

# Single-cell transcriptomics uncovers conserved cardiac cell types and regulatory adaptation across birds and mammals

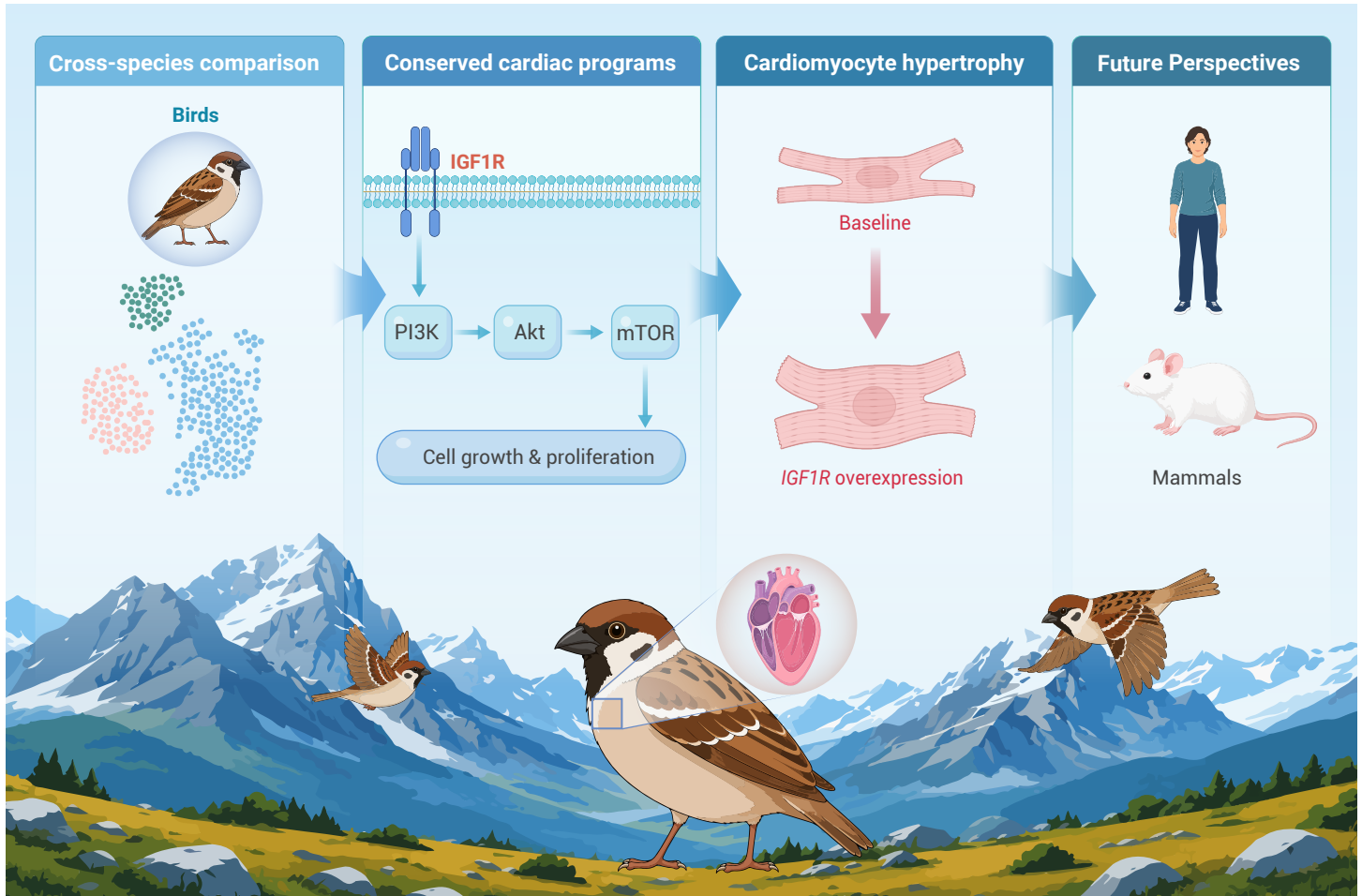
Yan Hao,<sup>1,9</sup> Xiang Zhou,<sup>2,3,9</sup> Qingshuo Zhao,<sup>1,9</sup> Huishang She,<sup>1</sup> Yun Fang,<sup>1</sup> Laikun Ma,<sup>4</sup> Zhengting Zou,<sup>1</sup> Weiwei Zhai,<sup>1,5</sup> Per G.P. Ericson,<sup>6</sup> Fumin Lei,<sup>1,5,\*</sup> Fanwei Meng,<sup>1,\*</sup> Shihua Zhang,<sup>3,7,8,\*</sup> and Yanhua Qu<sup>1,5,6,\*</sup>

\*Correspondence: quyh@ioz.ac.cn (Y.Q.); zsh@amss.ac.cn (S.Z.); mengfw@ioz.ac.cn (F.M.); leifm@ioz.ac.cn (F.L.)

Received: March 25, 2026; Accepted: September 4, 2026; Published Online: September 7, 2026; <https://doi.org/10.59717/j.xinn-life.2026.100251>

© 2026 The Author(s). This is an open access article under the CC BY license (<https://creativecommons.org/licenses/by/4.0/>).

## GRAPHICAL ABSTRACT



## PUBLIC SUMMARY

- Eleven major cardiac cell types were identified in adult and neonatal tree sparrows.
- Adult and neonatal birds exhibited a substantial difference in cell abundance and transcriptional divergence.
- Cardiac cell types and transcriptional programs were conserved across birds and mammals.
- Overexpression of insulin-like growth factor 1 receptor (*IGF1R*) in avian cardiomyocytes induced hypertrophy.

# Single-cell transcriptomics uncovers conserved cardiac cell types and regulatory adaptation across birds and mammals

Yan Hao,<sup>1,9</sup> Xiang Zhou,<sup>2,3,9</sup> Qingshuo Zhao,<sup>1,9</sup> Huishang She,<sup>1</sup> Yun Fang,<sup>1</sup> Laikun Ma,<sup>4</sup> Zhengting Zou,<sup>1</sup> Weiwei Zhai,<sup>1,5</sup> Per G.P. Ericson,<sup>6</sup> Fumin Lei,<sup>1,5,\*</sup> Fanwei Meng,<sup>1,\*</sup> Shihua Zhang,<sup>3,7,8,\*</sup> and Yanhua Qu<sup>1,5,6,\*</sup>

<sup>1</sup>State Key Laboratory of Animal Biodiversity Conservation and Integrated Pest Management, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China

<sup>2</sup>Guangdong Institute of Intelligence Science and Technology, Zhuhai 519031, China

<sup>3</sup>State Key Laboratory of Mathematical Sciences, Academy of Mathematics and Systems Science, Chinese Academy of Sciences, Beijing 100190, China

<sup>4</sup>Chengde Innovation Center for Exploitation and Ecological Protection of Distinctive Biological Resources, Hebei Minzu Normal University, Chengde 067000, China

<sup>5</sup>College of Life Science, University of Chinese Academy of Sciences, Beijing 100049, China

<sup>6</sup>Department of Bioinformatics and Genetics, Swedish Museum of Natural History, Stockholm SE-104 05, Sweden

<sup>7</sup>School of Mathematical Sciences, University of Chinese Academy of Sciences, Beijing 100049, China

<sup>8</sup>Key Laboratory of Systems Health Science of Zhejiang Province, School of Life Science, Hangzhou Institute for Advanced Study, UCAS, Hangzhou 310024, China

<sup>9</sup>These authors contributed equally

\*Correspondence: quyh@ioz.ac.cn (Y.Q.); zsh@amss.ac.cn (S.Z.); mengfw@ioz.ac.cn (F.M.); leifm@ioz.ac.cn (F.L.)

Received: March 25, 2026; Accepted: September 4, 2026; Published Online: September 7, 2026; <https://doi.org/10.59717/j.xinn-life.2026.100251>

© 2026 The Author(s). This is an open access article under the CC BY license (<https://creativecommons.org/licenses/by/4.0/>).

Citation: Hao Y., Zhou X., Zhao Q., et al. (2026). Single-cell transcriptomics uncovers conserved cardiac cell types and regulatory adaptation across birds and mammals. *The Innovation Life* 4:100251.

Birds and mammals possess highly efficient four-chambered hearts to meet the elevated metabolic demands of endothermy, yet the cellular and molecular basis of this convergent evolution remains poorly understood. To address this gap, we generated the first single-cell heart transcriptomic atlas of a wild bird, the Eurasian Tree Sparrow (*Passer montanus*), across neonatal and adult stages, and integrated it with existing human and mouse data. This cross-species comparison reveals broadly conserved transcriptional profiles: cardiomyocytes are the most transcriptionally conserved cell type across species and developmental stages, whereas non-myocyte populations (fibroblasts and endothelial cells) show greater transcriptional plasticity. These findings suggest that cardiac diversification and adaptation may occur primarily through regulatory changes within a shared cellular repertoire. Extending this framework, we investigate cardiac adaptation to high-elevation hypoxia in tree sparrows. Strikingly, the transcriptional programs significantly overlapped with genes previously linked to cardiac remodeling and disease-related phenotypes in humans and mice. To test the functional relevance of this overlap, we performed *in vitro* assays and found that *IGF1R* overexpression drives hypertrophy-like growth in avian cardiomyocytes, accompanied by PI3K-AKT-mTOR-associated transcriptional reprogramming, a pathway critically implicated in mammalian cardiac hypertrophy. Collectively, our findings demonstrate that birds and mammals share a conserved cardiac cellular blueprint, wherein regulatory programs are recurrently co-opted during development, adaptation to hypoxia, and pathological remodeling.

## INTRODUCTION

The independent evolution of endothermy in birds and mammals represents a landmark event in vertebrate history, enabling sustained high-energy activity and the colonization of diverse environments.<sup>1</sup> This breakthrough was accompanied by the parallel development of an advanced, four-chambered heart capable of supporting high-pressure pulmonary and systemic circulation,<sup>2</sup> a classic example of convergent evolution, as the avian and mammalian lineages diverged over 300 million years ago. While sharing this fundamental architecture, the avian cardiovascular system has undergone distinct specialization under the extreme metabolic demands of flight, resulting in proportionally larger hearts, greater stroke volumes, and enhanced capacities for hypoxia tolerance compared to mammals.<sup>3–5</sup> Together, birds and mammals thus provide a powerful comparative framework for understanding how two distinct lineages independently built circulatory systems capable of sustaining endothermy and intense aerobic performance.

As the central organ of the oxygen transport cascade, the heart is essential for meeting elevated metabolic demands, particularly under environmental hypoxia at high elevations.<sup>6</sup> This profound environmental challenge has driven convergent cardiovascular adaptations across air-breathing vertebrates, both birds and mammals. For example, high-elevation birds, non-

human mammals, and humans exhibit parallel cardiac responses, including increased heart mass (hypertrophy) and greater cardiac capillarization, which collectively enhance oxygen delivery in low-oxygen conditions.<sup>7</sup> These shared phenotypic changes, documented in species ranging from deer mice (*Peromyscus maniculatus*)<sup>8</sup> and Tibetan antelopes (*Pantholops hodgsonii*)<sup>9</sup> to highland chickens (*Gallus gallus domesticus*),<sup>10</sup> Andean and Tibetan humans (*Homo sapiens*),<sup>11</sup> Eurasian Tree Sparrows (*Passer montanus*)<sup>12</sup> and puna teals (*Spatula puna*),<sup>13</sup> reveal a consistent evolutionary strategy for sustaining aerobic performance in hypoxic environments.

Notably, this convergence extends beyond shared phenotypes to encompass their molecular regulation. Key genes governing cardiac contractility and hypertrophy, such as *CAMKII* and *MYH6*, show similar expression shifts in high-elevation birds, goats, and antelopes.<sup>9,12</sup> Likewise, *AKT1* and *IRS2*, which mediate cardiac hypertrophy in mice and humans,<sup>14</sup> are also implicated in hypoxia-induced cardiomyocyte hypertrophy in birds.<sup>15</sup> Together, these observations point to recurrent regulatory programs underlying hypoxia-associated cardiac remodeling across birds and mammals.

Despite these striking parallels, current insights remain fragmented across species and are largely limited to a handful of candidate genes. A systematic, cell-resolved understanding of the developmental and regulatory mechanisms underlying this convergence is still lacking. The vertebrate heart is a dynamic organ that undergoes defined transitions from embryonic patterning to postnatal maturation; therefore, comparisons based on a single developmental stage risk conflating species-specific differences in timing with fundamental differences in cell type composition or regulatory logic.<sup>16,17</sup> Sampling across developmental stages is essential to capture these dynamics and distinguish conserved, stage-specific transcriptional programs from lineage-specific adaptation. However, a systematic, cross-lineage comparison of cardiac cellular composition, gene regulatory networks, and developmental responses to hypoxia is still lacking. Addressing this gap is critical not only for understanding the repeatability of evolution but also for uncovering core mechanisms of cardiac resilience that may inform hypoxia-associated cardiac remodeling.

