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Author	Hyun Young Jung1, Minsu Kim1,2, Chang-Kyu Lee1,3, Cheorun Jo1,2,3
Affiliation	1 Department of Agricultural Biotechnology, Seoul National University, Seoul 08826, Korea 2 Center for Food and Bioconvergence, Seoul National University, Seoul 08826, Republic of Korea 3 Institute of Green Bio Science and Technology, Seoul National University, Pyeongchang, 25354 Gangwon-do, Republic of Korea
ORCID (for more information, please visit https://orcid.org)	Hyun Young Jung (https://orcid.org/0000-0002-4561-9304) Minsu Kim (https://orcid.org/0000-0001-7038-1732) Chang-Kyu Lee (https://orcid.org/0000-0001-6341-0013) Cheorun Jo (https://orcid.org/0000-0003-2109-3798)
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CORRESPONDING AUTHOR CONTACT INFORMATION

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Cheorun Jo
Email address – this is where your proofs will be sent	cheorun@snu.ac.kr
Secondary Email address	cheorun@gmail.com
Address	#200-5218, 1, Gwanak-ro, Gwanak-gu, Seoul 08826 Republic of Korea

Cell phone number	+82-10-3727-6923
Office phone number	+82-2-880-4804
Fax number	+82-2-873-2271

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Abstract

We investigated the biological and molecular characteristics of porcine muscle stem cells (MuSCs) derived from three anatomically distinct skeletal muscles [*triceps brachii* (TB), *longissimus dorsi* (LD), and *tensor fasciae* (TF)] in 2-week-old male Berkshire piglets. Myofiber typing revealed no significant differences in fiber composition among the muscles, indicating an early stage of muscle development. MuSCs were isolated using magnetic-activated cell sorting (MACS) and evaluated for cell yield, proliferation, differentiation, and gene expression profiles under *in vitro* conditions. Although the proportion of $CD29^+$ MuSCs was significantly lower in LD than in TB and TF, no differences were observed in proliferation rates, differentiation efficiency, or the expression of key myogenic regulatory factors (*PAX7*, *MYF5*, and *MYOD1*). Similarly, expression patterns of myosin heavy chain isoforms (*MYH1*, *MYH2*, *MYH4*, and *MYH7*) did not differ significantly among muscle sources. These findings suggest that, at this early developmental stage, the anatomical origin of skeletal muscle has minimal impact on the functional properties of MuSCs. This study provides foundational data for selecting MuSC sources in muscle biology research and emphasizes the need to standardize developmental timing in comparative studies.

Keywords: Anatomical origin, Muscle stem cells, Myogenesis, Muscle type, Berkshire

Introduction

Muscle stem cells (MuSCs), also referred to as satellite cells, play an essential role in postnatal skeletal muscle growth, repair, and regeneration [1]. These cells are located between the basal lamina and the sarcolemma of muscle fibers and typically remain in a quiescent state, but can be activated not only by injury or physiological stress, but also during normal tissue homeostasis [2, 3]. Once activated, MuSCs re-enter the cell cycle, proliferate, and differentiate into myogenic precursor cells that contribute to the formation or regeneration of muscle fibers [4]. Beyond their fundamental role in developmental and regenerative biology, MuSCs have garnered increasing interest in applied research fields, including regenerative medicine and cultured meat engineering [5, 6]. Although the molecular pathways regulating MuSC activation, proliferation, and differentiation have been extensively studied in rodent models, but direct interpretation of these findings is limited due to significant anatomical, metabolic, and developmental differences between rodents and larger mammals [7]. In contrast, pigs are physiologically more similar to humans in terms of skeletal muscle structure, fiber-type composition, and postnatal maturation patterns, making them a more appropriate model for studying MuSC biology in both biomedical and cellular food production contexts [8, 9].

Increasing attention has been directed toward the characterization of MuSCs using molecular markers that define distinct stages of the myogenic program [10]. CD29 (integrin β1) and CD56 (neural cell

adhesion molecule, NCAM) are commonly used to isolate MuSCs and to verify their early activation state. CD29 is broadly expressed across activated MuSC populations and is involved in cell–extracellular matrix adhesion, whereas CD56 plays a role in intercellular communication during early myogenesis [11]. Transcription factors further refine the classification of myogenic progression. *PAX7* and *MYF5* are typically expressed in quiescent or early-activated MuSCs, *MYOD1* marks cells committed to the myogenic lineage, and *MYOG* is upregulated during terminal differentiation [4]. Meanwhile, myosin heavy chain (MyHC) isoforms, including MYH1, MYH2, MYH4, and MYH7, serve as molecular indicators of fiber-type identity and maturation, reflecting the transition from undifferentiated progenitors to specific fast- or slow-twitch fiber phenotypes [12]. In particular, MYH1 and MYH2 reflect intermediate fibers (type 2a/2x), MYH4 reflects fast-twitch fibers (type 2b), and MYH7 reflects slow-twitch (type 1).

Despite the well-characterized molecular progression of myogenesis, limited information is available on how MuSC traits vary across different skeletal muscles within the same organism. Skeletal muscles differ in their embryological origin, physiological role, fiber-type composition, and mechanical loading environments, all of which can influence the abundance, activation state, and lineage potential of resident MuSCs [13, 14]. While numerous studies have investigated MuSCs derived from specific muscles such as the *longissimus dorsi* or *triceps brachii* [15-17], few have conducted direct comparisons across multiple anatomical locations under standardized conditions. Moreover, in biomanufacturing contexts such as cultured meat production, understanding whether the anatomical source of muscle influences MuSC function is essential for optimizing cell sourcing strategies.

