

Single-nucleus developmental atlas of the sheep adipose-skeletal muscle system reveals stem cell differentiation programs driven by core regulatory genes

Wannian Wang^{1,2}, Zhixu Pang¹, Sijia Chen¹, Ke Cai¹, Haiyang Wang⁴, Ruizhen Wang⁵, Yangyang Pan¹, Kaijie Yang¹, Liying Qiao¹, Jianhua Liu¹, and Wenzhong Liu^{1,2,3,*}

¹College of Animal Science, Shanxi Agricultural University, Jinzhong 030801, Shanxi, China

²Shanxi Key Laboratory of Livestock Genetic Resources Exploration and Biotech Breeding, Jinzhong 030801, Shanxi, China

³College of Modern Biological Breeding, Shanxi Agricultural University, Jinzhong 030801, Shanxi, China

⁴Medical School, Nanjing University, Nanjing 210093, Jiangsu, China

⁵Academy of Chinese Medical Sciences, Henan University of Chinese Medicine, Zhengzhou, 450046, Henan, China

*Corresponding author at: Department of Animal Genetics, Breeding and Reproduction, College of Animal Science, Shanxi Agricultural University, Taigu 030801, China

E-mail address: Wenzhong Liu, tglwzyc@163.com

ABSTRACT

Introduction: Adipose and skeletal muscle are interdependent for metabolism homeostasis, with their stem cells maintaining self-renewal and tissue formation through precise fate determination. However, the cellular developmental heterogeneity and stem cell differentiation regulation mechanisms of the sheep adipose-skeletal muscle system remain unclear.

Methods: Single-nucleus RNA sequencing was performed on 24 samples (four tissues from six female Hu sheep, three at 1 month and three at 5 months). Average depth >10 000 reads/cell, with median genes 755–2 122 per cell. Core regulatory genes were identified using combined machine learning and virtual knockout.

Results: This study constructed a single-nucleus developmental atlas of the sheep adipose-skeletal muscle system (155 012 cells, 19 467 features). Based on ensemble machine learning, we identified core regulatory genes (Subcutaneous adipose: *COL1A1*, *FBN1*, *MFAP5*; Perirenal adipose: *FABP4*, *ADIPOQ*, *PLIN1*; Biceps femoris muscle: *ACTA1*, *MYH1*, *TTN*; Longissimus dorsi muscle: *ACACB*, *AUTS2*, *FOXP1*) during the differentiation process of each tissue. Combining trajectory inference and virtual knockout, we uncovered key perturbation nodes and downstream effector pathways in the differentiation process driven by these regulatory genes. Furthermore, this study developed the SheepCellMap (<https://sheepcellmap.lwzlab.com/>) platform with marker search and visualization to support data for subsequent breeding practices.

Conclusion: This study creatively explains cross-tissue developmental logic of the adipose-skeletal muscle system at single-nucleus resolution and reveals stem cell differentiation programs driven by core regulatory genes. It provides single-cell portal and targets for adipose deposition and myofiber maturation.

Keywords: Single nucleus; Adipose; Skeletal muscle; Stem cell differentiation; Sheep

INTRODUCTION

Adipose tissue and skeletal muscle, as key organs in mammals, form an interdependent functional system in energy metabolism balance(Hargreaves & Spriet, 2020). Adipose tissue, as an energy storage site, regulates the systemic metabolic environment through various endocrine factors, and its deposition pattern directly affects the proportion of body fat(Scheja & Heeren, 2019). Skeletal muscle, as the main protein reserve, dominates basal metabolism and exercise capacity, and the composition of its muscle fibers drives the efficiency of material deposition and meat production(Smith et al., 2023). During development, both tissues interact across tissues through gene regulatory networks (*PPAR γ* and *FABP4* guide lipid accumulation and adipogenesis(Wang et al., 2022b), *MYOD1* and *MYOG* regulate myogenesis and myofibril maturation(Li et al., 2022), signaling pathways (TGF- β signaling is involved in regulating the lineage transition of lipids(Gao et al., 2024), Wnt signaling inhibits adipogenesis(Wang et al., 2024a) and intercellular communication(Schwalie et al., 2018), thereby jointly determining body composition shaping and growth efficiency. In sheep (*Ovis aries*), an important ruminant animal, differences in developmental patterns and regulation directly determine feed utilization efficiency, carcass quality, and the achievement of breeding goals(Chen et al., 2024). Compared to traditional model animals such as mice and pigs, sheep possess a unique adipose-muscle interaction pattern and energy metabolism regulation system, and their tissue development regulation mechanisms differ from those of monogastric animals(Louveau et al., 2016). Among them, the Hu sheep, as an excellent meat sheep breed, has the genetic advantages of fast growth, tender meat and moderate intramuscular adipose content, making it a unique model for studying stem cell differentiation and body composition regulation in ruminants. As a central driving force of development, stem cells maintain self-renewal while driving tissue formation and remodeling through precise fate determination(Ito & Ito, 2016). Adipose-derived stem cells are the starting point for fat cell generation, while satellite cells are the fundamental source of muscle fiber regeneration and muscle homeostasis. Stem cell fate is

constrained by multiple factors, including supporting cells, the local metabolic environment, extracellular matrix remodeling, and immune regulation(Sousa-Victor et al., 2022). Supporting cells maintain stem cell activity through growth factors (FGF2 maintains the proliferation and self-renewal of preadipocytes(Li et al., 2021), promotes the proliferation of myogenic stem cells(Mathes et al., 2021); VEGF supports the maintenance of adipocyte stemness by promoting microvascular homeostasis(Cox et al., 2021) and nutrient signals (amino acid-mTOR promotes muscle growth(Mao et al., 2025), glucose - AMPK regulates energy demand(Trefts & Shaw, 2021). ECM and metabolic status affect differentiation process(Dorst et al., 2023), while immune signals participate in cell function specialization and tissue maturation process(Chan et al., 2023).

With the development of single-cell RNA sequencing (scRNA-seq) and single-nucleus RNA sequencing (snRNA-seq) technology, the cell atlas of mammalian adipose and skeletal muscle tissues has been continuously improved(Han et al., 2025). White adipose tissue atlases of humans and mice show that high-fat diets and other metabolic stressors lead to significant changes in adipose morphology, physiology and cellular composition(Emont et al., 2022; Rosen & Spiegelman, 2014); specific cell types have been related to metabolic diseases, and preadipocyte subtypes enriched in patients with type 2 diabetes have been identified(Kahn et al., 2006). The multimodal cell atlas of skeletal muscle in human aging revealed that the ability of muscle stem cells to transition from a quiescent state to an activated state for tissue repair upon injury decreases significantly with age, and confirmed the increase in myofiber and signs of denervation in aged muscles(Lai et al., 2024). In addition, in livestock, scRNA-seq has been used to reveal the heterogeneity of early skin development in pigs(Wang et al., 2024b) and lipid dynamics in a model of skeletal muscle regeneration in pigs damaged by glycerol(Sun et al., 2024). However, research on cross-tissue integration in sheep is very limited, and to date, the cellular developmental heterogeneity of their adipose-skeletal muscle system and the regulatory mechanisms of stem cell differentiation remain unclear.

Therefore, this study aims to construct a single-nucleus framework of the sheep adipose-skeletal muscle system to identify molecular targets and signaling pathways that drive stem cell differentiation. Based on the unique high adipose deposition, excellent meat quality and stable genetic characteristics of Hu sheep, this study constructed a single-nucleus developmental atlas across tissues. We elucidated the cellular composition and functional heterogeneity of the adipose-skeletal muscle system, reconstructed the developmental lineage of each tissue, and identified multi-level shared core regulatory genes based on ensemble machine learning. Combining trajectory inference and virtual knockout, we clarified the expression dynamics and interactions of core genes in cell fate determination, identifying key nodes and effector pathways in the differentiation process. Based on this, we depicted a cross-tissue conserved developmental communication framework and tissue-specific regulatory patterns, thereby revealing the stem cell differentiation program driven by core regulatory genes in the adipose-muscle system. In addition, this study developed the SheepCellMap online platform, which provides visualized omics resources to support data for subsequent research and breeding practices.

MATERIALS AND METHODS

Animal and sample collection

This study focused on six female Hu sheep (three 1-month-old and three 5-month-old) as research subjects. They were uniformly raised at the animal husbandry station of Shanxi Agricultural University and fed a fully mixed diet during the experiment. The nutritional level met the feeding standards for sheep (metabolic energy 10.5–11.5 MJ/kg, crude protein 14%–16%), and they were free to eat and drink. The temperature of the sheep house environment is controlled at 15–25°C, with a relative humidity of 45%–65%, natural light and ventilation, and consistent feeding and management conditions. All individuals were uniformly sampled at the same time point in summer (1-month-old individuals and 5-month-old individuals were born from different batches, but their ages reached 1 month and 5 months respectively at the time of

sampling). Four tissue types were collected, including subcutaneous adipose tissue (SAT), perirenal adipose tissue (PAT), longissimus dorsi muscle (LD), and biceps femoris muscle (BF), resulting in a total of 24 tissue samples. Immediately after sampling, tissues were washed with pre-cooled phosphate-buffered saline (PBS) (Thermo Fisher, USA) to remove residual blood. They were then rapidly cut into approximately 5 mm³ tissue blocks using sterile surgical scissors and subjected to graded cryopreservation to maximize cell viability and RNA integrity. Meanwhile, retain a portion of the tissue samples for cell culture.

All experimental procedures in this study strictly followed the regulations for the husbandry and use of experimental animals at Shanxi Agricultural University and were approved by the Experimental Animal Ethics Committee of Shanxi Agricultural University. Animal handling and surgical procedures were performed in accordance with the relevant procedures of the College of Animal Science and Veterinary Medicine, Shanxi Agricultural University (Taiyuan, China). All experimental protocols in this study have passed institutional ethical review (Approval No.: SXAU-EAW-2024S.CR.009015380).

Single-nucleus suspension preparation and library construction

Frozen tissues were homogenized using a glass homogenizer in 3 mL of lysis buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.05% (v/v) NP-40 detergent). The samples were then incubated in 5 mL of lysis buffer for 5 minutes for lysis, followed by the addition of 5 mL of wash buffer (10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, 1% BSA, 1 mM MTT, 1 U/μl RNase inhibitor, and nuclease-free water). The samples were filtered through a 40 μm cell strainer and centrifuged at 500×g for 5 minutes. After centrifugation, the nuclear pellet was resuspended in 5–10 mL of wash buffer, gently pipetting up and down 8–10 times to ensure thorough dispersion. The suspension was washed 3 times, and the nuclei were then resuspended in 1 mL of wash buffer and mixed with 25% Optiprep. The mixture was spread on a 29% Optiprep pad and centrifuged at 10 000×g for 30 minutes. Finally, the nuclear pellet was resuspended in wash buffer and washed three more times to ensure purity. The cell (nuclear)

suspension was stained with 0.4% trypan blue, and cell viability was assessed under a microscope. Cells with a viability greater than 80% were used for library construction.

According to the manufacturer's protocol (DNBSEQ), snRNA-seq libraries were prepared using the DNBelabC series library construction kit and sequenced on the TaiM4-V3 platform using a 100bp paired-end sequencing (PE100) strategy. snRNA-seq in this study and the reagents and equipment used were provided by BGI Genomics Co., Ltd. in Shenzhen.

SnRNA-seq data analysis

In this study, the average sequencing depth per cell of all samples was over 10 000 reads. The median number of genes detected per cell ranged from 755 to 2 122, indicating satisfactory transcriptome coverage (**Supplementary Table S1**). The datasets could adequately reflect the gene expression profiles of cells with stable overall data quality.

This study used CellRanger (v.7.2.0) software to process its fastq file and compared it with the sheep reference genome (ARS-UI_Ramb_v3.0) to generate a matrix file for quantitative analysis. Downstream analysis was performed using the Seurat package (v.5.0.3) in R language (v.4.3.3). Cells with gene expression levels between 200 and 6 000 and mitochondrial gene content $\leq 15\%$ were retained, and applied DoubletDetection(v.2.5.2) to identify and remove cell doublets(Vijay et al., 2020). Cells with fewer than 200 detected genes were defined as low-quality cells, while those with more than 6 000 detected genes were regarded as potential cell doublets. A mitochondrial gene proportion above 15% generally indicates cell apoptosis or cellular stress. Combined with doublet removal, these filtering criteria effectively ensure the integrity and reliability of the data set.

This study used the NormalizeData function of Seurat (v.5.0.3)(Satija et al., 2015) for data standardization. Highly variable genes were identified using the FindVariableFeatures function, followed by variable gene identification and scaling. Dimensionality reduction was performed using the RunPCA function based on HVG, and the top 30 principal components were selected for uniform manifold approximation projection (UMAP). Batch effect correction was

performed using Harmony(Korsunsky et al., 2019), and cell clustering analysis was conducted using the FindNeighbors and FindClusters functions to identify cell clusters.

Marker genes for each cluster were identified using Seurat FindAllMarkers function (Wilcoxon rank-sum test) with $|\log_2FC| \geq 1.0$ and corrected $P < 0.01$. Cell clusters were annotated based on marker gene expression. Functional enrichment analysis was performed using clusterProfiler (v.4.4.4)(Yu, 2024). Gene Ontology (GO) entries with a Benjamini-Hochberg corrected $P < 0.05$ were considered significant.

Development-related genes were screened using hdWGCNA and PPI

To identify development-related genes in sheep, this study constructed a high-dimensional weighted gene co-expression network using the "hdWGCNA" package(Morabito et al., 2023). Specifically, a weighted gene co expression network was constructed and modules were tested for stem cell populations from various tissues based on developmental stages (1 month and 5 months of age). Core modules significantly associated with developmental progress were screened through module-trait association analysis. Hub genes in each core module were identified based on intramodal connectivity analysis, and the top 100 genes in the module with the highest correlation were identified as development-related candidate genes.

To further identify the regulatory roles of candidate genes at the protein level, this study used the STRING database(Szklarczyk et al., 2019) to perform protein-protein interaction (PPI) network analysis and viewed the network topology using Cytoscape(v.3.10.2) software. Based on the integrated results of 12 topology analysis algorithms, genes ranking in the top 50 in each algorithm score were identified as development-related genes.

Identification of key subtypes of stem cells and terminal cells

To assess the stemness and differentiation potential of stem cell subtypes in various tissues, this study used CytoTRACE(Gulati et al., 2020) to score stem cells. A higher CytoTRACE score indicates stronger stemness and higher differentiation potential, suggesting that these cells are positioned at the early stage of the differentiation trajectory.

