Matrix-Dependent Enhancement of Proliferation and Myogenesis in Chicken Muscle Stem Cell Monolayers and Their Taste-Related Metabolites

Running title: ECM Modulates Chicken Muscle Stem Cells Growth and Taste Metabolites

Minjun Kang[a,1], Minsu Kim[a,b,1], Hyun Young Jung[a], Eunil Kim[a], Soyoon Yun[a], Myung Yeo[a] and Cheorun Jo[a,b,c,d] *

[a]*Department of Agricultural Biotechnology, Seoul National University, Seoul 08826, Korea*
[b]*Center for Food and Bioconvergence, Seoul National University, Seoul 08826, Republic of Korea*
[c]*Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea*
[d]*Institute of Green Bio Science and Technology, Seoul National University, Pyeongchang, 25354 Gangwon-do, Republic of Korea*

[1] These authors contributed equally.

*To whom correspondence should be addressed:
Department of Agricultural Biotechnology, Seoul National University, Seoul 08826, Republic of Korea.
Phone: +82-2-880-4804; Fax: +82-2-873-2271; E-mail: cheorun@snu.ac.kr

Abstract

The choice of extracellular matrix (ECM) coating can profoundly affect the behavior of muscle stem cells (MuSCs), yet optimal substrates for cultured chicken meat remain unclear. Here, we compared collagen, fibronectin, fish gelatin, porcine gelatin, Geltrex, and laminin coatings for their ability to support proliferation and differentiation of MuSCs isolated from 18-day-old chicken embryos. Cells were expanded over passages 4–6 (three-day intervals) or induced to differentiate for four days at each passage. Serial passaging led to a consistent increase in the differentiating PAX7^{-}/MYOD^{+} population and a decrease in the self-renewing PAX7^{+}/MYOD^{+} cohort, regardless of coating, with only a late-passage difference between porcine and fish gelatin. During proliferation, *PAX7* mRNA remained stable across all substrates, whereas *MYOD* levels rose significantly on laminin and Geltrex at passages 5 and 6. Upon differentiation, laminin, followed closely by Geltrex, supported the thickest myotubes and the highest fusion indices, accompanied by elevated *MYOG* and *MYH1* expression. In contrast, analyses of free amino acids and nucleotides revealed no substantial coating-dependent differences, except for glycine, which was significantly higher in differentiated cells on laminin than on fish gelatin coatings. Nonetheless, cultured cells, regardless of substrate, retained profiles distinct from those of embryonic and adult chicken tissues. These data identify laminin as the most effective single-component coating for enhancing chicken MuSC expansion and myogenic maturation. However, the persistent gap in flavor-related metabolite content underscores the need to further optimize culture conditions—such as medium composition and differentiation protocols—to more closely recapitulate native muscle tissue.

Keywords: Chicken muscle stem cell, Extracellular matrix, Coating, Myogenesis, Free amino acid, Nucleotide compounds

Introduction

Cultured meat has emerged as a promising solution to meet growing global demand for protein while addressing resource scarcity, environmental impact, and animal welfare concerns [1]. By leveraging stem cell and tissue engineering technologies, cultured meat aims to replicate the taste, texture, and nutritional profile of conventional meat [2]. Achieving these goals requires not only an appropriate cell source and culture medium but also a scaffold that mimics the native muscle niche [3, 4].

Within this niche, the extracellular matrix (ECM), a network of proteins such as collagen, fibronectin, and laminin, provides critical biochemical and mechanical cues that regulate muscle stem cell (MuSC) behavior [5]. Collagen maintains tissue elasticity and stem cell quiescence [6], while its hydrolyzed form, gelatin, offers cost-effective biocompatibility and cell adhesion properties widely used in cultured meat scaffolds [7, 8]. Fibronectin promotes MuSC attachment and myofiber formation [9], and laminin, essential for myogenesis, cell alignment, and regeneration, activates key signaling pathways via α6 integrin [10]. Geltrex, a basement-membrane extract rich in laminin and other ECM components, further enhances MuSC attachment and differentiation *in vitro* [11]. Previous studies illustrate how ECM choice shapes MuSC outcomes: chitosan–alginate–collagen scaffolds improve adhesion and proliferation [12]; fibronectin-coated dishes drive a 23,600-fold expansion of bovine CD29$^+$ cells [13]; and laminin substrates yield more mature porcine myotubes than other coatings [14]. Yet, the optimal ECM varies by species and desired culture outcome. It should be noted, however, that Geltrex and laminin are not food-grade materials; therefore, their utility in cultured-meat applications will require translation to edible scaffolds such as gelatin, alginate, or plant/seaweed proteins.

Integrins, heterodimeric receptors that link ECM ligands to intracellular signaling, mediate these effects. In MuSCs, α1β1 engages collagen to support muscle development [15], α5β1 binds fibronectin during repair [16], and α6β1 anchors cells to laminin, promoting survival and regeneration [10]. Understanding these ECM–integrin interactions is therefore essential for scaffold design.

Beyond cell expansion and differentiation, flavor-related metabolites such as free amino acids and nucleotides critically determine meat taste. Cultured cells often exhibit altered metabolite profiles compared to native tissue, necessitating targeted analyses to guide process optimization [17].

In this study, we systematically evaluated five ECM coatings (collagen, gelatin, fibronectin, laminin, and Geltrex) for their ability to support proliferation, differentiation, and flavor-related metabolite accumulation in chicken MuSCs. By integrating cell-biology assays with metabolomic profiling, we aim to identify scaffold materials that maximize both the efficiency and sensory quality of cultured chicken meat.

Materials and methods

Animal care and use

This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University (Approval No.: SNU 230303-3). All experimental procedures followed the guidelines of the Institute of Laboratory Animal Resources at Seoul National University.

Cell harvesting

The isolation procedure was performed with reference to the method described in our previous study [18]. Fertilized eggs of the Hy-line breed were incubated at 37°C for 18 days, followed by euthanasia using CO_2. The pectoral muscles were harvested from chicken embryos, washed with

Dulbecco's phosphate-buffered saline (DPBS; Welgene, Gyeongsan, Korea) containing 2× antibiotic-antimycotic (AA; Gibco, Gaithersburg, MD, USA), and the fat, connective tissue, and blood vessels were removed. The muscles were minced using surgical scissors to a slurry consistency, followed by enzymatic digestion. Digestion was performed using 0.8 mg/mL pronase (Sigma-Aldrich, St. Louis, MO, USA) in DPBS at 37°C for 40 min, with vortexing every 10 min. The digested tissue was filtered through 100 µm and 40 µm cell strainers sequentially, and cells were collected by centrifugation at 1200×g for 3 min. The supernatant was discarded, and the cell pellet was resuspended in Dulbecco's Modified Eagle Medium F12 (DMEM-F12; Welgene) containing 20% (v/v) fetal bovine serum (FBS; Gibco), 1× AA (Gibco), 1× GlutaMAX™ (Gibco), and 1× β-mercaptoethanol (BME; Gibco). Cells were pre-plated for 1 h, and the supernatant containing MuSCs was collected. The isolated MuSCs were either used immediately for experiments or cryopreserved in liquid nitrogen using freezing medium containing 90% (v/v) FBS and 10% (v/v) dimethyl sulfoxide (Mylan, Dublin, Ireland).

Chicken muscle stem cell culture

The MuSCs were cultured on 0.1% (w/v) gelatin-coated dishes in growth medium (GM) consisting of DMEM-F12 with 20% (v/v) FBS, 1× AA, 20 µM SB203580 (Cayman, Ann Arbor, MI, USA), 1× GlutaMAX™, 10 ng/mL bFGF (R&D Systems, Minneapolis, MN, USA), and 1× BME at 43°C with 5% CO_2. This temperature was chosen based on previous studies reporting that 41 °C better supports proliferation and differentiation of avian muscle cells compared with the mammalian standard of 37 °C, and was further supported by our preliminary observations (data not shown), which indicated that MuSCs exhibited improved viability and proliferation at 43 °C relative to lower temperatures [19]. To obtain sufficient cell numbers, sub-culture was conducted every three days, with the experiment starting from passage 4.

Passage 4 cells were seeded at a density of 1.3×10^4 cells/cm² in each ECM-coated plate, as outlined in Table 1. Coating concentrations were selected based on the manufacturers' recommended ranges and our preliminary optimization experiments, which identified the doses that best supported chicken MuSC attachment and proliferation. All coatings were applied under their respective optimal conditions to ensure reproducible cell attachment. A total of 400 μL of coating solution was added to each well of a 12-well plate (SPL, Pocheon, Korea) and 200 μL to each well of a 24-well plate (SPL). The plates were gently agitated to ensure even distribution of the solution and incubated at 43°C with 5% CO_2 for 1 h. Cells were subcultured every three days for a total of three passages. Samples for proliferation analysis were collected on day 1 and 3. Differentiation was induced on day 3, when cells reached 80-90% confluency, using differentiation medium (DM) composed of DMEM-F12 with 10% (v/v) horse serum (HS; Gibco), 1× AA, 1× GlutaMAX™, and 1× BME. Differentiation was conducted for 4 days, with samples collected for qPCR and immunofluorescence staining.

Cell counting

On days 1 and 3 of proliferation, cells were incubated in 0.04% (v/v) trypsin (Welgene) in DPBS at 43°C with 5% CO_2 for 5 min followed by neutralization with DMEM-F12 containing 10% (v/v) FBS. The cell suspension was then centrifuged at 1200×g for 3 min, and the supernatant was discarded. The cell pellet was resuspended in 1 mL of GM, and the cell numbers were measured using the Countess 3 FL Cell Counter (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative reverse-transcription polymerase chain reaction (qPCR)

RNA was extracted from cells on day 3 of proliferation and day 4 of differentiation using TRIzol (Invitrogen, Carlsbad, CA, USA). cDNA synthesis was performed using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). qPCR was conducted using the DyNAmo HS SYBR Green qPCR Kit (Thermo Fisher Scientific) with a reaction volume of 10 µL, containing 1 pmol of each primer set (Table 2 and Fig. S1). Amplification and detection were carried out on the CFX96 Touch Real-Time PCR System (Bio-Rad, Hercules, CA, USA) under the following conditions: 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative gene expression levels were normalized to *GAPDH* using the delta-delta Ct method.