Here, we employ single-cell RNA sequencing (scRNA-seq) to construct a developmental cellular atlas of the heart in a wild bird: the Eurasian Tree Sparrow (hereafter tree sparrow). This human-commensal species colonized the high-elevation Qinghai-Tibetan Plateau approximately 2,600 years ago, evolving cardiovascular adaptations including increased heart mass, cardiomyocyte hypertrophy, and enhanced cardiac capillarization,<sup>12,18</sup> which parallel those observed in high-elevation mammals.<sup>7</sup> Recent work further indicates that the regulatory basis of cardiac hypertrophy in tree sparrows involves convergent molecular pathways shared with mice and humans.<sup>15</sup> Consequently, the tree sparrow provides a useful avian comparative model to dissect the cellular and molecular mechanisms underlying cardiac adaptation to hypoxia.

We profiled neonatal and adult tree sparrow hearts to map developmental

transitions in cellular composition and transcriptional states. This analysis revealed dynamic shifts in cell type proportions and transcriptional states across life stages. To assess the cross-species similarity of these patterns, we integrated our data with murine and human cardiac atlases. This cross-species comparison demonstrated that, while major cardiac cell types are broadly conserved across birds and mammals, cardiomyocytes exhibit the most profound transcriptional similarity across birds and mammals, regardless of life stages. Focusing on high-elevation adaptation, we identified cardiomyocyte hypertrophy-associated molecular programs and pinpointed *IGF1R* as a candidate regulator of cardiac hypertrophy. Functional validation confirmed that *IGF1R* overexpression drives hypertrophy-like growth in avian cardiomyocytes and triggers PI3K-AKT-mTOR-associated transcriptional changes, a conserved signaling axis central to mammalian cardiomyocyte growth. Taken together, this cross-species single-cell atlas reveals conserved transcriptional circuits that orchestrate heart development and cardiac hypertrophy. These findings suggest high-elevation tree sparrows can serve as a useful model for understanding cellular adaptations to hypoxia, potentially providing a foundation for exploring related mechanisms in human cardiovascular conditions.

## MATERIALS AND METHODS

### Sample collection

Tree sparrows, including adults and neonates (postnatal day 1), were collected from low-elevation (Cangzhou, 10 m a.s.l.) and high-elevation (Gahai, 3,400 m a.s.l.) sites between 2020 and 2021 (Table S1). In total, we collected four neonatal lowland, five neonatal highland, three adult lowland, and three adult highland individuals (Figure 1A). Following euthanasia, heart tissues were immediately dissected, preserved in GEXSCOPETM Tissue Preservation Solution (Singleron), and subsequently processed for single-cell and single-nucleus RNA library construction and sequencing.

### Single-cell isolation

Heart tissues of neonatal tree sparrows were rinsed three times with Hanks Balanced Salt Solution and dissected into small fragments (1–2 mm<sup>3</sup>). Tissue dissociation was performed by incubating the fragments in GEXSCOPETM Tissue Dissociation Solution (Singleron) at 37°C for 15 minutes. The resulting digestate was filtered through a sterile 40 µm cell strainer and centrifuged at 1,000 rpm (4°C) for 5 minutes. Following supernatant removal, the cell pellet was gently resuspended in 1 ml of 1× Phosphate-Buffered Saline (PBS). Cell viability and concentration were determined via Trypan Blue exclusion assay using a hemocytometer. Single-cell suspensions with a viability exceeding 80% were adjusted to a minimum concentration of 1 × 10<sup>5</sup> cells per mL for subsequent processing.

### Single-nucleus isolation

Given the large size of adult tree sparrow cardiomyocytes (approximately 100 µm in length and 40 µm in width), which precluded their use in standard single-cell workflows, we performed single-nucleus isolation from adult heart tissue. For each sample, tissue from the left ventricle was minced and homogenized in 1 ml of ice-cold Nuclear Lysis Buffer (0.1% NP-40, 10 mM Tris-HCl, 146 mM NaCl, 1 mM CaCl<sub>2</sub>, 21 mM MgCl<sub>2</sub>, 1 U/µl RNase inhibitor) for 7 minutes on ice. The lysis reaction was quenched by adding 1 ml of ST Wash Buffer (10 mM Tris-HCl, 146 mM NaCl, 1 mM CaCl<sub>2</sub>, 21 mM MgCl<sub>2</sub>, 0.01% BSA, 40 U/ml RNase inhibitor). The homogenate was filtered through a 40 µm cell strainer, and the filter was washed four times with 1 ml of ST Wash Buffer; the filtrates were combined. Nuclei were pelleted by centrifugation at 500 × g for 5 minutes at 4°C. The supernatant was discarded, and the pellet was washed once by resuspension in 5 ml of PBS with 1% BSA, followed by a repeat centrifugation. The final nuclear pellet was resuspended in 100 µl of PBS with 1% BSA. Nuclear integrity and concentration were assessed microscopically via Trypan Blue staining. Nuclear suspensions were diluted to a working concentration of 700–1,200 nuclei per µl for subsequent library preparation.

### Single-cell RNA sequencing library construction and sequencing

Libraries were prepared from single-cell suspensions (1–3 × 10<sup>5</sup> cells/mL in 1× PBS) using the GEXSCOPE® Single Cell RNA Library Kit (Singleron),

largely following the manufacturer's protocol and the methodology described by Dura et al.<sup>19</sup> Cells were partitioned into nanoliter-scale reaction wells using the Singleron Matrix® Single Cell Processing System. Within this microfluidic system, uniquely barcoded oligonucleotide beads were co-loaded and washed. Cells were lysed *in situ*, allowing polyadenylated mRNA transcripts to hybridize to the bead-bound primers during a 20-minute incubation at room temperature. The beads capturing cell-specific barcoded cDNA were recovered for downstream processing. Reverse transcription, cDNA amplification via PCR, and final library construction were performed according to the kit specifications. Libraries were size-selected, purified, and assessed for quality before being sequenced. Sequencing was performed on an Illumina NovaSeq 6000 platform, generating 150 bp paired-end reads. All procedures for scRNA-seq library preparation were conducted by Singleron Biotechnologies (Nanjing, China).

### Single-nucleus RNA sequencing

Single-nucleus suspensions (2.5–3 × 10<sup>4</sup> nuclei/µL) were processed using the Chromium Controller instrument (10x Genomics). Libraries were constructed following the manufacturer's protocol for the 10x Genomics Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Cat. #1000268). Briefly, nuclei were partitioned into Gel Bead-In-Emulsions (GEMs) within a microfluidic chip, where barcoded reverse transcription and cDNA amplification took place. The amplified cDNA was then used to construct sequencing libraries with the Chromium Single Cell 3' Library Construction Kit (Cat. #1000020). Final libraries were sequenced on an MGI-2000 platform, generating 150 bp paired-end reads. All procedures for snRNA-seq library preparation and sequencing were performed by OE Biotech Co., Ltd. (Shanghai, China).

### Processing of scRNA-seq raw sequencing data

Raw sequencing data were processed using the CellScope v1.14.1 (<https://github.com/singleron-RD/CellScope>). First, cell barcodes and unique molecular identifiers (UMIs) were extracted, and reads lacking a poly-T sequence were discarded. Low-quality reads and adapter sequences were then trimmed using Cutadapt v3.7.<sup>20</sup> The cleaned reads were aligned to the tree sparrow genome (<https://db.cngb.org/search/project/CNP0006114/>) using STAR v2.6.1a.<sup>21</sup> Finally, UMI and gene-level counts were quantified with featureCounts v2.0.1<sup>22</sup> to generate the raw count matrix. Detailed sequencing and mapping statistics for all samples are provided in Table S2.

### Processing of snRNA-seq raw sequencing data

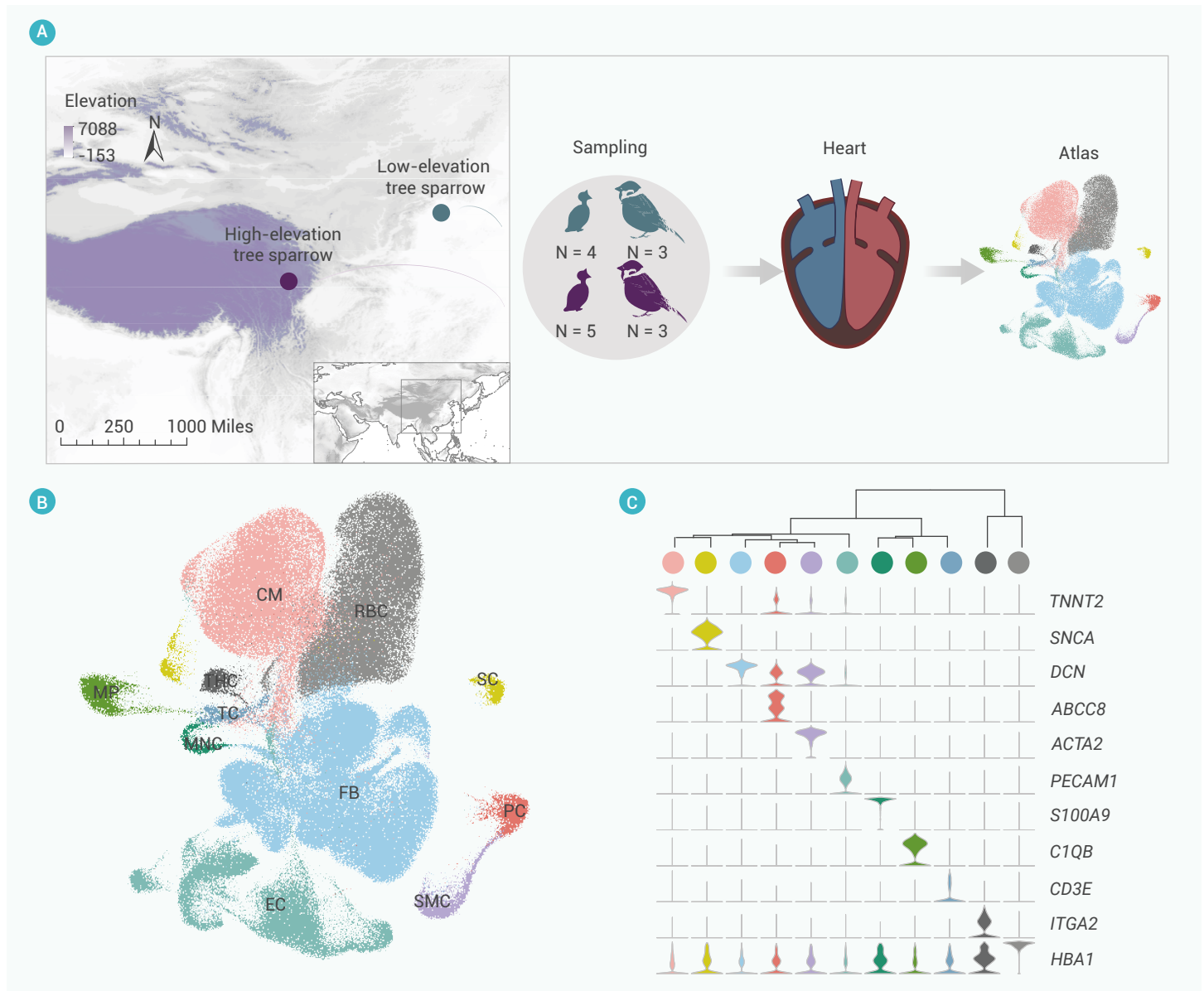
Single-nucleus RNA sequencing data were processed using Cell Ranger v7.1.0.<sup>23</sup> First, a custom pre-mRNA reference for the tree sparrow was constructed following the 10x Genomics reference preparation guidelines (<https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/advanced/references>). Raw sequencing files were demultiplexed and converted to FASTQ format using "cellranger mkfastq". Reads were then aligned to the custom pre-mRNA reference, and gene expression counts were quantified with "cellranger count" using default parameters. This pipeline generated a filtered gene-barcode matrix for downstream analysis. Detailed sequencing, alignment and cellular quality metrics are provided in Table S3.

### Quality control

Data from individual samples were processed and annotated separately using Seurat v4.0.1.<sup>24</sup> We first addressed ambient RNA contamination using SoupX v1.5.0,<sup>25</sup> estimating a contamination fraction ( $\rho$ ) for each sample based on the expression of bimodal genes enriched in empty droplets. Expression profiles were subsequently corrected using the estimated  $\rho$ .

Potential doublets were identified and removed with DoubletFinder v2.0.3.<sup>26</sup> For each sample, we generated artificial doublet profiles by randomly pairing cells ( $p_N = 0.25$ ). The optimal neighborhood size ( $p_K$ ) was determined via the mean-variance normalized bimodality coefficient (BCMVN). Cells predicted as doublets by the "doubletFinder\_v3" function were excluded from downstream analysis ( $n = 13,362$  doublets removed in total).

