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Pathogenesis of Germinal Matrix Hemorrhage: Insights from Single-Cell Transcriptomics

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Abstract

The germinal matrix harbors neurogenic niches in the subpallium of the prenatal human brain that produce abundant GABAergic neurons. In preterm infants, the germinal matrix is particularly vulnerable to developing hemorrhage, which disrupts neurogenesis and causes severe neurodevelopmental sequelae. However, the disease mechanisms that promote germinal matrix hemorrhage remain unclear. Here, we review recent advances using single-cell transcriptomics to uncover novel mechanisms that govern neurogenesis and angiogenesis in the germinal matrix of the prenatal human brain. These approaches also reveal the critical role of immune–vascular interaction that promotes vascular morphogenesis in the germinal matrix and how proinflammatory factors from activated neutrophils and monocytes can disrupt this process, leading to hemorrhage. Collectively, these results reveal fundamental disease mechanisms and therapeutic interventions for germinal matrix hemorrhage.

Keywords

germinal matrix; preterm birth; angiogenesis; inflammation; immune–vascular interaction

1. INTRODUCTION

Preterm birth poses a major challenge to public health and is the most common cause of neonatal death in the United States and worldwide (1, 2). Defined as all births delivered at less than 37 completed gestational weeks (GWs), the preterm birth rate in the United States ranged from 9.57% in 2014 to 10.49% in 2021 (3). Despite small variations, these numbers have remained remarkably stable since 1990 (4). Of the nearly 4 million births recorded in 2021 in the United States, 312,197 (8.52%) were of low birth weight (LBW) (less than 2,500 g) and 50,567 (1.38%) were of very low birth weight (VLBW) (less than 1,500 g)

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(3). Preterm infants with LBW or VLBW are often delivered before 30–32 GWs and have a 20–25% chance of developing germinal matrix hemorrhage (GMH) due to rupture of blood vessels in this rapidly expanding region in the prenatal human brain. Because the germinal matrix is located immediately adjacent to the lateral ventricles, separated by just a thin layer of immature ependyma and an actively neurogenic ventricular zone, GMH has a high probability of extending into the ventricle to become intraventricular hemorrhage (IVH), leading to complications including ventriculomegaly and white matter injury (5–7). Consequently, preterm infants who survive GMH-IVH have a higher propensity to develop cerebral palsy, cognitive impairments, and neurodevelopmental disorders (6–9). Although GMH-IVH has been a known risk for preterm infants, there is very limited information regarding disease mechanism, biomarkers, or therapeutic interventions that can prevent or mitigate hemorrhage. These barriers collectively present an unmet and urgent need to identify the underlying etiologies and targeted therapies.

Several hypotheses have been proposed for the pathogenesis of GMH-IVH in preterm infants, including hemodynamic and respiratory disturbances, immaturity in coagulation profile and vascular development, infection and inflammation, and genetic factors (7, 10–12). However, the mechanism(s) that promote GMH-IVH remain largely unknown. Most preclinical models are designed to deal with the consequences of, rather than the cause(s) leading to, hemorrhage. However, the recent advent of single-cell transcriptomics in combination with the use of high-quality prenatal human brain tissues has uncovered novel and fundamental principles that govern human brain development. These approaches are pushing the boundaries of our understanding on the developmental biology of the germinal matrix and the disease mechanism(s) for GMH-IVH. Given the tremendous progress made in these areas during the past few years, we believe the time is right to review these latest discoveries on the molecular and cellular mechanisms that govern neurogenesis, angiogenesis, and immune–vascular interactions in the germinal matrix. With the implementation of multiomics approaches to identify disease-specific biomarkers in biofluids from control and GMH-IVH cases, we hope that these lines of research will reveal new understandings of disease mechanisms and new breakthroughs in therapeutic interventions for GMH-IVH.

2. GERMINAL MATRIX

2.1. Germinal Matrix: The Intersection of Neurogenesis and Angiogenesis in Prenatal Human Brain

Broadly speaking, the germinal matrix refers to the symmetric, highly neurogenic and angiogenic regions lining the lateral ventricles in the mammalian brain during embryonic development. In the prenatal human brain, neuroimaging studies using magnetic resonance imaging (MRI) reveal that the volume of the germinal matrix reaches its maximum at 23–26 GWs (13–15). As the brain development progresses, the volume of the germinal matrix reduces, but remnants of the germinal matrix can be identified in the ventricular zone (VZ) and subventricular zone (SVZ) along the lateral ventricles at 36 GWs to near term (16). In general, histopathological examinations in postmortem brain tissues from preterm infants support the growth trajectories of the germinal matrix. They also reveal that this

brain region harbors neurogenic and gliogenic niches, which produce abundant GABAergic neurons, excitatory neurons, oligodendroglia, and astrocytes that populate the cerebral cortex and neighboring structures, such as the striatum and thalamus (17–20). The similarities in the organization and gene expression profiles between the germinal matrix in the prenatal human brain and the ganglionic eminences (GEs) in the embryonic mouse brain provide a convenient tool to investigate the mechanisms that govern neurogenesis and angiogenesis in this brain region. However, it remains unclear whether the mechanisms identified in rodents are conserved in the prenatal human brain. The uncertainty between mouse models and human diseases, however, has been addressed by a series of studies that leverage single-cell transcriptomics and fluorescence-activated cell sorting (FACS) to directly interrogate neurogenesis and angiogenesis in the prenatal human brain. In the following sections, we review recent progress on neurogenesis and angiogenesis in the germinal matrix during the second and third trimesters. These studies provide new insights into mechanisms that are more relevant to prenatal human brain development in health and diseases.

2.2. Neurogenesis in the Germinal Matrix

The germinal matrix, also referred to as the GE, generates a large and diverse number of cortical interneurons as well as glial cells in the mammalian brain. On the basis of recent studies using single-cell transcriptomics, it is estimated that between 40 and 60 different subtypes of GABAergic interneurons exist in the adult mouse cortex (21). Similar subtypes of GABAergic interneurons have been detected in the human brain, although the relative abundance of each subtype and their distribution, morphology, and gene expression profiles may vary (22, 23). The complexity of the GABAergic interneuron subtypes and their unique positions in different brain regions raise the intriguing question of what neurogenesis process produces such a diverse population of neurons. In the embryonic mouse brain, neurogenic niches in GEs reside in the VZ, where an enriched population of radial glia function as neural stem cells to give rise to GABAergic neuroblasts that undergo tangential migration to populate the cerebral cortex (Figure 1a). It is now well established that in the embryonic mouse brain, the neurogenesis process in GEs begins as early as embryonic day 11.5 (E11.5) with regional specialization of the lateral ganglionic eminence (LGE) via the expression of transcription factors Pax6 in the VZ of LGE and Pou3f1 (Oct6 or SCIP) and Sp8 in the SVZ of LGE (24–28). In contrast, the medial ganglionic eminence (MGE) is defined by the expression of transcription factors Nkx2–1, Dlx1/2/5/6, ASCL1 (Mash1), and Nr2f1 (COUP-TF1) in the VZ and/or SVZ (Figure 1a). As is evident from loss-of-function analyses for these transcription factors, the genetic cascade established by the combinatorial expression of these transcription factors provides precise regulatory mechanisms to control interneuron development (29). In addition to the spatial regulation of the neurogenic niche for GABAergic interneurons in the LGE and MGE, temporal regulation of cell identity within GEs is defined by the emergence of distinct populations of progenitors that give rise to early-born and late-born interneurons from GEs, similar to the temporal control of the development of pyramidal neurons in the pallium (30–32).

