SOX9-COL9A3–dependent regulation of choroid plexus epithelial polarity governs blood–cerebrospinal fluid barrier integrity

Keng Ioi Vong, Tsz Ching Ma, Baiying Li, Thomas Chun Ning Leung, Wenyang Nong, Sai Ming Ngai, Jerome Ho Lam Hui, Liwen Jiang, and Kim Ming Kwan

The choroid plexus (CP) is an extensively vascularized neuroepithelial tissue that projects into the brain ventricles. The restriction of transepithelial transport across the CP establishes the blood–cerebrospinal fluid (CSF) barrier that is fundamental to the homeostatic regulation of the central nervous system microenvironment. However, the molecular mechanisms that control this process remain elusive. Here we show that the genetic ablation of Sox9 in the hindbrain CP results in a hyperpermeable blood–CSF barrier that ultimately upsets the CSF electrolyte balance and alters CSF protein composition. Mechanistically, SOX9 is required for the transcriptional up-regulation of Col9a3 in the CP epithelium. The reduction of Col9a3 expression dramatically recapitulates the blood–CSF barrier defects of Sox9 mutants. Loss of collagen IX severely disrupts the structural integrity of the epithelial basement membrane in the CP, leading to progressive loss of extracellular matrix components. Consequently, this perturbs the polarized microtubule dynamics required for correct orientation of apicobasal polarity and thereby impedes tight junction assembly in the CP epithelium. Our findings reveal a pivotal cascade of SOX9-dependent molecular events that is critical for construction of the blood–CSF barrier.

In vertebrates, tight regulation of the brain extracellular microenvironment is fundamental to central nervous system (CNS) homeostasis (1,2). While the blood–brain barrier formed by junctional components between vascular endothelial cells is well perceived to provide insulation to the CNS, the importance of the choroid plexus (CP) has been neglected (3). Present in every brain ventricle, the CP acts as a physical barrier at the interface of systemic circulation and cerebrospinal fluid (CSF). The CNS is vulnerable to almost all undesired molecules present in the CSF (4); hence, failure in forming the blood–CSF barrier at the CP will conceivably elicit deleterious consequences to CNS function. However, the molecular control of blood–CSF barrier permeability remains largely elusive.

The CP consists of an outer layer of cuboidal epithelial cells enveloping a dense capillary core. Because the CP microvasculature is highly fenestrated and highly permeable, it has been suggested that the tight junctions between adjoining CP epithelial cells are the decisive parameter that governs the transepithelial permeability of molecules into the CSF (5). Consistent with this notion, loss of tight junction component MPDZ was linked to elevated permeability of the CP and neonatal hydrocephalus caused by an overabundance of CSF proteins (6). Studies in Drosophila or cultured mammalian cells suggest that tight junction formation often relies on the intricate interplay among cell polarity modules that designate the site of junctional assembly (7). Therefore, the establishment of a functional blood–CSF barrier is associated with epithelial polarity commitment in the CP. However, the vast majority of our current knowledge of epithelial cell polarization comes from simple in vitro cell-based systems (8).

Mechanisms that regulate apicobasal polarity are often more complicated in vivo and remain poorly understood (9,10). Interestingly, the polarized configuration of membrane proteins in the CP epithelium is apparently different than all other secretory epithelia, such as epithelia in kidney or intestine (11). For example, the “basolateral transporters,” such as Na+/K+ ATPase, NKCC1, and NHE1, are localized exclusively to the apical domain in the CP epithelium. Therefore, understanding the molecular basis that underlies this atypical epithelial polarity in the CP is key to determining the mechanisms that regulate blood–CSF barrier permeability.

SOX9 is a member of the high-mobility group transcription factor family that plays diverse roles in development (12). In humans, SOX9 mutations are commonly associated with congenital CNS anomalies, including ventriculomegaly and hydrocephalus, but the pathological mechanisms are unknown (13–15). Previous studies in mice suggest that Sox9 is essential for cell fate specification and differentiation in the neuroepithelium of neocortex.

Significance

Tight regulation of the brain microenvironment is fundamental to proper neurologic function. The restriction of molecule entry into the central nervous system from the brain vascular endothelium has been well studied; however, far less is known about the molecular events that control permeability across the choroid plexus (CP) epithelium at the interface between the systemic circulation and cerebrospinal fluid (CSF). Our study establishes an essential role for SOX9 in the regulation of CP permeability. SOX9 induces the transcription of Col9a3, which mediates the microtubule dynamics necessary for orienting cell polarity and thereby assembling epithelial tight junctions. Our findings lay the groundwork for the manipulation of blood–CSF barrier permeability and expand our understanding of epithelial tissue integrity.