To minimize biological variation, we focused our investigation on MuSCs isolated from 2-week-old piglets. This time point was chosen because young animals possess a higher proportion of proliferative and myogenic satellite cells, which gradually decline with age [18, 19]. In porcine skeletal muscle, the percentage of satellite cells significantly decreases between 1 and 7 weeks of age, and the percentage of myogenin positive cells, indicating differentiation characteristics, drops from approximately 31% at 1 week to approximately 14% at 7 weeks, and further to 9% by 21 weeks [15]. Selecting the 2-week time point thus balances biological accessibility with a still robust satellite cell population, enabling a reliable comparison of intrinsic MuSC properties before the onset of age-related decline. Muscles are in a transitional phase prior to full maturation, and environmental factors such as mechanical loading, neuromuscular remodeling, and metabolic specialization are not yet fully established [20, 21]. At this stage, myofiber composition remains relatively homogeneous, with slow-twitch (type 1) and intermediate (type 2a/x) fibers being predominant, while glycolytic type 2b fibers have not yet developed [22]. This allows the analysis of MuSCs in a relatively naïve and unprogrammed state. Furthermore, MuSCs isolated at this stage exhibit high proliferative capacity, enhanced viability, and stable marker expression *in vitro* [23], making them well suited for downstream characterization under controlled conditions. This

transition stage enables researchers to examine MuSCs in a relatively naive and unprogrammed state, maximizing the ability to detect intrinsic cellular properties without confounding tissue-specific adaptations [19, 24].

This study aimed to investigate the extent to which anatomical origin affects MuSC properties during a defined early postnatal stage. To minimize confounding by genetics and husbandry, we used a single, uniformly managed Berkshire breed. Because our objective was to examine how anatomical origin shapes MuSC characteristics during early postnatal development. MuSCs were isolated from three anatomically distinct skeletal muscles in pigs, *triceps brachii* (TB), *longissimus dorsi* (LD), and *tensor fasciae* (TF), and evaluated for differences in cell yield, proliferation behavior, differentiation efficiency, and the expression of myogenic transcription factors and MyHC isoforms. By integrating morphological, functional, and transcriptional analyses under uniform *in vitro* conditions, this study seeks to determine whether intrinsic differences exist among MuSCs isolated from distinct muscle regions, despite being collected from animals of the same age and breed. The findings are expected to contribute not only to basic muscle stem cell biology but also to the standardization of cell source selection in regenerative medicine and cellular agriculture.

Materials and Methods

Animal care

Ethical approval for the care and experimental use of pigs was granted by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University, under approval number SNU-230303-3. All experiments were strictly followed with the established protocol outlined by the Institute of Laboratory Animal Resources at Seoul National University. Three 2-week-old castrated male Berkshire piglets, each from a different dam, were used to minimize sex-related variability.

Isolation of pig MuSCs

Pig MuSCs were isolated from skeletal muscles of 2-week-old male Berkshire. All piglets were castrated male Berkshire obtained from a single commercial herd under uniform management. The pigs were euthanized in a humane manner using CO_2 inhalation followed by exsanguination. Among the muscles used in various previous studies, three muscles in anatomically different locations were selected. *triceps brachii* (TB), *longissimus dorsi* (LD), and *tensor fasciae* (TF) muscles were isolated and washed using Dulbecco's phosphate-buffered saline (DPBS; Welgene, Gyeongsan, Republic of Republic of Korea), supplemented with 2×antibiotic-antimycotic (AA; Gibco, Gaithersburg, MD, USA). Subsequent removal of excess connective tissues and blood vessels was carried out. The harvested tissues underwent thorough mincing via a meat grinder (LOCK&LOCK Co., Ltd, Seoul, Republic of Korea). Then they

were subjected to digestion using Pronase (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 0.8 mg/mL for 40 min at 37 °C, with intermittent vortexing every 10 min. Following centrifugation at 1200 × g for 15 min, the resulting pellets were resuspended in minimum essential medium supplemented with 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD, USA). The digested muscle tissues underwent centrifugation at 300 × g for 5 min to isolate the cell population containing pig MuSCs, with subsequent collection of the supernatant to separate undigested tissues. The supernatant underwent filtration using a 100 μm cell strainer, after which the pig MuSCs were harvested via centrifugation at 1200 × g for 15 min. Pig MuSCs were obtained through the positive sorting of CD29-expressing cells utilizing the magnetic-activated cell sorting (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany) [11]. The number of cells that could be separated from 1 g of tissue was counted before and after MACS, utilizing an automated cell counter (Countess3, Invitrogen, Waltham, MA, USA).

Pig MuSCs culture

The isolated pig MuSCs were cultured on 0.05% (w/v) laminin (11243217001, Sigma-Aldrich, Palo Alto, CA, USA)-coated 12-well plates (5×10^4 or 1×10^5 cells per well). The growth medium for culture, Skeletal Muscle Cell Growth Medium-2 BulletKit™ (Lonza, Basel, Switzerland), was prepared with 10% (v/v) FBS, 1× Glutamax™ (35050061, Gibco, Gaithersburg, MD, USA), 1×AA, 0.1 mM β-mercaptoethanol (Gibco, Gaithersburg, MD, USA), and 20 μM SB203580 (Cayman Chemical, Ann Arbor, MI, USA). Following 4 to 5 days of culture, the MuSCs reaching confluence were utilized for myogenic differentiation. The cells were cultured in differentiation media, comprising DMEM with 2% (v/v) horse serum (Biowest, Nuaillé, France), 1×Glutamax™, 1×AA, and 0.1 mM β-mercaptoethanol for 4 days. Daily medium changes were performed, and the cells were cultured in a humidified environment with 5% CO_2 at 37°C.

Cell growth rate

Cell counting was performed to calculate the doubling time of MuSCs. At 12, 24, 48, 72, 96, and 120 hrs after pig MuSCs seeding (5×10^4 per well), cells were collected with 0.04% trypsin-EDTA solution (Welgene) and counted using an automated cell counter (Countess3, Invitrogen). After that, the doubling time was calculated using the following equation. Cell population doubling time = $(T \times \log 2) / (\log N - \log N_0)$. T = cell culture time. N_0 = the initial number of cells being seeded. N = the final number of cells after culture.

