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Effect of Chicken Age on Proliferation and Differentiation Abilities of Muscle Stem Cells and Nutritional Characteristics of Cultured Meat Tissue

Abstract

This study aimed to investigate effects of chicken age on proliferation and differentiation capacity of muscle satellite cells (MSCs) and to determine total amino acid contents of cultured meat (CM) produced. Chicken MSCs (cMSCs) were isolated from hindlimb muscles of broiler chickens at 5-week-old (5W) and 19-embryonic-day (19ED), respectively. Proliferation abilities (population doubling time and cell counting kit 8) of cMSCs from 19ED were significantly higher than those from 5W ($p < 0.05$). Likewise, both myotube formation area and expression of myosin heavy chain heavy of cMSCs from 19ED were significantly higher than those from 5W ($p < 0.05$). After cMSCs were serially subcultured for long-term cultivation in 2D flasks to produce cultured meat tissue (CMT), total amino acid contents of CMT showed no significant difference between 5W and 19ED chickens ($p > 0.05$). This finding suggests that cMSCs from chicken embryos are more suitable for improving the production efficiency of CM than those derived from young chickens.

Keywords: muscle stem cell, chicken age, cultured meat, total amino acid

1. Introduction

Cultured meat (CM), an advanced meat technology where animal-derived stem cells are cultured on a large scale *in vitro* to produce meat products, has attracted attention as a promising alternative meat source. CM arises from adverse points associated with conventional livestock involving greenhouse gas emissions, resource inefficiency, animal welfare, and public health (Post, 2012). The CM industry is expected to have higher eco-friendly impacts than conventional meat from livestock as technology develops (Hong et al., 2021, Sinke et al., 2023). Muscle satellite cells (MSCs), myogenic stem cells located in muscle fibers, serve as the primary cell source for CM production. MSCs progress through a cascade of myogenesis for muscle generation in response to injury (Lepper et al., 2011). MSCs are activated for myogenic progression, involving a cascade of proliferation, differentiation, and fusion to form matured myofibers. A train of process muscle generated is regulated by myogenic transcription factors such as paired box protein 7 (pax7) and myogenic regulatory factors (Schmidt et al., 2019).

Current CM production faces challenges in terms of cost and efficiency. The transition from traditional 2D culture to 3D culture has been proposed as a method to address the issue of CM production (Ben-Arye et al., 2020, Alam et al., 2024, Kang et al., 2021;Pasitka et al., 2023). In addition, efforts are being made to accelerate the construction of serum-free media to address high costs of CM production (Messmer et al., 2022; Stout et al., 2022; Yu et al., 2023). However, fundamentally, research on the capabilities of the cells central to cultured meat production has not been extensively conducted. There are various types of cells that can be utilized in cultured meat production, and it is important to understand the characteristics of each cell type to effectively utilize them in production (Kang et al. 2024). In particular, the quality of the final cultured meat product as a food is expected to be determined by the diverse

characteristics of the cells and the choice of cell types used in the industry.

Meat quality could be influenced by several factors, including breed, sex, growth rate, nutrition, muscle types, and temperature (Joo et al., 2013). Similarly, the quality of CM can be determined by the characteristics of the cells used such as growth rate, nutrition and taste etc. Previous studies showed the difference in MSCs characteristic containing proliferation and differentiation capacities with bovine and porcine age (Kim et al., 2023; Mesires & Doumit, 2002). These capacities of MSCs could be changed by the age of the livestock, potentially affecting the production and quality of CM. However, there is a lack of studies on the impact of age on characteristics of chicken muscle stem cells (cMSCs) relatively than those of mammal cells. Also, research exploring the nutritional value of the chicken CM produced has to be conducted, which could be critical factors for CM. In particular, since the taste and nutritional components of cultured meat differ significantly from those of conventional meat, analysing amino acid profiles is necessary for good quality of cultured meat as food (Joo et al., 2022). We hypothesized that there would be variations in proliferation and differentiation capacities of MSCs and amino acid contents of chicken cultured meat tissue (CMT) based on the chicken age. Therefore, this study aimed to investigate effect of chicken age on proliferation and differentiation capacities and amino acid contents of CMT.

2. Materials & Methods

2.1. Animal care and experimental ethics

Procedures of animal use and treatment were approved by the Institutional Animal Care and Use Committee (IACUC) of Gyeongsang National University (approval no. *GNU-231017-C0196*). We followed all experimental processes in compliance with the IACUC

standard procedure.

