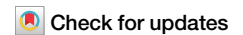


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Single-cell transcriptomics reveal mechanisms of skeletal muscle differentiation across duck embryonic development



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Skeletal muscle development is cornerstone of vertebrate locomotion, relies on the functionally distinct muscle fiber-type. Although the cellular dynamics in myogenesis have been extensively studied, the developmental origins and pathways governing fiber-type diversification remain unresolved. Furthermore, the evolutionary conservation of these mechanisms across vertebrates is poorly understood. Thus, we generate a comprehensive single-cell transcriptomic atlas of duck skeletal muscle across embryonic development to explore the trajectory from myogenic progenitors to myofiber. We identified a differentiation mechanism whereby slow-twitch type could transdifferentiate into the fast-twitch type, a process mediated by *LEF1*+(I) subtype. Comparative analysis of datasets across vertebrates (avian and mammalian) reveals that this fiber-type conversion program is phylogenetically conserved, suggesting homology in muscle adaptation mechanisms. Our study provides the transcription factors roadmap of vertebrate myofiber development, bridging gaps in developmental and evolutionary biology. These insights advance fundamental knowledge of tissue patterning and hold translational potential for regenerative medicine and agricultural biotechnology.

Skeletal muscle, a central component of the vertebrate motor system, is composed of multinucleated myofibers that are broadly classified into two types: slow-twitch/oxidative (type I) and fast-twitch/glycolytic (type II)^{1,2}. The type and proportion of myofibers directly determine the athletic ability and meat quality of muscle tissue³. The neurons that control fast type are thicker and mainly contribute to explosive movements. In contrast, slow type is thinner and mainly contributes to sustained movements⁴. Studies on major agricultural animals have consistently shown that the meat quality is basically dependent on myofiber characteristics^{5,6}. Therefore, an intensive analysis of the molecular phenotypic changes and genetic regulatory mechanisms of early myofiber development is crucial for efforts to improve animal meat quality and to gain a deeper understanding of athletic phenotypes.

Myogenesis begins in early embryonic development, first generating prenatal skeletal muscle progenitor cells (MPCs) that differentiate into myoblasts, which fuse into multinuclear myotubes and maturation to myofiber^{7–10}. The prenatal formation of vertebrate muscle fibers (MFs) comprises two successive waves: embryonic myoblasts differentiate into

primary fibers, while during fetal myogenesis, fetal myoblasts both fuse to primary fibers and fuse to one another to make secondary myofibers^{11–13}. Three distinct subtypes (fast, mixed slow/fast, and slow fiber-types) emerge during initial differentiation; then a later phase in which MFs are formed from fast or slow/fast fiber-types^{14,15}. Fast fiber-types become the predominant type, and this trend is prominent in the postnatal stage^{16,17}. The prenatal stage is crucial for myofiber formation, with both type and number changing dynamically throughout this period¹⁸.

Birds, as an important lineage of vertebrates, are considered an important model for studying muscle development due to their unique flying phenotype and high-quality meat supply¹⁹. During the hatching stage, avian skeletal muscle undergoes structural and functional maturation, with the total number of myofibers being determined before its final embryonic stage^{20–22}. A study on the skeletal muscle also promotes the understanding of the evolutionary mechanism of muscle biology²³. In mammals, due to the great value of humans, mice, and pigs in muscle diseases and agricultural production, single-cell sequencing technology has been widely used in the study of their muscle

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development, providing valuable insights into the heterogeneity of myogenic lineage cells^{7,24–26}. However, recent studies in birds have focused on a specific mechanism or cell type and have not provided a complete landscape of muscle development, especially in elucidating the molecular mechanisms of cell development pathways and myofiber formation, which highlights the need for further research.

Here, we constructed the comprehensive single-cell atlas of skeletal muscle development during the whole embryonic period of Pekin ducks, and elucidated the composition of skeletal muscle cell types, the differentiation dynamics of muscle subsets, and the developmental trajectories of myofiber subtypes. Our study pinpointed that *MYL9*⁺ mesenchymal stem cells (MSCs) are the embryonic source of MPCs, and further identified “slow-to-fast” fiber-type transition transcriptional regulators. The identification of 13 key transcription factors has delineated the molecular mechanisms governing skeletal muscle development. Additionally, cross-species comparative analysis between avians and mammals revealed both conserved and divergent features of muscle development across species (Fig. 1). This study not only expands our understanding of avian embryonic development but also identifies potential molecular targets for genetic improvement in agricultural animals and meat production.

Results

Single-cell transcriptomic atlas of the duck embryonic skeletal muscle

To comprehensively analyze the cellular heterogeneity and molecular changes in skeletal muscle during embryonic development, we analyzed the morphology and histology of Pekin duck embryos starting from embryonic day (E) 5 (Supplementary Figs. 1–3). The pectoral muscle (PM) was not observed before E7, and the morphologically clear muscle tissue was first observed at the E9 stage. At the same time, we measured the embryonic body weight (BW) and pectoral muscle weight (PMW) of Peking ducks during the whole embryonic period (from E9 to E27) and on the first day after hatching. The results showed that PMW and BW continued to increase before E21 (Supplementary Fig. 4), and after E21, the growth rate of PMW slowed down and showed a downward trend.

To gain a comprehensive view of skeletal muscle in the avian embryo, we profiled the transcriptomes of 76,916 high-quality cells from PM biopsies of 10 developmental time points (Fig. 2A, B, Supplementary Fig. 5A, Supplementary Data 1 and 2). Using unsupervised clustering analysis and lineage-specific marker gene identification (Fig. 2C, Supplementary Fig. 5B, Supplementary Data 3), these cells were clustered into 12 types, representing all major cell lineages involved in the development of skeletal muscle. The major mononucleated muscle-resident cell types include myogenic lineage

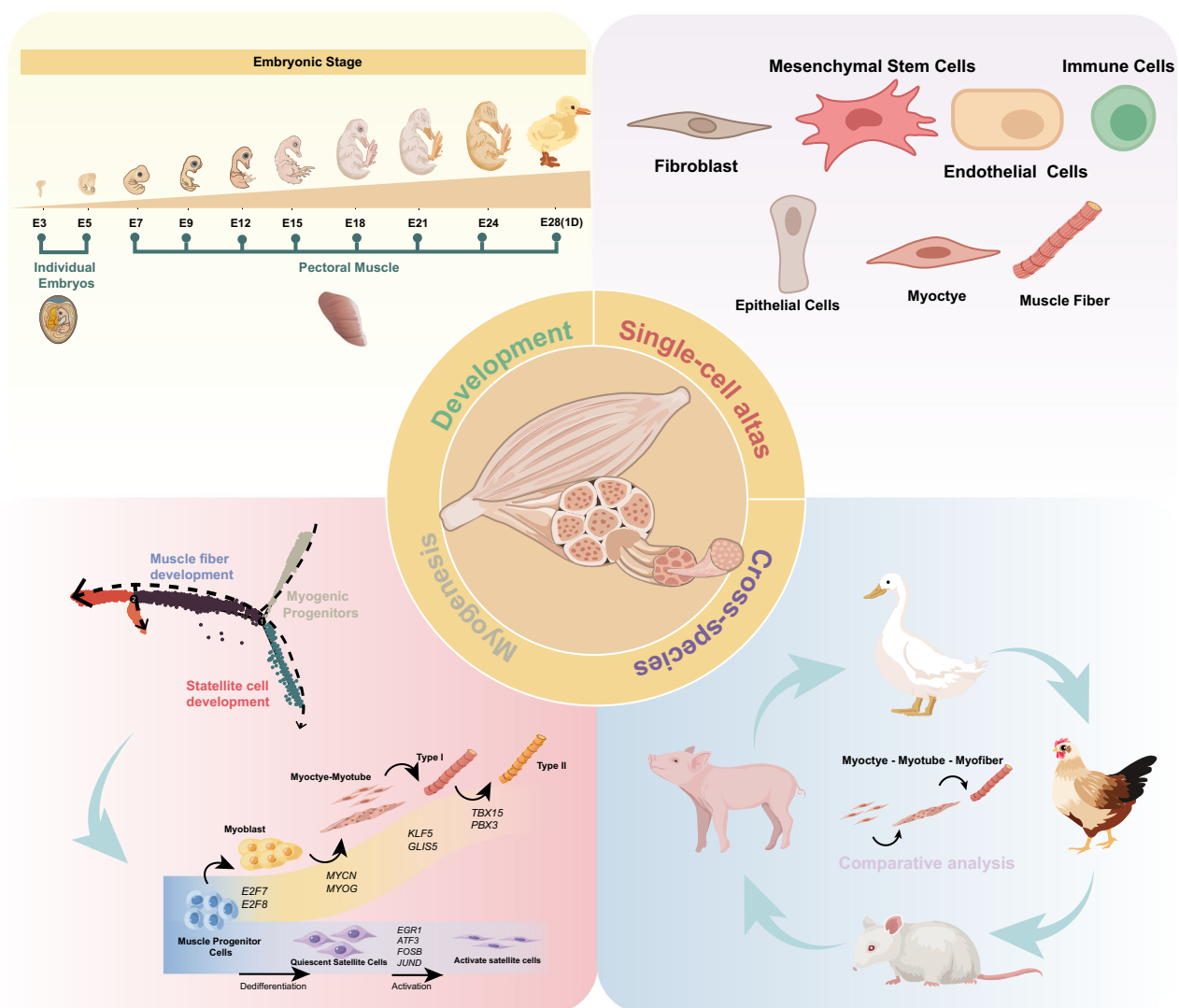


Fig. 1 | Study design overview. This schematic illustrates the sample collection and main analysis in the current study.