Immunofluorescence staining

Immunofluorescence staining was carried out following the protocol described in our previous study [20, 21]. Cells were fixed on day 1 of proliferation or day 4 of differentiation with 4% (v/v) paraformaldehyde (Sigma-Aldrich) in DPBS for 30 min at 4°C. The fixed cells were washed twice with DPBS, permeabilized with 0.2% (v/v) Triton X-100 (Sigma-Aldrich) for 15 min, and blocked with 10% (v/v) goat serum (Thermo Fisher Scientific) for 1 h at 4°C. Primary antibodies against PAX7 (1:500; R&D Systems), MYOD (1:500; Invitrogen), or myosin heavy chain (MHC, 1:500; R&D Systems) were added, and cells were incubated overnight at 4°C. Secondary antibodies (Alexa Fluor 488 and Alexa Fluor 568; Thermo Fisher Scientific) were applied overnight at 4°C. Nuclei were stained with Hoechst 33342 (1:1000; Molecular Probes) for 10 min at 4°C, followed by DPBS washes. Images were captured using an inverted fluorescence microscope (Eclipse TE2000-U; Nikon, Japan).

Free amino acid analysis

MuSCs were isolated from the pooled muscle tissue of seven individual chicken embryos and cultured. The samples were collected on the third day of proliferation or the third day of differentiation. Cells were washed twice with DPBS, harvested using a cell scraper, and transferred to 15 mL conical tubes before being stored at −80°C. In addition, muscle tissues from 18-day-old chicken embryos and commercially available chicken breasts (Harim Co., Ltd., Iksan, Korea), each pooled from seven individuals, were obtained and minced for 30 seconds and stored at −80°C. All samples were subsequently subjected to freeze-drying for further analysis. For free amino acid analysis, 70 mg of freeze-dried samples were weighed precisely and transferred to microtubes. The perchloric acid solution (0.1 M, Sigma-Aldrich) and 0.1% (v/v) metaphosphoric acid (Sigma-Aldrich) in distilled deionized water (DDW) was mixed at a 1:1 ratio (v/v). Subsequently, 1 mL of the mixed solution was added to a microtube containing the freeze-dried sample. The mixture was sonicated for 1 h at room temperature. After extraction, the solutions were filtered through a 0.22 μm membrane filter before analysis.

High-performance liquid chromatography (HPLC) analysis was performed using an Ultimate 3000 system (Thermo Fisher Scientific). o-Phthaldialdehyde (OPA)-derivatized samples were analyzed with a fluorescence detector (excitation: 340 nm, emission: 450 nm,), while 9-fluorenylmethoxycarbonyl (FMOC)-derivatized samples were analyzed with a fluorescence detector (emission: 305 nm, excitation: 266 nm). Additionally, UV detection was conducted at 338 nm. The separation was achieved using an Inno C18 column (4.6 mm × 150 mm, 5 μm; YoungJin Biochrom, Seongnam, Korea). The mobile phase consisted of solution A (40 mM sodium phosphate dibasic, pH 7, Sigma-Aldrich) and solution B (deionized water: acetonitrile: methanol = 10:45:45; acetonitrile and methanol were purchased from Sigma-Aldrich) with flow rate 1.5 mL/min. The injection volume was 0.5 μL, with the column temperature maintained at 40°C and the sample temperature at 20°C. The experimental groups were categorized as follows:

commercially available adult chicken, AC; chicken embryo, EC; proliferated MuSCs on fish gelatin coating, FGP; differentiated MuSCs on fish gelatin coating, FGD; proliferated MuSCs on laminin coating, LAMP; and differentiated MuSCs on laminin coating, LAMD.

Determination of nucleotide compounds

The sample preparation and pretreatment process required for nucleotide analysis were carried out in the same manner as those for free amino acid analysis. Nucleotide analysis was carried out using an Ultimate 3000 system equipped with a Phenomenex Synergi Hydro-RP column (4 µm, 250 × 4.6 mm; Phenomenex, Torrance, CA, USA). The mobile phases consisted of A (20 mM KH_2PO_4 buffer, pH 5.5, Sigma-Aldrich), B (methanol), and C (deionized water), with a gradient program as follows: 0–15 min, 100% A; 15–25 min, 80% A, 12% B, 8% C; 25–35 min, 100% A. The column temperature was maintained at 30°C, the flow rate was set to 1.0 mL/min, and the injection volume was 10 µL. Detection was performed using a UV/VIS detector at 254 nm. Standard solutions consisted of adenosine monophosphate (AMP; Sigma-Aldrich), guanosine monophosphate (GMP; Sigma-Aldrich), hypoxanthine (Sigma-Aldrich), inosine (Sigma-Aldrich), and inosine monophosphate (IMP; Sigma-Aldrich), and all solutions were filtered through a 0.2 µm syringe filter before being transferred into HPLC vials for analysis. The concentrations of nucleotides were calculated based on the peak areas and expressed as mg per 100 g of dried sample.

Statistical analysis

All experiments were conducted in triplicate, and the results were statistically analyzed using the mixed models, followed by Tukey's post-hoc test for multiple comparisons. The source of ECM coating and passage were set as fixed effects, while the three different individual chicks were designated as random effects. Statistical analysis was performed using SAS software (SAS, Release 9.4; SAS Institute Inc., Cary, NC). Significant differences between mean values were identified with a significance level of $p < 0.05$. The results were presented as the mean ± SEM.

Results and discussion

Proliferation capacity of chicken muscle stem cells with various ECM coatings

Cell counts were measured on days 1 and 3 of proliferation for passages 4–6 (Fig. 1). At passage 4 on day 1, cell counts on fibronectin, laminin, and Geltrex coated plates had significantly higher than that on fish gelatin ($p < 0.05$). A similar pattern held at passage 5, with fish gelatin resulting in the lowest counts ($p < 0.05$). By passage 6 on day 1, the cell counts were no longer different among the compared coatings ($p > 0.05$). Across all passages, day 3 counts mirrored day 1 trends, laminin and Geltrex remained superior to fish gelatin at passages 4 and 5 ($p < 0.05$). Although day 1 cell counts showed no significant differences across passages, day 3 counts decreased with increasing passage number for all coatings except collagen, indicating that replicative senescence progressively restricted proliferation and, consequently, attenuated substrate-dependent effects.

In summary, laminin and Geltrex demonstrated relatively high efficacy in promoting MuSC proliferation, whereas fish gelatin appeared suboptimal. These results align with previous findings with human myoblasts showing that they attach more readily and proliferate more rapidly on laminin-coated plates compared to other ECM coatings [22]. Similarly, Kim et al. (2024) reported that porcine MuSCs cultured on laminin-coated plates exhibited higher cell numbers at day 3 compared with those cultured on collagen-, fibronectin-, or porcine gelatin-coated plates [14]. Because Geltrex contains a high concentration of laminin, the results obtained with Geltrex were comparable to those obtained with laminin [23].

Impact of ECM coating on the heterogeneity of PAX7 and MYOD

Immunofluorescence profiling of PAX7 and MYOD across passages 4–6 (Fig. 2, Table 3) revealed a consistent loss of the quiescent MuSC pool on all ECMs, while the $PAX7^{+}/MYOD^{+}$ fraction declined progressively from passage 4 to 6 ($p < 0.05$). In parallel, the $PAX7^{-}/MYOD^{+}$ population, indicative of cells entering differentiation under additional myogenic regulatory factor influence, increased significantly over the same passages ($p < 0.05$). At passage 6, cells cultured on porcine gelatin showed a higher proportion of $PAX7^{-}/MYOD^{+}$ cells than those cultured on fish gelatin ($p < 0.05$), indicating a subtle substrate effect only at late passages.

The intermediate $PAX7^{+}/MYOD^{+}$ cohort, which balances self-renewal and commitment, also decreased with each passage ($p < 0.05$). These shifts mirror replicative senescence–associated changes reported in murine satellite cells, where aging drives loss of PAX7 expression and skews the population toward differentiation [24]. Crucially, despite this late-passage difference on porcine gelatin, all ECM coatings ultimately followed the same trajectory of declining self-renewal and rising differentiation. This universal trend in MuSC heterogeneity likely underlies the reduced proliferative capacity observed across coatings (Fig. 1).

Gene expression during the proliferation with different ECM coatings

To evaluate how ECM composition influences chicken MuSC activation and proliferation, we quantified mRNA levels of *PAX7*, *MYOD*, and key integrin subunits. *In vivo*, quiescent satellite cells express *PAX7* but not *MYOD*; upon injury they activate, upregulate *MYOD*, and downregulate *PAX7* [25, 26].

In our study, *PAX7* transcript levels remained constant across all coatings and passages ($p > 0.05$; Fig. 3A), indicating that ECM identity did not alter the quiescent marker at the mRNA level. In contrast, MYOD expression depended on both the coating material and the passage number. At passage 5, cells grown on Geltrex or laminin showed significantly higher MYOD expression than those on collagen, fibronectin, or fish gelatin ($p < 0.05$). At passage 6, MYOD expression remained higher in cells on laminin compared with all other substrates, except Geltrex. Notably, *MYOD* declined with successive passages on collagen and fish gelatin ($p < 0.05$), suggesting these matrices are less supportive of sustained myogenic activation. In contrast, *MYOD* expression remained elevated on laminin and Geltrex at later passages. We observed a different trend between *PAX7* mRNA and protein expression (Table 3), but it is a common phenomenon due to post-transcriptional regulation and species differences in expression kinetics [27]. Sampling at day 1 for protein versus day 3 for RNA may further contribute to this difference in chicken MuSCs.