Immunocytochemistry

Immediately after MACS, or day 3 of differentiation, MuSCs were fixed in 4% (v/v) paraformaldehyde (PFA) in DPBS for 30 minutes at 4°C. Subsequently, the fixed cells were washed twice with DPBS and

permeabilized with 0.2% (v/v) Triton™ X-100 (T8787, Sigma-Aldrich) for 15 min, followed by a blocking process with 10% (v/v) goat serum (Thermo Fisher Scientific, Waltham, MA, USA) for 1 hr. Primary antibodies against CD29 (1:200; MAB17783, R&D Systems, Minneapolis, MN, USA), CD56 (1:200; 710388, Thermo Fisher Scientific, Waltham, MA, USA), or MHC (1:1000; MAB4470, R&D Systems, Minneapolis, MN, USA) were added, and the cells were incubated at 4°C overnight. After removing goat serum and primary antibodies, cells were incubated with secondary antibodies (1:1,000; A-11001, A-11004, A-11008, Invitrogen, Waltham, MA, USA) at 4°C overnight. Nuclei were stained with Hoechst 33342 (1:1,000; Molecular Probes, Eugene, OR, USA) for 10 min at 4°C, followed by washing, and media replacement with DPBS. Stained images were captured using an inverted fluorescence microscope (Eclipse TE2000-U, Nikon, Konan, Tokyo, Japan). Counting was done manually. To maintain experimental reproducibility, three technical replicates (3 wells) were performed for three piglets, and five points were photographed for each well. The average value obtained from the five points was calculated, and the average value of the three technical replicates was calculated and used as one biological replicate. The differentiation ratio was determined as the percentage of MHC-positive cells relative to the total cell count.

Quantitative reverse-transcription polymerase chain reaction (qPCR)

Total RNA from MuSCs was extracted at five time points: after 1 and 4 days of proliferation (P d1, d4) and after 1 to 3 days of differentiation (D d1, d2, d3) using TRIzol reagent (Invitrogen, Carlsbad, USA). The RNA concentration and purity were assessed spectrophotometrically using a spectrophotometer (X-ma 3100, Human Co Ltd., Seoul, Korea), with A260/280 ratios confirmed to be between 1.8–2.0. cDNA synthesis was performed according to the protocol of the High-Capacity RNA-to-cDNA Kit (4387406, Applied Biosystems, Foster City, CA, USA). The cDNA was subjected to amplification in a 10 μL reaction volume using Applied Biosystems™ Power SYBR™ Green PCR Master Mix (4367659, Thermo Fisher Scientific, Waltham, MA, USA), along with 1 pmol of each primer set specified as follows: 5'-GTGCCCTCAGTGAGTTCGAT-3' (forward) and 5'-TCCAGACGGTTCCCTTTGTC-3' (reverse) for *PAX7*; 5'-AGTTCGGGGACGAGTTTGAG-3' (forward) and 5'-TCAAACGCCTGGTTGACCTT-3' (reverse) for *MYF5*; 5'-CTGCCCAAGGTGGAAATCCT-3' (forward) and 5'-GGGGGCCGCTATAATCCATC-3' (reverse) for *MYOD1*; 5'-GAGCTGTATGAGACATCCCCC-3' (forward) and 5'-GTGGACGGGCAGGTAGTTTT-3' (reverse) for *MYOG*; 5'-TTGACTGGGCTGCCATCAAT-3' (forward) and 5'-GCCTCAATGCGCTCCTTTTC-3' (reverse) for *MYH1*; 5'-CATTGAGGCCCAGAATAGGC-3' (forward) and 5'-TGCTTCCGTCTTCACTGTCAC-3' (reverse) for *MYH2*; 5'-GACTCTGGCTTTCCTCTTTGC-3' (forward) and 5'-GAGCTGACACGGTCTGGAAA-3' (reverse) for *MYH4*; 5'-CGTGGACTACAACATCATAGGC-3' (forward) and 5'-CCTTCTCAACAGGTGTGTCG-3' (reverse) for *MYH7*; 5'-

TGCTCCTCCCCGTTCGAC-3’ (forward) and 5′-ATGCGGCCAAATCCGTTC-3’ (reverse) for *GAPDH*; 5’-CCGGGACCTGACCGACTACC-3’ (forward) and 5’-TCGAAGTCCAGGGCGACGTA-3’ (reverse) for *ACTB*. Amplification and detection were performed using the Biorad CFX96 Real Time PCR Machine (Biorad, Hercules, CA, USA). Relative expression levels were calculated by normalizing the threshold cycle values of each gene to the geometric mean of the two-reference genes, *GAPDH* and *ACTB*. Specifically, for each sample, ΔCt was calculated as Ct (target) – Ct (ref_geo), where the Ct (ref_geo) equals the geometric mean of Ct (*GAPDH*) and Ct (*ACTB*).

Immunohistochemistry

For immunohistochemistry, the skeletal muscle tissues of three different anatomical origins were cut into 1 × 1 × 0.5 cm^3 pieces, immersed in 4% PFA, and stored at 4°C overnight for fixation. Following this, paraffin embedding was carried out to create a paraffin block. After the production of tissue sections, deparaffinization, and rehydration were performed. For antigen retrieval, the sections were immersed in 1 mM ethylenediaminetetraacetic acid (EDTA) buffer at 95°C for 10 minutes. Samples were then incubated with primary antibodies (5 μg/mL in DPBS, BA-D5 for type 1 muscle fibers, SC-71 for types 2a and 2x muscle fibers, and BF-F3 for type 2b muscle fibers; DSHB, Iowa City, IA, USA) overnight at 4°C. Secondary antibodies (1:1000; A-21140, A21121, A21426, Invitrogen, Waltham, MA, USA) were applied for 1 hour at room temperature. Sample images were captured using an inverted fluorescence microscope (Eclipse TE2000-U, Nikon, Konan). To analyze the proportion (%) of each muscle fiber type in each skeletal muscle, the number of stained muscle fibers was divided by the total number of muscle fibers. The number of muscle fibers was manually counted from the captured images.

Statistical analysis

For this study, cells were isolated from three independent animals (biological replicates, n = 3), and each biological replicate was assayed in triplicate (technical replicates). Data are presented as mean ± standard error of the mean (SEM). Between-muscle differences were evaluated using a one-way general linear model with Muscle (three levels: TB, LD, TF) as the fixed factor, the experimental unit was the piglet, and when multiple fields or sections were acquired, values were averaged per muscle per piglet before analysis. Post hoc comparisons employed Tukey’s multiple-range test with a significance threshold of $p < 0.05$. All analyses were performed in SAS (version 9.4; SAS Institute Inc., Cary, NC, USA).