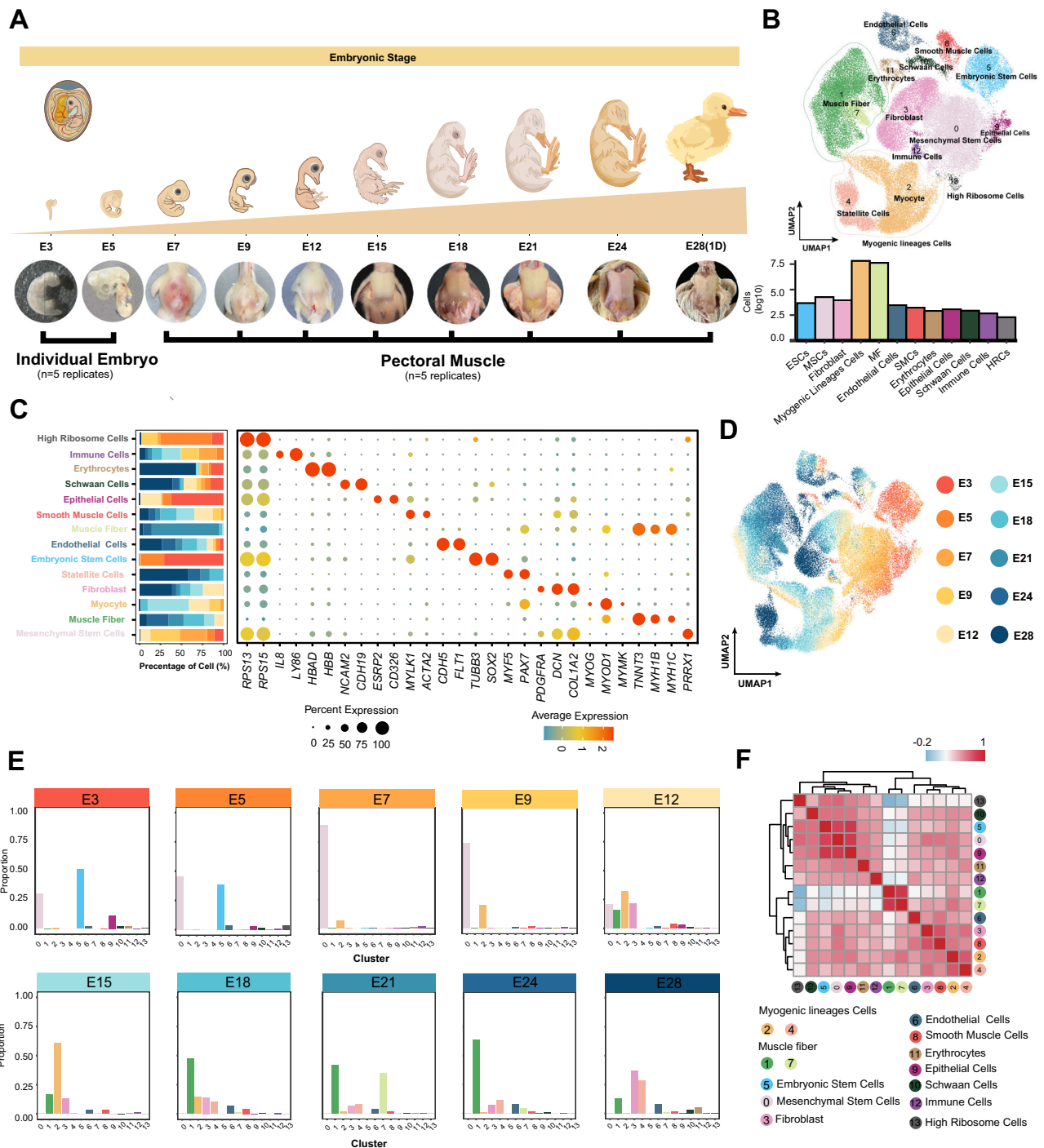


Fig. 2 | Single-cell transcriptomic profiling of duck skeletal muscle. A The diagram of skeletal development in a duck. Samples were obtained at ten development time points, E3, E5, E7, E9, E12, E15, E18, E21, E24, and E28. **B** UMAP analysis of 76,916 scRNA-seq profiles delineating 14 main cell populations; The number of cells sequenced for each cell type. Dots and bars are colored by cell type. **C** Bubble plot showing the representative marker genes for different cell clusters. The dot size

reflects the percentage of each cluster expressing the gene, while the color saturation indicates the relative expression level. **D** UMAP visualization showing the origin of cells at different stages. **E** Bar plot illustrating dynamic changes in cell population sizes across embryonic developmental stages (E3–E28). **F** Hierarchical clustering using the Pearson correlation coefficient (PCC) of a normalized transcriptome in cell type resolution ($n = 14$). The color intensity of the heatmap indicates the PCC values.

cells (e.g., myocytes, satellite cells), embryonic stem cells (ESCs), MSCs, fibroblasts, epithelial cells, pericytes (e.g., smooth muscle cells and endothelial cells), immune cells, glial cells (e.g., Schwann cells), erythrocytes, high-ribosomal cells, and, finally, multinucleated myofibers (MFs). Myogenic lineage cells and multinucleated MFs constituted the predominant cell populations.

The cell types and proportion in skeletal muscle at the embryonic stage were dynamically changing (Fig. 2D, E, Supplementary Fig. 5C, Supplementary Data 4). In detail, the earliest time points (E3 and E5) were composed of two stem cell populations: ESCs (E3: 30.22%, E5: 38.4%; *PAX3/STMN2/SIX3*), and MSCs (E3: 51.08%, E5: 45.81%; *MSX2/MYL9*). Cellular stemness assessment further validated that ESCs and MSCs

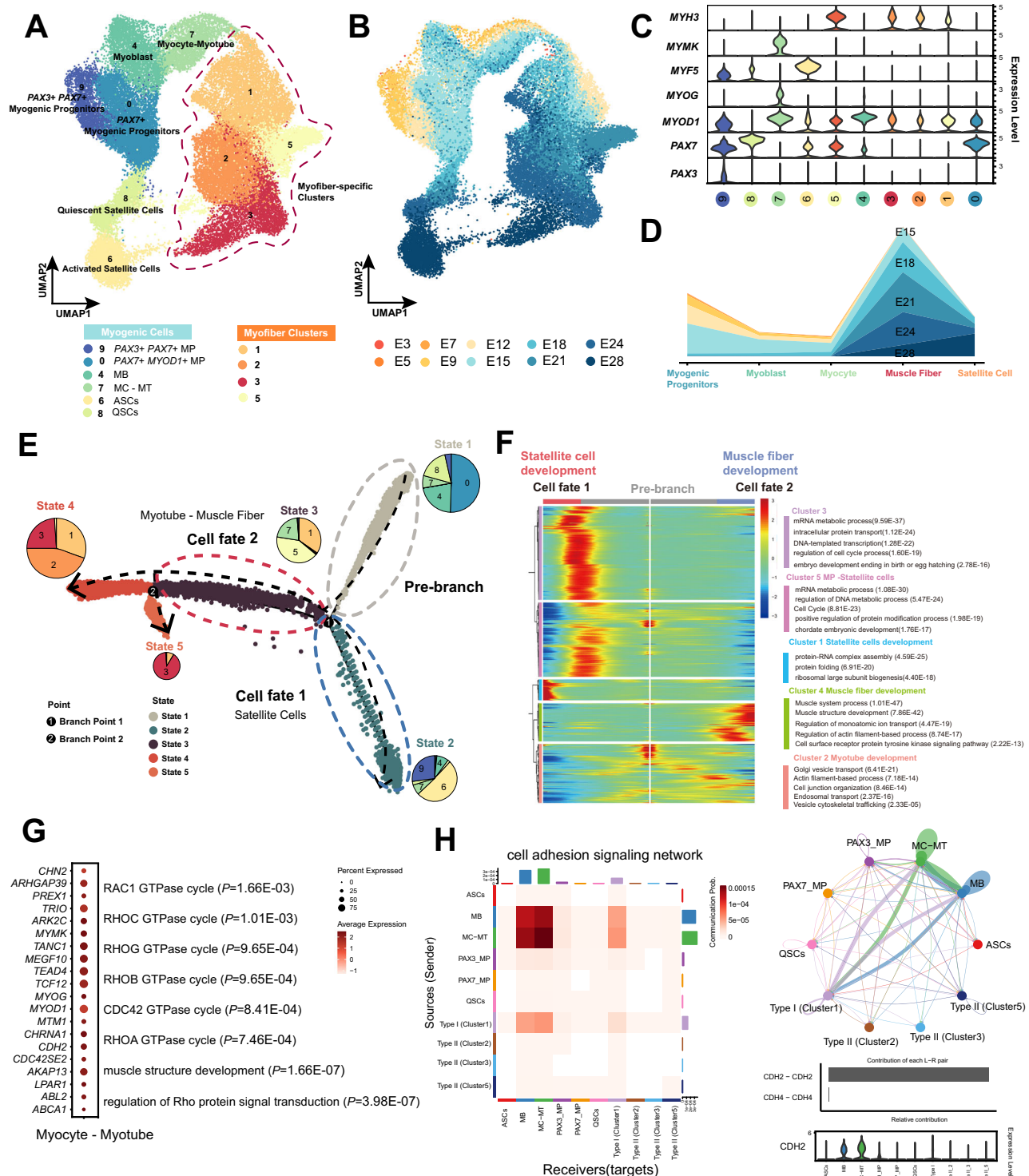


Fig. 3 | Reconstructing myogenic cells fate decision during embryonic development. **A** UMAP analysis of 36,128 cells delineating 10 main myogenic lineage cell subpopulations. **B** UMAP visualization showing the origin of myogenic lineage cells at different stages. **C** Violin plot showing the expression of the marker genes across myogenic lineage cells. The expression levels are shown for six marker genes in myogenic lineage cell subtypes. **D** Stacked chart showing the proportion of major cell types in ten stages. **E** Pseudo-time ordering analysis revealing all myogenic lineage cells as two branches and five states. The black dashed lines represent different evolutionary tracks, in which the light gray one represents pre-branch while dark purple and turquoise lines represent myofiber and satellite cells development,

respectively; the pie charts and bar charts show the proportions of cells from the 10 cell types at different states. **F** Heatmap illustrating DEG (differentially expressed genes) dynamics during the cell fates of muscle fiber and satellite cells development; the DEGs are clustered into five gene sets by functional enrichment analysis. **G** Bubble plots illustrate the DEGs enriched pathways in myocyte subpopulations. **H** Cell-cell interaction analysis of ligands and receptors in the myocyte and MF-I; The heatmap and circle plot depict signaling intensity within the CDH pathway, color gradient represents signal strength. The bar chart shows the contribution of each ligand-receptor pair to the CDH signaling pathway, and the Violin plot displays the expression of the CDH2 gene of CDH signaling pathway in the cell population.

exhibited the highest pluripotency scores, indicating their identity as highly plastic, pluripotent cell populations (Supplementary Fig. 5D, E). A significant correlation was observed between these two cell types ($r_s = 0.85$), suggesting a potential functional interplay during development (Fig. 2F). In addition to stem cells, epithelial cells, immune cells, and Schwann cells were identified as the earliest cell populations to acquire biological functionality during the initial phases of embryogenesis (Supplementary Fig. 5E).