Given the critical role of GABAergic interneurons in balancing the neural circuits, there has been tremendous interest in investigating the origin and developmental trajectories of GABAergic interneurons in the prenatal and early postnatal human brain. From more

clinical perspectives, it has been hypothesized that deficits in GABAergic interneurons likely contribute to the pathogenesis of neurodevelopmental and psychiatric disorders, including autism, epilepsy, cerebral palsy, and schizophrenia (33–35). Of note, epilepsy and cerebral palsy are common neurological sequalae in preterm infants who survive GMH (8, 9). Compared with that of rodents, the human brain is gyrencephalic and therefore requires a much larger number of cortical interneurons to populate the vastly expanded cerebral cortex. Since GEs in the embryonic mouse brain are transient, this raises the intriguing question as to how human GEs can produce sufficient interneurons for all brain regions. Using retroviral labeling and live imaging in organotypic slice cultures from the frontal cortex of prenatal human brain at 10–25 GWs, Letinic and colleagues (36) identified two distinct sources of GABAergic interneurons, one from the progenitors in the pallium (65%) and the other from the progenitors in GEs (35%). Consistent with these results, lentivirus-mediated barcoding and single-cell RNA-sequencing (scRNA-seq) of neural progenitors and mature neurons in the cerebral cortex from prenatal human brains at 15–18 GWs confirmed that neural progenitors within the cerebral cortex can indeed produce a small number (~25%) of GABAergic interneurons (37). While these results support the idea that the developing cerebral cortex can indeed produce GABAergic interneurons, the magnitude of these locally produced GABAergic interneurons seems small.

A major breakthrough in understanding the origin of GABAergic interneurons in the human brain came from two landmark studies that revealed the remarkable similarities in the domain organization and transcriptional regulation between the human and mouse LGE, MGE, and CGE, defined by region and cell type–specific transcription factors that are highly conserved across species (38–40) (Figure 1b). These studies revealed that GEs in the prenatal human brain from the late first trimester to the mid to late second trimester (12–24 GWs) show drastic expansion in size during this period and contain a massive number of SOX6, COUP-TFII, and SP8-expressing progenitors that are committed to the GABAergic fate. One of these studies combines BrdU labeling with cell type–specific markers and concludes that the human GEs are the major production sites of human GABAergic interneurons (38). In support of the conclusions from these two studies, scRNA-seq analyses using GEs from prenatal human brains and embryonic mouse brains at 9–18 GWs confirm the remarkable conservation of spatial and temporal transcriptional programs that regulate the development of GABAergic interneurons in human and mice (41).

So, what makes the human GE unique beyond its size and persistent presence during prenatal brain development? One intriguing feature of the human MGE noted in previous studies is that it contains distinct clusters of radial glial fibers and neural progenitors not seen in mouse GEs (38). However, the contents inside these clusters and how their organization contributes to the production of GABAergic interneurons remain unclear. In-depth histopathology characterizations of the LGE and MGE in prenatal human brains from 14–39 GWs using markers for neural progenitors and young neurons show that these clusters in the human MGE contain aggregates of DCX^+ neuroblasts, called DCX^+ cell–enriched nests (DENs) (42). There are several features suggesting that DENs are human brain–specific structures that can produce a large number of GABAergic interneurons throughout most, if not all, gestational ages. First, DENs are located in the outer subventricular zone (oSVZ) of the human MGE, where they are surrounded by

Nestin$^+$;SOX2$^+$ progenitors that extend from the inner subventricular zone (iSVZ) (Figure 1b). In contrast, the DCX$^+$ cells in the neighboring LGE are loosely organized. Remarkably, these DENs persist in the human MGE from the second to the third trimester. Even at full term (38–40 GWs), several DENs can still be identified in the remnants of the MGE (42). Second, compared with DCX$^+$ cells in the human LGE, ~20–25% of DCX$^+$ cells within DENs in the MGE are MKI-67$^+$, suggesting that they are actively proliferating. Remarkably, similar percentages of DCX$^+$ cells in DENs persist from the second trimester to the third trimester. In contrast, the number of MKI-67$^+$;DCX$^+$ cells in the LGE reduces drastically after 17 GWs, remains low from 22–27 GWs, and becomes undetectable from 34 GWs to perinatal stages (42). Finally, both transcriptomic and ultrastructural analyses show that the DCX$^+$ young neuroblasts within DENs exhibit unique cell adhesion properties that support the formation and maintenance of DENs. Specifically, DCX$^+$ cells inside DENs express cell adhesion-related nonclustered protocadherin-19 (PCDH19), whereas Nestin$^+$ progenitors surrounding DENs express PCDH10. In support of this unique property, when progenitor cells from the human MGE are transplanted to neonatal mouse brains, they can reorganize themselves to form DEN-like structures, disperse and migrate long distances, integrate, and mature into functional interneurons in mouse brains (42). Consistent with these properties, in the perinatal and early postnatal human brain, dense populations and diverse subtypes of GABAergic interneurons follow extensive chain migration from the VZ/SVZ to the periventricular white matter, forming a distinct structure called Arc, which can be visualized at T2 signal intensity in MRI (43).

Taken together, the organization of DENs in the human MGE and the persistent and highly proliferative properties of DCX$^+$ young GABAergic neuroblasts within DENs provide critical insights into how the human brain evolves to have sustained production of GABAergic interneurons. These results also shed light on how diseases in preterm infants, such as GMH, could interrupt this process and have permanent and devastating consequences on the production of GABAergic interneurons. On a positive note, these results offer the exciting prospect of transplanting human GABAergic progenitors, newly differentiated GABAergic neuroblasts, or mature GABAergic neurons as potential therapeutics for preterm infants that survive GMH (44–46).

2.3. Angiogenesis in the Germinal Matrix

The vasculature in the adult brain provides efficient blood supply to meet the high demand for oxygen and nutrients required by energy-intensive cognitive functions. Unlike the endothelial cells in peripheral organs, brain endothelial cells exhibit unique features, such as the absence of fenestration, the presence of specialized tight junctions, markedly reduced transcytosis, and extremely low permeability. Together with pericytes and astrocytes, brain-specific endothelial cells contribute to the formation of the blood–brain barrier (BBB) (47, 48). Using the embryonic mouse brain as a model system, several previous studies show that Wnt, Hedgehog, retinoic acid, and integrin-mediated signaling pathways are required for the early development of the vascular network and the initial formation of the BBB (49–55). However, it remains unclear whether the same signaling mechanisms are required to regulate vascular development in the prenatal human brain. It is also unclear why blood vessels in the germinal matrix are particularly vulnerable to rupture and develop hemorrhage

during the second trimester. Several mouse models exhibit spontaneous hemorrhage during the embryonic brain development (56–61). However, cerebral hemorrhage in these mouse models appears to be more widespread and not specific to the GEs. Hence, its relevance to germinal matrix hemorrhage in preterm infants remains unclear.

Notwithstanding the aforementioned caveats, it is important to review a large body of literature on the intersection of angiogenesis and neurogenesis in the GEs of the embryonic mouse brain. At E12.5, endothelial cells in the VZ and SVZ of GEs form an extensive vascular plexus that is different from the cortical plate where blood vessels are arranged perpendicular to the pia (62, 63). Despite the presence of these vascular plexuses, the VZ and SVZ of GEs in the embryonic mouse brain are generally hypoxic, with only 1–8% oxygen content (64, 65). This hypoxic microenvironment has been shown to promote neural stem cell differentiation through hypoxia-inducing factor-1α (HIF-1α), a transcription factor that activates angiogenic genes, such as vascular endothelial growth factor (*VEGF*) (65). The vascular plexuses within the hypoxic regions in the VZ and SVZ harbor a subtype of endothelial cells, called the tip cells, which make direct contact with the dividing neural progenitors to promote neurogenesis via a VEGF-mediated mechanism (62, 66). Disrupting the radial glia anchorage and contact with the periventricular vessels decreases radial glial progenitors and the production of GABAergic interneurons (67).

The advent of single-cell transcriptomics enables more in-depth investigations of the mouse brain vasculature at the molecular and cellular level. This is further assisted by using reporter mice for the visualization of vascular cells, such as *Tie2-GFP* for endothelial cells (68), *Pdgfrb*-GFP;*CSpg4*-DsRed for mural cells (69), *Tagln*-Cre;R26-stop-tdTomato for smooth muscle cells, and *Pdgfra*-H2BGFP for perivascular cells (70). These genetic tools increase the specificity to identify and characterize different vascular cell subtypes in the early postnatal and adult mouse brain. For instance, these results show that both mitotic endothelial cells and tip cells are identified in the mouse brain at postnatal day 7 (P7) (68) but are not detected in the adult mouse brain (70). These results also uncover a gradual and zonal distribution of transcriptomic signatures from arterial to capillary to venous endothelial cells along the arteriovenous axis (70, 71). Despite these advances, very limited data are available regarding the transcriptomic characteristics of vascular cells in the embryonic mouse brain. Compared with the wealth of literature on the mouse brain vasculature, much less is known about angiogenesis in the germinal matrix of the prenatal human brain. Historically, the vasculature within the germinal matrix in the second-trimester human brain have been described as primitive and thus could not be classified distinctly as arterioles, venules, or capillaries. Results from MRI and cranial ultrasound show that the arterial supply to the germinal matrix comes from multiple sources, including the Heubner's artery from the anterior cerebral artery and the lenticulostriate branches of the middle cerebral artery. In addition, the germinal matrix contains the terminal vein, which consists of the medullary, choroid, and thalamostriate branches. Together, the terminal zone of the arterial and venous vasculature creates a vascular end zone that has been postulated to be vulnerable to vascular injury.