2.2. Chicken muscle stem cell purification

Broiler chickens (Cobb) and their fertilized eggs were supplied by a local farm. The fertilized eggs were incubated under controlled conditions at 37.8°C and 80% relative humidity for a period of 19 days. As described below, chicken primary cells were isolated from 19-embryonic-day (19ED) and 5-week-old (5W) chickens each (Fig. 1A). Briefly, the hindlimb muscle was thoroughly sterilized with 70% ethanol and dissected into small pieces (~1 mm). These pieces were then treated with 0.1% collagenase type 1 (Worthington Biochemical, LS004194) at 37℃ for 1 hour with gentle shaking every 15 min. Dissociated tissues were then digested with 0.25% Trypsin-EDTA (Gibco, 25200072) for 10 min at 37℃. Dulbecco's Modified Eagle Medium (DMEM; Welgene, LM 001-08) supplemented with 10% fetal bovine serum (FBS; Welgene, S1-004) was added to cell suspension to block enzymatic digestion. The cell suspension was filtered with 100 and 40 μm cell strainers, centrifuged at 800×g for 5min, and treated with red blood cell lysis buffer (Invitrogen, 00443357) for 10 min at 4℃ to isolate primary cells. These cells were seeded into cell culture flasks in a CO_2 incubator, incubated at 41℃ with 5% CO_2, and purified with a pre-plating method (Kim et al., 2022) (Fig. 1B). In brief, isolated primary cells (pp1) were plated into a culture flask at a density of 5×10^6 cell/cm^2 and cultured for two hours. Suspension cells (pp2) were transferred into a new culture flask and cultured for one day. Next, adhesion cells (pp3) were detached with 0.05% trypsin-EDTA and cultured for two hours. Suspension cells (passage 1) were cultured for further sub-culture or cryopreservation.

2.3. cMSCs culture

cMSCs were cultured in growth media (GM) added with 20% fetal bovine serum (FBS), 1% GlutaMAX™ supplement (Gibco, 35050061), 1% antibiotic-antimycotic (Gibco, 15240062), 5 ng/mL basic fibroblast growth factor (R&D systems, 233-FB-025), and 10 μM p38 inhibitor (MedChemExpress, SB203580) in DMEM. These cells were seeded at a density of 5×10^3 cells/cm^2 on 0.1% collagen (Corning, 354236) coated flask. Cells were then subcultured or induced for differentiation when confluency reached over 70%.

2.4. Proliferation rate of cMSCs

EdU (5-ethynyl-2'-deoxyuridine) incorporation: EdU assay was conducted with a Click-iT Plus EdU Imaging Kit (Thermo Fisher Scientific, C10640) following the manufacturer's manual. Briefly, cMSCs were cultured on culture slides overnight and treated with 10 μM EdU for 2 h. Fluorescence images were obtained with a confocal laser scanning microscope (Nikon, AX R). Data were presented as means of five image fields randomly selected for statistical analysis.

Population doubling time (PDT): cMSCs were seeded into T25 flasks at a density of 5×10^3 cells/cm^2 and cultured for 3 days. The PDT was calculated using Equation 1:

$$PDT = \frac{dT}{log_2(\frac{N_1 - N_0}{N_0} + 1)}$$

Where PDT was population doubling time, N_0 was the number of cells at the beginning of culture, N_1 was the final number of cells following culture, and dT was the time of culture.

Cell counting kit-8 (CCK-8): cMSCs were seeded into 96-well plates at a density of 3

$\times 10^3$ cells/well and cultured overnight. The next day, 20 uL CCK-8 (Abbkine, KTA1020) was added to each well and the plate was incubated for 3 hours. UV absorbance was measured at 450 nm using a UV spectrophotometer (Thermo Fisher Scientific, Multiskan Sky).

2.5. Myogenic differentiation ability

cMSCs with confluency over 70% were differentiated with differentiation media (DM) containing 2% FBS, 1% GlutaMAX™ supplement, and 1% antibiotic-antimycotic in DMEM. The differentiation ability was calculated as fusion index indicating the percentage of nuclei in MHC-positive cells. Data are presented as mean of five randomly selected image fields for statistical analysis.

2.6. Immunofluorescence staining

cMSCs were fixed with 4% formaldehyde and permeabilized with 0.5% Triton X-100 for 15 min. These cells were blocked using 3% bovine serum albumin (BSA) in Dulbecco's Phosphate Buffered Saline (DPBS; Welgene, LB 001-02) at room temperature (RT) for 1 hour. They were then incubated with primary antibodies, mouse anti-pax7 (1:200; Santa Cruz Biotechnology, sc-81648) and mouse anti–myosin heavy chain (1:40; Developmental Studies Hybridoma Bank, MF20), at 4℃ overnight. They were then incubated with a secondary antibody at RT for 2 hours. Finally, cells were mounted using mounting media and DAPI and visualized using a fluorescence microscope (Olympus, CKX53).

2.7. Western blotting

cMSCs were lysed with RIPA lysis buffer (GenDEPOT, R4100-010) and harvested with a cell scraper on ice. Protein samples were electrophoresed on 10% SDS-PAGE and then transferred to 0.45 μm PVDF membrane using semi-dry blotting (Thermo Fisher Scientific, PB0013). The membrane was blocked using 3% BSA for 1 hour at RT and incubated with primary antibodies against myosin heavy chain (1:20) and beta actin (1:10000; Thermo Fisher Scientific, AC-15) overnight at 4℃. The membrane was incubated with HRP-conjugated secondary antibodies (1:10000; GenDEPOT, SA001-500) at RT for 2 h. The signal band was detected by ECL solution and visualized with a chemiluminescence imager (Thermo Fisher Scientific, A44116)

2.8. Fabrication of CMT

cMSCs were sub-cultured to reach 7 passages. They were then plated into ten 175T flasks at a density of 5×10^3 cells/cm^2 to obtain CMT. When the cell confluency reached over 70%, the culture medium was replaced with DM and cells were cultured for 3 days to induce myogenic differentiation. The CMT was harvested by physically detaching it using a cell scraper, followed by centrifugation at 1,000×g for 5 min and washing three times with DPBS.

