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INFLUENCE OF ANATOMICAL ORIGIN ON PIG MUSCLE STEM CELL DIFFERENTIATION CHARACTERISTICS

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I. INTRODUCTION

Skeletal muscle is essential for physiological functions and bodily movement, and can be categorized into red and white muscle. These types are distinguished by the relative composition of two muscle fiber types—fast-twitch and slow-twitch fibers. The ratio of these fiber types is influenced by various factors, including genetic background and exercise intensity, leading to differences in muscle function and metabolic characteristics [1]. During muscle development, there is a well-documented difference in the timing of fiber type formation. In the early stages of *in vivo* development, slow-twitch muscle fibers are formed first, followed by the differentiation of fast-twitch fibers [2]. This sequential development is crucial for the functional maturation of skeletal muscle. However, it remains unclear whether the anatomical origin of muscle stem cells (MuSCs) affects their differentiation tendency when cultured *in vitro*. Previous studies have not sufficiently addressed whether the original muscle source influences the type of muscle fibers that are formed during *in vitro* differentiation. In this study, we isolated MuSCs from three skeletal muscles located in different anatomical regions of the pig—*Triceps Brachii* (TB), *Longissimus Dorsi* (LD), and *Tensor Fasciae* (TF)—and compared *in vitro* differentiation patterns. By analyzing the differentiation characteristics and fiber type composition of these muscle-derived stem cells, we aim to provide foundational insights into muscle-specific differentiation tendencies. The findings of this study may serve as a valuable resource for future research in muscle biology using the pig as a model organism.

II. MATERIALS AND METHODS

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (Approval No. SNU-230303-3). All experiments strictly followed the established protocols outlined by the Institute of Laboratory Animal Resources at Seoul National University, Republic of Korea. Pig muscle stem cells (MuSCs) were isolated from the skeletal muscles of two-week-old male Berkshire pigs. The pigs were humanely euthanized by CO_2 inhalation followed by exsanguination. *Triceps Brachii* (TB), *Longissimus Dorsi* (LD), and *Tensor Fasciae* (TF) muscles were carefully dissected, and MuSCs were obtained through magnetic-activated cell sorting (MACS) using antibodies targeting CD29, a marker for muscle stem cells. Isolated MuSCs were cultured in 12-well plates pre-coated with 0.05% (w/v) laminin (11243217001, Sigma-Aldrich, Palo Alto, CA, USA). For proliferation, cells were maintained in Skeletal Muscle Cell Growth Medium-2 BulletKit™ (Lonza, Basel, Switzerland), supplemented with 10% (v/v) fetal bovine serum (FBS), 1× GlutaMAX™ (35050061, Gibco, Gaithersburg, MD, USA), 1× Antibiotic-Antimycotic (AA), 0.1 mM β-mercaptoethanol (Gibco), and 20 μM SB203580 (Cayman Chemical, Ann Arbor, MI, USA). For myogenic differentiation, the culture medium was switched to Dulbecco's Modified Eagle Medium (DMEM) containing 2% (v/v) horse serum (Biowest, Nuaillé, France), 1× GlutaMAX™, 1× AA, and 0.1 mM β-mercaptoethanol. Cell numbers were quantified using an automated cell counter (Countess 3, Invitrogen, Waltham, MA, USA). For immunocytochemistry, the following primary antibodies were used: CD29 (1:200; MAB17783, R&D Systems, Minneapolis, MN, USA), CD56 (1:200; 710388, Thermo Fisher Scientific, Waltham, MA, USA), and myosin heavy chain (MHC) (1:1000; MAB4470, R&D Systems). Quantitative real-time PCR (qPCR) was conducted to assess the expression of myosin heavy chain isoforms. Total RNA was extracted and reverse-transcribed, and gene expression was analyzed using the Biorad CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA). The following primers were used: *MYH1*: 5'-TTGACTGGGCTGCCATCAAT-3' (forward), 5'-GCCTCAATGCGCTCCTTTTC-3' (reverse); *MYH2*: 5'-CATTGAGGCCCAGAATAGGC-3' (forward), 5'-TGCTTCCGTCTTCACTGTCAC-3' (reverse); *MYH4*: 5'-GACTCTGGCTTTCCTCTTTGC-3' (forward), 5'-GAGCTGACACGGTCTGGAAA-3' (reverse); *MYH7*: 5'-CGTGGACTACAACATCATAGGC-3' (forward), 5'-CCTTCTCAACAGGTGTGTCG-3' (reverse); *GAPDH* (internal control): 5'-TGCTCCTCCCCGTTCGAC-3' (forward), 5'-ATGCGGCCAAATCCGTTC-3' (reverse). All qPCR assays were conducted in technical triplicates. Gene expression levels were normalized to GAPDH, and fold changes were calculated using the ΔΔCt method. Statistical

analyses were performed using one-way analysis of variance (ANOVA) with SAS 9.4 software (SAS Institute Inc., Cary, NC, USA), and significance was defined at $p < 0.05$.