Finally, we applied sample-specific quality filters based on library quality and cell integrity. For neonatal single-cell data, cells were removed if they met any of the following criteria: (1) fewer than 100 genes detected ( $n_{\text{Genes}} <$



**Figure 1. Single-cell atlas of the tree sparrow heart** (A) Geographic origins of neonatal and adult tree sparrows used for single-cell and single-nucleus RNA sequencing. Birds were collected from high-elevation (Gahai, 3,400 m a.s.l.) and low-elevation (Cangzhou, 10 m a.s.l.) localities. The map can be downloaded from <https://worldclim.org/>. (B) UMAP of the integrated single-cell transcriptomes showed 11 identified cell types clustered into three major groups. (C) Enrichment scores of canonical cell marker genes for the identified cell types.

100), (2) high mitochondrial gene content (mitoRatio  $\geq 40\%$ ), (3) high ribosomal gene content (riboRatio  $\geq 20\%$ ), and (4) low library complexity (log10GenesPerUMI  $\leq 0.7$ ). For adult single-nucleus data, thresholds were adjusted to: nGenes  $< 100$ , mitoRatio  $\geq 20\%$ , riboRatio  $\geq 20\%$ , and log10GenesPerUMI  $\leq 0.7$ . Genes expressed in fewer than 10 cells per sample were also filtered out. Distributions of these QC metrics and final cell statistics per sample are detailed in Figure S1 and Table S4.

#### Normalization, scaling, integration, and clustering

Normalization, integration and clustering analyses were performed using Seurat v4.0.1.<sup>24,27</sup> To correct for sequencing depth and stabilize variance, the raw UMI counts from each sample were normalized and variance-stabilized using the "SCTransform" function. For differential expression analysis and visualization purposes, counts were also normalized by the total UMI count per cell, scaled by a factor of 10,000, and log-transformed using the "NormalizeData" function.

To minimize technical variation associated with sequencing modality and sample processing, we integrated datasets using Seurat integration,<sup>24</sup> which is designed to align shared biological signals while reducing technical variation

across datasets. Briefly, we first identified 3,000 highly variable genes using "SelectIntegrationFeatures". Integration anchors were then identified using "PrepSCTIntegration" and "FindIntegrationAnchors", enabling the merging of samples into a single, batch-corrected dataset using "IntegrateData".

Principal component analysis (PCA) was conducted on the integrated data using "RunPCA". Dimensionality reduction for graph-based clustering was performed using the top principal components. Cell clusters were identified via the "FindNeighbors" and "FindClusters" functions. Marker genes for each cluster were identified with "FindAllMarkers", considering only genes with an adjusted  $P$  value  $< 0.05$  and an absolute log<sub>2</sub> fold change  $> 1$ . Cell types were manually annotated for each cluster based on the expression of established canonical marker genes (Table S5).

#### Comparison analysis of neonatal and adult hearts

To investigate developmental changes, we compared cellular composition and gene expression between neonatal and adult hearts. First, the relative abundance of each annotated cell type was quantified within each age group. We then assessed the transcriptomic similarity of each cell type across development by calculating the pairwise Spearman's correlation of gene

expression profiles between cells within that type for each age group separately. Finally, to determine whether these metrics changed significantly with age, we compared the relative abundances and cell-type-specific transcriptomic similarities (correlation coefficients) between neonates and adults using a non-parametric Wilcoxon rank-sum test. *P* values were adjusted for multiple comparisons using the false discovery rate (FDR) correction.

### Collection of cross-species heart single-cell datasets

To enable a comparative transcriptomic analysis, we collected publicly available single-cell and single-nucleus RNA sequencing datasets from murine and human hearts (Table S8). Here, mice were selected as a representative mammalian model due to their established role in cardiac disease research and their translational relevance to human physiology. By using mice and humans as a comparative benchmark, we assessed the extent of cellular and regulatory differentiation between birds and mammals, as well as within mammals themselves.

Given the substantial differences in lifespan, growth rate, and developmental timing among tree sparrows, mice and humans, we broadly categorized samples into two life stages for comparison: adults (sexually mature individuals) and neonates (neonatal birds, human infants, and mouse pups). Specifically, adult data consisted of single-nucleus data from human hearts (ages 40–65 years<sup>28</sup>) and mouse hearts (adults of unspecified age<sup>29</sup>). Neonatal data consisted of single-nucleus data from human fetal hearts (12–17 weeks<sup>30</sup>) and single-cell data from murine hearts (postnatal days 0–3<sup>31</sup>). We note that this comparative framework contains inherent technical and biological biases, including differences in sequencing strategies (scRNA-seq vs. snRNA-seq) and imperfect age-matching due to divergent species-specific developmental timelines.

For the cross-species analyses (here and below), we focused on the three major and consistently identifiable cardiac cell types: CMs, FBs, and ECs. These three cell types are available for both mice and humans, in both adults and neonates. It should also be noted that these three cell types constitute the structural and functional core of the vertebrate heart.<sup>28</sup>

### Cross-species cell-type mapping

To establish homology between cardiac cell types across species, we performed cross-species cell-type mapping using the Cross-species cell type Assignment and gene Module extraction (CAME) approach.<sup>32</sup> The annotated mouse and human datasets served as reference atlases (Table S8). We then projected the tree sparrow dataset (query) onto this integrated reference space to predict cell-type labels. The resulting correspondences between tree sparrow, mouse, and human cell types were visualized using a Sankey diagram.

### Quantification of transcriptomic heterogeneity

To quantify and compare cellular heterogeneity within defined cell types, we calculated transcriptomic entropy for each cell type across species and life stages, following the method of Ma et al.<sup>33</sup> To control for potential sampling bias arising from differing cell numbers per group, we computed two entropy values: real entropy from the observed expression data and unstructured entropy from randomly permuted data within each group. The final measure of transcriptomic heterogeneity for each cell type was defined as the residual entropy, calculated by subtracting the unstructured entropy from the real entropy.

### Quantification of transcriptomic similarity

For each set of homologous cell types identified across species, we quantified the degree of transcriptomic similarity between species pairs: tree sparrows vs. mice, tree sparrows vs. humans, and mice vs. humans. First, the average gene expression profile was computed for the cell type within each species. Pairwise species comparisons were then performed by calculating the Spearman's rank correlation coefficient (*r*) between these averaged expression vectors.

### Cross-species homologous gene identification

To enable direct comparative analysis of single-cell transcriptomes across species, we compiled a homology table linking mouse (GRCm39) and human

(GRCh38) genes using data from Ensembl (<http://ftp.ensembl.org/pub/release-105/>). The tree sparrow genome assembly and annotation were obtained from the China National GeneBank (<https://db.cngb.org/search/project/CNP0006114/>). Orthologous gene families were identified across the three species using OrthoFinder v2.5.4.<sup>34</sup> We extracted single-copy, one-to-one orthologs from the OrthoFinder output. This initial set was further refined using a reciprocal best-hit approach with BLASTp (v2.12.0+; E-value < 1e-10) and cross-referenced with established human-mouse homology data from the Ensembl BioMart web server (<http://www.ensembl.org/biomart/martview>).

### Quantification of cell-type-specific conserved gene expression

To identify conserved expression programs across species, we performed analyses on the set of high-confidence one-to-one orthologs. For each homologous heart cell type, we calculated the average expression of each ortholog across all cells for every sample (species and life stage). To mitigate confounding effects arising from direct comparisons of absolute expression levels across species and developmental stages, we transformed expression values into within-sample gene-rank scores.<sup>35</sup> Specifically, within each sample, genes were ranked by their average expression and assigned a normalized score between 0 and 1, with a floor of 0.5. Orthologs with rank scores below 0.8 in all samples were considered lowly expressed and filtered out. The resulting rank scores were used as input for downstream clustering analysis to identify evolutionarily stable co-expression modules.

The rank-transformed expression matrix for each homologous cell type was log-transformed and subjected to PCA using gmodels v2.18.1.<sup>36</sup> To identify patterns of co-expression, we performed hierarchical clustering on both genes and samples using a complete-linkage agglomerative method with a correlation-based distance metric. The results were visualized in a heatmap to depict expression patterns across species and life stages.

To systematically classify genes based on their evolutionary expression patterns, we applied k-means clustering to the rank-transformed expression data using the base R "kmeans" function. The optimal number of clusters (*k*) was determined by the elbow method. This approach enabled the identification of two key gene categories: (1) highly conserved genes (showing consistent high rank across species) and (2) species-specific genes (showing high rank in only one or a subset of species).

Functional enrichment analysis was performed on each gene cluster using ClueGO v2.5.10 with the biological process ontology from the GO\_Biological-Process-EBI-UniProt-GOA database (release 25.05.2022).<sup>37</sup> A right-sided hypergeometric test was applied, and *P* values were adjusted for multiple testing using the Benjamini–Hochberg method. For clusters of conserved genes, we used an adjusted *P* value (FDR) threshold of < 0.001.

### Compilation of cardiac phenotype-associated genes

Genes associated with human cardiac diseases and phenotypes were compiled from two primary sources. First, disease-associated susceptibility genes were retrieved from the DisGeNET database (v7.0, <https://www.disgenet.org/>).<sup>38</sup> Second, genes linked to specific heart-related phenotypes were obtained from the human phenotype ontology (HPO) database (<https://hpo.jax.org/>).<sup>39</sup> For comparative analysis, genes associated with murine cardiac phenotypes were collected from the mammalian phenotype ontology (MPO) via the Mouse Genome Informatics (MGI) database at Mouse Genome Informatics (<https://www.informatics.jax.org/>).<sup>40</sup>

### Correlation analysis of susceptibility gene expression

For each of the three susceptibility gene datasets (human DisGeNET, human HPO, and mouse MPO; see Table S10), we assessed the similarity of expression patterns between species pairs (tree sparrows vs. mice, tree sparrows vs. humans, mice vs. humans) using Spearman's rank correlation analysis performed with the "corr.test" function from the psych R package (v2.3.9). *P* values were adjusted for multiple comparisons across all gene sets and species pairs using the Benjamini–Hochberg FDR correction.

### Analysis of high-elevation-associated gene expression

We identified the differentially expressed genes (DEGs) between high- and low-elevation neonatal and adult tree sparrows, respectively. For each anno-

tated cell type, we performed the non-parametric Wilcoxon rank-sum test using the “FindMarkers” function. A gene was classified as a DEG if it was detected in > 10% of cells within that cell type, exhibited an absolute fold change > 2, and had a Bonferroni-adjusted  $P$  value < 0.05.

Subsequently, we examined the potential functional relevance of elevation-associated DEGs by testing for overlap with susceptibility genes linked to cardiac phenotypes in humans and mice. For each cell type and each susceptibility gene set, we calculated the proportion of DEGs that overlapped with susceptibility genes. To assess statistical significance, we generated a null distribution for each cell type through 2,000 genome-wide permutations. In each permutation, we randomly selected the same number of DEGs and the same number of susceptibility genes, then calculated the proportion of overlap between the two permuted datasets. The empirical proportion observed in the actual data was compared against this null distribution. An empirical proportion equal to or greater than the 95<sup>th</sup> percentile of the null distribution was considered significant ( $P < 0.05$ ).

### Recombinant adenovirus construction, production and titration

To explore how the mRNA expression level of the candidate gene, *i.e.*, *IGF1R*, regulated phenotypic changes in cardiomyocytes, we generated an IGF1R-3xFLAG/mNeonGreen expression vector by cloning a full-length coding sequence (4,089 bp, stop codon excluded) of tree sparrow *IGF1R* into the adenoviral shuttle vector pcADV-EF1-mNeonGreen-CMV-MCS-3xFLAG. An empty vector was used as the control. Recombinant adenoviruses expressing IGF1R-3xFLAG/mNeonGreen (AD-IGF1R-mNeonGreen) or 3xFLAG/mNeonGreen alone (AD-mNeonGreen) were generated in HEK293 cells using a third-generation adenoviral packaging system. Viral particles were amplified and purified by cesium chloride (CsCl) density gradient ultracentrifugation. Viral titers were determined by a hexon protein-based immunoassay and are reported as infectious units per milliliter (IFU/mL). The final titers were  $3.0 \times 10^{10}$  IFU/mL for AD-IGF1R-mNeonGreen and  $1.58 \times 10^{11}$  IFU/mL for AD-mNeonGreen.

### Primary chicken cardiomyocyte isolation, culture, and adenoviral infection

Primary cardiomyocytes were isolated from 10-day-old chicken embryos following an established protocol.<sup>41</sup> Briefly, embryonic hearts were dissected, minced, and subjected to sequential enzymatic digestion in D-Hanks solution containing 1.2 mg/mL pancreatin and 0.14 mg/mL collagenase at 37°C. Dissociated cells were pooled, centrifuged, and resuspended in Dulbecco's modified Eagle medium/F-12 (GIBCO, USA) supplemented with 15% fetal bovine serum and antibiotics. To enrich cardiomyocytes, cell suspensions were pre-plated for 1 hour to allow cardiac fibroblasts to adhere preferentially. The non-adherent cardiomyocytes were collected and seeded onto gelatin-coated culture dishes at  $1 \times 10^6$  cells/mL. Cells were maintained at 37°C in a humidified incubator with 5% CO<sub>2</sub>.