To assess the developmental maturity of adipocyte and muscle cell subtypes, this study used AddModuleScore(Wei et al., 2024) to construct a developmental score based on development-related genes. A higher developmental score indicates that the cell is closer to the terminal differentiation stage.

Multiple machine learning methods are used to screen core regulatory genes.

To robustly screen core regulatory genes that regulate stem cell differentiation, this study systematically used 14 representative machine learning algorithms and constructed 195 model combinations, aiming to maximize coverage of different modeling mechanisms and avoid the inherent biases and limitations of a single algorithm. These algorithms cover a wide range of categories and can be fully adapted to the high-dimensional, high-noise, and nonlinear regulatory data analysis characteristics of single-cell transcriptomes, including multilayer perceptron (MLP), logistic regression (LR), linear discriminant analysis (LDA), quadratic discriminant analysis (QDA), k-nearest neighbors (KNN), decision tree (DT), random forest (RF), XGBoost, ridge regression (RR), elastic network regression (ENR), support vector machine (SVM), gradient boosting machine (GBM), stepwise logistic regression (SLR), and Naive Bayes(Chu et al., 2023; Liu et al., 2025b; Wang et al., 2022a). Among them, linear models (RR, ENR, LR, SLR) excel at high-dimensional feature denoising and linear regulatory gene screening; discriminant analysis (LDA, QDA) can amplify differences in cell differentiation states and screen core genes with typing value; KNN and DT have broad adaptability and can fit nonlinear expression patterns; ensemble learning models (RF, XGBoost, GBM) have strong anti-overfitting ability and can mine weak, high-order gene regulatory signals; MLP is suitable for analyzing complex nonlinear regulatory relationships; and Naive Bayes can mine gene expression patterns based on probability distributions(Dong et al., 2025; Guo et al., 2023; Libbrecht & Noble, 2015).

After completing the unified preprocessing and integration of single-nucleus expression data in the early stage (removing batch effects, standardizing gene expression levels, and

normalizing features), the development-related gene expression data of each cell were extracted from the stem cell subtype with the highest differentiation potential, and the data set was constructed by using the developmental stage as the cell identity label. The training and test sets were split in an 8:2 ratio. Using developmental gene expression data to predict the developmental stage (1 month old or 5 month old) of each cell, each model was trained using 10-fold cross-validation to ensure generalization ability and optimize feature selection. We output the predicted identity labels (developmental stages) of the model in both the training and testing sets. Subsequently, we evaluated the prediction accuracy of the models on the training and test sets and calculated the mean area under the receiver operating characteristic (AUC) for each model. The models were ranked according to their mean AUC values, and the top 100 models were visualized. The combination of models with the highest AUC values was considered the optimal model. To enhance the interpretability of the model and clarify the importance of its features, we used the Shapley Additive Explanations (SHAP) framework (Qi et al., 2025) to quantify the contribution of each gene to the model. The top 3 genes were identified as core regulatory genes by multi-dimensional machine learning algorithms and SHAP values.

Developmental trajectory analysis

To reconstruct the developmental trajectory, this study used Monocle2 (Qiu et al., 2017) to infer the pseudotime sequence, identified developmental nodes using highly variable genes and cluster-specific markers, and used the `plot_genes_in_pseudotime` function to examine the static expression of core regulatory genes during the time sequence. Furthermore, to verify the role of core regulatory genes in differentiation, we calculated their correlation with stem cell subtype marker genes in the most differentiated stem cell subtypes. In terminally differentiated cells, we calculated their correlation with developmental scores.

Virtual knockout analysis

To clarify the perturbation genes and functional effects of core regulatory genes during stem cell differentiation, we performed a virtual knockout analysis using the scTenifoldKnk tool (Osorio et al., 2022). This method simulates the gene knockout process using single-nucleus transcriptome data. Filter the gene expression matrix to exclude features that are prone to noise. Cells with gene numbers between 200-6 000, cells with mitochondrial gene ratios < 15%, and at least 10 genes expressed in cells are retained to ensure data quality.

Based on the wild-type gene regulatory network (WT GRN) constructed using scTenifoldKnk, a virtual knockout gene regulatory network (KO GRN) was constructed for each target gene. Differentially expressed genes (DRGs) under WT and KO conditions were identified using manifold alignment, representing perturbations of core regulatory genes. DRGs with a significance level reaching the threshold (FDR-corrected $P < 0.05$) underwent functional enrichment analysis using DAVID.

In addition, to identify core perturbation genes, we performed PPI analysis on common perturbation genes of the three core regulatory genes using the STRING database and matched them with common perturbation pathways.

Cell-cell communication analysis

To clarify the differences in intercellular signaling across different tissues, this study used the CellChat (Jin et al., 2021) package (v.2.1.2) to analyze the communication patterns of cells within tissues and the interactions between cell subtypes of major cell types. The createCellChat function was used to construct objects, the computeCommunProb function was used to infer communication probabilities, and the filterCommunication function was used for filtering with the parameter set to "min.cells=10". Finally, the aggregateNet function was used to integrate the interaction networks.

Cell isolation, culture, and passaging

The four types of tissue samples were separately minced and then digested with dissociation solution in a water bath at 37°C with shaking. After digestion, complete medium was added to

terminate the reaction. The mixture was filtered through a cell strainer to remove undigested tissue fragments, and the filtrate was collected and centrifuged. The supernatant was discarded, and the cell pellet was resuspended to obtain primary cells. The isolated primary cells were seeded into culture dishes and maintained in a humidified incubator at 37 °C with 5% CO₂ (Thermo Fisher, USA) in DMEM medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (dual antibiotics) (Thermo Fisher, USA). The medium was replaced every 2 days, during which non-adherent cells and debris were removed. When the adherent cells reached 80%–90% confluence, they were passaged. Precursor cells from each tissue type with good growth status were then seeded into 6-well plates (Corning, USA) and cultured in the same incubator at 37 °C with 5% CO₂.

siRNA transfection and interference efficiency validation

When the cells in the 6-well plates reached 60%–70% confluence, the medium was replaced with DMEM complete medium without dual antibiotics for a 24 hours pretreatment. Three specific siRNA sequences targeting each core regulatory gene were designed (**Supplementary Table S2**), and a negative control (NC) was also included. All siRNAs were synthesized by Sangon Biotech (Shanghai) Co., Ltd. When the cell confluence reached approximately 80%, transfection was performed using the KeygenMAX·3000 (KeyGEN Biotech Co., Ltd, Jiangsu) transfection reagent. Briefly, siRNA (at a final concentration of 40 nM) and KeygenMAX·3000 were each diluted separately with Opti-MEM reduced-serum medium (Gibco, USA), incubated at room temperature for 5 min, then gently mixed and further incubated for 10 min to allow formation of transfection complexes, which were subsequently added to the cell culture. At 6 h post-transfection, the medium was replaced with fresh complete medium without antibiotics, and cells were cultured until 48 h after transfection. Cells from each group were then collected for total RNA extraction, and the expression level of the core regulatory gene was examined by real-time quantitative PCR (qPCR) to verify the interference efficiency.

Directed differentiation induction

Cells that showed satisfactory interference efficiency were, immediately after transfection, deprived of the complete medium and replaced with the corresponding differentiation induction medium to initiate adipogenic or myogenic differentiation. During the induction period, the differentiation medium was refreshed every 2 days, and the culture was continued until day 4 of differentiation. After differentiation, cells were harvested for total RNA extraction, and the expression changes of downstream target genes were examined by qPCR to evaluate the effects of core regulatory gene interference on these downstream targets.

Total RNA extraction, reverse transcription, and qPCR

Total RNA was extracted from cells before and after differentiation using RNAiso Plus reagent (9108, TaKaRa, China). The extracted RNA was then used for reverse transcription reactions, and cDNA synthesis was performed with the PrimeScript™ RT reagent Kit (Perfect Real Time) (RR037, TaKaRa, China) following the manufacturer's instructions.

Real-time quantitative PCR amplification was carried out using TB Green® Premix Ex Taq™ (Tli RNaseH Plus) reagents. The specific reaction mixtures and amplification protocols were strictly implemented according to the reagent manuals. The design, execution, and reporting of the qPCR experiments adhered to the MIQE guidelines to enhance the standardization and reproducibility of the experimental data. All primers used in this study were synthesized by Sangon Biotech (Shanghai) Co., Ltd., and the primer sequences for each gene are listed in **Supplementary Table S3**.

After qPCR detection, the Ct values of each sample were recorded. GAPDH was used as the internal reference gene to normalize the expression levels of target genes. The relative expression levels of target genes were calculated using the $2^{-\Delta\Delta Ct}$ method, which is suitable for comparing relative expression differences between experimental and control groups.

All experiments were performed in triplicate, and results are presented as mean±standard error of the mean (SEM). Data were collated and statistically analyzed using Microsoft Excel (v.16.83, Microsoft, USA). Differences between groups were compared using unpaired

Student's t-test, and $P < 0.05$ was considered statistically significant (* Indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$).

Building the SheepCellMap portal

SheepCellMap runs on Linux-based Apache (<https://httpd.apache.org>), using a Java web server (<https://www.java.com>) as the backend; the web interface is built using Vue (<https://vuejs.org>), HTML5, CSS, and JavaScript; and MySQL (<http://www.mysql.org>) is used as the relational database system for information storage. All code is developed using IntelliJ IDEA (<https://www.jetbrains.com>). SheepCellMap is hosted on Huawei Cloud's Elastic Cloud Server (ECS) (<https://www.huaweicloud.com>), providing stable network services. The website has been tested to ensure compatibility with various operating systems and web browsers, including Google Chrome, Firefox, and Microsoft Edge.

RESULT

Workflow of this study

Figure 1 shows the workflow of this study, which is divided into three parts: single nucleus atlas, differentiation lineage, and regulatory mechanism. To construct a single-nucleus developmental atlas of the sheep adipose-skeletal muscle system, we performed single-nucleus sequencing on four tissues (SAT, PAT, LD, and BF) from sheep at two developmental stages (1 month and 5 months of age). After rigorous quality control, we obtained a high-resolution dataset containing 155 012 high-quality single nucleus. The main cell types identified across tissues include adipocyte progenitors and stem cells(ASPC), adipocytes(ADC), muscle stem cells(MuSC), myofibers(Myof), and we further classified and characterized their subtypes. In addition, the data can be accessed through the SheepCellMap online portal we developed. To identify development-related genes in sheep development, this study used hdWGCNA to screen core modules significantly associated with the developmental process, selected the top 100 genes from the module with the highest correlation as candidate genes, and further identified development-related genes through the PPI network combined with 12 topology

algorithms, taking the genes ranked in the top 50 by all algorithms. On this basis, to assess the stemness and differentiation potential of stem cell subpopulations in each tissue, we used CytoTRACE to score the differentiation potential of ASPC and MuSC subtypes, while also using AddModuleScore to build a development score to evaluate the differentiation level of ADC and Myof subtypes. Then, to identify the core regulatory genes controlling ASPC and MuSC differentiation, this study combined various machine learning algorithms to confirm development-related genes. After 10-fold cross-validation, the best model was selected based on the average AUC, and the SHAP framework was used to quantify gene contributions, with the top 3 genes ranked as the core regulatory genes. At the same time, we reconstruct the developmental trajectories of different cell types, identify developmental nodes, analyze the expression dynamics of core regulatory genes along the pseudotime axis, and assess how they correlate with stem cell subtype marker genes and the developmental scores of terminally differentiated cells. Finally, to clarify the disruptive effects of core regulatory genes in stem cell differentiation, we used scTenifoldKnn for virtual knockout analysis to identify the perturbed genes and corresponding pathway changes.

This study performed PPI on the shared disturbance genes of core regulatory genes to screen the core disturbance genes, and mapped the shared disturbance genes to the corresponding pathways to identify the core disturbance pathways. At the same time, by analyzing the cellchat communication patterns of key cell types, identify the signaling pathways and receptor-ligand pairs that play a crucial role in various tissues. Ultimately, we revealed a stem cell differentiation regulatory program that is driven by core regulatory genes and mediated by core perturbed genes and their associated pathways.

Single-nucleus developmental atlas of the sheep adipose-skeletal muscle system

We generated a adipose–skeletal muscle system single-nucleus developmental atlas and comprehensively annotated all cell clusters using a combination of canonical cell type-specific markers and Gene Ontology (GO) functional enrichment results.

In the adipose tissue atlas study, a total of 69 112 single nucleus were obtained, including SAT (12 776) and PAT (24 890) cells from 1-month-old sheep, and SAT (11 594) and PAT(19 852) cells from 5-month-old sheep. Nine major cell types were represented, including ADC (36 090 cells, *ADIG*(Li et al., 2024), *G0S2*(Zhang et al., 2017), *GPAT4*(Cooper et al., 2015), *PLINI*(Wang et al., 2024c), ASPC (12 960 cells, *PDGFRA*(Angueira et al., 2021), *CD34*(Berry & Rodeheffer, 2013), *THY1*(Farup et al., 2021), *AEBPI*(Sauer et al., 2022), endothelial cells (6 068 cells, *FLT1*(Volpe et al., 2023), *KDR*(Van Craenenbroeck et al., 2013), *VWF*(Wong et al., 2023), *JAM2*(Luscinskas et al., 2002), lymphatic endothelial cells (1 532 cells, *RELN*(Liu et al., 2020), *PROX1*(Kazenwadel et al., 2023), *FLT4*(Kuonqui et al., 2023), *CCL21*(Collado-Diaz et al., 2026), lymphocytes (1 134 cells, *PTPRC*(Michalik et al., 2024), *CD247*(Rudemiller et al., 2014), *ITK*(Wang et al., 2015), *IL7R*(Oliveira et al., 2022), macrophages (5 281 cells, *CSF1R*(Lv et al., 2024), *CIQA*(Purves-Tyson et al., 2020), *CD86*(Ibrahim et al., 2025), *CIQB*(Yan et al., 2024), mesenchymal stromal cells (897 cells, *LAMA2*(Zhu et al., 2020), *TNXB*(Alcaraz et al., 2014), *FBNI*(Martín-López et al., 2021), *DCN*(Kershner et al., 2022), pericytes (4 541 cells, *PDGFRB*(Daneman et al., 2010), *ABCC9*(Nelson et al., 2015), *ITGA7*(Malhotra et al., 2012), *RGS5*(Bondjers et al., 2003), and smooth muscle cells (609 cells, *TMEM178*(Xie et al., 2018), *CDH19*(Yang et al., 2026), *SCN7A*(Saleh et al., 2005), *CADMI*(Moiseeva et al., 2013) (**Figure 2A**). The bubble plot shows the top 10 marker genes for each cluster in fat tissue (**Supplementary Figure S1A; Supplementary Table S4**).