Several previous studies have examined the developing vasculature in the prenatal human brain and shown that vascular density is higher in the germinal matrix compared with that

in the gray matter and white matter (72–74). However, there are many critical barriers and unanswered questions that preclude in-depth understanding of the region-specific vulnerability of the vasculature in the germinal matrix. Using a large collection of prenatal human brains from the second and third trimesters, Crouch and colleagues (75) show that vascular density in the germinal matrix is significantly higher in the VZ and SVZ (zone 1), which is within 100 μm from the ventricular surface (Figure 2a). Similar to the VZ/SVZ in the GEs of the embryonic mouse brain, the VZ and SVZ in the GEs of the second-trimester prenatal human brain contain an enriched population of Nestin$^+$ and PDGFR-β$^+$ neural progenitors (zone 1). However, unlike the embryonic mouse brain, GEs in the prenatal human brain undergo significant expansions in the region between 100 μm and 1 mm from the VZ/SVZ (zone 2), which now contains abundant immature neuroblasts that are organized in linear patterns in the LGE and clustered into DENs in the MGE (Figure 1). Because of the tremendous expansion of young proliferating SOX2$^+$ neural progenitors and DCX$^+$ neuroblasts, this region is also called the oSVZ (42) (Figure 1c). Finally, zone 3, which is defined as 500 μm distal to zone 2, contains streams of migrating neuroblasts and glial progenitors that exit GEs into neighboring structures (75) (Figure 1c).

There are several distinct features of the nascent vasculature in the germinal matrix in the second-trimester human brain. First, at the early stages of the second trimester (14–17 GWs), blood vessels within the VZ/SVZ neurogenic niches (zone 1) form an interconnected network of vascular plexuses and have a higher density of MKI-67$^+$;CD31$^+$ proliferating endothelial cells (Figure 2b). These vascular plexuses are covered by MKI-67$^+$;NG2$^+$ mural cells. As brain development progresses, the density of proliferating vascular cells reduces significantly at 21–25 GWs. Second, in addition to increased cell proliferation, the nascent vasculature in the second trimester displays angiogenic properties, such as increased branch points, in the VZ/SVZ (75). Third, many CD31$^+$ endothelial cells in the vascular plexus in the VZ/SVZ in zone 1 contain abundant filopodia, many interdigitating with NG2$^+$ mural cells and making direct contacts with PDGFR-β$^+$ radial glia fibers (Figure 2b). Finally, transmission electron microscopy of the blood vessels in the VZ, SVZ, and oSVZ in the MGE at 17 and 23 GWs shows endothelial cells exhibiting characteristic features, including junctional complexes and Weibel-Palade bodies, and many are covered by mural cells. At 17 GWs, most blood vessels contain discontinuous basal lamina, a feature of an immature BBB. Many of these blood vessels have an absence of endothelial lumen, suggesting they are young “sprouts,” which become perfused starting at 23 GWs. Collectively, these results reveal the dynamic angiogenesis process in the germinal matrix of the second-trimester human brain where nascent vasculature in this brain region interact with neural progenitors. These results are reminiscent of those in the GEs of the embryonic mouse brain, suggesting that angiogenesis and neurogenesis could have mutual interactions in the germinal matrix.

Although histological characterizations provide exquisite details about the angiogenesis process in the germinal matrix, they offer limited information regarding the mechanisms that promote angiogenesis in this brain region. The relatively low abundance of vascular cells compared with that of neurons and glia in the mammalian brain further makes it challenging to characterize the molecular properties of these cells. This obstacle has been overcome by an integrated FACS-based approach that enriches vascular cells from the germinal matrix and cortex in the second-trimester human brain, followed by single-cell transcriptomics

using these enriched vascular cells to provide new insights into age-dependent and region-specific properties of these cells during human brain development (75, 76). Specifically, CD31 and CD13 (ANPEP) are used as respective markers for endothelial cells and mural cells in FACS to isolate these cells for scRNA-seq. Clustering of these vascular cells on the basis of their transcriptomic profiles shows that, as early as the second trimester, both the germinal matrix and the cortex in the prenatal human brain contain an ensemble of endothelial cells, including mitotic endothelial cells, tip cells, arterial endothelial cells, venous endothelial cells and capillary endothelial cells, and mural cells, including smooth muscle cells, pericytes, and mitotic mural cells (Figure 2). In addition to endothelial cells and mural cells, there is a distinct and significant population of fibroblasts in prenatal human brain at 15 and 23 GWs.

When combined with the bioinformatics tool RNA velocity, these approaches further delineate the developmental trajectory of the endothelial cells. At 15 GWs, the mitotic endothelial cells are predicted to give rise to venous endothelial cells, which progress to become capillary endothelial cells and tip cells and finally differentiate into arterial endothelial cells. Similar analyses on the 15-GW mural cell clusters predict that the mitotic mural cells give rise to smooth muscle cells, fibroblasts, and a very small number of pericytes. As the brain matures at 23 GWs, the composition of endothelial cells shows more arterial endothelial cells and fewer capillary endothelial cells and tip cells, whereas the composition of mural cells at 23 GWs shows fewer mitotic mural cells and fibroblasts but a significant expansion of pericytes. Analyses of the differentially expressed genes reveal age-dependent bioenergetic properties in the endothelial cells at 15–18 and 20–23 GWs. The younger endothelial cells (15–18 GWs) are more enriched with mitochondrial genes related to oxidative phosphorylation and respiratory chain complex than the older endothelial cells (20–23 GWs). Interestingly, ultrastructural analyses show that young endothelial cells (17 GWs) have smaller mitochondrial mass when compared with those from 21 GWs. Seahorse assays show that the young endothelial cells (16 GWs) have a greater reserve capacity for respiration and are less dependent on glycolysis for energy production than older endothelial cells (24 GWs).

Further analyses of the scRNA-seq data reveal the signaling pathways that endothelial cells and mural cells employ to promote angiogenesis. Among the most overrepresented signaling pathways are ligand–receptor pairs involved in the formation of the extracellular matrix (ECM), including collagen, laminin, and fibronectin (Figure 2c). Another highly enriched signaling pathway involves growth factor midkine (MDK), which promotes hypoxia-induced angiogenesis as well as the proliferation of neural progenitors (77). In support of these results, immunostains and scRNA-seq show the expression of MDK receptors integrin B1 (ITGB1) and syndecan 2 (SDC2) in endothelial and mural cells during the second trimester in the prenatal human brain (Figure 2c). Finally, transplantation of vascular cells isolated from the second-trimester human brain into induced pluripotent stem cell–derived cortical organoids significantly increases the number of $NeuN^+$ and $RBFOX3^+$ ($CTIP2^+$) neurons and reduces cellular stress within the cortical organoids. Interestingly, within the highly neurogenic environment in the cortical organoids, most transplanted endothelial cells differentiate into tip cells, whereas as transplanted mural cells differentiate into smooth muscle cells.

Collectively, the single-cell transcriptomic data on vascular cells from the second-trimester prenatal human brain unravel the complexities of vascular development and highlight the critical mutual interactions of angiogenesis and neurogenesis at this stage during human brain development. These data also provide an important blueprint for many future studies focusing on vascular development and maturation in the human brain in the third trimester, the perinatal and early postnatal stages, and the aging process (75). These future studies will provide a more complete understanding of the molecular and cellular mechanisms that govern the development of brain vasculature in health and diseases (78). There are, however, a few caveats and unanswered questions that still need to be addressed for angiogenesis in the second trimester. Most notably, despite the differences in the vascular density between the VZ and SVZ in the pallium and subpallium during the second trimester, these single-cell transcriptomic data do not show any definitive differences in endothelial or mural cells from these regions in morphology, transcriptomes, or subtype classifications, suggesting that region-specific factors from nonvascular cells may dictate the timing and the extent of angiogenesis in different parts of the developing brain.

