contrast, hemorrhagic transformation in adult mice usually occurs during the subacute stage within days. These results suggested that hemorrhagic transformation in human PAIS patients might be underestimated because it might occur at the acute ischemic stage, which is more difficult to detect and diagnose.

At 6 h of occlusion, more severe hemorrhagic transformation took place in rat pups than in mouse pups, as evidenced by the 89% prevalence of parenchymal hematoma PH-type transformation in rats versus 17% in mice (Supplementary Fig. 7a, c ‘vehicle group’ versus Fig. 4e-6h, f-6h). These results demonstrated that the phenomenon of hemorrhagic transformation after brain ischemic stroke is evolutionary conserved across mice, rats, and humans. Thus, PAIS modeled in rodent animals by SIMPLeR and SIMPLE mimicked the clinical observations of ischemia-induced hemorrhagic transformation, and they are reliable models that merit broad applications in the basic research field of PAIS.

There have been very few comparative epidemiology studies that systematically analyze the age-dependent incidence of hemorrhage transformation after focal cerebral ischemia across the age categories of perinatal fetuses, preterm and full-term infants, children, and adults. We studied the hemorrhagic transformation rate, specifically at 6–10 h occlusion, crossing the different ages restricted to P4, P15, and P80 mice. We
discovered that there was a neat, negative correlation between brain maturation status and hemorrhagic transformation rate (Fig. 4g, h). The more mature the brain is, the lower the hemorrhagic transformation rate at the acute phase. This finding seems to echo the findings in humans that the hemorrhagic transformation rate is 5-fold higher in children than in adults.3,33

Our previous publication implied that damaged SMCs are cleared by migrating macrophage-like cells.36 Additionally, it is well known that the removal of mural cells, including SMCs, leads to vascular disruption and bleeding.34,35 We hypothesize that macrophages are involved in hemorrhagic transformation occurrence after ischemic insult. Minocycline, a macrophage inhibitor, ameliorated the severity of microbleeds (Supplementary Fig. 7a–c). Our experiment showed that 58% of rats in the minocycline-treated group had parenchymal hematoma (PH) type (7/12 rats), compared to 89% of rats in the vehicle-treated group (8/9 rats, Supplementary Fig. 7c). Alternatively, we used the strategy of macrophage deletion to further test our hypothesis. As previously reported, long-term inhibition of colony-stimulating factor 1 receptor (CSF1R) by feeding PLX 5622 for 2 weeks resulted in nearly full depletion of circulating monocytes/macrophages and brain resident macrophages, including microglia and border associated macrophages, in mice.36 Notably, hemorrhagic transformation was completely prevented by this pretreatment in mouse pups (Fig. 4i, j). We further found that the rate of hemorrhagic transformation was tightly and reversely correlated with the residual macrophage density in the cerebral cortex (Fig. 4k). Two weeks of feeding PLX 5562 was less potent in rats than in mice (Supplementary Fig. 7d–f). In rats, PLX 5622-treatment did not decrease the overall rate of hemorrhagic transformation but significantly ameliorated the severity of microbleeds (Supplementary Fig. 7g–i). Taken together, our findings demonstrate that CSF1R-positive macrophages, including but not limited to microglia, are the crucial cell types that mediate focal cerebral infarction-induced hemorrhagic transformation.

Discussion
As a method to establish an embolic stroke model in perinatal mice, SIMPLeR has several strengths over the traditional approaches. First, SIMPLeR can induce in situ embolization by aggregating red blood cells in vivo. This cannot be achieved by conventional strategies in which a red embolus is pre-formed in vitro.37 The vascular microenvironment for embolus formation in SIMPLeR is more natural than the plastic cannular tube used for embolus induction in the classical approaches. Although the photothermal stroke model can also induce de novo occlusive obstruction in vessels, the phototoxic dye (Rose Bengal) results in a pure platelet occlusion that has few RBCs, which, however, is out of scope of modeling a red embolic stroke. Second, SIMPLeR requires no surgical operations on arteries or skulls. These operations are indispensable in traditional strategies that require transarterial lodging of the embolus or local injection of thrombin after opening a cranial window on the skull, which may cause a secondary injury. More importantly, it is extremely challenging to implement these surgical operations on perinatal mice that have light body weights (1–3 g). The third strength of SIMPLeR is to reversibly occlude vessels by precisely controlling the occlusion duration, whereas conventional approaches either produce permanent occlusion or unpredictable spontaneous reperfusion.38 Overall, embolus introduction and final lodgment of the embolus within the cerebral vasculature make the previously established embolic models difficult to manage, leading to variations in infarct size and affected brain regions. SIMPLeR can address all these difficulties by controlling embolism with spatiotemporal precision.

