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Letter to the editor Open Access

Single-cell RNA sequencing reveals gonadal dynamic changes during sex differentiation in hermaphroditic protogynous orange-spotted grouper (*Epinephelus coioides*)

DEAR EDITOR,

Sex differentiation is a complex process that requires many factors to regulate gonadal proliferation, differentiation, development, and organization. In teleosts, the molecular mechanisms of sex differentiation are diverse and unclear, especially in hermaphrodites. In the present study, single-cell RNA sequencing (scRNA-seq) was used to identify 15 cell types, including germ, follicle, and theca cells, in the gonads during sex differentiation in the hermaphroditic orange-spotted grouper (*Epinephelus coioides*). Two pre-follicle cell types were defined, and the differentiation trajectories of follicle cells were outlined. Notably, male-related genes were highly expressed in both pre-follicle and follicle cells, including *amh*, *sox9*, and *dmrt3* in pre-follicle cells and *dmrt1* in follicle cells. Oocytes exhibited two distinct states, with high expression of oocyte development-related genes in one state and spermatogenesis-related genes in the other state. Our findings provide novel insights into cell type and lineage tracing in the gonads during sex differentiation in a hermaphroditic species.

The reproductive strategies of teleosts are complex, including both gonochoristic and hermaphroditic species. Hermaphroditic fish can reverse sex during their life cycle, with the capability of producing mature male and female gametes within a single individual. Female-first maturation is defined as protogynous and male-first maturation is defined as protandry (Nagahama et al., 2021). The orange-spotted grouper (*E. coioides*), an important maricultural species in Asia, is a protogynous hermaphrodite and considered a good model for studying sex differentiation and sex reversal (Liu & De Mitcheson, 2009). Most orange-spotted groupers will develop into females after sex differentiation, with only a small proportion developing into males. The origin of germ cells and the reasons for this preference toward female-first maturation remain unknown and have long been of interest to scientists. Many genes are involved in sex differentiation (Han et al., 2019; Huang et al., 2019; Sun et al., 2017), but the mechanism underlying sex differentiation in groupers remains unclear. Therefore, to better understand how sex differentiation is regulated at the cellular level, we performed scRNA-seq and revealed gene expression patterns in sex differentiation.

We collected gonads from orange-spotted groupers at three developmental stages: undifferentiated gonads (80 days post-hatching (dph)), differentiating gonads (120 dph), and differentiated gonads (180 dph). Histological examination was conducted to identify the time point of sex differentiation. In the 80 dph grouper gonads, blood vessels had emerged, a hallmark of paired gonads (Figure 1A), and primordial germ cell (PGC)-like cells were found due to their unique morphology (Porras-Gómez et al., 2021). In the 120 dph grouper gonads, several germ cells were detected, and the ovarian cavity was formed (Figure 1A). In the 180 dph grouper gonads, primary growth stage oocytes were observed, indicating completion of sex differentiation (Figure 1A). Subsequently, we prepared single-cell suspensions from mixed and freshly dissected gonads at 80 dph (n=30), 120 dph (n=20), and 180 dph (n=5), each with two biological replicates (Figure 1B). In the sequencing data, 83 160 cells were profiled with approximately 37 000 reads per cell, and the median number of genes detected per cell was 1 112 (Supplementary Table S1 and Figure S1).

To further define the major cell types of the six samples, canonical correlation analysis (CCA, Stuart et al., 2019) was used to minimize batch effects, and all cells were combined from the three developmental stages. The aggregated cells were clustered into 25 clusters using uniform manifold approximation and projection (UMAP, Figure 1C; Supplementary Figure S2A), and cell distribution was changed in the three developmental stages (Supplementary Figure S2B). In our experiment, all clusters were divided into two classes, i.e., parenchymal and stromal cells. Parenchymal cells were further divided into follicle, theca, and germ cells based on the following marker genes: follicle cells (*gsdf*, *amh*, and *fshr*), theca cells (*cyp11a1*, *cyp17a1*, and *star*), gonocytes (*ddx4*, *nanos2*, *piwil1*, and *piwil2*), and oocytes (*h1foo*, *zar1*, and *lhx8*). As stromal cells accounted for over 90% of the total detected cells, they were further divided into four populations, i.e., vasculature, epithelial, immune, and uncharacterized stromal cells. The following marker genes were used to