Mesenchymal cells were considered to provide the positional cues to MPCs from the somite²⁷. We revealed the classification of MSC lineages during early embryonic development and identified chondrogenic MSCs (ChondroMSCs, *TNMD* + MSCs and *ACAN* + MSCs), dermal MSCs (DermMSCs, *FLNB1* + MSCs, *WIF1* + *APCDD1* + MSCs), *MSX2* + MSCs, and *MYL9* + MSCs during the embryonic period (Supplementary Fig. 6A). *TNMD* and *ACAN*, which are the markers of cartilage, were expressed in ChondroMSCs at E12 (Supplementary Fig. 6B, C). DermMSCs showed high activity of numerous WNT signaling-associated gene including *WNT5B*, *WIF1*, and *ARPPC1*, which are associated with activation in the dermis and subsequently in the epidermis (Supplementary Fig. 6C, D)²⁸. According to the developmental stage and Monocle3 analysis, *MYL9* + MSCs represented a start point in the trajectory analysis predictions, MPCs were last, and *MSX2* + MSCs were located between these two cell types (Supplementary Fig. 6E). Therefore, we predicted *MYL9* + MSCs as an early MSC progenitor, and it may differentiate into chondrocytes, dermis, and muscle progenitors during the embryonic period.

Deciphering the developmental trajectories of avian embryonic skeletal muscle

To further investigate the cellular heterogeneity and fate determination of myogenic cells, mononucleated myogenic cell lineages and multinucleated MF were extracted for subclustering (36,128 cells, clusters 1, 2, 4, and 7 in Fig. 2B). Using unsupervised clustering (UMAP), we defined a hierarchy of myogenic lineages that included four myogenic cell subtypes: myogenic progenitors (MPs) marked by expression of *PAX3*, *PAX7*; myoblasts (MBs) marked by *MYOD1*; myocytes (MCs) by *MYOG*; satellite cells (SCs) by *PAX7*. Myofiber clusters (MF) by *ENSAPLG00020013072* (*MYH3*) (Fig. 3A, C, Supplementary Data 5).

The proportions of identified myogenic lineage cell populations exhibited dynamic changes from E7 to E28 (Fig. 3B, D, Supplementary Fig. 7A). Comparison of the proportion of myogenic cells at different stages, the myogenic population mainly consisted of *PAX3* + MPs during the early embryonic period (E7–E9), and MPs increased *PAX7* while decreasing *PAX3* expression from E7 to E15 (Supplementary Fig. 7B). Initiation of *MYMK* expression in myocyte (MCs) ($p < 2.22E-16$) at E12 triggers the myoblast fusion program essential for multinucleated myofiber formation during myogenesis (Supplementary Fig. 7C)²⁹. The number of MBs and MCs decreased at the mid-embryonic period (E15–E18), potentially due to the incorporation of most of the differentiated MCs into MFs. MFs gradually increased in proportion from early-to-mid-embryonic period (E7–E21; 3.58–88.16%) that decreased during later embryonic development (E24–E28; 84.42–33.28%) (Fig. 3E, Supplementary Fig. 7D). Interestingly, Satellite cells remained mostly quiescent until mid-embryogenesis, then shifted to proliferation (63.12%) by E28 (Supplementary Fig. 7D). Compared to their quiescent satellite cells (QSCs), activated satellite cells (ASCs) exhibited significantly higher expression levels of *MYOD1* ($p = 4.7E-261$) (Supplementary Fig. 7E). This finding is consistent with the gene expression patterns previously observed in satellite cells of neonatal pig muscle³⁰.

Pseudotime trajectory analyses divided myogenic cells into five developmental states on two cell lineage branches. Myogenic progenitor and myocytes (State 1) were set as the initial stages of the developmental process. The developmental trajectory of MPs was divided into two branches (Fig. 3E, Supplementary Fig. 7F). The dedifferentiation of MPs, which facilitated their ultimate conversion into SCs, was designated cell fate 1 (State 2). Another branch that the differentiation of MPs into MCs and their subsequent fusion into myofibers was designated cell fate 2 (State 3). We visualized dynamic changes in gene expression along pseudotime

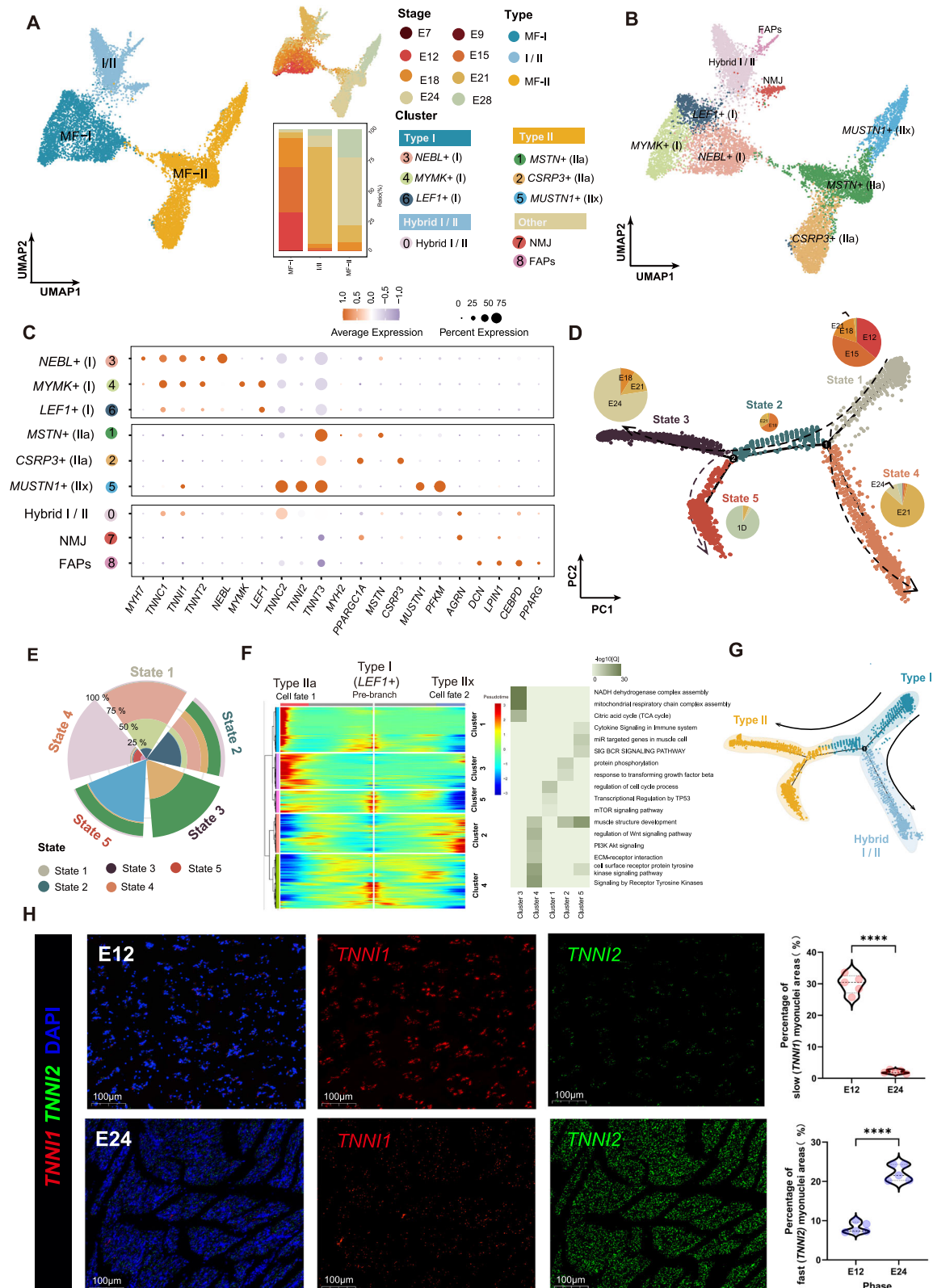
trajectories in muscle cells (Fig. 3F). Based on the gene expression profiles along the trajectory, the dedifferentiation process of MPs (cell fate 1) involves gene expression reprogramming and dynamic regulation of protein-related processes (Cluster 5), supported by the pathways such as mRNA metabolism ($p = 1.08E-30$), cell cycle regulation ($p = 8.81E-23$), and protein modification ($p = 1.98E-19$), which together promote the transition from the progenitor state to the satellite cell state. Pseudotime profiling reveals that both *FOSB* and *TSC22D3* are uniquely upregulated during satellite cell activation, suggesting their involvement in the quiescence-to-proliferation switch (Supplementary Fig. 7G). *FOSB* is induced during the early activation process and plays a critical role in regulating the initial activation of satellite cells³¹. *TSC22D3* inhibits myogenic differentiation by modulating the transcriptional activity of *MYOD1* and *HDAC1*, thereby reducing the expression of *MYOG* and mediating the anti-myogenic effects of glucocorticoids³². Additionally, the fusion of myotubes and the formation of myofibers rely on fundamental biological processes (cell fate 2, Cluster 2), including Golgi vesicle transport ($p = 6.41E-21$), endosomal transport ($p = 2.37E-16$), and vesicle cytoskeletal trafficking ($p = 2.33E-05$), which are essential for maintaining the muscle cytoskeleton. Based on the above functional analysis of myocyte-myotube subcluster (Cluster 7), the biological processes related to Rho protein signal transduction ($p < 0.01$) contribute to myocyte fusion, Rho family members are localized to vesicular compartments, and would be directly involved in driving actin-based vesicle motility (Fig. 3G)^{33,34}. Furthermore, we analyzed the cell signaling mechanisms that promote the transition of myocytes to myotubes through ligand-receptor (LR) cell-cell interactions. Myocytes were predicted to signal via cell adhesion, including *CDH2-CDH2* (Fig. 3H, Supplementary Data 6). Cell adhesion is involved in myoblast interaction and fusion, which has been reported to *CDH2* trigger signaling events that promote myogenesis through the regulation of Rho family members' activities^{35,36}.

