Rotation report

Meningeal lymphatics in zebrafish

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Abstract

Meninges are no longer perceived as a sheer mechanical shielding of the brain. This three-layer structure is essential for normal cerebral activity and may well emerge to be a decisive player in neurodegenerative diseases, for example due to inappropriate immune response or deficient waste clearance through the newly discovered brain lymphatics. Larval zebrafish is transparent, which makes it a useful model to study dynamic processes happening in the brain such as clearance mechanisms. While it is unclear if fish have lumenised meningeal lymphatic vessels, they do possess a population of brain lymphatic endothelial cells (BLECs) which fulfils some lymphatic functions. Specific genetic markers are lacking to thoroughly study meningeal cell types in zebrafish, including the BLECs. Here, we show that *ependymin* (*epd*) is not a marker for the BLECs but may be a marker for the leptomeningeal fibroblasts which support the BLECs. If confirmed, this opens the way to the use of *epd* as a control for experiments involving the BLECs or as a marker to directly manipulate leptomeningeal fibroblasts. We conclude by presenting other BLEC candidate markers and discussing the potential function of the BLECs based on our imaging of the zebrafish inner endomeningeal layer.

Background

Meninges and neurodegeneration

Meninges are layers of tissue between the skull and the brain. In human, they are a three-layer structure. From the skull to the brain parenchyma, they consist in the dura, the arachnoid mater and the pia. The two latter are called together the leptomeninges[1]. The subarachnoid space is filled with cerebrospinal fluid (CSF) and contains the subarachnoid trabeculae, a loose mesh of collagen fibrils and fibroblasts that holds the arachnoid and the pia together[2]. Pial blood vessels are also traversing the trabeculae, before diving down into the brain parenchyma[3]. The pia is a thin membrane that closely adheres to the brain[3].

Recent discoveries have profoundly changed the view of meninges as merely a protective coating of the brain[1] and they are now seen as a potential key player in neurodegenerative diseases[4].

Brain metabolism produces waste proteins prone to form toxic aggregates, such as tau or amyloid-beta, which need to be cleared. This is done by various overlapping clearance mechanisms. Succinctly, waste metabolites can be degraded by secreted enzymes, taken up by cells, exported into the blood or lymph, or recirculated into the cerebrospinal fluid (CSF)[5]. Presumably, meninges are essential for some or most of these pathways, including the glymphatic system which may account alone for up to 55% of amyloid-beta clearance[6]. The newly discovered meningeal lymphatic vessels[7,8] are also likely to appear paramount to our understanding of neurodegenerative diseases, both through clearance systems and regulation of immune response[9].

Brain lymphatics in zebrafish

Being optically transparent, larval zebrafish are convenient tools to study dynamic processes like brain clearance systems. For example, dynamics of fluorescently-tagged amyloid-beta injected into the brain can be imaged in a live fish down to subcellular resolution (**Figure 1**).