Results

Muscle fiber type composition of pig muscle tissues

To determine whether intrinsic differences in muscle fiber composition exist among the three anatomically distinct skeletal muscles at an early postnatal stage, we first performed immunofluorescence staining for myosin heavy chain isoforms specific to type 1 (slow-twitch), type 2a/2x (oxidative fast-twitch), and type 2b (glycolytic fast-twitch) fibers (Figure 1A). Representative images from TB, LD, and TF muscles showed that type 1 fibers (blue) and type 2a/2x fibers (green) were predominant in all muscle types. Notably, type 2b fibers (red) were absent in all samples examined.

Quantitative analysis revealed no statistically significant differences in the relative proportions of type 1 and type 2a/2x fibers among TB, LD, and TF muscles (Figure 1B). Type 1 fibers accounted for approximately 42–44% of total fibers, while type 2a/2x fibers comprised around 55–57%, with no observable type 2b fiber presence across all groups. These findings are consistent with the developmental stage of the piglets, as previous studies have reported that type 2b fiber specification occurs later in postnatal development. The absence of mature type 2b fibers at this stage supports the interpretation that these muscles have not yet completed fiber-type maturation.

Together, these results confirm that the three muscles analyzed, despite differences in anatomical location and function, share a similar muscle fiber composition at 2 weeks of age. This uniformity in fiber type distribution reduces a potential confounding variable and ensures that subsequent analyses of MuSC characteristics are not biased by pre-existing differences in the muscle microenvironment.

Isolation efficiency and marker expression of pig MuSCs

To evaluate the efficiency and consistency of MuSC isolation across different anatomical origins, we employed enzymatic dissociation followed by magnetic-activated cell sorting (MACS), using CD29 as a MuSC surface marker [11]. $CD29^+$ and $CD29^-$ cell fractions were successfully isolated from the TB, LD, and TF muscles (Figure 2A). Quantification of total isolated cells revealed no significant differences among the three muscle types ($p > 0.05$; Figure 2B). However, the proportion of $CD29^+$ cells differed significantly depending on muscle origin. TB showed the highest percentage of $CD29^+$ cells (~35%), followed by TF (~28%), whereas LD displayed the lowest (~20%) ($p < 0.05$; Figure 2C). These differences were also reflected in the absolute number of $CD29^+$ cells per gram of muscle tissue (Figure 2D).

To confirm the identity of the $CD29^+$ cells as MuSCs, immunofluorescence staining was performed for CD29 and CD56, two canonical markers of porcine MuSCs. All $CD29^+$ cell populations from TB, LD, and TF expressed both markers, with comparable cell morphology across groups (Figure 3). This confirms that the isolated cells are indeed MuSCs, regardless of anatomical origin. These results indicate that while the abundance of $CD29^+$ MuSCs varies significantly between muscles, their phenotypic identity is conserved. The lower MuSC proportion observed in the LD muscle is consistent with previous

studies reporting regional differences in MuSC density [14] and underscores the importance of anatomical source selection in studies of muscle stem cell biology and applications in biomanufacturing.

In vitro proliferation and differentiation capacity of pig MuSCs

To investigate whether MuSCs isolated from different skeletal muscles exhibit distinct growth behavior *in vitro*, we first assessed their proliferation capacity. $CD29^+$ MuSCs (5×10^4 cells) were seeded in 12-well plates and cultured for 5 days (Figure 4A). Cell numbers were quantified at 12-hour intervals. As shown in Figure 4B, all groups demonstrated comparable growth performances, with no statistically significant differences in cell number at any time point. Doubling time calculations further confirmed that MuSCs from TB, LD, and TF shared similar proliferative capacities (Figure 4C).

To evaluate their myogenic differentiation potential, MuSCs were cultured under low-serum conditions to induce myotube formation. Preliminary observations indicated that differentiation progressed effectively up to day 3. However, by day 4, partial detachment of cells from the culture surface was observed in all groups (Figure 5). Consequently, all subsequent analyses were conducted on day 3 to ensure consistency and data reliability.

Immunofluorescence staining for myosin heavy chain (MHC) revealed extensive myotube formation in all muscle-derived MuSC cultures (Figure 6A). Quantitative analysis showed that over 90% of cells were MHC-positive regardless of anatomical origin, with no significant intergroup differences (Figure 6B). These findings indicate that the differentiation capacity of MuSCs is maintained across distinct muscle types at this developmental stage.

Together, these results demonstrate that MuSCs isolated from TB, LD, and TF muscles exhibit comparable proliferative and myogenic differentiation capacities *in vitro*, suggesting that functional properties of MuSCs are preserved across anatomical origins under standardized culture conditions.

Myogenic gene expression profiles of pig MuSCs

To further characterize the myogenic progression of MuSCs, we examined the expression levels of key myogenic regulatory factors (*PAX7*, *MYF5*, *MYOD1*, *MYOG*) and myosin heavy chain (MyHC) isoform genes (*MYH1*, *MYH2*, *MYH4*, *MYH7*) during *in vitro* proliferation and differentiation (Figure 7).

PAX7 expression peaked on day 4 of proliferation (P d4) in all groups, indicating the active maintenance of the stem cell population prior to differentiation (Figure 7A). *MYF5* expression was highest at P d1 and declined thereafter (Figure 7B), while *MYOD1* expression gradually increased and reached its peak at D d2 (Figure 7C), reflecting ongoing lineage commitment during early differentiation. In contrast, *MYOG* expression sharply increased at D d1 and decreased thereafter, consistent with the onset and tapering of terminal differentiation (Figure 7D).

The expression profiles of MyHC isoform genes exhibited temporally distinct activation patterns. *MYH1* and *MYH2* showed peak expression at D d2, and *MYH4* expression increased notably at the same time point (Figures 7E–G). *MYH7* expression, indicative of slow-twitch fiber specification, rose from D d1 and peaked at D d2 (Figure 7H).

Although minor variations in expression levels were observed between muscle types, no statistically significant differences were detected at any time point. MuSCs derived from the TB, LD, and TF exhibited comparable transcriptional dynamics throughout the proliferation and differentiation process. These results suggest that, under standardized *in vitro* conditions, MuSCs from distinct anatomical origins undergo similar gene expression programs during myogenic progression.

