Single-cell transcriptomic analysis of neuroepithelial cells and other cell types of the gills of zebrafish (*Danio rerio*) exposed to hypoxia

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The fish gill is a multifunctional organ involved in numerous physiological processes, such as gas exchange and sensing of hypoxia by respiratory chemoreceptors, called neuroepithelial cells (NECs). Many studies have focused on zebrafish (*Danio rerio*) to investigate the structure, function and development of the gills, yet the transcriptomic profile of most gill cells remains obscure. We present the results of a comprehensive transcriptomic analysis of the gills of zebrafish using single-cell RNA sequencing (scRNA-seq). Gill cells from *ETvmat2:EGFP* zebrafish were individually labelled before scRNA-seq library construction using 10× Genomics Chromium technology. 12,819 cells were sequenced with an average depth of over 27,000 reads per cell. We identified a median of 485 genes per cell and 16 cell clusters, including NECs, neurons, pavement cells, endothelial cells and mitochondrion-rich cells. The identity of NECs was confirmed by expression of *slc18a2*, encoding the vesicular monoamine transporter, Vmat2. Highly differentially-expressed genes in NECs included *tph1a*, encoding tryptophan hydroxylase, *sv2* (synaptic vesicle protein), and proteins implicated in O₂ sensing (*ndufa4l2a*, *cox8a1* and *epas1a*). In addition, NECs and neurons expressed genes encoding transmembrane receptors for serotonergic, cholinergic or dopaminergic neurotransmission. Differential expression analysis showed a clear shift in the transcriptome of NECs following 14 days of acclimation to hypoxia. NECs in the hypoxia group showed high expression of genes involved in cell cycle control and proliferation. The present article provides a complete cell atlas for the zebrafish gill and serves as a platform for future studies investigating the molecular biology and physiology of this organ.

The fish gill is a complex, multifunctional organ that orchestrates numerous physiological processes, including respiratory gas exchange, ionic and acid–base regulation, and excretion of nitrogenous compounds. The gills are organized into four branchial arches (in teleosts) with numerous filaments, each of which contains afferent and efferent arteries and gives rise to a series of respiratory lamellae. The gills are covered by an epithelial layer comprised of a multitude of cell types. Those that are well documented include pavement (respiratory) cells, chemoreceptors, neurons, mitochondrion-rich cells (ionocytes) and pillar cells. Despite decades of studies on the gills, including those of the model vertebrate, zebrafish (*Danio rerio*), the regulation of gene expression in gill cells remains largely unknown. We present the first transcriptomic analysis of cells from the zebrafish gills using single-cell RNA sequencing (scRNA-seq). We identified hundreds of genes within each cell and defined 16 separate cell types based on differential gene expression. The present study provides a complete atlas of gill cells from zebrafish with a particular focus on cells associated with O₂ sensing and the control of respiration.

As part of its role as a respiratory organ, the gill filaments are the primary site for respiratory chemoreceptors—called neuroepithelial cells or neuroendocrine cells (NECs)—due to their resemblance to neurons, their
Figure 1. Transcriptomic atlas of gill cells dissociated from the gills of Erimacrus isenbeckii. (A) Unsupervised uniform manifold approximation projection (UMAP) plot in which gill cells were subdivided into 16 clusters. Colours correspond to separate cell clusters whose identities were defined by the major cell type present within the cluster. Identified cell clusters included (organised by decreasing abundance) endothelial cells, pavement cells, leukocytes, mucus cells, Na⁺/K⁺-ATPase-rich cells (NaR cells), immune cells, tuft cells I, H⁺-ATPase-rich cells (HR cells), haematopoietic stem and progenitor cells (HSPC), proliferating cells, tuft cells II, neurons, neuroepithelial cells (NECs), fibroblasts, epithelial neural progenitor cells (ENPC) and platelets. (B) UMAP visualization of cell-cycle phases of the aforementioned clusters. G1, G1 phase; G2M, G2 and metaphase; S, synthesis phase. (C) Violin plot showing the number of unique genes in each cell cluster. (D) Violin plot showing the proportion of mitochondrial genes present within each cell cluster. (E) Heatmap of the top 32 genes that were differentially enriched in each cell cluster. Two representative genes from each cluster are indicated by an arrowhead. Scale gene expression is shown by colours, where yellow indicates relatively high expression and purple indicates relatively low expression.

Despite recent advances in delineating the role of gill NECs in the hypoxic response, specific mechanisms through which NECs sense, transduce and transmit hypoxic signals remain ambiguous. Conventional molecular approaches, such as polymerase chain reaction (PCR) analysis, allow only few gene candidates to be examined at a time, making it difficult to draw connections between different components of the signal pathway. With respect to the carotid body, microarray analysis and/or real-time quantitative PCR was used to characterize genes involved in the O₂-sensing pathway in both mouse22 and human23, whereas scRNA-seq analysis revealed the first transcriptomic profile of mouse chemoreceptor cells, highlighting genes likely to be important in chemosensory function16. A comprehensive transcriptomic analysis, such as RNA sequencing, provides more specificity in gene detection, identifies more protein-coding genes, and provides a wider range of gene expression changes when compared to microarrays24. In addition, NECs make up only a small proportion of gill cells—about 0.79–1.17% of all dissociated cells from filaments in zebrafish, depending on whether animals are exposed to normal levels of O₂ or hypoxia16. Consequently, the whole-tissue approach to transcriptomic analysis would limit collection of sufficient amounts of RNA from the relatively small population of gill NECs.

In the present study, we employed the droplet-based scRNA-seq approach to profile individual cells dissociated from the gills of zebrafish under control conditions or acclimated to 2 weeks of hypoxia. This approach was adopted to capture transcriptomes of individual NECs, sensory neurons involved in the hypoxia signalling pathway, and other cell types of the gill. Cell types were identified based on marker gene expression, and a gill cell atlas was generated from our dataset. Cross examination of different cell clusters uncovered genes that were highly differentially expressed in NECs, many of which are shared by chemoreceptor (type I) cells of the mammalian carotid body. Our data provide evidence that NECs exhibit the machinery for chemosensory transduction of hypoxic signals. Our results also reveal the expression of 5-HT, ACh and DA receptors in distinct cell types in the gills, indicating their potential roles in O₂-chemosensory transmission. Lastly, 2 weeks of exposure to hypoxia affected the population size and transcriptome of gill cells, including NECs. NECs had increased expression of genes involved in protein synthesis, cell growth and control of the cell cycle.