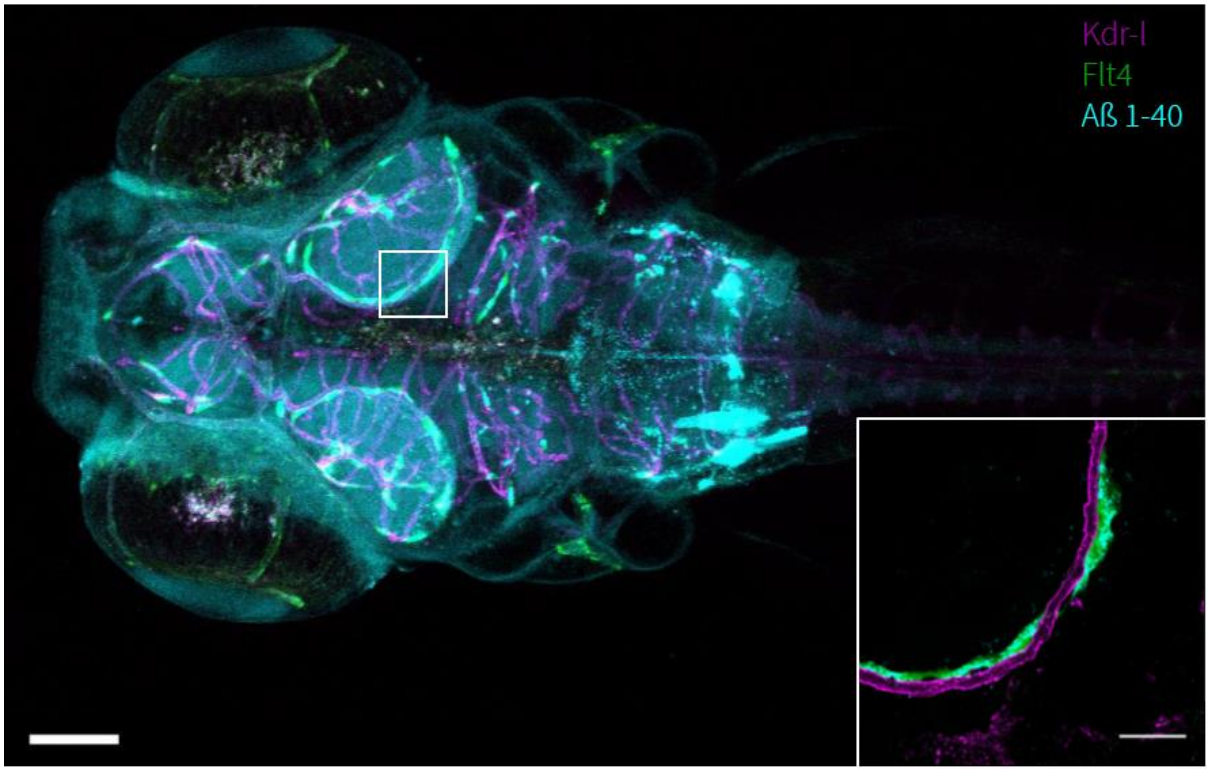

Figure 1 | BLECs take up fluorescently-tagged amyloid-beta 1-40 injected into the hindbrain. Blood vasculature is highlighted in purple (*kdrl-l:mCherry*); lympho-venous cells in green (*flt4:mCitrine*); and fluorescently-tagged amyloid-beta 1-40 in blue. Note the colocalization of amyloid-beta inside the Flt4-positive BLECs. The fish is 7 dpf (days post-fertilisation). Images are representative of two experiments. Left scale bar is 100 µm, right scale bar is 20 µm (Shibata-Germanos, unpublished data).

In teleost fish, meninges are composed of two main layers: the ectomeninx and the endomeninx. The ectomeninx is the outer layer, which seems homologous to the dura. The space between the ectomeninx and the endomeninx contains the perimeningeal fluid (PMF) and the intermeningeal adipose tissue. The endomeninx is comprised of three sublayers: the outer (OL), intermediate (IML) and inner endomeningeal layers (IL). The inner endomeningeal layer is the functional homolog of the subarachnoid space. As in mammals, it contains blood vessels, cerebrospinal fluid (CSF), and a loose network of collagen fibrils and leptomeningeal fibroblasts[10].

When zebrafish were investigated as a potential model for the study of brain lymphatics, rather than obvious lumenised vessels, a novel population of lymphatic cells was discovered. These were variably characterised as brain lymphatic endothelial cells (BLECs)[11], meningeal mural lymphatic endothelial cells (muLECs)[12], or fluorescent granular perithelial cells (FGPs)[13].

The BLECs are loosely connected lymphatic endothelial cells located at the inner endomeningeal layer. They express typical lymphatic markers (*flt4*, *prox1a*, *lyve1*, among others[13]) but do not seem to form lumenised vessels[11]. They are flat elongated cells around 40 µm in length that sometimes project long processes[11,13]. They contain electron-dense spherical inclusions which can be larger than 2 µm in diameter[11].

These lymphatic cells sprout from the venous endothelium of the optic choroidal vascular plexus around 56 hours post-fertilisation (hpf). Starting at 3 days post-fertilisation (dpf), they form a loop along the mesencephalic vein over each optic tectum (**Figure 1**). In adulthood, BLECs cover most surfaces of the brain except for the dorsal surface of the telencephalon[11], and are mostly found in close proximity to blood vessels[11,12].