2.9. Total amino acid contents

The CMT was freeze-dried for 24 hours. Then 10 mg of lyophilized sample was hydrolyzed with 6N HCl in a dry oven at 110℃ for 16 h. After evaporating the solution, the hydrolysate was diluted with 0.1 N HCl and filtered through a 0.22 μm membrane filter. Total amino acid

was analyzed according to the amino acid analysis manual produced by Agilent (Henderson et al., 2000).

2.10. Statistical analysis

All statistical analyses were conducted using GraphPad Prism 10 version 10.0.2 (San Diego, CA, USA). Student's *t*-test was performed to compare two groups of data. Data were collected from three independent experiments and presented as mean ± standard deviation. Statistical significance was considered when p-value was less than 0.05 ($p < 0.05$).

3. Results and Discussion

3.1. Purification of cMSCs from two different growth stage

To purify cMSCs, chicken hindlimb muscles were dissociated with collagenase. Isolated cells were sorted using a pre-plating technique (Fig. 1A and 1B). These isolated cMSCs were identified with pax7, a specific marker of muscle stem cells, to confirm the purity of cMSCs in isolated groups (Fig. 1C). The purity of cMSCs was observed to be at 90% in both of groups ($p > 0.05$). Pre-plating is a simple and low-damaging method of isolating cells using different cell adhesion speeds of cells to be purified. This technique allows purification of MSCs from many species by slightly applying various time processes. In this study, cMSCs with a high purity in both groups were obtained with the above method. The total cell number after isolation showed a significant difference ($p < 0.001$) between 19ED and 5W groups (Fig. 1E).

MSCs are currently the main cell source used to produce CM (Kang et al., 2024; Lee &

Choi, 2024). In previous studies, pools of MSCs varied with age. Previous studies have reported that the number of MSCs is decreased with age in mammals, including humans, mice, porcine, and bovine (Kim et al., 2023; Mesires & Doumit, 2002; Ogura et al., 2020; Verdijk et al., 2014). The present study showed that the number of MSCs decreased with age in chickens. This indicates that MSCs from embryos could allow more MSCs to be obtained from the same muscle mass. Thus, less time is required to obtain sufficient MSCs. MSCs of avians can be obtained with relatively easier preparation than mammals even at the embryonic stage. However, despite the variety of biopsy methods available, performing a biopsy on small chick embryos can inflict fatal damage. From an animal ethics perspective, which method is more ethical between aged animals at post-natal and embryos at pre-natal to obtain avian cell sources for CM production should be further discussed.

3.2. Proliferation ability of cMSCs

To measure the proliferation ability of cMSCs, we cultured cMSCs in GM for 3 days (Fig. 2A). The percentage of EdU-positive cells among total cells was not significantly ($p > 0.05$) higher in the 19ED group than in the 5W group (Fig. 2B and 2C). However, the PDT was significantly ($p < 0.05$) lower in the 19ED group than in the 5W group (Fig. 2D). There was no significant difference in absorbance at 450 nm between 1 day of culture and 2 days of culture, whereas a significant difference ($p < 0.05$) was found after 3 days of culture between 19ED and 5W groups. This result indicated that cMSCs isolated from 19ED showed a higher proliferative ability than those isolated from 5W.

The proliferative ability of cMSCs is a crucial parameter as it is correlated with the yield and cost of CM production. The proliferative ability of cMSCs is a crucial parameter as it is

correlated with the yield and cost of CM production. According to Zelenyuk, productivity is negatively correlated with the cost and time required for production (Zelenyuk, 2023). This implies that higher productivity can be achieved from the greater proliferative capacity of cMSCs, indicating in lower input of time and materials. The proliferative capacity of the cells could be directly related to productivity as time input means doubling time of the cells in this study (Hauser et al., 2024). When CM was first introduced in 2013 by Mark Post, the production cost was about $300,000 (Post, 2014). The culture medium accounts for the largest portion of CM production cost. As the technology and process of cultured media production are further developed, the production cost of cultured media could reach a maximum of $0.24/L (Garrison et al., 2022; Specht & Scientist, 2020). However, for the CM market to grow into a competitive industry, not only culture media, but also labor costs and equipment should be significantly reduced (Garrison et al., 2022). A strategy to reduce production costs could include selecting cell sources with high proliferative capacity as a cell source for CM. MSCs with high proliferative ability could produce a greater quantity of CM per unit of culture medium, reducing the cost of production per kilogram. In studies using primary MSCs, animal age could directly affect CM production yield. This is because regenerative and proliferative abilities of MSCs are decreased with increasing age due to age-related loss of function (Liu et al., 2022). Some research studies about CM have argued about the relation between animal age and the proliferation capacity of MSC (Ono et al., 2010; Parker, 2015; Yin et al., 2013). In bovine, the proliferative capacity of MSCs derived from younger calves exhibits markedly high proliferative potential with up-regulated expression levels of key myogenic genes including pax7, myoD, and myoG (Kim et al., 2023). $NCAM^+$ MSCs showed a decrease in proliferative capacity as the age of porcine increased from 1 to 21 weeks old (Mesires & Doumit, 2002). In this study, embryonic-derived cMSCs had a higher proliferative capacity than adult-derived ones. It means that

cMSCs have potential to enhance the production efficiency of CM products.