Results
Construction of the gill cell atlas. Gill cells were dissociated from a total of 8 zebrafish: 4 fish exposed to hypoxia and 4 fish in normoxia as controls. After quality filters and doublet removal, a total of 12,819 cells were contained in the dataset. We then generated a gill cell atlas with 16 cell identities using our single-cell sequencing data (Fig. 1A,B). The data had a mean of 27,312 reads per cell, a median of 485 genes per cell and a median of 2844 unique transcript molecules per cell. Following demultiplexing of sample barcodes, the multidimensional dataset was mapped onto a two-dimensional graph using the Uniform Manifold Approximation Projection (UMAP) method. This atlas served as a tool to demonstrate relationships between cell clusters and visualize relative gene expression.

The cell atlas was built with integrated data from both treatment conditions to ensure consistent clustering and cell identification between conditions. Unsupervised clustering analysis compared cells based on transcriptomic similarities and returned 22 clusters. The resulting cell atlas contained 16 cell identities, including NECs, neurons and other cell types, as shown in Fig. 1A (see also Supplementary Table S1). Based on our knowledge of gill cell morphology and gene expression, certain cell clusters were annotated with a broad identity without further specification. For example, two computationally-defined cell clusters expressing endothelial cell markers could not be further specified based on gene expression. These clusters were therefore combined into a single cluster.

presence within the epithelial layer, and their secretory activity. NECs detect changes in O₂ and CO₂/H⁺ and display K⁺ channel inhibition, membrane depolarization and synaptic vesicle recycling under conditions of hypoxia or hypercapnia/acidosis3–8. These cellular responses are believed to initiate reflex physiological changes, such as hyperventilation, thus maintaining internal O₂ homeostasis9,10. NECs contain the neurotransmitter, serotonin (5-hydroxytryptamine, 5-HT), stored in synaptic vesicles and are innervated by sensory nerve fibres11,12. The first gill arch and associated NECs display evidence of homology with the carotid body, the primary O₂ sensing organ in mammals9, though recent evidence suggests that NECs may be homologues of pulmonary neuroepithelial bodies, which function as respiratory chemoreceptors during the perinatal period13,14. Like mammalian chemoreceptors, NECs display pronounced plasticity when acclimated to hypoxia, such as hypertrophy3,6,15, proliferation and changes in gene expression16. In addition to 5-HT, other neurotransmitters, such as acetylcholine (ACh), adenosine triphosphate (ATP) and dopamine (DA), appear to be involved in hypoxic signalling in the gill17–21; however, the location or expression of corresponding receptor targets have not been identified.
Annotated cell populations had a variable number of unique genes and mitochondrial gene content (Fig. 1C,D). Moreover, the 16 cell identities differed greatly in gene expression. Their highly differentially expressed genes are depicted in the heatmap in Fig. 1E.

The annotation of cell clusters was mostly based on previously established molecular markers and known functional characteristics of gill cells. For example, NECs displayed particularly high expression for tph1a (Fig. 1E), the gene encoding the rate-limiting enzyme in the synthesis of serotonin. NECs are the major source of serotonin in the zebrafish gill12. Endothelial cells were identified based on their high expression of endothelial PAS domain protein (epas1a and epas1b), basal cell adhesion molecule (bcam) and endothelin 1 (edn1)26,27. The endothelial cell cluster may include pillar cells that form the vascular channels of the respiratory lamellae. Pillar cells are a modified form of endothelial cells6. The gene, ptprc, encoding the protein, tyrosine phosphatase receptor type C or CD45 antigen, was used to identify the broad leukocyte population28. The cluster of mucus cells did not have high expression of specific cell markers but was identified based on expression of mucins (muc5.1 and muc13a).

For clusters without established gene markers, the top 32 significantly enriched genes were examined in more detail to reveal their identity. For instance, respiratory pavement cells were identified by their high expression of cldna, cldhb, and cldnc (Fig. 1E), genes encoding claudin proteins important for establishing tight junctions. These genes are orthologous to the human gene, CLDN4, which is highly expressed in respiratory type I and type II alveolar cells in lung epithelium29. Immune cells were assigned this identity due to their high expression of lymphocyte antigen ly6m5 (Fig. 1E) and chemokines, such as ccl19b (Fig. 1E), cxcl8a and cxcl2b. In addition, proliferating cells were identified based on their high expression of proliferative markers, such as mki67 and top2a (Fig. 1E). Results from cell cycle scoring confirmed that cells in the proliferating cell cluster were in growth phase 2 or metaphase (G2/M) of the cell cycle (Fig. 1B). Moreover, two stem and progenitor cell populations were identified in our data. They are the cluster of hematopoietic stem and progenitor cells (HSPC), identified by their expression of grn1 (Fig. 1E), grn2 and thy1/cd9030,31, and the cluster of epidermal neural progenitor cells (ENPC), which form ionocytes in zebrafish embryos, identified by the expression of ifng (Fig. 1E), notch1b, grhl1 and foxi3b32,33.

Two populations of ionocytes, cells involved in ion transport in the gills, were identified. H+-ATPase-rich cells (HR cells) and Na+/K+-ATPase-rich cells (NaR cells) were identified in our dataset through their expression of foxi3a and foxi3b (Fig. 1E). These genes were present in both computationally-defined clusters. Close examination of the top differentially expressed genes in these two cell clusters showed that one expressed a high level of atp6v1c1b and atp6v1aa encoding H+-ATPase subunits. These cells were further identified as HR cells34. The other cluster expressed a high level of atp1b1b and atp1a1a.1 for Na+/K+- ATPase subunits, markers for NaR cells35 and were annotated as such. Moreover, NaR cells showed high mitochondrial gene content, as shown in Fig. 1D, and this feature is consistent with the high mitochondrial content of mitochondrion-rich ionocytes1.

Furthermore, we have identified novel cell populations in the gills. Two cell clusters expressing significant levels of the transcription factor, pou2f3, advilin (avil) and villin 1 (vill1) were annotated with identities, tuft cells I and tuft cells II. These cells were given such identities due to their resemblance in gene expression to mammalian tuft cells, which display a tuft-like brush of microvilli36. Tuft cells found in the respiratory tract, in close proximity to pulmonary neuroendocrine cells in lungs, represent an epithelial cell type with both sensory and secretory properties37. Genes orthologous to mammalian tuft cell markers, such as mydgf, pgs2b, and gng13a were highly expressed in tuft cells I, whereas mydgf, pgs2b, trpm4a and alox5a were highly expressed in tuft cells II. Both clusters shared distinctly high expression of pleg2.