Similarly to lymphatics in other organs, BLECs selectively endocytose macromolecules up to at least 150 kDa when these are injected into the brain (optic tectum) or the CSF (hindbrain ventricle)[11], including amyloid-beta (**Figure 1**). They also actively take up low density lipoproteins (LDLs) from the blood[12]. When the fish is fed a high cholesterol diet, BLECs become elongated and accumulate lipid droplets[13].

Finally, BLECs express endothelial growth factors and ligands, both angiogenic and lymphangiogenic, and were shown to be essential for normal meningeal vascularisation[12].

BLECs are labelled by various non-specific markers (see above). While that may be satisfactory for some imaging applications, having a specific marker would allow

precise control of their function in an experimental context. It would be possible, for instance, to specifically inhibit macromolecule uptake to study the contribution of BLECs to clearance pathways[14], or conditionally ablate them at different stages to study how meningeal blood vasculature development is affected[15].

Materials and methods

Zebrafish strains

Animal work followed guidelines of the animal ethics committee of the University College London, UK. The *Tg(kdr-l:HRAS-mCherry-CAAX)^{s916}; Tg(flt4^{BAC}:mCitrine)^{hu7135}* double transgenic line, herein denoted *Tg(kdr-l:mCherry);Tg(flt4:mCitrine)*, was used in the experiments reported here[16].

Embryos, larval and adult zebrafish (*Danio rerio*) were kept at the University College London's fish facility (at 28°C with a 14 hrs light and 10 hrs dark cycle). Experimental procedures were conducted under the project licence awarded to Jason Rihel from the UK Home Office, according to the UK Animals (Scientific Procedures) Act 1986.

Colorimetric whole-mount in situ hybridisation

Anti-sense RNA probes were generated from 24 hpf cDNA using direct amplification and transcription with T3 RNA polymerase. In situ hybridization was carried out as described previously[17,18]. For the detection of *epd* mRNA, 150 ng of the probes generated from the *epd* primer pair (**Table 1**) was hybridized to *Tg(kdr-l:mCherry);Tg(flt4:mCitrine)* fish with a *nacre (mitfa)$^{w2/w2}$* mutant background.

Table 1 | Primers used for synthesis of in situ hybridisation probes.

Primer pair	Gene / Region	Sequence	Probe length
Forward primer	*epd* / 3'UTR	AGTGTCTGGACCTGAAGAAAGT	820 bp
Reverse primer	*epd* / 3'UTR	AGGTAAGATAGAAAGTGCAAGCA	

Fluorescent whole-mount in situ hybridization followed by immunostaining

Fluorescent in situ hybridisation followed by immunostaining was performed as described previously[11]. Here, 150 ng of probe generated from the primer pair (**Table 1**) were use and the anti-dig POD antibody (Roche #11207733910 TSA Cyanine 3 Tyramide Reagent Pack) was used at 1:500 dilution. For the mCitrine immunostaining, the

primary antibody used here was rabbit α-GFP (Amsbio, #TP401) used at 1:250 dilution. The secondary antibody was goat α-Rabbit 488 (Lifetech, #A-11034) used at 1:100 dilution. TOTO™-3 iodide (642/660) nuclear stain (ThermoFisher, #T3604) was applied at the same time as the secondary antibody at a 1:5000 dilution.

Microscopy and image processing

Images of embryonic fish and adult brain whole mounts were imaged with a Leica SP8 microscope using 10x or 20x dry objectives and 25x and 40x water immersion objectives. Confocal stacks were processed using Fiji-ImageJ version 1.51s. Colorimetric in situ hybridizations were imaged with a Nikon Eclipse Ni using 10x and 20x objectives. The Extended Depth of Field ImageJ plugin[19] was used for some of the images in **Figure 3**. Images and figures were assembled using Microsoft Power Point and Adobe Illustrator.