3.3. Differentiation capacity of cMSCs

Growth medium for cultured cMSCs was replaced with DM and cells were induced to undergo myogenic differentiation for three days. MSCs were fused and elongated on day 1 and differentiated into myotubes over three days (Fig. 3A). Myogenic differentiation exhibited accelerated progression for MSCs derived from 19ED. Differentiated myofibers were stained with MHC, a muscle-specific marker. Myotube formation area (MFA) was then measured to determine myogenic differentiation ability. Results showed a significantly ($p < 0.05$) higher MFA in MSCs derived from 19ED than those from 5W (Fig. 3B and 3C). In addition, the relative expression level of MHC was observed to be significantly ($p < 0.05$) lower in 19ED than in 5W (Fig. 3D and 3E).

The age at which animals are slaughtered impacts meat quality such as texture, sensory, and nutritional value (Baéza et al., 2012; Li et al., 2021). Broiler chickens generally undergo muscle growth, differentiation, and development during a rearing period of 5-7 weeks after hatching, ultimately being processed into meat. Over the duration of chicken growth, both elongation and enlargement of myofiber could control muscle growth, especially enlargement of muscle fibers in diameter of muscle fibers, which continued until 35 weeks of age (Ono et al., 1993). Additionally, meat quality could vary depending on the growth stage and slaughter time of chicken (Baéza et al., 2012). A significant portion of differences in muscle fiber growth and meat quality characteristics can be attributed to the growth and differentiation of myofibers. Therefore, muscle fiber growth and differentiation might also play a crucial role in CM. However, there is a lack of research on how these differentiation

properties affect the quality of CM and whether they play a pivotal role in precisely mimicking the flavor of conventional meat, as meat taste does not merely arise from muscle protein (Melzener et al., 2023). Thus, this study aimed to explore whether the level of muscle fiber growth and differentiation also could play a significant role in CM.

It was observed that after MSCs were induced to differentiate into myotubes *in vitro*, myotubes reached their maximum formation at 3 - 4 days of differentiation. However, myotubes were detached from culture flasks after 3 - 4 days, notwithstanding repeated experiments. This occurred because myotubes struggled to develop into fully matured muscle fibers in an *in vitro* culture They face challenges for long-term myogenic maturation due to either minor physical impacts or spontaneous detachment over time (Denes et al., 2019; Romagnoli et al., 2021). Thus, rapid differentiation could be time-efficient and economically advantageous for CM production by quickly forming a large number of myotubes. The process of CM production involves differentiation and maturation of animal cells, following a large-scale culture of cells proliferating in a bioreactor to fabricate the CM. A limit of our investigation lies in the absence of comparative analysis regarding differentiation into fully matured muscle fibers due to an insufficient duration of differentiation resulting from myotube detachment. In this aspect, we plan to research the differentiation potential when muscle fibers are fully matured through a long-term culture. Further studies are needed to develop techniques for differentiating MSCs into muscle fibers for long-term maturation, along with investigating culture duration required to limit.

3.4. Amino acid contents of CMT

The CMT was fabricated on large-scale culturing in 175T flasks and then harvested as

described below (Fig. 4A and 4B). The relative production yield (RPY) is a parameter comparing the relative weight of CMT produced per identical culture area. The RPY of CMT was significantly ($p < 0.05$) higher in the 5W group than in the 19ED group (Fig. 4D). Subsequently, we measured total amino acid contents in CMT produced by each group. Results revealed that amino acid contents showed no significant ($p > 0.05$) difference between the two groups (Fig. 4E).

Remarkably, the yield of CMT per culture area from 19ED chickens was lower than that from 5W, and the differentiation into muscle fibers was also less progressed relatively than 5W. This observation could potentially be ascribed to the fact that cMSCs from 19ED exhibited rapidly declined stemness and myogenic potential compared to those at 5W during long-term culture. However, this rationale could not entirely elucidate the pronounced decline in differentiation capabilities observed in cMSCs from 19ED. The main cause could be fibroblast contamination during cell sorting. The pre-plating isolation method revealed that fibroblasts proliferated at a pace exceeding that of muscle stem cells. In this study, extended culture periods for promoting excessive growth of these quicker proliferating fibroblasts might elucidate distinctive outcomes recorded for 19ED. Regarding the fabrication of CM, it is critical that stem cells retain their differentiation characteristics during long-term culture. Recent studies have focused on inducing spontaneous immortalization of primary cells or pluripotent stem cells as a strategy to overcome these limitations. In 2022, CM produced from immortalized chicken fibroblasts received approval from the FDA as no harmful factors for consumption were identified (Fasan et al., 2022). Therefore, further discussion should be given to the use of immortalized cell lines to ensure consistent maintenance of myogenic potential during long-term cultivation for cultured meat production.