3. GERMINAL MATRIX HEMORRHAGE

3.1. Germinal Matrix Hemorrhage: Definition and Prevalence

Globally, an estimated 15 million babies are born prematurely before 37 GWs every year (1, 2). Despite the concerted efforts to prevent preterm birth, its prevalence has remained stable over the past decades. For preterm infants born before 30 GWs, 20–40% develop GMH, which leads to devastating neurodevelopmental sequelae (5, 9, 79). GMH and IVH are large medical burdens, affecting 12,000 premature infants every year in the United States (12). In premature infants weighing 500–750 g, GMH-IVH occurs at a staggering rate of 45%. While more than 80% of infants with hemorrhage limited to the germinal matrix survive, the rate drastically drops to 50% in those with IVH (5). Consequences of GMH include cerebral edema, leukocyte infiltration, and calcification in the germinal matrix and the periventricular white matter. Longer-term consequences include hydrocephalus, cerebral palsy, and epilepsy.

Since preterm infants have a higher propensity to develop a myriad of comorbidities, the etiology for GMH is most likely multifactorial. Indeed, possible mechanisms for GMH include immaturity of the brain vasculature, incomplete development of the BBB, hypoxic-ischemic injury, hemodynamic instability, and inflammation. Once hemorrhage occurs, the influx of peripheral immune cells and the release of blood-related factors produce mediators of toxic byproducts, including thrombin, hemoglobin, iron, and complement proteins, which can perpetuate a vicious cycle that negatively impacts the proliferation of neural progenitors (19) and further aggravates hemorrhage and tissue damage (5). For instance, thrombin is a blood coagulation enzyme that can increase inflammation, leading to apoptosis in periventricular neurons and ventricular dilation via increased flow of cerebrospinal fluid from the choroid plexus. Hemoglobin and iron released from lysed red blood cells are cytotoxic, resulting in oxidative damage of neurons and glia. Complements such as C3a can induce neutrophil infiltration and expand infarct size. Finally, degradation of hemoglobin produces free iron that can generate reactive oxygen species, which can lead

to broader damage to lipids, proteins, and nucleic acids. Due to its close proximity to the periventricular white matter, GMH can have negative impacts on the migration of neurons and glia cells. In the following sections, we review the current literature on experimental models of GMH, emphasizing recent discoveries using single-cell transcriptomics to reveal the role of immune cells in the development of blood vessels in the prenatal human brain and how aberrant inflammation promotes hemorrhage in the germinal matrix.

3.2. Genetic Models of Embryonic Brain Hemorrhage

There are few animal models where spontaneous hemorrhage occurs in the GEs during embryonic brain development. Of these genetically engineered mouse models that do produce brain hemorrhage during embryonic development, all support that perturbations to angiogenesis and/or immaturity in the BBB could increase the risk of developing cerebral hemorrhage. Most of these studies highlight the critical role of cell–cell interactions and ligand–receptor-mediated signaling pathways in promoting angiogenesis in embryonic brain development. For instance, canonical Wnt-β-catenin signaling, mediated by Wnt7a and Wnt7b, is required for the proper growth of endothelial cells (50, 51). Mice lacking *Wnt7a* and *Wnt7b* exhibit profound hemorrhage phenotypes in the embryonic brain and spinal cord (50). Integrins are transmembrane heterodimeric receptors, consisting of α and β subunits, that link the ECM and the actin cytoskeleton in the mammalian cells (60). Several studies show that integrins alphav (αv) and beta8 (β8) are critical to maintain vascular growth and integrity in GEs at E12.5–13.5 in the embryonic mouse brain. Mice lacking αv or β8 subunits show no defects in the initial vascular growth in GEs, but starting from E12.5, blood vessels in αvβ8 mutant mice exhibit increases in vascular sprouting, branching, and proliferation, leading to vascular dysplasia and hemorrhage (56, 61, 80–83). The mechanism that promotes hemorrhage phenotypes in integrin β8 mutants appears to be mediated by the essential role of β8 in activating TGFβ1-TGFBR2 signaling in the endothelial cells but is not related to BBB disruption or the lack of pericyte coverage (56). Parenthetically, mice with loss-of-function mutations in platelet-derived growth factor-B/PDGF receptor-β (PDGF-B/PDGFRβ) signaling lack pericyte coverage in the nascent brain vasculature and have defective BBBs but show no evidence of hemorrhage (56, 84, 85). Another line of evidence supporting the idea that perturbations to angiogenesis and vascular maturation in GEs could contribute to GMH is that of the inducible transgenic mouse model where VEGF expression can be temporarily upregulated in the VZ during embryonic development. This approach produces widespread hemorrhage affecting the VZ in both GEs and the cortical plate (59). The relevance of these results to GMH in preterm infants is underscored by the findings that VEGF expression is more abundant in the germinal matrix compared with that in the subcortical white matter and cortical plate and that VEGFR2 inhibitor ZD6474 can reduce the incidence of GMH in premature rabbit pups (86).

Aside from integrin-mediated angiogenesis, disrupting the signaling pathways regulated by G protein–coupled receptors (GPCRs) sphingosine 1-phosphate receptor (S1PR1) and GPR124 could disrupt angiogenesis in the embryonic mouse brain, leading to hemorrhage. The lysophospholipid sphingosine 1-phosphate (S1P) is a platelet-derived metabolic product of cell membrane sphingolipids enriched in the circulating fluids (87). By binding to extracellular chaperones, S1P establishes a gradient and interacts with S1PRs to regulate

diverse functions, including vascular development and immune cell trafficking (88). Within the GEs of the embryonic mouse brain, S1PR1-mediated endothelial cell sprouting of filopodia regulates vascular morphogenesis and proliferation of neural progenitors in the VZ (66). Although loss of S1PR1 in endothelial cells does not lead to overt hemorrhage phenotype in GEs, it promotes the extravasation of $IBA1^+$ cells into GEs and upregulates the expression of CD16 and CXCL16 in these extravascular immune cells (89). Similar to S1PR1, another GPCR, GPR124, also known as adenosine GPCR A2 (ADGRA2) or tumor endothelial marker 5 (TEM5), is highly expressed in brain endothelial cells. Compared with the phenotypes in endothelial cell–specific S1PR1 conditional mutant mice, mice with complete or endothelial cell–specific GPR124 deletion show profound angiogenic sprouting defects in the vascular plexus near the VZ and SVZ, leading to diffuse hemorrhage in the developing forebrain (58). Interestingly, GPR124 loss-of-function phenotypes in the endothelial cells can be rescued by restoring the canonical Wnt-β-catenin signaling (57, 90).

Like integrins, collagens are important components in the ECM and have well-documented roles in regulating angiogenesis. Mutations in the human *COL4A1* gene, which encodes procollagen type IV alpha 1 subunit, lead to a rare neurodevelopmental disease called porencephaly or cystic encephalomalacia with the formation of fluid-filled cysts involving the cerebral cortex and subcortical white matter (91). Mice that carry the homozygous allele of the semidominant mutation in the *Col4a1* gene ($Col4a1^{\Delta ex40/\Delta ex40}$) die around midgestational age, whereas ~50% of heterozygous ($Col4a1^{+/\Delta ex40}$) mice develop spontaneous hemorrhage in the cerebral cortex due to vascular defects caused by inhibition of secretion in both mutant and normal collagen (91). However, the pattern and the timing of brain hemorrhage in $Col4a1^{+/\Delta ex40}$ mutant mice are very different from those in mutant mice where the Wnt, integrin, or GPCR signaling pathway is disrupted. Specifically, the hemorrhage in $Col4a1^{+/\Delta ex40}$ mutant mice occurs at P0 and primarily affects the cerebral cortex due to inconsistent and highly variable basement membranes in the cerebral vasculature.

Collectively, the brain hemorrhage phenotypes in the mutant mice discussed in this section reveal the critical roles of the Wnt, integrin, sphingosine, GPCR, and collagen pathways in the development of the cerebral vasculature during embryonic and early postnatal life. However, these models do not include many key features in GMH-IVH, suggesting that there are critical species-specific differences in cerebrovascular development in mice and humans.