RNAseq data

RNAseq raw counts were downloaded from Gene Expression Omnibus (GEO) (accession code GSE97650 for the lyve1-positive BLECs[12]; GSE97651 for the pdgfrβ-positive pericytes[12]; GSE97421 for the mrc1a-positive BLECs, the kdr-l-positive blood vasculature and the whole fish[13]). All subsequent data processing and plotting was done in R v3.3.3. After merging the datasets, raw counts were normalised using the DESeq2 R plugin[20]. In the plots (**Figure 2**, **Figure 5**), one asterisk indicates a p-value ≤ 0.05; two asterisks a p-value ≤ 0.01; three asterisks a p-value ≤ 0.001, as computed by Welch's t-test.

Results

Ependymin-expressing cells gradually cover the zebrafish brain

From RNAseq data by Bower et al., 2017[12] and expression data available on the ZFIN database[21], *epd* (*ependymin*) was initially identified as a candidate marker for the BLECs (**Figure 2**). Wild type colorimetric in situ hybridisation at different developmental

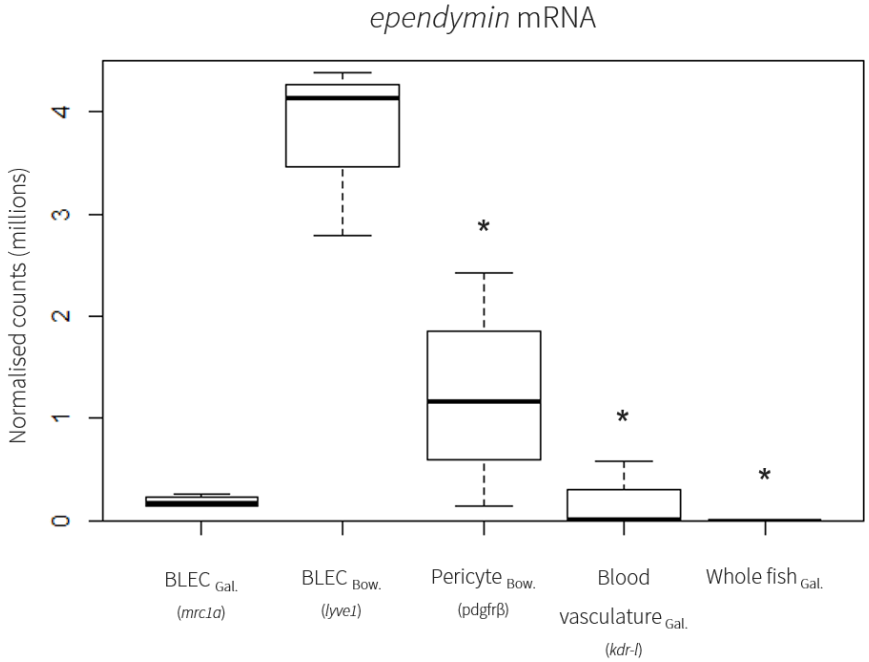

Figure 2 | Normalised *epd* mRNA counts in BLECs, pericytes, blood vasculature, and whole fish. The marker used for FACsorting is indicated below the label. *Gal.* indicates that the RNAseq data are from Galanternik et al., 2017; *Bow.* indicates that the RNAseq data are from Bower et al., 2017. Asterisks indicate significant difference with the BLEC counts from Bower et al., 2017.

stages and adulthood revealed staining patterns that seemed consistent, although more extensive, with what we previously observed in *Tg(kdr-l:mCherry);Tg(flt4:mCitrine)* double transgenic embryos (eg. **Figure 1**). Namely, *epd*-positive cells cannot be observed at 24 hpf. In later larval stages (3 and 5 dpf), they form a ring over the distal periphery of each optic tectum and an arborizing structure ventrally. They are also present laterally in two dense zones on the sides of the telencephalon and over the sides of the midbrain and hindbrain. At 15 dpf, what appear to be pial blood vessels can clearly be distinguished in negative, both dorsally and ventrally. In adult fish, most of the brain is covered with a dense layer of *epd*-positive cells (**Figure 3**). No staining could be observed anywhere else than the brain at any developmental stage (data not shown).