After 48 hours in culture, cardiomyocytes were infected with AD-IGF1R-mNeonGreen or AD-mNeonGreen at a multiplicity of infection (MOI) of 300. The virus-containing medium was replaced with fresh complete medium 12–24 hours post-infection to minimize cytotoxicity. Cells were harvested at 72 hours post-infection for downstream analyses.

### Immunofluorescence staining and cell size quantification

Cardiomyocytes were fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.5% Triton X-100 for 20 minutes, and blocked with 5% goat serum for 30 minutes. Cells were then incubated overnight at 4°C with anti- $\alpha$ -actin primary antibody (Rabbit clone, Proteintech; 1:200 dilution) and Alexa fluorophore-conjugated secondary antibody. Nuclei were stained with DAPI. Images were acquired under identical acquisition settings across all groups with a Zeiss LSM 880 laser confocal microscope.

Cardiomyocyte surface areas were quantified from raw images in ImageJ after scale calibration by manual tracing of individual cell boundaries. From three independent biological replicates, ten randomly selected, non-overlapping fields per group were analyzed, yielding approximately 200 cells per condition. All quantitative analyses were performed on raw images prior to any adjustment, and representative images are shown with scale bars indicated. Because cell area data were non-normally distributed, group compar-

isons were performed using the Wilcoxon rank-sum test.

### Transcriptomic analysis of IGF1R-overexpressing chicken cardiomyocytes

Total RNA was isolated from AD-IGF1R-mNeonGreen (IGF1R-overexpressing) and AD-mNeonGreen (control) chicken cardiomyocytes 72 hours post-infection. Transcriptome sequencing was performed on the DNBSEQ-T7 platform to generate paired-end reads. Raw sequencing reads were processed to remove adapter sequences, low-quality reads and poly-N reads using fastp v0.20.1 with default parameters.<sup>42</sup> The high-quality, trimmed reads were then aligned to the chicken reference genome (assembly GCF\_016699485.2, GRCg7b) using STAR v2.7.6a.<sup>21</sup> Gene-level expression quantification was performed with RSEM v1.3.0,<sup>43</sup> generating normalized transcripts per kilobase (TPM) values for all annotated genes. Differential gene expression analysis between the IGF1R-overexpression and control groups was conducted using edgeR v3.32.1.<sup>44</sup> Genes were considered differentially expressed if they satisfied the following criteria: (i) an expression level > 1 count-per-million (CPM) in at least two samples, (ii) a fold-change  $\geq 1.25$  between AD-IGF1R-mNeonGreen (IGF1R-overexpressing) and AD-mNeonGreen (control), and (iii) a false discovery rate (FDR)-adjusted  $P$ -value < 0.05. To investigate the biological processes and pathways associated with *IGF1R* overexpression, functional enrichment analysis of the differentially expressed gene set was performed using clusterProfiler v4.12.0,<sup>45</sup> interrogating Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Additionally, to identify genes whose expression profiles were closely associated with *IGF1R* expression across all samples, we calculated pairwise Pearson correlation coefficients between *IGF1R* and every other gene. Correlations with an FDR-corrected  $P$ -value < 0.05 were deemed statistically significant.

## RESULTS

### Construction of a single-cell atlas of neonatal and adult tree sparrow hearts

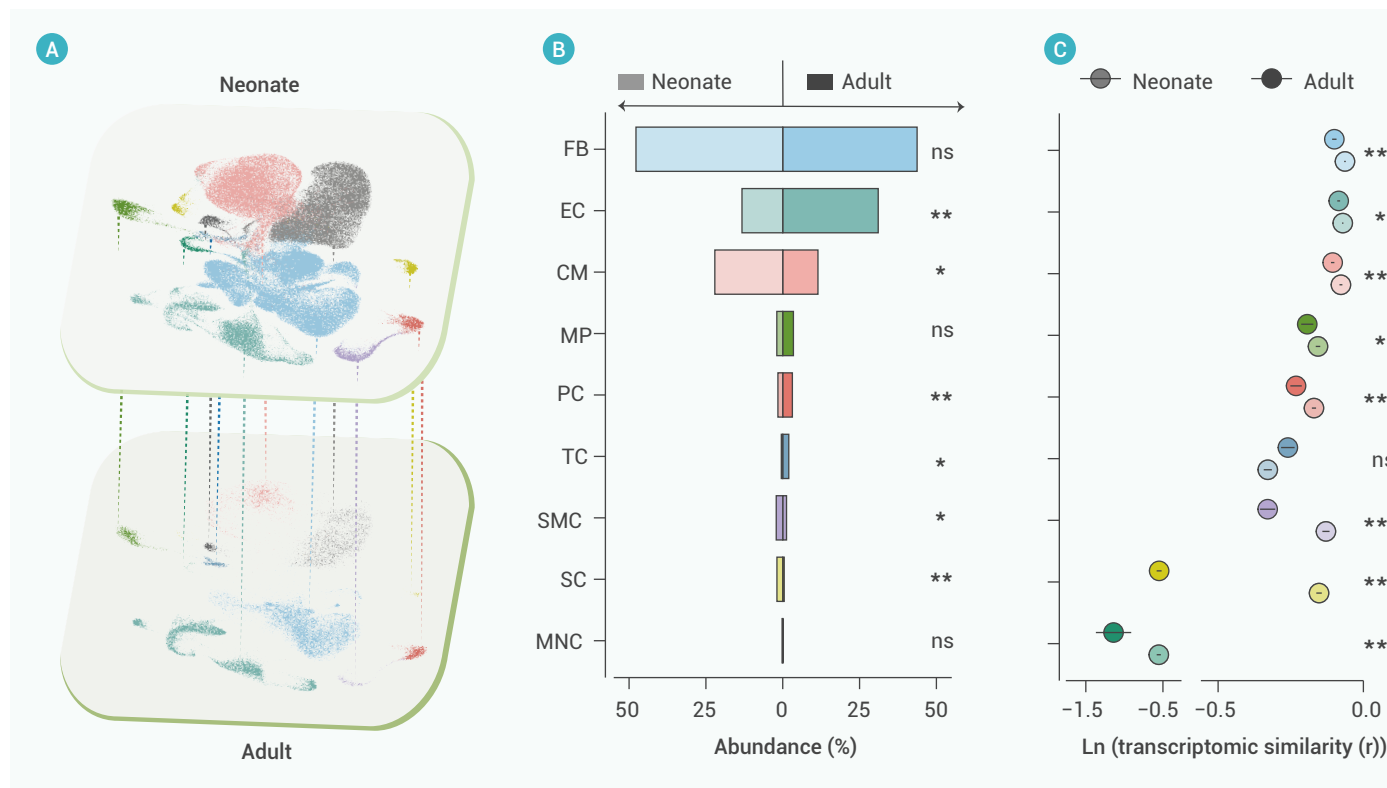
We generated single-cell transcriptomic atlases of the avian heart from neonatal ( $n = 9$ ) and adult tree sparrows ( $n = 6$ ) (Figure 1A & Table S1). To effectively capture the large adult cardiomyocytes (~100  $\mu$ m in length), we applied single-nucleus RNA sequencing (snRNA-seq) for adult tissues and scRNA-seq for neonatal hearts (see Materials and Methods). After stringent quality control, our dataset comprised 168,634 neonatal cells and 33,694 adult nuclei (Figure S1 & Tables S2–S4).

Unsupervised clustering of neonatal and adult datasets independently revealed concordant cell type annotations despite platform differences (Figure 1B). Using previously described cardiac cell-type markers (Table S5),<sup>28</sup> we identified 11 major cardiac cell types. These included six cell types spanning myocardial (cardiomyocytes (CMs)), mesenchymal (fibroblasts (FBs)), pericytes (PCs), and smooth muscle cells (SMCs)), endothelial (endothelial cells (ECs)), and neural (Schwann cells (SCs)) lineages; three immune populations—macrophages (MPs), T cells (TCs), and monocytes (MNCs); and two non-immune blood cell types—thrombocytes (THCs) and red blood cells (RBCs). Cell-type contributions were balanced across biological replicates, and clustering remained robust upon replicate subsampling (Figures S2 & S3). Gene Ontology analysis of marker genes confirmed expected functional specializations; for example, “cardiac muscle tissue development” was enriched in CMs, “angiogenesis” in ECs, and “extracellular matrix organization” in FBs (Figure S4).

The cellular composition of the heart was found to be highly conserved between neonatal and adult stages. Homologous cell types from each life stage co-clustered closely in integrated Uniform Manifold Approximation and Projection (UMAP) visualizations (Figure S5). Furthermore, canonical marker genes exhibited similarly high enrichment scores in both neonates and adults, with no evidence of life-stage-specific cell populations (Figures 1C & S6). Together, these data establish the first single-cell heart transcriptomic atlas of a wild bird across two life stages, extending beyond existing embryonic atlases in domestic birds.<sup>46</sup>

### Transcriptomic similarity of heart cell types between neonatal and adult birds

The conserved cellular identities between neonatal and adult hearts



**Figure 2. Cellular abundance and transcriptomic similarity between neonatal and adult stages** (A) Heart cell types were well-aligned between neonatal and adult birds. (B) Proportional abundance of heart cell types, demonstrating a shift in cellular composition between neonatal and adult stages. (C) Transcriptomic similarity between neonates and adults, assessed by expression correlation of homologous heart cell types. Data are presented as median  $\pm$  SEM. In panels (B) and (C), statistical significance was tested using the Wilcoxon rank-sum test with False Discovery Rate (FDR) correction for multiple comparisons, ns, not significant; \*, adjusted- $P < 0.05$ ; \*\*, adjusted- $P < 0.01$ ; \*\*\*, adjusted- $P < 0.001$ .

enabled a direct comparison of their transcriptomic profiles across life stages (Figure 2A). We observed significant shifts in cellular abundance: for example, the proportion of CMs decreased from 22% in neonates to 11% in adults, whereas ECs increased from 13% to 31% (Figure 2B & Table S6). Concomitant with the higher cardiomyocyte proportion in neonatal birds, neonatal hearts showed elevated levels of mitochondria-encoded RNAs, consistent with higher early-stage metabolic demand (Figure S7). Conversely, mitochondrial RNA content was higher in adult immune cells, aligning with their increased abundance in mature hearts (Figure S7).

We next assessed transcriptomic similarity between neonatal and adult hearts by calculating expression correlations for each cell type. This analysis revealed that while CMs, FBs and ECs showed the greatest transcriptional similarity, the SCs, SMCs and MNCs exhibited the least transcriptional similarity between life stages (Figure 2C). Notably, overall gene expression similarity was generally lower within adult cell types than within neonatal ones, indicating greater transcriptional heterogeneity in mature hearts (Table S7). Together, these results demonstrate that shifts in both cellular abundance and transcriptional regulation contribute to heart cell type heterogeneity across developmental stages.

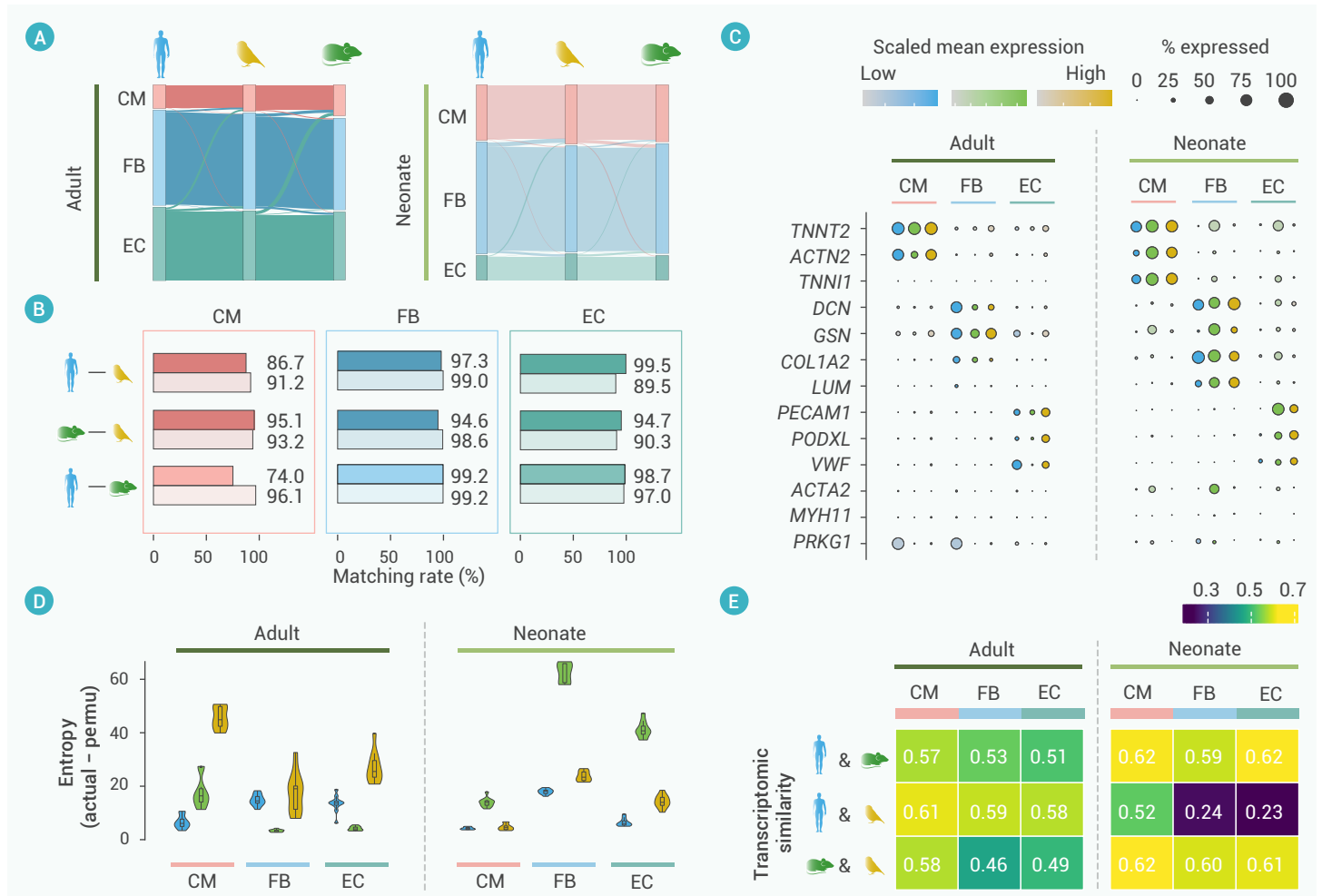
### Cellular heterogeneity and transcriptomic similarity of cardiac cell types across tree sparrows, mice and humans