Each cell type exhibited its marker gene expression characteristics, and the corresponding GO enrichment results further confirmed the functional roles of each cell population in the adipose tissue microenvironment. ADC is significantly enriched in fatty acid metabolic process and lipid catabolic process, while ASPC is significantly enriched in stem cell population maintenance and regulation of stem cell differentiation (**Figure 2B**). From a developmental dynamics perspective, the proportion of SAT in ADC goes up significantly from 24.9% at 1 month to 65.4% at 5 months, while the proportion of ASPC drops from 40.7% at 1 month to

14.6% at 5 months, suggesting that SAT undergoes active adipogenic differentiation during development to achieve functional maturation of fat tissue (Smith & Kahn, 2016). In comparison, PAT has a higher proportion of ADC at 1 month old (58.4%), which isn't much different from 5 months old (54.4%); ASPC is similar at 1 month (13.0%) and 5 months (14.2%), suggesting that ADC development in PAT mostly happens before birth, which might be related to its early role in energy storage and endocrine regulation (Lopreiato et al., 2018). The two show different developmental windows and tissue maturation patterns (**Figure 2C**). In the study of muscle tissue atlases, a total of 85 900 single nucleus were collected, including LD (17 539) and BF (17 980) cells from 1-month-old sheep, and LD (25 634) and BF (24 747) cells from 5-month-old sheep. Ten major cell types were represented, including ADC (5 946 cells, *ADIPOQ* (Wang et al., 2024d), *PLIN1* (Wang et al., 2024c), *FABP4* (Leung et al., 2025), *PPARG* (Adam et al., 2023), cycling cell (357 cells, *MKI67* (Deprez et al., 2020), *TPX2* (Liu et al., 2025a), *SMC4* (Wei-Shan et al., 2019), *TOP2A* (Arroyo et al., 2025), endothelial cells (3 594 cells, *PECAMI* (Privratsky & Newman, 2014), *VWF* (Wong et al., 2023), *EMCN* (Zhang et al., 2022), *KDR* (Van Craenenbroeck et al., 2013), fibroblasts (11 270 cells, *COL6A3* (Madsen et al., 2023), *DCN* (Kang et al., 2025), *COL6A2* (Yang et al., 2025), *PDGFRA* (Gao et al., 2022), lymphocytes (1,253 cells, *PTPRC* (Michalik et al., 2024), *CD247* (Rudemiller et al., 2014), *CD226* (Weulersse et al., 2020), *ITK* (Wang et al., 2015), MuSC (3 650 cells, *NCAMI* (Stadelmann et al., 2022), *ITGA7* (Dominici & Richard, 2022), *PAX7* (Sincennes et al., 2021), *GFRA1* (Yoo et al., 2024), myeloid cells (1,831 cells, *SYK* (Torres-Hernandez et al., 2019), *MRC1* (Kramer et al., 2022), *VSIG4* (Zheng et al., 2024), *CSF1R* (Stahl et al., 2025), Myof (54 327 cells, *MYOMI* (Ruan et al., 2026), *MYBPCI* (Zhao et al., 2023), *ACTN2* (Jordan et al., 2021), *NEB* (Ranu et al., 2022), pericytes (2 377 cells, *STAC* (Takata et al., 2019), *PDGFRB* (Daneman et al., 2010), *RGS5* (Bondjers et al., 2003), *NOTCH3* (Tefft et al., 2022), and smooth muscle cells (1 295 cells, *ITGA9* (Huang et al., 2020), *CAVIN2* (Kasahara et al., 2023), *EMPI* (Somjen et al., 2010), *PRKCZ* (Wu et al., 2021) (**Figure 2D**). The bubble plot

shows the top 10 marker genes for each cluster in muscle tissue (**Supplementary Figure S1B; Supplementary Table S5**).

The significant expression patterns of marker genes clearly distinguished these cell types, and the GO enrichment analysis of differentially expressed genes functionally supported this classification result. MuSC is significantly enriched in pathways like muscle cell differentiation and skeletal muscle tissue regeneration, while Myof is significantly enriched in items like muscle system process and muscle contraction (**Figure 2E**). The dynamics of muscle tissue development show that the proportion of Myof in BF (1 Month: 41.9%, 5 Month: 69.2%) and LD (1 Month: 66.3%, 5 Month: 69.8%) both increase with age, and the increase is more noticeable in BF. This suggests that trunk muscles develop more fully during the embryonic stage, while limb muscles still have strong myocyte differentiation and proliferation activity after birth (Zhang et al., 2024). The proportion of MuSCs in both types of skeletal muscle gradually decreases with development (BF: from 3.9% to 2.8%; LD: from 6.2% to 4.4%), which happens alongside the increasing trend of mature muscle cells (**Figure 2F**).

Furthermore, this study reviewed the differences among ASPC, ADC, MuSC, and Myof at the tissue level and during development. In adipose tissue, comparing ASPCs in different tissues showed that ASPCs in SAT are enriched in extracellular matrix organization and collagen fiber assembly, while ASPCs in PAT are enriched in pathways like cell migration. Comparing different ages, 1-month-old ASPCs are enriched in ribosome biogenesis and translation, mainly focusing on proliferation reserves; 5-month-old ASPCs are enriched in cell adhesion, migration, differentiation, and growth factor response, shifting towards executing signaling (**Supplementary Figure S1C**). In ADC comparisons, ADCs in PAT are enriched in metabolic pathways related to adaptive thermogenesis and temperature regulation, while ADCs in SAT are enriched in cytoplasmic translation, ribosome biogenesis, and collagen fiber assembly. This suggests that terminal cells in PAT lean towards metabolic functions, whereas terminal cells in SAT focus more on protein synthesis storage and ECM maintenance. Comparing different ages,

1-month-old ADCs are enriched in steroid hormones and oxidative stress, showing a stress-adaptive state; 5-month-old ADCs are enriched in fatty acid and fatty acyl-CoA metabolism and cell-substrate adhesion regulation, shifting toward mature lipid metabolism functions (**Supplementary Figure S1D**). When comparing muscle tissues, MuSCs in BF are enriched in classic muscle function pathways such as muscle system processes, muscle contraction, myofibril assembly, and myocyte development, while MuSCs in LD are enriched in the BMP signaling pathway and leucine transport. Comparing different ages, 1-month-old MuSCs are enriched in muscle system processes and the breakdown of carbohydrates and nucleotides, mainly for energy supply and adaptive reserves; 5-month-old MuSCs, however, are enriched in actin filament organization, myofibril organization, and filament organization, shifting toward refining the structure of the contractile apparatus (**Supplementary Figure S1E**). In the tissue comparison of Myof, Myof in BF is enriched in typical mature muscle fiber functional pathways like muscle contraction and muscle tissue development. Myof in LD is also enriched in muscle system processes and myocyte development, but additionally enriched in immune-related pathways, suggesting that terminal muscle fibers in LD may not only execute contraction functions but also participate in regulating the local immune microenvironment. In the comparison across ages, 1-month-old Myof is enriched in growth regulation and muscle adaptation, being in a growth response state; whereas 5-month-old Myof is enriched in myocyte differentiation and muscle tissue development, continuing to progress toward a mature phenotype (**Supplementary Figure S1F**).

Cell subtype atlas and their functional heterogeneity

To elucidate the internal composition and functional differences of key cell types, this study performed subtype annotation on cell types (ASPC, ADC, MuSC, Myof) that play a key role in development. Combining the specific expression patterns and functional enrichment of marker genes, we found significant heterogeneity in differentiation degree and metabolic state within each cell type.

ASPCs were divided into four subpopulations (ASPC1–ASPC4) (**Figure 3A; Supplementary Figure S2A; Supplementary Table S6**). ASPC1 is enriched in extracellular matrix organization related pathways and expresses genes such as *DACT2*, *CMKLR2*, and *CARD10*, suggesting it might maintain stem cell traits by shaping the cellular microenvironment, thereby keeping the potential to differentiate into mature adipose cells. ASPC2 is highly expressed in *TTN*, *ACTA1*, and *MYH1*, enriched in pathways related to cell proliferation and growth factor response, and functionally focuses on responding to proliferation and differentiation signals. Developmental dynamics revealed that ASPC2 accounted for a relatively small proportion at 1 month of age, but its percentage increased markedly by 5 months. This might be because this subgroup doesn't take part in the early development process, but is gradually recruited and activated later on in fat tissue growth to carry out specific functions that support fat tissue expansion and remodeling. It likely initiates proliferation and differentiation programs in response to growth factor signals, thereby contributing to functional maturation and continuous remodeling of adipose tissue, and supporting postnatal adipogenesis and tissue expansion. ASPC3 is enriched in cell-matrix adhesion and its regulatory pathways, showing traits like promoting focal adhesion assembly and cell migration. Its high expression in genes like *MYO10* and *SLC6A6* suggests it might play a role in supporting angiogenesis and cell movement. The functional enrichment of ASPC4 focuses on pathways like cytoplasmic translation and ribosome biogenesis, showing active protein synthesis and ribosome assembly. Its marker genes include *KRT19*, *CD24*, and *SPPI*, suggesting that this subgroup is in a highly proliferative state, maintaining efficient material production to keep the cell cycle running (**Figure 3B**).

ADCs were further subdivided into three main subpopulations (ADC1–ADC3) (**Figure 3C; Supplementary Figure S2B; Supplementary Table S7**), exhibiting a functional progression from homeostasis maintenance to signal activation. ADC1 was enriched in ribosome and protein translation-related pathways, suggesting that it is in a state of metabolic maintenance

and active protein synthesis, and is related to energy metabolism and maintenance of basic functions; ADC2 was enriched in cell-cell adhesion and synapse organization, indicating that it plays a key role in intercellular communication and tissue structure stability; ADC3 was enriched in the PI3K/AKT signaling and cell growth related pathways. This pathway is a key signaling axis that regulates cell growth, proliferation, and adipose deposition, suggesting that ADC3 might be in an active proliferation or signaling state. Developmental dynamics shows that the proportion of ADC3 was extremely low at 1 month of age but increased markedly by 5 months. This change may be related to the body entering a rapid growth phase at 5 months, when tissues need a lot of new adipose to meet energy storage and endocrine function demands. Consequently, ADC3 appears to be progressively activated and expanded during this period **(Figure 3D)**.

MuSCs were subdivided into two functionally distinct subpopulations (MuSC1–MuSC2) **(Figure 3E; Supplementary Figure S2C; Supplementary Table S8)**. MuSC1 was enriched in extracellular matrix-related pathways, such as extracellular matrix organization and basement membrane organization, and significantly expressed *TAGLN3*, *PIEZO1*, and *ITGA8*, suggesting its important role in the localization and microenvironment interaction of muscle stem cells. MuSC2 are enriched in pathways such as muscle tissue development, Wnt signaling pathway, and actin filament organization. *MYO16*, *ELMO1*, and *ACTC1* are highly expressed in MuSC2, indicating that MuSC2 is in an activated state and is involved in the regulation of muscle tissue development and differentiation **(Figure 3F)**.

Myofibers were identified into two distinct subtypes (Type 1 Myof and Type 2 Myof) **(Figure 3G; Supplementary Figure S2D; Supplementary Table S9)**. Type 1 Myof characteristically expresses *MYH7B* and *TNNT1*, and is enriched in skeletal muscle contraction and glucose metabolism-related pathways, suggesting that it maintains a cellular state prioritizing energy metabolism efficiency, with glucose metabolism and sustained contractile capacity synergistically driving the contractile function of endurance myof fibers. In contrast, Type 2

Myof expresses *ACTN3* and *TNNT3*, and is enriched in sarcomere organization and myofibril assembly, indicating that it is primarily characterized by sarcomere construction and rapid contractile capacity, consistent with the fast-twitch muscle fiber phenotype (**Figure 3H**).

Identification of development-related genes of sheep adipose and muscle tissues

In order to identify genes related to sheep development, this study constructed hdWGCNA for stem cells (ASPC, MuSC) from various tissues based on developmental stages (1 month and 5 months of age). Selecting an appropriate soft threshold ensures that the network conforms to scale-free properties to identify gene modules that are highly co-expressed during development. Subsequently, core modules that were significantly related to the development process were screened out through module-trait correlation analysis, and hub genes were identified based on the gene connectivity within the modules. The top 100 genes in the modules with the highest correlation were identified as development-related candidate genes. At the same time, PPI and Cytoscape were used to integrate 12 topological analysis algorithms to identify development-related genes (**Supplementary Table S10**).

Specifically, SAT identified 9 modules, with the red module showing the highest correlation with developmental progress ($r=0.24$). The red module genes are significantly enriched in growth-related pathways such as Insulin secretion (promoting growth hormone), FoxO signaling pathway (regulating cell proliferation, differentiation, and metabolism), and Glucagon signaling pathway (regulating catabolism). Among the top 100 genes in this module, 57 genes showed prominence in at least one topology analysis, with 35 genes exhibiting high network centrality across all analysis methods, considered developmentally relevant genes (**Supplementary Figure S3A; Supplementary Figure S4A**). PAT detected 11 modules, with the turquoise module showing the highest correlation ($r=0.46$). The turquoise module gene is significantly enriched in growth-related pathways such as positive regulation of fat cell differentiation (driving adipocyte proliferation) and PPAR signaling pathway (adipogenic differentiation). A total of 76 genes showed importance in at least one method, with 14 genes

exhibiting the highest network centrality, confirming their crucial role in development (Supplementary Figure S3B; Supplementary Figure S4B). BF identified 16 modules, with the turquoise module showing the highest correlation ($r=0.43$). The turquoise module gene is significantly enriched in growth-related pathways such as insulin-like growth factor receptor binding (driving muscle growth) and skeletal muscle thin filament assembly. 71 genes exhibited network importance, with 21 genes being central across all analysis methods (Supplementary Figure S3C; Supplementary Figure S4C). LD detected 17 modules, with the brown module showing the most significant correlation with developmental progress ($r=0.63$). The brown module genes are significantly enriched in growth-related pathways such as insulin receptor binding and phosphatidylinositol 3-kinase binding. 54 genes showed importance in at least one algorithm, with 41 genes exhibiting high centrality within the network (Figure 3I; Supplementary Figure S3D; Supplementary Figure S4D).

