Oviduct Epithelial Cells Constitute Distinct Lineages from the Ampulla to the Isthmus Related to High-Altitude Adaptation in Yak (Bos grunniens)

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Research Article

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Abstract

High-altitude adaptation is viewed as a biologically based process during which yaks respond to the harsh condition of coldness and low oxygen. Although high-altitude adaptation has been studied intensively, there is a lack of multi-omics analysis on its physiological mechanism. Therefore, in this study, 10X Genomics single-cell technology were adopted to identify cellular heterogeneity and trajectory differentiation in different parts of the yak oviduct, thus further exploring new cell markers and ligand-receptors. Besides, based on the comparison of oviduct epithelial cells from the ampulla and isthmus between the yak and bovine, genes and pathways related to altitude adaptation were discussed by bulk-seq. Single cell RNA sequencing (scRNA-seq) revealed 14 cell types defined by multiple specific molecular markers, the main cell types are epithelial cells, ciliated cells, secretory cells, basal cells, mesenchymal stem cells, T cells, B cells, monocytes, NK cells, macrophages, and fibroblasts, among which basal cells could be differentiated into secretory cells and ciliated cells. Apart from that, immune cells were the apex products of cell development, while ciliated cells had the characteristics of de-differentiation, and the new ligand-receptors CD74-COPa and CD74-APP were found. In addition, by bulk-seq, it was found that in the ampulla, there were 5149 differential genes (1875 up-regulated genes and 3274 down-regulated genes). YME1L1 was down-regulated significantly, whereas PP1B, PCBD, CYTB, RPL24, RPS5, and UBL5 were up-regulated to a great extent. In the isthmus, there were 6313 differential genes (2146 up-regulated genes and 4167 down-regulated genes). Among them, YME1L1, GSTA4, DNTTIP2, TMC06, LRBA, and MYH14 were down-regulated obviously, while SNRPD3, CYTB, ADIPOQ, RNH1, PRDX5, DPF1, CRYAB, and PRXL2B were up-regulated dramatically. Through the comparison, it was observed that GSTA4 and YME1L1 were down-regulated, while CYTB was up-regulated in the ampulla and the isthmus. Beyond that, the oxidative phosphorylation pathway was explored. In conclusion, it was demonstrated that ciliated cells were featured with dedifferentiation, and new ligand receptors CD74-COPa and CD74-APP were discovered. Furthermore, GSTA4, YME1L1 and CYTB were main molecules of altitude adaptation, and the oxidative phosphorylation pathway was the key pathway of high-altitude adaptation. This study provides basic data on the heterogeneity and cell trajectory of yak oviduct from the single-cell sequence, and provides the key genes and pathways of yak high-altitude adaptation, as well as the theoretical basis and the data for revealing the low pregnancy rate and genetic breeding of yaks.

Introduction

Yaks are characterized by the strong high-altitude adaptation, which can help them live in the anoxic environment of high altitude successfully. There are studies that have been compared lung, muscle, spleen and spleen tissues of the yak and cattle, finding that mitochondrion is the most important organelle in hypoxia adaptation. Other than that, insulin related pathways and thermogenic related pathways play a major role in high-altitude adaptation[1]. Wangsheng Zhao et al. made a comparison concerning abnormal expression of ER associated by comparing epididymides mRNA between the yak and cattleyak, showing abnormal expression of genes would disrupt the protein processing pathway of Cattleyak [2] and participate in sperm generation and maturation. Moreover, there is a comparison about
the heart between the yak and cattle, and then it is found that six mRNAs (MAPKAPK3, PXN, NFATC2, ATP7A, DIAPH1, and F2R), eight miRNAs (including miR-195), and 15 circRNAs (including novel-cic-017096 and novel-circ-018073) were defined as important genes in adaptive regulation of the yak heart plateau[3]. The study on Hb adaptation to hypoxia in plateau fish has always been involved, it was found that long-term maintenance of high Hb concentration may be detrimental to physiological adaptation to high altitude hypoxia[4]. The high-altitude deer (Peromyscus maniculatus) is the animal with the highest mass-specific lipid oxidation rates observed on land [5]. The mechanisms of these high rates of oxidation will help us understand the regulation of lipid metabolism. Other than that, the expression of apoptosis-related genes[6], ERK1/2[7], DDIT3[8], HIF-1α[9], and FSHR[10] in reproductive organs of female yaks has been studied. Currently, studies on high altitude adaptation of hearts, lungs, epididymides, muscle, spleens and other organs in yaks have been carried out, but there is still a lack of studies on the oviduct to provide the evidence about the relation between low pregnancy rate and high-altitude adaptation in yaks.