Slow-to-fast fiber-type transition during skeletal muscle development

The composition of fast and slow skeletal muscles directly determines the athletic ability and meat quality. Deciphering the myogenic commitment trajectory of MPs is key to unraveling the dynamics of avian myofiber (MF) formation. The heterogeneity of subpopulations classification was based on previously identified markers associated with myofiber-specific type¹. Three subtypes represented type I cluster (slow-switch myofiber/MF-I, *TNNI1* +), type II cluster (fast-switch myofiber/MF-II, *TNNI2* +), and hybrid I/II (*TNNC1* + *TNNC2* +) (Fig. 4A, Supplementary Data 7). In addition, with two known MF-II subtypes: one expressing *MYH2* (type IIa) and the other expressing *MYH1B* (type IIx). MF-I generally decreased, while MF-II increased in relative abundance during embryonic development, and this trend was further confirmed by the single-nucleus RNA sequencing (snRNA-seq) results. (Fig. 4A, Supplementary Figs. 8 and 9A).

All 9 subtypes were identified based on the expression of cell type markers (Fig. 4B, Supplementary Data 8). Further subclustering identified two specialized subtypes: neuromuscular junction (NMJ) and fibro-adipogenic progenitors. NMJ showed enrichment in a gene associated with axon guidance (*AGRN*). Fibroblast with adipocyte-like properties co-expressed adipogenic markers (*PPARG*, *LPIN1*, *CEBPD*) and fibroblast markers (*DCN*), expand upon muscle injury and promote myogenesis (Fig. 4C)³⁷. Notably, MF-I (marked by *MYMK*, *NEBL*, and *LEF1*) were predominantly enriched during mid-embryonic development (E12–E18; ~86.98%) (Supplementary Fig. 9B). In contrast, MF-II showed progressive dominance during mid-to-late development (E12–E28), with *MSTN* + (II) and *CSRP3* + (IIa) detectable at E12 and E18, respectively, and persisting through E28 (*MSTN*: mid, 7.42%; late, 24.56%; *CSRP3*: mid, 1.46%; late, 23.67%), while *MUSTN* + (IIx) emerged predominantly by E28 (~79.2%) (Supplementary Fig. 9B).

We analyzed the pseudotime trajectories, observing a defined path with development in both MF-I and MF-II. The trajectory analysis was divided into five developmental states, with E12–15 located at the initial State 1, E28 at State 5, and E18 at intermediate states along the trajectory (Fig. 4D). MF-I



were predominantly distributed in the early stages of the pseudotime trajectory, including *MYMK*+ (I) and *NEBL*+ (I) (Supplementary Fig. 9C, D). *MYMK*+ (I) served as the starting point in the trajectory analysis predictions, and expressed myocyte fusion marker (*MYBP3*) and cardiac muscle marker (*MYBP3*) in E12. *NEBL*+ (I) was enriched in genes related to cardiac contraction, such as *MYL2*, which has been previously shown to be highly

expressed in MF-I, supporting their sustained contraction and fatigue-resistant properties (Supplementary Fig. 9D)^{38,39}. In summary, early-stage MF-II exhibits characteristics of cardiomyocytes.

Focusing on branch point 1, representing cell differentiation from MF-I (*MYMK*+, *NEBL*+) to *LEF1*+ (I) and hybrid I/II (States 2 and 4) (Supplementary Fig. 9E). State 2 marked a transitional phase in the type I to II

Fig. 4 | Cell and transcriptional heterogeneity in myofibers. **A** UMAP visualization showing subtype in scRNA-seq, colored according to myofiber-type-specific classification and different stages. Stacked bar plots illustrating the evolving subpopulations landscape. **B** UMAP visualization showing fiber-type subpopulations. **C** Dotplot showing the expression of the marker gene in fiber-type subtypes. **D** Pseudotime trajectory revealing all fiber-type populations as two branches and five states. The black dashed lines with arrows represent different evolutionary tracks, including I/II hybrid and type II development; each dot represents one cell, and is colored by states; the pie charts show the proportions of cells at each state. **E** Stacked

Rose Diagram shows the proportions of 9 subpopulations at each cell state. **F** Heatmap illustrating differentially expressed genes dynamics during the cell fates of Type IIa (Cell fate 1) and Type IIx (Cell fate 2) development; the DEGs are clustered into five gene sets by functional enrichment analysis. **G** Remodeling of trajectory development from type I to type II formation. **H** *asmFISH* analysis of *TNNI1*+(slow) and *TNNI2*+(fast) on FFPE sections. Violin plot showing percentage of slow and fast fiber-type (E12 versus E24, five windows). Student's *t*-test was used to perform statistical analysis, $n = 4$ independent experiments. Scale bar, 100 μm . *P* value: *t*-test. **** $P < 0.0001$.

fiber-type conversion, predominantly characterized by *LEF1*+(I) (~42.59%), *LEF1* expression in E18 was confirmed by *asmFISH* (Supplementary Fig. 10A). State 4 included hybrid I/II (~79.37%) and derived subtype specialized (Fig. 4E). We analyzed gene expression patterns along the stages of the fiber-type transformation and identified five gene expression modules, while combined with temporal gene expression levels, most modules reflected fundamental biological processes associated with muscle cell differentiation and actin cytoskeleton formation (Supplementary Fig. 10B). During the specification of hybrid I/II, we observed significant enrichment of TGF β signaling pathway activity (Supplementary Fig. 10B). These hybrid I/II were predominantly detected at E21, a developmental stage marking the onset of PMW regression, as confirmed by histological analyses. Given the established role of TGF β signaling in restricting myoblast fusion to control syncytia formation and muscle regeneration⁴⁰, it may mediate the later-stage PM regression program through modulation of myogenic fusion dynamics.

Branch point 2 delineates Type I (*MYMK*+, *NEBL*+) formed two differentiation branches through *LEF1*+(I) toward *MSTN*+(IIa), *CSRP3*+(IIa), and *MUSTN*+(IIx). Differential expression analysis between the two branches revealed that "PI3K-Akt signaling pathway ($p = 1.77\text{E-}08$)", "Signaling by Receptor Tyrosine Kinases ($p = 8.1\text{E-}15$)" and "regulation of Wnt signaling pathway ($p = 1.98\text{E-}10$)" were enriched at the cell fate decision point, indicating the importance of these signaling pathways for regulating fiber-type transition (Fig. 4F). Consistent with pseudotime cell trajectories, a general decrease in MF-I subtypes accompanied by a relative increase in MF-II subtype in embryonic late-stage, which translated into type changes in the myofibers, as confirmed by *asmFISH* analysis (Fig. 4G, H, Supplementary Fig. 10C, D, Supplementary Data 9).

In summary, these results suggest a "slow-to-fast" fiber-type transition during skeletal muscle embryonic development, and *LEF1*+(I) was identified as the new state during myofiber transformation.

Activation of transcription factor regulatory network underlies myogenesis

To investigate the transcriptional regulatory programs during muscle development, we identified key regulatory factors involved in the fate determination of different muscle cell types at each stage. We have predicted 13 detected transcription factors during myogenesis using pySCENIC (Fig. 5A). In addition to known key regulators of myogenesis (*MYOG*, *MYCN*, *TCF4*, *PBX3*), we predicted *in silico* *E2F7* and *E2F8* may be involved in myoblast differentiation, given their function in cell cycle control, control of central regulators of cell division and cell fate decisions^{41,42}. We constructed a gene expression network for myocyte differentiation and identified six transcription factors that play an important role in early myocyte differentiation and myotube fusion. It is worth noting that during the further differentiation of myoblasts, the target gene *RREB1* is co-regulated by four transcription factors (*TCF4*, *PBX3*, *MYCN*, *MYOG*) (Supplementary Fig. 11A). *EGR1*, *ATF3*, *FOSB*, and *JUND* were identified as specific transcription factors during satellite cell activation, which have been reported to be involved in the early activation of MuSCs (ex vivo) was dependent on the transcription factors *EGR1*, *FOSB*, and *JUND*⁴³. *ATF3* is an *EGR1* transcriptional target, suggesting its involvement in the activation control of MuSCs⁴⁴. Heterogeneous muscle cell clusters that showed strong cluster-specific gene expression modules, we analyzed module 2 as a specific

expression module for ASCs, and these genes were upregulated during the formation of ASCs (Fig. 5B, Supplementary Fig. 11B, Supplementary Data 10). We explored potential genes driving satellite cell activation and constructed a transcription factor regulatory network (Supplementary Fig. 11C). Among these, the target genes *DUSP1*, *CREM*, *NR4A3*, and *NR4A2* were regulated by three core transcription factors. Notably, *NR4A3* and *NR4A2* are homologs of H3K27 methyltransferases, and previous studies have confirmed that *NR4A2* activation enhances oxidative myofibers⁴⁵.