In addition, SIMPLeR demonstrated its superiority over SIMPLE in mimicking embolic stroke, while it preserved the strengths of SIMPLE, such as reversibility and the ability to generate the model without operating surgically on the skull or arteries. First, SIMPLeR is more clinically relevant than SIMPLE because the former relies almost exclusively on biological materials such as RBCs and fibrins to cause stroke and the latter utilizes mechanical occlusion with MNPs. Second, SIMPLE uses much fewer MNPs than SIMPLE because RBCs are thousands of times larger than the nanoparticles we used. Using a low dose of MNPs is of great importance because we found that injection of an extremely high dose of MNPs between 150–200 mg kg$^{-1}$ caused significant lethality in adult mice, demonstrating that injecting an appropriate MNP dosage is crucial. We also found MNP accumulation in perinatal mouse internal organs following SIMPLE but not in SIMPLeR (Supplementary Fig. 3; Movies 8–11). The drawback of both SIMPLE and SIMPLeR is that neither of them can induce infarction in the deep brain regions because of the limitation of penetration depth of the magnetic field.

Hemorrhagic transformation is a natural evolution of cerebral infarction and causes secondary brain damage.20,21 Both humans and adult rodents manifest hemorrhagic transformation at the subacute stage after ischemic stroke,30,33 but when hemorrhagic transformation takes place in PAIS remains unclear. Our study...
implies that PAIS patients may have hemorrhagic transformation at a very early time window following ischemia onset. There is controversy regarding the possibility of a macrophage-dependent mechanism mediating hemorrhagic transformation in adult ischemic stroke\textsuperscript{24,30}. This study clearly demonstrated that inhibition or depletion of macrophages remarkably prevented hemorrhagic transformation in mouse and rat pups at postnatal days 3 to 5 after arterial occlusion by showing a linear relationship between macrophage density and hemorrhagic transformation probability (Fig. 4). However, it is still not clear whether circulating macrophages or brain resident central nervous system (CNS) macrophages play a major role, which merits further investigation. Hence, our research demonstrated that PAIS may have distinct early pathology from adult ischemic stroke, which,
in turn, suggests the need for a specialized treatment strategy for PATS.

**Methods**

**Animals.** All animal experiments were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) at the School of Life Sciences, Westlake University. Wild-type C57BL/6j mice were purchased from the Laboratory Animal Resources Center of Westlake University. Sprague Dawley rats (250–290 g) were purchased from SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Standard chow and water were provided to mice ad libitum. Four mice or two rats were housed in each cage. All animals were housed in a standard animal room with a 12/12-hour light/dark cycle at 25 °C. Both male and female mice and rats were used in this study.

**Synthesis of SiO2-coated Fe3O4-core MNPs.** To obtain MNPs, 0.75 g of FeCl3·6H2O was fully dissolved in 25.0 mL of ethylene glycol, followed by the addition of 0.18 g of sodium citrate tribasic dihydrate. After the complete dissolution of sodium citrate tribasic dihydrate, 1.2 g of sodium acetate was added to the above solution. After vigorous stirring for 0.5 h, the as-prepared mixture was slowly poured into a Telfon-lined stainless-steel autoclave reactor (capacity: 50.0 mL) and heated at 200 °C for 10 h. After the surface temperature of the autoclave cooled to room temperature, the reactor was carefully removed. Then, the as-prepared products, which were black in color, were thoroughly washed with ethanol and distilled water several times to eliminate the byproducts and finally dispersed in deionized water for further usage.