The sexual maturity of yaks happens at the age of 2–3. They have two oestrous cycles every year, each of which lasts for 21 days, and the pregnancy time is about 255 days. Besides, they can give birth to 4–5 fetuses in life. Its low pregnant rate greatly limits the expansion of the yak population. At present, it is urgent to solve the problem of low conception rate. While the oviduct is an important conduit for transporting sperm, oocyte and oosperm, which is made up of three parts: the infundibulum, ampulla, and isthmus, which have similar histological structure, but completely different physiological functions. The infundibulum is the conduit for the transport of mature oocyte, and the ampulla provides sites for the fertilization. The isthmus is the conduit for sperm storage and oosperm transportation, while oviduct epithelial cells are mainly composed of ciliated cells and secretory cells, and a small number of basal cells and peg cells can be detected. In addition, the mitotic phase and lymphoid cells of basal cells could be observed in oviduct epithelial cells in early pregnancy in yaks. Besides, there is the presence of basal cells and peg cells in the isthmus of oviduct epithelial cells in early pregnancy in sheep [11]. Oviduct secreting cells can synthesize and secrete oviduct specific glycoprotein (OGP)[12], and the OGP synthesis sites of various animals are different in species, such as the infundibulum and ampulla in cattle[13] and sheep[14], and the isthmus in rats[15]. Other than that, oviduct intertubal stem cells inhibit T cells from induced apoptosis by down-regulating Fas receptor and Fas ligand on their surface [16], and they can also restrict DC differentiation, maturation and function, the formation of B lymphocytes and other pathways to play an immunosuppressive role [17]. IFN-γ stimulation of mesenchymal stem cells leads to an increase in the expression of HLA-I molecules on their surface, which makes them more resistant to the cytotoxic effects of NK cells[18]. Furthermore, the immune system of the oviduct can adapt to allogeneic sperms and semi-allogeneic embryos until trophoblast growth and interferon-s[19], IL-1b, and TNFa [20] activate the maternal immune system. However, many experiments are transcriptome sequencing, and it is impossible to deeply explore the mechanism of high-altitude adaptation. Therefore, it is urgent to carry out research on high-altitude adaptation of yak oviduct using single-cell sequencing techniques.
Single-cell sequencing is the first accurate typing of cell subtypes and mining of new cell types that can be used to construct differentiation trajectories of cell lineages and create molecular maps of cell developmental lineages. At present, single-cell RNA sequencing was performed to analyze the transcriptional pathways of breast epithelial cells[21], human pluripotent stem cells[22], and zebrafish embryonic development[23]. In addition, insulin-like growth factor 1 (IGF1) was defined as the target gene of the oviduct by mouse E_2 after single-cell RNA sequencing. Beyond that, E_2 can regulate epithelial, stromal, and muscle cells of the oviduct[24].

High-altitude adaptation is a very complex phenotype, and it is difficult to fully clarify its adaptation mechanism by using the single omics. In this study, single-cell sequences and bulk-seq were used to analyze cellular heterogeneity of the yak oviduct and explore the relationship between population differences and cell differentiation. Then, key molecules of yak high-altitude adaptation have been found. The study on the gene and pathway mechanism of high-altitude adaptation provides basic information for improving the pregnancy rate of yaks, and serves as theoretical basis for altitude medicine and prevention of hypoxia injury.

**Materials And Methods**

**Animals**

In this study, single-cell experiment samples were collected from yaks in Xining, Qinghai Province, China, featured with the altitude of about 3800 ~ 4500m above sea level. Adult female yaks (5 years old, n = 2) with the weight of 310kg and 326kg respectively were selected. After clinical and physical examination, both yaks were found to have normal reproductive status without any pathophysiological diseases. Besides, the animals were euthanized by intravenous injection of pentobarbital sodium (200 mg/kg). Meanwhile, all experimental procedures were approved by the Animal Research Ethics Committee of Gansu Agricultural University.

**Preparation of cell suspension**

The oviduct samples were tested within 2 hours after isolation. Besides, the oviduct tissue was dissected under a microscope and washed with Dulbecco phosphata-buffered saline (DPBS) for three times. The tissue was cut into pieces with aseptic small ophthalmic scissors to 1mm³, and then digested with type I collagenase (GIBCO) and pancreatin (GIBCO) at 37°C for 25 min(containing 2 mM EDTA). After that, the digestion was stopped with 1mL serum, and the tissue was filtered with the 40µm polyester mesh. The cell suspension was centrifuged 600r/min for 6min, and the supernatant was completely sucked out. The red blood cell lysis buffer was added for eliminating red blood cells, while the cells were incubated at room temperature for 1min, with 1200r/min 5min. Afterwards, the supernatant was sucked out, and 0.04% 5mL BSA and DPBS were added so as to terminate the reaction. Furthermore, the cells were washed twice with 10% BSA (Bovine Serum Albumin) in DPBS to obtain a single cell suspension, and trypan blue staining was performed on the cell suspension with cell viability > 90%.
**scRNA-Sequencing Processing** Cell suspension was processed with the Single Cell 3’v3 Reagent Kit (10× Genomics, Pleasanton, CA, United States). The 33.4µL single-cell Master Mix, including 20.0µL RT Reagent, 3.1µL Template Switch Oligo, 2.0µL Reducing Agent B and 8.3µL RT Enzyme C was prepared. Then, cell suspension was added into Master Mix to make the volume 80µL, and the mixture of 75µL Master Mix and single-cell suspension was extracted, while 40µL Gel Beads and 280µL Partitioning Oil were added into chips that were put in 10X Genomics to function. After that, transfer GEMS and GEMS-RT Incubation, followed by GEMS-RT magnetic beads being purified and cDNA being amplified.