Contrary to expectations, integrin transcript levels varied with ECM substrate rather than strictly following ligand specificity. *ITGA1* (collagen receptor) unexpectedly peaked at passage 5 on fibronectin ($p < 0.05$; Fig. 3B). ITGA5 (a fibronectin receptor) showed higher expression on collagen than on fibronectin or porcine gelatin at passage 4 ($p < 0.05$), and its expression was also relatively higher on porcine gelatin than on most other ECMs at passages 5 and 6 ($p < 0.05$), except for laminin at passage 5 ($p > 0.05$). Furthermore, while ITGA5 expression decreased with successive passages on collagen ($p < 0.05$), it gradually increased on porcine gelatin ($p < 0.05$), despite being primarily associated with fibronectin. *ITGA6* (laminin receptor) steadily declined on Geltrex over successive passages, and *ITGB1* expression remained unaffected by coating. These results indicate that, although individual integrins exhibit transient and substrate-dependent expression, no single ECM consistently upregulates its cognate integrin during extended culture. Further studies such as integrin protein quantification and functional adhesion assays are needed to clarify how ECM–integrin interactions regulate chicken MuSC proliferation.

Impact of ECM coating on myotube formation

Differentiation was induced and myotube fusion was quantified on day 4 at passages 5 and 6 (Fig. 4, Table 4). Morphological analysis by microscopy showed that myotubes on Geltrex and laminin were broader and thicker than those on collagen, fibronectin, porcine gelatin, or fish gelatin. At passage 5, the fusion index of myotubes on laminin was significantly higher than those on collagen, fibronectin, or fish gelatin ($p < 0.05$). At passage 6, laminin again supported greater fusion than fish and porcine gelatin ($p < 0.05$), while Geltrex remained statistically indistinguishable from laminin ($p > 0.05$). Across all coatings, the fusion index declined progressively from passage 5 to 6, mirroring established observations that MuSCs lose differentiation capacity with repeated passaging [28, 29]. Taken together, these data demonstrate that laminin, and by extension, Geltrex with its high laminin content, most effectively drive myotube formation in chicken MuSCs under our culture conditions.

Effects of ECM coating on the regulation of gene expression during the differentiation

We next examined how ECM substrates influence transcriptional programs during myogenic differentiation by measuring *MYOG* and *MYH1*, key markers of early differentiation and fast-twitch fiber maturation, respectively (Fig. 5A). On day 4 of differentiation at passage 5, both Geltrex and laminin coatings induced significantly higher *MYOG* levels compared with fibronectin or fish gelatin ($p < 0.05$). By passage 6, laminin continued to support elevated *MYOG* compared to all other coatings ($p < 0.05$), reflecting its superior capacity to trigger the commitment phase of myogenesis. *MYH1* expression followed a similar pattern to *MYOG*. At passage 5, Geltrex outperformed fibronectin ($p < 0.05$), while at passage 6 laminin induced significantly greater *MYH1* than collagen, fish gelatin, and porcine gelatin ($p < 0.05$). This progressive enhancement of *MYH1* on laminin-rich substrates parallels the thicker, more extensive myotubes observed in Fig. 4 and Table 3, suggesting that these ECMs not only initiate differentiation but also promote subsequent myofiber maturation.

We also tracked mRNAs for the principal integrin subunits (*ITGA1*, *ITGA5*, *ITGA6,* and *ITGB1*) to probe how ECM engagement evolves during differentiation (Fig. 5B). *ITGA1* levels rose modestly on porcine gelatin at passage 6 compared with Geltrex ($p < 0.05$), although the pure collagen surprisingly did not boost its own receptor transcript. *ITGA5* expression was highest on collagen at passage 4 compared with Geltrex and laminin ($p < 0.05$). At passage 5, its expression peaked on porcine gelatin, and this pattern persisted at passage 6, where *ITGA5* levels on porcine gelatin remained higher than those on most other ECMs except fish gelatin. Moreover, *ITGA5* expression increased with successive passages on both porcine and fish gelatin, showing a trend consistent with the proliferation results. *ITGA6* transcripts tended to be higher on laminin at passages 5 and 6 than on other coatings, consistent with laminin's role in promoting sarcomere assembly. Finally, *ITGB1* expression increased significantly with passage on Geltrex ($p < 0.05$), whereas no passage-dependent change was observed on fibronectin. Nevertheless, at passage 6, *ITGB1* expression was higher on fibronectin than on collagen or Geltrex, suggesting that β1 pairing with various α-subunits contributes broadly to fusion but is not exclusively driven by any single ECM.

Overall, during differentiation the transcriptional responses of both myogenic markers and integrins become more ligand-specific than in the proliferation phase. Laminin and Geltrex consistently upregulate genes critical for early commitment (*MYOG*), fiber maturation (*MYH1*), and their corresponding receptors (*ITGA6*), underscoring their central roles in orchestrating chicken MuSC differentiation. Further protein-level and functional adhesion assays will be essential to confirm how these mRNA changes translate into enhanced myofiber formation and maturation.

Influence of laminin and fish gelatin on flavor-related compounds in chicken MuSCs

To explore how ECM coating and cell state affect flavor precursors, we compared free amino acids and nucleotides in proliferating versus differentiated chicken MuSCs grown on laminin (LAMP, LAMD) or fish gelatin (FGP, FGD), alongside adult (AC) and embryonic (EC) tissue samples (Table 5). Among all measured amino acids, only glycine differed significantly. Differentiated MuSCs on laminin (LAMD) and fish gelatin (FGD) contained more glycine than their proliferating counterparts (LAMP, FGP), with LAMD exceeding FGD ($p < 0.05$). Elevated glycine, reported to decline in aged or diseased muscle (Fazelzadeh et al., 2016; Martins-Bach et al., 2012), likely reflects the more advanced differentiation state of LAMD. In contrast, glutamate and alanine, key contributors to umami and sweetness, respectively, showed no coating- or state-dependent changes, nor did they approach levels found in AC or EC, underscoring a persistent gap between *in vitro* cultured cells and native tissue.

Nucleotide contents followed a similar pattern to the result of free amino acids. No significant differences among cultured groups, and all were substantially lower than in adult tissue (Table 6). This aligns with prior reports of reduced nucleotide levels in cultured meat models [17, 30].

Despite laminin's superior support of proliferation and differentiation, neither ECM coating enhanced the accumulation of taste-related metabolites in MuSCs. These findings highlight the necessity of optimizing scaffold composition or supplementing culture media to more closely recapitulate the metabolite profile of real meat and improve the sensory quality of cultured products.

This study compared the effects of various ECM coatings on chicken MuSC proliferation and differentiation. Both Geltrex and laminin enhanced cell growth and upregulated *MYOD*, *MYOG*, and *MYH1* during myogenic differentiation, with laminin yielding the highest fusion index and most robust myotube formation. Although most free amino acids and nucleotides showed no coating-dependent differences, glycine was significantly higher in differentiated cells on laminin than on fish gelatin, indicating a modest substrate effect on specific metabolites. Nonetheless, overall metabolite levels remained substantially lower than those in native tissue, underscoring the need to optimize culture conditions to improve flavor-related compounds. It should be noted that ECM coatings were applied at concentrations recommended by the respective manufacturers and optimized in preliminary experiments to ensure efficient MuSC attachment and proliferation. However, variations in coating doses or surface adsorption among materials may have partially influenced the observed outcomes, which should be considered when interpreting ECM-dependent effects.

Beyond the cellular level, these findings have broader implications for the development of cultured chicken meat. The promotion of myogenic differentiation and myotube maturation by laminin may contribute to improved muscle fiber structure and texture, while the relatively low abundance of flavor precursors highlights the need for strategies that enhance metabolic maturation. Furthermore, identifying an ECM coating that supports both robust proliferation and differentiation could facilitate scalable production, serving as a foundation for scaffold design and bioprocess optimization in cultured meat manufacturing.

Competing interests

The authors have declared no conflict of interest.

Acknowledgments

This research received financial support from the Korea Evaluation Institute of Industrial Technology (KEIT, Project No. 20012411) and a research grant provided by the National Research Foundation of Korea, funded by the Korean government (Grant No. 2022R1A2C1005235).

Author`s Contributions

Minjun Kang: conceptualization, data curation, formal analysis, investigation, methodology, writing – original draft, and writing – review & editing. **Minsu Kim:** conceptualization, data curation, methodology, validation, writing – original draft, and writing – review & editing. **Hyun Young Jung:** investigation. **Eunil Kim:** investigation. **Soyoon Yun:** investigation. **Myung Yeo:** investigation. **Cheorun Jo:** conceptualization, project administration, resources, supervision, and writing - review & editing.

Ethics approval

This experiment was approved by the Institutional Animal Care and Use Committee (IACUC) at Seoul National University (Approval No.: SNU 230303-3). All experimental procedures followed the guidelines of the Institute of Laboratory Animal Resources at Seoul National University.

