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Islet β-cells physiological difference study of old and young mice based on single-cell transcriptomics

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ABSTRACT

Aims/Introduction: Body aging is a universal biological process. With aging, cells undergo a series of physiological changes. The main feature is cell proliferation decline, although the cells still have normal functions. Pancreatic β-cells are no exception. However, the physiological senescence of β-cells, and the resulting function and transcriptome changes have rarely attracted attention. The specific senescence phenotype of β-cells remains unknown.

Materials and Methods: Pancreatic samples from three female C57BL/6 mice with aged 2.5 months (young) mice and 20 months (old) were digested to a single-cell suspension and analyzed, with 10× Genomics single-cell ribonucleic acid sequencing, β-cells were determined by biosynthesis analysis, and differences between old and young mice were identified.

Results: A total of 47 differential genes with significant and statistical significance were screened in β-cells (fold change >1.5, $P < 0.05$). In old mice, 27 genes were upregulated and 20 genes were downregulated. Genes *Mt1*, *Mt2*, *Pyy*, *Gcg* and *Pnlip*, and mitochondrial genes *mt-Nd1*, *mt-Nd3*, *mt-Co1*, *mt-Co2* and *mt-Co3* were found to be involved in cellular senescence. Transcription factors *Jund* and *Fos* were important regulators of senescence.

Conclusions: An overall difference was found between the pancreatic β-cells of old and young mice. Transcription factors facilitate transitions between pancreatic β-cells. These findings are worthy of deep exploration, and provide new resources and directions for the research of pancreatic aging in mice.

HIGHLIGHTS

- Through 10× Genomics single-cell ribonucleic acid sequencing techniques, we found that the genes *Mt1*, *Mt2*, *Pyy*, *Gcg*, *Pnlip* and the rest, are highly expressed in old mice. The transcription factors, *Jund* and *Fos*, were important regulators of the senescence process.
- When reactive oxygen species production is excessive, the anti-oxidant mechanism of metallothionein is decompensated, and reactive oxygen species further affects the mitochondrial function and protein structure of β-cells, leading to abnormal cell function.

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INTRODUCTION

Senescence is the decline in the function of organs and tissues over time, and is the biggest risk factor for many diseases, including several neurodegenerative diseases and cardiovascular diseases[1]. Characterization of molecular and cellular changes associated with aging could provide insights into this complex process and highlight opportunities to slow or reverse its progress, thereby preventing or treating age-related pathology. Many studies used model organisms to support this feasibility and proved that in addition to lifespan, the integrity of multiple tissues can be adjusted by discrete molecular modifications[2,3].

Diabetes mellitus is a pancreatic endocrine dysfunction mainly caused by autoimmune destruction (type 1 diabetes mellitus)[4] or impaired tissue insulin sensitivity, insulin resistance and glucolipotoxicity that leads to the progressive dysfunction of insulin-producing pancreatic β-cells (type 2 diabetes

mellitus)[5]. To date, no treatment can stop or reverse the progression of this disease. Cell senescence refers to a relatively stable state in which cells break away from the cell cycle and show irreversible growth arrest, but still maintain metabolic activity and survival[6]. Pancreatic β-cells play an important role in the occurrence and development of type 2 diabetes mellitus. When the body has impaired glucose tolerance, the β-cells can maintain the balance of glucose metabolism by increasing insulin secretion; however, this compensation might be restricted by β-cell proliferation decline and impaired function with vertebrate aging. Cyclin-dependent kinase inhibitor p16Ink4a encoded by the Cdkn2a gene is a senescence marker for β-cells[7,8]. The increase in p21Cis1 (another effector of cell senescence) marks the entry of early senescence, which leads to an increase in p16Ink4a expression. The cells maintain the senescence state, and senescence-associated secretory profile is finally expressed[9]. Just a few studies focused on the biological function changes brought about by β-cell physiological senescence. The specific senescence phenotype of β-cells remains unknown.

In the present study, the similarities and differences between the pancreatic cells of old and young mice were determined using the 10× Genomics (Pleasanton, CA, USA) high-throughput single-cell ribonucleic acid sequencing (scRNA-seq) technique. Differences in cell regeneration potential and possibility of switching between cells were also investigated. For all major cell populations, scRNA-seq provides a comprehensive dataset of genes and pathways whose transcription profiles change with age. Computational analysis showed that the pancreatic cells do not undergo the same changes with aging. This finding suggests that despite the presence of overlapping features, the effects of aging on cells are not universal. With the involuntary changes in cells regulating aging-related changes[3], the ligand–receptor interaction in almost all types of senescent pancreatic cells was also described in detail. This research provides resources for promoting molecular- and cellular-based research on the aging of mouse pancreas.