**Identification of O2-chemoreceptive neuroepithelial cells and their expression of unique markers.** Neuroepithelial cells of the gills were identified based on expression of the reporter genes, EGFP and slc18a2, the latter of which encodes the vesicular monoamine transporter, Vmat2 (black arrowheads, Fig. 2A). We previously established that EGFP recapitulates the expression of slc18a2 in the ETvmat2:EGFP transgenic line, and expression of both genes in the gills was limited only to serotonergic NECs16. Moreover, high expression of tph1a and DOPA decarboxylase (ddc) indicates a serotonergic phenotype in these cells. tph1a and ddc were expressed predominantly in the NEC cluster (Fig. 2A) with an average log-fold increase of 3.42 and 1.29, respectively, in NECs compared with other gill cells. In addition, some cells in the neuron cluster adjacent to NECs in the UMAP plot also expressed low levels of tph1a (red arrowhead, Fig. 2A), consistent with serotonergic neurons of the gill filament32. Moreover, the synaptic vesicle glycoprotein 2A (sv2a) and the serotonin transporter, SERT (slc6a4a), were highly differentially expressed in NECs (Fig. 2A), providing additional verification.

In the cell atlas, NECs were separated from other cell clusters, illustrating their unique transcriptomic profile. Differential gene expression (DE) analysis of NECs against all other gill cells in the dataset indicated that 617 genes were differentially expressed in NECs (based on adjusted P-value < 0.05). The most differentially expressed genes in NECs are listed in Table 1. Among these, regulators of G-protein signaling, such as slc:ch211-196h16.12 (encoding regulator of G-protein signaling 5-like, Rgs5al), rgs5a and rgs4, were among the most differentially expressed and were up to a 6 log-fold increase from the global average. A few G-protein genes, such as slc:rd2-27j5.5 and rasd1, were also highly expressed in NECs. NECs also had exclusively high expression of gastrin releasing peptide, gpr, and retinaldehyde dehydrogenase 2, aldha2a2, for neuropeptide signaling and retinoic acid production, respectively. In addition, a few transcription factors, zgc:56628, pbx1a, met2a1, ascl1a and bx7.1, were uniquely expressed in NECs. Moreover, the glial cell line-derived neurotrophic factor receptor, gfra3, was found to be highly expressed. NECs also expressed genes of the kcn family of voltage-gated K+ channels, including kcn2d and kcnip3b, the latter of which encodes the channel-interacting protein, calsenilin (Supplementary Table S1).

Gill NECs are known for their O2 sensing properties3–4. To investigate potential molecular O2 sensors within zebrafish NECs, we examined top expressing mitochondrial genes in the NEC cluster since a signature profile...
of mitochondrial gene expression has been linked to the exquisite O₂ sensitivity of carotid body chemoreceptor type I cells. We found the NADH dehydrogenase (ubiquinone) subunit \( (\text{ndufa4l2a}) \) and the cytochrome c oxidase subunit 8A-like \( (\text{si:dkey-85n7.8}) \) to be highly expressed in NECs (Fig. 2B; Table 1). \text{ndufa4l2a} \) was ranked as the fifth differentially expressed gene with an average log-fold increase of 3.34, and \text{si:dkey-85n7.8} \) was ranked seventh with an average log-fold increase of 2.79 in NECs. As demonstrated in the cell atlas, these high expression values were unique to NECs, as their expression in other cell clusters was much lower (Fig. 2B). Moreover, NECs also showed high expression of hypoxia inducible factor genes, \text{epas1a/hif-2aa} \) and \text{epas1b/hif-2a} \). In particular, \text{epas1a/hif-2aa} \) was highly differentially expressed in NEC and endothelial cell clusters, and was found at lower levels of expression in other cell types (Fig. 2B). The genes, \text{rgs4} \) and \text{rgs5a}, encode regulator of G-protein signaling proteins and were highly expressed in NECs (see Fig. 1E and Table 1). Both \text{rgs4} \) and \text{rgs5} \) were shown to be targets of HIF in carotid body type I cells, and \text{rgs5a} \) a target of Hif2α in O₂-sensitive conditions.

**Figure 2.** Expression of signature genes found in neuroepithelial cells (NECs). (A) UMAP plots showing the log-normalized counts of selective genes involved in identification of NECs. Colour intensity is proportional to the expression level of a given gene. Scales indicate relative gene expression. Black arrowheads indicate high expression in the NEC cluster; red arrowhead indicates expression in the neuron cluster. (B) UMAP plots showing the normalized counts of representative genes that may be involved in O₂ sensing in NECs. See text and Table 1 for gene description.
Differential expression (DE) analysis of neurons against all other gill cells showed 501 genes, top differentially expressed genes in neurons (Fig. 3). GFRAL and SCG3 were shown to be widely expressed in in the two clusters, while the expression level was significantly higher in NECs. Furthermore, a number of genes snap25a for exocytotic neurotransmitter release was exclusively found in NECs and neurons showed that they shared gene expression characteristics of excitable or neurosecretory cells. Close examination of the subset data containing only NECs and neurons showed that they shared gene expression characteristics of excitable or neurosecretory cells. Although more prevalent in NECs, both cell clusters exhibited profound expression of membrane transporters, the two clusters were extracted for comparison (Fig. 4A). Close examination of the subset data containing only NECs and neurons showed that they shared gene expression characteristics of excitable or neurosecretory cells. Although more prevalent in NECs, both cell clusters exhibited profound expression of membrane transporters, such as subunits of Na+/K+-ATPase, atp1a3b, atp1b2a (Fig. 4B). Genes encoding the calcium binding protein synaptotagmins, syt1a and syt11a, were expressed highly in NECs and neurons (Fig. 4C). In addition, synaptosomal-associated protein (snap25a) for exocytotic neurotransmitter release was exclusively found in the two clusters, while the expression level was significantly higher in NECs. Furthermore, a number of genes adrenal medullary chromaffin cells. Thus, high expression of rgs4 and rgs5a in zebrafish NECs may be related to high endogenous expression of hif2a.

NECs also expressed slc16a3 at a significantly higher level than other cell types, though at a lower log-fold change of 0.53 (Supplementary Table S1). This gene encodes the monocarboxylate transporter MCT4, which has recently been implicated in a lactate-sensing mechanism in carotid body type I cells, and there is evidence that gill NECs participate in lactate sensing in rainbow trout (Oncorhynchus mykiss).