Discussion

This study investigated whether the anatomical origin of skeletal muscle influences the biological characteristics of MuSCs during the early postnatal period. MuSCs were isolated from three anatomically distinct muscles (TB, LD, and TF) in 2-week-old piglets. Despite the known differences in function and fiber-type composition among these muscles in mature animals, MuSCs from all three regions exhibited highly comparable biological behaviors *in vitro*. Specifically, we observed similar proliferation performances, differentiation capacity, and gene expression profiles of myogenesis-related markers across all groups. Although the LD muscle yielded a significantly lower percentage of $CD29^+$ cells, no differences were detected in the downstream functional properties of these cells. Collectively, these findings suggest that during early postnatal development, the intrinsic developmental status of MuSCs predominates over tissue-specific factors, leading to a functionally synchronized myogenic phenotype across anatomically distinct skeletal muscles.

One of the key observations supporting this conclusion was the absence of type 2b muscle fibers in all three muscles examined. In adult pigs, each muscle displays a characteristic fiber-type composition that reflects its physiological role [25, 26]. For example, the LD muscle, which contributes to postural support and short-duration, high-force contractions, typically contains a high proportion of type 2b glycolytic fibers. In contrast, limb muscles such as TB and TF, which are engaged in more sustained activity, generally harbor a greater proportion of type 1 and type 2a oxidative fibers [13, 27]. The lack of such fiber-type specialization at 2 weeks of age is consistent with previous studies showing that fiber-type transitions are still underway during this developmental window [24]. Specifically, type 2b fibers emerge later in postnatal life, following the sequential conversion from type 1 to type 2a and type 2x. Our findings, therefore, reflect the immature status of the muscles examined and highlight the need to consider developmental timing when interpreting muscle histology or designing experiments involving muscle progenitor cells.

Although the proportion of $CD29^+$ MuSCs was significantly lower in LD muscle, the functional capacity of these cells was maintained. All $CD29^+$ populations expressed canonical MuSC markers (CD29 and CD56), proliferated at comparable rates, and formed myotubes with similar efficiency, as indicated by >90% MHC positivity after three days of differentiation. These results support the idea that stem cell yield and functional competence can be uncoupled, particularly during early development. This observation is also consistent with previous reports indicating that MuSC density may be influenced by factors such as metabolic demand, fiber-type composition, and regenerative necessity. Muscles that undergo frequent dynamic contraction or have higher oxidative capacity tend to maintain larger pools of quiescent MuSCs [14, 28]. In contrast, postural muscles like LD may require fewer regenerative events and thus sustain a smaller stem cell reservoir. Nevertheless, under standardized *in vitro* conditions, the intrinsic proliferative and differentiation programs of MuSCs appear to be preserved irrespective of their anatomical origin.

The transcriptional profiles of myogenic regulatory factor genes further support the functional equivalence of MuSCs from different muscles. *PAX7*, *MYF5*, and *MYOD1* were upregulated during proliferation and declined upon differentiation, while *MYOG* was transiently upregulated during the early phase of myotube formation. Similarly, MHC isoform expression changed over time in all groups. However, gene expression levels showed no significant differences between muscle types at any time points, indicating that the transcriptional responses during myogenesis were largely conserved across anatomical origins. These data suggest that MuSCs from distinct anatomical origins undergo a similar temporal sequence of transcriptional activation during early myogenesis when cultured *in vitro*, reflecting a common intrinsic program.

The lack of significant functional or molecular divergence among MuSCs from different anatomical regions in this study is likely attributable to the *in vitro* environment. *In vivo*, MuSCs reside within a complex and dynamic niche composed of myofibers, extracellular matrix, vascular and immune cells, and motor neurons, all of which provide spatially and temporally regulated cues that shape MuSC behavior [29, 30]. In contrast, monolayer culture conditions eliminate most of these environmental inputs, potentially masking context-dependent differences in MuSC phenotype. Therefore, while the data presented here provide strong evidence for the functional similarity of MuSCs from different muscles *in vitro*, these findings may not fully capture the heterogeneity that exists *in vivo*. Future studies should incorporate more physiologically relevant culture systems, such as 3D scaffolds, co-culture with niche-associated cells, or *in vivo* lineage tracing to evaluate how anatomical origin influences MuSC fate and behavior in situ. Moreover, longitudinal studies spanning multiple developmental stages may help identify the point at which anatomical specialization of MuSCs begins to emerge.

The results of this study have important implications for basic research and applied domains such as regenerative medicine and cultured-meat production. By showing that MuSCs isolated from different

muscles can be used largely interchangeably at two weeks of age, our findings provide flexibility in tissue sourcing and support the feasibility of scalable MuSC-based biomanufacturing. At the same time, the relative immaturity of MuSCs at this stage may limit their suitability for modeling adult muscle function or for generating fiber-type-specific tissues, underscoring the need to consider both anatomical origin and developmental stage when establishing muscle models for research or industrial use. Taken together, our data indicate that, at two weeks of age, anatomical origin exerts only a limited influence on MuSC properties in vitro, and that developmental timing is the dominant factor defining early postnatal myogenesis. Because we intentionally used a single breed to control for genetic background, these conclusions relate to the effects of anatomical origin during early development and should not be overly generalized to the breed. Future comparative studies across breeds, particularly those utilizing *in vivo* lineage tracing or 3D niche models, will be valuable in establishing translational relevance and elucidating how breed, age, and microenvironment interact to shape MuSC heterogeneity and functional specialization.