3.3. Other Experimental Models of Germinal Matrix Hemorrhage/Intraventricular Hemorrhage

The essential role of collagens in angiogenesis and the maintenance of cerebral vasculature have provided a convenient target to disrupt vasculature in the embryonic and perinatal mouse brain and to model the sequalae of hemorrhage. To this end, several studies use stereotactic injection of collagenase into the VZ/SVZ adjacent to the caudate-putamen in neonatal rats or mice to induce hemorrhage, determine the neurological deficits, and examine the efficacy of therapeutic interventions (92–95). Another model is the glycerol-induced GMH-IVH in preterm rabbit pups (96). This model has been thought to be closer

to preterm human infants because of the neuroanatomical similarities in the germinal matrix in preterm rabbits and the prenatal human brain. Moreover, approximately 12.5–25% of preterm rabbits delivered at 29 gestational days (full term is 31–32 days) will develop spontaneous hemorrhage in the germinal matrix. The incidence of GMH-IVH can be further enhanced by intraperitoneal injection of glycerol. In addition to collagenase and glycerol, stereotactic injection of blood into the mouse neonatal GEs also captures aspects of GMH. Intracranial injection of autologous blood collected from the tail tip of P0 mouse pups led to ventricular dilation within 4 days, but parenchymal disruption was limited to the germinal zone (97). This injury leads to dysregulated neuronal and oligodendroglial differentiations. A recent study confirmed that intracerebroventricular injection of age-matched blood at E14.5 led to robust ventriculomegaly (98).

While the results from the genetic and other experimentally induced models offer insights into the pathophysiology of and potential therapeutic targets for GMH-IVH, it remains unclear how relevant these models are to GMH in preterm infants. As mentioned above, the hemorrhage phenotypes in these genetic models tend to be widespread compared with the more neuroanatomically restricted nature of GMH. Furthermore, on the basis of the single-cell transcriptomic data of vascular cells from the second-trimester prenatal human brain, the top signaling pathways that promote the development and interactions between endothelial cells and mural cells are overrepresented by mechanisms related to collagen, laminin, MDK, Notch, and CD99, with much smaller contributions from angiogenic factors, such as VEGF, ANGPT, or PDGF (75). It remains unclear whether genetic or other experimentally induced animal models of brain hemorrhage capture alterations in these signaling pathways.

3.4. Immune-Vascular Interface in the Germinal Matrix

As described in the previous sections, single-cell transcriptomics reveals that the germinal matrix in the second trimester exhibits active angiogenesis driven by diverse endothelial and mural cells that utilize a repertoire of signaling mechanisms to facilitate cell–cell communication and maturation of the nascent vasculature (75, 78). However, the mechanism(s) rendering nascent vasculature in the germinal matrix more vulnerable to rupture remain unclear. Interestingly, a large retrospective study based on more than 3 million mother–infant dyads shows that maternal immune activation significantly increases the risk of brain hemorrhage and neurological comorbidities (99). Although bacterial infection can activate the microglia inflammasome pathway in the neonatal brain (100), it remains unclear how inflammation disrupts the homeostatic interaction between microglia and blood vessels in the prenatal human brain in a region-specific and stage-dependent manner. It is also unclear which immune cell subtypes and what proinflammatory factors produced by these cells could contribute to GMH (101, 102). Nonetheless, these results raise the intriguing possibility that aberrant activation of immune cells can adversely affect angiogenesis and BBB integrity in preterm infants.

Microglia are brain-resident macrophages implicated in many functions during brain development, including synapse formation, neurogenesis, and angiogenesis (103, 104). Our understanding of microglia is further expanded by fate-mapping and scRNA-seq to elucidate the origins of microglia, which are not born in the brain parenchyma. Instead, they

originate from erythromyeloid progenitors (c-Kit^{+};CD45^{-};F4/80^{-};CD206^{-}) in the yolk sac and colonize the mouse brain between E8.5 and E9.5, as extensively proven using lineage tracing, parabiosis, and neonatal bone marrow transplant studies. In the developing human brain, microglia are observed at relatively earlier developmental stages at 4.5–5.5 GWs close to the meninges and the choroid plexus and in the ventricular zone (105). During the second trimester, more microglia with diverse morphology and gene expression profiles can be identified in the telencephalon (106–108). It is now recognized that parenchymal microglia are not the only immune cells in the brain. For instance, there are several well-documented populations of border-associated macrophages, including macrophages in the choroid plexus, meninges, and perivascular spaces. Focusing on perivascular macrophages, they differ from homeostatic microglia in their origins, marker genes/proteins, and milieu during development. In mice, perivascular macrophages are derived from perinatal meningeal macrophages shortly after birth, and their differentiation depends on integrins and the presence of arterial vascular smooth muscle cells (109). According to studies in mice, perivascular macrophages can be distinguished from microglia using markers such as *Mrc1, Lyve1, Cd163*, CD45^{high}, and MHC-II^{high}. In contrast, microglia express markers such as *Tmem119, Hexb, P2ry12, Sall1*, CD45^{low}, and MHC-II^{low} (103, 109). Finally, perivascular macrophages and microglia occupy different niches during development. CD206^{+} perivascular macrophages are rarely found in the perivascular compartment until P10 in mice and 41 GWs in humans (109). Instead, the perivascular compartment is occupied by P2RY12^{+} and CD206^{-} microglia in the embryonic brain.

Since microglia appear before vascularization in the brain and are tightly associated with blood vessels during development, they are hypothesized to play active roles in angiogenesis. In mice, early postnatal microglia at P7–P14 are recruited to blood vessel areas void of astrocytic end feet and remain highly motile (110). These microglia preferentially contact PDGFRβ^{+} SMA^{-} capillaries. In PU.1 and CSF1R mutant mice with microglia and macrophage depletion, the complexity of vasculature is significantly reduced (111). Specifically, microglia and macrophage enable endothelial tip cell anastomosis to form branch points downstream of VEGF in the embryonic mouse hindbrain. In another model with monocyte depletion, vascular complexity remains normal, suggesting the specific role of microglia and macrophages in endothelial branch point formation. Live imaging in the developing zebrafish showed that Pu.1^{+} macrophages migrate to tip cells to facilitate their fusion (111).

Given the intriguing results on the role of microglia in the embryonic mouse brain, several studies have leveraged FACS and single-cell transcriptomics to characterize CD45^{int};CD11b^{+};DRAQ5^{+} cells from the human brain at 9–18 GWs (112) and CD45^{lo};CD11b^{+};CX3CR1^{hi};CD64^{+} cells from the brain stem and cortex at 10–12 GWs (113). While these studies reveal the transcriptomic and epigenomic profiles of microglia and myeloid cells in the prenatal human brain as early as the first and second trimesters, the exact role of these immune cells in angiogenesis remains unclear. To address this, Chen and colleagues (89) performed histopathology analyses on how immune cells interact with blood vessels in the prenatal human brain in the second trimester (14–23 GWs). These results show that a higher density of immune cells is associated with the vascular plexus in the germinal matrix than with the capillaries in the cortical plate. Most of the

immune cells in the germinal matrix are inside the vasculature, attached to the outer walls of the vascular plexuses, or intermingled with neural progenitors in the SVZ or oSVZ. Interestingly, similar results are identified in the GEs of the embryonic mouse brain, where microglia are associated with nascent vasculatures. Indeed, genetic or pharmacological ablation of myeloid cells using CSF1R inhibitor PLX5622 in the embryonic mouse brain results in region-specific and age-dependent defects in the formation of the vascular network in the GE but not in the cortical plate or in the VZ/SVZ of the pallium (89). Consistent with these results, bulk RNA-seq shows that $CD45^+$ immune cells from human brains at 20–23 GWs exhibit transcriptomic features that are proangiogenic. In addition, $CD45^+$ immune cells from 20–23 GWs are more effective in promoting vascular morphogenesis in human umbilical vein endothelial cells (HUVECs) in 3D Matrigel than those from 14–19 GWs (Figure 3a).