**Synthesis of streptavidin-MNPs.** To prepare Fe3O4@SiO2 nanoparticles, we began by fully dispersing 50.0 mg of MNPs in a solution consisting of 160.0 mL of ethan, 40.0 mL of H2O, and 4.0 mL of aqueous ammonia (25–28 wt%). The mixture was treated by ultrasonication at 25 °C for 30 min. Then, 2.0 mL of tetraethyl orthosilicate (TEOS) was gradually added to the well-dispersed solution with magnetic stirring at 25 °C. After 6 h, the obtained Fe3O4@SiO2 nanoparticles were washed with ethanol and deionized water several times to remove byproducts. To prepare sulfhydryl-functionalized MNPs, the necessary quantity of Fe3O4@SiO2 nanoparticles were immersed in 3-mercaptopropyltrimethoxysilane (MPTMS) solution (4% in anhydrous ethanol) and stirred for approximately 1 h at 30 °C. Next, the modified nanoparticles were washed with ethanol and dimethyl sulfoxide (DMSO) to remove the uncombined MPTMS. Then, the N-succinimidyl 4-malimidobutylate (GMBS) solution (7.0 mM in DMSO) was added to the dispersed nanoparticle solution and allowed to react for approximately 45 min at 30 °C. After the reaction, the MNPs were washed with DMSO and phosphate-buffered saline (PBS) to remove the excess GMBS. Subsequently, the modified MNPs were dipped in streptavidin (50.0 μg·mL−1 in PBS) for 2 h at room temperature to coat their surfaces with streptavidin. Finally, the mouse RBC-specific biotinylated anti-Ter119 monoclonal antibody was incubated with streptavidin-MNPs at room temperature in a shaker for 2 h to obtain antibody-functionalized MNPs. Some of the SIMPLER experiments used streptavidin-MNPs, whereas others used MNPs coated with streptavidin-MNPs (Cat# Mag301005 Beijing Zhongkeleiming Daojin Technology Co., Ltd.)

**Procedure for generating biotin-mRBCs.** Allogeneic peripheral blood from young and healthy adult WT mice (4-6 weeks) was collected and washed with sterile PBS. Forty million RBCs were incubated with biotin-conjugated anti-Ter119 antibody (Cat# 13-5921-82, Thermo Fisher Scientific) diluted at the concentration of 10 μg·mL−1 in ice for 30 min and then washed and centrifuged at 200 for 10 min at 4 °C. Next, these biotin-RBCs were mixed with streptavidin-MNPs at various doses as indicated in the Results. Streptavidin-MNPs with an average size of 300 nm were produced by our collaborators at Westlake University (Dr. Botao Ji’s laboratory and Dr. Bobo Dang’s laboratory). The mixture of biotin-RBCs and the streptavidin-MNPs was kept on a shaker for another 20 min, and then the procedure of generating magnetized biotin-mRBCs was completed. Unmagnetized RBCs underwent the same treatment, but without streptavidin-MNPs. For each single step, the RBC quality was closely monitored by microscopic inspection. Once the erythrocytes that lost a biconcave shape accounted for more than 10%, we ceased the process and initiated a separate mRBC preparation.

**mRBC enrichment assay (mREA).** We created the following mREA means for statistical analysis of magnetization efficiency. A drop of 20 μL RBC-nanoparticle mixture, including unmagnetized RBCs and mRBCs, was placed on the foil with a rectangular magnet underneath (length x width x height = 5 cm x 2 cm x 0.5 cm). The center of the liquid drop was aligned with the edge of the magnet. Upon this magnetic gradient, mRBCs moved into a line that aligned with the edge of the magnet, whereas unmagnetized RBCs did not move (Fig. 1g, i-g). A similar principle, as described above, of quantifying RBC magnetization efficiency by mREA is to use a 1-well magnet frame (Cat# 18000, EasySep® Magnet), which is a piece of chemistry equipment consisting of a magnetic bead separation tool, to separate mRBCs and unmagnetized RBCs. We calculated the percentage of unmagnetized RBCs compared to the total number of RBCs we used. Finally, the mRBC percentage was determined by subtracting the unmagnetized RBC percentage. Thus, the magnetization efficiency was quantified.

**Scanning electron microscopy (SEM) for RBCs.** RBCs were fixed with 2.5% glutaraldehyde solution and then postfixed with 1% osmic acid for another 1.5 h. After each fixation, the samples were washed three times with 0.1 M, pH 7.4 PBS for 15 min. The samples were dehydrated in increasing concentrations of ethanol (30, 50, 70, 80, 90, 95, and 100%) for 15 min at each step. The samples were dried and coated with aurum before being viewed either with a Nova 450 or with a Zeiss Gemini 350 scanning electron microscope or a Nova 450 scanning electron microscope.

**Transmission electron microscopy (TEM).** The unmagnetized RBCs, mRBCs, and occluded distal MCAs were fixed as described above and contrasted with 2% uranyl acetate for 30 min at room temperature. The samples were gradually dehydrated by successive baths in 50%, 70%, 90%, and 100% ethanol. Next, the samples were washed twice with 100% acetone and embedded in resin Epon. Ultrathin sections at 60 nm thickness were obtained and deposited on copper grids to be examined with a Talos L120C G2 transmission electron microscope or Tecnai T10 transmission electron microscope.
Laser speckle contrast imaging (LSCI). The theories and techniques of LSCI have been documented in the literature. LSCI provides a measure of blood flow velocity and the extent of blockage of dynamic speckles caused by the motion of red blood cells through the vessels. Briefly, mice were anesthetized with isoflurane and placed under a RF-LIII device (RWD Life Sciences). The skull over both hemispheres was exposed by making an incision along the midline of the scalp. When a 785 nm laser was used to illuminate the brain, it produced a random interference pattern that represented blood flow in the form of a speckle pattern. Scattering light was detected by a charge-coupled device (CCD) camera, and the images were acquired by custom software from RWD Life Sciences Company. For each acquisition, a total of 160 images, each of which measured 2048 × 2048 pixels, were collected at 16 Hz.