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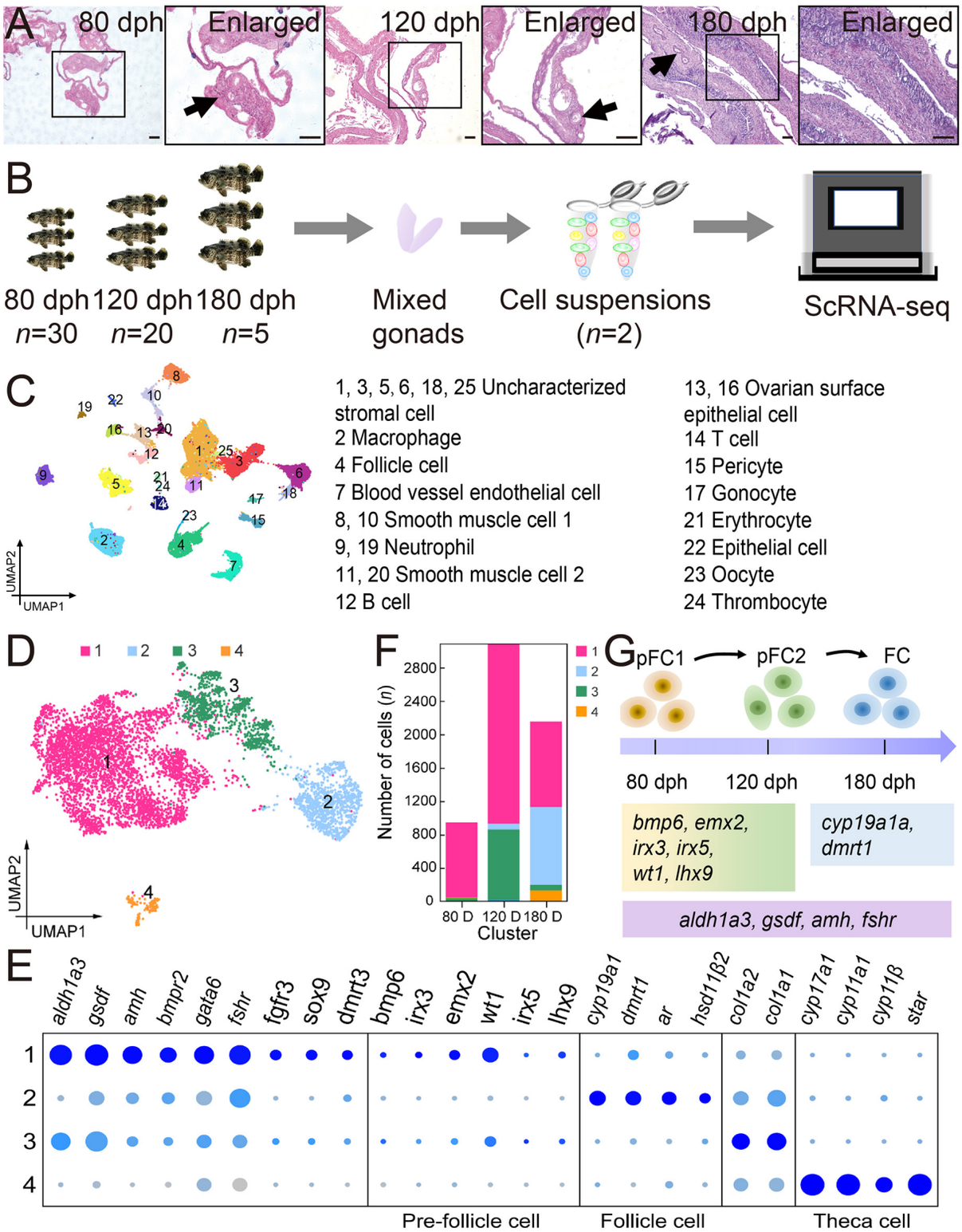

Figure 1 ScRNA-seq reveals dynamic changes during sex differentiation in orange-spotted grouper gonads