Without a doubt, high-quality meat is one of the most nutritious foods available, containing 20-30% protein generally. It is also rich in essential amino acids that human beings require (Ahmad, 2018). In this study, total amino acid contents in CMT have been found to be lower than those of traditional meat in previous study (Bhawana et al., 2023). The differences are thought to be due to varying differentiation periods and levels of myogenic developmen of myofiber. However, in this experiment, there was no difference in amino acid content between 19ED and 5W, which were differentiated over the same period. This could be attributed to the relatively short-term cultivation for in vitro differentiation and maturation of MSCs. Namely, the period is too short to differentiate the MSCs and make the difference in amino acid contents with age. To bridge this nutritional gap, it becomes imperative to pioneer and implement technologies that ensure complete maturation of muscle stem cells into fully developed muscle fibers subsequent to their differentiation phase. Furthermore, since there was no significant difference in the amino acid composition between cultured tissues produced using the same amount of 19ED and 5W cells, it may be advantageous to use MSCs from 19ED, which proliferate and differentiate more rapidly, for cultured meat production.

4. Conclusions

cMSCs from 19ED had higher proliferation and differentiation capacities than those derived from 5W chickens, although there were no significant differences in total amino acid contents produced between the two groups. This finding suggests that cMSCs from 19ED could be suitable for enhancing the production efficiency of CMT. However, the stemness and

myogenic potential of cMSCs from 19ED diminished more rapidly than those from 5W during long-term cultivation. Therefore, it is necessary to maintain characteristics of MSCs over prolonged culture for CMT fabrication.

Author contribution

Conceptualization: Kim CJ, Joo ST; Data curation: Kim CJ, Kim SH, Lee SY, Joo ST; Formal analysis: Kim CJ, Kim SH; Methodology: Kim CJ, Kim SH, Hwang YH; Software: Lee EY; Validation: Kim SH; Investigation: Kim SH, Hwang YH; Writing - original draft: Kim CJ; Writing - review & editing: Kim CJ, Kim SH, Lee EY, Hwang YH, Lee SY, Joo ST

Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure Legends :

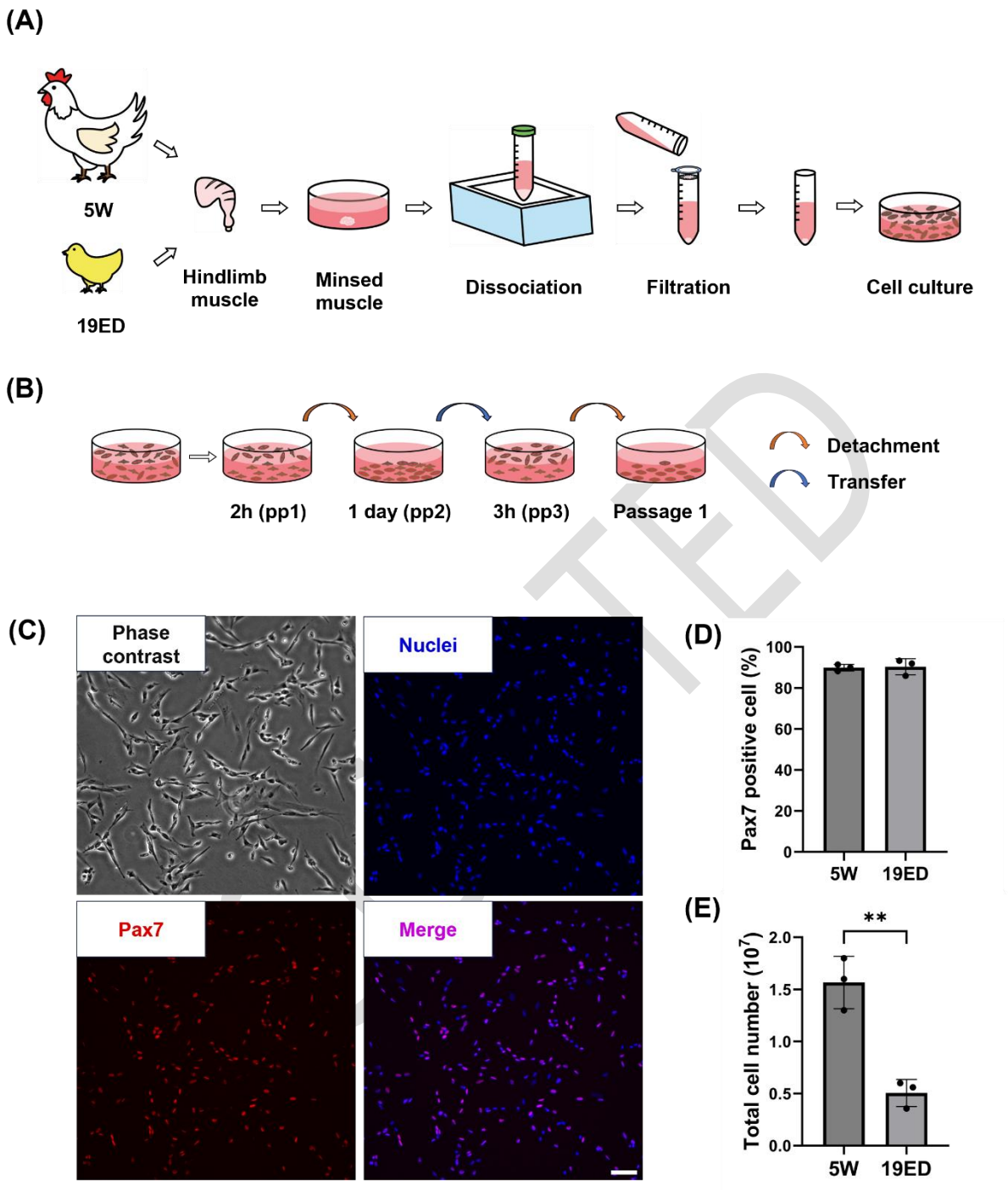
Figure 1. Purification of cMSCs from chicken hindlimb muscles. (A). Schematic illustration of muscle primary cell isolation from 5W and 19ED chickens. (B). Schematic illustration on purification of cMSCs by pre-plating technique. (C). Representative images for characterization of cMSCs; Upper right (nuclei; blue), lower left (pax7; red), lower right (merge; purple). Scale bar = 100 μm. (D). Pax7 positive cell percentage of 5W and 19ED chickens. (E). Total cell number of 5W and 19ED chickens after muscle primary cell isolation.