To investigate the cellular and transcriptional basis of cardiac specialization in endothermic vertebrates, we compared single-cell and single-nucleus RNA sequencing data from tree sparrows with publicly available datasets from mice and humans (Table S8). We first employed CAME<sup>32</sup> to align three major cardiac cell types: CMs, FBs, and ECs, across species for neonatal and adult stages independently. Using mouse and human data as references, CAME assigned tree sparrow cell types with high accuracy (Figure 3A). Matching rates between tree sparrows and both mammalian species were consistently high for adults (86.7–99.5% for tree sparrow/human; 94.6–95.1% for tree sparrow/mouse) and neonates (89.5–99.0% for tree sparrow/human; 90.3–98.6% for tree sparrow/mouse). These interspecies

alignment rates were similar to those of the mouse-human comparisons (74.0–99.2% for adults and 96.1–99.2% for neonates; Figure 3B). Cell type identities were further confirmed by marker gene expression (Figure 3C). Collectively, these results demonstrate that the major heart cell types are conserved and well-aligned in tree sparrows, mice and humans.

The conserved identity of major heart cell types across species enabled a comparative analysis of their transcriptional states. We quantified within-cell-type heterogeneity using transcriptomic entropy, where higher entropy indicates greater gene expression variability. This analysis revealed distinct, species-specific patterns of heterogeneity across life stages. In humans, cellular heterogeneity remained relatively stable from neonatal to adult stages. In contrast, murine FBs and ECs showed significantly higher entropy in neonates than in adults. Tree sparrows exhibited the opposite trend, with CMs and ECs displaying greater transcriptional heterogeneity in adults compared to neonates (Figure 3D).

To further evaluate the similarity of transcriptional profiles in homologous cell types across species, we analyzed expression correlations using Spearman's coefficients based on highly variable genes. Despite substantial evolutionary divergence between birds and mammals, and differences in developmental stage, homologous cell types exhibited robust transcriptomic similarity across species. For CMs, transcriptomic similarity between adult and neonatal mouse/human ranged from 0.57 to 0.62, comparable to values observed between adult and neonatal human/tree sparrow (0.52 to 0.61) and between adult and neonatal mouse/tree sparrow (0.58 to 0.62) (Figure 3E). In FBs and ECs, correlation values between adult mouse and human (0.51 to 0.53) were in a range similar to those between adult human/tree sparrow (0.58 to 0.59) and mouse/tree sparrow (0.46 to 0.49) (Figure 3E). However, cross-species comparisons of neonatal FBs and ECs revealed varying degrees of similarity: while human/mouse and mouse/tree sparrow correlations were 0.59–0.62 and 0.60–0.61, respectively, human/tree sparrow correlations were notably lower (0.23–0.24). These results indicate that developmental and evolutionary regulation of heart transcriptional programs is cell-type dependent, with core contractile cells under strong functional



**Figure 3. Cross-species analysis of cardiac cell-type heterogeneity and transcriptomic similarity** (A) Alignment of major cardiac cell types: cardiomyocytes (CM), fibroblasts (FB), and endothelial cells (EC), across tree sparrows, mice and humans. (B) Cardiac cell type matching rates are similar between mice/humans and either mice/tree sparrows or human/tree sparrows. (C) Expression of cell type marker genes was stable across the species. (D) Transcriptomic heterogeneity within each cardiac cell type, quantified as entropy. Higher entropy indicates greater transcriptional diversity among individual cells. Neonatal and adult stages are shown separately for each species. (E) Similarity in gene expression profiles between species for each homologous cardiac cell type. Neonatal and adult stages are shown separately.

constraint and stromal/vascular cells displaying greater regulatory plasticity across species and developmental stages.

### Conserved gene expression in cardiac cell types cross species

To identify evolutionarily conserved transcriptional features, we defined homologous genes expressed in CMs, ECs, and FBs across all three species. After filtering lowly expressed genes, we analyzed a set of 5,189–5,677 genes. Unsupervised k-means clustering for each cell type revealed nine gene clusters, each containing a core subset of genes with highly similar cross-species expression profiles (Figures 4A & S8–S10).

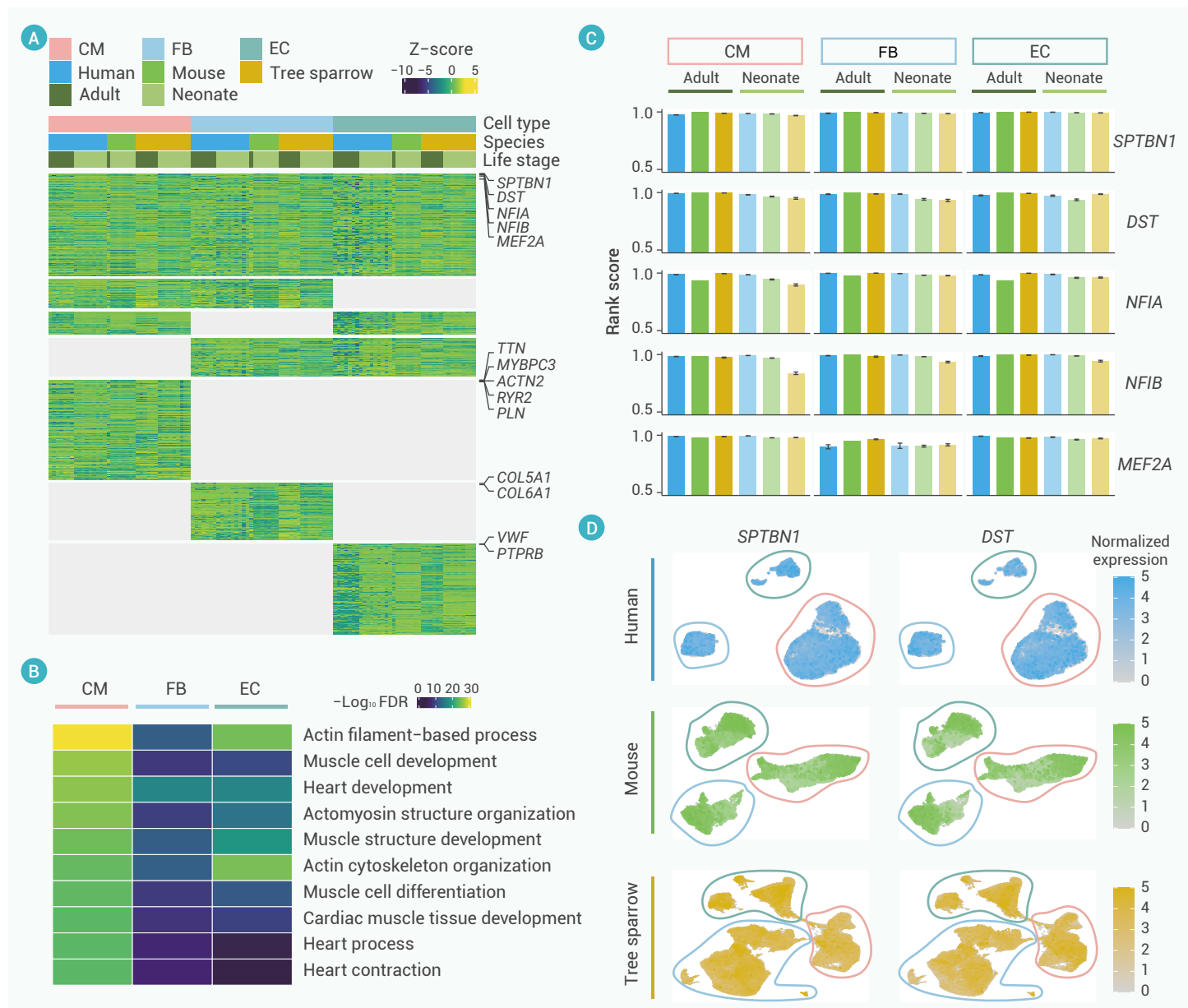
Notably, a substantial proportion of the identified cross-species conserved genes (40–45%, 244 out of 541–606; Figure S11) are shared among CMs, FBs and ECs. These shared genes are significantly enriched for cardiac-specific biological processes, including heart and muscle development (Figures 4B & S12 & Table S9). Representative conserved genes (e.g., *SPTBN1* and *DST*; Figures 4C & D) are involved in actin cytoskeleton,<sup>47–49</sup> collagen synthesis and assembly (*COL4A1*, *COL5A1*, *COL1A2*, *DCN*),<sup>50</sup> insulin-like growth factor signaling (*IGFBP7*),<sup>51</sup> and muscle-specific transcriptional regulation (*NFIA* and *NFIB*).<sup>52</sup> Notably, mutations in these genes are associated with cardiac developmental disorders and structural defects in humans and model organisms,<sup>47–52</sup> underscoring their essential role in heart biology.

Given that these conserved genes are critical for cardiac homeostasis, we reasoned that their dysregulation might also contribute to cardiovascular disease. If this hypothesis holds, then many conserved genes should overlap with known cardiac disease susceptibility genes. To test this, we analyzed the expression patterns of known cardiac disease susceptibility genes from

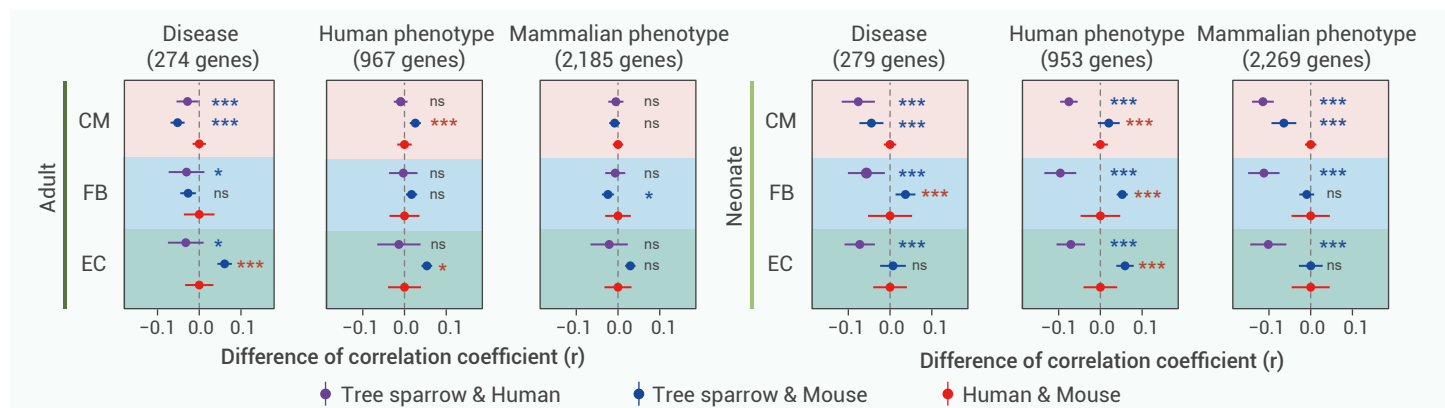
humans and mice (human heart diseases;<sup>38</sup> human and murine heart phenotypic changes;<sup>39,40</sup>) across species (Table S10). We observed strong correlation in the expression profiles of these disease-related genes across species, particularly in adult hearts (Figure 5). For example, correlations between tree sparrows and both mice ( $r_s = 0.53–0.66$ ) and humans ( $r_s = 0.52–0.72$ ) were high (3 out of 18 comparisons) and, in most cases (10 of 18 comparisons), not significantly lower than those between mice and humans ( $r_s = 0.58–0.75$ , post-hoc Tukey's Honest Significant Difference (HSD) tests,  $P > 0.05$ ; Figure 5). In neonatal hearts, however, correlations between birds and mammals were frequently lower than those between mice and humans (11 of 18 comparisons, post-hoc Tukey's HSD tests,  $P < 0.05$ ; Figure 5), suggesting greater developmental divergence at early stages. This trend was consistent across combined gene sets (Figure S13). These results reveal that genes critical for cardiac development and function, including many implicated in heart disease, exhibit conserved expression between avian and mammalian lineages, particularly in mature hearts, underscoring the shared regulatory programs that govern disease-associated loci.