### Identification of the gill neuron cluster.

The neuron cluster in the cell atlas expressed high levels of neuronal markers, such as pvalb8 and nrnx2a (arrowhead, Fig. 3). pvalb8 encodes parvalbumin 8 and is highly expressed in GABAergic interneurons in the rodent brain. However, we did not detect significant expression of genes involved in gamma-aminobutyric acid (GABA) synthesis or uptake in the neuron cluster. Neurons also showed high expression of transcription factors, lmx1a and lmx1b, and their mammalian orthologues were shown to be important to survival of dopaminergic neurons in mammals. Differential expression (DE) analysis of neurons against all other gill cells showed 501 genes, whose expression was significantly higher in neurons (adjusted P-value < 0.05). The top differentially expressed genes in neurons are listed in Table 2. Other genes that were highly expressed in neurons include prr15la, vill and stom. Neurons also uniquely expressed transcription factors, foxa, foxa1 and foxa3. In addition, genes encoding Ca2+-binding proteins, ano2b and anxa5b, were highly differentially expressed in neurons.

### Contrasting expression profiles between NEC and neuron clusters.

The NEC and neuron clusters were positioned closely in the UMAP reduction and had overlapping gene expression. To examine this in detail, the two clusters were extracted for comparison (Fig. 4A). Close examination of the subset data containing only NECs and neurons showed that they shared gene expression characteristics of excitatory or neurosecretory cells. Although more prevalent in NECs, both cell clusters exhibited profound expression of membrane transporters, such as subunits of Na+/K+-ATPase, atp1a3b, atp1b2a and atp1a3a (Fig. 4B). Genes encoding the calcium binding protein synaptotagmins, syt1a and syt11a, were expressed highly in NECs and neurons (Fig. 4C). In addition, synaptosomal-associated protein (snap25a) for exocytotic neurotransmitter release was exclusively found in the two clusters, while the expression level was significantly higher in NECs.

### Table 1. The top 25 most abundant genes in the neuroepithelial cell (NEC) cluster. Average log-fold change indicates higher expression over other cell types. The adjusted P-value for each gene was < 0.05. See Supplementary Table S1 for the full list of genes within each cell cluster.

| Rank | Gene | Gene name or encoded protein | Avg. log-fold change |
|------|------|-----------------------------|----------------------|
| 1    | slc21a1-196h16.12 | Regulator of G protein signaling 5-like | 6.67 |
| 2    | rgs5a | Regulator of G protein signaling 5a | 4.23 |
| 3    | grp  | Gastrin-releasing peptide | 3.48 |
| 4    | tph1a | Tryptophan hydroxylase 1 (tryptophan 5-monooxygenase) a | 3.42 |
| 5    | ndufa4l2a | NADH dehydrogenase [ubiquinone] 1a subcomplex subunit 4-like 2a | 3.34 |
| 6    | abhd1a2 | Retinal Dehydrogenase 2 | 2.82 |
| 7    | sdc2-357n7.8 | Cytochrome c oxidase subunit 8A, mitochondrial-like | 2.79 |
| 8    | sdc2-27j5.5 | GTP-binding protein Rhos | 2.63 |
| 9    | scgn | Secretagogin, EF-hand calcium binding protein | 2.62 |
| 10   | CARZ01030107.1 | CARZ01030107.1 (Clone-based (Ensembl) gene) | 2.41 |
| 11   | atp1a3b | ATPase Na+K+ transporting subunit alpha 3b | 2.37 |
| 12   | col18a1a | Collagen type XVIII alpha 1 chain a | 2.36 |
| 13   | atp1b2a | ATPase Na+K+ transporting subunit beta 2a | 2.26 |
| 14   | rgs4 | Regulator of G protein signaling 4 | 2.16 |
| 15   | zgc:56628 | LIM domain transcription factor LMO4 | 2.16 |
| 16   | vamp2 | Vesicle-associated membrane protein 2 | 2.02 |
| 17   | syt1a | Synaptotagmin Ia | 1.97 |
| 18   | pbxla | Pre-B-cell leukemia homeobox 1a | 1.94 |
| 19   | rads1 | RAS, dexamethasone-induced 1 | 1.93 |
| 20   | scg3 | Secretogranin III | 1.86 |
| 21   | gfrn3 | GDNF family receptor alpha 3 | 1.83 |
| 22   | cplx2 | Complex 2 | 1.82 |
| 23   | tnnclb | Troponin C type 1b (slow) | 1.81 |
| 24   | meis2a.1 | Meis homeobox 2a | 1.78 |
| 25   | qdpra | Quinoid dihydropteridine reductase a | 1.76 |

*Note: The table includes the top 25 most abundant genes in the neuroepithelial cell (NEC) cluster. Average log-fold change indicates higher expression over other cell types. The adjusted P-value for each gene was < 0.05. See Supplementary Table S1 for the full list of genes within each cell cluster.*
involved in synaptic vesicle docking or fusion preceding neurotransmitter release, including stx1b, vamp2 and cplx2, were also highly expressed in both clusters (Fig. 4D). We compared the transcriptomes of both clusters to further investigate unique expression in NECs. DE analysis between the two clusters revealed genes that were differentially expressed in either cluster, and the top 20 genes are presented in a heatmap (Fig. 4E). The results showed that, when compared to neurons, the top differentially expressed genes found in NECs were consistent with previous DE analysis against all other cells. These genes include rgs5a, rgs5al, tph1a, ndufa4l2a, and grp. It was also revealed that epas1a and epas1b were significantly highly expressed in NECs compared to neurons. Moreover, the gene encoding the Ca²⁺-binding protein, secretagogin (scgn), was highly differentially expressed in NECs. On the other hand, neurons had high expression of epithelial cell adhesion molecule (epcam), claudins (cldnh, cldnb and cldn7b), delayed rectifier K⁺ channel subunit, kcnc1a, and plac8 onzin related protein 6 (ponzr6). When compared to neurons, these genes were down-regulated in NECs.

Expression of receptors involved in neurotransmission or modulation. The present study also mapped the distribution of genes encoding neurotransmitter receptors in gills. Only receptors showing significant expression were selected to be shown in Fig. 5A–C. 5-HT receptors were found throughout many clusters. In particular, CR759836.1 (encoding 5-HT₃A-like receptor) was highly expressed in neurons, occupying more than 90% of the cells in that cluster (arrowhead, Fig. 5A). The average expression of CR759836.1 in neurons was also significantly higher than in other gill cells, with a difference of more than a 4 log-fold change (Table 2). On the other hand, htr3a was found in some endothelial cells, tuft cells and pavement cells. Moreover, htr2b was expressed in many endothelial cells, pavement cells and tuft cells. The high expression of htr2b appeared to concentrate in the endothelial cell cluster (arrow, Fig. 5A). The gene for inhibitory 5-HT₁A receptor, htr1ab, was found to be most abundant in NECs (arrow, Fig. 5A) and platelets, but nearly absent in other clusters.