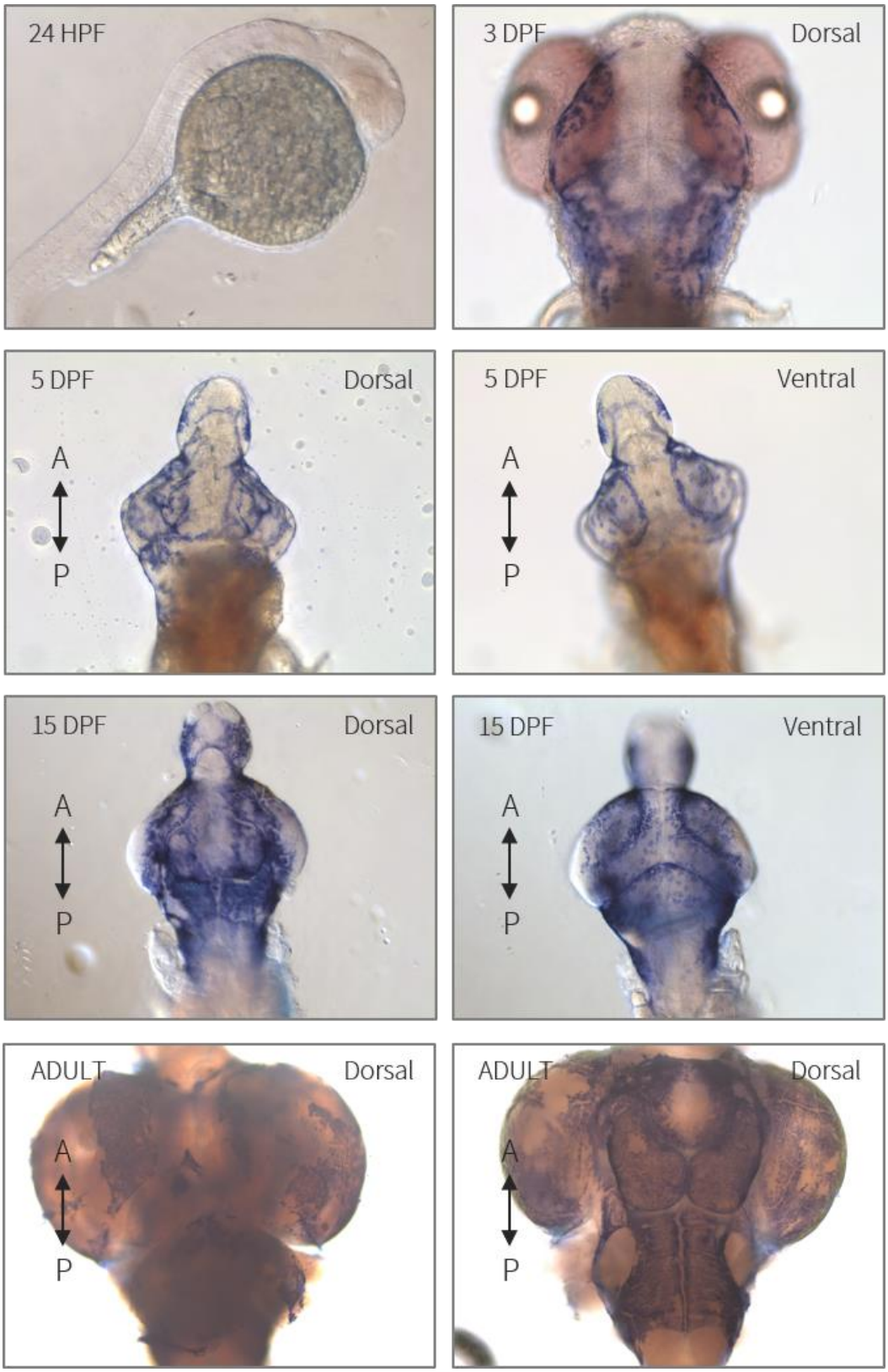

Figure 3 | *epd*-expressing cells gradually cover the zebrafish brain. Purple staining labels *epd* mRNA at different ages (24 hpf, 3 dpf, 5 dpf, 15 dpf, adult). Adults were one year and 7 months. Images are representative of at least 10 animals for each age, except for adults which were in duplicates. Areas without staining in adults are mostly artefacts due to tearing off of the meninges.