### Regulatory basis underlying cardiac adaptation in high-elevation tree sparrows

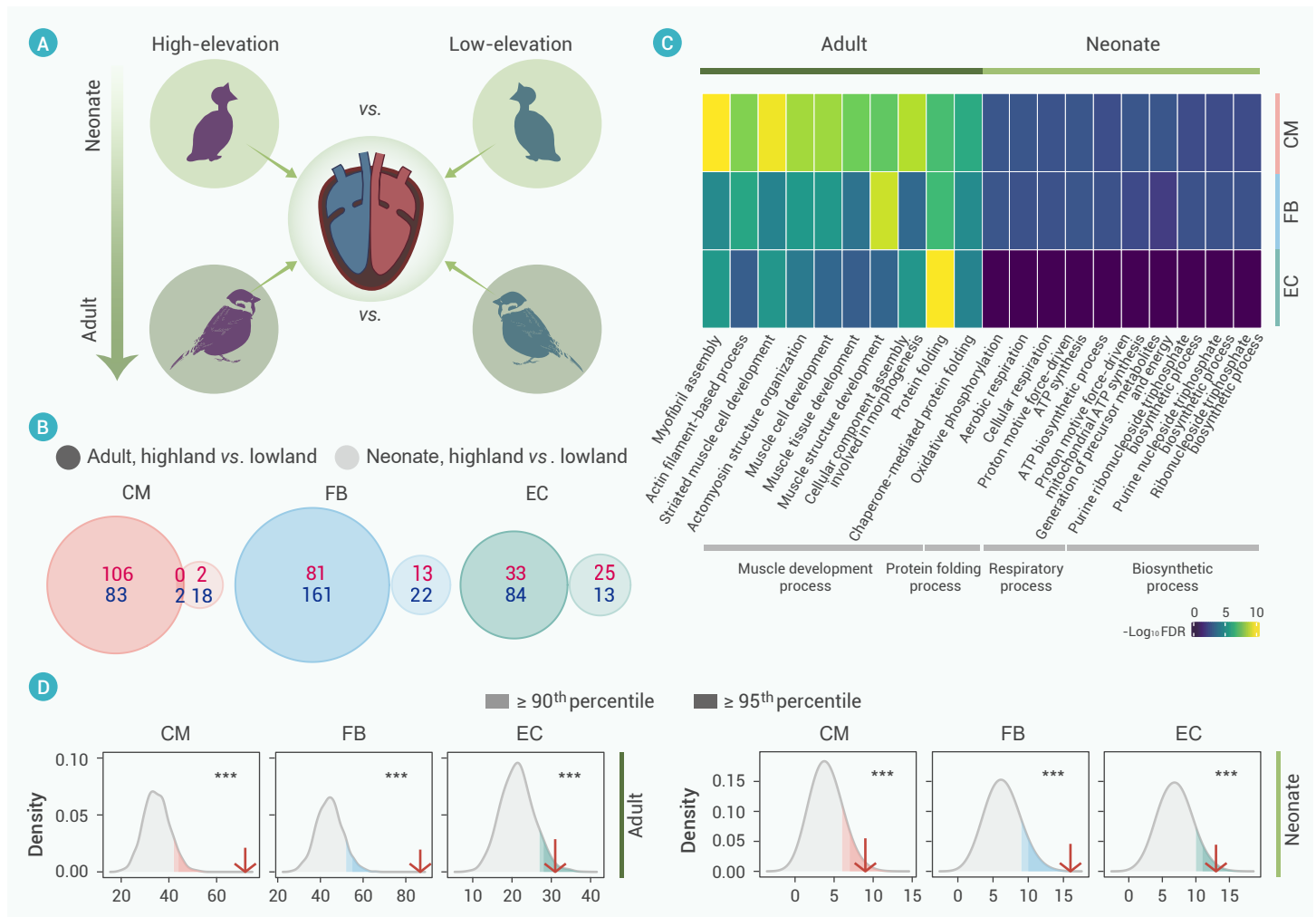
The heart is highly sensitive to chronic hypoxia, which can induce phenotypic changes such as cardiac hypertrophy. Based on our previous observations of cardiac hypertrophy in high-elevation tree sparrows,<sup>12,15</sup> and the current observation of conserved transcriptional regulation of heart cell types across species, we hypothesized that the regulatory basis of cardiac hypertrophy of tree sparrows is shared with mice and humans. We tested this by



**Figure 4. Conserved transcriptional programs in cardiac cell types across species** (A) Identification of gene expression programs conserved across tree sparrows, mice and humans versus those specific to each lineage. (B) Enrichment analysis of conserved genes revealed significant overrepresentation of biological processes related to heart and muscle development. (C) Expression patterns of the highly conserved genes across species. (D) The genes *SPTBN1* and *DST* exhibit pan-cardiac expression, conserved across species.



**Figure 5. Cross-species correlation of expression for cardiac disease susceptibility genes** Pairwise comparisons (tree sparrows/mice, tree sparrows/humans, mice/humans) were assessed using Spearman's rank correlation. Correlation coefficients for the mouse-human comparison were used as benchmarks and compared with those for the tree sparrow-mouse and tree sparrow-human comparisons using post-hoc Tukey's Honest Significant Difference (HSD) tests, \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ ; ns, non-significant. Red stars denote species pairs (tree sparrow/mouse and tree sparrow/human) with higher correlation coefficients than the mouse/human pair. Blue stars denote pairs with lower correlation coefficients.



**Figure 6. Cardiac adaptation in the high-elevation tree sparrows** (A) Schematic of the comparison design. Differentially expressed genes (DEGs) were identified for each cardiac cell type between high-elevation and low-elevation tree sparrows. (B) Analysis of cell type-specific DEGs from high-elevation adults versus neonates. Few DEGs were shared between the high-elevation adult and neonatal tree sparrows. (C) Distinct functional enrichment for DEG sets between high-elevation adult and neonatal tree sparrows. (D) DEGs were enriched for cardiac disease susceptibility genes. Empirical values were compared with permuted distributions generated from 2,000 random samplings and a threshold of the 95<sup>th</sup> percentile of permuted distributions.

analyzing single-cell data from high-elevation (Gahai, 3,400 m a.s.l.) and low-elevation populations (Cangzhou, 10 m a.s.l.) of tree sparrows (Figure 6A).

We first identified cell-type specific DEGs in high-elevation neonatal and adult birds. Very few DEGs were shared between adult and neonatal birds, and adults exhibited far more DEGs than neonates (Figure 6B). For example, adult CMs showed 191 DEGs compared to only 22 in neonates, with just two genes in common. Functional enrichment also differed: adult DEGs were associated with cardiac muscle development, whereas neonatal DEGs were linked to energy metabolism (Figure 6C). Similar patterns were observed for FBs and ECs, indicating distinct physiological constraints on the developing versus mature heart in hypoxic environments.

We next examined whether these high-elevation adaptation-related DEGs overlap with known cardiac disease susceptibility genes from human and mouse databases. Specifically, 26–39% of adult high-elevation DEGs and 34–46% of neonatal DEGs, corresponded to mammalian cardiac disease genes, a significant enrichment over random expectation (permutation test,  $P < 0.05$ ; Figures 6D & S14). This indicates that genes involved in hypoxia adaptation in high-elevation tree sparrows are strongly enriched for those implicated in mammalian cardiac pathology.

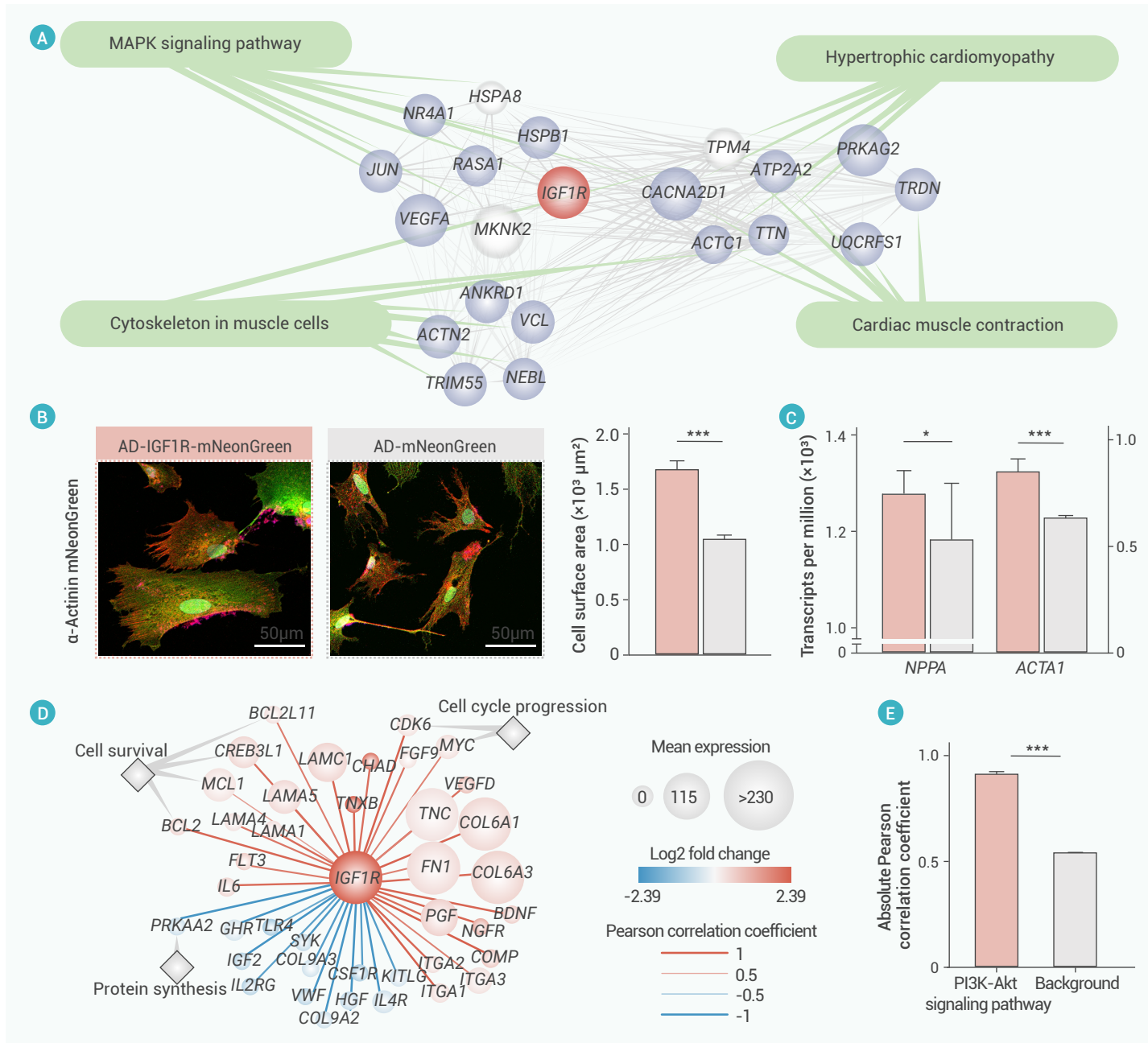
#### Regulatory changes of *IGF1R* underlying cardiomyocyte hypertrophy

Given our prior finding of cardiac hypertrophy in high-elevation tree sparrows,<sup>12,15</sup> we specifically examined cardiomyocyte-specific DEGs. These genes were significantly enriched in pathways associated with hypertrophic cardiomyopathy, MAPK signaling pathway, cytoskeleton in muscle cells and

cardiac muscle contraction (Figure 7A). Among them, we identified the insulin-like growth factor 1 receptor (*IGF1R*), which encodes a tyrosine kinase that activates the IGF1-PI3K-AKT-mTOR pathway, a central regulator of cardiac growth in mammals.<sup>14,53</sup> *IGF1R* expression is upregulated in hypertrophied hearts in mice and humans,<sup>53,54</sup> and similarly, we observed elevated *IGF1R* expression in cardiomyocytes of high-elevation sparrows. Based on its functional role, we selected *IGF1R* for functional validation to determine whether its regulatory changes contribute to avian cardiac hypertrophy.

To elucidate the functional role of *IGF1R* in the avian heart, we established a primary neonatal chicken cardiomyocyte culture model. Cardiomyocytes were transduced with an adenovirus-mediated overexpression of *IGF1R* tagged with green fluorescent protein (AD-IGF1R-mNeonGreen), while control cells received an mNeonGreen-only adenovirus (AD-mNeonGreen). Evaluation at 72 hours post-transduction revealed that *IGF1R* overexpression induced a significant increase in cardiomyocyte surface area compared with the AD-mNeonGreen control (Wilcoxon rank-sum test,  $P < 2.2 \times 10^{-16}$ ; Figure 7B). This hypertrophic morphology was accompanied by a concomitant upregulation of canonical molecular markers of cardiac hypertrophy. Specifically, quantitative analysis confirmed elevated mRNA levels of atrial natriuretic peptide (*NPPA*) and  $\alpha$ -skeletal actin (*ACTA1*) in AD-IGF1R-treated cells relative to controls (Figure 7C).