Library construction: Fragmentation Mix was prepared on ice: 5µL fragmentation buffer, 10µL Fragmentation Enzymes, and 10µL purified cDNA products were taken to an EP tube, while 25µL buffer EB and 15µL Fragmentation Mix were added. Afterwards, the samples were centrifuged and put into the PCR machine for reaction: 32°C for 5 min, 65°C for 30 min, 4°C for hold, and 65°C. Then, fragment screening, purification of ligand and product fragment screening were conducted. Apart from that, 60µL SPRlselect was added into each sample, incubated for 5 min at room temperature, and the EP tubes were placed in a magnetic force on the shelf, and kept in the solution state until it became clear, followed by 150µL supernatant fluid being transferred to the new EP tubes. After that, 20µL SPRlselect was added into each sample, and incubated for 5 min at room temperature, while the EP tubes were placed in a magnetic force on the shelf, and kept in the solution state until it became clear, before 165µL clear liquid absorption was removed. Furthermore, 200µL fresh mixture of 80% ethanol solution was added and kept for 30s, followed by the ethanol being removed. Moreover, the High Sensitivity DNA Assay Kit (Agilent Technologies) was used for library quality inspection, and then ABI StepOnePlus Real-Time PCR System (Life Technologies) was applied to analysis and pooling, according to PE150 modeling of Hiseq2500 to sequence.

**Bulk RNA-Sequencing Processing**

GSE124110 microarray datasets of GPL22316 Platform (Illumina HiSeq 1500, Bos taurus) were obtained in GEO (http://www.ncbi.nlm.nih.gov/geo), and they were carried out by Gene Center Munich in Germany, while here, the oviduct epithelium of the ampulla and isthmus was selected for this experiment. Apart from that, Munich has an altitude of about 520m. The differences in DESeq2 software analysis 10X cell subgroup expression and the bulk-seq gene expression were employed, and FDR < 0.05 and | log₂FC | > 1 genes were screened for significant differences.

**The analysis of the DEGs**

In order to better analyze the gene expression of differential genes, GO (Gene Ontology, GO) functional enrichment and KEGG (Kyoto Encyclopedia of Genes and Genomes, KEGG) signal pathway enrichment analyses were performed, when GO contained molecular functions (MF), biological processes (BP), and cellular components (CC), and P-value < 0.05 was defined as statistical significance, while the KEGG database included biological functions, diseases, chemicals, and drugs. Here, it should be noted that this study focuses on the pathway function module.
Results

Single-cell RNA sequencing (scRNA-seq) reveals cell heterogeneity in yak oviduct.

Oviducts were obtained from two female yaks, and single-cell suspension was prepared. A total of 40781 cells were collected to get high quality cells (Supplementary Fig. 1). Finally, 28913 cells were sequenced, and the relationships among nUMI, nGene and pMito were found (Fig. 1A). The quality control requirements are that the number of genes identified in a single cell is between 500 and 4000, and that the total number of UMIs in a single cell is < 8000, while the proportion of mitochondrial gene expression of UMIs in a single cell is < 20%. Then, the results were demonstrated based on cell subgroup classification, and those of single cell subgroup classification were further visualized with the nonlinear clustering method of t-SNE (t-SNE, T-Distributed Neighbor Embedding). In addition to that, the subtypes of cells in each sample were classified (Fig. 1B ~ 1G). Cell subtype 0 represents T cell, Cell subtype 1, 4, 14 represents NKT cell, Cell subtype 7, 10 represents NK cell, Cell subtype 2 represents macrophage, Cell subtype 5, 11, 17 represents Monocyte, Cell subtype 13, 15, 20 represents B cell, Cell subtype 19 represents neutrophil, Cell subtype 3 represents mesenchymal stem cell, Cell subtype 8, 9 represents ciliated cells, Cell subtype 6 represents epithelial cell, Cell subtype 16 represents basal cell, Cell subtype 18 represents fibroblast, Cell subtype 12 represents secretory cell.