Author contributions: K.I.V., S.M.N., J.H.L.H., L.J., and K.M.K. designed research; K.I.V., S.M.N., J.H.L.H., L.J., and K.M.K. performed research; K.I.V., T.C.M., B.L., T.C.N.L., W.N., S.M.N., J.H.L.H., L.J., and K.M.K. analyzed data; and K.I.V. and K.M.K. wrote the paper.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi/10.1073/pnas.2009568118/-/DCSupplemental.

Published February 1, 2021.
retina, and spinal cord during embryogenesis (16–18). Here we reveal a role for SOX9 in the developing CNS as an essential regulator of blood–CSF barrier function. Our findings demonstrate a series of cellular defects following the ablation of Sox9 in the CP epithelium that eventually results in CSF electrolyte imbalance and aberrant hyperpermeability of the blood–CSF barrier. Mechanistically, SOX9 regulates the synthesis of collagen IX, deprivation of which markedly increases the vulnerability of the basement membrane, leading to the progressive loss of extracellular matrix (ECM) components. This disrupts ECM–cytoskeleton interactions and consequently perturbs the polarized microtubule dynamics required for maintenance of epithelial apicobasal polarity. Our results thus unveil a model of SOX9-dependent ECM signals that lead to cytoskeleton rearrangement to reinforce the apicobasal polarity in the CP epithelium and thereby establish the functional blood–CSF barrier.

Results

SOX9 Is Required for Blood–CSF Barrier Integrity. SOX9 expression was detected in the hindbrain CP once it emerged from the roof plate and persisted throughout gestation (SI Appendix, Fig. S1A). Immunohistochemistry showed that SOX9 was expressed exclusively in the CP epithelium and not in the underlying stroma (SI Appendix, Fig. S1 B and C). This expression pattern made SOX9 a candidate molecule that governs the acquisition of blood–CSF barrier function. To derive CP epithelium-specific Sox9 conditional knockout mice (hereinafter referred as Sox9 CKO), we crossed mice harboring Sox9 floxed alleles with a Pax2-Cre transgene that showed robust activity in the rhombomere (r)-1–derived hindbrain

Fig. 1. Conditional inactivation of Sox9 led to blood–CSF barrier dysfunction. (A) Representative images of coronal sections of E12.5 or E18.5 control and Sox9 CKO immunostained for SOX9. n = 3 per genotype. (B–G) Representative histological images showing the cytoarchitecture of hindbrain CP of control and Sox9 CKO at E12.5 (B) or E18.5 (E) and CP epithelial cell size and circularity at E12.5 (C and D) and E18.5 (F and G). n ≥ 150 cells from three independent pairs of control and Sox9 CKO, Kolmogorov–Smirnoff test. (H and I) Analysis of blood–CSF barrier permeability by in utero injection of rhodamine-conjugated 10-kDa dextran (H) or 70-kDa dextran (I) into E18.5 embryos. Loss of Sox9 resulted in the penetration of both 10-kDa and 70-kDa dextran into the CP epithelial cell layer. Dextran was detected in the extracellular space between adjacent epithelial cells (arrows) or even taken up into the cytoplasm (filled arrowheads). n = 3 per genotype. (J) BCA protein assay analysis of protein abundance in CSF from E17.5 control and Sox9 CKO. n = 4 per genotype. Data are mean ± SEM. **P < 0.01; ***P < 0.001. (Scale bars: 50 μm in A; 100 μm in B and E; 20 μm in H and I.)
CP epithelium (SI Appendix, Fig. S2A). Immunohistochemistry confirmed the removal of SOX9 expression in the Pax2-Cre lineage of the CP (Fig. 1A and SI Appendix, Fig. S2B). Despite Sox9 ablation, the epithelial morphology of the CP was comparable between control and Sox9 CKO at E12.5 (Fig. 1B–D). In situ hybridization indicated that Transcriptin (Tr), a differentiation marker of CP epithelial cells, was expressed in the Sox9-deleted CP and maintained as development proceeded (SI Appendix, Fig. S3A). These results suggested that CP epithelial specification and differentiation were not perturbed by Sox9 inactivation. However, histological examination of the Sox9 CKO mutants at E18.5, which is the latest timepoint available for analysis due to perinatal lethality, revealed the absence of distinct frond-like folds projecting into the fourth ventricle (Fig. 1E). Moreover, analysis of cell size and shape indicated conspicuous morphometric irregularities of the Sox9 CKO epithelium compared to controls (Fig. 1F and G).

To investigate whether the aberrant cytarchitecture hampers the integrity of the CP epithelium and abolges blood–CSF barrier function, we delivered the albumin-binding azo dye Evans Blue into the circulation of Sox9 CKO and control littersmates by in utero injection into the fetal livers at E18.5. Evans Blue was confined within the vascular capillaries in control embryos but stained the brain parenchyma of Sox9 CKO embryos (SI Appendix, Fig. S3B). To define the route of dextran leakage, the brains were stained for the enzyme following dye injection. Remarkably, the tracers extensively infiltrated the lateral intercellular space between adjoining CP epithelial cells or even permeated the entire cytoplasm in Sox9 CKO mutants (SI Appendix, Fig. S3B).