Transcriptomic profiling further delineated the signaling mechanism associated with this phenotypic change. *IGF1R* overexpression induced significant alterations within its canonical downstream IGF1-PI3K-AKT-mTOR signaling cascade (Figure 7D). Among 362 genes annotated to the PI3K-AKT



**Figure 7. Overexpression of *IGF1R* mediates cardiomyocyte hypertrophy** (A) Cardiomyocyte-specific DEGs are significantly enriched for pathways related to cardiac hypertrophy. *IGF1R*, a key hub gene selected for functional validation, is highlighted in red. The susceptibility genes involved in cardiac diseases of humans and mice are indicated in blue. (B) Functional validation of *IGF1R* in vitro. Representative images and quantification show that adenovirus-mediated *IGF1R* overexpression (AD-IGF1R-mNeonGreen) increases cardiomyocyte size compared to controls (AD-mNeonGreen). Scale bar, 50 μm. Data are median ± SEM;  $n = 3$  biological replicates; Wilcoxon rank-sum test, \*\*\*,  $P < 0.001$ . (C) *IGF1R* overexpression upregulates hypertrophy markers. Transcriptomic analysis shows elevated mRNA levels of *NPPA* (ANP) and *ACTA1* (α-skeletal actin) in AD-IGF1R-mNeonGreen cardiomyocytes relative to AD-mNeonGreen controls. Data are median ± SEM. \*, FDR-adjusted  $P < 0.05$ ; \*\*\*, FDR-adjusted  $P < 0.001$ . (D) *IGF1R* overexpression altered the expression of 41 genes in the PI3K-AKT signaling pathway (absolute fold-change > 1.25, FDR-adjusted  $P < 0.05$ ). (E) Pathway-specific co-regulation. Expression of the 41 DEGs in the PI3K-Akt pathway shows significantly stronger correlation with *IGF1R* expression (median  $r = 0.91$ ) than a background set of all expressed genes (median  $r = 0.51$ ; Wilcoxon rank-sum test,  $P < 2.2 \times 10^{-16}$ ).

signaling pathway, 41 were differentially expressed (absolute fold-change > 1.25, FDR-adjusted  $P < 0.05$ ). The transcriptional response of this pathway appeared highly coordinated: the expression levels of these 41 pathway-related DEGs showed a significantly stronger correlation with *IGF1R* expression than a background set of all expressed genes (median correlation: 0.91 vs. 0.51; Wilcoxon rank-sum test,  $P < 2.2 \times 10^{-16}$ ; Figure 7E). In particular, genes associated with cell growth, survival, and hypertrophic remodeling (e.g., *BDNF*, *BCL2*, *CDK6*, and *MYC*) were upregulated, whereas the mTOR suppressor *PRKAA2* was significantly downregulated (Figure 7D). Although transcriptomic data alone cannot establish the functional contribution of every individual DEG, the overall expression pattern is consistent with a coordinated hypertrophy-associated signaling response following *IGF1R* overex-

pression. Collectively, these results suggest that *IGF1R* upregulation is sufficient to induce hypertrophy-like morphological and molecular features in avian cardiomyocytes and trigger PI3K-AKT-mTOR-associated transcriptional changes, broadly consistent with its known roles in mammals.<sup>53,54</sup>

## DISCUSSION

Understanding the convergent evolution of the avian and mammalian heart requires a comparative analysis of their cellular and molecular compositions. To address this, we generated the first single-cell transcriptomic atlas of a wild bird heart across neonatal and adult stages (Figure 1B), extending beyond previous embryonic studies in domestic chickens.<sup>46</sup>

Our analyses reveal that although the core repertoire of cardiac cell types is

shared between neonatal and adult hearts, their relative abundances undergo substantial remodeling. The proportion of cardiomyocytes decreases by half in adulthood, whereas endothelial cells expand markedly (Figure 2B & Table S6). This shift is consistent with the transition from early postnatal growth to lifelong maintenance: the juvenile heart requires a high density of proliferative cardiomyocytes, whereas the mature heart relies on a robust vascular network to support sustained metabolic demand and tissue homeostasis.<sup>28</sup> More broadly, this pattern echoes the evolutionary trajectory of endothermic hearts, where increased vascularization has accompanied the rise of high-pressure, high-output circulatory systems. At the transcriptional level, cell types such as smooth muscle cells and monocytes exhibit greater divergence in adults (Figure 2C & Table S7), suggesting that functional specialization, particularly in vascular regulation and immune surveillance, intensifies with maturation, possibly reflecting the increased physiological demands and disease susceptibility of the adult heart.

These compositional and transcriptional changes are accompanied by stage-specific metabolic signatures. Neonatal cardiomyocytes show elevated mitochondrial RNA, likely reflecting the high energetic demand of rapid postnatal growth, while adult immune cells display increased mitochondrial RNA content corresponding to their greater abundance (Figure S7). This observation aligns with a fundamental principle of vertebrate cardiac development: energy metabolism shifts from glycolysis in the immature heart to oxidative phosphorylation in the adult, a transition that also supports the higher oxygen requirements of endothermic species. Collectively, our findings demonstrate that avian cardiac maturation is driven not by the emergence of novel cell types, but by dynamic shifts in cellular abundance and transcriptional programs.

Our cross-species comparison reveals that the endothermic vertebrates' hearts are constructed from a conserved set of cellular building blocks, yet exhibit dynamic, cell-type-specific patterns of transcriptional regulation across lineages and life stages. Cardiomyocytes emerge as the most transcriptionally conserved cell type across all species and developmental stages (Figure 3E). This high degree of conservation reflects stringent functional constraints on the heart's electrophysiological, contractile and secretory processes.<sup>28</sup> In contrast, non-myocyte populations, such as fibroblasts and endothelial cells, display greater plasticity and heterogeneity across species and developmental stages (Figure 3E). This divergence likely reflects adaptive tuning of supportive roles: fibroblasts modulate extracellular matrix remodeling in response to species-specific mechanical demands, while endothelial cells adjust angiogenic and immune functions to match metabolic needs.

We identified a set of conserved genes stably expressed across the major cardiac cell types in birds, mice, and humans (Figure 4A). These genes function as essential, multifunctional regulators of core cardiac processes, including structural maintenance and cellular signaling. Intriguingly, this conservation extends to the molecular basis of cardiac disease. In adult hearts, the expression patterns of genes linked to human cardiac pathologies are conserved among tree sparrows, mice and humans (Figure 5), indicating that fundamental pathways of cardiac dysfunction are shared across endothermic vertebrates. Given the established utility of mice as models for human cardiac disease, the strong correlation observed between tree sparrows and humans, comparable to that between mice and humans, may suggest that avian models could offer valuable insights into cardiac disease mechanisms. Although this correlation was somewhat diminished in neonatal hearts, likely reflecting developmental plasticity, the overall pattern highlights a cross-species conserved molecular blueprint. Collectively, our findings tentatively suggest that the hearts of endothermic vertebrates are constructed and maintained by a conserved genetic program, and that disruptions to this program may lead to similar pathological remodeling across even evolutionarily divergent species.

Comparative analysis of high- and low-elevation tree sparrows revealed that cardiac adaptation to chronic hypoxia is life-stage specific. Adults and neonates exhibit largely non-overlapping sets of differentially expressed genes (DEGs), with distinct functional enrichments: adult DEGs were associated with cardiac muscle development, whereas neonatal DEGs were linked to energy metabolism (Figures 6B & C). This divergence reflects fundamentally different physiological demands: neonates prioritize energy production

under low oxygen, while adults remodel cardiac structure to optimize contractile efficiency and oxygen diffusion. Notably, 26–39% of adult adaptation-related DEGs corresponded to known cardiac disease susceptibility genes in humans and mice, a statistically significant enrichment beyond random chance (Figures 6D & S14). This similarity suggests that the core molecular pathways regulating the heart's response to environmental stress, such as hypoxia, may be evolutionarily conserved and that these same pathways, when dysregulated, could also contribute to common cardiac diseases.

Examination of cardiomyocyte-specific DEGs in high-elevation tree sparrows identified significant enrichment for hypertrophic pathways, including the key gene *IGF1R*. To validate its functional role, we overexpressed *IGF1R* in a chicken cardiomyocyte model, which induced cellular hypertrophy, supporting a contributory role of *IGF1R* in avian cardiomyocyte hypertrophy. Transcriptomic profiling revealed that *IGF1R* overexpression induces the expression of a core set of genes within the canonical IGF1-PI3K-AKT-mTOR pathway, mirroring the pathway enrichment observed in high-elevation bird hearts (Figure 7D).<sup>15</sup> These results are consistent with the possibility that the *IGF1R*-associated signaling cascade, a central driver of physiological cardiac hypertrophy, is conserved between birds and mammals,<sup>14,53</sup> and they suggest a potentially shared molecular toolkit for cardiac growth.

Several limitations in the present study should be recognized regarding the cross-life-stage and cross-species analyses. First, neonatal and adult single-cell data were generated using different technical platforms (scRNA-seq vs. snRNA-seq), which may introduce bias when comparing developmental stages. To minimize these platform-specific effects, we integrated datasets using Seurat integration,<sup>24</sup> a method designed to align shared biological signals while reducing technical variation. Nevertheless, we acknowledge that neonate-adult comparisons remain subject to inherent technical and biological biases. Second, the neonatal and adult categories represent coarse approximations; precise developmental equivalence across such phylogenetically distant species is unattainable due to vast differences in absolute age, growth rates, and developmental milestones. Consequently, observed transcriptional differences must be interpreted with caution, as they may reflect a combination of true developmental differences, evolutionary divergence, technical artifacts arising from modality-specific biases, and residual stage-mismatch. An additional limitation is the absence of *in vivo* experiments. Our *in vitro* overexpression experiments show that enhanced *IGF1R* expression is sufficient to induce hypertrophy and PI3K-AKT activation in avian cardiomyocytes, but they do not demonstrate that *IGF1R* signaling is necessary for cardiac hypertrophy *in vivo*. Therefore, *IGF1R* should be considered a plausible contributor, not yet a required mediator, of the adaptive phenotype in high-altitude tree sparrows.

## CONCLUSION

This study provides the first single-cell heart atlas of a wild bird across two life stages. It suggests a potentially conserved cellular and molecular blueprint shared between birds and mammals, which may be associated with both adaptive and pathological remodeling. This convergence points to a shared regulatory logic in the hearts of endothermic animals and hints that birds could serve as a useful model for certain aspects of cardiac biology. Future studies might build on this atlas to explore how conserved genetic programs are integrated into species-specific gene regulatory networks and to investigate the translational potential of adaptive mechanisms, such as high-elevation cardiac hypertrophy. Such studies could also offer new perspectives on hypoxia-mediated cardiovascular diseases in humans, particularly given that birds are highly tolerant of hypoxia to support flight at high elevation.<sup>55,56</sup>

## REFERENCES

1. Hillenius Willem J. and Ruben John A. (2004). The evolution of endothermy in terrestrial vertebrates: Who? when? why? *Physiol. Biochem. Zool.* **77**:1019–1042. DOI:10.1086/425185
2. Stephenson A., Adams J. W. and Vaccarezza M. (2017). The vertebrate heart: An evolutionary perspective. *J. Anat.* **6**:787–797. DOI:10.1111/joa.12687
3. Shiels H. A. (2022). Avian cardiomyocyte architecture and what it reveals about the evolution of the vertebrate heart. *Philos. Trans. R. Soc. Lond., B, Biol. Sci.* **377**:20210332. DOI:10.1098/rstb.2021.0332
4. Ivy C. M. and Scott G. R. (2015). Control of breathing and the circulation in high-