The t-SNE method usually presents more detailed results for the classification of different subsets of cells (Fig. 2A). In total, 14 types of cells: epithelial cells, ciliated cells, secretory cells, basal cells, mesenchymal cells, T cells, B cells, monocytes, NK cells, macrophages, and fibroblasts were identified. Besides, the three parts of the oviduct have the same cell types, and most of them are T cells and NK cells, 7966 and 6475 respectively, while the least is basal cells and neutrophils, 163 and 70 respectively. For the infundibulum, ampulla and isthmus of the oviduct, it was found that T cells and NK cells were the most in all parts (Fig. 2B). For ciliated cells and secretory cells, the most ciliated cells were in the isthmus and the least in the ampulla; whereas, the secretory cells were the most in the ampulla of the oviduct and least in the isthmus of the oviduct. The first 20 up-regulated genes were used to depict the heat map in each cluster. To illustrate the gene expression pattern, the marker genes of each cluster were screened for the purpose of constructing the heat map and point map set of all cells. According to the expression level of the marker genes, the expression and distribution of each marker gene in each cell of oviduct tissue are shown through expression and distribution heat maps and bubble maps. In summary, our data are sufficient to reflect the entire transcriptome of the oviduct (Fig. 2C). Among them, in the target cell or control cell group, the gene was expressed in more than 25% of the cells, and the P value was ≤ 0.01; the gene expression fold log$_2$FC was ≥ 0.360674, that is, the fold up-regulation of the gene was ≥ 1.28. Moreover, T cells are labeled with the CD96 protein and CD37, while NK cells were labeled with TNFRSF9, CACYBP, IL7R, and CD40LG. Apart from that, macrophages were labeled with CDHR5, ARHGAP20, SLC8A1, MEGF10, and AXL, whereas mesenchymal stem cells were ANPEP, VCAN, FGFR2, and BMPR1B; beyond that, B cells were BLK, RSAD2, ISG15, and CD79B, while epithelial cells were WFDC2, SFTA2, and PLA2G10. Furthermore, ciliated cells were DTHD1, DNAH9, ATP6V1G3, and CCDC153, and secretory cells
were CCN1, CRYAB, FAM71A, and MMP7. To sum up, this study successfully identified the main cell types in yak oviduct fertilization.

**scRNA-seq discovers differentiation trajectory and novel ligand-receptors in yak oviduct**

The gene expression matrix generated by 10x Genomics was introduced into monocles to construct the cell differentiation locus, and the cell differentiation trajectories of each sample of oviducts can be clearly seen. The monocle can use the signals of gene expression levels in all cells to analyze the differences in gene expression of different types of cells (different cells or developmental time points, etc.; the screening condition is FDR < 1e-5) to find the key genes related to the developmental differentiation process. Since the cell lineage pseudo-temporal differentiation trajectory was successfully recapitulated, natural killer T (NKT) cells, ciliated cells, secretory and basal cells, and secretory and ciliated cells were located at the beginning of quasi-temporal differentiation, while immune cells were at the end. Besides, basal cells are present widely and can be evaluated as basal cells that can differentiate into secretory and ciliated cells, while immune cells are the apical products of cell development (Fig. 3A). Furthermore, the relationship between immune cells and basal cells was discussed. NKT cells, monocytes, B cells, and NK cells were at the early stage of development, and macrophages were at the end of cell development, suggesting that macrophages are late cells in the reproductive system and are higher immune cells, while basal cells are present throughout development (Fig. 3B). Beyond that, ciliated cells are in the early stages of development, and secretory cells are slightly later than ciliated cells, while mesenchymal stem cells are in the middle of ciliated cells and secretory cells (Fig. 3C).

To explore the interaction between cells, the relationship between monocytes, macrophages, ciliated cells, secretory cells, and basal cells was investigated by receptor ligand relationship. Among them, ligand-receptors namely CD74-COPA and CD74-APP were found using macrophages as ligands and ciliated cells, monocytes, basal cells, and secretory cells as receptors (Fig. 3D). Besides, monocytes are the ligands, and ciliated, secretory, and basal cells are the receptors (Fig. 3E). Moreover, ligand-receptors CD74-COPA and CD74-APP are found, while new ligand-receptor parings namely ADIPOQ-ENSBMUG00000010022, ENSBMUG00000001906-DPP4, and ENSBMUG00000001906-ENSBMUG00000025858 are also found.

**Comparison of mRNA in oviduct epithelial cells by bulk-seq**

Epithelial cells from three parts of the oviduct were extracted, and the mRNAs were compared. The screening criteria are |log2FC| ≥ 1 and q ≤ 0.01. Besides, the comparison between the isthmus and ampulla revealed 81 differentially expressed genes, including 27 up-regulated genes and 54 down-regulated genes, among which C3, ID1, TFPI2, CDC42EP1, ADAM28, and EFEMP1 had significant differences (Fig. 4A). After Go analysis, the biological process was mainly in the cellular process, cellular component organization or biogenesis, biological regulation, regulation of the biological process, the metabolic process, response to stimuli, the multicellular organismal process, the developmental process,
and localization. Cellular components were mainly responsible for cells, cell parts, organelles, organelle parts, membranes, membrane parts, protein-containing complexes, and the membrane-enclosed lumen. Moreover, molecular functions were focused on binding, catalytic activities, molecular function regulators, and molecular transducer activities (Fig. 4B). KEGG analysis showed that pathways were mainly enriched in cell adhesion molecules (CAMs), antigen processing and presentation, and Hippo signaling pathways (Fig. 4C).