To rule out the possibility that the albumin leakiness was due to an impairment of transport machineries, we also analyzed CP permeability using rhodamine-conjugated 10-kDa and 70-kDa dextran, which are inert polysaccharides. Consistent with our observations with Evans Blue, pronounced fluorescence was detected in the CP epithelial cells of Sox9 CKO mutants, whereas the tracers were excluded from control CP epithelium (Fig. 1H–I). Notably, the fluorescent dextran was not observed around the brain vasculature but instead was present in proximity to the ventricular surface in the Sox9 CKO mutants (SI Appendix, Fig. S3 C and D). Immunofluorescent staining of vascular junction markers CD31 and Claudin-5 indicated that neither the vascular structure in the brain nor that in the CP was affected by Sox9 ablation (SI Appendix, Fig. S3E and F). Moreover, the ultrastructure of the vascular endothelium appeared to be unaltered in Sox9 CKO (SI Appendix, Fig. S3 G and H). These findings support the notion that tracer leakiness arises from a hyperpermeable CP epithelium and penetrates the neural tissues via the CSF in Sox9 CKO mutants.

To corroborate this possibility, we measured the total protein concentration in the CSF using the bicinchonic acid (BCA) protein assay. Remarkably, the total protein abundance in the CSF was increased by more than 2-fold at E17.5 on Sox9 ablation in the CP (Fig. 1J).

Loss of Sox9 Perturbed CP Epithelial Apicobasal Polarity and Tight Junction Assembly. Because tight junctions are the key structures that restrict transepithelial permeability, we investigated whether the malfunction of the blood–CSF barrier in Sox9 CKO mutants was accompanied by tight junction defects in the CP epithelium. For this purpose, we performed transmission electron microscopy (TEM) on E18.5 CP from control and Sox9 CKO mutants. TEM demonstrated the typical tight junction ultrastructure in control CP at E18.5, which is characterized by linear electron-dense bands that form apical “kissing points” between opposing epithelial cells. Remarkably, the intercellular space between adjoining CP epithelial cells in Sox9 CKO was substantially wider and without any electron-dense structures (Fig. 2A).

These observations suggest the absence of tight junctions between CP epithelia cells on Sox9 ablation. Therefore, we examined the expression of the tight junction scaffold protein zonula occludens 1 (ZO-1) by immunohistochemistry. In control CP epithelial cells, ZO-1 was localized at the apical-most surface. In sharp contrast, in the absence of SOX9, ZO-1 was dispersed broadly along the entire apicobasal axis (Fig. 2B). Polarized ZO-1 distribution depends on PAR protein complexes that specify the apical pole (19). Interestingly, PAR-3 failed to reach the apical region but instead mislocalized to the basolateral membrane in the CP epithelium of Sox9 CKO mutants (Fig. 2C).

In line with these observations, the adherens junction components E-cadherin and β-catenin were abnormally present on the apical cell surface in the mutants (Fig. 2D and E). Interestingly, apicobasal polarity in Sox9 CKO mutant CP epithelium was not disrupted until after E16.5 (SI Appendix, Fig. S4 A–C). These data suggest that SOX9 is required for the maintenance of correct apicobasal polarity in the CP epithelium.

Ablation of CP Epithelial Polarity Disrupts CSF Electrolyte Balance. To evaluate the impact of perturbations in apicobasal polarity, we first examined the directional transport system in the CP. Consistent with previous reports, by immunostaining of the Na+ /K+ ATPase α1 subunit (ATP1A1), we showed that the Na+/K+ ATPase was positioned at the apical surface of the CP epithelium facing the brain ventricle. However, ATP1A1 was aberrantly diffused toward the basal side and colocalized with β-catenin in the Sox9 CKO CP epithelium (Fig. 2F and SI Appendix, Fig. S4D). On the other hand, the basolateral transporter anion exchanger 2 protein (AE2) was displaced from the basal membrane and mislocalized to the apical domain following Sox9 ablation (Fig. 2G). Although the overall abundance of ATP1A1 and AE2 appeared to be unaffected by the loss of Sox9, their availability at the apical membrane was substantially reduced in Sox9 CKO (SI Appendix, Fig. S4G). Quantitative plots of transporter subcellular distribution across the apical-basal axis revealed sharp peaks of fluorescent signals corresponding to ATP1A1 and AE2 at the apical and basal poles, respectively, in controls. In contrast, the immunosignals of both apical and basal transporters were essentially uniform across the entire apicobasal axis in Sox9 CKO mutants (Fig. 2H and I). A similar phenomenon was observed for the Na+ /K+ /Cl− cotransporter (NKCC1), suggesting the elimination of membrane transporter asymmetry (SI Appendix, Fig. S4 E–H).