To further investigate how immune cells regulate angiogenesis in a brain region–specific manner, Chen and colleagues (89) performed scRNA-seq on FACS-isolated $CD45^+$ cells from the germinal matrix and the cortical plate in second-trimester (15–23 GWs) control prenatal human brain tissues. This approach identifies 11 subtypes of immune cells, including homeostatic microglia, white matter–associated microglia, proliferating microglia, vasculature-associated microglia (VAM), monocytes, human leukocyte antigen (HLA^+) myeloid cells, neuron-associated microglia, T cells, and B cells (89). The diversity of these immune cells, confirmed by high-dimensional flow cytometry using 16 different cell surface markers, is much more comprehensive compared with those reported in other studies (112, 113). Moreover, these results reveal region-specific distribution of immune cells, with VAM, HLA^+ myeloid cells, and monocytes shown to be more enriched in the germinal matrix. Furthermore, the bioinformatic tools CellPhoneDB and NicheNet predict that the most prominent ligand–target gene link is integrin β2 (ITGB2) from monocytes and intercellular adhesion molecule 2 (ICAM2) receptor from endothelial cells (114). Additional ligands employed by HLA^+ myeloid cells and monocytes to interact with endothelial cells include VEGFB, IGF1, TGFB1, TNF, CXCL16, and IL1B (89). Together, these results provide direct evidence as to how microglia and other key immune cells can communicate with vascular cells to promote angiogenesis in the germinal matrix in the second trimester (Figure 3a). Compared with previously published datasets from prenatal human and mouse brains (112, 113, 115, 116), results from this study not only capture the developmental trajectory of $CD45^+$ cells transitioning from intravascular space to parenchymal microglia but also provide insights into the molecular mechanisms employed by subsets of $CD45^+$ cells to promote angiogenesis in the GEs (Figure 3a). These datasets provide valuable resources for future studies to investigate how these immune cells might impact other critical aspects of brain development, including myelination, neuronal migration, and differentiation.

3.5. Proinflammatory Milieu Contributes to Germinal Matrix Hemorrhage

Past work has examined how systemic inflammation affects BBB permeability in the adult brain. In lipopolysaccharide (LPS)-injected mice, an increased number of microglia expressing *Cldn5* migrate to blood vessels via CCR5 (117). LPS exposure also increases the permeability of the BBB. Intriguingly, microglia seem to apply a two-stage effect on the integrity of the BBB. Initially, vasculature-associated microglia maintain the BBB. However,

during sustained inflammation, these microglia become phagocytic and consume astrocytic end feet, impairing BBB function (117). Considering these results, Chen and colleagues (89) performed scRNA-seq using FACS-isolated $CD45^+$ cells in the GE and cortex from two GMH cases at 24–25 GWs and two age-matched controls. This approach revealed a drastic reduction of homeostatic microglia in the germinal matrix of preterm infants with GMH. In addition, $CD45^+$ cells from preterm infants with GMH harbor activated neutrophils and monocytes, indicating potential changes in homeostatic immune–vascular interactions. At least two mechanisms could explain how perturbations to immune–vascular interactions contribute to GMH. First, the significantly expanded neutrophils produce bactericidal factors, including elastase (ELANE) and azurocidin 1 (AZU1), that could disrupt the integrity of nascent vasculature in the germinal matrix of GMH cases (Figure 3b). In support of this mechanism, AZU1 has been shown to increase vascular endothelial permeability and recruit monocytes during inflammation (118). Furthermore, histopathological examinations reveal many $ELANE^+$ neutrophils traveling across capillaries into the brain parenchyma. Finally, engineered microvessels and 3D Matrigel assays show that AZU1 can disrupt human microvascular endothelial cell (HMVEC)-formed barrier permeability as well as VEGF and $CD45^+$ cell–mediated HUVEC tube morphogenesis.

Second, aside from the bactericidal factors produced by activated neutrophils, $CD45^+$ cell subtypes critical for vascular development in the germinal matrix exhibit prominent transcriptomic changes that suggest their immune activation. Among the signaling pathways identified in activated $CD45^+$ cell subtypes, upregulation of CXCL16 in monocytes most likely creates a proinflammatory milieu to disrupt vascular integrity (119, 120). Indeed, CXCL16, combined with ELANE, disrupts vascular endothelial cadherin junctional complexes of endothelial cells in 3D engineered microvessels. In Matrigel assays, CXCL16 alone is sufficient to inhibit VEGF-mediated branch morphogenesis of HUVECs. Bioinformatic analysis revealed that upregulated expression of S1PR1 in endothelial cells could be a potential downstream effector of CXCL16 in GMH cases. This most likely disrupts the S1P gradient required for the migration of immune cells across vascular barriers (88, 121). In support of this idea, endothelial cell–specific knockout of S1PR1 in embryonic mice leads to prominent infiltration of peripheral immune cells, including many that express monocyte marker CD16 and CXCL16. These results suggest that CXCL16 and S1PR1 may constitute reciprocal interactions between immune cells and endothelial cells. As such, upregulation of CXCL16 in subsets of $CD45^+$ cells and disruption of S1PR1 in endothelial cells could propagate a vicious cycle that exacerbates the egress of activated peripheral immune cells across the primitive BBB in GMH. Interestingly, intraperitoneal (IP) injection of ELANE/CXCL16 in pregnant dams at E12.5 disrupts vascular integrity and leads to hemorrhage in the VZ of the GE but not in the VZ/SVZ of the pallium or in the cortical plate at E13.5 (Figure 3b). Several ELANE/CXCL16-injected mouse embryos exhibit IVH. In contrast, IP injections of ELANE/CXCL16 at E13.5 and E15.5 did not produce the same hemorrhage phenotypes at E17.5. While the cause for the stage-dependent vulnerability to ELANE and CXCL16 remains unclear, this may be related to the immaturity of the BBB at E12.5–E13.5.

Collectively, these results provide supporting evidence on how inflammation perturbs the homeostatic immune–vascular interaction in the germinal matrix to promote hemorrhage.

There are, however, several caveats that deserve our attention. First, it is important to note that both GMH cases used in this study are preterm infants born at 23–24 GWs with significant complications, such as lung immaturity and necrotizing enterocolitis. Thus, it is possible that these complications could aggravate inflammation, infection, and/or hypoxic-ischemic injury that could disrupt angiogenesis in the germinal matrix. Second, given the high prevalence of comorbidities in preterm infants (99, 122, 123), the causes for GMH are likely to be multifactorial. Indeed, histopathology in several preterm infants with GMH shows low numbers of $ELANE^+$ neutrophils close to those in age-matched controls (89). These results suggest that other factors, such as immaturity of the brain vasculature and/or hypoxic-ischemic injury to endothelial cells and mural cells, could also contribute to the pathogenesis of GMH.

4. THERAPEUTIC INSIGHTS FROM SINGLE-CELL TRANSCRIPTOMICS

Advances in using single-cell transcriptomics to directly investigate neurogenesis, angiogenesis, and immune–vascular interactions in the second-trimester human brain provide critical insights into the functional properties of neural progenitors for GABAergic interneurons, endothelial and mural cells for brain vasculature, and the diverse repertoire of immune cells that contribute to neurogenesis and angiogenesis. Comparative studies using brain tissues from control preterm infants and age-matched cases with GMH will further identify important spatial and temporal mechanisms that interfere or disrupt brain vasculature specifically in the germinal matrix. These results will also offer new directions to diagnose, prevent, and treat GMH in preterm infants.

Single-cell transcriptomic data can be leveraged to monitor and identify risk factors that promote GMH in preterm infants. For instance, highly sensitive ELISA assays can be implemented in preterm infants to determine whether higher levels of bactericidal factors, AZU1 and ELANE, and chemokine CXCL16 in the serum samples (between 20 and 30 GWs) positively correlate with the development of GMH. To further expand our understanding of the mechanism of neuroinflammation beyond AZU1, ELANE, and CXCL16, it is feasible to perform quantitative proteomic analyses using blood samples from preterm infants and correlate these results with the single-cell transcriptomics data from $CD45^+$ immune cells isolated from patients with GMH. On the other hand, neuroinflammation can serve as a potential therapeutic target for preterm infants with GMH. For instance, it is conceivable that neutralizing antibodies for neutrophil-derived bactericidal factors AZU1 and ELANE can be used to block their roles in acute inflammation. In addition, anti-CXCL16 antibodies are actively being developed to mitigate inflammation associated with chronic human immunodeficiency virus infection (120), inflammation (124) or cancer immunotherapy (119, 125). One can leverage these resources to determine whether blocking neuroinflammation can indeed protect brain vasculature from AZU1, ELANE, or CXCL16. The abilities to modulate neutrophil lifespan, target neutrophil recruitment, inhibit the production of bactericidal factors, or reprogram neutrophils are potential therapeutic interventions (126). Finally, vascular cells, including endothelial cells and mural cells, from the germinal matrix of the second-trimester human brain can also be further developed as potential therapeutic interventions (75, 76). These primary brain endothelial cells can be propagated in culture for >10 passages without losing their inherent

properties. Since endothelial cells are the primary target of proinflammatory factors AZU1, ELANE and CXCL16, it is possible to transplant genetically engineered endothelial cells to protect and repair vasculature damaged by activated neutrophils and monocytes and the proinflammatory factors they produce.