MATERIALS AND METHOD

Animals

Six healthy female C57BL/6 mice (three aged 2.5 months and three aged 20 months) were purchased from the Sino-British SIPPR/BK Lab., Animal Ltd. (Shanghai, China). The animals were maintained in a room with controlled temperature (20–25°C), humidity (40–70%) and illumination (12-h light/dark cycle), and fed with regular high-pressure mice diet and free-accessed tap water.

Preparation of single-cell suspensions

Islet cell suspension was transported rapidly to the research facility. Laparotomy was carried out to expose the pancreas of mice under anesthesia with 10% chloral hydrate (300 mg/kg). The bile duct was ligated out of the liver and infused with 2 mL of 0.5 mg/mL collagenase (C-7657; Sigma-Aldrich, St. Louis, MO, USA) to fill the pancreas from the opening of the cholangiopancreatic duct in duodenum. The animal was killed, and the pancreatic tissues were rapidly separated and stored in a 37°C stationary bath for digestion for 16.5 min. Hanks (with 0.1% bovine serum albumin) was then added to terminate the digestion, and the samples were centrifuged at 12.3g for 2 min at 4°C. The supernatant was removed without disturbing the cell pellet three times. After large pieces of undigested tissues were removed, Biocoll separating solution (L-6155; Biochrom GmbH, Berlin, Germany) was used for islet purification to obtain islet cell pellets, which were then washed three times with Hanks (with 0.1% bovine serum albumin) added with 0.5 mL of 0.25% Trypsin-ethylendiaminetetraacetic acid (25200072; Gibco, Grand Island, NY, USA), digested for 14 min, and added with 1640 medium (12633012; Gibco, Grand Island, NY, USA) to stop the digestion. Afterward, 1× phosphate-buffered saline (calcium and magnesium free) containing 0.04% weight/volume bovine serum albumin (400 μg/mL) was added, and the specimen was centrifuged at 12.3g at 4°C for 5 min. The cell pellets were resuspended in 1 mL of red blood cell lysis buffer and incubated for 10 min at 4°C. The samples were then filtered over Scienceware Flowmi 40 μm cell strainers. Cell concentration and cell viability were determined by hemocytometer and Trypan Blue staining, respectively.

scRNA-seq

scRNA-seq libraries were prepared with Chromium Single cell 3′ Reagent v3 Kits (Shanghai OE Biotech Co., Ltd., Pujiang, Shanghai, China) in accordance with the manufacturer's protocol. Single-cell suspensions were loaded on the Chromium Single Cell Controller Instrument (10× Genomics) to generate single-cell gel beads in emulsions. Reverse transcription reactions were then engaged barcoded full-length complementary deoxyribonucleic acid (cDNA), followed by the disruption of emulsions using the recovery agent and cDNA clean up with DynaBeads Myone Silane Beads (Thermo Fisher Scientific, Pudong New Area, Shanghai, China). The cDNA was then amplified by polymerase chain reaction with appropriate cycles depending on the recovery cells. The amplified cDNA underwent fragmentation, terminal repair, A-tail ligation, index linker ligation and library amplification. The libraries were then sequenced on the Illumina sequencing platform (HiSeq X Ten) to generate 150 bp paired-end reads.

scRNA-seq data preprocessing

scRNA-seq analysis produced demultiplexed cellular barcodes and mapped reads to mouse genome (version mm10) with Cell Ranger pipeline (version 3.3.0) provided by 10× Genomics. Seurat (version 2.3.4; Satija Lab, New York City, NY, USA) was used for subsequent analysis. First, low-quality cells and multiplet captures were removed by applying a criterion to filter out cells with UMI/gene numbers out of the limit of mean value ±2-fold of standard deviations under the assumption of Gaussian distribution for each cell's UMI/gene numbers. Cells with

>20% mitochondrial-derived UMI counts were removed. Second, principal component analysis was caried out to reduce the dimensionality on the log-transformed gene-barcode matrices of top variable genes. For t-distributed stochastic neighbor embedding projection and clustering analysis, the cells were clustered using a graph-based clustering approach and visualized in two dimensions using t-distributed stochastic neighbor embedding.

RESULTS

scRNA-seq identifies multiple cell populations in the mouse pancreas

Islet cells were collected from three female 8-week-old C57/BL6 mice and three female 18-month-old C57/BL6 mice to investigate the differences in islet cells between young and old mice. A total of 59,346 cells were obtained from the raw data and divided into 23 clusters (Figure 1a). According to previous studies, the following cell type-specific markers were identified[10,11]:β-cells (*Ins1* and *Ins2*), β-like cells (*Ins1* and *Ins2*), α-cells (*GCG*), γ-cells (*PPY*), δ-cells (*SST*), acinar cells (*PRSS1*), ductal cells (*KRT19*), endothelial cells (*Pecam1* and *Cdh5*), T cells (*CD3d* and *CD3e*), B cells (*Cd79a* and *Cd79b*) and myeloid cells (*Cd14* and *Cd68*; Figure 1b–e). The bimodal distribution of Ins2 expression was applied to distinguish β-cells and β-like cells (Figure 1f)[10,11].