**YME1L1 and CYTB of high-altitude adaptation screened by Bulk-Seq**

Bulk-seq was performed on yak oviduct cells from 10X Genomics and bovine oviduct cells. In the ampulla, there were 5149 differential genes, including 1875 up-regulated differential genes and 3274 down-regulated differential genes (Fig. 5A). According to these differential genes, the volcano map and the heat map (Fig. 5B, C) were made. PP1B, PCBD2, CYTB, RPL24, RPS5, and UBL5 rose significantly, while GSTA4 and YME1L1 lowered dramatically.

In the isthmus, there were 6313 differential genes, including 2146 up-regulated differential genes and 4167 down-regulated differential genes (Fig. 5A). According to these differences in genes, the volcano and heat map was mapped (Fig. 5D, E). SNRPD3, CYTB, ADIPOQ, RNH1, PRDX5, DPF1, CRYAB, and PRXL2B rose obviously, while YME1L1, GSTA4, DNTTIP2, TMCO6, LRBA, and MYH14 lowered significantly. In this case, it was found that GSTA4 and YME1L1 were down-regulated, while CYTB was up-regulated in both the ampulla and the isthmus.

**Oxidative phosphorylation pathway of high-altitude adaptation screened by Bulk-Seq**

Enrichment analysis of up-regulated and down-regulated genes was conducted respectively, and the top 20 genes with significant differences were selected because there were too many differentially expressed genes. Up-regulated genes in the ampulla, the biological process, the peptide metabolic process, translation, and the peptide biosynthetic process were featured with obvious differences. In the cellular component, ribosome, ribonucleoprotein complexes, and intracellular parts were in a significant difference. In molecular function, the structural constituent of ribosome and structural molecule activity featured huge difference (Fig. 6A), while oxidative phosphorylation was the key pathway (Fig. 6B). These differential genes were analyzed by regulatory network, RPL34, RPL38, RPL13, RPS3A and other RPSs family gene and RPLs family genes are the hub genes of the regulatory network. In addition, LSMs family gene, PSMs family gene and NDUFs family gene are also involved in the regulation of related genes (Fig. 6C).

Down-regulated genes in the ampulla, biological process, cellular component organization, cellular component organization or biogenesis, and organelle organization were significantly different. In the cellular component, cell parts, cells, and intracellular parts were noticeably different. In molecular
function, binding, protein binding, and enzyme binding were significantly different (Fig. 7A). In the KEGG pathway, the longevity regulating pathway – mammals, AMPK signaling pathway and Hippo signaling pathway were key ones (Fig. 7B). CREBBP CARM1, AKT1 TSC2, EP300, STAT3, ERBB3 are the key nodes in the regulation of network, in addition, the PIAS1, SOS1, LRP6, CDH1, SP3, CDK6 genes are involved in the gene regulation (Fig. 7C).

Up-regulated genes in the isthmus, biological process, peptide biosynthetic process, translation, and peptide metabolic process were different to a great extent. In the cellular component, there were obvious differences in ribosome, ribonucleoprotein complexes, and mitochondria. Besides, in molecular function, the structural constituent of ribosomes and structural molecule activity were significantly different (Fig. 8A). Furthermore, it should be mentioned that oxidative phosphorylation was the key pathway (Fig. 8B). RPLs family gene and PSMs family gene at the hub genes of the control network, in addition, NDUFB7, NDUFB9, GTF2B, INTS10, POLR2K, LSM3 genes are involved in the gene regulation (Fig. 8C).

Down-regulated genes in the isthmus, biological process, cellular component organization, cellular component organization or biogenesis, and cellular protein modification process were dramatically different. In the cellular component, cell parts, cells and intracellular parts were extremely different. Whereas, in molecular function, dramatic differences exist in binding, protein binding and enzyme binding (Fig. 9A). Furthermore, the longevity regulating pathway – mammals and the thyroid hormone signaling pathway were key ones (Fig. 9B). STAT1, NCOA2 SMAD2, STAT3, CBL, TSC2 in the regulatory networks of key nodes, in addition, NBR1, SQSTM1, PRKCZ, DMD, EIF3A, RB1CC1 genes are involved in gene regulation (Fig. 9C).