We predicted 25 transcription factors potentially regulating MF-type formation (Fig. 5C, Supplementary Fig. 11D). Among these, *TBX15* and *PBX3* were identified as top candidate TF driving the type I-to-type II myofiber transition, consistent with *TBX15* expressed in glycolytic myofibers (type II), which suggests their pivotal roles in modulating skeletal myofiber-type determination^{46,47}. We next reconstructed interaction networks across the prioritized transcription factors (*TBX15*, *PBX3*) to resolve co-regulatory relationships (Supplementary Fig. 11E, F). Both involved PI3K-Akt signaling pathway (*TBX15*: $p = 1.01\text{E-}05$; *PBX3*: $p = 5.03\text{E-}04$) and cell surface receptor protein tyrosine kinase (RTK) signaling pathway (*TBX15*: $p = 1.31\text{E-}12$; *PBX3*: $p = 2.52\text{E-}07$), which are known to be enriched at the cell fate decision point about slow-to-fast fiber-type transition (Fig. 5D). RTKs are the common upstream activators for PI3K-AKT signaling pathways⁴⁸. Interaction networks across the prioritized transcription factors (*TBX15*, *PBX3*) were reconstructed to resolve their co-regulatory relationship by integrating time-series gene expression patterns with regulons. *PTK2* (*FAK*) emerged as a key regulator of integrin-mediated survival signaling activation triggered downstream PI3K/Akt pathway signaling, which was predicted to be regulated by *TBX15* and *PBX3* (Fig. 5E)⁴⁹.

Our analyses predicted 13 key transcription factors that potentially regulate the differentiation of MPCs into myoblasts, myocytes/myotubes, Type I, and Type II fibers. This prediction elucidates a key regulatory network in skeletal muscle development. (Fig. 5F).

Conserved myogenesis changes in avian and mammal

Cross-species comparative analysis of single-cell transcriptome data is essential for exploring the similarities and differences in early embryonic skeletal muscle development between birds and mammals, as well as their underlying molecular mechanisms. We integrated publicly available single-cell transcriptomic data from embryonic skeletal muscle of pig (E16, E18, E21, E28, D1) and chicken (E10, E14, E18, D5)^{26,50,51}. In integrated UMAP data, the distribution of same cell types in three species was relatively consistent, indicating that cell types and gene expression patterns are conserved across vertebrates (Fig. 6A). Species-independent cluster analysis showed that the major cell types particularly MSCs, fibroblasts, muscle lineage cells, and endothelial cells within mononucleated populations, were highly conserved across chicken, pig, and duck (Fig. 6B, Supplementary Fig. 12A). These results suggest that the evolutionary conservation of skeletal muscle might underlie the shared characteristics of muscle cell types in vertebrates.

Homologous genes played an indispensable role in cross-species comparative analysis, and a total of 11,467 homologous genes were identified (Supplementary Fig. 12B). We compared differentially expressed genes (DEGs) in five conserved cell populations between birds and mammals, identifying 341 shared cell markers (Fig. 6C, Supplementary Data 11).

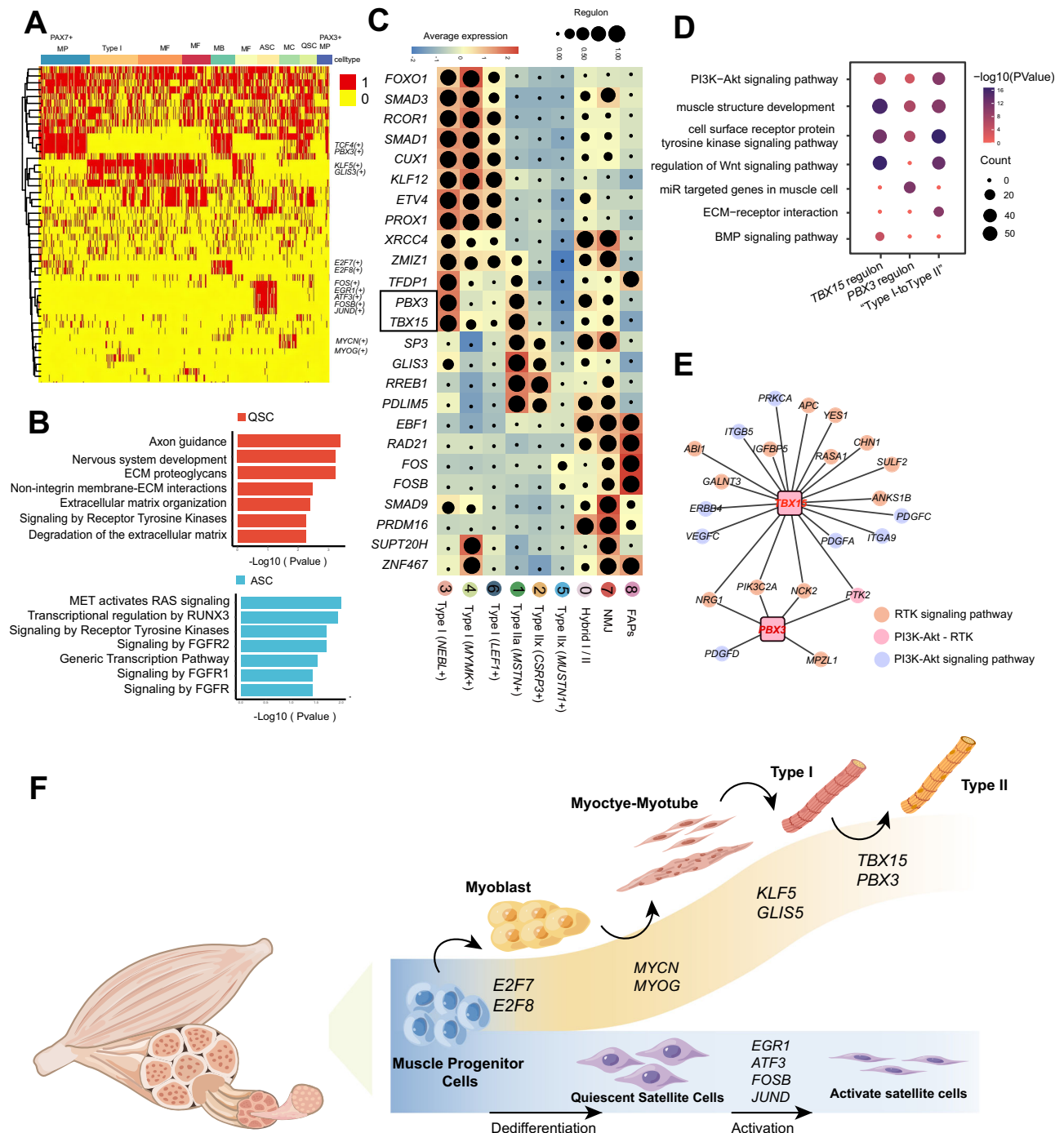


Fig. 5 | Activation of transcription factor regulatory network underlies myogenesis. **A** Binary plots depicting active regulons in myogenic lineage cells; Regulons and cells are ordered by hierarchical clustering. **B** Genes specifically enriched in QSC (cluster 6) are module 2, and ASC (cluster 8) are module 6. The numbers at the bottom of the figure represent cell clusters, and the numbers on the right side of the figure represent the 20 modules. DEGs enriched functional pathway for the genes in

modules 2 and 6. **C** SCENIC predicted transcription factor expression (box color). The dot size shows target gene accessibility (regulon/AUCell). **D** Gene set enrichment analysis for pathway across *TBX15* regulons, *PBX3* regulons, and “Type I-to-Type II” time-series genes. **E** GRN showing regulation between *TBX15* and *PBX3*, inferred using pySCENIC. **F** A model of the early cell fate transition during myogenesis.

Additionally, higher conservation of cell type-specific marker genes was observed in MSCs, muscle cells, and myofibers of chicken and duck compared to pig (Fig. 6D, Supplementary Data 12). Conserved gene expression patterns among these organisms, although their proportions exhibited some differences.

Marker genes for MSCs (*COL26A1+*), fibroblasts (*LUM+*, *DCN+*, and *COL1A1+*), and endothelial cells (*PLVAP+* and *EGFL7+*) showed consistent expression patterns across all three species. In contrast, certain

marker genes were species-specific, such as *PRRX1+* MSCs (chicken and duck), *COL3A1+COL6A3+* fibroblasts (pig and chicken), and *KRT8+EMCN+* epithelial cells (pig and duck) (Fig. 6E). In pig and chicken muscle cells, we identified highly conserved muscle subpopulations, including myoblasts, myocytes, and satellite cells (Fig. 6F). Myogenic cell marker genes demonstrated universal applicability across species. The three species shared multiple conserved developmental regulatory genes, such as *MYOD1*, *MYOG*, and *MYF5*, which play critical roles in myoblast