References

1. Lee HJ, Yong HI, Kim M, Choi Y-S, Jo C. Status of meat alternatives and their potential role in the future meat market—A review. Asian-Australasian journal of animal sciences. 2020;33(10):1533. https://doi.org/10.5713/ajas.20.0419
2. Post MJ, Levenberg S, Kaplan DL, Genovese N, Fu J, Bryant CJ, et al. Scientific, sustainability and regulatory challenges of cultured meat. Nature Food. 2020;1(7):403-15. https://doi.org/10.1038/s43016-020-0112-z
3. Choi KH, Yoon JW, Kim M, Lee HJ, Jeong J, Ryu M, et al. Muscle stem cell isolation and in vitro culture for meat production: A methodological review. Comprehensive Reviews in Food Science and Food Safety. 2021;20(1):429-57. https://doi.org/10.1111/1541-4337.12661
4. Lee D-K, Kim M, Jeong J, Lee Y-S, Yoon JW, An M-J, et al. Unlocking the potential of stem cells: Their crucial role in the production of cultivated meat. Current Research in Food Science. 2023:100551. https://doi.org/10.1016/j.crfs.2023.100551

5. Thomas K, Engler AJ, Meyer GA. Extracellular matrix regulation in the muscle satellite cell niche. Connective tissue research. 2015;56(1):1-8. https://doi.org/10.3109/03008207.2014.947369

6. Schüler SC, Liu Y, Dumontier S, Grandbois M, Le Moal E, Cornelison D, et al. Extracellular matrix: Brick and mortar in the skeletal muscle stem cell niche. Frontiers in Cell and Developmental Biology. 2022;10:1056523. https://doi.org/10.3389/fcell.2022.1056523

7. Li Y, Liu W, Li S, Zhang M, Yang F, Wang S. Porcine skeletal muscle tissue fabrication for cultured meat production using three-dimensional bioprinting technology. Journal of Future Foods. 2021;1(1):88-97. https://doi.org/10.1016/j.jfutfo.2021.09.005

8. Said M, editor Role and function of gelatin in the development of the food and non-food industry: A review. IOP Conference Series: Earth and Environmental Science; 2020: IOP Publishing. https://doi.org/10.1088/1755-1315/492/1/012086

9. Lukjanenko L, Jung MJ, Hegde N, Perruisseau-Carrier C, Migliavacca E, Rozo M, et al. Loss of fibronectin from the aged stem cell niche affects the regenerative capacity of skeletal muscle in mice. Nature Medicine. 2016;22(8):897-905. https://doi.org/10.1038/nm.4126

10. Rayagiri SS, Ranaldi D, Raven A, Mohamad Azhar NIF, Lefebvre O, Zammit PS, et al. Basal lamina remodeling at the skeletal muscle stem cell niche mediates stem cell self-renewal. Nature Communications. 2018;9(1):1075. https://doi.org/10.1038/s41467-018-03425-3

11. Teo YX, Lee KY, Goh CJH, Wang LC, Sobota RM, Chiam K-H, et al. Fungus-derived protein particles as cell-adhesive matrices for cell-cultivated food. npj Science of Food. 2023;7(1):34. https://doi.org/10.1038/s41538-023-00209-y

12. Li L, Chen L, Chen X, Chen Y, Ding S, Fan X, et al. Chitosan-sodium alginate-collagen/gelatin three-dimensional edible scaffolds for building a structured model for cell

cultured meat. International Journal of Biological Macromolecules. 2022;209:668-79. https://doi.org/10.1016/j.ijbiomac.2022.04.052

13. Naraoka Y, Mabuchi Y, Yoneyama Y, Suto EG, Hisamatsu D, Ikeda M, et al. Isolation and Characterization of Tissue Resident CD29-Positive Progenitor Cells in Livestock to Generate a Three-Dimensional Meat Bud. Cells. 2021;10(9):2499. https://doi.org/10.3390/cells10092499

14. Kim M, Jung HY, Kim B, Jo C. Laminin as a key extracellular matrix for proliferation, differentiation, and maturation of porcine muscle stem cell cultivation. Food Science of Animal Resources. 2024;44(3):710-22. https://doi.org/10.5851/kosfa.2024.e27

15. Zeltz C, Gullberg D. The integrin–collagen connection–a glue for tissue repair? Journal of Cell Science. 2016;129(4):653-64. https://doi.org/10.1242/jcs.180992

16. Boppart MD, Mahmassani ZS. Integrin signaling: linking mechanical stimulation to skeletal muscle hypertrophy. American Journal of Physiology-Cell Physiology. 2019;317(4):C629-C41. https://doi.org/10.5713/ajas.20.0419

17. Kim M, Jung HY, Jo C. Fundamental study on structural formation, amino acids and nucleotide-related compounds of cultivated meat from 3D-cultured pig muscle stem cells. Food Science and Biotechnology. 2025;34(2):457-69. https://doi.org/10.1007/s10068-024-01793-9

18. Jung HY, Kim M, Lee C-K, Yeo M, Sohn SH, Jo C. Comparative evaluation of myogenic potential of purebred chicken muscle stem cells. Current Research in Biotechnology. 2024;8:100241. https://doi.org/10.1016/j.crbiot.2024.100241

19. Gregg CR, Hutson BL, Flees JJ, Starkey CW, Starkey JD. Effect of standard and physiological cell culture temperatures on in vitro proliferation and differentiation of primary broiler chicken pectoralis major muscle satellite cells. Frontiers in Physiology. 2023;14:1288809.

20. Choi K-H, Kim M, Yoon JW, Jeong J, Ryu M, Jo C, et al. Purification of pig muscle stem cells using magnetic-activated cell sorting (MACS) based on the expression of cluster of differentiation 29 (CD29). Food Science of Animal Resources. 2020;40(5):852. https://doi.org/10.5851/kosfa.2020.e51

21. Choi K-H, Yoon JW, Kim M, Jeong J, Ryu M, Park S, et al. Optimization of culture conditions for maintaining pig muscle stem cells *in vitro*. Food Science of Animal Resources. 2020;40(4):659-67. https://doi.org/10.5851/kosfa.2020.e39

22. Chowdhury SR, Ismail AB, Chee SC, Laupa MSb, Jaffri FB, Mohmad Saberi SE, et al. One-step purification of human skeletal muscle myoblasts and subsequent expansion using laminin-coated surface. Tissue Engineering Part C: Methods. 2015;21(11):1135-42. https://doi.org/10.1089/ten.tec.2015.0015

23. Mollica PA, Booth-Creech EN, Reid JA, Zamponi M, Sullivan SM, Palmer X-L, et al. 3D bioprinted mammary organoids and tumoroids in human mammary derived ECM hydrogels. Acta Biomaterialia. 2019;95:201-13. https://doi.org/10.1016/j.actbio.2019.06.017

24. Shefer G, Van de Mark DP, Richardson JB, Yablonka-Reuveni Z. Satellite-cell pool size does matter: defining the myogenic potency of aging skeletal muscle. Developmental Biology. 2006;294(1):50-66. https://doi.org/10.1016/j.ydbio.2006.02.022

25. Kuang S, Kuroda K, Le Grand F, Rudnicki MA. Asymmetric self-renewal and commitment of satellite stem cells in muscle. Cell. 2007;129(5):999-1010. https://doi.org/10.1016/j.cell.2007.03.044

26. Olguin HC, Olwin BB. Pax-7 up-regulation inhibits myogenesis and cell cycle progression in satellite cells: a potential mechanism for self-renewal. Developmental Biology. 2004;275(2):375-88. https://doi.org/10.1016/j.ydbio.2004.08.015

27. Lackner DH, Bähler J. Translational control of gene expression: from transcripts to transcriptomes. International Review of Cell and Molecular Biology. 2008;271:199-251. https://doi.org/10.1016/S1937-6448(08)01205-7

28. Park S, Park G, Oh S, Park Y, Kim Y, Kim J, et al. Investigating proliferation and differentiation capacities of Hanwoo steer myosatellite cells at different passages for developing cell-cultured meat. Scientific Reports. 2023;13(1):15614. https://doi.org/10.1038/s41598-023-40800-7

29. Shahini A, Choudhury D, Asmani M, Zhao R, Lei P, Andreadis ST. NANOG restores the impaired myogenic differentiation potential of skeletal myoblasts after multiple population doublings. Stem Cell Research. 2018;26:55-66. https://doi.org/10.1016/j.scr.2017.11.018

30. Joo S-T, Choi J-S, Hur S-J, Kim G-D, Kim C-J, Lee E-Y, et al. A Comparative study on the taste characteristics of satellite cell cultured meat derived from chicken and cattle muscles. Food Science of Animal Resources. 2022;42(1):175-85. https://doi.org/10.5851/kosfa.2021.e72

Tables

Table 1. Concentrations and solutions for each ECM coating

ECM	Concentration	Solution
Collagen	50 µg/mL	0.2% (v/v) acetic acid
Fibronectin	50 µg/mL	DPBS
Fish gelatin	10 mg/mL	DDW
Geltrex	150 µg/mL	DPBS
Laminin	50 µg/mL	DPBS
Porcine gelatin	1 mg/mL	DDW

All reagents were purchased from the following suppliers: Collagen (Sigma-Aldrich, St. Louis, MO, USA), Fibronectin (Roche, Basel, Switzerland), Fish gelatin (Sigma-Aldrich), Geltrex (Gibco, Gaithersburg, MD, USA), Laminin (Sigma-Aldrich), Porcine gelatin (Sigma-Aldrich).

Table 2. Primers used for quantitative reverse-transcription polymerase chain reaction

Gene		Primer sequence (5'→3')	Product size (bp)	Accession No.
ITGA1	F	CGTATTGCATCCGGTGGAGA	124	NM_205069.2
	R	GCCACATCACGAGACCAGAA		
ITGA5	F	TTACTTCTGGCAAGGGCAGG	122	XM_040693851.2
	R	AACTGTCATCGTAGGTGGCG		
ITGA6	F	GTGGATCATCGCAGTGGCTA	139	NM_205289.2
	R	AGCATGGATCTCAGCCTTGT		
ITGB1	F	GCGTGGTTGCTGGAATTGTT	135	NM_001039254.3
	R	TTTCACCCGTATCCCACTTG		
PAX7	F	CTCCTGCCAACCACATGAAT	108	NM_205065.1
	R	TGTTCAAGGCTATGGTGAGGTT		
MYOD	F	ATTCCTTCCCCCACCAACTG	145	NM_204214.3
	R	GGTCTTGGAGCTTGGCTGAA		
MYH1	F	TCCGCAAGATCCAACACGAA	150	XM_046929488.1
	R	ATGCCACTTTGTTGTCACGA		
MYOG	F	ATGGGGAAAACTTCCTGGGC	109	NM_204184.1
	R	TTCTCCTCCAAAGCCCCTCT		
GAPDH	F	CGTCCTCTCTGGCAAAGTCC	132	NM_204305.2
	R	TTCCCGTTCTCAGCCTTGAC		

ITGA1, integrin α1; *ITGA5*, integrin α5; *ITGA6*, integrin α6; *ITGB1*, integrin β1; *PAX7*, paired box 7; *MYOD*, myoblast determination protein 1; *MYH1*, myosin heavy chain 1; *MYOG*, myogenin; *GAPDH*, glyceraldehyde 3-phosphate dehydrogenase.