Conclusion

In summary, this study systematically compared the biological and molecular characteristics of muscle stem cells (MuSCs) derived from different anatomical locations in 2-week-old pigs. Despite the distinct physiological functions and developmental trajectories of the muscles examined, no significant differences were observed in myofiber composition, MuSC abundance, proliferative performance, or differentiation capacity under *in vitro* conditions. These findings suggest that, during early postnatal development, the functional properties of MuSCs are largely conserved across muscle types and primarily shaped by developmental stage rather than anatomical origin. However, the immature status of MuSCs at this age may limit their capacity to fully represent adult muscle characteristics. Therefore, future studies should incorporate both developmental stage and *in vivo* environmental context when evaluating MuSC heterogeneity and functional specialization. To gain a more comprehensive understanding of MuSC heterogeneity, future research should integrate mRNA expression profiling with protein-level analyses such as Western blotting or proteomics, which may reveal subtle yet functionally relevant differences that are not evident at the transcript level. The results presented here provide foundational data for the development of standardized muscle model systems and may contribute to advancing applications in muscle regeneration, disease modeling, and cultured meat production.

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Figure captions

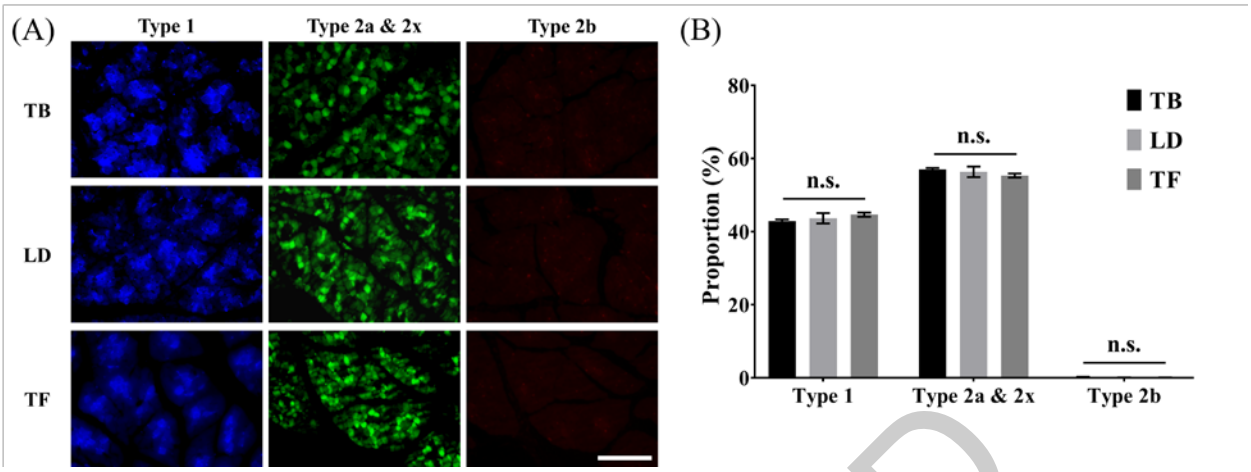

Fig. 1. Muscle fiber type distribution across the skeletal muscles of different anatomical regions. (A) Immunohistochemical staining for type 1 (blue), type 2a & 2x (green), and type 2b (red) fibers. (B) Comparative analysis of muscle fiber types. TB: *triceps brachii*, LD: *longissimus dorsi*, TF: *tensor fasciae*, n.s.: not significant. Data presented as mean ± SEM (n = 3). Scale bar = 200 μm.

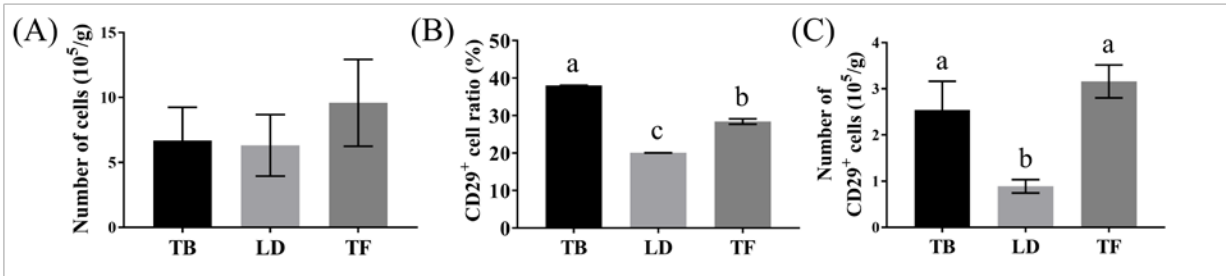

Fig. 2. Confirmation of porcine muscle stem cell isolation efficiency. (A) Number of cells isolated from 1 g of muscle tissue. (B) Proportion of CD29 positive cells within the isolated population. (C) Total number of CD29 positive cells that can be isolated from 1 g of muscle tissue. TB: *triceps brachii*, LD: *longissimus dorsi*, TF: *tensor fasciae*. Data presented as mean ± SEM (n = 3). $^{a\text{-}c}$ Different letters indicate significant differences ($P < 0.05$).