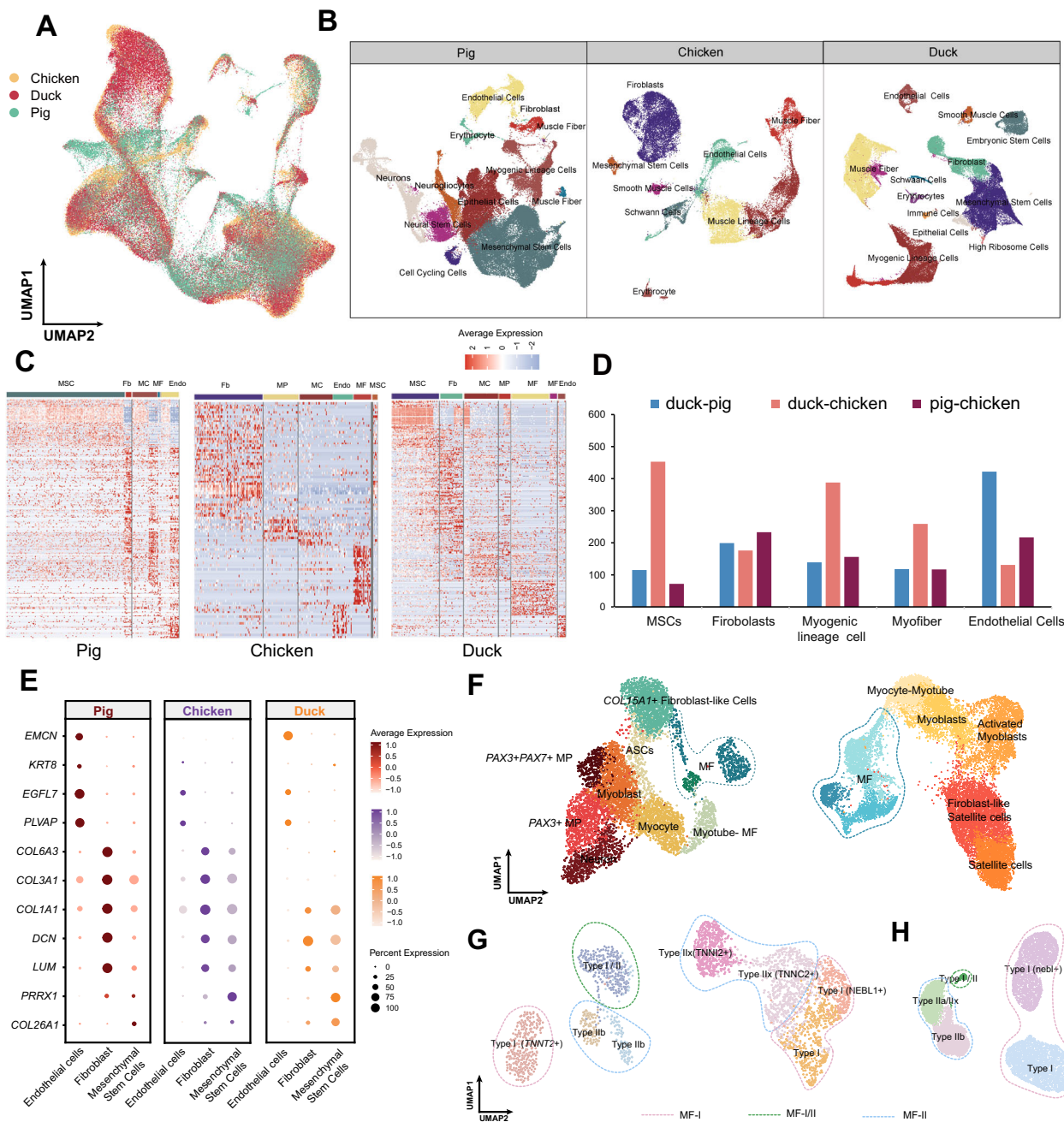


Fig. 6 | Cross-species cell atlas of ducks, chickens, pigs, and mice. **A** UMAP visualization of integrated scRNA-seq data from pigs, chickens, and ducks. **B** UMAP plot revealing cellular heterogeneity with major cell types in the muscle tissues of ducks, chickens, and pigs. Different colors represent different cell clusters. **C** Heatmap showing scaled expression levels of representative genes of pig skeletal muscle, chicken skeletal muscle, and duck skeletal muscle, with genes ordered into those common to the species. **D** Bar charts showing the number of conserved cell-

differentiation and muscle maturation. In contrast to ducks, a fibroblast-like cell subtype was identified in pig and chicken, characterized by high expression of fibroblast-associated markers (*COL5A1*) (Supplementary Fig. 12C).

We visualized muscle cell populations in pig and chicken embryonic skeletal muscle across different developmental stages (Fig. 6F). The results showed that type I myofibers emerged at E16, gradually decreased during development, and disappeared completely by postnatal 1 day (D1) in pig. The proportion of type II myofibers increased over time. Hybrid I/II

type marker genes between ducks and pigs (blue), ducks and chickens (pink), and pigs and chickens (fuchsia). **E** The bubble plot showing cross-species comparison of selected top markers for all major cell types profiled across all species. **F** UMAP visualization showing subclusters of pig myogenic cell types, chicken myogenic cell types. **G** UMAP visualization showing subclusters of pig and chicken myofiber cell types. **H** UMAP visualization showing subclusters of mouse myofiber cell types.

myofibers appeared at E18 and persisted postnatally (Supplementary Fig. 12D). Both pig and duck myofibers displayed consistent expression patterns, with hybrid I/II myofibers coexisting alongside conventional type I and II myofibers (Fig. 6G). Unlike pig and duck, where hybrid I/II myofibers were identified, only type I and type IIX myofibers were identified in the chicken skeletal muscle (Supplementary Data 13, Fig. 6H). Similar trends were observed in mouse embryonic myofibers during development. We observed a conserved pattern in the developing Peking duck PM, where the embryonic myosin gene *MYH3* was highly expressed throughout mid-

embryogenesis but was sharply downregulated around the early postnatal stage (Fig. 6H, Supplementary Fig. 12E, F, Supplementary Data 14)^{16,52}. These findings suggest that both mammals and birds undergo a “slow-to-fast” fiber-type transition during skeletal muscle development.

Discussion

Myofibers, the fundamental units of skeletal muscle, undergo profound transformations during early embryonic development, marked by the reduction of specific subpopulations, the emergence of novel cell types, and dynamic shifts in gene expression programs and regulatory networks^{53,54}. While prior research has established a foundational understanding of cell lineage development in early vertebrate embryonic skeletal muscle^{55,56}, the mechanisms governing the formation and transformation of early myofiber types remain largely elusive. In this study, we generated a comprehensive single-cell atlas of avian embryonic skeletal muscle development, systematically delineating the heterogeneity and dynamic evolution of skeletal muscle cells. Our findings elucidate the molecular framework driving myofiber formation and transcriptional regulation, which advances our understanding of early myofiber development and provides a theoretical foundation for the classification of avian myofiber types.

We identified a distinct subpopulation characterized by elevated expression of ribosomal protein genes in our integrated analysis of the embryonic myogenesis process in Beijing ducks. Importantly, our finding is not without precedent; a recent study by Li et al.⁵⁷ independently identified an analogous ribosome-high subpopulation, suggesting this may represent a potentially rare or context-specific biological state. Ribosomal biogenesis is a hallmark of highly metabolically active cells. Therefore, we hypothesize that this subpopulation may represent a state of elevated protein synthesis, potentially linked to the high metabolic demands of rapid muscle differentiation and growth during duck embryogenesis. Future studies are needed to validate this hypothesis and explore the functional role of this cell state across different biological systems.

During avian myofiber formation, we observed notable dynamic changes, including transitions in subtypes and reprogramming of gene expression. CDH2(N-cadherin) triggers signaling events that promote myogenesis through the regulation of Rho family members' activities. N-cadherin-mediated cell–cell adhesion is involved in both the commitment of muscle precursors and their terminal differentiation⁵⁸. The high expression of cardiac-related genes (*MYL2*, *MYBPC3*) in early embryonic type I myofibers may reflect shared developmental mechanisms, the functional demands of slow-twitch fibers, and the coordinated expression of genes across tissues. Previous studies have shown that genes expressed in cardiac muscle are also expressed in type I myofibers. This shared gene expression may support the common requirements of both muscle types in contraction mechanisms and energy metabolism⁵⁹. As embryonic development progressed, the emergence of *MSTN*+ (IIa) introduced inhibitory regulation of skeletal muscle growth and proliferation. *MSTN* inhibits skeletal muscle growth and proliferation and is enriched in type IIa^{60,61}. Morphological and anatomical results revealed noticeable atrophy in the PM after E21, which may be associated with *MSTN* expression in type IIa. In related medical research, inhibiting *MSTN* gene expression or activity has been proposed as a key strategy for treating muscle atrophy diseases, resembling the muscle atrophy phenotype observed in late embryonic development^{62,63}. Meanwhile, the co-expression of slow-type genes (*TNNC1*, *TNNI1*) in type II myofibers highlighted the unique characteristics of I/II hybrid myofibers.

Other studies also indicated that the PI3K-Akt and mTOR signaling pathways are critical for myogenesis, with PI3K-Akt and mTOR signaling being responsible for myocyte differentiation and muscle hypertrophy^{64,65}. Previous indicated *FHL3* facilitates bovine skeletal muscle cells proliferation and impedes differentiation by augmenting the phosphorylation levels within the PI3K/Akt signaling pathway, but the shift in myofiber types was not mediated by this pathway⁶⁶. Skeletal muscle heterogeneity is a multi-dimensional process influenced by a complex interplay of transcriptional, structural, and functional factors⁶⁷. This network model proposes *TBX15* and *PBX3* as primary candidates within the PI3K/Akt signaling pathway,

rather than as the sole drivers of myofiber transition. Consequently, these two transcription factors are prioritized as the top targets for future functional validation to definitively elucidate their roles in the fiber-type switching process.

These results suggest a “slow-to-fast” fiber-type transition during skeletal muscle development, accompanied by the conversion of a subset of type I into I/II hybrid states, which may serve as an important mechanism for maintaining slow-twitch fiber function postnatally. Pseudotime analysis positioned the *LEF1*+ (I) subtype as an intermediate state. We hypothesize that it may represent a transitional population during the slow-to-fast myofiber switching process. It is important to note that their biological mechanism remain to be fully elucidated. The functional validation of these candidates through in vitro models, such as gain- or loss-of-function studies in myogenic cell lines, represents an indispensable next step. Such experiments would be crucial to definitively confirm their necessity and sufficiency in driving the transcriptional programs underlying fiber-type transitions and to map their position within the broader regulatory network.

Furthermore, cross-species comparative analysis revealed that birds and mammals share similar patterns in the development and transition of fast- and slow-twitch myofibers. The expression dynamics of *MYH3* in duck PM closely mirrors the conserved embryonic pattern observed in mice, demonstrating transient activation followed by downregulation⁶⁸. Regulatory network analysis suggests this conserved program involves both canonical myogenic factors⁶⁹ (e.g., *MYOD1*, *NFAT*, and *MEF2*) and novel candidates (e.g., *PPP2R3B*, *PDLIM5*).