III. RESULTS AND DISCUSSION

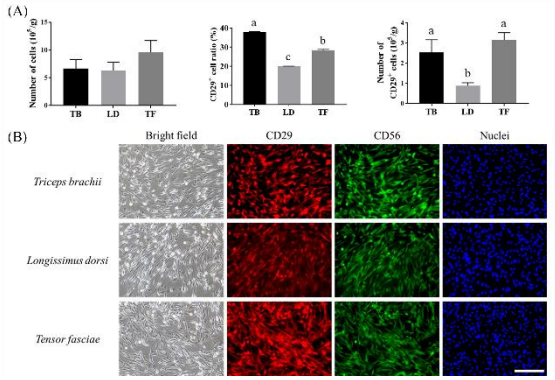


Figure 1. Confirmation of muscle stem cell identity. (A) Quantification and characterization of $CD29^+$ muscle stem cells isolated from muscle tissue. (B) Verification of muscle stem cell identity via expression of cell surface markers. TB: *Triceps Brachii*, LD: *Longissimus Dorsi*, TF: *Tensor Fasciae*. Data are presented as mean ± SEM. Different letters (a–c) indicate statistically significant differences ($P < 0.05$).

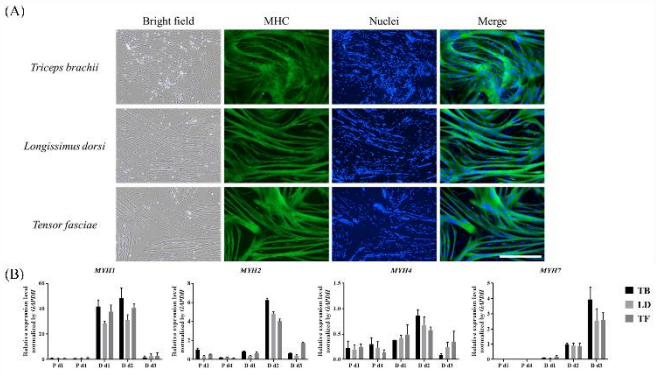


Figure 2. Myogenic differentiation of muscle stem cells isolated from anatomically distinct skeletal muscles. (A) Cell morphology and immunofluorescence staining of myosin heavy chain (MHC, green). Nuclei were counterstained with Hoechst (blue). (B) mRNA expression patterns of myosin heavy chain isoforms during proliferation (P) and differentiation (D) phases. Expression levels of *MYH1*, *MYH2*, *MYH4*, and *MYH7* were normalized to *GAPDH*. TB: *Triceps Brachii*, LD: *Longissimus Dorsi*, TF: *Tensor Fasciae*. Data are presented as mean ± SEM.

MuSCs were successfully isolated from three distinct skeletal muscles of pigs. While there were no statistically significant differences in the total number of cells isolated per gram of muscle tissue among the three muscle types ($p > 0.05$), the yield of $CD29^+$ cells obtained *via* MACS showed significant variation depending on the anatomical origin. Notably, LD-derived cells yielded significantly fewer $CD29^+$ MuSCs compared to TB and TF ($p < 0.05$; Fig. 1A). To confirm the identity of the isolated cells as MuSCs, immunofluorescence staining for muscle stem cell-specific surface markers CD29 and CD56 was performed. All three populations showed strong and consistent expression of both markers, supporting the successful isolating of MuSCs regardless of muscle origin (Fig. 1B). To assess the differentiation characteristic of these MuSCs, cells were cultured under myogenic conditions to induce myotube formation. All groups demonstrated robust myogenic differentiation, forming multinucleated myotubes expressing MHC, indicating high myogenic potential irrespective of anatomical origin (Fig. 2A). Furthermore, gene expression analysis of myosin heavy chain isoforms (*MYH1*, *MYH2*, *MYH4*, and *MYH7*) was conducted during the proliferation and differentiation phases. Across all time points, no significant differences were observed in the expression levels of these isoforms among the three muscle-derived MuSC populations (Fig. 2B). This suggests that, despite differences in initial $CD29^+$ cell yield, the differentiation capacity and myogenic program of MuSCs are largely conserved across different skeletal muscle origins.

IV. CONCLUSION

The results indicate that while MuSC yield varies by muscle origin, their identity and differentiation capacity remain consistent. This suggests that MuSCs from muscles with different anatomical origins can serve as reliable models not only for muscle biology but also for meat science research, particularly in understanding muscle development and traits related to meat quality.

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