scRNA-seq analysis shows β-cells associated transcriptomic changes in old mice and young mice

Different gene expression between young and old β-cells was determined. A total of 47 genes were confirmed as differentially expressed between the β-cells of young and old mice ($P < 0.05$, fold change >1.5), including 27 upregulated and 20 downregulated genes. The heatmap of differentially expressed genes is shown in Figure 2a. Mitochondrial genes *mt-Co1*, *mt-Co2*, *mt-Co3*, *mt-Nd1* and *mt-Nd3* associated with metabolic diseases were highly expressed in the pancreas of old mice[12]. *CTRL*, which encodes pancreatic chymotrypsin, was lowly expressed in young β-cells. *CTRL* is a minor chymotrypsin isoform with no important role in cerulein-induced pancreatitis in mice[13]. The differentially expressed genes were further analyzed by Gene Ontology enrichment. The top 30 significantly enriched Gene Ontology terms of upregulated genes ($P < 0.05$) are shown in Figure 2b, including nitric oxide-mediated signal transduction, aerobic respiration, oxidation-reduction process, hormone activity and neuropeptide hormone activity. The top-ranking significant Gene Ontology terms enriched by downregulated genes included proteolysis, digestion, lipid catabolic process, lipid metabolic process and serine-type peptidase activity (Figure 2c). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping based on the encyclopedia's ortholog terms was also carried out to assess the aging-related pathways of β-cells. The significantly enriched KEGG pathways of upregulated genes ($P < 0.05$) are shown in Figure 2d, including influenza A, thermogenesis and neuroactive ligand–receptor interaction. For downregulated differentially expressed genes in old β-cells, the most significantly enriched KEGG pathways were related to pancreatic secretion, protein digestion and absorption, and fat digestion and absorption (Figure 2e). These results showed that these genes play an important role in the aging of β-cells. In addition, senescent and young cells show comprehensive difference in pancreatic β cells.

Pseudotime analysis of the young mice β-cells and old mice β-cells

Pseudotime analysis with Monocle2 was used to reconstruct the gene expression profiles and evolution of β-cells during aging. Pseudotime ordering of β-cells yielded the changes of β-cells with aging (Figures 3a,b). Five genes related to mitochondria, namely, *mt-Co1*, *mt-Co2*, *mt-Co3*, *mt-Nd1* and *mt-Nd3*, showed a special expression pattern. The expression of these genes increased in the early stage, then weakened in the middle stage, and increased in the later stage. The gene-encoding metallothionein is at the initial stage of the cell. When the mitochondrial gene expression level was high, the expression levels of *Mt1* and *Mt2* were low. At the end stage, when the mitochondrial genes were highly expressed, *Mt1* and *Mt2* also showed a trend of high expression (Figure 3c). The different expression levels of mitochondrial genes, *mt-Co1*, *mt-Co3*, *mt-Co2*, *mt-Nd3*, *mt-Nd1*, *Mt2* and *Mt1*, indicated that the metabolic difference of β-cells is caused by their varying mitochondrial functions (Figure 3d). Wiley *et al.*[14] found that accumulation of mitochondrial reactive oxygen species (ROS) has been linked to cellular senescence, and the mitochondrial dysfunction-associated senescence phenotype is of great importance. In addition, the old mouse β-cells had low expression of the lipolytic enzyme-ending genes, *Pnlip*, *Clps* and *Pnliprp1*, and high expression of the gene, *Acly*, which is a key enzyme for lipid synthesis. These results showed that aging affects the aerobic metabolism, oxidation–reduction, immune metabolism cell stability, signal transduction and lipid metabolism of β-cells.

Estimating transcription regulator activity by the single-cell algorithm, Single-Cell Regulatory Network Inference and Clustering

Pancreatic cells undergo reversible switching between different cell stages, and this plasticity drives metastasis. The regulatory activity of all transcription factors (TFs) was analyzed to understand the specific gene regulation of young and old β-cell types. TF profiles were analyzed by Single-Cell Regulatory Network Inference and Clustering (SCENIC) to identify the potential regulon activity score, co-expression module and their associated cis-regulatory elements. The identified TFs were classified into five main modules according to the distribution of old and young mouse β-cells (Figure 4a). The most overrepresented motifs include *Fos*, *Jund* and *Sox9*, all of which were enriched in young mouse β-cells and grouped into module 5 (Figure 4b). Matsuoka *et al.*[15] reported that the overexpression of these TFs can transdifferentiate embryonic or mature α-cells