A: Gonadal histological morphology of orange-spotted grouper by hematoxylin-eosin (H&E) staining at 80 dph, 120 dph, and 180 dph. Enlarged figure shows higher magnification view of boxed area in previous figure. Black arrows represent blood vessels. Dph, day post-hatching. Scale bar: 50 μm. B: Overview of whole experiment. Fish were dissected from the cloacal aperture, with gonads and connective tissue then removed. Gonads were dissociated into single-cell suspensions for sequencing (*n*=2) and analysis. C: Visualization of 15 cell types among six samples in UMAP plot. Colors mark 25 distinct clusters identified by unsupervised clustering and characterized with differential gene expression and gene set enrichment analyses. Each dot represents a cell. D: Cluster 4 subcluster UMAP plot, with cells color-coded by computationally determined cell subtypes. E: Gene expression plots of selected genes. Cells expressing indicated genes are in blue, and relative intensity indicates relative expression levels. F: Histogram of distribution of cell clusters in three developmental stages. G: Schematic representation of potential differentiation of cluster 4 subclusters from 80 dph to 180 dph. FC, follicle cell; pFC, pre-follicle cell.

classify stromal cell subgroups: (1) vasculature cells: blood vessel endothelial cells (*vmf* and *fli1*), smooth muscle cells (*mylk*, *myh11*, *des*, and *smtnl1*), pericytes cells (*notch3*); (2) epithelial cells: ovarian surface epithelium (*stm* and *gata3*), epithelial cells (*fabp* and *sox10*); (3) immune cells: macrophages (*csf1r2* and *mpeg1*), neutrophils (*lce* and *cebpe*), B cells (*iglc5* and *iglc6*), T cells (*gfi1* and *il2rb*), erythrocytes (*hba* and *hbad*), thrombocytes (*thbs1* and *mpl*); and (4) uncharacterized stromal cells (*col1a1a* and *dcn*) (Supplementary Figure S3 and Supplementary Materials).

Cluster 17 cells were identified as gonocytes based on the expression of germ cell marker genes *ddx4*, *piwil1*, and *piwil2* and germ stem cell marker gene *nanos2* (Supplementary Figure S3). To improve the reliability of the dataset, *Ddx4* and *Piwil1* antibodies were used for immunohistochemical (IHC) analysis during sex differentiation (Supplementary Figure S4A–L), and proliferating cell nuclear antigen (PCNA) was used to detect the proliferative potential of the gonads (Supplementary Figure S4M–R). Detailed information on the antibodies used is provided in Supplementary Table S2. Results showed that *Ddx4* signals were located in all germ cells, especially in the PGC-like cells in 80 dph gonads (Supplementary Figure S4A, D). *Piwil1* signals were also detected in germ cells, especially in oogonia and oocytes in 180 dph gonads (Supplementary Figure S4I, L). PCNA signals were detected in the nuclei of PGC-like cells and oogonia, indicating that these cells had more proliferative activity (Supplementary Figure S4P, R). In addition, three encoded Tudor domain-containing (TDRD) protein genes (*tdrd6*, *tdrd7b*, and *tdrd9*) showed specific expression in the gonocytes, and may therefore be potential markers of gonocytes (Supplementary Figure S5). These results should facilitate future characterization and profiling of gonocytes.

We also analyzed the expression trends of all genes during sex differentiation when comparing gonocytes in the three developmental stages. We identified 2 555 genes that showed consistent increases across the three developmental stages (profile 7, Supplementary Figure S6A), including many reproductive and developmental genes, such as *ddx4*, *piwil1*, *piwil2*, *tdrd6*, *tdrd7*, and *tdrd9* (Supplementary Figure S6B). Profile 7 genes were mainly enriched in DNA replication and mTOR signaling pathway (Supplementary Figure S6C).

Cluster 23 cells were identified as oocytes based on the expression of many oocyte development-related genes (Supplementary Figure S3). However, trajectory analysis revealed that a small portion of cells in cluster 23 expressed many spermatogonia proliferation and differentiation-related genes, such as *zbtb16*, etv5, *sox9*, and *tdrd5* (Supplementary Figure S7; Supplementary Materials). These cells exhibited a distinct developmental state, suggesting that oogonia may possess differential potencies.