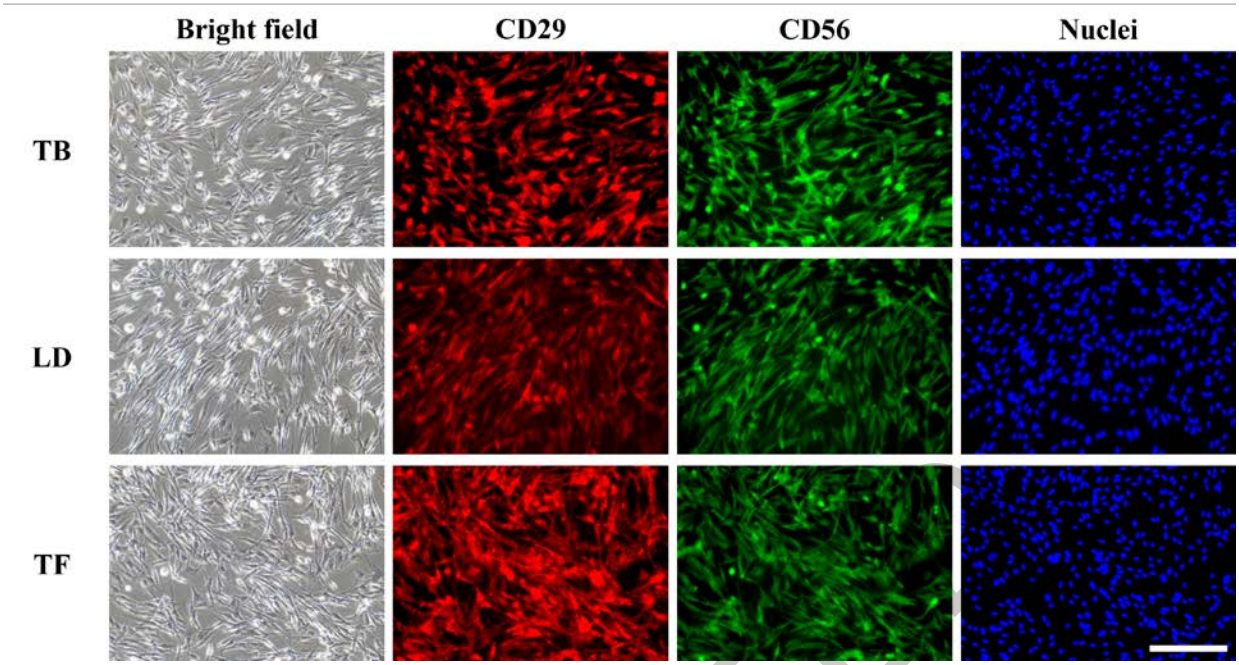

Fig. 3. Verification of muscle stem cell-specific cell surface marker. Immunofluorescence staining of CD29 (red) and CD56 (green) in muscle stem cell cultures. Nuclei were stained with Hoechst (blue). TB: *triceps brachii*, LD: *longissimus dorsi*, TF: *tensor fasciae*. Scale bar = 200 μm.

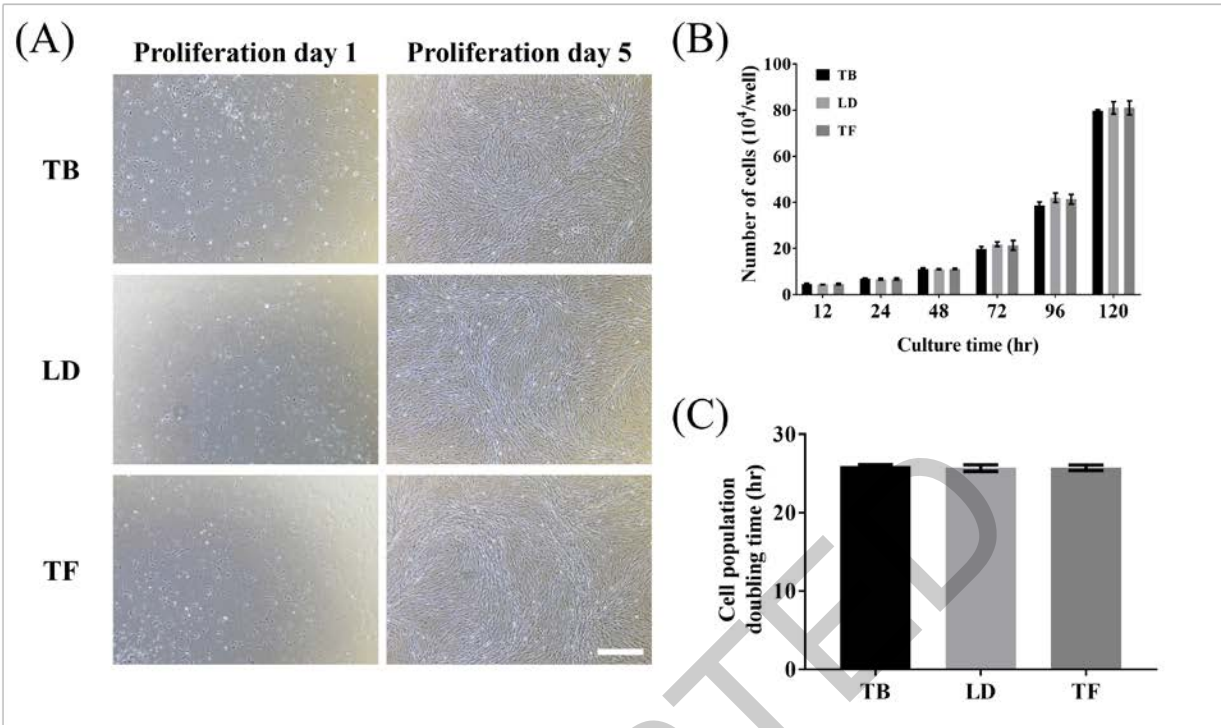

Fig. 4. Proliferation of muscle stem cells isolated from skeletal muscles of different anatomical regions. (A) Morphology of cultured cells at days 1 and 5. (B) Number of muscle stem cells from different portions of skeletal muscles during culture. (C) Doubling times of muscle stem cells from skeletal muscles of different anatomical regions. TB: *triceps brachii*, LD: *longissimus dorsi*, TF: *tensor fasciae*. Data presented as mean ± SEM (n = 3). Scale bar = 500 μm.

