Supplementary Materials and Methods

Sampling, phenotypic information collection, and ethical approval

Peripheral whole blood was collected from 24 healthy captive TMs from the Jianyang Monkey breeding farm, which is affiliated with the Zoological Institution of the Provincial Hospital of Sichuan, on 10 January 2017. Sample collection and utility protocols were carried out in strict adherence to the guidelines of the Management Committee of Experimental Animals of Sichuan Province, China (SYXK (Sichuan) 2018-020). This study was approved by the Ethics Committee of College of Life Sciences, Sichuan University (No. 20200529001). The selected macaques included 12 males and 12 females, with ages ranging from 3 to 18 years according to their birth date or age-related morphological characteristics. All individuals were randomly sampled, and their non-relatedness was confirmed based on relatedness distance calculated from SNPs obtained in RNA-Seq data.

Due to the limited studies on TM age, researchers often divide groups based on age information obtained from cynomolgus and rhesus monkeys (Kemnitz, 2011; Wu et al., 2014). Here, the TMs were divided into four developmental stages, i.e., childhood (0–3 years old), adolescence (4–7 years old), post-adolescence (8–14 years old), and aged (>15 years old). The males were coded as 1 and females were coded as 0 in downstream analysis. Sample information is listed in Supplementary Table S1.

RNA extraction, sequencing, and mapping

Whole blood was drawn and temporarily preserved in specialized blood collection tubes (PAXgene Blood RNA tubes), after which white and red blood cells were separated. All operations were performed surrounded by dry ice (solid form of carbon dioxide). Total RNA was extracted following the manufacturer's PAXgene Blood RNA kit manual (Becton, Dickinson and Company, USA). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Total RNA was treated with a GlobinZero kit (Epicentre, Illumina, Madison, WI, USA) and purified with a modified Qiagen RNeasy MinElute (Qiagen Inc., Valencia,
CA, USA) cleanup procedure or ethanol precipitation. The RNA integrity number (RIN) for all samples was above 7.5, except for sample TM16 (6.5). Single-stranded RNA was transformed into cDNA using a reverse transcript polymerase chain reaction (PCR) approach with a strand-specific kit from Epicentre (ScriptSeq v2 Library Prep kit, Illumina, Madison, WI, USA) with ScriptSeq Index PCR primers (Epicentre, Illumina, Madison, WI, USA). After quantification with the KAPA SYBR Fast qPCR library quantification kit (Kapa Biosystems Inc., Wilmington, MA, USA), double-stranded cDNA was sequenced following previously described methods (Fan et al., 2014). Briefly, each cDNA sample was sonicated into 300–500 bp sized fragments and applied to paired-end library generation, with the libraries then sequenced using the Illumina Hiseq 2000 system at Novogene (Beijing). Library preparation and all sequencing runs were performed following the manufacturer’s instructions. The raw data were deposited in the NCBI Sequence Read Archive (SRA) database under SRA project accession number PRJNA516976.

Raw data quality control was performed using NGS QC Toolkit v2.3 by filtering out reads containing more than 20% low-quality bases (PHRED quality score <20). Adaptors were auto-detected and filtered out by setting the Paired-End DNA Library. We chose the rhesus macaque genome (Ensembl, MMUL_8.0.1.90, unmasked) as our reference genome, given that it is well annotated and the species shares a close relationship with TMs (divergence ~3 Mya) (Fan et al., 2014). Clean data were mapped to the reference genome with hisat2. The output bam files were sorted and indexed with SAMtools v1.6 before expression level estimation. HTSeq-count v0.9.1 (Simon et al., 2015) was applied to qualify the expression level of each gene using union mode.

**DEG analysis**

As recommended in the typical RNA-Seq workflow (Dillies et al., 2013), expression values were normalized in the R package DESeq2 by introducing two variables: i.e., dispersions for a gene × sample matrix, and size factors for a single number per sample. Mapped fragments of less than 10 in more than 50% of samples (50% cut-off
threshold was set to ensure a Y-linked DEG detection as there were 12 (50%) male individuals in the study) were discarded in subsequent analyses (Love et al., 2014). DESeq2 was then used in DEG detection using negative binomial generalized linear models and Wald statistics (Love et al., 2014). A multi-factors method was implemented for model fitting with the formula, gene expression ~ age + sex + RIN (RNA integrity number) + rank (RNA quality rank), to estimate significance of the continuous factor of age. The effects of age on transcriptome-wide gene expression were assessed based on an FDR significance threshold of <0.05, given the limited sample number. In addition, DEGs previously reported from human blood (Peters et al., 2015) were used to compare with blood DEGs identified in our TM data.

**Weighted gene co-expression network analysis (WGCNA)**

All genes were analyzed using the WGCNA package in R (Horvath et al., 2012). Gene connectivity was measured by the K-within metric produced by the intramodularConnectivity function in WGCNA with parameters soft threshold=3, TOMType="unsigned", minModuleSize=30, cutreeDynamic cuttree. The edges and nodes of each gene-set module were exported to files using the function exportNetworkToCytoscape. Visualization and subsequent analysis were performed in Cytoscape v3.5.1 (Shannon et al., 2003). The NetworkAnalyzer tool integrated with Cytoscape was implemented to obtain attributes of each node, such as NeighborhoodConnectivity, NumberOfDirectedEdges, and stress centrality. Nodes with directed edges, neighborhood connectivity (above 60% maximum score), and high-stress scores (over 5000) were considered important nodes and treated as core genes in the network.

**Enrichment analysis**

Both Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment were performed in Kobas3 (Xie et al., 2011) and the R package clusterProfiler. Ensembl IDs of genes associated with age (FDR<0.05) were set as query, and all genes analyzed (N=18656) were set as background. Query lists were
further divided into significantly up-regulated and down-regulated genes. The minimum allowed overlap between query genes and genes belonging to a GO term was set to 2. P-values were corrected using the Benjamini-Hochberg FDR method (Benjamini & Hochberg, 1995). The enrichment pathways were selected at FDR<0.05 in GO enrichment analysis and FDR<0.05 in KEGG enrichment analysis. The web enrichment tools g:profiler and panther were also used to confirm the results (Mi et al., 2019).

REFERENCES


Supplementary Figures and Tables

Supplementary Figure S1 Saturation test of RNA-Seq data from 24 Tibetan macaques.
Supplementary Figure S2 Assessment of effects of variables on gene expression based on linear correlation analysis. PCAn (Principal Component Analysis component n) higher than dotted line is the significant component (<=n<=24).

Supplementary Figure S3 Correlation relationships among metadata variables. Darker color and flatter shape of ellipse indicate a more significant correlation. Positive and negative correlations are determined by ellipse direction.
Figure S4 Neighbor genes of Foxo3 and Foxo4 found in positive age-related WGCNA gene module.

Supplementary Table S1 Sample information on 24 Tibetan macaques

Supplementary Table S2 Age-associated DEGs (2523 in Tibetan macaques)

Supplementary Table S3 KEGG enrichment analysis of genes in blue module

Supplementary Table S4 KEGG enrichment analysis of genes in royal-blue module

Supplementary Table S5 GO analysis of down-regulated DEGs

Supplementary Table S6 KEGG analysis of down-regulated DEGs

Supplementary Table S7 GO analysis of up-regulated DEGs

Supplementary Table S8 KEGG analysis of up-regulated DEGs

Supplementary Table S1–S8 is listed as a separate excel file.