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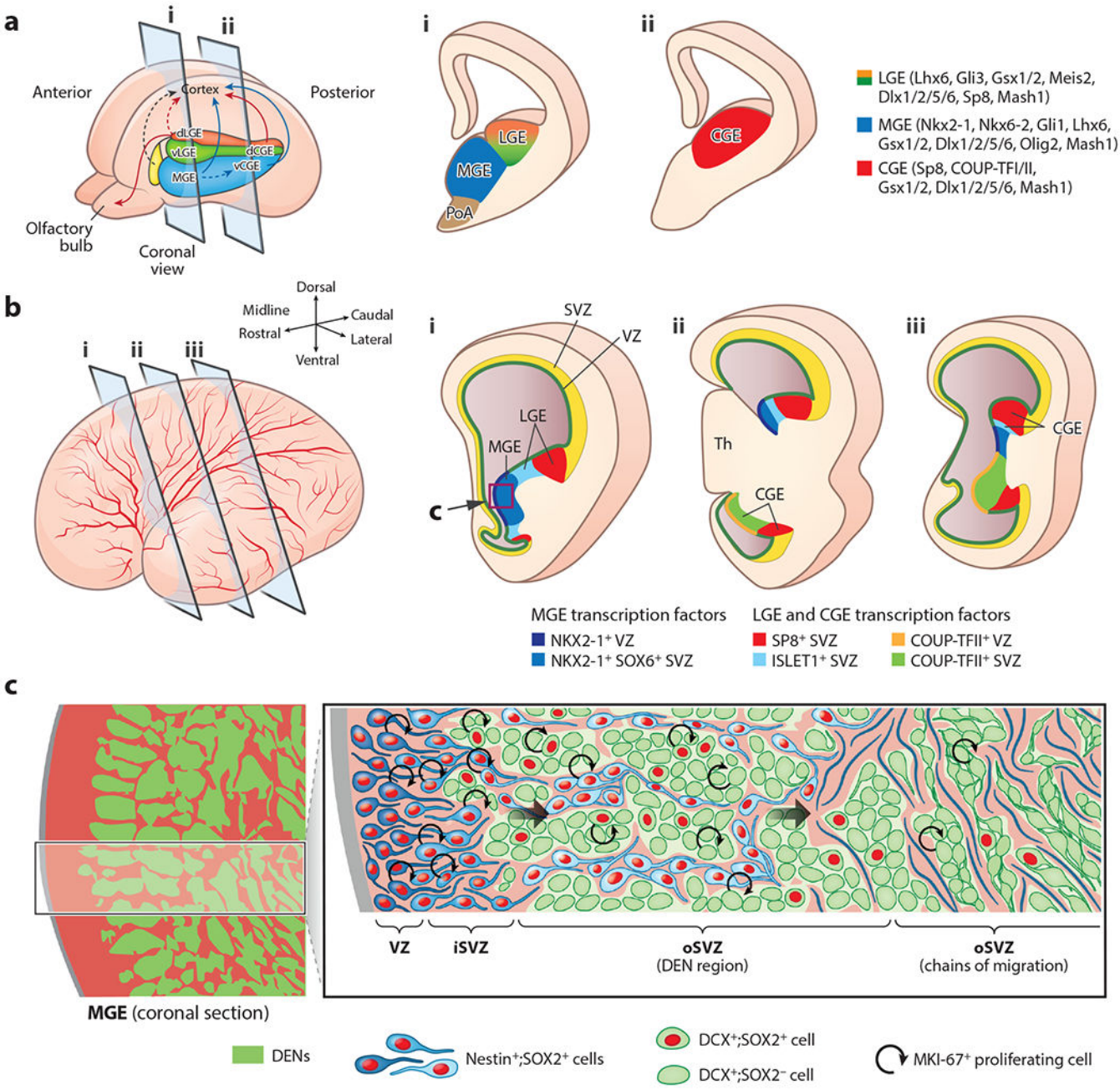

Figure 1.

Generation of GABAergic interneurons in mouse and human GEs. (*a*) A schematic diagram of the LGE, MGE, and CGE in the developing mouse brain at embryonic days 11.5–12.5 and the representative transcription factors that are expressed in these regions. (*b*) A schematic diagram of the LGE, MGE, and CGE in the prenatal human brain at 21 GWs and the representative transcription factors that are expressed in these regions. (*c*) A schematic diagram showing a zoomed-in view of the human MGE in the second trimester, highlighting the unique organization of DENs that are surrounded by neural progenitors. (*Left panel*) Overview of the MGE at 22 GWs showing the organization of progenitors (*red*

regions) and DCX^+ neuroblasts (green regions). (Right panel) Higher magnification view of the boxed area in the left panel. $Nestin^+$;$SOX2^+$ cells are present in the MGE within the VZ, iSVZ, and oSVZ. These cells have proliferative abilities, especially within the iSVZ. DCX^+ cells are also present as cell-dense clusters (i.e., DENs) separated by $Nestin^+$ fibers. A subpopulation of DCX^+ cells express SOX2, and some can divide within DENs. Abbreviations: CGE, caudal ganglionic eminence; dCGE, dorsal CGE; DEN, DCX^+ cell–enriched nest; dLGE, dorsal LGE; GE, ganglionic eminence; GW, gestational week; iSVZ, inner subventricular zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; oSVZ, outer subventricular zone; PoA, pre-optic area; Th, thalamus; vCGE, ventral CGE; vLGE, ventral LGE; VZ, ventricular zone. Panels *a*, *b*, and *c* adapted with permission from References 28, 40, and 42, respectively.