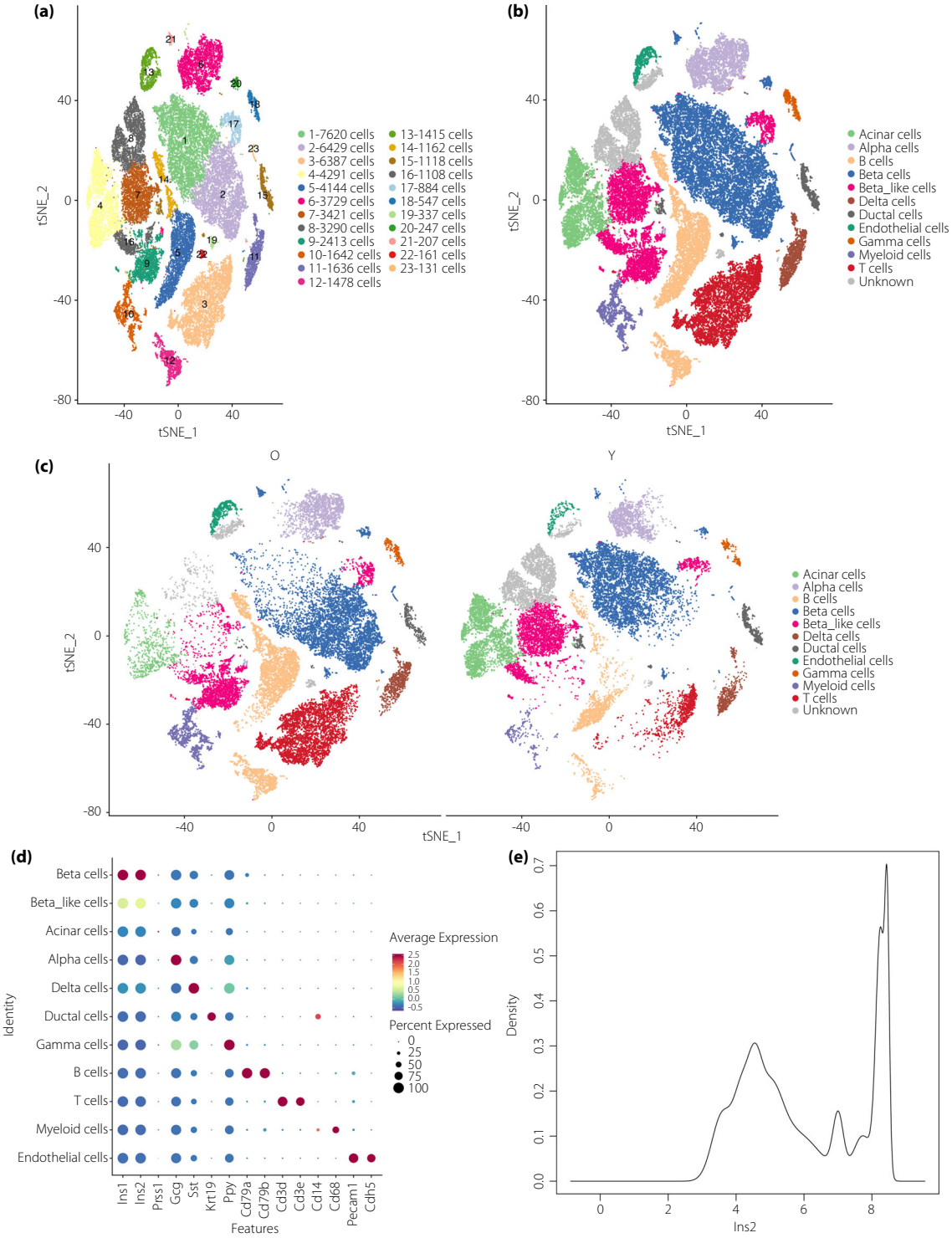

Figure 1 | Integrated single-cell ribonucleic acid sequencing analysis of young mice pancreatic cells and old pancreatic cells. (a) Cells on the t-distributed stochastic neighbor embedding plot of 23 clusters. (b) Cellular populations identified. (c) Cells on the t-distributed stochastic neighbor embedding plot of all six samples were colored as originating either from young mice pancreatic cells and old pancreatic cells. (d) Canonical cell markers were used to label clusters by cell identity as represented in the t-distributed stochastic neighbor embedding plot. Cell types were classified as β-cells (C1, C2, C19, C20, C21, C23), β-like-cells (C7, C9, C16, C17), α-cells (C6), γ-cells (C18), δ-cells (C11), acinar cells, ductal cells (C15), endothelial cells (C13), T cells (C3), B cells (C5, C12, C14) and myeloid cells (C10). (e) Bimodal distribution of Ins2 expression.