Table 3. The percentage of different PAX7 and MYOD heterogeneity on day 1 of proliferation Data are presented as mean ± SEM (n=3).

ECM	$\text{PAX7}^{-}/\text{MYOD}^{+}$ (%)			$\text{PAX7}^{+}/\text{MYOD}^{+}$ (%)		
	Passage			Passage		
	4	5	6	4	5	6
Collagen	41.2 ± 23.84^{B}	51.4 ± 23.16^{A}	60.0 ± 21.88^{Aab}	51.6 ± 23.89^{A}	42.2 ± 24.22^{AB}	35.1 ± 22.05^{B}
Fibronectin	43.0 ± 22.32^{B}	50.2 ± 24.22^{B}	59.4 ± 25.37^{Aab}	53.2 ± 22.23^{A}	46.3 ± 24.06^{AB}	36.1 ± 25.32^{B}
Fish gelatin	40.5 ± 22.08^{B}	49.3 ± 23.84^{AB}	54 ± 23.78^{Ab}	52.0 ± 18.86^{A}	43.3 ± 24.62^{AB}	38.3 ± 25.39^{B}
Geltrex	39.7 ± 22.66^{B}	49.4 ± 22.94^{AB}	56.4 ± 23.89^{Aab}	57.2 ± 22.58^{A}	47.5 ± 22.16^{AB}	40.8 ± 25.32^{B}
Laminin	41.4 ± 22.94^{B}	48.8 ± 23.39^{B}	59.4 ± 25.69^{Aab}	54.8 ± 22.82^{A}	47.6 ± 23.16^{AB}	39.5 ± 26.67^{B}
Porcine gelatin	40.3 ± 22.50^{C}	54.3 ± 24.90^{B}	64.8 ± 19.97^{Aa}	56.0 ± 22.73^{A}	42.7 ± 24.67^{B}	30.5 ± 21.05^{C}

Data are presented as mean ± SEM (n=3).

$^{A-C}$Significantly different among passages in same ECM coating treatment group ($p < 0.05$).

$^{a, b}$Significantly different among ECM coating treatment within the same passage group ($p < 0.05$).

Table 4. Fusion index (%) during chicken MuSCs differentiation across various ECM proteins and passages on day 4 of differentiation

Data are presented as mean ± SEM (n=3).

ECM	Passage		
	4	5	6
Collagen	13.6 ± 3.56^{A}	5.9 ± 2.81^{Bb}	4.0 ± 2.05^{Bab}
Fibronectin	14.8 ± 6.43^{A}	5.5 ± 2.40^{Bb}	2.9 ± 1.01^{Bab}
Fish gelatin	13.7 ± 4.66^{A}	4.7 ± 2.17^{Bb}	1.8 ± 0.74^{Bb}
Geltrex	15.1 ± 3.47^{A}	10.3 ± 4.83^{ABab}	7.0 ± 3.66^{Bab}
Laminin	19.4 ± 6.02^{A}	13.3 ± 5.17^{Ba}	8.2 ± 3.58^{Ba}
Porcine gelatin	12.4 ± 4.11^{A}	7.8 ± 3.67^{ABab}	1.8 ± 0.61^{Bb}

Data are presented as mean ± SEM (n=3).

Fusion index was calculated as the percentage of nuclei incorporated in the myotubes relative to the total number of nuclei.

$^{A-C}$Significant differences among passages in same ECM coating treatment group ($p < 0.05$).

$^{a, b}$Significant differences among ECM coating treatment within the same passage group ($p < 0.05$).

Table 5. Free amino acid content (mg/100 g dry matter) of tissues from chicken embryo and adult chicken, and cultured cells

Free amino acid	Treatments					
	AC	EC	FGP	FGD	LAMP	LAMD
Alanine	11.95 ± 1.373^{ab}	7.36 ± 1.227^{b}	11.38 ± 1.285^{ab}	13.07 ± 0.402^{a}	13.03 ± 0.913^{a}	15.05 ± 1.461^{a}
Arginine	6.25 ± 0.718	6.51 ± 1.160	3.52 ± 0.612	5.50 ± 1.271	3.87 ± 1.129	4.68 ± 0.354
Asparagine	2.87 ± 0.333^{ab}	4.16 ± 0.692^{a}	1.22 ± 0.455^{b}	1.82 ± 0.457^{ab}	1.43 ± 0.770^{b}	2.34 ± 0.189^{ab}
Aspartic acid	7.18 ± 0.798^{a}	3.97 ± 0.951^{ab}	2.88 ± 0.410^{b}	4.54 ± 0.684^{ab}	3.04 ± 0.669^{b}	5.20 ± 0.343^{ab}
GABA	na.	0.30 ± 0.163	0.04 ± 0.043	0.04 ± 0.036	0.06 ± 0.057	0.06 ± 0.061
Glutamic acid	8.78 ± 1.036^{b}	24.95 ± 4.224^{a}	19.35 ± 1.794^{a}	18.18 ± 1.066^{ab}	20.61 ± 1.449^{a}	25.81 ± 1.751^{a}
Glutamine	23.56 ± 5.301	59.73 ± 24.539	11.47 ± 5.397	28.98 ± 13.155	10.90 ± 5.623	32.29 ± 14.576
Glycine	6.76 ± 0.713^{b}	11.98 ± 1.534^{a}	2.41 ± 0.499^{c}	7.40 ± 0.296^{b}	2.55 ± 0.837^{c}	11.71 ± 0.442^{a}
Histidine	2.82 ± 0.378^{a}	3.64 ± 0.752^{a}	0.79 ± 0.151^{b}	1.00 ± 0.151^{b}	0.77 ± 0.219^{b}	0.89 ± 0.156^{b}
Isoleucine	3.50 ± 0.392^{a}	2.63 ± 0.467^{ab}	1.72 ± 0.173^{b}	2.26 ± 0.365^{ab}	1.77 ± 0.326^{b}	1.84 ± 0.268^{b}
Leucine	6.91 ± 0.740	7.40 ± 1.711	5.88 ± 1.293	7.97 ± 1.396	6.39 ± 1.976	7.63 ± 0.994
Lysine	8.32 ± 0.894^{a}	6.34 ± 0.970^{ab}	2.87 ± 0.877^{b}	4.73 ± 1.435^{ab}	3.46 ± 1.619^{ab}	4.54 ± 0.808^{ab}
Methionine	2.41 ± 0.243	1.73 ± 0.405	0.72 ± 0.424	1.28 ± 0.665	0.85 ± 0.589	1.10 ± 0.483
Phenylalanine	3.80 ± 0.389	3.99 ± 0.911	3.24 ± 0.764	4.02 ± 0.723	3.45 ± 1.098	3.97 ± 0.888
Proline	4.66 ± 0.454^{ab}	7.46 ± 1.119^{a}	1.75 ± 0.237^{c}	2.31 ± 0.359^{bc}	2.17 ± 0.445^{bc}	2.35 ± 0.525^{bc}
Serine	7.53 ± 0.904^{ab}	11.26 ± 1.959^{a}	2.03 ± 0.373^{c}	3.22 ± 0.715^{bc}	2.14 ± 0.818^{c}	3.66 ± 0.092^{bc}
Taurine	24.07 ± 12.570^{ab}	45.15 ± 7.968^{a}	7.82 ± 2.228^{b}	11.19 ± 1.602^{b}	7.49 ± 2.428^{b}	12.87 ± 1.408^{b}
Threonine	6.24 ± 0.687	7.84 ± 1.413	4.42 ± 0.673	4.84 ± 0.275	4.60 ± 0.880	4.97 ± 0.571
Tryptophan	0.99 ± 0.103^{a}	0.53 ± 0.102^{ab}	0.35 ± 0.070^{b}	0.58 ± 0.150^{ab}	0.38 ± 0.116^{b}	0.51 ± 0.067^{ab}
Tyrosine	4.11 ± 0.434^{ab}	4.95 ± 0.897^{a}	1.97 ± 0.376^{b}	2.72 ± 0.565^{ab}	2.12 ± 0.591^{b}	2.45 ± 0.467^{ab}
Valine	4.46 ± 0.470	4.42 ± 0.802	2.02 ± 0.323	2.98 ± 0.510	2.20 ± 0.537	2.72 ± 0.446

Data are presented as mean ± SEM (n=3).

AC, Commercially available adult chicken; EC, Chicken embryo; FGP, Proliferated MuSCs on fish gelatin coating, FGD, Differentiated MuSCs on fish gelatin coating; LAMP, Proliferated MuSCs on laminin coating; LAMD, Differentiated MuSCs on laminin coating.

[a-c]Significant differences in the same amino acid among adult, embryonic, and culture conditions.

Table 6. Nucleotide content (mg/100 g dry matter) of tissues from chicken embryo and adult chicken, and cultured cells

Data are presented as mean ± SEM (n=3).

Nucleotide	Treatments					
	AC	EC	FGP	FGD	LAMP	LAMD
AMP	5.45 ± 5.454	na.	na.	na.	na.	na.
IMP	243.43 ± 15.616^{a}	5.13 ± 0.329^{b}	7.04 ± 0.452^{b}	2.79 ± 0.179^{b}	2.63 ± 0.169^{b}	26.4 ± 1.694^{b}
Inosine	147.13 ± 9.438^{a}	21.32 ± 1.368^{b}	12.48 ± 0.801^{b}	10.13 ± 0.65^{b}	14.92 ± 0.957^{b}	11.58 ± 0.743^{b}
Hypoxanthine	27.85 ± 2.158^{a}	13.09 ± 4.968^{b}	1.45 ± 0.714^{c}	3.82 ± 1.514^{bc}	1.16 ± 0.468^{c}	5.89 ± 0.399^{bc}

Data are presented as mean ± SEM (n=3).

AC, Commercially available adult chicken; EC, Chicken embryo; FGP, Proliferated MuSCs on fish gelatin coating, FGD, Differentiated MuSCs on fish gelatin coating; LAMP, Proliferated MuSCs on laminin coating; LAMD, Differentiated MuSCs on laminin coating.