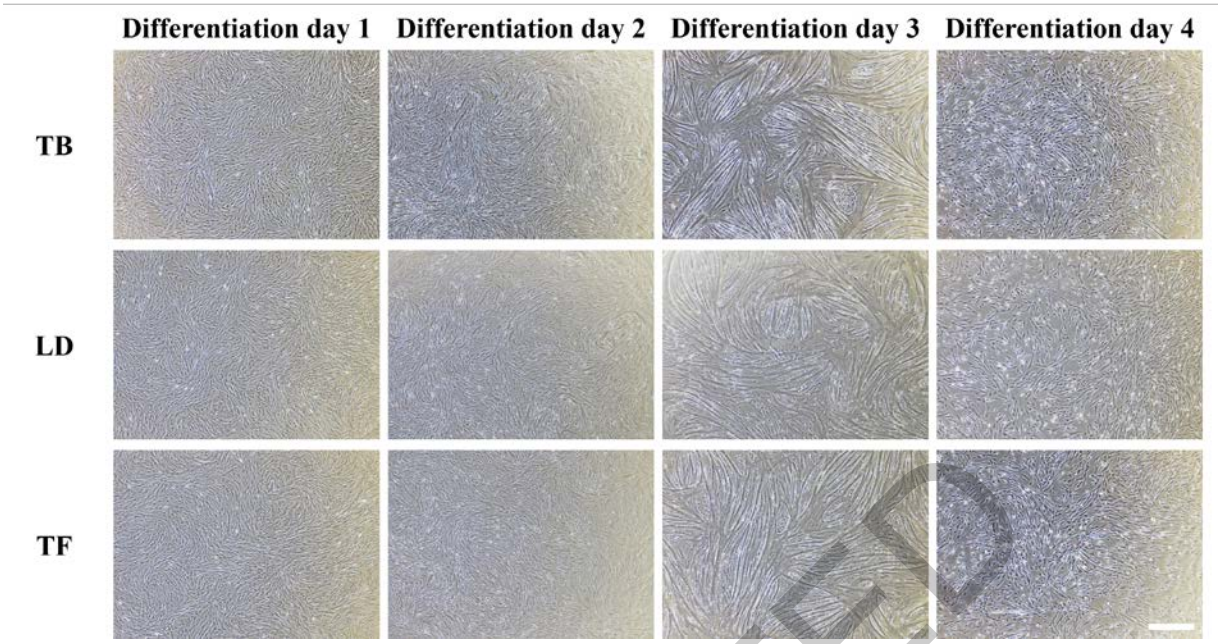

Fig. 5. Myotube formation in muscle stem cells isolated from skeletal muscles of different anatomical regions. Maximum myotube formation were confirmed on differentiation day 3, and myotube deprivation was confirmed on day 4. TB: *triceps brachii*, LD: *longissimus dorsi*, TF: *tensor fasciae*. Scale bar = 500 μm.

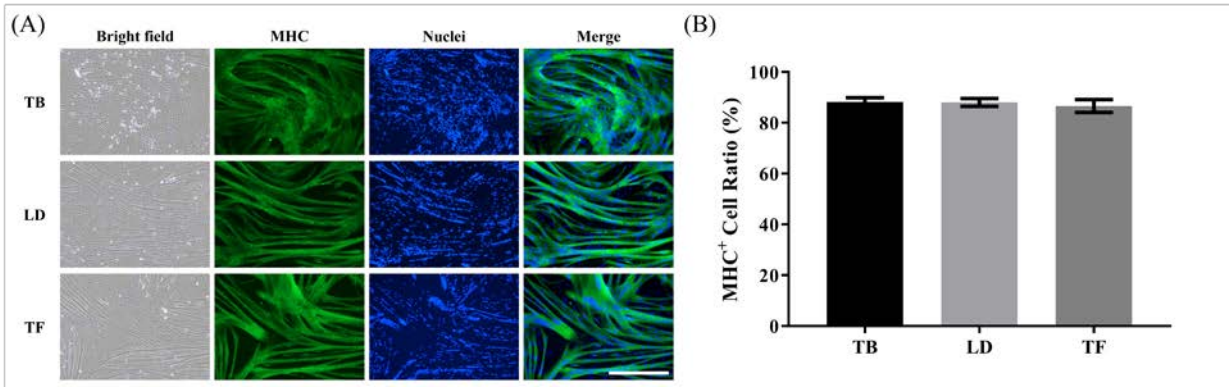

Fig. 6. Differentiation in muscle stem cells isolated from skeletal muscles of different anatomical regions. (A) Cell morphology and immunofluorescence staining of myosin heavy chain (MHC, green) in muscle stem cells. Nuclei were stained with Hoechst (blue). (B) Percentage of MHC positive cells within the muscle stem cell population. Cells were stained at day 3 of differentiation. TB: *triceps brachii*, LD: *longissimus dorsi*, TF: *tensor fasciae*. Data presented as mean ± SEM (n = 3). Scale bar = 500 µm.

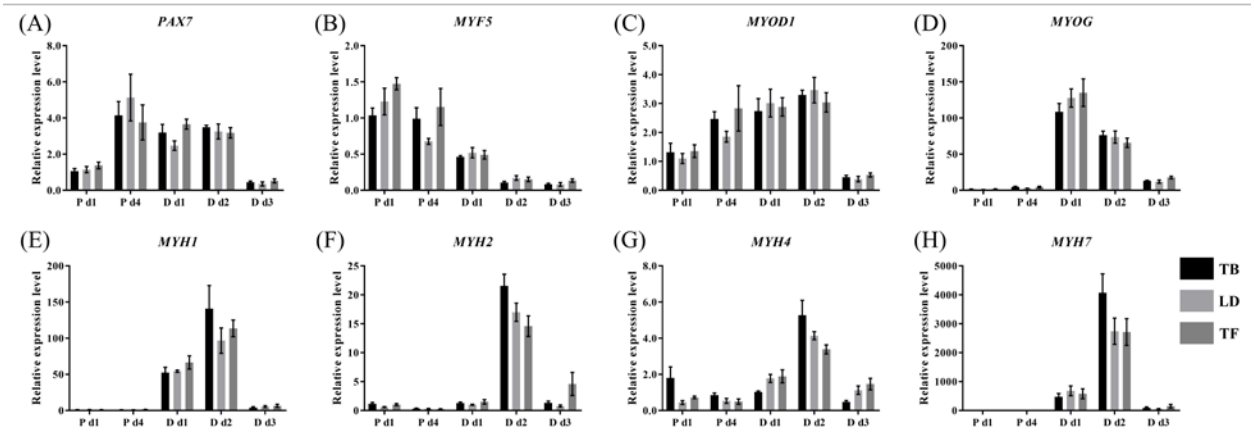

Fig. 7. The mRNA expression patterns of myogenic regulatory factors in muscle stem cells isolated from skeletal muscles of different anatomical regions during culture. The expression patterns of (A) *PAX7*, (B) *MYF5*, (C) *MYOD1*, (D) *MYOG*, (E) *MYH1*, (F) *MYH2*, (G) *MYH4* and (H) *MYH7*. All mRNA expression levels were normalized to the geometric mean of the expression levels of *GAPDH* and *ACTB*. On the X axis, P stands for proliferation period, and D stands for differentiation period. TB: *triceps brachii*, LD: *longissimus dorsi*, TF: *tensor fasciae*. Data presented as mean ± SEM (n = 3).