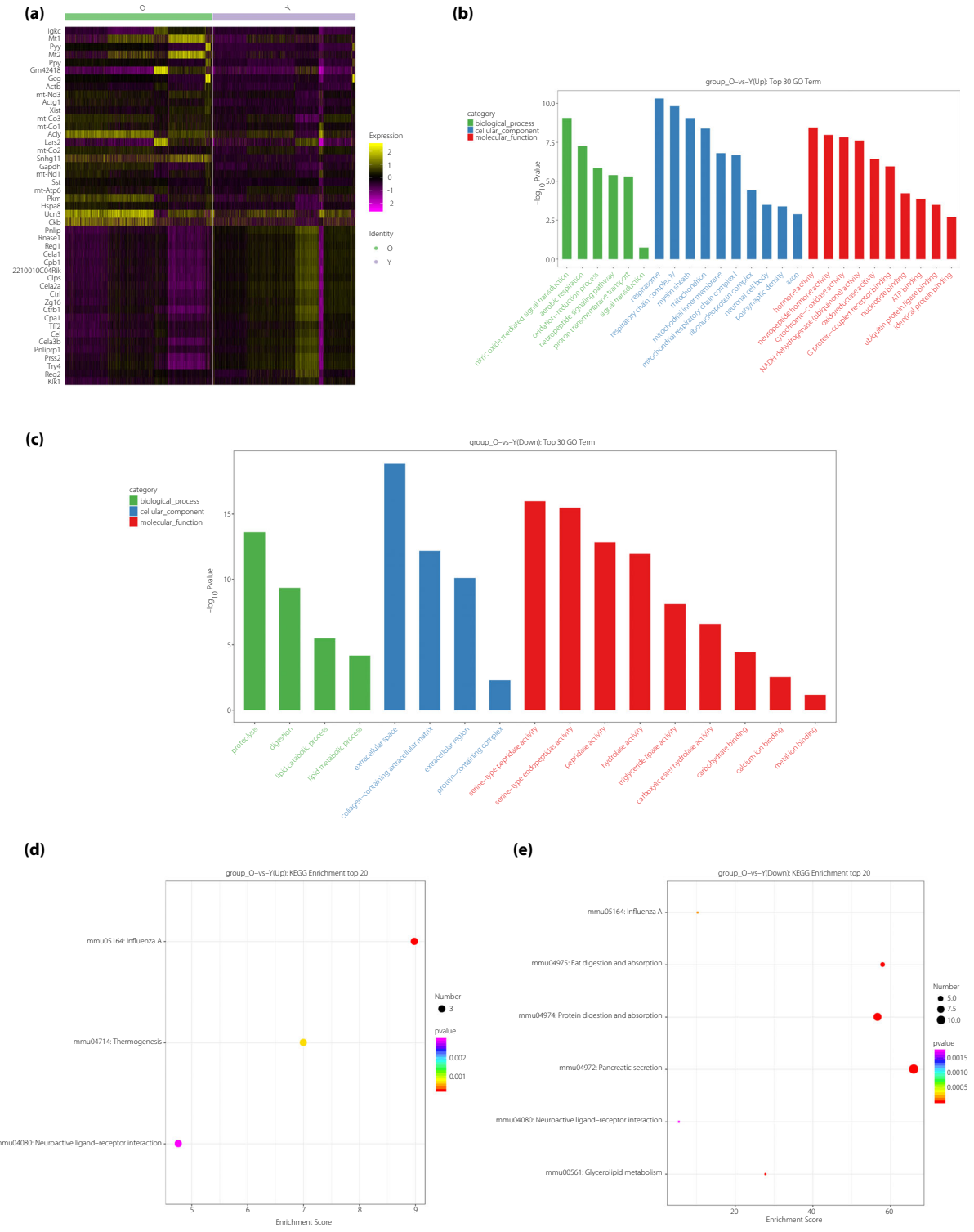

Figure 2 | Differential expression analysis of single-cell ribonucleic acid sequencing data from old and young pancreas identifies genes characteristic of β-cells. (a) Heatmaps are shown representing the upregulated and downregulated genes in β-cells. (b,c) Functional enrichment analysis with Gene Ontology (GO) was carried out using GOrilla with the significantly upregulated and downregulated genes in pancreatic β-cells from old mice compared with young mice. Top 30 significantly enriched GO processes are shown for pancreatic β-cells. (d,e) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of significantly upregulated and down-regulated genes in pancreatic β-cells. Top 20 KEGG enrichment terms using cis method.