**Discussion**

Hypoxic adaptation is an enduring topic, scRNA-seq and bulk-seq were performed to elucidate high-altitude adaptation in the yak oviduct. In this study, single-cell technique was adopted to analyze the heterogeneity, locus differentiation and ligand receptor pairs in the infundibulum, ampulla and isthmus of the oviduct in yaks. At present, there are few studies on the oviduct using single-cell technology, but some results demonstrate that E2 shows differential expression in the transcription profile of mouse oviduct ciliated cells and secretory cells[24], and there are differences in the differentiation of mouse epithelial cells at the distal and proximal end of the oviduct[25]. In the above studies, detailed researches on the three parts of the oviduct were not carried out. In this experiment, it was found that the most ciliated cells were observed in the isthmus, and the least ciliated cells were seen in the ampulla. Most secretory cells were discovered in the ampulla, while the least in the isthmus of the oviduct. Therefore, more ciliated cells in the isthmus may contribute to sperm movement, and more secretory cells in the ampulla may be conducive to the combination of sperms and oocyte. These results can provide theoretical basis for reproductive development and embryo transfer in mammals.

Through this experiment, 14 cell subtypes and corresponding new marker genes in the oviduct of mammalian yaks were found. Among them, 14 cell subtypes were mainly ciliated cells, secretory cells,
oviduct epithelial cells, T cells, B cells, NK cells, basal cells, oviduct mesenchymal stem cells, neutrophils, macrophages, and fibroblasts, when ciliated cells and secretory cells are abundant in epithelial cells of the yak oviduct, which is also the focus of our study. Other than that, WFDC2 and PLA2G7 were found to be epithelial markers by the cell marker database. WFDC2 can maintain the integrity of tight junctions between colon epithelial cells in vivo, and prevent symbiosis bacterial invasion and mucosal inflammation[26], whereas PLA2G7 is also a type of high molecular weight keratin, and may regulate immune pathways through macrophages[27]. Besides, ATP6V1G3 is the identification protein of ciliated cells, and is a surface marker of renal cell carcinoma[28]. Furthermore, the upregulation of ATP6V1G3 genes may play an important role in the occurrence of recurrent abortion[29]. In addition, CCN1 was used as an identification protein of secretory cells for the first time in this experiment, and it can also induce protein secretion in human dermal fibroblasts and promote skin connective tissue aging[30]. Moreover, CCN1 binds to its receptor α6β1 to activate nuclear factor-κB and facilitate atherosclerosis[31]. Apart from that, immune cells were studied, and it was found that CD96 protein and CD37 are surface markers of T cells, and CD37 is also a surface marker of lymphoma[32]. TNFRSF9, CACYBP, IL7R and CD40LG are identification proteins of NK cells, where TNFRSF9, CD40LG and IL-17 are a regulator of NK cell effector function[33], an effector molecule associated with NK cell toxicity[34], and a factor that enhances the sensitivity of U-2 OS cells to NK cell lysis[35] respectively. Moreover, CACYBP is involved in the immune response of NFAT transcription factors[36]. Besides that, CACYBP, IL7R and CD40LG were also identified as tubal protein molecules for the first time. Oviduct mesenchymal stem cells are identified by ANPEP, VCAN, FGFR2, and BMPR1B, and ANPEP is the characteristic protein of human testicular stem cells[37]. Mir-223 regulates the lipid and osteogenic differentiation of mesenchymal stem cells through the C/EBPs/Mir-223/FGFR2 regulatory feedback loop[38], whereas Mir-125b regulates the osteogenic differentiation of human bone marrow mesenchymal stem cells by targeting BMPR1b[39]. FGFR2 and BMPR1b are also the first surface markers of mesenchymal stem cells. In this experiment, the mining of new marker genes can better identify cells and enrich the database of biomarkers.

Lymphocytes are distributed in both ovaries and oviducts, most of which are T lymphocytes, and a few are macrophage monocytes. There are lymphocyte colonies in the basal layer of the endometrium, when there are mainly T lymphocytes and a small number of B lymphocytes. In this study, 10x Genomics was used to analyze the cell heterogeneity. It was found that there were more lymphoid cells in the infundibular part of the oviduct than in the isthmus and the ampulla. Specifically, there were more T cells in the isthmus than in the ampulla and the ampulla. Studies have shown that the local immunity of the chicken oviduct is enhanced during sexual maturation and may decrease during aging, and the effects of estrogen and progesterone on T and B cells are also different according to the location of the oviduct[40]. Besides, oviduct mesenchymal stem cells also have certain immune effects. Moreover, ectomesenchymal stem cell research has manifested that the oviduct of people has certain immunosuppressive function, while is weak in the bone marrow mesenchymal stem cells. In addition, it is the main source of mesenchymal stem cells between people, and umbilical cord mesenchymal stem cells in the abdominal cavity injection in the treatment of rats with acute salpingitis can reduce serum inflammatory factors, and resume the function of oviduct structure and fertility. The results showed that
the mesenchymal stem cells in yaks' oviduct were the least in the isthmus and the most in the ampulla, suggesting that the ampulla mesenchymal stem cells were related to the combination of sperms and germ cells. The regular contractions of muscles and the flow of oviduct fluid support the transport of sperms, oocytes and germ cells. Moreover, the muscle layer in the isthmus was the thickest, and the ampulla was the thinnest. Segmental contraction and peristalsis of the thick muscular layer at the isthmus of the oviduct facilitate the transport of germ cells and germ cells. Reverse flow of oviduct fluid is conducive to sperm transport, which increases the success rate of sperm transport to the ampulla. Besides, scanning electron microscopy demonstrated that the oviduct had complex folded shape and the isthmus was curved and narrow. After the sperm enters the oviduct and stays in the isthmus, special active reaction is produced between cilia cells of the epithelium and sperm flagellum [41], which reduces the movement speed of the sperm, is conducive to sperm adhesion and slows down the rapid migration of the ovum to the uterine cavity.