In contrast to 5-HT receptors, expression of cholinergic receptors, targets of ACh, was less abundant and generally restricted to NECs and neurons. The nicotinic ACh receptor α2b subunit gene, chrna2b, was limited to NECs, neurons and two tuft cell clusters (arrowheads, Fig. 5B). The α6 subunit, chrna6, showed expression primarily in NECs (Fig. 5B). The β4 and β3a subunit genes for ACh receptors, chrnb4 and chrnb3a, were present mainly in neurons and NECs, respectively (Fig. 5B).

The presence of all DA receptors was investigated; however, only genes for receptors in the D₂-like family were detected. The expression of D₁ receptor, ddrk1a, was found in a few cell clusters, including NECs, endothelial
and htr1d receptors were less abundant but present in cell populations, e.g. expression of socs3a, cldni, tmsb1, cfl1l, apoeb, are expected to contribute to the structure or growth of cells, including gadd45bb, gadd45ba, Other differentially expressed genes in regulation of the cell cycle, such as gadd45ga. and cluster. Among the top differentially expressed genes in hypoxia across all cell types, many were directly involved in Supplementary Table S2. Differentially expressed genes in hypoxia were mostly present in the endothelial cell shown in the heatmap in Fig. 6C. A full list of all genes that were differentially expressed in hypoxia is provided expressed genes in hypoxia compared to normoxia were selected and their expression in each cell identity is expression analysis between hypoxia and normoxia with all cell clusters in the dataset. The top 40 differentially cells, tuft cells I, neurons and ENPC remained largely unchanged in their relative frequency. In addition, mucous types were NECs, endothelial cells and platelets. In contrast, leukocytes, immune cells, HR cells, HSPC, proliferating cells, tuft cells II and fibroblasts showed a decrease in relative frequency in hypoxia. Changes of cell proportion and transcription in chronic hypoxia. The integrated data contained cells from animals acclimated to hypoxia and normoxia. After demultiplexing the attached MultiSeq barcodes, cells from different treatments could be distinguished. Results showed that the 16 cell identities were present in both treatments. However, cells from fish acclimated to hypoxia had different proportions of cell identities compared to fish in normoxia. As shown in the stacked bar plot in Fig. 6A, endothelial cells and pavement cells together accounted for less than 50% of the overall cell population. However, in normoxia, endothelial cells and pavement cells together accounted for more than 50% of the two largest cell populations in both groups. When compared to normoxia, the hypoxia group had greater proportions of endothelial cells and pavement cells that, when combined, accounted for more than 50% of the overall cell population. However, in normoxia, endothelial cells and pavement cells together accounted for less than 50% of the overall cell population. The proportions of individual cell identities are presented in the bar plots in Fig. 6B. Results indicated that 3 cell identities showed an increase in cell proportion in hypoxia. These cell types were NECs, endothelial cells and platelets. In contrast, leukocytes, immune cells, HR cells, HSPC, proliferating cells, tuft cells II and fibroblasts showed a decrease in relative frequency in hypoxia. In addition, mucus cells, tuft cells I, neurons and ENPC remained largely unchanged in their relative frequency. To determine the effects of chronic hypoxia at the transcriptional level, we conducted a differential gene expression analysis between hypoxia and normoxia with all cell clusters in the dataset. The top 40 differentially expressed genes in hypoxia compared to normoxia were selected and their expression in each cell identity is shown in the heatmap in Fig. 6C. A full list of all genes that were differentially expressed in hypoxia is provided in Supplementary Table S2. Differentially expressed genes in hypoxia were mostly present in the endothelial cell cluster. Among the top differentially expressed genes in hypoxia across all cell types, many were directly involved in regulation of the cell cycle, such as gadd45bb, gadd45ba, and gadd45ga. Other differentially expressed genes are expected to contribute to the structure or growth of cells, including cldni, tmsb1, cfl1l, apoeb, and socs3a. Interestingly, NECs showed the highest expression of several genes that were upregulated by hypoxia, including

| Rank | Gene | Gene name or encoded protein | Avg. log-fold change |
|------|------|-----------------------------|----------------------|
| 1    | CR759836.1 | 5-Hydroxytryptamine receptor 3A-like | 4.06 |
| 2    | pvalb8 | Parvalbumin 8 | 3.74 |
| 3    | siCh211-152c2.3 | Secretogranin III | 3.14 |
| 4    | socs3a | Potassium voltage-gated channel, Shaw-related subfamily, member 1a | 2.56 |
| 5    | prr15la | Proline rich 15 like a | 2.29 |
| 6    | hmx1bb | LIM homeobox transcription factor 1, beta b | 2.24 |
| 7    | vill | Villin-like | 2.13 |
| 8    | stom | Stomatina | 1.99 |
| 9    | ano2b | Anoactinin 2b | 1.96 |
| 10   | siCh211-207j7.2 | Protein IWS1 homolog A-like | 1.90 |
| 11   | syt1a | SYNAPTOTAGMIN 1a | 1.89 |
| 12   | zgc:112408 | zgc:112408 | 1.85 |
| 13   | hepacam2 | HEPACAM family member 2 | 1.79 |
| 14   | gfnl1 | GDNF family receptor alpha like | 1.77 |
| 15   | neunx2a | Neurexin 2a | 1.66 |
| 16   | rfxon1 | RNA binding fox-1 homolog 1 | 1.65 |
| 17   | zgc:101731 | SNARE_SNAP25N and SNARE_SNAP23C domain-containing protein; synaptoosomal-associated protein 25 | 1.62 |
| 18   | anks4b | Ankyrin repeat and sterile alpha motif domain containing 4B | 1.61 |
| 19   | hipkca | Inositol-trisphosphate 3-kinase Ca | 1.60 |
| 20   | ret | Ret proto-oncogene receptor tyrosine kinase | 1.59 |
| 21   | foxa | Forkhead box A sequence | 1.58 |
| 22   | siCh211-56a11.2 | Protein phosphatase 1 regulatory subunit 17 | 1.54 |
| 23   | pclob | Piccolo presynaptic cytomatrix protein b | 1.52 |
| 24   | ggtcb | Gamma-glutamylcyclotransferase b | 1.43 |

Table 2. The top 25 most abundant genes in the neuron cluster. Average log-fold change indicates higher expression over other cell types. The adjusted P-value for each gene was < 0.05.
Figure 4. Comparison of gene expression between the NEC and neuron clusters. (A) UMAP plot showing the subset of the cell population containing NECs and neurons only. (B) UMAP plots showing the log-normalized expression of genes encoding membrane transporters. (C) UMAP plots showing the log-normalized expression of genes involved in Ca\(^{2+}\)-mediated signals. (D) UMAP plots showing the log-normalized expression of genes involved in neurotransmitter release. (E) Heatmap of the top 20 highly differentially expressed genes in NECs and neurons. Scaled gene expression is shown by colours, where yellow indicates relatively high expression and purple indicates relatively low expression. See text and Tables 1 and 2 for gene description.
the HIF genes, epas1a and epas1b, and the G-protein signaling regulator, si:ch211-196h16.12. We did not detect significant hypoxic upregulation of gene expression in neurons.