Developmental origin and differentiation trends of stem cell subtypes

To further elucidate the developmental origin and differentiation trends of stem cell subtypes in different tissues, we investigated their lineage relationships and differentiation states. CytoTRACE analysis revealed significant heterogeneity among stem cell subtypes in various tissues. ASPC1 exhibits the highest stemness in SAT and PAT, indicating an earlier developmental stage with stronger self-renewal and multilineage differentiation potential. In contrast, MuSC2 exhibited the highest stemness in both BF and LD, indicating a more primitive cellular state during myogenic development (Figure 4A).

Developmental scoring reveals tissue-specific terminal differentiation trends. In SAT and PAT, ADC1 had the highest development score, indicating that it was closer to the mature adipocyte stage than other subtypes and showed obvious terminal differentiation characteristics; while in BF and LD, Type 2 Myof showed a higher development score, indicating that it was in the later stages of differentiation (Figure 4B).

Integrating machine learning and SHAP models to identify core regulatory genes

In order to identify core genes that may play key regulatory roles during development, this study used 14 machine learning algorithms to construct 195 feature selection models, and screened development-related genes in stem cell subtypes (ASPC1 and MuSC2) with the highest developmental potential. The importance of each gene to the model output was then quantified using the SHAP interpretation framework, and the top three genes with SHAP contribution values were identified as core regulatory genes. Models ranked in the top 100 by AUC were showcased.

Specifically, in SAT, the RF (mtry=10, 25%p) model performed best (AUC=0.906), with prediction accuracies of 1.000 and 0.811 on the training and test sets, respectively. SHAP analysis identified *COL1A1*, *FBN1*, and *MFAP5* as the core regulatory genes driving SAT developmental regulation (**Supplementary Figure S5**). In PAT, the RF (mtry=8, 50%p) model performed best (AUC=0.921), with accuracies of 0.993 and 0.850 on the training and test sets, respectively. SHAP analysis showed that *FABP4*, *ADIPOQ*, and *PLIN1* contributed the most to the model output, indicating their core roles in adipogenic regulation (**Supplementary Figure S6**). In BF, the RF (mtry=16, 75%p) model achieved the highest predictive performance (AUC=0.925), with a training set accuracy of 1.000 and a test set accuracy of 0.850. SHAP analysis showed that *ACTA1*, *MYH1*, and *TTN* contributed most significantly to the model output and were identified as core regulatory genes (**Supplementary Figure S7**). In LD, the RF (mtry=30, 75%p) model performed best (AUC=0.925), with training and test set accuracies of 1.000 and 0.850, respectively. SHAP analysis identified *ACACB*, *AUTS2*, and *FOXPI* as the core candidate genes with the greatest regulatory potential during tissue development (**Supplementary Figure S8**). Notably, in all four tissue types, the random forest model with different parameter settings outperformed other algorithms, and the average AUC exceeded 0.9, demonstrating its stability in differentiating developmental stages and selecting core genes (**Figure 4C**).

Expression characteristics of core regulatory genes in differentiation trajectories

This study used pseudo-temporal trajectory analysis to clarify the cell development process and the expression characteristics of core regulatory genes. The results showed that the stem cell subtype (ASPC1 in SAT and PAT; MuSC2 in BF and LD) with the highest developmental potential was located at the beginning of the trajectory, while the mature terminal cell subtype (ADC1 in SAT and PAT; Type 2 Myof in BF and LD) was distributed at the end, forming a continuous lineage structure from stem cells to terminal cells.

Further analysis of the expression patterns of core regulatory genes in the pseudotime trajectory revealed that the expression changes of these genes are highly coupled with the cell differentiation process, but exhibit differentiated regulatory trajectories and temporal characteristics in different tissues. In SAT, key regulatory genes (*COL1A1*, *FBN1*, *MFAP5*) are highly active at the start of the pathway, then gradually decrease as differentiation progresses (**Supplementary Figure S9A**). In PAT, main regulatory genes (*FABP4*, *ADIPOQ*, *PLIN1*) show gradually increasing expression levels in the mid and late stages (**Supplementary Figure S9B**). In BF, *ACTA1* rises early on but keeps going down in the mid-to-late stages; *MYH1* stays stable at first, then drops; *TTN* keeps going up (**Supplementary Figure S9C**). In LD, *ACACB* and *FOXP1* increase in expression in the mid-to-late stages, while *AUTS2* keeps decreasing (**Supplementary Figure S9D**).

Besides, to more directly understand the dynamic changes of core regulatory genes in stem cell subtypes with the highest developmental potential and in mature terminal cell subtypes, we checked their temporal expression patterns in ASPC1, ADC1, MuSC2, and Type 2 Myof. In SAT, *COL1A1*, *FBN1*, and *MFAP5* were highly expressed at the ASPC1 stage of trajectory initiation, and then gradually decreased as cells differentiated into terminal cells at ADC1 (**Figure 4D**), suggesting that they mainly mediate early cytotromal construction and stemness maintenance. In PAT, the expression of *FABP4*, *ADIPOQ*, and *PLIN1* gradually increased with trajectory progression, reaching a peak at ADC1 (**Figure 4E**), indicating that their core roles may be concentrated in lipid droplet accumulation, enhanced endocrine function, and adipocyte

maturation. In BF, *ACTA1* briefly increased in the early stages of the trajectory before decreasing, while *MYH1* and *TTN* continued to increase during the differentiation into Type 2 Myof (Figure 4F), revealing the functional transition from cytoskeleton construction to the maturation of myofibril contractile apparatus during skeletal muscle development. In LD, *ACACB* and *FOXP1* were upregulated in the early stages of differentiation and then decreased, while *AUTS2* expression continued to decrease along the trajectory (Figure 4G), suggesting the rhythm of myofibril metabolic remodeling and functional specialization, reflecting the characteristics of tissue-specific time-programmed regulation.

Correlation of core regulatory genes with stem cell characteristics and terminal cell development scores

To further clarify the functions of core developmental genes in cell differentiation, this study conducted a correlation analysis. First, regarding the regulatory role of stem cell subtypes, we performed a correlation analysis between core regulatory genes and stem cell subtype marker genes. The results showed significant correlations between them (Supplementary Figure S10). Notably, regardless of whether the correlation was positive or negative, this significant association suggests that core genes may be involved in maintaining stem cell characteristics, including self-renewal capacity and multi-lineage differentiation potential. Second, regarding the regulatory associations of terminal cells, we calculated the correlation between core regulatory genes and AddModuleScore, finding a significant correlation between core gene expression levels and terminal cell developmental scores (Figure 5A). Similarly, both positive and negative correlations indicate that core genes are involved in the development of terminal cells. This indicating that these genes not only participate in maintaining stem cell status but also have regulatory potential in the differentiation and maturation process of terminal cells.

Perturbation nodes and functional impacts of core regulatory genes during differentiation

Pseudo-time series analysis shows that core regulatory genes are highly correlated with cell differentiation. To further elucidate the functional targets and regulatory pathways of core regulatory genes in stem cell differentiation, this study conducted virtual knockout analysis in stem cell subtypes with the highest differentiation degree in various tissues, identifying key nodes (**Supplementary Table S11**) and core perturbation pathways (**Supplementary Table S12**) that drive differentiation through key regulatory genes.

In SAT, knockout of *COL1A1* (**Figure 5B**), *FBN1*, and *MFAP5* (**Supplementary Figure S11**) identified 16 common perturbation genes (**Figure 5D**), mainly involving ECM structural components such as *COL1A2* and *COL3A1*. The common perturbation pathways included extracellular matrix organization and ECM-receptor interaction (**Figure 5C and 5E**), with *COL1A2* located at the core of the PPI network and a key node in the ECM-receptor interaction pathway (**Figure 5F**). These results indicate that SAT rely on an intact ECM structure to maintain stem cell stemness in the early stages of development (Johnston and Abbott, 2022), and the loss of core regulatory genes leads to the disruption of their signaling architecture, becoming an important source of disturbance that causes stem cells to drift toward differentiation.

In PAT, knockout of *FABP4* (**Figure 5B**), *ADIPOQ*, and *PLIN1* (**Supplementary Figure S11**) identified 51 common perturbation genes (**Figure 5D**), encompassing multiple gene classes including *COL3A1* and *RPS29*. These common perturbation pathways included collagen fibril organization, integrin-mediated signaling pathway, platelet-derived growth factor binding, and ribosome-related pathways (**Figure 5C and 5E**). The PPI network revealed *COL3A1* as a central node involved in the integrin-mediated signaling pathway (**Figure 5F**). These results indicate that collagen fiber remodeling and integrin signaling-mediated cell adhesion drive the establishment of adipocyte phenotypes during PAT differentiation.

In BF, knockout of *ACTA1* (**Figure 5B**), *MYH1*, and *TTN* (**Supplementary Figure S11**) identified 30 common perturbation genes (**Figure 5D**), with *ACTN2* located at the core of the

PPI network. These common perturbation pathways are concentrated in skeletal muscle architecture and contractile capacity formation, including sarcolemma, calcium signaling pathway, skeletal muscle tissue development, and actin cytoskeleton organization (**Figure 5C and 5E**). *ACTN2* is located at a key node in actin cytoskeleton organization (**Figure 5F**), suggesting that actin skeletal remodeling and calcium signaling synergistically drive myofiber functional maturation.

In LD, virtual knockout of *ACACB* (**Figure 5B**), *AUTS2*, and *FOXPI* (**Supplementary Figure S11**) revealed 17 common perturbation genes (**Figure 5D**), including *FOXO1*. Eight common perturbation pathways were identified, including oas04820 (Cytoskeleton in muscle cells) and GO:0015629 (actin cytoskeleton). *FOXO1* plays a role in oas04068 (FoxO signaling pathway), indicating that cytoskeleton remodeling and the FoxO signaling pathway synergistically regulate differentiation (**Figure 5F**); the loss of core regulatory genes will disrupt this synergistic network, thereby hindering the progression of myofibers to the terminal maturation stage.

Conserved developmental communication framework and tissue-specific regulatory patterns

To systematically analyze the tissue-specific transcellular signaling regulation during stem cell differentiation into terminal cells, we constructed cell communication networks using various subtypes of stem cells and terminal cells in the four tissues (**Figure 6A**), and identified 1 026 (SAT), 1 377 (PAT), 172 (BF), and 138 (LD) significant cell interactions, respectively (**Figure 6B; Supplementary Table S13**), indicating that stem cells and terminal cells maintain continuous and intensive dynamic signal exchange during development.

After statistical analysis of cell interaction pathways, 44 (SAT), 51 (PAT), 21 (BF), and 16 (LD) significant pathways were detected, of which 14 pathways were shared across all four tissues (**Figure 6C**). These shared pathways encompass multiple key biological regulatory dimensions, including extracellular matrix and adhesion regulation signals (COLLAGEN,

LAMININ, HSPG, CADM), developmental fate and lineage determination signals (BMP, NOTCH, SLIT, SEMA3, SEMA6), growth and metabolism regulation signals (IGF), and neural synapse-related signals (MPZ, NCAM, PTPRM). These results indicate that despite differences in tissue origin and terminal cell type, adipose tissue and muscle share a conserved transcellular developmental regulatory framework during the transition from stem cells to mature cells.

To further identify key regulatory axes determining tissue specificity, we selected the top 10 interacting pathways from four tissues and performed intersection analysis (**Figure 6D**). The results showed that core regulatory pathways exhibited high tissue dependence. COLLAGEN was the strongest shared pathway between SAT and PAT, indicating an ECM remodeling-driven differentiation pattern (**Figure 6E and 6F**). In SAT, COLLAGEN produced a total of 36 receptor-ligand pairs (with COL4A1_CD44 having the most interactions at 45), while in PAT it covered 43 receptor-ligand pairs (with COL1A1_SDC4 having the most interactions at 38)(**Supplementary Table S13**). Following closely behind, LAMININ ranked second in both SAT and PAT, suggesting that collagen laminin signaling is a stable regulatory module during adipocyte maturation. In contrast, FGF interaction strength in PAT was significantly higher than in SAT (**Figure 6G**), reflecting enhanced fibroblast repair signaling characteristics in PAT, possibly related to its stronger inflammatory-fibrotic microenvironment. SAT-specific pathways included APP, BMP, EGF, and FN1, while PAT-specific pathways included NRXN, TENASCIN, THBS, and Glutamate, indicating that although the two adipose tissue types share an ECM-dominated differentiation regulatory base, their external signal input and microenvironment response strategies differ significantly.

LAMININ is the strongest shared pathway between BF and LD, indicating that the maturation process from MuSC to myofibrils depends on strong ECM anchoring and signaling for maintaining sarcomere homeostasis (**Figure 6E and 6F**). LAMININ produced a total of 10 receptor-ligand pairs in BF (with LAMA2_DAG1 having the most interactions at 22), and

covered 8 receptor-ligand pairs in LD (with LAMA2_DAG1 having the most interactions at 21)(**Supplementary Table S13**). Meanwhile, the interaction probability of SEMA6 in LD is significantly higher than in BF (**Figure 6G**), reflecting the more active synaptic structures and refined signaling requirements during LD development. The specific pathways of muscle tissue are also clearly identifiable. The ADGRL and CADM pathways play a specific role in BF, while LD has CDH and SLIT pathways, which further support the signaling requirements of different muscle groups in performance building.

Cell communication networks of various tissues

To explore potential cellular interactions within tissues, we constructed cellular communication networks for each tissue (**Supplementary Figure S12A**) and identified 2 627 (SAT), 2 645 (PAT), 1 941 (BF), and 2 113 (LD) significant cellular interactions, respectively (**Supplementary Table S14**). These results suggest that the scale of cellular communication in the two types of adipose tissues is similar, and the total number of interactions is higher than that in skeletal muscle, reflecting tissue-specific differences in the complexity of inter cellular signal communication.

After statistical analysis of cell interaction pathways, 58 (SAT), 66 (PAT), 54 (BF), and 49 (LD) significant pathways were detected, of which 39 pathways were present in all four tissues. These shared pathways encompass multiple key biological regulatory dimensions, including extracellular matrix and cell adhesion regulatory signals (COLLAGEN, LAMININ, HSPG), developmental fate and lineage determination signals (BMP, NOTCH, TGFb, SLIT, SEMA3, SEMA6, ADGRA, ADGRE, ADGRG, ADGRL), growth and metabolic regulatory signals (IGF, ANGPT, ANGPTL, PDGF, VEGF, FGF, PTN, VISFATIN, ADIPONECTIN), immune regulation and inflammation signals (CD45, CSF, THBS, APP), axon guidance and synapse-related signals (Netrin, EPHB, MPZ, NCAM, PTPRM), and neurotransmitter/hormone signals (DHEA, GAP) (**Supplementary Figure S12B**).