AMP, Adenosine monophosphate; IMP, Inosine monophosphate.

$^{a\text{-}c}$Significant differences in the same amino acid among adult, embryonic, and culture conditions.

Figure captions

Fig. 1. Effect of ECM protein coating treatment on cell numbers. $^{A, B}$Significantly different among passages in the same ECM coating treatment ($p < 0.05$). $^{a-c}$Significantly different among ECM coating treatment within the same passage group ($p < 0.05$). Data are presented as mean ± SEM (n = 3).

Fig. 2. Effect of different ECM proteins on PAX7 and MYOD heterogeneity during proliferation. Immunofluorescence staining images showing PAX7 (green) and MYOD (red) were merged. Scale bar = 200 μm.

Fig. 3. Effect of ECM protein coating treatment on myogenic marker gene expression. (A) Relative mRNA expression of proliferating myogenic markers (*PAX7* and *MYOD*) and (B) integrin family (*ITGA1, ITGA5, ITGA6*, and *ITGB1*) in MuSCs on day 3 of each passage. $^{A, B}$Significant differences among passages in same ECM coating treatment group ($p < 0.05$). $^{a, b}$Significant differences among ECM coating treatment within the same passage group ($p < 0.05$). Data are presented as mean ± SEM (n = 3).

Fig. 4. Effect of different ECM coatings on myogenic differentiation. Immunofluorescence staining image of nuclei (blue) and MHC (green) were merged together. Scale bar = 100 μm.

Fig. 5. Myogenic potential of chicken MuSCs with various ECM protein coatings. (A) Relative mRNA expression of differentiating myogenic markers (*MYOG* and *MYH1*) and (B) integrin family (*ITGA1, ITGA5, ITGA6*, and *ITGB1*) in MuSCs on differentiation day 4 of each passage. $^{A, B}$Significantly different among passages within same ECM coating treatment group ($p < 0.05$). $^{a-c}$Significant differences among ECM coating treatment within the same passage group ($p < 0.05$). Data are presented as mean ± SEM (n = 3).

Fig. 1.

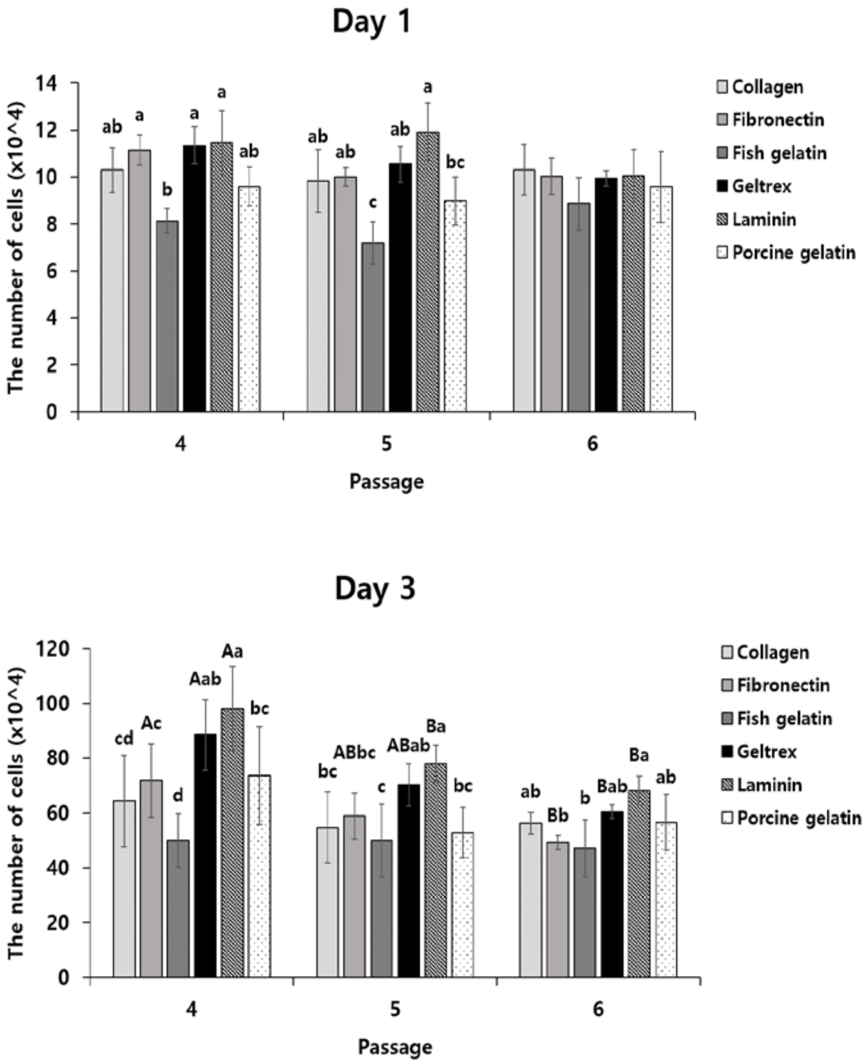

Day 1
The number of cells (x10^4)
14
12
10
8
6
4
2
0
ab
a
b
a
a
ab
ab
ab
c
ab
a
bc
4
5
6
Passage
Collagen
Fibronectin
Fish gelatin
Geltrex
Laminin
Porcine gelatin
Day 3
The number of cells (x10^4)
120
100
80
60
40
20
0
cd
Ac
d
Aab
Aa
bc
bc
ABbc
c
ABab
Ba
bc
ab
Bb
b
Bab
Ba
ab
4
5
6
Passage
Collagen
Fibronectin
Fish gelatin
Geltrex
Laminin
Porcine gelatin

Fig. 2.

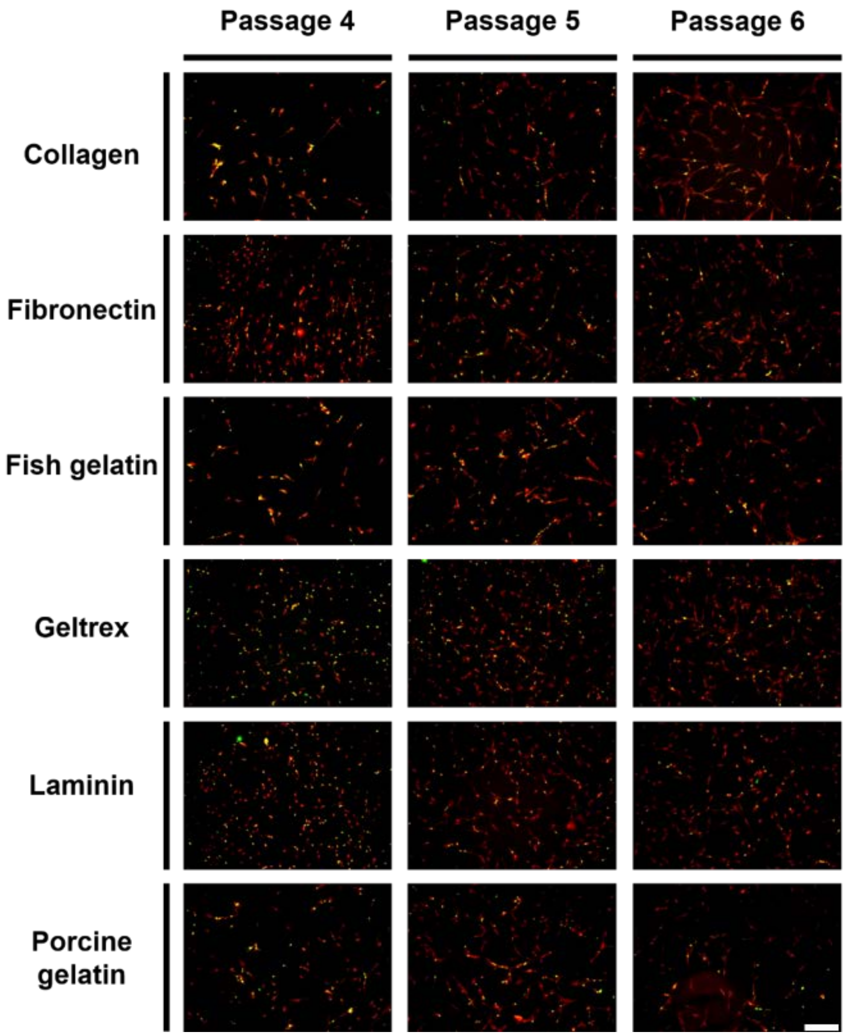
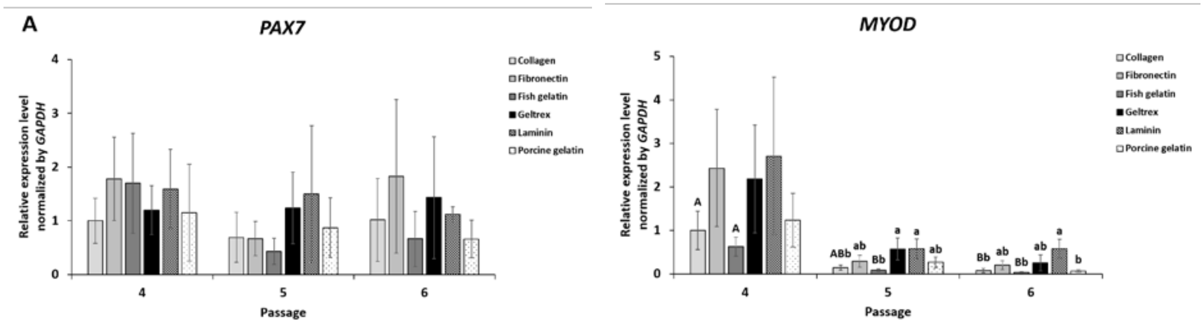

Passage 4
Passage 5
Passage 6
Collagen
Fibronectin
Fish gelatin
Geltrex
Laminin
Porcine gelatin

Fig. 3.