Figure 2. Proliferation ability of MSCs between 5W and 19ED chickens. (A) Representative image of cMSCs cultivation for 3 days for proliferation. (B) Representative image of EdU positive cell of cMSCs (nuclei = blue, EdU = cyan, and merge = sky). Scale bar = 100 μm. (C) EdU positive cell percentage of 5W and 19ED chickens. (D) Population doubling time of MSCs of 5W and 19ED chickens. (E) CCK-8 absorbance of MSCs during 3 days of culture between 5W and 19ED chickens.

Figure 3. Difference in myogenic differentiation of P3 MSCs between 19ED and 5W chickens. (A) Representative image of cMSCs cultivation during 3 days for myogenic differentiation. (B) Representative image of difference in myogenic differentiation of MSCs on differentiation for 3 days between 5W and 19ED chickens. Scale bar = 50 μm. (C) Myotube formation area of MSCs of 5W and 19ED chickens. (D) Representative western blot image of MHC expression between 5W and 19ED chickens. (E) Relative MHC expression of differentiated myofiber on 3 days between 5W and 19ED chickens.

Figure 4. Representative images of inducing differentiation of P7 satellite cells for manufactured CMT. (A) Schematic illustration of CMT fabrication using cMSCs. (B) A representative image showing differentiation of cMSCs after 5 days of cultivation until reaching 100% confluency for cultured meat production. Scale bar = 100 μm. (C) Representative image of

harvested CMT. (D) Relative production yield of CMT harvested in 5W and 19ED chickens. (E) Total amino acid contents of CMT produced in 5W and 19ED chickens.