Through cell communication analysis, the number and signal intensity of receptor-ligand pairs between cells within various tissues were systematically quantified. In adipose tissue, SAT identified a total of 215 significant receptor-ligand pairs, with the highest number of EREG_EGFR mediated interactions (40) and followed by COL1A1_ITGA1_ITGB1 (36). PAT identified a total of 272 receptor-ligand pairs, with the most abundant COL1A1_ITGA1_ITGB1 mediated interactions (42). From the perspective of stromal signaling family, COLLAGEN plays a dominant role in cell communication in both types of adipose tissue: SAT identified a total of 877 cell interactions mediated by COLLAGEN, covering 40 receptor-ligand pairs; PAT identified a total of 742 interactions mediated by this signal, involving 45 receptor-ligand pairs. In skeletal muscle tissue, LAMININ becomes the most central signal source. BF identified a total of 229 receptor-ligand pairs, with 615 LAMININ mediated interactions covering 42 receptor-ligand pairs, with the highest proportion of LAMC1_DAG1-mediated interactions (54); LD identified a total of 192 receptor-ligand interactions, with 645 LAMININ mediated interactions covering 35 receptor-ligand pairs, with the highest being HSPG2_DAG1 (67) and LAMC1_DAG1 (54) (**Supplementary Table S14**). The above results suggest that COLLAGEN is a core component mediating stromal-cellular communication in adipose tissue, while LAMININ plays a more crucial role in the regulation of extracellular matrix signaling in skeletal muscle (**Supplementary Figure S12B**); corresponding to the different functional requirements of the two types of tissues for basement membrane integrity and cell-stromal adhesion patterns.

The stem cell differentiation process driven by core regulatory genes

The results of this study show that stem cell differentiation exhibits a three-tiered tissue-specific regulatory pattern: core regulatory genes, core perturbation genes, and core perturbation pathways. Adipose and muscle tissues share some core matrix components but also possess their own unique molecular characteristics. Adipose tissue (SAT and PAT) relies on a COLLAGEN network as the main matrix support, while muscle tissue (BF and LD) uses

LAMININ as its core framework. Furthermore, FGF signaling has a stronger effect in PAT, while SEMA6 signaling has a significant regulatory advantage in LD (**Figure 7**).

SAT differentiation is centered on ECM specific assembly, primarily regulated by *COL1A1* (type I collagen core subunits constructing the ECM framework), *FBNI* (elastic fiber assembly to maintain matrix mechanical properties), and *MFAP5* (involved in ECM component cross-linking). Core regulatory genes, through mediating genes such as *COL1A2*, drive specific ECM remodeling, ultimately achieving SAT-directed differentiation.

The differentiation of PAT occurs simultaneously with the specialization of visceral fat metabolism phenotype. The core regulatory gene cluster includes *FABP4* (intracellular transport of fatty acids to support PAT lipid metabolism function), *ADIPOQ* (homeostatic regulation of energy metabolism), and *PLIN1* (maintaining lipid droplet homeostasis to preserve the visceral fat phenotype of PAT). It mediates genes such as *COL3A1* to drive the integrin signaling pathway, enabling the directed differentiation of PAT. Notably, pathways enriched with multiple ribosomal genes, such as *RPS29*, also play a crucial role.

In terms of muscle tissue differentiation, the differentiation of the BF is centered on the specialized construction of the myocytoskeleton, which, together with *ACTA1* (encoding actin), *MYH1* (for myofiber contraction), and *TTN* (for maintaining the structural integrity of sarcomeres), shapes the muscle tissue specificity of biceps femoris. It drives biceps femoris differentiation by mediating *ACTN2*, which works in conjunction with the calcium signaling pathway. Furthermore, LAMININ, as the core matrix framework of this process, supports the structural specialization of myocytes.

The differentiation of the LD depends on the synergistic effect of transcriptional regulation and cytoskeleton regulation. *ACACB* (regulation of energy metabolism in myocytes), *AUTS2* (regulation of muscle differentiation through epigenetic modification), and *FOXP1* (driving the maturation of myofibrils) mediate the directional differentiation of LD by *FOXO1* in the FoxO signaling pathway.

Effects of interference with core regulatory genes on downstream target gene expression

To clarify the regulatory role of core regulatory genes on downstream target genes, we specifically interfered with the selected core genes in precursor cells derived from different tissues using siRNA, and examined the expression changes of corresponding downstream target genes by qPCR. The results showed that interference with *COL1A1* in SAT significantly downregulated the expression of its downstream target *COL1A2* ($P<0.01$) (**Supplementary Figure S13A**); interference with *FABP4* in PAT significantly reduced the expression of *COL3A1* ($P<0.05$) (**Supplementary Figure S13B**); interference with *TTN* in BF markedly suppressed the expression of *ACTN2* ($P<0.001$) (**Supplementary Figure S13C**); whereas in LD, interference with *FOXPI* led to a significant increase in *FOXO1* expression ($P<0.05$) (**Supplementary Figure S13D**). These findings suggest that the core genes identified in this study can effectively participate in and influence the corresponding differentiation processes by regulating the expression of their downstream target genes.

SheepCellMap Portal

To further elucidate the mechanisms of sheep fat and muscle development, this study constructed a single-cell portal, SheepCellMap (<https://sheepcellmap.lwzlab.com/>). Currently, the platform is divided into six sections: adipose tissue atlas, adipose stem cell atlas, adipocyte atlas, muscle tissue atlas, muscle stem cell atlas, and muscle cell atlas (**Supplementary Figure S14**). Each section provides the following interactive content:

- a) UMAP visualization of the atlas.
- b) List of marker genes for each cell type.
- c) Violin plots of marker gene expression levels.
- d) Word cloud chart of marker genes.

SheepCellMap provides a linked display of cell atlas, marker genes, and expression levels, allowing users to filter by cell type, tissue origin, and developmental stage. It enables rapid location of target cell subpopulations and viewing of corresponding differentially expressed

genes, offering researchers a one-stop tool for key cell screening and rapid capture of core sheep breeding genes.

DISCUSSION

In the life process of mammals, adipose tissue and skeletal muscle are not only the core organ for energy storage and metabolic regulation, but also key tissues that affect body size, growth, development and production performance(Hargreaves & Spriet, 2020). This study systematically analyze the cross-tissue development process of sheep adipose-skeletal muscle system at the single-cell scale, and comprehensively characterize the cell composition and functional heterogeneity including adipose stem cells, adipocytes, muscle stem cells and myofibers. By reconstructing the developmental origin and differentiation trajectory of stem cell subtypes in each tissue, this study identified core regulatory genes and their associated signaling networks that drive stem cell differentiation into terminal cells, and revealed the dynamic expression characteristics of these core genes in the process of cell fate determination. Simultaneously, this study depicted a cross-tissue-shared developmental communication framework and tissue-specific regulatory patterns, and further elucidated the molecular mechanisms by which key regulatory genes drive stem cell differentiation in the adipose-muscle system. In addition, the SheepCellMap online platform developed in this study provides visualized omics resources that can be used for resource sharing and breeding practices. Single-cell atlas reveal the fine stratification and functional specialization of cell types in the adipose-skeletal muscle system. The functional differences among ASPC subtypes are determined by their respective mitochondrial activity and lipid metabolism(Gu et al., 2019). In this study, the ASPC subgroups exhibited a functional gradient along their differentiation trajectory, gradually transitioning from an early state of high expression of genes related to ECM construction and cell adhesion to a terminal differentiation direction dominated by lipid droplet generation, lipid metabolism, and endocrine regulation. Different subgroups not only showed a continuous progression in transcriptional characteristics but also exhibited

871 differences in their ability to sense microenvironmental cues(Liao et al., 2022). The ADC
872 subgroups exhibit a functional continuity from homeostasis maintenance to signal activation.
873 ADC1 is enriched in ribosome and protein translation pathways, indicating that it is mainly
874 responsible for basal metabolic homeostasis. ADC2 is enriched in intercellular adhesion and
875 synaptic tissue-related pathways, mediating intercellular signal transduction and maintaining
876 the integrity of tissue spatial structure, suggesting that ADC2 ensures the coordination of
877 adipose tissue as a functional whole by building intercellular interaction bridges. ADC3
878 regulates cell proliferation and metabolic stress through the PI3K/AKT pathway. This indicates
879 that the regulatory pattern of sheep adipose tissue contains functional subsets of adipocytes
880 similar to those in humans and mice(Emont et al., 2022; Rosen & Spiegelman, 2014). MuSCs
881 exhibit a developmental stage distribution. MuSC1 is enriched in extracellular matrix
882 organization, suggesting that it is mainly responsible for sensing the microenvironment and
883 maintaining cell localization and structural stability. This characteristic is consistent with
884 studies showing that MuSCs rely on ECM to regulate the resting state(Auletta et al., 2025).
885 MuSC2 is enriched in muscle tissue development and the canonical Wnt signaling pathway,
886 and upregulated *MYO16* and *ACTC1*, indicating that MuSC2 is in the pre-activation stage of
887 differentiation transition, which matches the classic mechanism of Wnt signaling-driven
888 myocyte stem cell fate transition. Myofibers subtypes exhibit a high degree of contrast in
889 metabolic patterns and kinetic capabilities. Type 1 Myof is enriched in glucose metabolism and
890 sustained contraction-related functions and expresses *MYH7B* and *TNNT1*, demonstrating a
891 metabolic strategy focused on energy efficiency and endurance. In contrast, Type 2 Myof is
892 enriched in sarcomereorganization and myofibrilassembly and expresses *ACTN3* and *TNNT3*,
893 exhibiting rapid contraction and explosive output characteristics. This molecular and functional
894 contrast is consistent with the classic physiological differences between fast and slow muscle
895 fibers in mammals, and also indicates the stable solidification of myofibril types at the
896 transcriptional regulatory level after development(Wischnewski et al., 2024).

In addition, this study also compared how the functions of four main cell types change over time. In fat tissue, 1-month-old ASCs are dominated by ribosome biosynthesis and translation, indicating that early fat stem cells are in a highly active protein synthesis state, mainly building the necessary biological macromolecules for future differentiation. By 5 months, ASCs shift to cell adhesion, migration, and growth factor responses, suggesting that as development progresses, stem cells transition from proliferation reserves to signal sensing and interaction with their microenvironment(Emont et al., 2022). ADCs show even more dramatic changes over time. At 1 month, ADCs are enriched in steroid hormone response and oxidative stress pathways, reflecting that early fat cells are in a stress-adaptive state, likely due to the drastic hormonal changes in early postnatal fat tissue (like fluctuations in glucocorticoid and sex hormone levels)(Massier et al., 2023). By 5 months, ADCs shift entirely to mature lipid metabolism functions, including fatty acid and CoA metabolism and regulation of cell-substrate adhesion. In muscle tissue, the timing changes show a strategy different from fat. MuSCs shift from being driven by carbohydrate and nucleotide breakdown metabolism at 1 month old to focusing on actin cytoskeleton organization, sarcomeres, and fine assembly of myofilaments by 5 months. This change reveals that early MuSCs prioritize energy metabolism and material breakdown to provide the energy and raw materials for large-scale structural protein synthesis later, while later on they concentrate on the fine construction of the contractile machinery(Kim et al., 2026). At the level of mature Myof, 1-month-old Myof are mainly enriched in pathways regulating growth and muscle adaptation, meeting the rapid growth demands after birth; by 5 months, Myof further enhance muscle cell differentiation and muscle tissue development functions, continuously advancing the maturation of the contractile phenotype.

This study clarified the developmental origin and differentiation trend of stem cell differentiation process by temporal trajectory, and screened out the core regulatory genes driving differentiation by combining various machine learning algorithms and SHAP model.

923 Core regulatory genes are closely linked to the biological mechanisms of adipose and muscle
924 development. In SAT, *COL1A1* is a core structural component of the extracellular matrix. It
925 regulates preadipocyte proliferation and differentiation initiation via integrin-mediated cell-
926 matrix adhesion, and participates in adipose tissue development and remodeling(Liu et al.,
927 2025c). *FBNI* is a core constituent of extracellular matrix microfibrils. It maintains the
928 mechanical integrity and elasticity of adipose tissue. It also modulates preadipocyte
929 differentiation and tissue expansion by regulating TGF- β signaling(Walji et al., 2016). *MFAP5*
930 encodes microfibrillar associated protein 5. This protein interacts with collagen to mediate
931 extracellular matrix fiber assembly. It further regulates adipocyte differentiation and tissue
932 homeostasis via stromal microenvironment remodeling(Vaittinen et al., 2011). In PAT, *FABP4*
933 mediates intracellular fatty acid transport. It directly drives lipid droplet formation and
934 accumulation. It also supports the establishment of lipid metabolic function in terminally
935 differentiating adipocytes(Yu et al., 2024). *ADIPOQ* encodes adiponectin. This adipokine
936 regulates systemic insulin sensitivity and energy homeostasis. It further modulates adipocyte
937 differentiation and lipid storage via autocrine feedback(Xu et al., 2024). *PLIN1* encodes
938 perilipin 1. The protein specifically localizes to the surface of lipid droplets in mature
939 adipocytes. It preserves lipid droplet structural integrity through surface coating. It also fine-
940 tunes the dynamic balance between lipolysis and lipid storage(Wang et al., 2024c). In BF,
941 *ACTA1* encodes skeletal muscle α -actin, a core component of the myocyte cytoskeleton. This
942 protein dominates cytoskeleton construction and morphogenesis in the early differentiation
943 stage. As myocytes mature, it gradually shifts to participate in sarcomeric contractile structure
944 assembly(Garg et al., 2024). *MYH1* encodes myosin heavy chain, the core contractile protein
945 of fast-twitch skeletal muscle fibers. This protein directly mediates force generation and rapid
946 contraction(Ochala et al., 2021). *TTN* encodes titin. This protein maintains sarcomeric
947 structural integrity and elasticity. It coordinates myofilament sliding and contraction processes.
948 It also governs sarcomere assembly and contractile apparatus maturation in the late

developmental stage(Lakdawala et al., 2025). In LD, *ACACB* encodes acetyl-CoA carboxylase β , a key rate limiting enzyme for fatty acid oxidation. It reshapes myocyte energy metabolism patterns by regulating fatty acid metabolic flux. It also mediates early metabolic reprogramming to support myofiber differentiation and structural formation(Yoon et al., 2020). *FOXP1* is a member of the forkhead box transcription factor family. It modulates myofiber fate determination and differentiation progression by regulating the temporal expression of downstream myogenic genes(Schonk et al., 2025). *AUTS2* drives actin cytoskeleton remodeling through activation of the Rac1 signaling pathway. It participates in myocyte morphogenesis and myofibril assembly during the structural construction phase of skeletal muscle development(Hori & Hoshino, 2017). Of particular note, all four types of tissues achieved the best performance with the random forest model, suggesting that the developmental molecular regulatory network has highly nonlinear characteristics(Green et al., 2017).