Next, to investigate whether the loss of transporter polarization in CP impaired the homeostatic ionic balance, we measured the concentration of CSF electrolytes by atomic absorption spectrophotometry. Although the level of K+ in CSF was only subtly increased following Sox9 deletion in CP, that of Na+ ions was remarkably reduced, by 34%, in the Sox9 CKO mutants (Fig. 2J and K).

SOX9 Activates Col9α3 Transcription for Collagen IX Synthesis. We sought to understand how SOX9 as a transcriptional regulator is able to influence the apicobasal polarity. In a search for molecular pathways bridging the loss of Sox9 and polarity defects, we compared the transcriptome of E16.5 CP from control and Sox9 CKO mice by performing RNA-sequencing. Interestingly, only a small set of genes were differentially expressed in the CP of Sox9 CKO mutants (27 down-regulated and 13 up-regulated). The transcript levels of genes critical for establishing apicobasal polarity, including aPKC, Crb3, Par-3, Scrib, as well as major junctional complex members Cldn1 and ZO-1, were not significantly altered by Sox9 inactivation, as confirmed by RT-qPCR analyses (SI Appendix, Fig. S5 A and B). This implied that apicobasal polarity was not regulated by SOX9 at the transcriptional level, but instead that additional mechanisms are required for mediating SOX9 function. Sox9 often transcriptionally regulates multiple genes encoding ECM components in different organs (20), but, quite
unexpectedly, loss of Sox9 in the CP specifically abolished the expression of Col9a2 and Col9a3, which encode the α2 and α3 chains of collagen IX, respectively (SI Appendix, Table S1). RT-qPCR analyses verified the dramatic down-regulation of Col9a2 and Col9a3 in Sox9 CKO CP (Fig. 3A and C).

To determine whether SOX9 directly binds to Col9a2 and Col9a3 loci in the CP, we performed in vivo chromatin immunoprecipitation (ChIP) with E16.5 wild-type CP. While SOX9 did not significantly associate with the Col9a2 enhancer element, SOX9 displayed strong affinity to a dimeric consensus regulatory motif located within intron 1 of Col9a3. The same region was not pulled down by a nonspecific negative control IgG and SOX9 antibody did not preferentially immunoprecipitate a random region in the Col9a3 3′ UTR (Fig. 3B and D). Meanwhile, previously reported SOX9 targets Col2a1 and Col4a2 were not regulated by SOX9 in the CP (SI Appendix, Fig. S5C and D). Remarkably, Col9a3 was expressed exclusively in the CP epithelium, as demonstrated by RNAscope in situ hybridization (SI Appendix, Fig. S6A and B). Col9a3 expression was completely abrogated from the majority of CP epithelial cells in the Sox9 mutants at both E14.5 and E18.5 (Fig. 3E and F). Lineage tracing with the ROSA26-YFP reporter suggested that those few cells which express Col9a3 were not descended from Pax2-Cre-labeled rhombomere 1 and thus are SOX9-positive (SI Appendix, Fig. S6C). Collectively, our results suggest that SOX9 physically binds to the Col9a3 promoter and activates its transcription in the CP epithelium.

Collagen IX Is Essential for the Anchorage of ECM Macromolecules at the CP Subepithelial Basement Membrane. We proceeded to interrogate whether the loss of Col9a3 has a causative linkage to
CP hyperpermeability in Sox9 CKO mutants. To this end, we performed in utero electroporation to deliver siRNA against Col9a3 into the hindbrain CP of wild-type embryos at E14.5, before establishment of the blood–CSF barrier (SI Appendix, Fig. S7A). Following electroporation, Col9a3 expression levels were significantly reduced, as shown by RT-qPCR analyses (SI Appendix, Fig. S7B).
Fig. S7B) and RNAseq (SI Appendix, Fig. S7C). Silencing Col9a3 expression caused the tight junction scaffold protein ZO-1 to become randomly scattered in the cytoplasm (Fig. 4A and SI Appendix, Fig. S7D), showing striking resemblance to the phenotypes of Sox9 CKO mutants. Evaluation of CP permeability by tracer extravasation assay demonstrated that the CP epithelium was extensively infiltrated by 10-kDa or 70-kDa dextran on Col9a3 silencing, although to a milder extent compared to that in Sox9 CKO mutants (Fig. 4B and C). In agreement with the CP hyper-permeability observed, Col9a3 knockdown (KD) increased the total protein abundance in CSF by 42% (Fig. 4D). Like in the Sox9 CKO, the apicobasal polarity was lost in the Col9a3 KD CP epithelium (Fig. 4E). Correspondingly, neither the apical transporter ATP1A1 nor the basal transporter AE2 was correctly targeted to their respective membrane domains on Col9a3 KD (Fig. 4F–I). Moreover, Na⁺ concentration in the CSF dropped significantly following Col9a3 KD in the CP (SI Appendix, Fig. S7 E and F).