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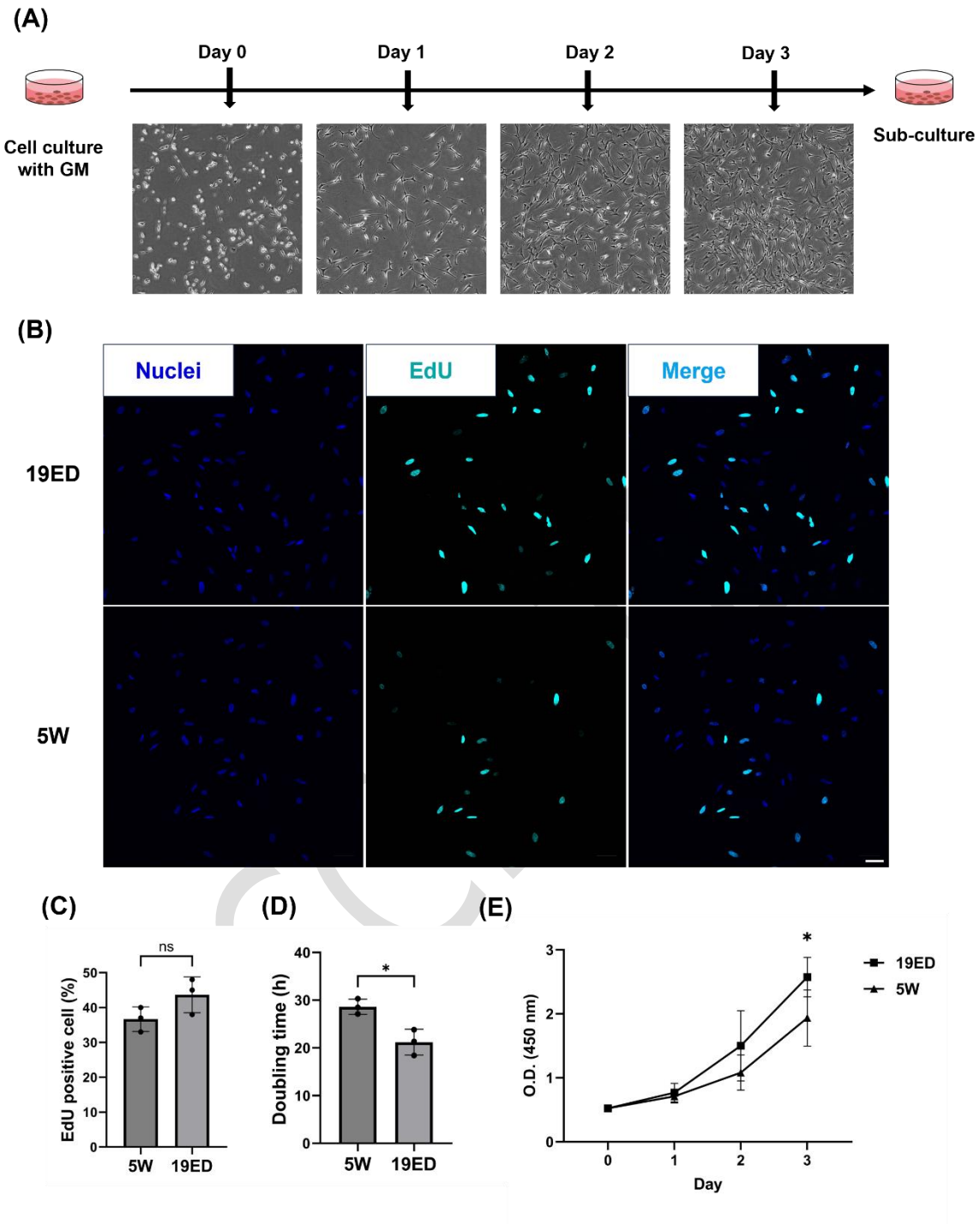

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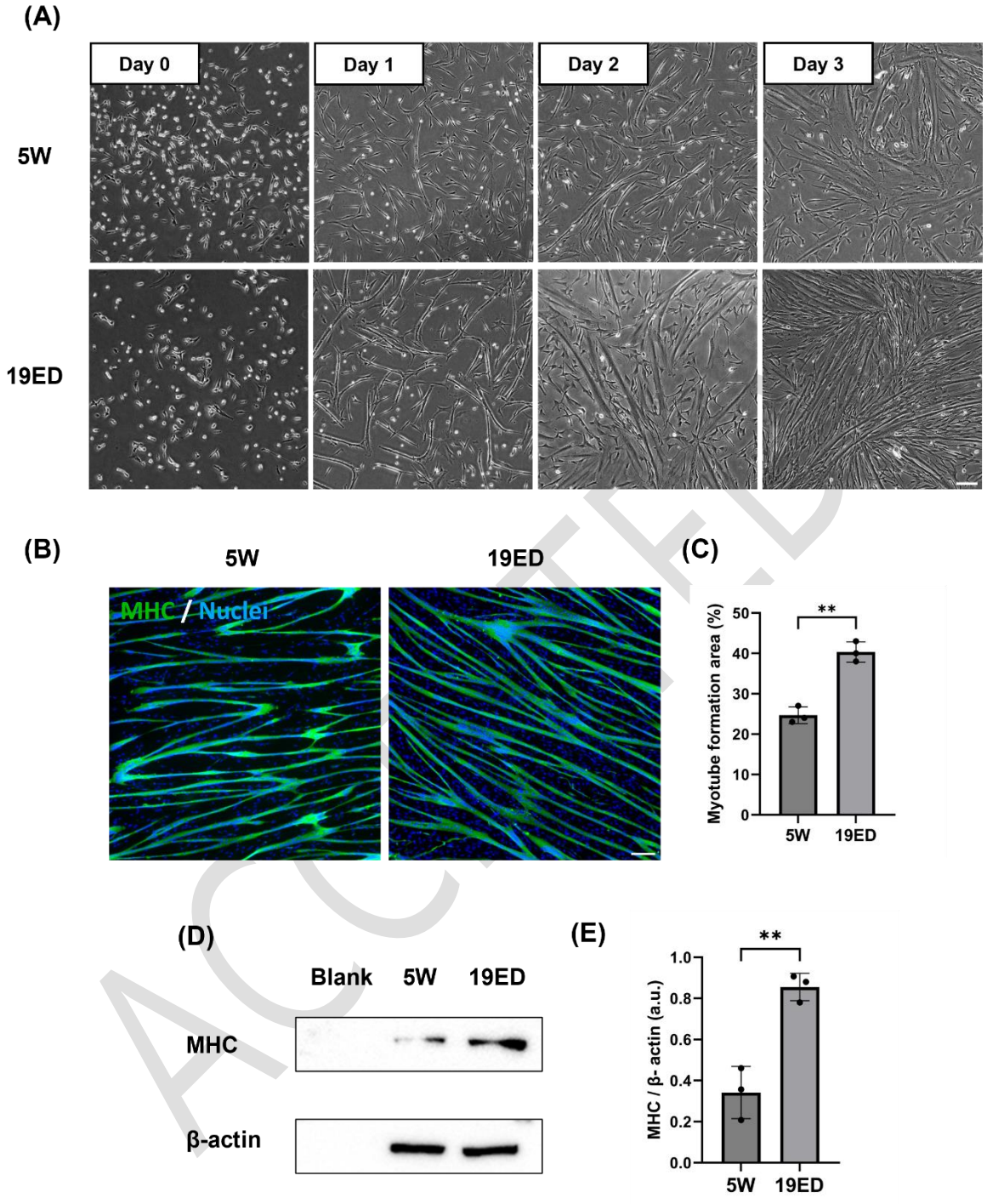