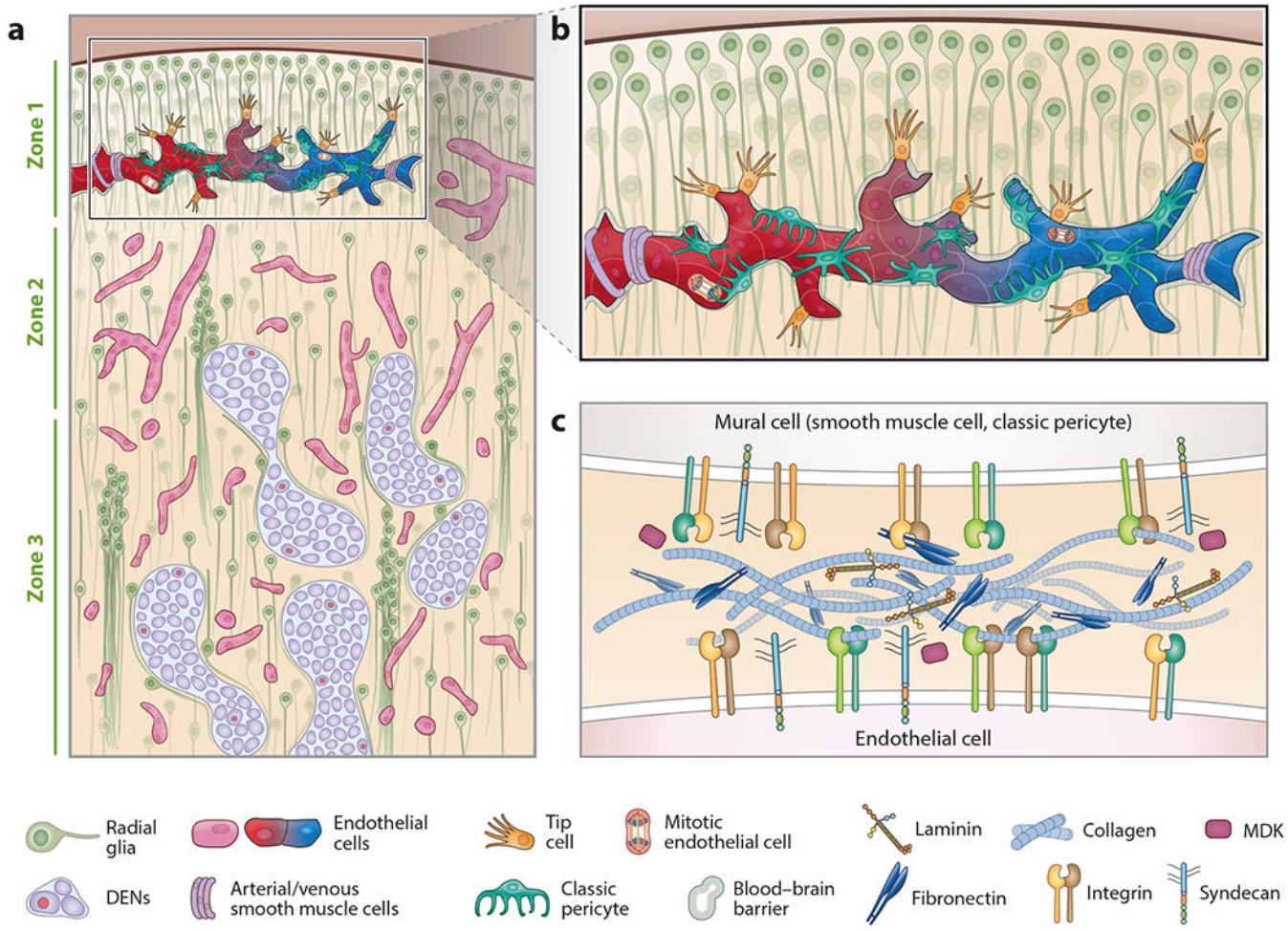

Figure 2.

Neurovascular interactions in the germinal matrix of the prenatal human brain during the second trimester. (*a*) A schematic diagram showing the newly formed vasculature and its interaction with neural progenitors, including radial glia and DENs, in the germinal matrix of the prenatal human brain in the second trimester (14–23 gestational weeks). Zone 1 refers to the VZ and the iSVZ, and both zone 2 and zone 3 belong to the oSVZ in the germinal matrix. (*b*) A zoomed-in view of the vasculature in the VZ/iSVZ of the highlighted area in the germinal matrix in panel *a*. This diagram shows an ensemble of several key vascular cell types in the VZ/SVZ, including endothelial cells, tip cells, mitotic endothelial cells, arterial and venous smooth muscle cells, and classic pericytes. (*c*) A schematic diagram showing the key molecular pathways that regulate the interactions between endothelial cells and mural cells, as revealed by single-cell transcriptomics. These include collagens, laminin, MDK, fibronectin, and syndecan. Abbreviations: DEN, DCX^+ cell–enriched nest; iSVZ, inner subventricular zone; MDK, midkine; oSVZ, outer subventricular zone; VZ, ventricular zone. Illustrations by Sarah Pyle.

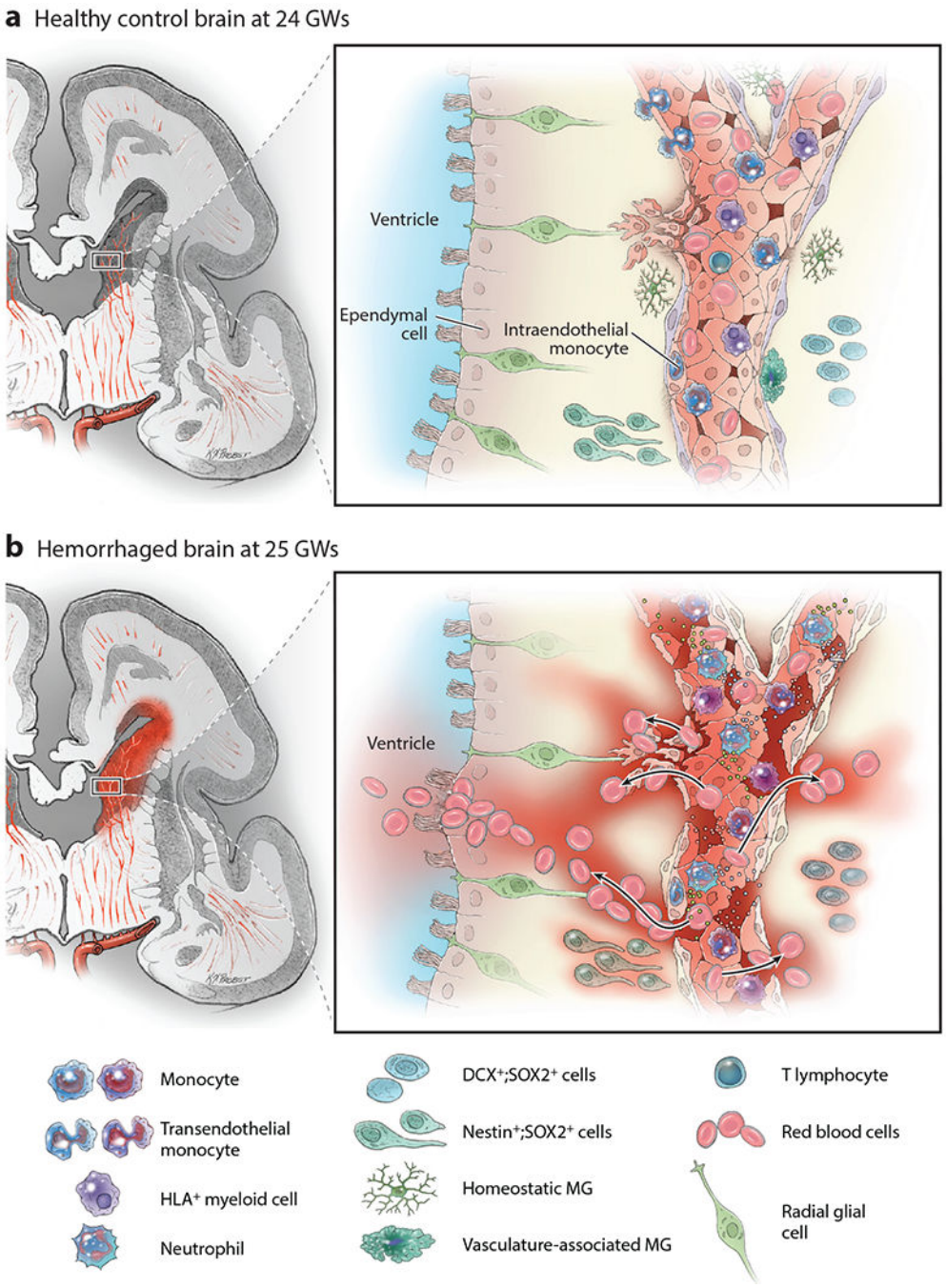

Figure 3.

Roles of immune cells in vascular morphogenesis in the germinal matrix of the second-trimester human brain in control and in GMH. (*a, left*) A graphical representation of a hemicoronal section of prenatal human brain at 24 GWs. (*a, right*) An enlarged view of the highlighted area in the germinal matrix in the left panel, depicting how subsets of $CD45^+$ immune cells, including monocytes (*blue*), HLA^+ myeloid cells (*purple*), and vasculature-associated microglia (MG) (*light green*), interact with the nascent vasculature to promote angiogenesis. (*b, left*) A graphical representation of a hemicoronal section

of a prenatal human brain at 25 GWs with GMH. (*b, right*) An enlarged view of the highlighted area in the left diagram, depicting that activated neutrophils produce bactericidal factors, such as ELANE and AZU1, whereas activated monocytes produce CXCL16 to create a proinflammatory milieu that disrupts nascent vasculature and promotes GMH. Abbreviations: AZU1, azurocidin 1; ELANE, elastase; GMH, germinal matrix hemorrhage; GW, gestational week; HLA, human leukocyte antigen; MG, microglia. Illustrations by Ken Probst.