In the analysis of skeletal muscle-resident cell lineages, we found that MSCs and fibroblasts shared highly similar cell markers, consistent with previous findings in mammals that fibroblasts represent a more differentiated state of MSCs. Notably, the high expression of ribosomal genes in avian and mammalian MSCs supports the synthesis of specific differentiation-related proteins, which may be a key mechanism for cell fate determination and functional specialization during skeletal muscle development. Additionally, we observed that avian skeletal muscle-resident cell types were more similar to those in chickens, although chickens lacked an epithelial cell subpopulation, suggesting unique features of the avian skeletal muscle microenvironment. Epithelial and immune cells, as highly pluripotent cell populations, play crucial roles in maintaining skeletal muscle microenvironment homeostasis. Epithelial cells contribute to somite formation, laying the foundation for the development of skeletal muscle, cartilage, and dermis, while immune cells maintain tissue microenvironment stability through phagocytosis, supporting normal muscle cell differentiation and tissue construction. This cross-species analysis should be interpreted with the understanding that differences in muscle anatomy, developmental timing, and sequencing technology represent inherent limitations. Our objective was not to ignore these issues, but to identify conserved transcriptional programs that are enough to be defined despite these technical and biological variations.

Our results show that PM is mainly glycolytic (type II fibers) during embryogenesis and early post-hatch stages, aligning with established findings in poultry muscle biology^{70,71}. However, the postnatal development of dark meat color involves a substantial upregulation of myoglobin, which coincides with increased aerobic activity after hatching⁷². While this study details embryonic muscle heterogeneity, future work should extend single-cell transcriptomics to adult stages and across species to fully understand muscle development and meat color formation. Additionally, expanding single-cell transcriptomic datasets across multiple species and developmental stages would provide deeper insights into cross-species evolutionary analyses.

Methods

Ethics statement

The experiments were approved by the Institutional Animal Care and Use Committee of China Agricultural University (Permit number: SYXK 2007-0023). All experiments were performed in accordance with the relevant guidelines and regulations. Upon hatching (D1/E28), ducklings were

immediately transferred to a controlled, biosecure rearing facility. Standard conditions included: Maintained at 30–32 °C using brooder lamps, with free movement to warmer or cooler zones. Provided ad libitum access to a commercial starter diet formulated for waterfowl and clean drinking water. Water was provided in shallow containers to prevent drowning risk while allowing natural drinking behavior. The euthanasia method was selected to ensure rapid and painless death, strictly following the recommendations for poultry in the AVMA Guidelines (2020). Ducklings were first placed in a sealed induction chamber and anesthetized using a rising concentration of isoflurane (5%) delivered in oxygen until loss of consciousness was confirmed by the absence of reflex responses (e.g., toe pinch, corneal reflex). Following deep anesthesia, a physical method (decapitation) was performed promptly by trained personnel to ensure death. This two-step method is considered acceptable and humane for neonates and poultry by the AVMA.

Embryo tissue collection

Fertilized Pekin duck eggs for these experiments were provided by Beijing Nankou Duck Breeding Technology Ltd. (Beijing, China). Fertilized Pekin duck eggs were incubated at 37.5 °C and 55% humidity, with the eggs turned every 2 h. Three hundred fertilized eggs were used in this study, and the first 150 eggs were hatched to determine the approximate time range for the desired developmental stages. The embryos were observed every hour for the first 72 h of incubation, then every 12 h from day 5 to 9, and then every 24 h for day 10 to hatching. The next 100 eggs were then used to identify via H&E staining to classify the PM (M) development stage as early, mid and late phase, that ten developmental time points representing distinct embryogenic stages: (1) Early phase: E3, E5, E7, E9; (2) Intermediate phase: E12, E15, E18; (3) Late phase: E21, E24, E28 (1 day-post-hatching). At least 5 biological replicate samples of PM for each stage were dissected, starting from E7 to E28, and embryos were collected in E3 and E5 for scRNA-seq.

Morphological analysis of the pectoral muscle

The fresh PM samples from 10 embryo stages were fixed in 10% neutral buffered formalin at room temperature for 24–48 h (tissue volume ≤10% of fixative). Fixed tissues were dehydrated through a graded ethanol series (70%, 80%, 95%, and 100% ×2), cleared in xylene (2 changes, 1 h each), and infiltrated with molten paraffin wax (58–60 °C, 2 changes, 2 h each) using an automated tissue processor (Leica TP1020). Tissues were embedded in paraffin blocks with optimal orientation using metal molds. Blocks were trimmed and sectioned at 4–5 µm thickness using a rotary microtome (Leica RM2235). Sections were floated on a 40 °C water bath to remove wrinkles, mounted onto charged slides, and dried at 60 °C for 1 h. Deparaffinization was performed in xylene (2 × 5 min), followed by rehydration in graded ethanol (100% → 70%). Sections were stained with Mayer's hematoxylin (5 min), rinsed in tap water, counterstained with eosin (30 s), dehydrated, cleared in xylene, and cover slipped with DPX mounting medium. All sections were photographed using a digital microscope (Nikon).

Preparation of a single-cell suspension

The whole embryo and muscle tissues from the early embryonic stage were carefully minced with surgical scissors, then transferred to 5 mL conical tubes. The bio sample was digested with protease (0.17%, Sigma-Aldrich, Louis, MO, USA) for 45 min and collagenase-type II (0.1%, Sigma-Aldrich) for 45 min at 37 °C in a thermostatic shaker (90 r min⁻¹) and filtered with 70 µm and 40 µm nylon cell strainer. Single-cell suspensions were subjected to exclude debris and dead cells, as well as ambient RNA. The quality of the single-cell suspension was detected by AO/PI fluorescence staining. The single-cell suspension was used to construct a single-cell library after the following conditions: cell activity > 90%, cell agglomeration rate < 5%, and total number of cells > 10⁵. The 10x Genomics Cell Preparation Guide describes best practices and general protocols for washing, counting, and concentrating cells from both abundant and limited cell suspensions (greater than or less than 10,000 total cells, respectively) in preparation for use in 10x Genomics Single Cell Protocols⁷³.

Preparation of single-nucleus suspension

The frozen muscle tissue samples from mid and later embryonic stages were thawed, minced, and homogenized in a 2 mL Dounce homogenizer (Sigma-Aldrich, D8938) with 15 strokes (loose pestle) followed by 15 strokes (tight pestle) in 1 mL nuclei lysis buffer containing 250 mM sucrose, 4 mM MgCl₂, 1% RNase inhibitor, and 0.1% NP40 in nuclease-free water. Homogenates were filtered through 40-µm cell strainers and centrifuged at 500 × g for 5 min at 4 °C, and the supernatant was discarded. Pellets were resuspended in nuclei suspension buffer (PBS without Ca²⁺/Mg²⁺, 1% BSA, 0.2 U/µL RNase inhibitor). This was followed by centrifugation at 500 × g for 5 min at 4 °C to pellet the nuclei. After centrifugation, the nuclei were then washed with 1x PBS supplemented with 0.5% BSA, and a final concentration of 1000 nuclei per µL was used for capture and library generation.

sc/snRNA library and sequencing

The suspension was loaded into Chromium microfluidic chips with 3' (v3) chemistry and barcoded with a 10x Chromium Controller (10X Genomics). RNA from the barcoded cells was subsequently reverse-transcribed and sequencing libraries constructed with reagents from a Chromium Single Cell 3' v3 reagent kit (10X Genomics) according to the manufacturer's instructions. Sequencing was performed with Illumina (NovaSeq X Plus) according to the manufacturer's instructions (Illumina).

Raw data processing and quality control

The raw sequencing data of each sample were processed and aligned to the CAU_mallard (2.0) reference genome using the Cell Ranger pipeline (release: 6.1.2)⁷⁴. The pipeline generated a raw unique molecular identifier (UMI) count matrix for each sample, which records the number of UMIs per gene associated with each cell barcode. A total of 116,712 cells were captured. Cells with an abnormally high number of UMIs (≥500) or mitochondrial gene percent (≤10%) were filtered. In addition, artificial doublets were generated using the DoubletFinder tool (v2.0.3), and the proportion of artificial k-nearest neighbors (pANN) for each cell was determined using the principal component distance (PC distance) and filtered doublet GEMs based on the expected number of doublets. After cell quality control, a total of 76,916 high-quality cells were retained for further analysis.

Batch effect correction across duck embryonic stages

Single-cell batch correction was performed using the fastMNN algorithm through the SeuratWrappers (v.0.3.0). Briefly, log-normalized counts were input to "RunFastMNN" with default parameters to identify mutual nearest neighbors (MNNs) across batches. Corrected low-dimensional embeddings were returned and integrated for downstream analysis.

Identification and annotation of clusters

After quality control, the scaled data were generated using Seurat for cell normalization and regression based on the expression matrix⁷⁵. "Log-Normalize" and "FindVariableFeatures" were applied to normalize and find variable features within the single-cell gene expression data. The "RunPCA" function in the Seurat package to perform the principal component analysis (PCA) on the single-cell expression matrix with genes restricted to highly variable genes was then employed. Based on the PCElbowPlot, a certain number of principal components (PCs) for the clustering analysis, when that number reached the baseline of the standard deviation of PC, was picked. We then cluster the cells using the "FindClusters" function, and cell clusters were visualized using UMAP plots. To identify the DEGs of a cluster relative to all other clusters, the "FindAllMarkers" function in Seurat was used. The cell populations were annotated based on the expression pattern of DEGs and the well-known cellular markers from the previous studies or the CellMarker2.0 website (<http://117.50.127.228/CellMarker/>)^{59,76}. Parameters provided for this function were set as follows: differential expression threshold of 0.25 log fold change using the Wilcoxon rank sum test with $P < 0.05$ following Bonferroni correction.