**Differential gene expression analysis in NECs exposed to chronic hypoxia using DEsingle.** To further examine the effects of chronic hypoxia on transcription in NECs with a higher level of accuracy, differential gene expression analysis was conducted using DEsingle with the subset data containing only NECs. The results revealed that 30 genes were significantly differentially expressed in cells from hypoxia compared to
normoxia (adjusted P-value < 0.05). Among these genes, 21 were upregulated (within red bracket, Fig. 7) and 9 were down regulated (within blue bracket, Fig. 7) in hypoxia. The top highly regulated genes in hypoxia included apoe, krt91, cfd, phlda2, junba; ribosomal proteins, such as rp7, rpl10a, rpl6, rps6; and elongation factors, eef1g and eef1b2. In particular, phlda2 is believed to be involved in the positive regulation of apoptosis45, whereas junba is a proto oncogene, which could contribute to cell proliferation46. Most of the genes that were highly upregulated were encoding ribosomal proteins or elongation factors. On the other hand, genes that were down regulated in hypoxia included lgals17, isg15, zgc:152791, dnaese14.1, irf1b, vill1, rasd1, scgrn and hist2h2l. Aside from rasd1 and scgrn, no other genes involved in the chemosensory transduction of O2 signalling were found to be differentially regulated after 2 weeks of chronic hypoxia.

Discussion
Investigating the mechanisms of O2 sensing and chemotransduction in the fish gill has been limited due to our lack of understanding of the cell types involved and the molecular basis of these processes. Using a transgenic zebrafish, the present study employed single-cell RNA sequencing and developed a transcriptomic atlas of cells from the gills. The single cell atlas presents information regarding gene expression profile, cell identification, and transcriptomic similarities among 16 different cell populations, including chemoreceptive NECs, neurons, endothelial cells and ionocytes, using unbiased clustering analysis. We were able to identify expression of numerous genes implicated in O2 sensing and chemosensory signaling, including those involved in neurotransmission, neurosecretion, and those encoding ion channels and mitochondrial proteins. Moreover, we demonstrated transcriptional changes in gill cells following exposure to chronic hypoxia.

Transcriptomic profiling of chemoreceptive neuroepithelial cells. In the cell atlas, chemoreceptive NECs were transcriptionally similar to the neuron population and separated from other gill cells. NECs of the

Figure 6. Effects of chronic hypoxia on gill cell composition and global gene expression. (A) Stacked bar graph shows the relative difference in % cell composition between normoxic and hypoxic conditions. Cell types are coded by colour and are indicated at the right. (B) Bar graphs showing changes in relative cell frequency in normoxia (green) vs. hypoxia (orange) in each cell cluster. (C) Heatmap showing the distribution of the top 40 highly differentially expressed genes in chronic hypoxia compared to normoxia. Scaled gene expression is shown by colours, where yellow indicates relatively high expression and purple indicates relatively low expression.
Fish gill are considered to be neuron-like, or paraneurons. NECs showed a unique transcriptome and genes involved in 5-HT production, such as *tph1a*, were among those highly expressed. In zebrafish, three paralogs of *tph* genes are present in the central nervous system: *tph1a*, *tph1b* and *tph2*. In a recent report, *tph1a* expression was described in cutaneous NECs and cells of the pharyngeal arch in zebrafish. The present results of *tph1a* expression in O2-sensitive NECs of the gill filaments provide evidence that 5-HT is produced within NECs, and support accumulating evidence that 5-HT is an important neurotransmitter involved in O2 sensing in the gill. In addition, the high expression of *slc6a4a*, which encodes the serotonin transporter (SERT) and displays broad expression in the zebrafish brain, may indicate reuptake of 5-HT by NECs from the synaptic cleft after the release of 5-HT during signal transmission. Furthermore, the gill cell atlas recapitulated expression of *slc18a2* in the NEC cluster, confirming the identity of these cells. The *slc18a2* gene has previously been characterized in gill NECs in zebrafish and encodes a vesicular monoamine transporter involved in storage of 5-HT.

In a recent study, in which single-nuclei RNA sequencing was used to obtain a transcriptomic profile of gill cells in Atlantic salmon, a cell cluster was assigned the identity of “neuroepithelial cells.” In that study, however, the authors did not identify markers associated with chemosensory NECs of the gill epithelium, but instead characterized the cell cluster based on expression of genes associated with cells derived from the neuroepithelium that are uniquely involved in neural development. Such cells are also called neuroepithelial cells but, unlike gill NECs, are involved in the production of migratory cells of the neural crest. While there is evidence of neural crest cells in the gills of zebrafish, chemosensory gill NECs appear not to be associated with the neural crest. It is presently unclear whether the cell cluster described in West et al. would fit with our model of chemosensory NECs.

In the present article, NECs showed high expression of mitochondrial genes, *ndufa4l2a* and *sidkey-85n7.8* or *cox8al* (cox8a-like subunit). The mitochondrial electron transport chain (ETC) subunits, NDUF4L2, COX4I2 and COX8B, were highly expressing genes found in mammalian chemoreceptive type I cells. COX4I2 and COX8B subunits were suggested to interact to regulate O2 diffusion across the mitochondrial inner membrane, a process that can be highly sensitive to a decrease in PO2, marking a potential site of acute O2 sensing in these cells. The highly expressed cox8a-like subunit in NECs in zebrafish could pose a potential site of O2 sensing. In mouse, the COX8B gene contained HIF binding sites in the promoter region and was proposed to be over-expressed due to high expression of HIF2α genes. In addition, expression of *Epas1*, which encodes HIF2α, was necessary for acute O2 sensing by carotid body type 1 cells as well as the ventilatory sensitivity to hypoxia in mice. In our dataset, we observed concurrent high expression of the HIF2α genes, *epas1a* and *epas1b* in zebrafish NECs, suggesting a key role of HIF2α in acute O2 sensing during vertebrate evolution.