The remarkable recapitulation of the CP cellular defects in Sox9 CKO mutants by silencing Col9a3 expression prompted us to question further how the reduction of collagen IX would compromise blood–CSF barrier integrity. Previous studies reported that Col9a3 is responsible for maintaining matrix integrity in cartilage by anchoring ECM macromolecules (21–23). In the CP, laminin was deposited as a discrete continuous border of the basement membrane underlying the epithelia. Despite early ablation of laminin expression and down-regulation of Col9a3, the emergence of CP, laminin expression was initially normal in the basement membrane of Sox9 CKO and did not become substantially altered until E16.5 (SI Appendix, S8 A, C, and E). Examination of collagen I deposition in the Sox9 mutant CP across this temporal window led to similar observations, suggesting that the progressive displacement of ECM proteins from the basement membrane was not limited to specific matrix components (SI Appendix, Fig. S8 B, D, F, and G). Importantly, similar to what was observed in Sox9 CKO mutants, only scattered and discontinuous patches of laminin deposition were detected in the CP 3 d after the KD of Col9a3 (Fig. 4 J and K).

These findings together suggest that the loss of collagen IX exasperated the structural integrity of CP epithelial basement membrane and resulted in the progressive elimination of ECM molecules. Interestingly, the destabilization of ECM molecules temporally precedes the disruption of apicobasal polarity in the Sox9 CKO mutants, suggesting that deprivation of ECM molecules leads to disruption of apicobasal polarity (Fig. 2 D and E and SI Appendix, Fig. S4 D–F). Furthermore, the epithelium-specific laminin receptor integrin α6 was markedly obliterated from the basal membrane of both Col9a3 KD and Sox9 CKO CP (SI Appendix, Fig. S9 A and B). Therefore, we sought to directly demonstrate the effect of blocking integrin-ECM communication on cell polarity. Specifically, CP primary epithelial cells were incubated with normal IgG or function-blocking antibody against integrin α6 prior to seeding onto a laminin-coated surface. ZO-1 and β-catenin colocalized together at the cell-cell boundaries in control experiments, whereas interruption of integrin α6 binding to laminin caused the mislocalization of both proteins (SI Appendix, Fig. S9C). Thus, our results pinpoint the importance of integrin-ECM interactions in the regulation of epithelial polarity in the CP.

Collagen IX-Dependent ECM Signals Mediate Microtubule Dynamics Necessary for the Maintenance of Apicobasal Polarity in the CP Epithelium. Given that previous in vitro studies demonstrated that integrin-ECM communication may provide extrinsic cues for cytoskeleton remodeling necessary for epithelial polarity (24, 25), we first examined whether the loss of Sox9 or the silencing of Col9a3 would perturb the polarized cytoskeletal organization. Phalloidin staining indicated that the actin filaments were concentrated apically in the CP epithelium of both control and Sox9 CKO mutants (Fig. 5A).

In contrast, the acetylated tubulin became broadly distributed without clear nucleation origins on Sox9 ablation (Fig. 5B). Notably, similar disruption of microtubule organization was observed following KD of Col9a3 (Fig. 5C). We hypothesized that this apical cytoskeletal polarity may reflect perturbations in microtubule dynamics. To evaluate this hypothesis, we performed ex vivo microtubule reorganization assay with CP tissues isolated from E17.5 control and Sox9 CKO mice. In control CP epithelial cells, nascent microtubules focally emanated from the apex and displayed a typical astral configuration. In stark contrast, nascent microtubules were randomly nucleated in the cytoplasm of Sox9 CKO epithelia (Fig. 5D). Quantification of α-tubulin fluorescence intensity showed that the number of nascent microtubules per unit area was significantly reduced in Sox9 CKO CP epithelial cells (Fig. 5E). Moreover, we repeated the same experiments with Col9a3 KD CP. Like what we observed in Sox9-deleted cells, following the silencing of Col9a3, nascent microtubules did not nucleate apically but instead emerged from widespread regions within the CP epithelial cells (Fig. 5 F and G). Because the typical array of noncentrosomal microtubules aligned along the apico- basal axis of epithelial cells is derived from the Golgi apparatus (26), we questioned whether the dispersed nucleation of microtubules was due to aberrant Golgi positioning. In the control CP epithelium, Golgi complexes were positioned in the apical cortex above the nucleus. A wind rose diagram illustrates that the Golgi was most frequently found at <30° to a reference line drawn parallel to the apico basal axis (Fig. 5 H and I). In stark contrast, the apical positioning of the Golgi was lost and became randomly oriented to the basal or lateral side in the Sox9 CKO CP epithelial cells (Fig. 5 J and K). These findings suggest that basement membrane-derived extrinsic cues are crucial for maintaining proper Golgi orientation and the polarized microtubule scaffold in CP epithelial cells.