The essence of cell differentiation is the selective expression of the genome in time and space, through the expression of different genes on and off, resulting in the production of signature proteins. 10x Single cell RNA-seq is the best way to explore cell lineages at this stage. It was found that basal cells can differentiate into ciliated cells and secretory cells, when basal cells are the most important self-renewing stem cells in the respiratory tract of rats and hamsters[42]. At the same time, locus differentiation of tubal mesenchymal stem cells, basal cells, ciliated cells and secretory cells was demonstrated. Other than that, ciliated cells were discovered at both the beginning and middle of the cell differentiation tree, indicating that ciliated cells were at the earliest stage of cell development, suggesting that ciliated cells had undergone dedifferentiation. Besides, studies have shown that in novel coronavirus infection using the regenerative human bronchial epithelial cell model, the expression of DNAH7, FOXJ1 and RFX3, and the key genes regulating cilia production in lungs, were down-regulated, revealing cilia dedifferentiation. FOXJ1 down-regulation can further induce cilia loss. Changes in cilia directly lead to the weakened ability of the respiratory tract to remove foreign bodies [43]. Therefore, it is speculated that the dedifferentiation of ciliated cells occurred in this experiment, and the low conception rate of yaks may be related to the decreased swinging ability of ciliated cells after dedifferentiation. In addition, basal cells and oviduct mesenchymal stem cells are progenitor cells that can evolve into ciliated cells, secretory cells, and other immune cells, among which macrophages are apical products of immune cells.

Ligand-receptor interaction is a major tool in cell communication research in recent years. In this study, CD74 was found to be a key molecule in signal transduction between immune cells and non-immune cells in the oviduct. CD74 is the high-affinity receptor of the macrophage migration inhibitory factor (MIF) on the cell membrane, and MIF and CD74 are involved in the occurrence and development of ARDS [44; 45]. In addition, CD74-ICD mediates the maturation of B cells through the nF-KB pathway [46], CD74 may play a role in innate immune system responses and the potential of immunotherapy[47], while the macrophage migration inhibitory factor activates the production of main angiogenic factors in human ectopic endometrium cells through CD44, CD74 and MAPK signaling pathways [48]. Then, it was found that CD74-COPA is a key pair of ligand receptors, and COPA syndrome is an autosomal dominant
inherited immune disorder caused by COPα gene variation. Moreover, the role of COPA in the oviduct need to be further studied.

Studies on high-altitude adaptation have been carried out in both humans and animals[49]. Three causal genes SNPs of EPAS1, which were defined as key adaptive gene[50], in high altitude (≥ 1800 m) and low altitude (< 1500 m) cattles, ACSS2, ALDOC, EPAS1, EGLN1 and NUCB2 may be involved in hypoxia adaptation[51]. In the study of Tibetan pigs, EPAS1, CYP4F2 and THSD7A play an important role in hypoxia circulation, while THSD7A plays an important role in the evolution of Tibetan pigs[52]. In this study, it was observed that GSTA4 and YME1L1 were down-regulated, while CYTB was up-regulated in the ampulla and the isthmus. YME1L1 is a zinc-dependent mitochondrial metalloproteinase that regulates mitochondrial morphology. HSF1 mediates oxidative stress resilience response and changes of mitochondria morphology through YME1L1 in a heat stress independent way [53]. Besides, aerobic exercise induced mitochondrial imbalance and increased protein levels of HSP60, Lonp1 and Yme1L1 in Gastrocnemius Muscle of aged mice [54], whereas YME1L1 was down-regulated in the ampulla and isthmus in yaks, which was consistent with the results from previous studies. Apart from that, CytB, ATP 6 and ATP 8 genes are widely used to search for interspecies or intraspecific phylogenetic rules. Especially, the CytB gene has been reported to the greatest extent [55; 56; 57]. In this study, it was revealed that the CytB gene was up-regulated in the ampulla and isthmus of yaks. The levels of Pdk4 and CytB genes involved in mitochondrial function and metabolism regulation were also partially up-regulated [58], suggesting that the mitochondrial oxidative stress pathway in the yak oviduct was activated. Previous studies on hypoxia adaptability of yaks showed that the oxidative phosphorylation pathway was also a key pathway for hypoxia adaptability[59], which was consistent with the results of this study. In the study of high-elevation phrynocephalus lizards[60], galliform birds[61], Chinese snub-nosed monkeys[62] and rat lung[63], oxidative phosphorylation pathway were also an important pathway for hypoxia adaptability. These results were in line with the significantly different oxidative phosphorylation pathways, implying that the hypoxia adaptability of high-altitude animals were relevant to mitochondrial oxidative stress, especially the activation of oxidative phosphorylation pathways.