A
PAX7
MYOD
Relative expression level normalized by GAPDH
Passage
4
5
6
Collagen
Fibronectin
Fish gelatin
Geltrex
Laminin
Porcine gelatin
A
A
ABb
ab
Bb
a
a
ab
Bb
ab
Bb
ab
a
b

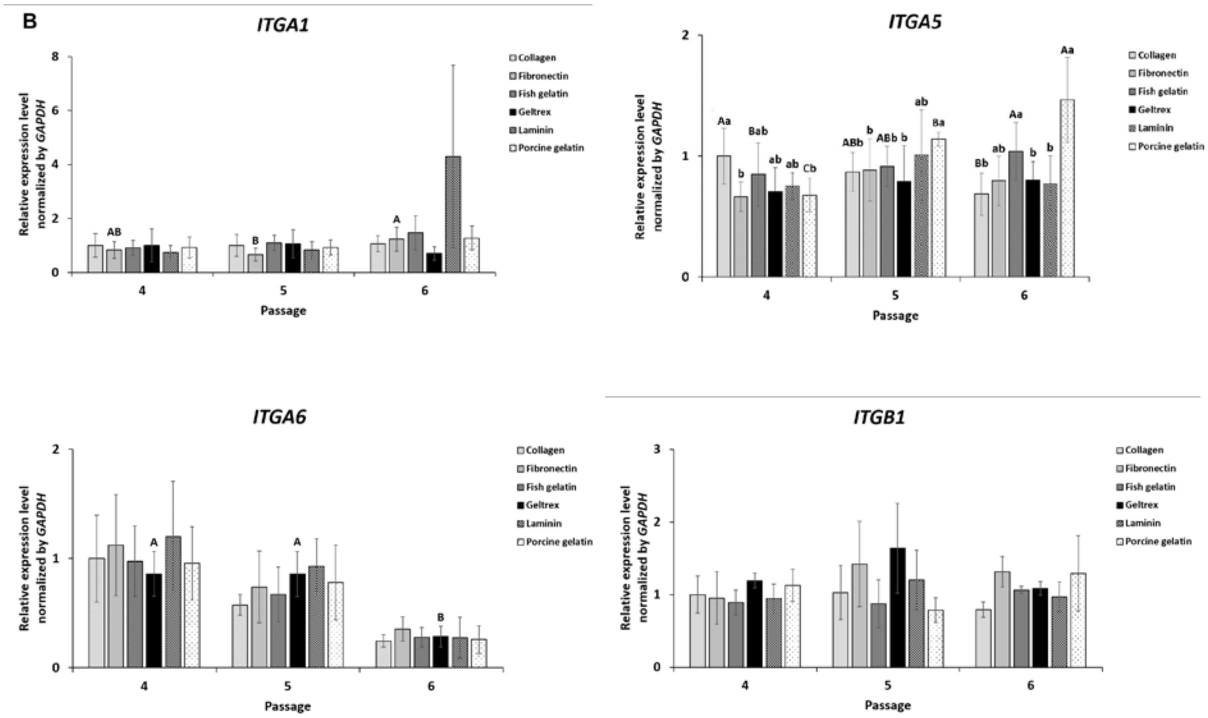

B
ITGA1
ITGA5
ITGA6
ITGB1
Relative expression level normalized by GAPDH
Passage
4
5
6
Collagen
Fibronectin
Fish gelatin
Geltrex
Laminin
Porcine gelatin
AB
B
A
Aa
b
Bab
ab
ab
Cb
ABb
b
ABb
b
ab
Ba
Bb
ab
Aa
b
b
Aa
A
A
B

Fig. 4.

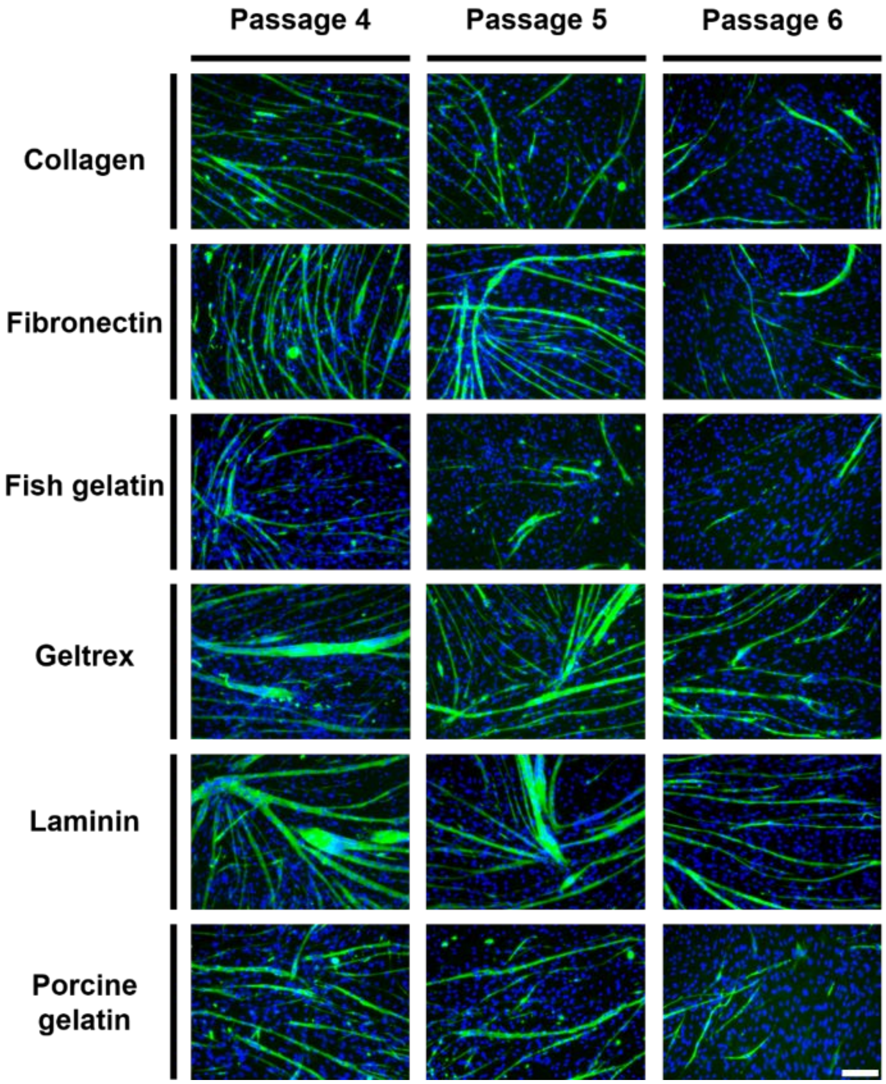
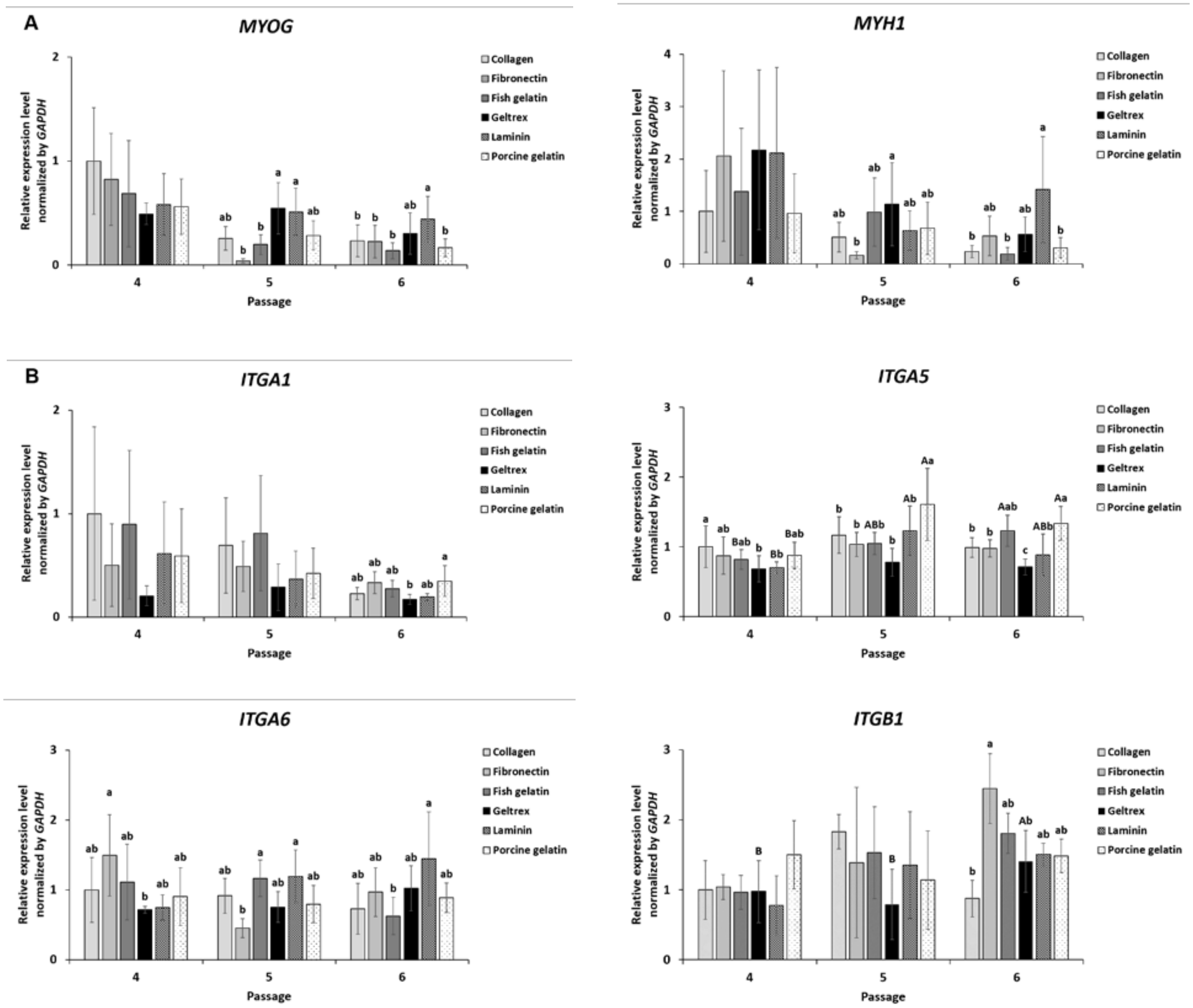

Passage 4
Passage 5
Passage 6
Collagen
Fibronectin
Fish gelatin
Geltrex
Laminin
Porcine gelatin

Fig. 5.