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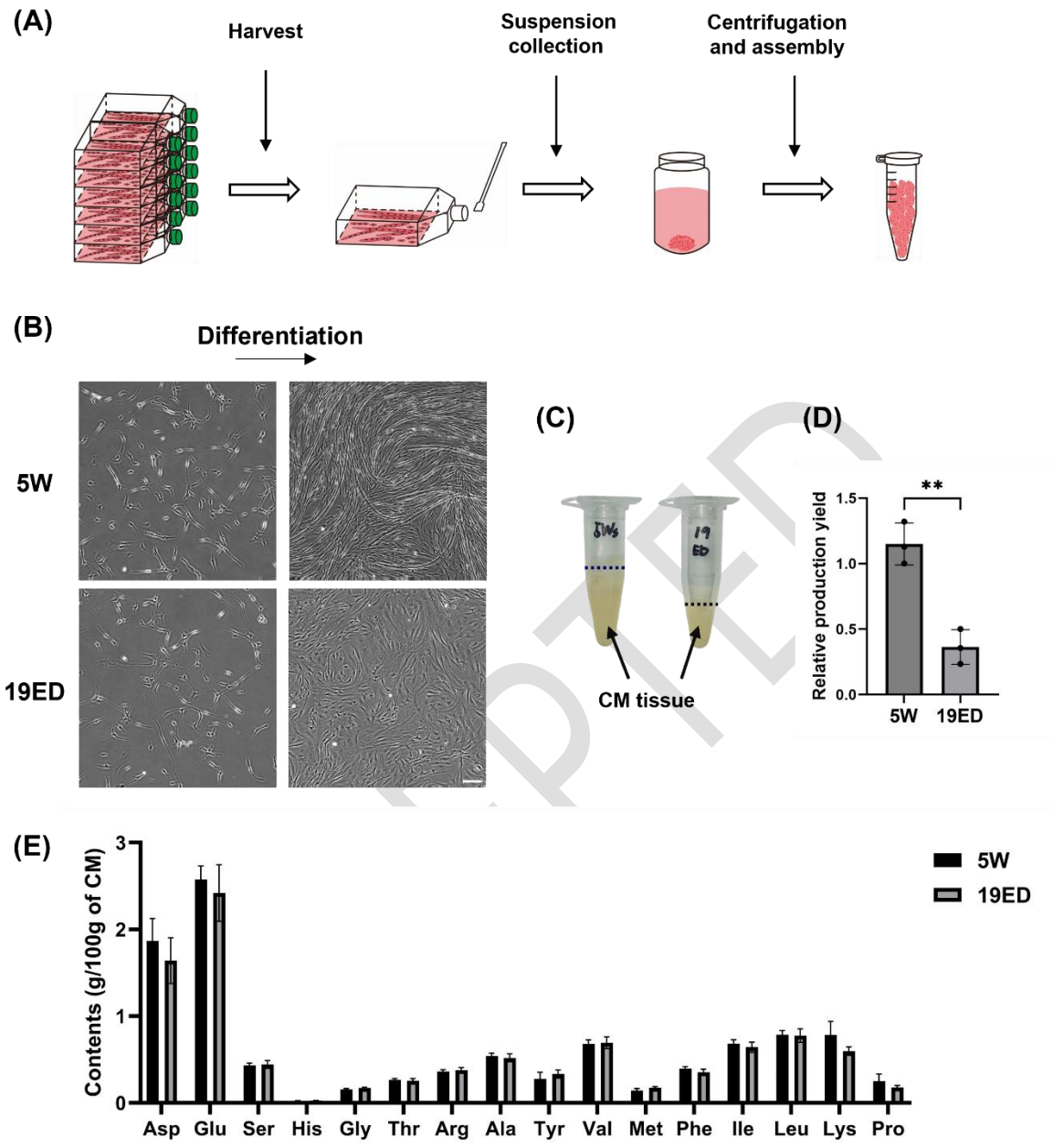

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