In light of these findings, we examined the possibility that Sox9-dependent phenotypes were elicited by non–cell-autonomous ECM deficiency. Using the ROSA26-YFP reporter mice as an indicator for Pax2-Cre mosaicism in the CP, we found that laminin was abolished not only from the YFP⁺ cells, but also from cells that were YFP⁻ and thus presumably SOX9⁻ (SI Appendix, Fig. S10A). Likewise, the asymmetry of apical/basal transporters was impaired in Sox9 CKO irrespective of YFP reporter expression (SI Appendix, Fig. S10B). Indeed, the aberrant transporter polarity was evident in some of the Sox9 CKO cells that expressed Col9a3 transcripts (SI Appendix, Fig. S10C). Together, these observations imply that the apicobasal polarity was regulated by Sox9 in a non–cell-autonomous manner.

Sox9 Deletion in the CP Altered CSF Protein Composition. To investigate whether these cellular defects disturbed CSF composition, we compared the proteome of CSF obtained from control and Sox9 CKO mice by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Interestingly, our results indicated that the loss of Sox9 substantially altered the CSF protein composition (Fig. 6A and SI Appendix, Fig. S11 A–D and Table S2). To identify the key biological processes and pathways that are affected, we performed functional annotation clustering and Gene Ontology (GO) enrichment analysis for the three GO categories—biological processes (BP), cellular components (CC), and molecular functions (MF)—using Database for Annotation Visualization and Integrated Discovery (DAVID) (Fig. 6B). The key GO terms significantly enriched in BP, MF, and CC are protein folding, RNA binding, and extracellular exosomes, respectively.

Since exosomes are involved in the long-range delivery of signaling molecules, we assessed how this may impact the role of CSF as the signaling niche for CNS development. To this end, we tested the proliferative response of neural progenitors to CSF from wild-type mice or Sox9 CKO mutants. While adding control CSF to neural progenitors of cerebellum suppressed their proliferation,
Fig. 4. Knockdown of Col9a3 in the hindbrain CP epithelium. (A) Immunostaining analysis revealing the loss of apical localization of ZO-1 on Col9a3 KD. \( n = 3 \) per genotype. (B and C) Representative images of CP showing the distribution of 10-kDa dextran (B) and 70-kDa dextran (C) in control and Col9a3 KD embryos. \( n = 3 \) independent experiments. (D) Analysis of CSF protein content in control and Col9a3 KD mice by BCA assay. Data are mean ± SEM. *P < 0.05. (E) Immunostaining of PAR3 revealed CP epithelial cells failed to maintain apicobasal polarity following Col9a3 KD. \( n = 3 \) independent experiments. (F and G) Representative images demonstrating the alteration of ATP1A1 (F) and AE2 (G) subcellular localization in Col9a3 KD epithelial cells. (H and I) Plots of signal intensity of ATP1A1 (H) and AE2 (I) along the apicobasal axis of individual CP epithelial cells in control and Col9a3 KD embryos. At least 30 cells from three independent experiments were analyzed; multiple t tests. Shaded area around the curves represents SEM. *P < 0.05. (J and K) Immunostaining showed that the basal lamina of the CP epithelium was deprived of laminin following genetic ablation of Sox9 (yellow arrows) (J) or Col9a3 KD (K). White arrows and filled arrowheads indicate laminin expression at the subepithelial basement membrane and the vascular endothelium, respectively. \( n = 3 \) independent experiments or per genotype. (Scale bars: 20 \( \mu \)m in A–C, E–G, J, and K.)
Fig. 5. Disruption of polarized microtubule scaffold by loss of Sox9. (A) Representative images of phalloidin staining demonstrating similar F-actin distribution in CP of control and Sox9 CKO. (B and C) Examination of microtubule organization in the CP epithelium of E17.5 control and Sox9 CKO (B) or Col9a3 KD embryos (C) by immunostaining for acetylated tubulin (Ac-tubulin). (D–G) Microtubule regrowth assay showing that nascent microtubules were focally emanated from the apical domain in normal CP epithelial cells. (D) Conditional Sox9 deletion or (F) the KD of Col9a3 led to aberrant nucleation of MTs from multiple loci in the cytoplasm, n = 3 per group. Quantification of α-tubulin intensity indicates reduced microtubule nucleation in Sox9 CKO (E) or Col9a3 KD CP (G). n = 150 cells. (H–K) Analysis of Golgi orientation in CP epithelium by immunostaining for TGN46 in controls (H) and Sox9 CKO mutants (J). Wind rose diagram showing the orientation of Golgi apparatus to a reference line drawn parallel to the apicobasal axis and across the nucleus in controls (I) and Sox9 CKO mutants (K). n = 150 cells. ***P < 0.001. (Scale bars: 50 μm in A; 10 μm in B and C; 20 μm in D, F, H, and J.)
Fig. 6. Removal of Sox9 from CP altered CSF protein composition. (A) Quantitative analysis of proteins in CSF from control or Sox9 CKO by LC-MS/MS. CSF protein composition represents pooled samples from at least three mice per genotype. Hierarchical clustering was performed using the average linkage clustering method and the Pearson metric with Proteome Discoverer. Proteins enriched in CSF of Sox9 CKO or control are indicated in regions A and B, respectively. (B) The differentially expressed proteins were subjected to analysis with DAVID for annotation enrichment analysis. The 10 most significantly enriched GO terms in BP, MF, and CC terms are presented. All the Benjamini- adjusted P values of the terms were negative 10-based log-transformed. (C and D) Immunostaining for PH3 and quantification of PH3+ cells in the cerebellar external granular layer (EGL) (C) and dorsal telencephalon (D) of control and Sox9 CKO. At least eight sections from each embryo were analyzed. n = 3 per genotype. (Scale bars: 20 μm.) (E) Schematic diagram of the proposed working model of SOX9 in the regulation of blood–CSF barrier function. Data are mean ± SEM. ***P < 0.001. ns, not statistically significant.
The molecular pathways that regulate blood–CSF barrier permeability were previously unknown. Here we provide evidence resulting from analyzing niches for diverse purposes (38–43). Interestingly, the number of PH3+ cells in the developing neocortex (44) was significantly increased in the CP epithelium, the deficits of collagen IX deposition in the basement membrane destabilized ECM molecules derived from both Sox9-deleted cells and the neighboring Sox9-expressing cells. Consequently, the epithelial polarity was globally disrupted in the mosaic Sox9 CKO mutants. Sox9 mutations in human neonates commonly manifest hydrocephalus accompanied by ventriculomegaly, but the mechanistic linkage is unclear (13–15). Hydrocephalus can be caused by dysfunction of ependymal cells lining the ventricles, impairment of CSF dynamics, or overabundance of CSF proteins (6, 36, 37). Therefore, the elevated levels of CSF proteins on loss of Sox9 function provides a possible explanation for the pathophysiology of hydrocephalus in human patients. Nevertheless, the perinatal lethality of Sox9 CKO mice hinders further verification of such a premise. The CP has drawn increasing attention recently because of its ability to dynamically modulate CSF signaling niches for diverse purposes (38–43). Interestingly, the responses to CSF appeared to be heterogeneous across different compartments of the CNS. A previous study indicated significant differences in the transcriptome of CP from different brain ventricles, such that CSF composition is regionalized albeit with continuous CSF flow between brain ventricles (44). Our results suggest the likelihood that such region-specific CSF microenvironments is essential for exercising target-specific responses at the functional level.