Functional annotation

We identified homologous genes of duck using the biomaRt package, and analyzed the gene functional enrichment in the Metascape online tool with default parameters⁷⁷.

Trajectory analysis and CytoTRACE

To construct the differentiation trajectory of myogenesis, nine myogenic cell clusters (MPs, MB, and myofiber cluster) were selected for pseudotime analysis, which was performed with Monocle2 (v2.22.0)⁷⁸. DEGs were identified with the “differentialGeneTest” function. The top 950 genes with the lowest *q* value were used to construct the pseudotime trajectory. Then the cell differentiation trajectory was inferred after dimension reduction and cell ordering with the default parameters, except method was specified = “DDRTree” in the reduceDimension function. The root state was set according to Seurat’s identified cell cluster label, and the “BEAM” function was used to calculate branch-specific expressed genes. We used Monocle2’s implemented “plot_genes_branched_heatmap” function to plot a branch-specific expression heatmap. Each cell was assigned a pseudotime value on the basis of its position along the trajectory. Marker expression changes along pseudotime were generated by the “plot_genes_in_pseudotime” function in Monocle2. *MYMK*+(I), *NEBL*+(I), *LEF1*+(I), *MSTN*+(IIa), *CSRP3*+(IIa), *MUSTN*+(IIx), and other specialized subclusters (NMJ) were also done using Monocle2. DEGs across different development conditions were identified with the “differentialGeneTest” function. The top 1000 genes with the lowest *q* value were used to construct the pseudotime trajectory. Then, the cell differentiation trajectory was inferred after dimension reduction and cell ordering with the default parameters. The remaining steps were identical to those used in the pseudotemporal ordering of myogenic cells.

The developmental and differentiation trajectories of all muscle-resident cells were analyzed using Monocle3 (v1.3.1)⁷⁹. The analysis integrated the results of cell clustering and identification during dimensionality reduction. A trajectory graph was then generated by applying the “learn_graph” function. Following the manual specification of the root node based on prior knowledge, pseudotime was computed for each cell along the trajectory.

After the trajectory construction, the cells in each state were mapped back onto the original object. CytoTRACE was used to predict the differentiation potential of the cells⁸⁰. Cells with higher CytoTRACE scores represented higher stemness (less differentiation) within the given dataset, and vice versa.

Ligand-receptor cell communication

CellChat (v1.6.1) in predicting ligand-receptor interaction and cell-cell communication networks from single-cell transcriptome data was employed. From the CellChat results, cell-cell communication in different pathways was obtained⁸¹.

Single-cell regulatory network inference and clustering

We used pySCENIC (v 0.11.2) to infer the regulatory network and regulatory activity of target cells⁸². The ranking databases and the Motif-to-TF annotation database for human and mouse were downloaded from the cisTarget databases (<https://resources.aertslab.org/cistarget/>). The count matrices were used as input to identify co-expression modules by the GRNBoost2 algorithm. And pySCENIC finds enriched motifs for a gene signature and optionally prunes targets from this signature based on cisTarget databases. Using the AUCell function, the functional activity score of the regulon is measured as the area under the recovery curve (AUC). Then, the binary regulon activity score was obtained using the binarize function. Finally, 87 regulons in ducks were identified, respectively.

FISH assay procedure for RNA detection

RNA amplification-based single molecule in situ hybridization (asmFISH) assays for markers of MF populations (*TNNI1*, *TNNI2*) and marker *LEF1* was performed on FFPE sections from E12, E18 and E21 in embryo stage⁸³,

which hereby was performed using SEERNA® FISH RNA Fluorescence in Situ Hybridization Kit (Dynamic Biosystems, Suzhou, China) under the guidance of standard protocol of the manual. The label probe was conjugated to Alexa Fluor 488, Cy3, Texas Red, Cy5, or Alexa Fluor 750 (Life Technologies, Thermo Fisher Scientific, Shanghai, China).

The sections in 5-μm thickness were baked in an oven at 60 °C for 30–60 min, followed by deparaffinization in xylene for 15 and 10 min, respectively. Then the slides were rehydrated in two cylinders of 100% absolute ethanol, each for 2 min. Next, the slides were submerged in diethyl pyrocarbonate-treated H₂O (DEPC-H₂O) for 2 min. The tissue sections were then fixed by 4% (w/v) paraformaldehyde in DEPC-PBS for 10 min and washed three times with Wash Buffer. The sections were then permeabilized in 0.1 M HCl containing 0.3 mg/mL pepsin (Sigma, Shanghai, China) in a 42 °C incubator for 30–60 min, and then washed three times with Wash Buffer. The sections were then incubated in order with the following solutions: target probes in hybridization mix at 42 °C for 4 h, ligation mix at 42 °C for 30 min, splint primers in the circularization mix at 42 °C for 30 min, amplification mix for rolling circle amplification at 42 °C for 2 h. Slides were washed with Wash Buffer (Dynamic Biosystems, Suzhou, China) three times after each step. After that, the sections were incubated in hybridization buffer containing the label probes in a 42 °C incubator for 30 min. Finally, the slides were mounted with SlowFade Gold Antifade Mountant (Thermo Fisher Scientific, Shanghai, China) medium containing 0.5 μg/mL DAPI (Sigma, Shanghai, China), which were then ready for image acquisition using the 3D-Hitech Pannoramic®MIDI II equipped with a pco.edge 4.2bi camera using the 40X or 20X objective lens.

Digital image acquisition and processing

The images were then analyzed with the open-source image analysis software Fiji (ImageJ, 1.54g) (<https://imagej.net/ij/>). All images were exported as a single channel. tif files subjected to ImageJ analysis. For three-channel images, the Bio-Formats plugin was used to split individual fluorescence channels (Image > Color > Split Channels). Background subtraction was performed using a rolling ball algorithm (Process > Subtract Background) with a radius of 50–100 pixels to correct for uneven illumination. Images were converted to 8-bit grayscale (Image > Type > 8-bit) and thresholded using the MaxEntropy or Li algorithm (Image > Adjust > Threshold). Parameters, including spot count, area, and integrated fluorescence intensity, were recorded. For each FFPE tissue section, five representative windows were randomly selected and quantified. The area of DAPI, *TNNI1*, *TNNI2*, and *LEF1* staining was calculated by normalizing the positive-signal area to the total imaged area in Fiji.

Statistics and reproducibility

No statistical methods were used to predetermine sample size. Sample sizes were chosen based on standard practices in the field and our previous experimental experience. The number of biological replicates (*n*) for each experiment is stated in the respective figure legend. Here, *n* refers to independent biological samples. Data are presented as mean ± SEM unless otherwise noted. Statistical analyses were performed using GraphPad Prism v1.0. Normality was assessed using the Shapiro-Wilk test. For comparisons between two groups, an unpaired two-tailed Student’s *t*-test was used for normally distributed data. A *p* value of less than 0.05 was considered statistically significant. All experiments were successfully repeated at least 3 times with consistent results.

Comparison of cell types among ducks, chickens, mice, and pigs

To compare developing embryonic muscle across species, publicly available scRNA-seq datasets from pig, chicken, and mouse embryonic muscle samples were also downloaded. The pig skeletal muscle dataset was derived from Longissimus Dorsi muscle (E16, E18, E21, E28, D1), the chicken skeletal muscle dataset was derived from leg muscle (E10, E14, E18) and breast muscle (D5), whereas the mouse muscle data were obtained from embryonic limb (E9.5, E10.5, E11.5, E12.5, E13.5) and Soleus muscles (D10). To project the duck single-cell data onto the chicken, mouse, and pig datasets,

expression matrices were exported from Seurat. The homologous genes of human, mouse, and pig were obtained by the Ensemble BioMart release 115, and genes with gene names were only retained. It identified 11467 conserved homologous genes, and all genes that were not present in all four species were excluded from the matrices. For comparisons of cell transcriptomes, duck, chicken, and pig datasets were integrated using the Harmony function⁸⁴. For identifying differentially expressed and conserved gene expression, three individual objects were made from three species: duck-chicken, duck-pig, and chicken-pig. The “FindMarkers” function was used to find conserved and divergent cell type-specific markers in a pairwise fashion. Then the marker genes identified in previous research were used to define cell types. The integrated dataset was embedded and visualized using UMAP and labeled according to annotations in the separately processed datasets.

To quantify myosin heavy chain genes (*MYH3*, *MYH7*, *MYH2*) expression across duck embryonic developmental stages, we analyzed single-cell RNA sequencing data using the following approach. The expression matrix was transformed to a long format containing gene expression values for all cells. Statistical summaries were then calculated, grouped by developmental stage (10 phases). For each stage, we computed: (1) mean expression level, (2) percentage of cells with detectable myosin heavy chain gene expression (expression >0), and (3) total cell counts per stage. These calculations were derived from the myogenic cell populations within each defined developmental stage to characterize myosin heavy chain gene expression dynamics during development. Regulatory transcription factors (TFs) for *MYH3*, *MYH7*, and *MYH2* were predicted using the GENIE3 package (v1.20.0) in R.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

The additional data files and scRNA-seq sample matrix have been deposited at Figshare (<https://doi.org/10.6084/m9.figshare.29069636.v1>). The scRNA-seq data for the duck skeletal muscle have been deposited at the GSA database with accession number CRA037446. The chicken leg muscle scRNA-seq dataset is available at GEO, under accession code GSE251682⁵⁰. The pig Longissimus Dorsi muscle scRNA-seq dataset is available at GEO, under accession code GSE206914 and GSE247753^{26,51}. The mouse limb muscle sci-RNA-seq3 and snRNA-seq dataset is available at GEO, under accession codes GSE119945 and GSE147127^{16,52}. All other data are available from the corresponding author (or other sources, as applicable) on reasonable request. The numerical data underlying all graphs and charts can be found in Supplementary Data 1–14.

Code availability

All packages and software used in this study were obtained from publicly available sources, as detailed in the “Methods” section. No custom code was developed.

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Competing interests

The authors declare no competing interests.

Additional information

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