Ependymin is a marker of the inner endomeningeal layer

In order to assess *epd* as a specific BLEC marker, we performed a fluorescent in situ hybridisation (FISH) followed by *flt4:mCitrine* immunostaining on the *Tg(kdrl:mCherry);Tg(flt4:mCitrine)* double transgenic line at different developmental stages (3 dpf, 5 dpf, 15 dpf, adult). Flt4 is a lympho-venous marker which labels the BLECs (eg. **Figure 1**). Confocal imaging revealed that the *flt4:mCitrine*-positive cell and the *epd*-expressing cells were in fact two distinct populations. The *epd*-expressing cells form a loose mesh, cut across by lumenised vessels. The BLECs, on the other hand, sit in the intercellular spaces formed by the *epd*-expressing cells. This mesh covers most of the brain in adult fish.

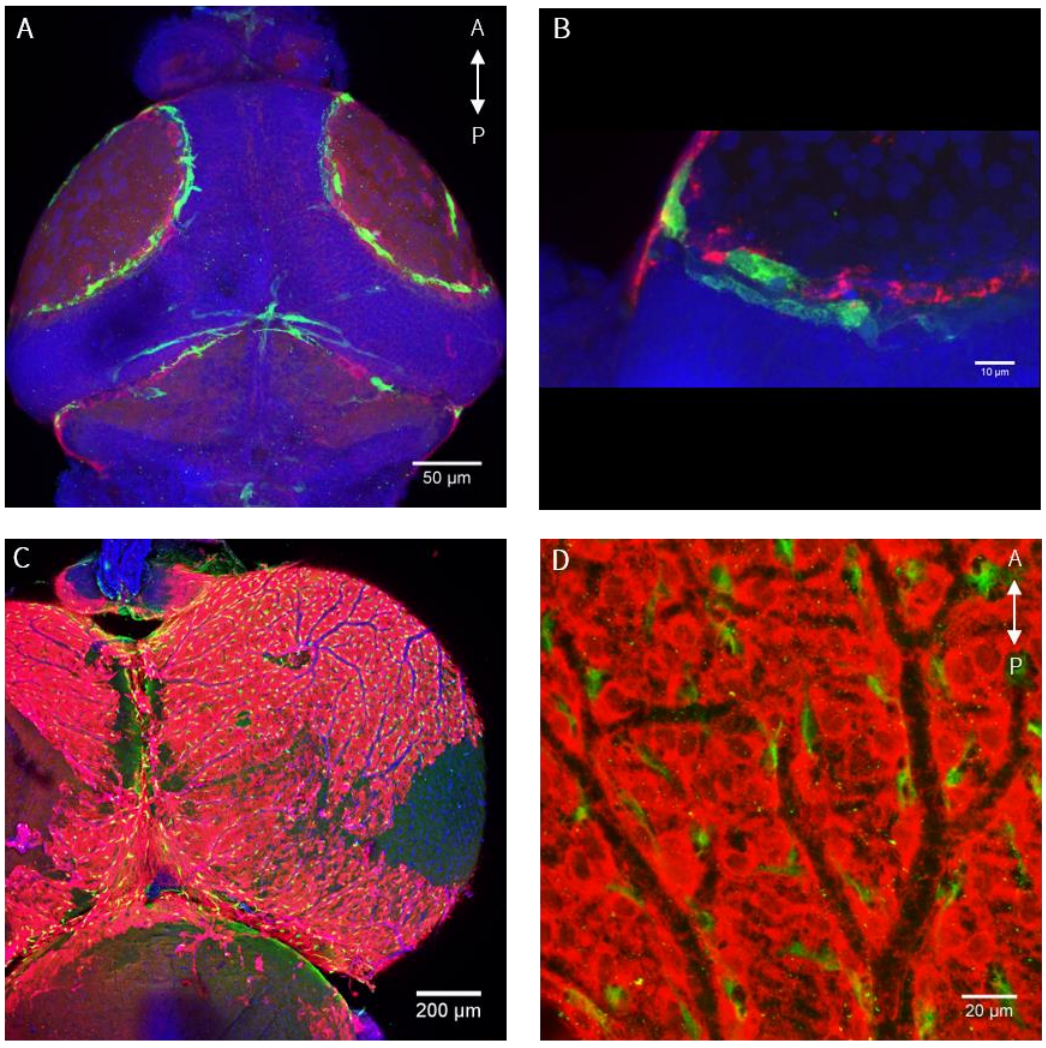

Figure 4 | Flt4-positive BLECs (green) and *epd*-expressing cells (red) are two distinct populations. **(A)** BLECs and *epd*-expressing cells form a ring over each optic tectum at 3 dpf. **(B)** Close inspection of the ring at 5 dpf reveals that BLECs and *epd*-expressing cells do not colocalise. **(C)** Most of the adult brain is covered by a dense zone of *epd*-expressing cells. Meninges were removed on the right to show that *epd*-positive cells form a thin layer of leptomeningeal cells. **(D)** Zoom on the adult optic tectum reveals that *epd*-expressing cells form a loose mesh traversed by blood vessels. BLECs sit in the extracellular spaces of this net.

epd-expressing cells may be the leptomeningeal fibroblasts of the inner endomeningeal layer. This is consistent with the anatomical localisation of the BLECs at the inner endomeningeal layer[11], the morphological description of the loose mesh of fibroblasts at this layer, and the high level of epd synthesis by these same fibroblasts[10].