In addition, the core regulatory genes in the differentiation trajectory do not function independently, but are co expressed in the form of functional modules, and their overall trend is closely coupled with the functional requirements and temporal characteristics of each tissue development stage. In SAT, *COL1A1*, *FBNI*, and *MFAP5* were highly expressed at the early trajectory and gradually decreased as cells matured. *COL1A1* provides mechanical support and adhesion points, *FBNI* regulates ECM tension through microfibrils, and *MFAP5* controls the assembly of elastic fibers and communication between cells and the matrix. The three together form the ECM structural regulatory module, which synergistically upregulates during the initial stage of developmental trajectory and synchronously decreases with the progression of differentiation. This indicates that ECM integrity promotes self-renewal of early adipose stem cells. As cells gradually differentiate into mature adipocytes, their dependence on ECM structural signals decreases, and the demand for ECM synthesis decreases accordingly.(Broutier et al., 2016).

975 In PAT, *FABP4*, *ADIPOQ*, and *PLINI* form a lipid metabolism functional execution module,
 976 all of which are at low expression levels at the beginning of the trajectory. As the differentiation
 977 trajectory progresses, the three are synchronously and continuously upregulated until reaching
 978 their peak at the end of development, indicating that the lipid metabolism functional module
 979 gradually strengthens during development. *FABP4* improves fatty acid transport and lipid
 980 droplet utilization efficiency, *ADIPOQ* regulates insulin sensitivity and energy metabolism,
 981 and *PLINI* maintains the stability and lipid storage capacity of lipid droplets by coating them.
 982 The continuous increase of the three promotes the continuous accumulation of lipid droplets,
 983 enabling adipocytes to gradually mature and form stable physiological characteristics(Morigny
 984 et al., 2021).

985 In BF, the regulation module presents a more complex overall trend of stages. *ACTAI* rises
 986 briefly in the early stage of differentiation and then falls back, while *MYHI* and *TTN* continue
 987 to rise from early to late. The three together form a continuous functional transition module
 988 from the construction of the cytoskeleton to the maturation of contractile function. In the early
 989 stages, the construction of the cytoskeleton, represented by *ACTAI*, was dominant, while in the
 990 later stages, the focus was on the assembly of contractile devices, represented by *MYHI* and
 991 *TTN*. The temporal differentiation of the expression trends of the three suggests a shift in the
 992 functional focus during the development of BF(Narici et al., 2016).

993 In LD, *ACACB*, *FOXPI*, and *AUTS2* showed an overall downward trend (*ACACB* and *FOXPI*
 994 were upregulated early and then decreased, while *AUTS2* continued to decline). *ACACB*
 995 promotes energy retention by inhibiting fatty acid oxidation, and *FOXPI*, as a transcriptional
 996 regulator, affects myofibrillary fate. *AUTS2* promotes actin cytoskeleton remodeling by
 997 activating the Rac1 signaling pathway(Hori et al., 2014). After these early tasks are gradually
 998 completed, the expression of the three is synergistically downregulated, releasing
 999 differentiation programs to allow for the comprehensive unfolding of muscle fiber maturation.

1000 The overall downward trend of this module suggests that the developmental regulation of LD

focuses on early metabolic programming(Shao et al., 2024). Furthermore, core regulatory genes showed significant correlations with stemness marker genes, suggesting that these genes may play a regulatory role in stemness maintenance and fate decision-making. A significant correlation also existed between core regulatory genes and terminal cell development scores, indicating their potential involvement in terminal cell maturation(Massier et al., 2023).

Stem cell differentiation is not an isolated intracellular event. The four tissues share 14 communication pathways covering ECM/adhesion, lineage determination, metabolic growth and neural synaptic signaling modules, indicating that the stem cell differentiation process of adipose and muscle tissues shares a conserved differentiation communication framework. In addition, each tissue still forms a strong regulatory bias. Adipose tissue uses the collagen-lamin axis as the core of signal input, with COLLAGEN being the dominant pathway in both SAT and PAT. SAT centers around the COL4A1_CD44 receptor-ligand pair. COL4A1 is the main collagen component of the basement membrane, and its receptor CD44, which binds to hyaluronic acid and collagen, is widely expressed on the surface of mesenchymal stem cells and adipocyte progenitors. When they bind, they can activate the FAK/ERK signaling pathway, promoting cell adhesion, migration, and proliferation(Zick, 2022). In comparison, the dominance of COL1A1_SDC4 in PAT suggests a different signaling logic. COL1A1 is the most abundant fibrillar collagen in the stromal matrix, and its receptor SDC4, a transmembrane proteoglycan, not only mediates the physical connection between cells and the ECM but also regulates PKC α and Rac1 signaling through its intracellular domain, playing a role in cell stress and fibrosis responses under inflammatory conditions(Jimenez et al., 2001). The differences in this ligand-receptor pair are totally in line with previous studies, which showed that SAT development depends on the mechanical properties of the extracellular matrix and fiber structure stability, while PAT is more prone to inflammation and fibrosis(Kolb, 2022). This study found that FGF signaling was significantly enhanced in PAT, supporting its stronger injury-related ECM pattern and tendency to fibrosis remodeling.

1027 In contrast, LAMININ is essential for muscle integrity(Holmberg & Durbeej, 2013). The
1028 muscle tissue communication network exhibits an architecture with LAMININ as the absolute
1029 center, with interactions mainly mediated by LAMA2_DAG1. This suggests that the
1030 development of MuSCs toward a mature state mainly relies on ECM anchoring and signals for
1031 sarcomere homeostasis, implying that laminin is a key component of the basement layer
1032 surrounding muscle stem cells and adult cells. Previous studies have shown that mutations in
1033 the gene encoding the laminin $\alpha 2$ chain (LAM2) lead to congenital muscular dystrophy type
1034 1A(Allamand & Guicheney, 2002), and most LAMA2 gene mutations result in a complete lack
1035 of the laminin $\alpha 2$ chain(Allamand et al., 1997). DAG1 is the core component of the muscle
1036 dystrophy protein-glycoprotein complex. It links the basement membrane laminin to the
1037 intracellular cytoskeleton and plays an irreplaceable role in keeping the muscle membrane
1038 stable and resisting mechanical stress damage. Furthermore, these laminin isoforms play a key
1039 role in the maintenance of neuromuscular junctions and the organization of synaptic vesicle
1040 release sites. They organize neuromuscular junctions by binding receptors, including integrins,
1041 low-glucose, and voltage-gated calcium channels. Disruption of these laminin-receptor
1042 interactions occurs in neuromuscular diseases including Pearson syndrome and Lambert-Eaton
1043 myasthenia gravis(Rogers & Nishimune, 2017). Interventions that maintain appropriate
1044 laminin levels and their receptor interactions may be helpful in treating neuromuscular diseases
1045 and age-related neuromuscular junction degeneration. Furthermore, the significantly enhanced
1046 SEMA6 signaling in LD suggests differentiated requirements for neural innervation and
1047 shaping signals during the development of different muscle groups. Notably, synaptic
1048 pathways (such as NCAM, MPZ, and PTPRM) are frequently observed in both fat and muscle,
1049 suggesting that tissue innervation participates in regulation early in development, rather than
1050 only functioning during maturation. This indicates that neural signals are not a late-stage
1051 regulator of energy metabolism and motor function, but rather an indispensable early
1052 component of the developmental trajectory(Perez-Tilve et al., 2011). It is worth noting that

1053 HSPG2_DAG1 frequently appears in cell communication within LD tissues. HSPG2 not only
1054 serves as a structural component of the basement membrane but can also bind and concentrate
1055 various growth factors (such as FGF and VEGF) through its heparan sulfate side chains,
1056 suggesting that the basement membrane signaling regulation in LD has a stronger capacity for
1057 integrating growth factor signals, which may be related to the higher metabolic demand and
1058 degree of vascularization in LD(Johnson et al., 2024).

1059 In SAT, the function of core regulatory genes (*COL1A1*, *FBNI*, *MFAP5*) is not simply ECM
1060 synthesis, but rather the construction of a unique signal transduction framework for SAT
1061 through the synergistic regulation of collagen fiber assembly, elastic fiber conformational
1062 remodeling, and ECM cross-linking networks. Previous studies have constructed adipose-
1063 derived ECM models by coating decellularized human tissue matrix onto polyacrylamide gels
1064 of different hardness, and found that the low-hardness environment can significantly increase
1065 the expression of adipogenesis markers in human-derived adipose stem cells(Young et al.,
1066 2013); 3D-printed bio-inks based on porcine adipose matrix encapsulated human ASPCs have
1067 also successfully induced their differentiation into the adipose lineage(Hou et al., 2021);
1068 significant adipogenesis accompanied by host tissue infiltration was also observed after
1069 subcutaneous injection of adipose ECM containing fibroblast growth factor into mice(Lu et al.,
1070 2014). These findings are consistent with this study, indicating that adipose-derived
1071 extracellular matrix can adequately provide the necessary signals to drive adipogenesis and
1072 differentiation. In PAT, the core regulatory genes (*FABP4*, *ADIPOQ*, *PLIN1*) exhibit a
1073 metabolic-driven regulatory pattern, but are accompanied by active integrin and ribosome-
1074 related pathways, indicating that the terminal maturation of PAT not only requires lipid droplet
1075 homeostasis, but also requires the enhancement of protein translation ability(Wang et al., 2020).

1076 In muscle tissue, the co-perturbation of the core regulatory genes *ACTA1*, *MYH1*, and *TTN* in
1077 BF significantly points to *ACTN2*, suggesting that *ACTN2* is a key response node in the
1078 myofiber fate regulatory network. This phenomenon is independently supported in the

cardiomyocyte system. A meta-analysis of heart failure including a large-scale GWAS identified a new chromosome 1 locus associated with heart failure, and its functional analysis showed that the regulatory region corresponding to this locus is specifically activated during cardiomyocyte differentiation and has significant three-dimensional chromatin interactions with *ACTN2*(Arvanitis et al., 2020). Genome editing experiments in human embryonic stem cell-derived cardiomyocytes further confirmed that this regulatory region affects the cardiomyocyte maturation process by regulating *ACTN2* expression(Arvanitis et al., 2020). LD exhibits a completely different regulatory mechanism. Its core regulatory genes (*ACACB*, *AUTS2*, *FOXPI*) act simultaneously on energy metabolism, epigenetic modification, and transcriptional regulation, driving *FOXO1* to participate in the FoxO pathway to regulate the differentiation program of muscle stem cells. Previous studies have shown that overexpression of *FOXO1* significantly inhibits myoblast differentiation. Insulin and IGF-I are key factors regulating the subcellular localization of *FOXO1* and strongly promote myogenic differentiation through a PI3K-Akt-dependent mechanism(Pirskanen et al., 2000). *FOXO1* inhibits the generation of insulin-positive cells(Bouchi et al., 2014), and insulin treatment significantly restores the inhibitory myogenic differentiation induced by *FOXO1*. On the other hand, in contrast to the view that *FOXO1* negatively regulates muscle cell differentiation, there are opposing observations that muscle cell fusion requires the nuclear accumulation of *FOXO1*. A study by Hakuno et al.(Hakuno et al., 2011) reported that the expression of a *FOXO1* dominant-negative mutant lacking 256 N-terminal residues (including Akt phosphorylation sites and transcription activation domains) resulted in a significant decrease in the expression of myogenic markers (including myopoietin and *MyHC*) in L6 cells, indicating that myogenic differentiation was inhibited by this *FOXO1* dominant-negative mutant form. This study also has some limitations. First, although we explored the differentiation process of the adipose-skeletal muscle system, it may not fully cover all developmental stages of all adipose and muscle tissues. Second, while we identified core regulatory genes as important

drivers of stem cell differentiation, further exploration of other regulatory pathways is needed to evaluate the comprehensive regulatory network of stem cell differentiation.

CONCLUSION

This study systematically elucidated the cross-tissue developmental logic of the adipose-skeletal muscle system at the single-cell scale, revealing the high coupling relationship between stem cell lineage origin, differentiation trajectory, and key regulatory gene networks. It clarified the expression dynamics of core regulatory genes in cell fate determination, analyzed key perturbation nodes and downstream effector pathways in the differentiation process driven by core regulatory genes, and further revealed the stem cell differentiation program driven by key regulatory genes in the adipose-skeletal muscle system. It is worth emphasizing that all the core regulatory genes identified in this study exhibited strong perturbation effects in virtual knockout experiments. Molecular validation also provides reliability for the function of core regulatory genes. These results provide high-resolution evidence for understanding the molecular basis of fat deposition and myofibril maturation, and also provide clear molecular targets for regulating fat deposition or improving muscle performance.

Ethics statement

All experimental procedures in this study strictly followed the regulations for the husbandry and use of experimental animals at Shanxi Agricultural University and were approved by the Experimental Animal Ethics Committee of Shanxi Agricultural University. Animal handling and surgical procedures were performed in accordance with the relevant procedures of the College of Animal Science and Veterinary Medicine, Shanxi Agricultural University (Taiyuan, China). All experimental protocols in this study have passed institutional ethical review (Approval No.: SXAU-EAW-2024S.CR.009015380).

Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The snRNA-seq data generated in this study have been deposited in the National Genomics Data Center (NGDC) under the accession number PRJCA066891, and are also accessible via the SheepCellMap (<https://sheepcellmap.lwzlab.com/>) platform developed in this study. All data generated in this study are included in the results and supplementary files.

Supporting Information

Supplementary data to this article can be found online.

Authorship contributions

W.W. contributed to investigation, data curation, formal analysis, visualization, and writing original draft preparation. Z.P. and S.C. participated in investigation, data curation, and formal analysis, with S.C. also contributing to validation. K.C. was responsible for methodology and software. H.W. assisted with investigation and data curation. R.W., Y.P., K.Y., and J.L. provided resources and validation. L.Q. conceived and supervised the study. W.L. acquired funding, supervised the overall project, and reviewed and edited the manuscript. All authors have read and approved the final manuscript.