Taken together, our findings suggest a model of blood–CSF barrier permeability regulation that depends on a Sox9-COL9A3 microtubule functional axis, which may lead to strategies that manipulate CP epithelial polarity or the dynamics of CSF composition for therapeutic and pharmacologic purposes.

Materials and Methods

Experimental Animals. Pax2-Cre, Sox9flox/flox, and R26R-YFP mouse lines were described previously (45–47). Pax2-Cre;Sox9flox/flox mice are referred to as Sox9 CKO. Mice with the Pax2-Cre;Sox9flox/flox genotype were used as controls unless otherwise defined. All animal procedures were conducted with the approval of the Animal Experimentation Ethics Committee of The Chinese University of Hong Kong.

Blood–CSF Barrier Permeability Assay. In utero liver injection of tracers was performed as described previously (48). Timed-pregnant mice were anesthetized, and uterine horns were exposed. Fluorescent tracers (5 μL of 2 mg/ml 10-kDa or 70-kDa tetramethylrhodamine-conjugated dextran in PBS or 1% Evans Blue in PBS) were injected into the livers of embryos. Tracers were allowed to circulate for 10 min before sacrifice of the embryos.

In Utero Electroporation. Timed-pregnant ICR mice at E14.5 were anesthetized, and uterine horns were exposed. Then 1 μL of ON-TARGETPlus SMARTpool siRNA targeting Col9a3 (50 nM) or siGLO nontargeting control (Dharmacon; GE Healthcare) was microinjected into the fourth ventricle with a fire-polished glass capillary connected to a micro syringe pump controller (Micro4; World Precision Instruments). To perform electroporation, five 50-ms pulses of 35 V at 950-ms intervals were delivered across the CP (ECM830; BTX).

CSF isolated from Sox9 CKO constrained progenitor expansion to a markedly greater extent (SI Appendix, Fig. S11E). However, neither CSF from controls nor CSF from Sox9 CKO mice produced a significant effect on progenitors isolated from the developing neocortex (SI Appendix, Fig. S11F).