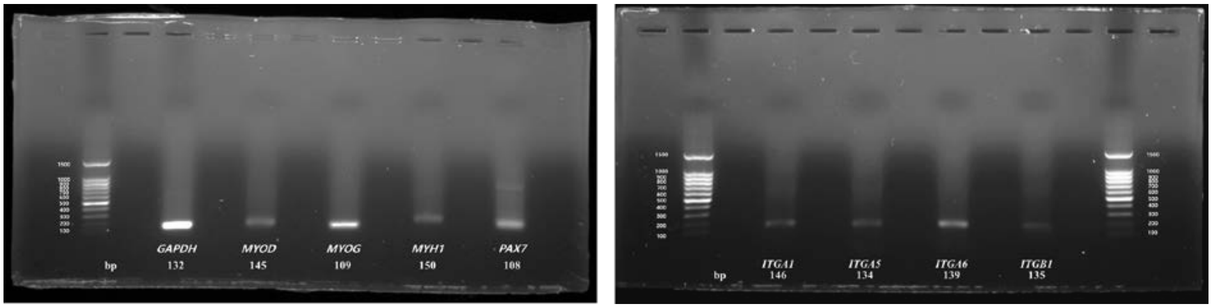

A
MYOG
MYH1
B
ITGA1
ITGA5
ITGA6
ITGB1
Relative expression level normalized by GAPDH
Passage
4
5
6
Collagen
Fibronectin
Fish gelatin
Geltrex
Laminin
Porcine gelatin
0
1
2
3
4
a
b
ab
c
Aa
Ab
Aab
ABb
Bab
Bb
B

Supplementary figure captions

Fig. S1. Validation of primers used in this study. (A) Agarose gel electrophoresis showing amplicon sizes for each primer pair. (B) Melt curve analysis confirming the specificity of amplification. (C) Standard curve used to calculate amplification efficiency for each primer.

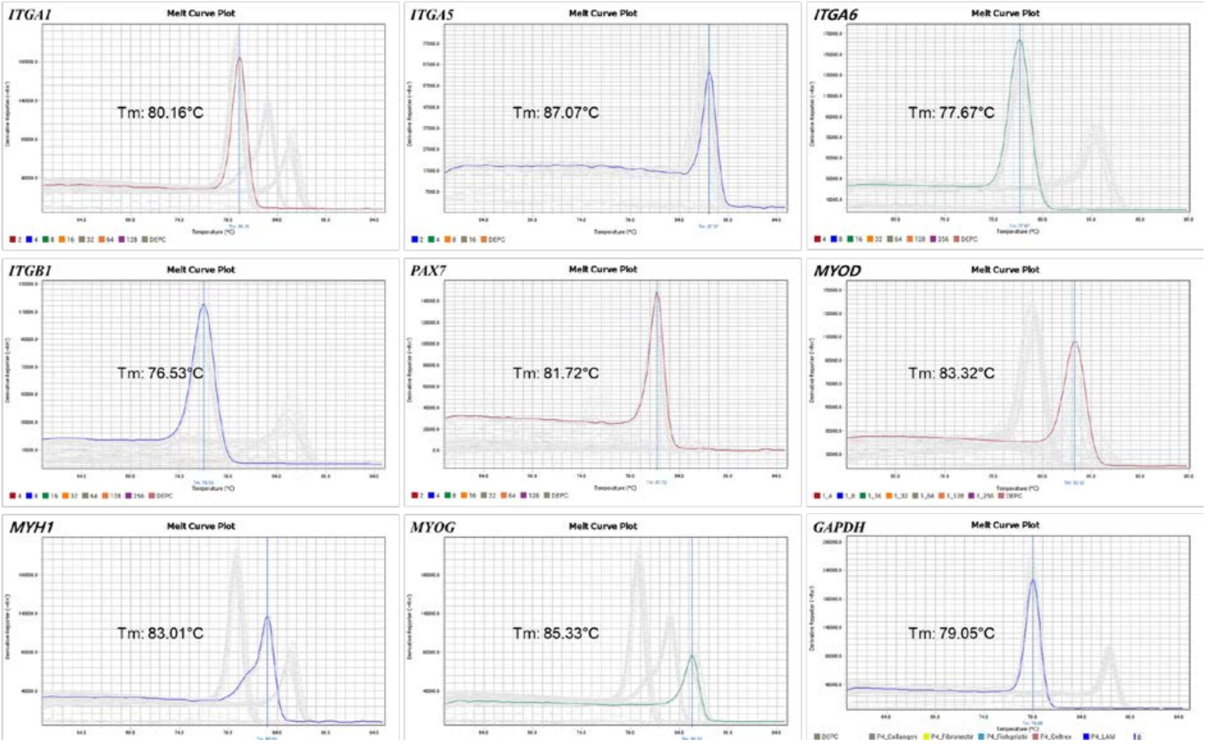
Fig. S1

A



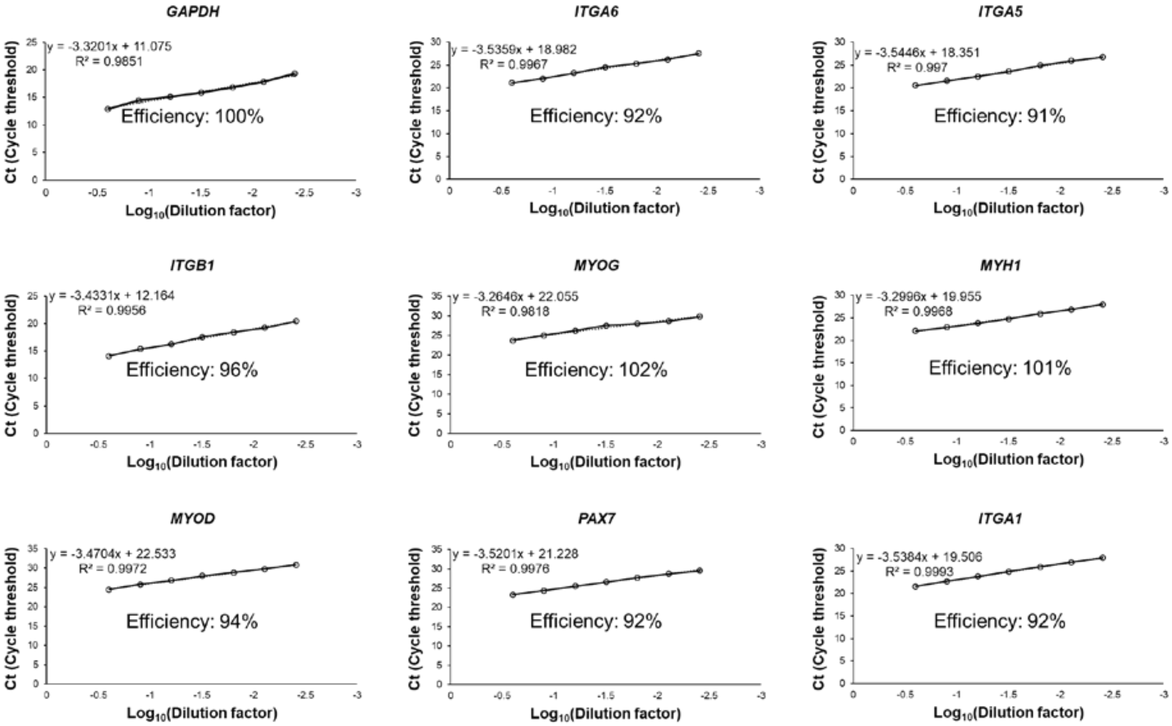
bp
GAPDH 132
MYOD 145
MYOG 109
MYH1 150
PAX7 108
bp
ITGA1 146
ITGA5 134
ITGA6 139
ITGB1 135

B



ITGA1
Melt Curve Plot
Tm: 80.16°C
ITGA5
Melt Curve Plot
Tm: 87.07°C
ITGA6
Melt Curve Plot
Tm: 77.67°C
ITGB1
Melt Curve Plot
Tm: 76.53°C
PAX7
Melt Curve Plot
Tm: 81.72°C
MYOD
Melt Curve Plot
Tm: 83.32°C
MYH1
Melt Curve Plot
Tm: 83.01°C
MYOG
Melt Curve Plot
Tm: 85.33°C
GAPDH
Melt Curve Plot
Tm: 79.05°C
Temperature (°C)
Derivative Reporter (-Rn')

C


GAPDH
y = -3.3201x + 11.075
R² = 0.9851
Efficiency: 100%
ITGA6
y = -3.5359x + 18.982
R² = 0.9967
Efficiency: 92%
ITGA5
y = -3.5446x + 18.351
R² = 0.997
Efficiency: 91%
ITGB1
y = -3.4331x + 12.164
R² = 0.9956
Efficiency: 96%
MYOG
y = -3.2646x + 22.055
R² = 0.9818
Efficiency: 102%
MYH1
y = -3.2996x + 19.955
R² = 0.9968
Efficiency: 101%
MYOD
y = -3.4704x + 22.533
R² = 0.9972
Efficiency: 94%
PAX7
y = -3.5201x + 21.228
R² = 0.9976
Efficiency: 92%
ITGA1
y = -3.5384x + 19.506
R² = 0.9993
Efficiency: 92%
Ct (Cycle threshold)
Log10(Dilution factor)
0
-0.5
-1
-1.5
-2
-2.5
-3