- altitude mammals and birds. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **186**:66–74. DOI:10.1016/j.cbpa.2014.10.009
5. Monge C. and Leon-Varlarde F. (1991). Physiological adaptation to high altitude: Oxygen transport in mammals and birds. *Physiol. Rev.* **71**:1135–1172. DOI:10.1152/physrev.1991.71.4.1135
  6. Ostadal B. and Kolar F. (2007). Cardiac adaptation to chronic high-altitude hypoxia: Beneficial and adverse effects. *Respir. Physiol. Neurobiol.* **158**:224–236. DOI:10.1016/j.resp.2007.03.005
  7. She H. and Qu Y. (2025). Cardiovascular plasticity and adaptation of high-altitude birds and mammals. *Integr. Zool.* DOI:10.1111/1749-4877.12996
  8. Velotta J. P., Ivy C. M., Wolf C. J., et al. (2018). Maladaptive phenotypic plasticity in cardiac muscle growth is suppressed in high-altitude deer mice. *Evolution* **72**:2712–2727. DOI:10.1111/evo.13626
  9. Rong C., Yan M., Zhen-Zhong B., et al. (2012). Cardiac adaptive mechanisms of Tibetan antelope (*Pantholops hodgsonii*) at high altitudes. *Am. J. Vet. Res.* **73**:809–813. DOI:10.2460/ajvr.73.6.809
  10. Cueva S., Sillau H., Valenzuela A., et al. (1974). High altitude induced pulmonary hypertension and right heart failure in broiler chickens. *Res. Vet. Sci.* **16**:370–374. DOI:10.1016/S0034-5288(18)33737-8
  11. Penalzoa D. and Arias-Stella J. (2007). The heart and pulmonary circulation at high altitudes: Healthy highlanders and chronic mountain sickness. *Circulation* **115**:1132–1146. DOI:10.1161/CIRCULATIONAHA.106.624544
  12. Qu Y., Chen C., Xiong Y., et al. (2020). Rapid phenotypic evolution with shallow genomic differentiation during early stages of high elevation adaptation in Eurasian Tree Sparrows. *Natl. Sci. Rev.* **7**:113–127. DOI:10.1093/nsr/nwz138
  13. Laguë S. L., Ivy C. M., York J. M., et al. (2020). Cardiovascular responses to progressive hypoxia in ducks native to high altitude in the Andes. *J. Exp. Biol.* **223**:jeb211250. DOI:10.1242/jeb.211250
  14. Heineke J. and Molkentin J. D. (2006). Regulation of cardiac hypertrophy by intracellular signaling pathways. *Nat. Rev. Mol. Cell Biol.* **7**:589–600. DOI:10.1038/nrm1983
  15. She H., Scott G. R., Fang Y., et al. (2025). Divergent cardiovascular adaptations and gene regulation in high-elevation natives and recent colonizers of the Qinghai-Tibetan Plateau. *Mol. Biol. Evol.* **42**:msaf103. DOI:10.1093/molbev/msaf103
  16. Burggren W. W., Christoffels V. M., Crossley I. D. A., et al. (2014). Comparative cardiovascular physiology: Future trends, opportunities and challenges. *Acta Physiol.* **210**:257–276. DOI:10.1111/apha.12170
  17. Burggren W. W. and Reyna K. S. (2011). Developmental trajectories, critical windows and phenotypic alteration during cardio-respiratory development. *Respir. Physiol. Neurobiol.* **178**:13–21. DOI:10.1016/j.resp.2011.05.001
  18. Sun Y.-F., Ren Z.-P., Wu Y.-F., et al. (2016). Flying high: Limits to flight performance by sparrows on the Qinghai-Tibet Plateau. *J. Exp. Biol.* **219**:3642–3648. DOI:10.1242/jeb.142216
  19. Dura B., Choi J.-Y., Zhang K., et al. (2019). scFTD-seq: Freeze-thaw lysis based, portable approach toward highly distributed single-cell 3' mRNA profiling. *Nucleic Acids Res.* **47**:e16. DOI:10.1093/nar/gky1173
  20. Martin M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **17**. DOI:10.14806/ej.17.1.200
  21. Dobin A., Davis C. A., Schlesinger F., et al. (2013). STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics* **29**:15–21. DOI:10.1093/bioinformatics/bts635
  22. Liao Y., Smyth G. K. and Shi W. (2014). featureCounts: An efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**:923–930. DOI:10.1093/bioinformatics/btt656
  23. Zheng G. X. Y., Terry J. M., Belgrader P., et al. (2017). Massively parallel digital transcriptional profiling of single cells. *Nat. Commun.* **8**:14049. DOI:10.1038/ncomms14049
  24. Hao Y., Hao S., Andersen-Nissen E., et al. (2021). Integrated analysis of multimodal single-cell data. *Cell* **184**:3573–3587.e3529. DOI:10.1016/j.cell.2021.04.048
  25. Young M. D. and Behjati S. (2020). SoupX removes ambient RNA contamination from droplet-based single-cell RNA sequencing data. *GigaScience* **9**:giaa151. DOI:10.1093/gigascience/giaa151
  26. McGinnis C. S., Murrow L. M. and Gartner Z. J. (2019). DoubletFinder: Doublet detection in single-cell RNA sequencing data using artificial nearest neighbors. *Cell Syst.* **8**:329–337.e324. DOI:10.1016/j.cels.2019.03.003
  27. Ahlmann-Eltze C. and Huber W. (2021). glmGamPoi: Fitting Gamma-Poisson generalized linear models on single cell count data. *Bioinformatics* **36**:5701–5702. DOI:10.1093/bioinformatics/btaa1009
  28. Litviňuková M., Talavera-López C., Maatz H., et al. (2020). Cells of the adult human heart. *Nature* **588**:466–472. DOI:10.1038/s41586-020-2797-4
  29. Zhang Y., Gago-Lopez N., Li N., et al. (2019). Single-cell imaging and transcriptomic analyses of endogenous cardiomyocyte dedifferentiation and cycling. *Cell Discov.* **5**:30. DOI:10.1038/s41421-019-0095-9
  30. Cao J., O'Day D. R., Pliner H. A., et al. (2020). A human cell atlas of fetal gene expression. *Science* **370**:eaba7721. DOI:10.1126/science.aba7721
  31. Feng W., Bais A., He H., et al. (2022). Single-cell transcriptomic analysis identifies murine heart molecular features at embryonic and neonatal stages. *Nat. Commun.* **13**:7960. DOI:10.1038/s41467-022-35691-7
  32. Liu X. Y., Shen Q. L. and Zhang S. H. (2023). Cross-species cell-type assignment from single-cell RNA-seq data by a heterogeneous graph neural network. *Genome Res.* **33**:96–111. DOI:10.1101/gr.276868.122
  33. Ma S., Skarica M., Li Q., et al. (2022). Molecular and cellular evolution of the primate dorsolateral prefrontal cortex. *Science* **377**:eabo7257. DOI:10.1126/science.abo7257
  34. Emms D. M. and Kelly S. (2019). OrthoFinder: Phylogenetic orthology inference for comparative genomics. *Genome Biol.* **20**:238. DOI:10.1186/s13059-019-1832-y
  35. Geirsdottir L., David E., Keren-Shaul H., et al. (2019). Cross-species single-cell analysis reveals divergence of the primate microglia program. *Cell* **179**:1609–1622.e1616. DOI:10.1016/j.cell.2019.11.010
  36. Warnes G., Bolker B., Lumley T., et al. (2022). gmodels: Various R programming tools for model fitting. <https://CRAN.R-project.org/package=gmodels>
  37. Bindea G., Mlecnik B., Hackl H., et al. (2009). ClueGO: A Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* **25**:1091–1093. DOI:10.1093/bioinformatics/btp101
  38. Piñero J., Ramírez-Anguaita J. M., Saüch-Pitarch J., et al. (2020). The DisGeNET knowledge platform for disease genomics: 2019 update. *Nucleic Acids Res.* **48**:D845–D855. DOI:10.1093/nar/gkz1021
  39. Gargano M. A., Matentzoglou N., Coleman B., et al. (2024). The Human Phenotype Ontology in 2024: Phenotypes around the world. *Nucleic Acids Res.* **52**:D1333–D1346. DOI:10.1093/nar/gkad1005
  40. Smith C. L. and Eppig J. T. (2009). The Mammalian Phenotype Ontology: Enabling robust annotation and comparative analysis. *Wires Syst. Biol. Med.* **1**:390–399. DOI:10.1002/wsbm.44
  41. Liu C., Xue R., Wu D., et al. (2014). *REDD1* attenuates cardiac hypertrophy via enhancing autophagy. *Biochem. Biophys. Res. Commun.* **454**:215–220. DOI:10.1016/j.bbrc.2014.10.079
  42. Chen S., Zhou Y., Chen Y., et al. (2018). fastp: An ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* **34**:i884–i890. DOI:10.1093/bioinformatics/bty560
  43. Li B. and Dewey C. N. (2011). RSEM: Accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinform.* **12**:323. DOI:10.1186/1471-2105-12-323
  44. Robinson M. D., McCarthy D. J. and Smyth G. K. (2010). edgeR: A bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**:139–140. DOI:10.1093/bioinformatics/btp616
  45. Xu S., Hu E., Cai Y., et al. (2024). Using clusterProfiler to characterize multiomics data. *Nat. Protoc.* **19**:3292–3320. DOI:10.1038/s41596-024-01020-z
  46. Mantri M., Scuderi G. J., Abedini-Nassab R., et al. (2021). Spatiotemporal single-cell RNA sequencing of developing chicken hearts identifies interplay between cellular differentiation and morphogenesis. *Nat. Commun.* **12**:1771. DOI:10.1038/s41467-021-21892-z
  47. Cousin M. A., Creighton B. A., Breaux K. A., et al. (2021). Pathogenic *SPTBN1* variants cause an autosomal dominant neurodevelopmental syndrome. *Nat. Genet.* **53**:1006–1021. DOI:10.1038/s41588-021-00886-z
  48. Yoshioka N., Kabata Y., Kuriyama M., et al. (2020). Diverse dystonin gene mutations cause distinct patterns of *Dst* isoform deficiency and phenotypic heterogeneity in *Dystonia musculorum* mice. *Dis. Model. Mech.* **13**:dmm041608. DOI:10.1242/dmm.041608
  49. Jiang Y. L., Jiao B., Liao X. X., et al. (2020). Analyses mutations in *GSN*, *CST3*, *TTR*, and *ITM2B* genes in Chinese patients with Alzheimer's disease. *Front. Aging. Neurosci.* **12**:581524. DOI:10.3389/fnagi.2020.581524
  50. Myllyharju J. and Kivirikko K. I. (2001). Collagens and collagen-related diseases. *Ann. Med.* **33**:7–21. DOI:10.3109/07853890109002055
  51. Zhang L., Smyth D., Al-Khalaf M., et al. (2022). Insulin-like growth factor-binding protein-7 (*IGFBP7*) links senescence to heart failure. *Nat. Cardiovasc. Res.* **1**:1195–1214. DOI:10.1038/s44161-022-00181-y
  52. Harris L., Genovesi L. A., Gronostajski R. M., et al. (2015). Nuclear factor one transcription factors: Divergent functions in developmental versus adult stem cell populations. *Dev. Dyn.* **244**:227–238. DOI:10.1002/dvdy.24182
  53. Nakamura M. and Sadoshima J. (2018). Mechanisms of physiological and pathological cardiac hypertrophy. *Nat. Rev. Cardiol.* **15**:387–407. DOI:10.1038/s41569-018-0007-y
  54. Kim J., Wende A. R., Sena S., et al. (2008). Insulin-like growth factor I receptor signaling is required for exercise-induced cardiac hypertrophy. *Mol. Endocrinol.* **22**:2531–2543. DOI:10.1210/me.2008-0265
  55. West J. B. (2009). Comparative physiology of the pulmonary blood-gas barrier: The unique avian solution. *Am. J. Physiol.-Regul. Integr. Comp. Physiol.* **297**:R1625–R1634. DOI:10.1152/ajpregu.00459.2009
  56. Parr N., Wilkes M. and Hawkes L. A. (2019). Natural climbers: Insights from avian physiology at high altitude. *High Alt. Med. Biol.* **20**:427–437. DOI:10.1089/ham.2019.0032

## FUNDING AND ACKNOWLEDGMENTS

This research was funded by the Third Xinjiang Scientific Expedition Program (2022xjkk0205), the National Natural Science Foundation of China (NSFCU23A20162 and NSFC32570498) and State Key Laboratory of Animal Biodiversity Conservation and Integrated Pest Management (Grant No. SKLA2502). We thank Kesheng Wang, Yuehan Xue, Nan Fang, Hongjiu Zhang, and Liang Ma for discussions and technical support in cell

isolation and library preparation for single-cell sequencing. Elements of the graphical abstract were created with BioRender. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

#### AUTHOR CONTRIBUTIONS

This study was conceived by W.Z., S.Z., P.E., and Y.Q. Research was supervised by Z.Z., W.Z., F.M., F.L., S.Z., and Y.Q. Data analyses were carried out by Y.H. and X.Z. Experiments were performed by Q.Z. and F.M. Field work was carried out by H.S., L.M., and Y.F. The manuscript was written by Y.Q. with contributions from W.Z., Z.Z., P.E., and S.Z. All authors contributed to the manuscript and approved the final version.

#### DECLARATION OF INTERESTS

The authors declare no competing interests.

#### ETHICAL STATEMENT AND PATIENT CONSENT

All sampling procedures were approved by the Institutional Animal Care and Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences (IOZ20160049).

#### DATA AND CODE AVAILABILITY

The single-cell data for heart tissue of the tree sparrow that support the findings of this study have been deposited into CNGB Sequence Archive (CNSA) (<https://db.cngb.org/cnsa/>) of China National GeneBank DataBase (CNGBdb) with accession number CNP0006422. Analysis scripts are available on GitHub (<https://github.com/haoyanioz/Cross-species-single-cell-transcriptomic-analysis-for-heart>).

#### SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-life.2026.100251>.