Differential expression analyses showed that NECs had high expression of genes involved in membrane potential regulation, Ca2+ signaling and neurosecretion, compared to other cell clusters. These observations are...
aligned with the putative \( O_2 \)-sensory and neurosecretory roles of NECs\(^3\). Many highly differentially expressed genes in NECs overlapped with those found in carotid body \( O_2 \)-chemoreceptive type 1 cells\(^{18}\), and support the functional homology between these chemoreceptors. Mammalian type 1 cells expressed high levels of G-proteins (such as \( \text{Gnas} \) and \( \text{Gnbi21} \)) and a G-protein regulator (\( \text{Rgs4} \)). Similarly, in NECs, pronounced expression of G-proteins (\( \text{gpg13b, rasd1} \)) and G-protein regulators (\( \text{rgs5a, rgs4} \)) were observed, G-protein subunits and coupled receptors have been found in NECs of the obligate air-breathing fish, \( \text{Arapaima gigas} \)\(^{46}\), though G-protein-mediated signaling in fish species remains poorly studied. In the mammalian carotid body, G-protein-coupled receptors, including those activated by 5-HT, were observed to modulate type 1 cell membrane potential and hypoxic sensory transduction or transmission\(^{57,58}\). Our observations of high expression of G-protein regulators could suggest sensory modulation by G-proteins in NECs. In addition, NECs had an exclusively high expression of retinaldehyde dehydrogenase (\( \text{aldh1a2} \)). Retinaldehyde dehydrogenase catalyzes the chemical reaction to produce retinoic acid, which is important for the growth and development of many cells\(^{59}\). The retinoic acid receptor gene, \( \text{rarg} \), was found in all clusters in our dataset. The specific role of retinaldehyde dehydrogenase in NECs is unclear but its high expression could indicate a potential retinoic acid signaling pathway through which NECs communicate with other cells.

**Receptor expression in neuroepithelial cells and neurons represents potential targets for neurotransmission or modulation.** Our analysis revealed a distinct neuron population, whose transcriptome was similar to NECs. Their high expression of membrane Na\(^+/K\)\(^+\) pumps, Ca\(^{2+}\) binding proteins and synaptic proteins are indicators of critical processes involved in neuronal function, such as regulation of transmembrane ionic gradients, and Ca\(^{2+}\)-dependent neurotransmission. This cluster of cells may include serotoninergic neurons that have been described previously in the zebrafish gill filaments\(^{11,16}\), as some cells within the neuron cluster showed expression of 5-HT synthesizing enzyme genes, \( \text{tph1a} \) and \( \text{ddc} \). However, the specific function and role of the whole neuron cluster would require further investigation.

Despite their ambiguous identity, these neurons are candidates for involvement in post-synaptic, hypoxic signal transmission or regulation, as they express a variety of neurotransmitter receptors. The most highly differentially expressed gene in neurons was \( \text{CR759836.1} \), encoding 5-HT\(_3\)-like receptors. Because at least one signal transmission or regulation, as they express a variety of neurotransmitter receptors. The most highly differentially expressed gene in neurons was \( \text{CR759836.1} \), encoding 5-HT\(_3\)-like receptors. Because at least one

**Transcriptional changes following chronic hypoxia.** Our results from DE analysis revealed that endothelial cells were most affected by hypoxia. Endothelial cells have long been recognized as part of a vascular \( O_2 \)-sensing system\(^{25}\). Their high expression of HIF genes, \( \text{epasa1} \) and \( \text{epasa1b} \), in the present study indicates their potential ability to respond to hypoxia. Chronic hypoxia induces proliferation and vascular remodeling\(^{26}\), and our results of increased expression of cell cycle regulator genes and cell structural components support this notion. Differential expression analysis using the DEsingle method revealed that chronic hypoxia upregulated expression of genes encoding ribosomal proteins or elongation factors in NECs. An increase in expression of these genes could indicate ongoing cell growth or proliferation, as we observed with the increased proportion of NECs in the hypoxia group. Two weeks of chronic hypoxia was previously shown to increase the proportion of EGFP-labeled NECs, cell size and EGFP expression\(^{16}\). Although the increase in EGFP expression, which reflects an increase in \( \text{slc18a2} \), could not be detected in our data, possibly due to the lack of sequencing depth, our results of increased proliferation agree with previous findings. This increased proliferation of NECs in chronic hypoxia
may well be associated with the observed high expression of the Hif2α genes, epas1a and epas1b. In this regard, it is noteworthy that exposure to chronic hypoxia also causes hyperplasia of mammalian carotid body type I cells and this response is dependent on endogenous HIF2α expression in these cells\textsuperscript{66}. The DEsingle analysis did not show transcriptional changes in genes expected to be involved in chemotransduction. These may have occurred before the end of the 2 weeks hypoxic treatment, possibly even during the first few days, as 36 h of hypoxic exposure has previously been shown to cause changes in the HIF pathway in zebrafish\textsuperscript{66}.

Conclusion

The current study has presented the transcriptomic profiles of NECs, neurons and other important cell types of the zebrafish gill using single-cell RNA sequencing under normoxic and hypoxic conditions. We confirmed the identity of NECs in our data by using a transgenic line in which slc18a2 expression could be identified and matched with expression of other genes that were not previously identified in NECs. In addition, differential gene expression analysis revealed potential pathways of hypoxic signal transduction via mitochondrial signature features and markers of neurotransmission in NECs. Identification of the expression of neurotransmitter receptors found in different cell populations provided evidence for putative synaptic or paracrine signaling mechanisms. The gill cell atlas generated in this article presents a valuable resource for future studies on O2 sensing in zebrafish and other aspects of the biology of fish gills.

Methods

Animals. Transgenic ETvma2:EGFP zebrafish (Danio rerio) used to identify O2-sensitive NECs\textsuperscript{16} were bred and raised to 12 months before experiments. Fish were maintained at 28 °C on a 14 h-10 h light–dark cycle in the Laboratory for the Physiology and Genetics of Aquatic Organisms at the University of Ottawa. All procedures for animal care and experimentation were performed according to protocol BL-1760, which was approved by the University of Ottawa Animal Care Committee, and following guidelines provided by the Canadian Council on Animal Care (CCAC). In addition, these procedures followed the guidelines provided by ARRIVE (Animals in Research: Reporting In Vivo Experiments).