Overall, the 10X cRNA-seq data and bulk-seq reveal high-altitude adaptation genes and pathways. This study presents key insights into the transcriptional landscape of the yak oviduct, defining different cellular substates and subtypes, and illustrating transcriptional implementation in major cell fate determining processes, thus providing reference for yak high-altitude adaptation.

**Conclusion**

This study found that CD74 is an important molecule for signal communication between immune and non-immune cells. YME1L1 and CYTB were main molecules of altitude adaptation, and the oxidative phosphorylation pathway was the key pathway of high-altitude adaptation. This study provides the theoretical basis and the data for revealing the yak high-altitude adaptation.

**Declarations**
DATA AVAILABILITY

All sequences have been deposited to GSA with accession number CRA007411.

CONFLICT OF INTEREST

The authors have stated explicitly that there are no conflicts of interest in connection with this article.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Research Ethics Committee of Gansu Agricultural University.

AUTHOR CONTRIBUTIONS

J.Z and S.J.Y conceived the project; J.Z and YY.P wrote the manuscript with the help of all authors; Y.C. and J.L.W performed scRNA-seq, while M.W conducted the electron microscopy. All authors contribute to writing and approval of the submitted version.

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Figures
Figure 1

Distribution diagram of basic information in each sample. (A) Distribution of basic cell information of each sample before and after filtration. (B) Cell type distribution of infundibular sample 1. (C) Cell type distribution of infundibular sample 2. (D) Cell type distribution of ampulla sample 1. (E) Cell type distribution of ampulla sample 2. (F) Cell type distribution of isthmus sample 1. (G) Cell type distribution of isthmus sample 2.
Figure 2

Identification of cell clusters in the yak oviduct by single-cell RNA sequencing (scRNA-seq). (A) t-SNE cell classification. (B) Heat map of marker gene expression: each column in the figure represents a cell, and each row represents a gene. (C) Map of marker gene expression distribution.
Figure 3

Prediction of cell differentiation and receptor-ligand.

(A) Differentiation of immune cells and ciliated cells, secretory cells, basal cells.

(B) Differentiation of basal cells and immune cells. (C) Differentiation of oviduct mesenchymal stem cells.

(D) Interaction of macrophages as ligands. (E) Interaction of monocytes as ligands
Figure 4

Comparison of oviduct epithelial cells between ampulla and isthmus. (A) Differential genes of ampulla and isthmus. (B) GO analysis of differential genes between ampulla and isthmus. (C) KEGG analysis of differential genes between ampulla and isthmus.
Figure 5

Bulk-seq study on the hypoxia adaptation genes between yak and bovine

(A) Differential gene of ampulla and isthmus. (B) Heatmap of differential genes in ampulla. (C) Heat map of differential genes in ampulla. (D) Volcanic map of differential genes in isthmus. (E) Volcanic map of differential genes in isthmus
Figure 6
Analysis of up-regulated differential genes in ampulla. (A) GO analysis of up-regulated differential genes in ampulla. (B) KEGG analysis of up-regulated differential genes in ampulla. (C) Regulation analysis of up-regulated differential genes in ampulla.

Figure 7
Analysis of up-regulated differential genes in ampulla. (A) GO analysis of down-regulated differential genes in ampulla. (B) KEGG analysis of down-regulated differential genes in ampulla. (C) Regulation analysis of down-regulated differential genes in ampulla.

Figure 8
Analysis of up-regulated differential genes in isthmus. (A) GO analysis of up-regulated differential genes in isthmus. (B) KEGG analysis of up-regulated differential genes in isthmus. (C) Regulation analysis of up-
regulated differential genes in isthm

Figure 9

Analysis of down-regulated differential genes in isthmus. (A) GO analysis of down-regulated differential genes in isthmus. (B) KEGG analysis of down-regulated differential genes in isthmus. (C) Regulation analysis of down-regulated differential genes in isthmus.

Supplementary Files

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