TTC staining. At 10 h after focal arterial ischemic stroke, the brains of mouse pups were dissected. Whole brain or 1 mm thick transverse brain sections were incubated with 2% TTC (Cat# A61058-0005, BBI) dissolved in PBS at 37 °C for 15 min. Next, brain sections were fixed with 4% PFA overnight. Images were taken with a fluorescence stereo zoom microscope (Zeiss Axio Zoom V16).

Immunostaining. Briefly, mice were euthanized by injection of an overdose of pentobarbital sodium anaesthetic and fixed via heart perfusion with 4% paraformaldehyde (PFA). Brains were dissected out and postfixed in 4% PFA for another 2–4 h. Brain dehydration was achieved by soaking in sucrose gradients of 10%, 20%, and 30%. Brain cryosections at various thicknesses (20 μm) were incubated with 2% TTC (Cat# A61058-0005, BBI) dissolved in PBS at 37 °C for 15 min. Next, brain sections were fixed with 4% PFA overnight. Images were taken with a fluorescence stereo zoom microscope (Zeiss Axio Zoom V16).

MCA occlusion. Focal cerebral ischemia was induced by MCA occlusion as described previously. Briefly, adult mice (P60) were anesthetized with sodium pentobarbital (80 mg kg⁻¹) and placed on a heating pad to maintain body temperature. Under a dissecting microscope, the right common carotid artery and external carotid artery were exposed via an incision along the midline of the neck. A filament (Cat# 602356PK5, Docol) was inserted into the lumen of the carotid artery and advanced to the MCA. The occlusion lasted for 6 h. Mouse brains were dissected for hemorrhagic transformation evaluation.

Macrophage depletion with PLX5622. Macrophage depletion was achieved by administering the CSF1R inhibitor PLX 5622, as previously described. The pregnant dams starting at embryonic day 6 (E6) were fed a PLX 5622-formulated AIN-76A diet (1.2 g PLX 5622 per kilogram of diet, Plexxigon) ad libitum. Control dams received a control diet (AIN-76A, Research Diets) accordingly. After macrophage depletion, the diet was continuously provided to mother mice for an additional 2–3 days when perinatal arterial ischemic stroke was modeled.

Macrophage activity inhibition with minocycline. Minocycline (45 mg/kg, Cat# M9511, Sigma) was intraperitoneally injected into neonatal SD rats (P2 – P4) twice, at 1 h prior to and at 3 h after SIMPLE onset. At 6 h after SIMPLE, the rat pup brains were dissected for hemorrhagic transformation evaluation.

Statistics and Reproducibility. The quantified data in all figures were analyzed with GraphPad Prism 7.0 (La Jolla, CA, USA) and presented as the mean ± SEM with individual data points shown. Unpaired two-tailed Student’s t-test was used for assessing the statistical significance between two groups. Statistical significance was determined by calculation of p-value (*p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001; ns: not significant). The absolute values of effect sizes are reported in this study. The repetition of our data is independent biological replicates, the number of replicates for each experiment noted in the corresponding figure legend.

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

Data availability. The source data underlying the graphs and charts shown in the figures and tables are provided in Supplementary Data 1. All data generated or analyzed during this study are included in this published article (and its supplementary information file).

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In utero photothermohem. The principle of photothermohemorrhage induction was according to a previous report. However, we modified the procedure to induce brain damage in mouse embryos. Rose bengal (80 mg kg⁻¹, 350000 Sigma) was intraperitoneally injected into a female mouse at gestational day 18. A pregnant mouse was anesthetized with isoflurane and the embryo uterus gauzes were applied to avoid dryness of the uterus. Analgesia was provided by intraperitoneal injection of 0.2% meloxicam. At 24 h after photothermohemorrhage, the brains of the illuminated embryos were dissected out, and bright-field images were taken by a Stereo Zoom Microscope (Zeiss Axio Zoom. V16) to examine the hemorrhagic transformation. Sham control was conducted with the same procedure, except intraperitoneal injection of vehicle instead of rose bengal.
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