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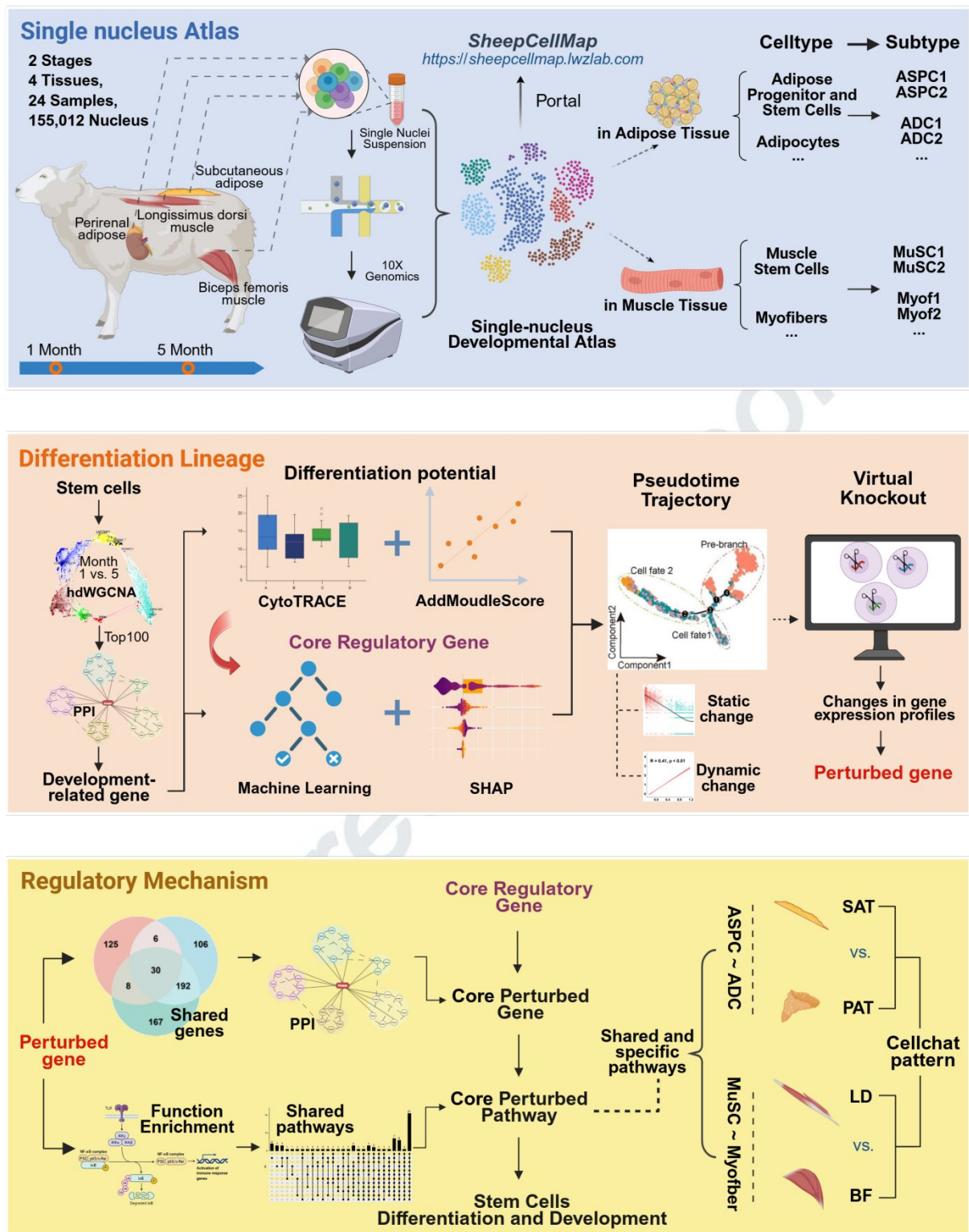


Figure 1. Schematic view of the procedures for data collection and analyses in this study.

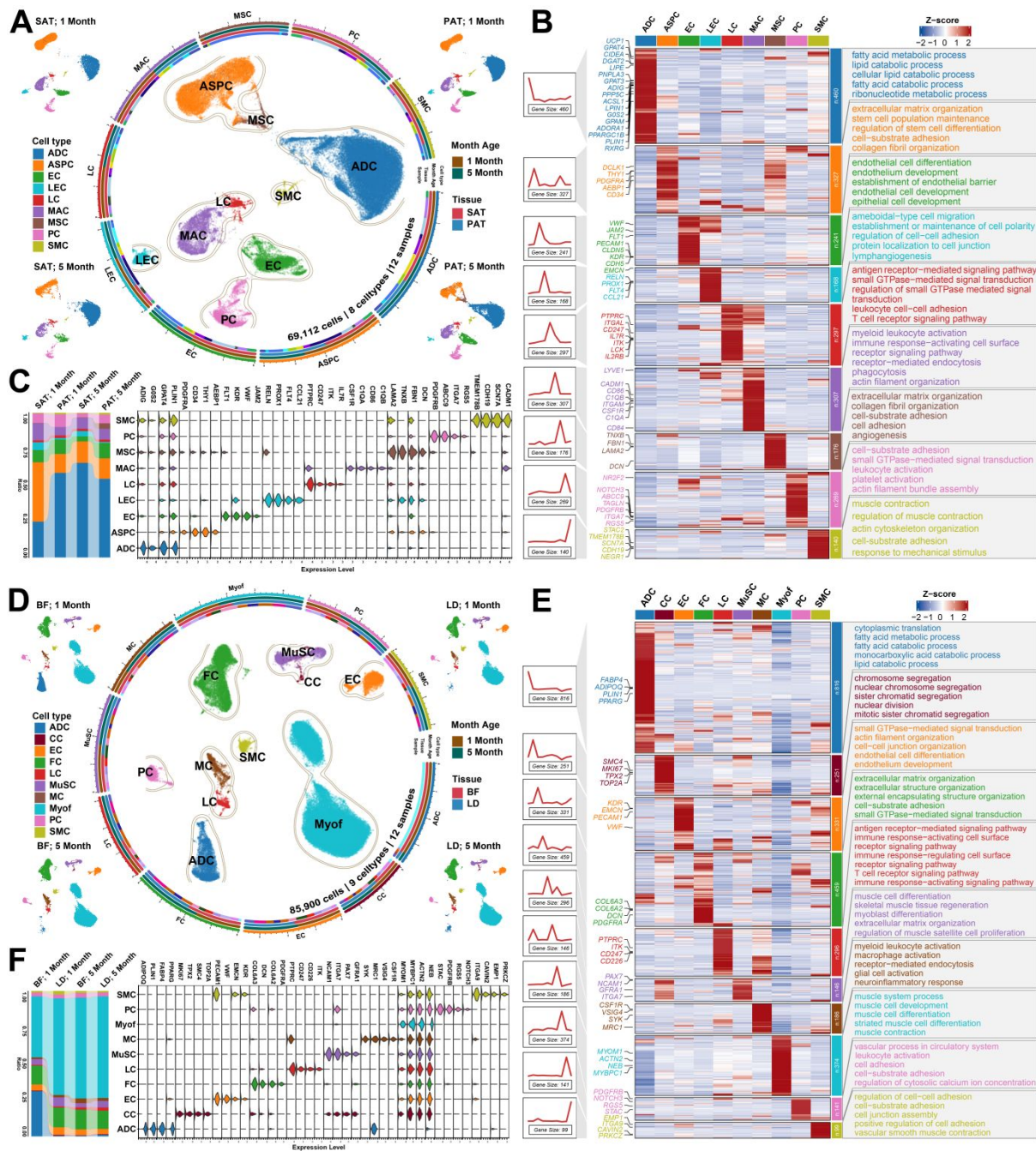


Figure 2. Single-nucleus developmental atlas and functional enrichment of the adipose-skeletal muscle system.

A: UMAP analysis of 69,112 single nucleus from SAT and PAT. In the UMAP map, eight cell types were marked with different colors. B: Heatmap showing the differentially expressed genes for each cell types in adipose tissue and their corresponding enriched Gene Ontology biological processes. C: Proportion of cell types and expression of marker genes in adipose tissue. D: UMAP analysis of 85,900 single nucleus from BF and LD. In the UMAP map, eight

cell types were marked with different colors. E: Heatmap showing the differentially expressed genes for each cell types in muscle tissue and their corresponding enriched Gene Ontology biological processes. F: Proportion of cell types and expression of marker genes in muscle tissue.

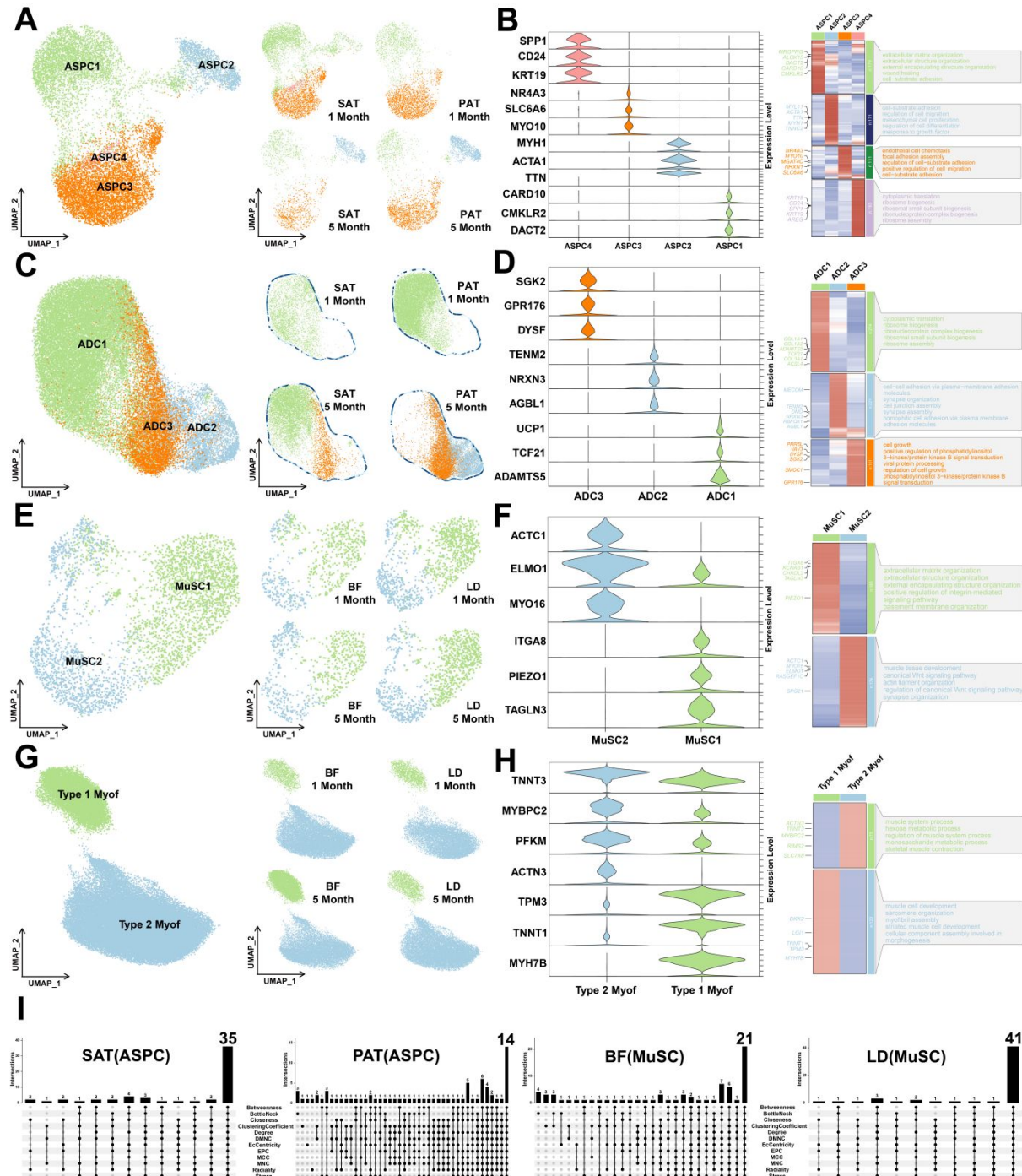


Figure 3. Cell subtype atlas and identification of development-related genes in various tissues.

A: UMAP plot of ASPC subtypes in adipose tissue. B: Marker genes and their functional enrichment in ASPC subtypes. C: UMAP plot of ADC subtypes in adipose tissue. D: Marker

genes and their functional enrichment in ADC subtypes. E: UMAP plot of MuSC subtypes in adipose tissue. F: Marker genes and their functional enrichment in MuSC subtypes. G: UMAP plot of Myof subtypes in adipose tissue. H: Marker genes and their functional enrichment in Myof subtypes. I: Development-related genes in sheep stem cells were identified using hdWGCNA and PPI.