Chronic hypoxia. For chronic hypoxia treatment, adult fish were housed in closed, static 2 l aquaria for 14 days. Fish were fed and given water changes twice per day. A Pegas 4000 MF gas mixer (Columbus Instruments, Columbus, OH, USA) was used to mix compressed air and 100% N2 and generate the appropriate partial pressure of O2 (PO2) to deliver to aquaria through suspended air stones. In the hypoxia group, 4 fish were acclimated to severe hypoxia through a gradual decrease in PO2 from 158 to 35 mmHg over the course of 1 week. Fish were then kept at 35 mmHg for an additional week. This PO2 level was above the critical O2 tension for zebrafish (~ 20 mmHg) and has been shown to induce increases in cell size and number in NECs\textsuperscript{16}. As a control, the normoxia group had 4 fish kept in aquarium with continuously aerated water throughout the treatment period.

Cell dissociation sample preparation. After 14 days of hypoxia treatment, adult fish were stunned by a blow to the head and decapitated for tissue collection. Techniques for tissue extraction and cell dissociation were adapted from previous studies\textsuperscript{3,16} and carried out under sterile conditions. In a laminar flow hood, whole gill baskets were removed from the head and quickly immersed in ice-cold phosphate-buffered solution (PBS) containing (mM): NaCl 137, NaHPO4 15.2, KCl 2.7 and KH2PO4 1.5; pH 7.8. Gill baskets were rinsed in wash solution containing 1% FBS before resuspension in PBS. Cells were counted manually with a hemocytometer and viability above 89% were submitted to the StemCore Laboratories at the Ottawa Hospital Research Institute (Ottawa, Canada) for preparation and sequencing. For single-cell cDNA library preparation, barcoded cells were centrifuged again and dispersed in fresh PBS.

Cell samples of two treatments were individually “barcoded” to allow samples to be combined prior to library preparation and sequencing. The multiplexing procedure was carried out according to the MULTI-seq protocol\textsuperscript{10}. Cells were first centrifuged to obtain the cell pellet. 150 µl of 200 nM anchor/200 nM barcode solution was added to cells and cells were resuspended. The lipid-modified DNA oligonucleotide anchor and unique oligonucleotides served as barcodes and were bound to cells during this process. Each sample received a different barcode sequence, which allowed computational demultiplexing later on. After 10 min of incubation at room temperature, 200 nM of co-anchor was supplemented to stabilize anchoring of barcodes at the membrane. Cells were incubated on ice for 5 min, then centrifuged at 1300 rpm for 3 min. The cell pellet was washed twice with PBS containing 1% FBS before resuspension in PBS. Cells were counted manually with a hemocytometer and samples were pooled to reach a final concentration of 500–1000 cells/µl.

10x genomics single-cell library preparation and sequencing. Samples of dissociated cells with a viability above 89% were submitted to the StemCore Laboratories at the Ottawa Hospital Research Institute (Ottawa, Canada) for preparation and sequencing. For single-cell cDNA library preparation, barcoded cells from hypoxic and normoxic treatments were pooled. 3 samples of 15,000 cells (i.e. a total of 45,000 cells) were then processed with the Chromium Single Cell 3’ Assay (10x Genomics, San Francisco, CA, USA). cDNA synthesis and library construction were carried out according to the manufacturer’s protocol and the resulting...
cDNA libraries were amplified using PCR. Samples were sequenced on a NextSeq 500 sequencing system (Illumina, San Diego, USA) with a target read of 18,000 per cell.

**Bioinformatic analysis of single-cell RNA sequencing data.** The resulting sequencing data was first analyzed using the Cell Ranger pipeline (10x genomics) to demultiplex raw base cell files, perform alignment, aggregate outputs and generate feature-barcode matrices. The reference genome used in alignment was constructed using the Ensembl release 101 Danio genome annotation and the EGFP sequence. Matrices containing gene features and counts of single cells were further analyzed using the deMULTiplex software package to demultiplex sample barcodes in order to differentiate samples of different treatment groups.

**Seurat unsupervised analysis.** Bioinformatic analysis of the data was carried out using the Seurat package v3.2.169 within R v4.0.2. Cell quality was assessed through a combination of quality control metrics, including total number of expressed genes, number of unique genes and mitochondrial gene content. Outlier cells were defined if they were found outside of three absolute deviations from the median for any metric and excluded from subsequent analyses. Data were normalized and scaled in Seurat using NormalizeData and log normalized using a scale factor of 10,000. Principal component analysis (PCA) was performed on the scaled data and the first 30 principal components were used in the “FindNeighbors” function to determine the Shared Nearest Neighbour (SNN). Using the “FindClusters” function with a resolution of 0.2, cells showing similar expression were clustered. The multidimensional dataset was visualized in a UMAP graph generated with the first 30 principal components. UMAPs allowed graphical presentation of cell clusters with identity labels and visualization of marker gene expression. To determine unique marker genes of each cluster, Wilcoxon sum tests were performed using the “FindAllMarkers” function. Positive markers from the analysis were used to determine cell identity and new cluster identities were assigned using the “RenameIdents” function. In addition, cells were assigned a score for cell cycle using the “CellCycleScoring” function to determine their mitotic state. Heatmaps and dot-plots showing gene expression were generated using built-in functions in Seurat.

**Doublet removal.** An additional doublet removal was performed using the DoubletFinder package70, after the quality control was performed in Seurat. DoubletFinder performed PCA and used the principal component distance matrix to find each cell’s proportion of k nearest neighbors to predict doublets. A total of 1,148 predicted doublet cells were removed to reduce aggregated cells in the dataset. The returned output was used in subsequent analyses with Seurat.

**Differential expression analysis using DEsingle.** The Bioconductor package, DEsingle v1.10.0, was used to infer transcriptomic changes in hypoxia treatment in comparison to normoxia. DEsingle uses the zero-inflated negative binomial model to discriminate dropout zeros to detect differentially expressed genes with high accuracy71. For each cell cluster examined, an expression matrix containing gene counts and a group vector containing group information were created. These two inputs were passed to the “DEsingle” function to conduct the differential gene expression analysis. Only genes with statistical significance (adjusted P-value below 0.05) between hypoxia and normoxia were selected to be presented in heatmaps.

**Data availability**

The datasets generated during the current study are available in the Gene Expression Omnibus (GEO) repository, National Center for Biotechnology Information (NCBI) [Accession Identification GSE198044].

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Author contributions

WP and M.G.J. conceived and designed research; WP carried out the experimental procedures, performed RNA sequencing analysis and statistical testing; WP and M.G.J. interpreted results; R.S.G. and D.P.C. contributed to experimental design and RNA sequencing analysis; A.L.S. and C.A.N. provided the transgenic line and contributed to research design; W.P. and M.G.J. wrote and edited the manuscript; all authors have reviewed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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