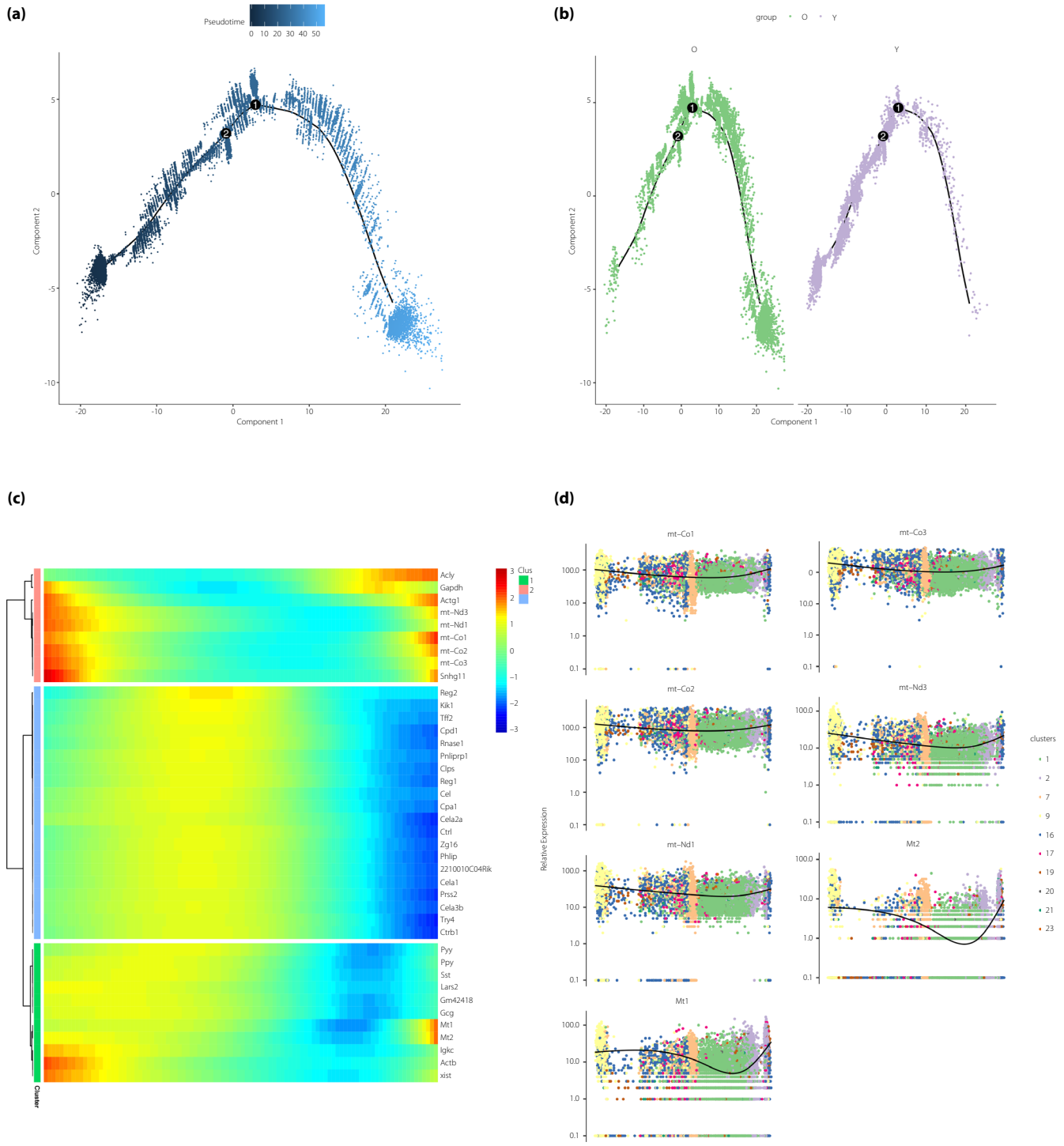

Figure 3 | Pseudotime analysis of the old mice β-cells and young mice β-cells. Each dot represents one cell. (a,b) *In silico* pseudotime ordering of β-cells shows that 'young β-cells' are along the main path of the left state. Whereas 'old β-cells' are along the right state. (c) Clustering of differentially modulated genes by pseudotime progression of β-cells shows distinct kinetics of gene responses to cell conversion. (d) Gene expression kinetics along pseudotime progression of representative genes.

from diabetic mice into insulin-producing β-like cells. β-Cell growth and differentiation are regulated by TFs. Understanding these signals is important for the *in vitro* endocrine induction of stem cells, the differentiation of β-cells, and the determination of trigger factors for regeneration *in vivo*[16]. Furthermore, unraveling the regulation of cell differentiation and senescence

by different TFs allows us to fully understand the mechanism of senescence and therefore control this process.