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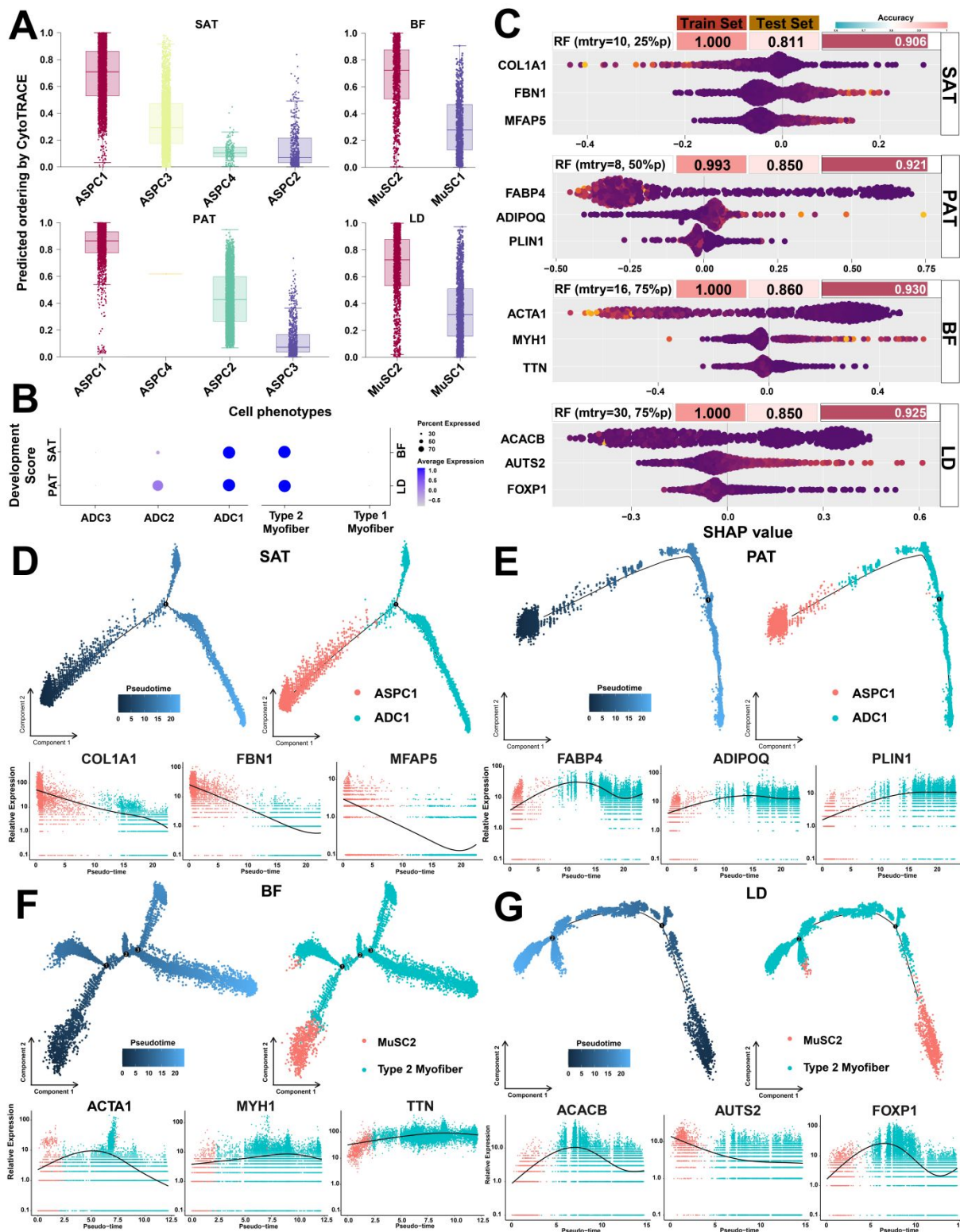


Figure 4. Identification of core regulatory genes and their expression characteristics in differentiation trajectories.

A: CytoTRACE identifies the developmental origin of stem cells. B: Developmental scores determine the differentiation endpoints of each tissue. C: Core regulatory genes in various

A

SAT

COL1A1: $R = 0.57, p < 0.01$

FBNI: $R = 0.41, p < 0.01$

MFAP5: $R = 0.18, p < 0.01$

ACTA1: $R = 0.67, p < 0.01$

MYH1: $R = 0.26, p < 0.01$

TTN: $R = -0.25, p < 0.01$

PAT

FABP4: $R = 0.63, p < 0.01$

ADIPOQ: $R = 0.36, p < 0.01$

VIM: $R = 0.55, p < 0.01$

ACACB: $R = 0.56, p < 0.01$

APOE2: $R = 0.13, p < 0.01$

FOXO1: $R = 0.69, p < 0.01$

BF

LD



Figure 5. Perturbation nodes and functional impacts of core regulatory genes.

A: Correlation between core regulatory genes and developmental scores. B: Top 10 perturbation genes after virtual knockout of core regulatory genes. C: Functional enrichment analysis of perturbation genes. D: The intersection of perturbation genes obtained by knocking out core regulatory genes in various tissues. E: Intersection of functional enrichment of perturbation genes in various tissues. F: PPI is used to screen key perturbation nodes of core regulatory genes.

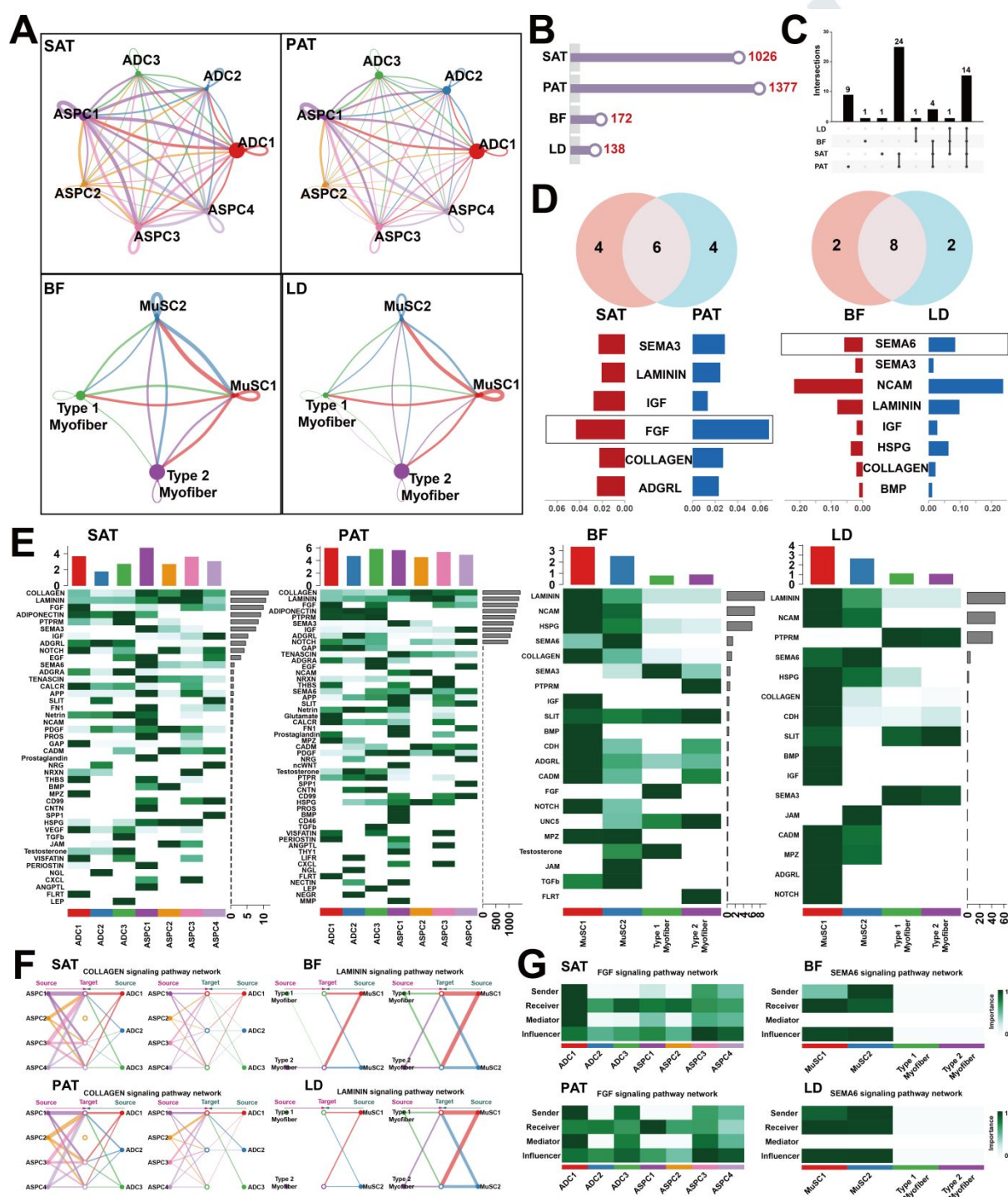


Figure 6. Cell communication patterns of stem cells and terminal cell subtypes in various tissues.

A: Cellular communication networks of various tissues. B: Changes in the total number of interactions between cells in various tissues. C: Upset plot of cell interaction pathways in various tissues. D: Differences in interaction probability among the top 10 pathways with the strongest interaction strength. E: Outgoing signaling of all pathways across the each tissue, with the x-axis representing different cell types and the y-axis representing signaling pathways. F: Interaction patterns of the pathways with the strongest interaction strength in various tissues. G: Interaction patterns of the pathways with the greatest interaction differences.

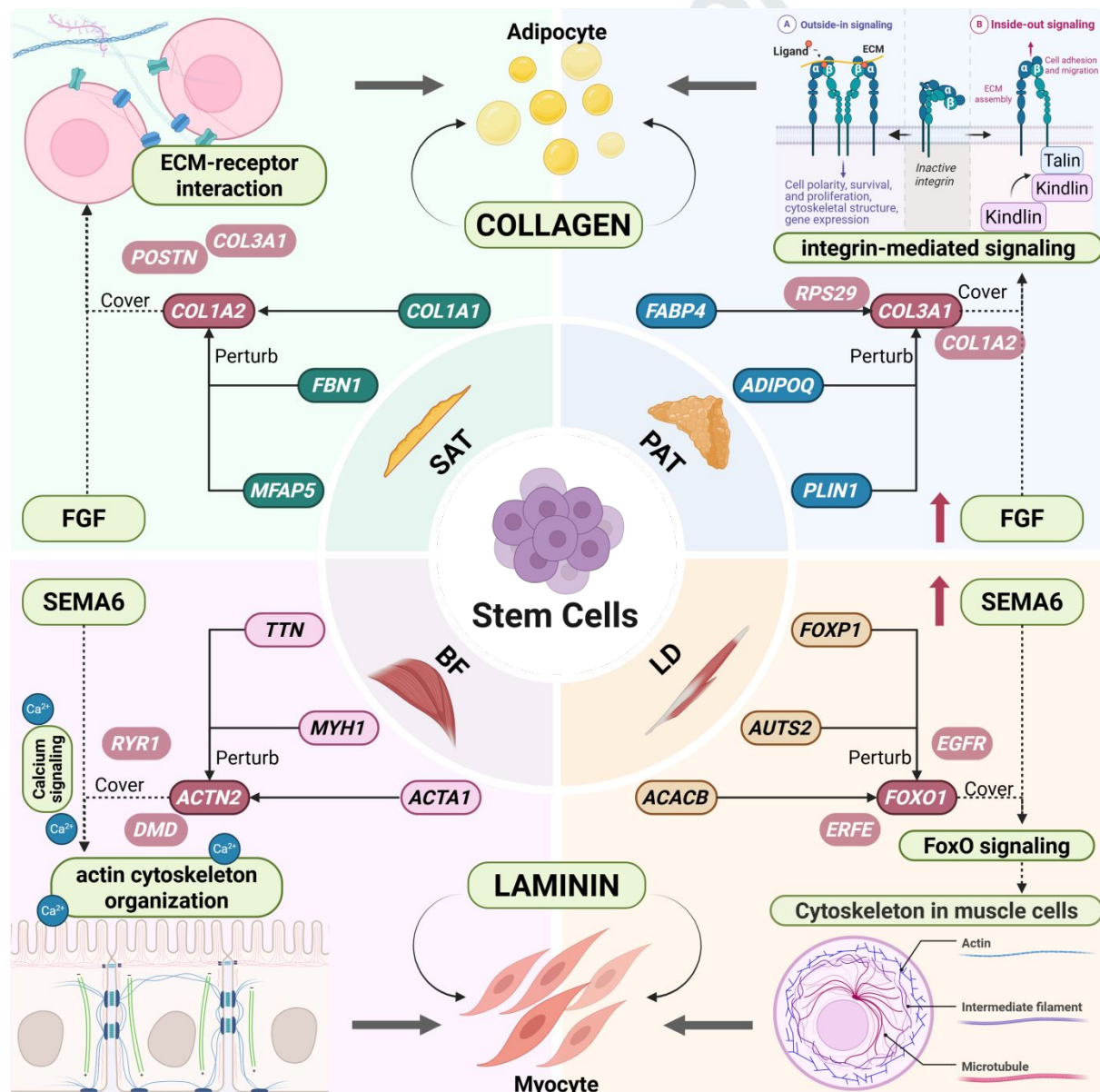


Figure 7. The stem cell differentiation process driven by core regulatory genes.

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绵羊脂肪-骨骼肌系统的单核发育图谱揭示了由核心调控基因驱动的干细胞分化程序

王万年^{1,2}, 庞志旭¹, 陈思佳¹, 蔡柯¹, 王海洋⁴, 王瑞珍⁵, 潘洋洋¹, 杨凯捷¹, 乔利英¹, 刘建华¹, 刘文忠^{1,2,3,*}

¹山西农业大学动物科学学院, 晋中 030801, 山西, 中国

²畜禽遗传资源发掘与生物育种技术山西省重点实验室, 晋中 030801, 山西, 中国

³山西农业大学现代种业学院, 晋中 030801, 山西, 中国

⁴南京大学医学院, 南京 210093, 江苏, 中国

⁵河南中医药大学中医药科学院, 郑州 450046, 河南, 中国

*通讯作者: Wenzhong Liu, tglwzyc@163.com

摘要

引言：脂肪和骨骼肌在代谢稳态中相互依存，其干细胞通过精准的命运决定以维持自我更新和组织形成。然而，绵羊脂肪-骨骼肌系统的细胞发育异质性及其干细胞分化调控机制尚不清楚。

方法：对 24 个样本（6 只雌性湖羊的 4 个组织，3 只 1 月龄，3 只月龄）进行单核 RNA 测序。平均深度大于 10000 次读取/细胞，每个细胞的基因中位数为 755-2122。使用机器学习 and 虚拟敲除相结合的方法鉴定了核心调控基因。

结果：本研究构建了绵羊脂肪骨骼肌系统的单核发育图谱（155 012 个细胞，19 467 个基因）。基于集成机器学习确定了每个组织的分化过程中的核心调控基因（皮下脂肪：*COL1A1*, *FBN1*, *MFAP5*；肾周脂肪：*FABP4*, *ADIPOQ*, *PLIN1*；股二头肌：*ACTA1*, *MYH1*, *TTN*；背阔肌：*ACACB*, *AUTS2*, *FOXP1*）。结合轨迹推断和虚拟敲除确定了核心调控基因驱动分化过程中的关键扰动节点和下游效应通路。此外，本研究还开发了 SheepCellMap(<https://sheepcellmap.lwzlab.com/>)门户，该平台具有标记搜索和可视化功能，为后续育种实践提供数据支持。

结论：本研究以单核分辨率创造性地解释了脂肪-骨骼肌系统的跨组织发育逻辑，并揭示了由核心调控基因驱动的干细胞分化程序。为脂肪沉积和肌纤维成熟提供了单细胞门户和靶点。

关键词：单核；脂肪；骨骼肌；干细胞分化；绵羊