What are the next top BLEC marker candidates?

Bower et al. found high *epd* mRNA counts when supposedly sequencing BLECs' RNA. This is contradictory with our in situ data (**Figure 3**). A likely explanation is that the BLEC population was contaminated by RNA from the surrounding fibroblast-like cells during FACsorting, which would explain the reported high *epd* mRNA counts.

Galanternik et al. performed RNAseq on BLECs FACsorted using *mrc1a* as marker[13]. As they found low *epd* mRNA counts in BLECs (**Figure 2**), it is unlikely that the sorted cells were contaminated by the *epd*-expressing fibroblast-like cells. We thus believe this dataset may be more reliable and we used it to extract the next BLEC candidate markers. These were defined as the genes whose counts were ten-fold higher in BLECs than other cells types and whole fish, with a minimum median BLEC count of 1000 (**Figure 5**). This yielded 31 genes, which were manually searched for expression data in the ZFIN database[22]. Among those, 15 had expression data that did not seem consistent with the BLECs, such as staining before 56 hpf or in other regions than the brain; and 16 had no or unclear expression data. Future studies will probably want to investigate these 16 genes as potential specific BLEC markers.

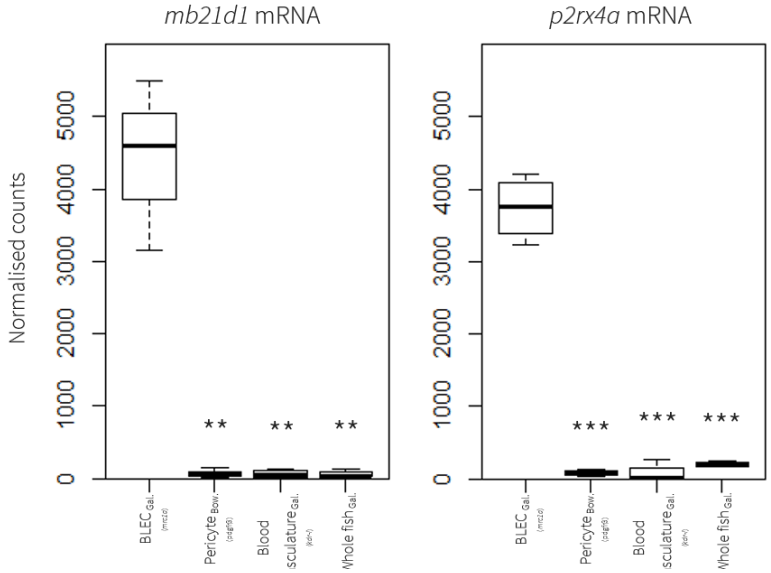

Figure 5 | mRNA counts of two candidate BLEC markers. These two genes were randomly picked as examples from the 16 candidate markers that showed no or unclear expression data on the ZFIN database. See Figure 1 legend for axis labels. Asterisks indicate statistical significance compared to the BLECs RNA counts from Galanternik et al, 2017.