DISCUSSION

The complexity of mouse pancreatic cells was studied, and the results showed that the identity and composition of these cells are usually associated with aging. When quantified as a fraction of the total number of pancreatic cells, the cell number of most cell types did not fundamentally change with age. This finding might show additional changes in cell subtypes, especially those occurring in specific areas of the pancreas. scRNA-seq in the pancreas is still at an early stage. With its development, high-throughput transcriptome analysis across cell types, states and subpopulations has become viable[17]. Here, a unique resource of single-cell transcriptomes was generated for the pancreatic islets of six mice. Data analysis can identify cell types and prove that cell type-specific gene expression programs are the strongest determinants of cell transcriptomes. Even with huge amounts of atypical cells, we are able to simultaneously define the transcriptional characteristics of rich and rare cell types in the pancreas, including α, β, β-like, δ, γ, ε, myeloid, immune, endothelial, acinar and ductal cells.

Many factors can cause β-cell dysfunction, and β-cell mitochondrial dysfunction is a key factor in its function impairment. 10× Genomics scRNA-seq results showed that in the elderly β-cells, the related mitochondrial genes, *mt-Co1*, *mt-Co2*, *mt-Co3*, *mt-Nd1*, and *mt-Nd3* were upregulated. *mt-Co1*, *mt-Co2* and *mt-Co3* encoded cytochrome oxidase, and *mt-Nd1* and *mt-Nd3* encoded nicotinamide adenine dinucleotide dehydrogenase. Nicotinamide adenine dinucleotide dehydrogenase is involved in the aerobic oxidation of the respiratory chain complex I, and cytochrome oxidase is involved in the composition of complex III. Oxidative phosphorylation produces many by-products, including ROS, which is mainly produced by