We further examined progenitor expansion in vivo by immunostaining for phospho-histone H3 (PH3), which labels cells undergoing mitosis. Interestingly, the number of PH3+ cells in the external granular layer of cerebellum was decreased remarkably following Sox9 ablation in the CP (Fig. 6C). As Sox9 is not expressed in granule cells, this difference was presumably due to alterations in the CSF proteome (26, 27). In contrast, the number of mitotic progenitors in the dorsal telencephalon appeared to be similar in control and Sox9 CKO (Fig. 6D). These results suggest that inactivation of Sox9 in the CP led to changes in the CSF environmental niche that ultimately altered neural progenitor behavior in a region-specific manner across different CNS compartments.

Discussion

The molecular pathways that regulate blood–CSF barrier permeability were previously unknown. Here we provide evidence that the maintenance of apicobasal polarity in the CP epithelium is fundamental to restricting transepithelial permeability and therefore defining functional blood–CSF barrier. Mechanistically, Sox9 transcriptionally regulates the expression of Col9a3, which is required for the intricate interplay between ECM molecules and microtubule dynamics necessary for polarity orientation and tight junction assembly. The correct apicobasal polarity of epithelial cells is the basis of organ function (28). Pioneering in vitro studies have implied an instrumental role of basement membrane ECM molecules in defining the apical versus basal pole of epithelium (25); however, the in vivo relevance of ECM signals to epithelial polarity has remained largely ambiguous and controversial (29, 30). Studies defining the apical versus basal pole of epithelium (25); however, the precise localization of epithelial apicobasal polarity: although Col9a3 was exclusively expressed in CP epithelium in the CNS. Since the cellular anomalies observed following KD of Col9a3 are essentially phenocopies of Sox9 CKO mutants, it is likely that the CP hyperpermeability defects are caused predominantly by a deficiency in collagen IX. This may explain the non–cell-autonomous Sox9 regulation of CP epithelial apicobasal polarity: although Sox9 CKO mutants showed mosaicism of Sox9 deletion in the CP epithelium, the deficits of collagen IX deposition in the basement membrane destabilized ECM molecules derived from both Sox9-deleted cells and the neighboring Sox9-expressing cells. Consequently, the epithelial polarity was globally disrupted in the mosaic Sox9 CKO mutants. Sox9 mutations in human neonates commonly manifest hydrocephalus accompanied by ventriculomegaly, but the mechanistic linkage is unclear (13–15). Hydrocephalus can be caused by dysfunction of ependymal cells lining the ventricles, impairment of CSF dynamics, or overabundance of CSF proteins (6, 36, 37). Therefore, the elevated levels of CSF proteins on loss of Sox9 function provides a possible explanation for the pathophysiology of hydrocephalus in human patients. Nevertheless, the perinatal lethality of Sox9 CKO mice hinders further verification of such a premise. The CP has drawn increasing attention recently because of its ability to dynamically modulate CSF signaling niches for diverse purposes (38–43). Interestingly, the responses to CSF appeared to be heterogeneous across different compartments of the CNS. A previous study indicated significant differences in the transcriptome of CP from different brain ventricles, such that CSF composition is regionalized albeit with continuous CSF flow between brain ventricles (44). Our results suggest the likelihood that such region-specific CSF microenvironments is essential for exercising target-specific responses at the functional level.

Taken together, our findings suggest a model of blood–CSF barrier permeability regulation that depends on a Sox9-COL9A3-microtubule functional axis, which may lead to strategies that manipulate CP epithelial polarity or the dynamics of CSF composition for therapeutic and pharmacologic purposes.
Quantification and Statistical Analysis. The brightness and contrast of the confocal images were adjusted with Leica Application Suite X (LAS X; version 3.0.2.16120) or Olympus FV10-ASW (version 04.02.02.09). Fluorescence intensity and Golgi orientation were analyzed using Fiji/ImageJ version 1.43. All statistical analyses were performed using GraphPad Prism 8 software, and significance was accepted at P < 0.05. All images are representative of at least three independent experiments unless specified otherwise. Data analysis was not performed blinded to the mouse genotype, because the phenotype often could be obviously recognized.

Additional information of study methodology is provided in SI Appendix, Materials and Methods.

Data Availability. Transcriptome data have been deposited in the National Center for Biotechnology Information database through the BioSample submission portal (accession nos. SAMN16774154 and SAMN16774155). All other study data are included in the article and SI Appendix.

ACKNOWLEDGMENTS. We thank Richard R. Behringer (University of Texas MD Anderson Cancer Center) for providing the Sox9 conditional mouse line, helpful advice, and comments on the manuscript. We also thank Sze-nim Lim and Freddie Kwok for their excellent technical support. The work described in this paper was supported by grants from the Research Grant Council of the Hong Kong Special Administrative Region (GRF 14105418, CNF 4012-16E, and AoE/M-05/12) and a Chinese University of Hong Kong Direct Grant for Research.