Discussion

Although largely overlooked until recently, meninges are likely to be key players in neurodegenerative diseases. Newly discovered lymphatic vessels in the mouse dura mater, for instance, are a potential clearance pathway for toxic waste proteins like tau and amyloid-beta. Meninges also represent exciting new opportunities for therapy, such as boosting CNS drainage with lymphatic growth factors[4]. Meningeal structures are fairly conserved in zebrafish[10], which has the considerable advantage of being translucent. Although it is unclear if the fish meninges contain homologous lymphatic vessels, they do include brain lymphatic endothelial cells (BLECs). These share common functional features with proper lymphatics in other organs, including drainage of macromolecules from the underlying tissue. As such, they are interesting subjects for the study of brain lymphatics and clearance systems. Nonetheless, specific markers for meningeal cell types are lacking, including for the newly discovered BLECs and for the leptomeningeal fibroblasts of the inner endomeningeal layer.

Here, we show that *ependymin* (*epd*), a gene we initially studied as a candidate BLEC marker, appears in fact to be a marker of the inner endomeningeal layer, potentially labelling the leptomeningeal fibroblasts. If confirmed, this should allow the use of leptomeningeal fibroblasts as control for experiments involving the BLECs. For example, epd can be used to negatively FACsort the BLECs, or check that a RNAseq dataset is not contaminated by the surrounding *epd*-expressing fibroblasts (eg. **Figure 1**). This is how we identified that Galanternik et al.'s BLEC RNAseq dataset was probably more reliable than Bower et al.'s, allowing us to extract 16 genes that could be the next marker candidates. *epd* could also serve to study the interactions between the BLECs and the fibroblasts, which may be extensive as they develop in strikingly similar patterns. For instance, it would be possible to specifically ablate the leptomeningeal fibroblasts at different developmental stages to observe how BLECs' development is affected.

Future directions

Imaging for the first time the BLECs sitting within the loose mesh of leptomeningeal fibroblasts at the inner endomeningeal layer (**Figure 4C, D**) raises questions in regards with their function. This layer is homologous to the mammalian subarachnoid space: it is filled with CSF that flows through the mesh. By sitting at the extracellular spaces of the net, BLECs are in an ideal position to be in extensive contact with CSF. Yet, we

know through previous studies[11–13] that BLECs endocytose macromolecules injected into the CSF, such as dyes or amyloid-beta (Shibata-Germanos, unpublished data). It therefore raises the question that BLECs may represent a specific clearance pathway for CSF. After endocytosis, BLECs could enzymatically degrade the CSF filtrate, dispose of it in the surrounding blood vessels, or use their long thin processes to pass it to neighbouring BLECs until peripheral populations can dispose of it in putative meningeal lymphatic vessels. It should be possible to provide some answers by imaging for long time periods fluorescent dyes or fluorescently-tagged proteins injected into the brain parenchyma or the CSF, as previously performed at shorter time scales.

The glymphatic system, which may account for up to 55% of amyloid-beta clearance in mouse[6], is essentially only active during sleep or anaesthesia[23]. Incidentally, clearance of metabolic waste is one of the main hypothesis for the function of sleep[6]. In the present context, it would be interesting to study if BLECs are also upregulated during sleep compared to wakefulness. Larval zebrafish is a convenient model for the study of sleep. For instance, several live larvae can be imaged for hours during day versus night under a confocal microscope and the sleeping patterns of hundreds of larvae can be monitored at once. Zebrafish are also convenient for studies involving drugs as these can simply be added into the water. Therefore, it would be possible to test the effects of drugs such as melatonin, that quickly induces sleep, or caffeine, that induces enhanced wakefulness, on the BLECs uptake of fluorescent tracers. Similarly, glymphatic function has been shown to decrease by up to 90% in aged mouse, it would therefore be informative to similarly assess BLECs function in aged fish, and if it is possible to rescue it with drugs.

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