Cluster 4 cells were initially identified as follicle cells due to the high expression of follicle cell markers. To explore cluster 4 subtypes during sex differentiation, we performed re-clustering analysis and revealed the existence of four subclusters (sc4.1, sc4.2, sc4.3, and sc4.4; Figure 1D). Sc4.1, sc4.2, and sc4.3 showed high expression of many follicle cell markers, including *amh*, *fshr*, and *gsdf*. However, both sc4.1 and sc4.3 expressed pre-follicle cell-specific genes (*bmp6*, *emx2*, *irx3*, *irx5*, *wt1*, and *lhx9*), as reported in zebrafish (Liu et al., 2022). Sc4.3 showed a similar gene expression pattern to sc4.1, as well as enrichment of collagen-related genes, such as *col1a1* and *col1a2* (Figure 1E). Therefore, sc4.1 and sc4.3 cells were identified as pre-follicle cells (pFC1 and pFC2, respectively), while sc4.2 cells were identified as follicle cells with high *cyp19a1a* expression in the early ovary, consistent with previous research (Dranow et al., 2016; Nakamura et al., 2009). Notably, both pre-follicle and follicle cells showed high expression of male-related genes, including *amh*, *dmrt3*, and *sox9* in pre-follicle cells and *dmrt1* in follicle cells. In addition, *cyp11a1*, *cyp17a1*, and *star* were all expressed in sc4.4 of cluster 4, and thus sc4.4 cells were identified as theca cells (TC, Figure 1E; Supplementary Materials).

Our results showed that pFC1 was the dominant cell type at 80 dph, pFC1 and pFC2 were the dominant cell types at 120 dph, and all four subclusters were present at 180 dph (Figure 1F). Depending on the emergence points of the three subclusters, we predicted the developmental trajectories of the follicle cells. The earliest cell type (pFC1) may be a precursor of follicle cells, while pFC2 may be an intermediate state between pFC1 and follicle cells (Figure 1G).

In addition to its expression in follicle cells, *cyp19a1* was also expressed in uncharacterized stromal cells (cluster 3). Cluster 3 was divided into two subclusters (sc3.1 and sc3.2) based on different marker genes and time of occurrence (Supplementary Figure S8A, B). Sc3.1 specifically expressed *cldn7a*, *tnfsf15*, *tacstd2*, *ccxl4*, *f2rl1*, and *fndc5*, whereas sc3.2 strongly expressed *cyp19a1a*, *fshr*, *gsdf*, and *foxl2* (Supplementary Figure S8C, D; Supplementary Materials), indicating that stromal cells in sc3.2 may also participate in hormone production. These results are consistent with previous research on zebrafish (Liu et al., 2022). We also outlined the developmental trajectories of cluster 3 during sex differentiation. First, a small number of progenitor cells from 80 dph preferentially developed into sc3.1 at 120 dph. Subsequently, number of sc3.1 cells decreased and number of sc3.2 cells increased sharply at 180 dph (Supplementary Figure S8E). The reason for this change may be the transition from sc3.1 to sc3.2 from 120 dph to 180 dph.

In this study, we performed the first scRNA-seq analysis of the orange-spotted grouper, providing a precise map of the gonads during sex differentiation. From the scRNA-seq data, we identified 15 cell types in the early developing gonads, as well as various novel cell markers. Furthermore, we defined two pre-follicle cell types and one follicle cell type and confirmed the order in which they appear during sex differentiation. In addition, transcriptomic differences in gonocytes were revealed across the three developmental stages. This study provides an important dataset and new avenues for research in sex differentiation. This study also reveals a previously unanticipated complexity of the developing gonad and provides a comprehensive resource for future systematic analysis of sex differentiation.

DATA AVAILABILITY

Datasets generated in this study are available in the GSA at the National Genomics Data Center (CRA004056 and CRA007271), NCBI Gene Expression Omnibus (GSE218835), and Science Data Bank (DOI: 10.57760/sciencedb.06672).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

X.C.L., Z.N.M., and X.W. conceived and supervised the study. X.W. and Y.Y. analyzed the data. C.Y.Z., T.W., and Y.H.T., performed the experiments. X.W., C.Y.Z., and Y.Y. wrote the manuscript. All authors read and approved the final version of the manuscript.

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