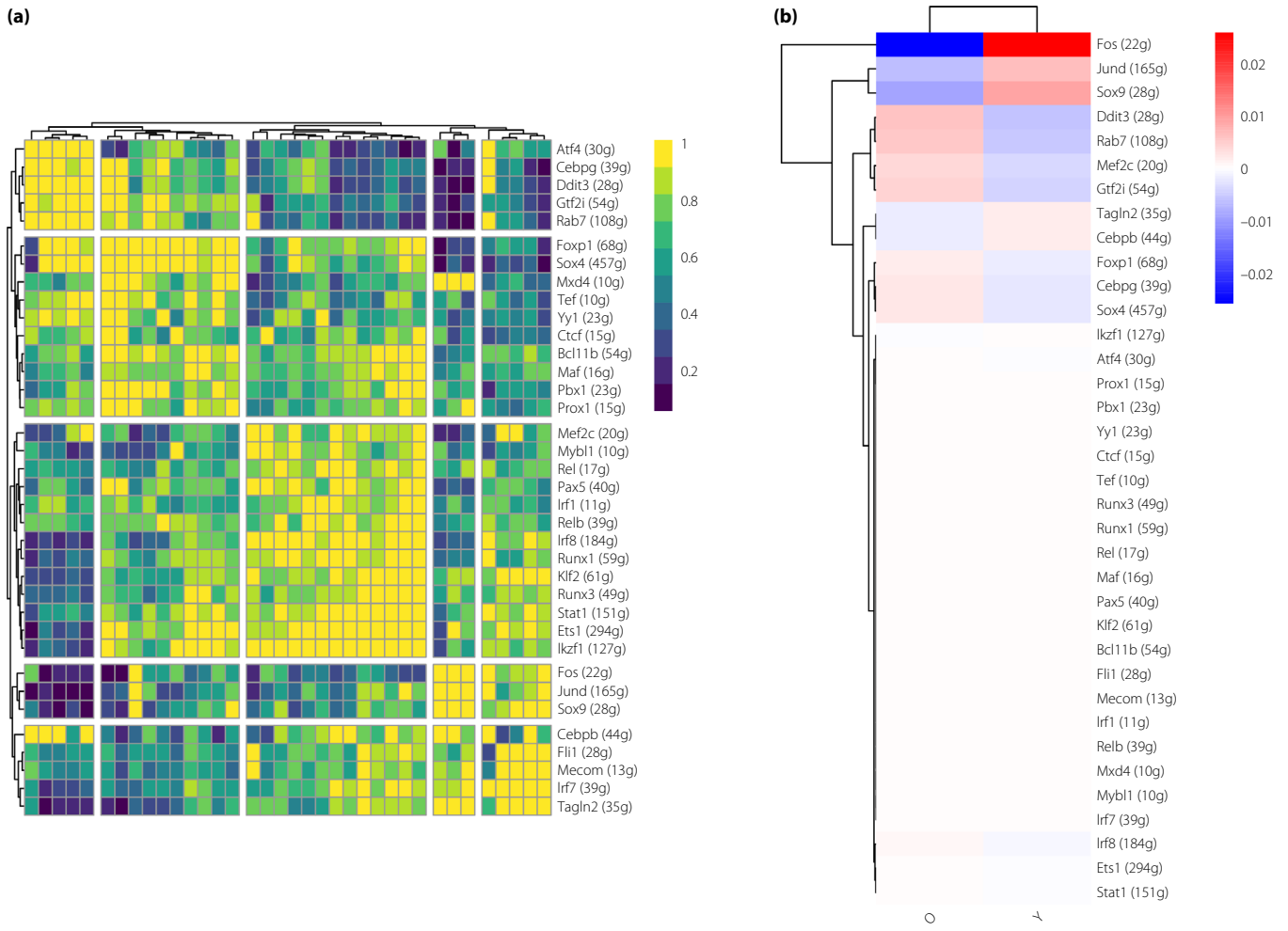

Figure 4 | Single-cell algorithm Single-Cell Regulatory Network Inference and Clustering (SCENIC) estimate transcription regulator activity. (a) Identification of regulon modules based on the regulon matrix of the old mice β-cells and the young mice β-cells. According to the connection specificity index, TF regulons are grouped into five major modules. (b) TF regulons activity heatmap.

complexes I and III[18]. With the high expression of these five mitochondrial genes in the β-cells of old mice, the activity of the aerobic oxidative respiratory chain complexes I and III is increased, which ultimately intensifies ROS production. According to the pseudotime gene expression heat map, the high expression of these mitochondrial genes increases ROS production in the early stage of β-cells, and the appropriate amount of ROS is an important driving force for β-cell proliferation and β-cell mass establishment in the early postnatal period[19]. Over time, metabolic stress stimulates the high expression of mitochondrial genes again, thus increasing the ROS production in elderly β-cells. The oxidative stress caused by direct exposure to ROS at the end of the cell can damage the function of β-cells[20], thus triggering the anti-oxidant mechanism of the β-cell itself, and increasing the expression of genes *Mt1* and *Mt2* encoding metallothionein. Given the low content of anti-oxidant enzymes in β-cells[21,22], metabolic pressure increases with age. When ROS production is excessive, the anti-oxidant mechanism of metallothionein is decompensated, and ROS further affects the mitochondrial function and protein structure of β-cells, leading to abnormal cell function. The key lipolytic enzymes genes, *Pnlip*, *Clps* and *Pnliprp1*, were lowly expressed in old mouse β-cells, thus allowing easy lipid accumulation in senescent β-cells. This finding also explains why lipid metabolic disorders are likely to occur with aging. *Gcg* and *Sst*, which are expressed by pancreatic islet α- and δ-cells, are highly expressed in elderly β-cells, indicating that senescent β-cells might have a certain trend of transdifferentiation. These new findings are worthy of further study.

Accumulating evidence suggests the differential roles of various TFs on β-cell differentiation and functions. Compared with those in old mouse β-cells, *Jund*/*Fos* TFs were enriched in young mouse β-cells. These TFs regulate β-cell growth and differentiation. Good *et al.*[23] found that as a stress response factor of β-cells, *Jund*, promotes redox imbalance and apoptosis in stress processes related to pathophysiology. This TF also plays a maladaptive role in β-cells during prolonged metabolic stress[24]. Experimental verification showed that immature β-cells have high metabolic activity, and reactive oxygen species and *Fos* TF drive β-cell proliferation and massive expansion after birth[25]. This result further explains that young mouse β-cells show greater abilities to cope with oxidative stress and proliferate than old mouse β-cells. A new direction for cell senescence control through TF regulation could be developed.

Although single-cell sequencing can achieve high efficiency, high throughput, high capture rate and short cycle characteristics, the current scRNA-seq islet research has certain limitations, such as partial capture of the cellular transcriptome, wide range of gene expression levels within the same cell and influence of the sample preparation on the transcriptomes. To date, improvements to this methodology are ongoing, including the recent development of 'molecular crowding SCRB-seq,'[26] single-cell sequencing methodology that can effectively narrow the gap between cell function and transcriptology, and combined whole-cell patch clamp with scRNA-seq[27]. Due to the individual differences between species, the results of the current study could not be completely consistent across other species. Although one of the advantages of 10× Genomics scRNA-seq is that it makes small clinical samples accessible to quantitative analysis, the findings of the present study should be considered alongside the study limitations, which include the small sample size and only one species.

In conclusion, the single-cell transcriptomes of mouse pancreatic cells at 8 weeks (young) and 18 months (old) were characterized. Differences in β-cells between old and young mice were identified. Genes, *Mt1*, *Mt2*, *Pyy*, *Gcg*, *Sst* and *Pnlip*, are highly expressed in old β-cells. However, the regulation of these genes during pancreatic senescence and homeostasis must be further studied. The regulating role of TFs for growth and senescence in pancreatic β-cells was also discussed. These data are worthy of deep exploration, and provide new resources and directions for the research of pancreatic aging.

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DISCLOSURE

This study was approved by the Animal Care and UtilizationCommittee of Fujian Academy of Medical Sciences, approval number: DL2020-01, 23 January 2020. All animal experiments were carried out following the national guidelines and the relevant national laws on the protection of animals.

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