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Myogenesis of Porcine Muscle Satellite Cells by Extracellular Matrix From Fibrotic Adipose Tissue-Derived Mesenchymal Stem Cells

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Abstract: Cultivated meat relies on the production of muscle tissue using cell culture techniques. Myogenesis is a cellular differentiation process in which muscle satellite cells (SC) transform into myoblasts, or myotubes. The extracellular matrix (ECM) plays a pivotal role in meat quality, myogenesis, SC proliferation, and muscle tissue development to replicate meat texture. Because fibrotic differentiation and ECM are involved in the myogenic process, we aimed to induce myogenesis in SC using fibrotic differentiation of adipose tissue-derived mesenchymal stem cells (ADSC). ADSC, fibroblasts (FC), and SC were isolated from the back fat, ear margin tissues, and femur muscle tissues, respectively, of a 1-year-old Berkshire piglet. ADSC were treated with connective tissue growth factor (CTGF; 0, 12.5, 25, or 50 ng/mL) for 72 h to induce fibrotic differentiation. Conditioned media from differentiated ADSC and FC were prepared and labeled CA-CM and F-CM, respectively. SC were exposed to CA-CM or F-CM to assess their effects on myogenesis. The results demonstrated α-smooth muscle actin and collagen I as the optimal markers for assessing fibrotic differentiation in ADSC. CTGF significantly elevated both mRNA and protein expression of α-smooth muscle actin and collagen I ($P < 0.05$), suggesting that CTGF acts as an inducer of fibrotic differentiation in ADSC. Moreover, CTGF elevated the expression of ECM components (laminin, fibronectin, and collagen I) in ADSC ($P < 0.05$). mRNA expression of myogenesis-related genes (*MyoG, MyoD, Myf*5, and *Myf6*) increased in SC exposed to both CA-CM and F-CM ($P < 0.05$). Our data revealed that fibrotic differentiation of ADSC increased ECM production, and ECM-containing conditioned medium induced myogenesis in SC. These findings indicate that ECM-containing conditioned medium is a good option for enhancing myogenesis in SC, with potential implications for cultivated meat production.

Key words: cultivated meat, myogenesis, adipose-derived stem cell, fibrotic differentiation, muscle satellite cell
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Introduction

Cultivated meat, also known as *in vitro*, synthetic, or laboratory meat, is produced from adipose tissue-derived mesenchymal stem cells (ADSC), muscle satellite cells (SC), or induced pluripotent stem cells (Datar and Betti, 2010; Jairath et al., 2021). Cultivated meat is actively being studied as a viable alternative to traditional meat because of its potential to reduce the need for animal slaughter, lower the risk of animal diseases and epidemics, and minimize water and land use (Zhang et al., 2020; Hong et al., 2021). However, obtaining a product that completely replicates the taste, texture, and nutritional composition of traditional meat remains challenging.

Myogenesis, involving both embryonic and postnatal stages, is the complex process of generating skeletal muscle tissue from mesenchymal stem cells, crucial in cultivated meat production (Chriki and Hocquette, 2020; Ahmad et al., 2023). During

embryonic myogenesis, embryonic somites undergo differentiation into muscle progenitor cells facilitated by the Notch and Wnt signaling pathways (Grefte et al., 2007). Subsequently, certain muscle progenitor cells transition into postnatal muscle development, either directly differentiating into myoblasts or giving rise to a subset of postnatal muscle stem cells referred to as SC (Luo et al., 2021). This phase entails the activation and proliferation of SC, along with the fusion of differentiating myoblasts to form fully developed myofibers under the regulation of myogenic regulatory factors (MRF) (Hawke and Garry, 2001). Furthermore, myogenesis is regulated by myogenic factors, such as Pax7, Myf5, MyoD, MyoG, and Myf6 (Le Grand and Rudnicki, 2007).

The texture of meat is primarily influenced by the structures and composition of skeletal muscle (Dransfield et al., 1984). Skeletal muscle is mainly composed of muscle fibers and intramuscular connective tissues, including the extracellular matrix (ECM) (Nishimura, 2010). In the process of producing cultured meat, the ECM plays a critical role in ensuring the quality of the meat, facilitating the differentiation and proliferation of SC, and fostering muscle tissue development to mimic the texture of conventionally farmed meat (Yue, 2014; Ahmad et al., 2021). For instance, the mechanical characteristics of meat depend on factors such as the density of cross-linked chains per unit volume and the crystallinity of collagenous fibers (Lepetit, 2008).

The ECM not only provides structural support to the muscle but also facilitates force transmission, mediates cell-to-cell adhesion, and enhances the binding of molecules, thereby activating signaling pathways that regulate myogenesis (Bayne et al., 1984; Boonen and Post, 2008). Additionally, the ECM supports the development of myotubes during the early stages of myogenic differentiation (Zhang et al., 2021). For example, collagen I promotes the migration and myogenic differentiation of mouse myoblast cells (C2C12) by releasing interleukin (IL)-6 through activation of the FAK/NF-κB p65 pathway (Liu et al., 2020). Therefore, increasing ECM production is essential as it promotes myogenesis and enhances the overall quality of meat. To replicate an ECM environment that supports animal muscle cells during their growth and development, both *in vitro* and *in vivo* ECM models have been employed. Decellularized ECM (dECM) serves as a valuable *in vitro* model for exploring the multifaceted functions of ECM due to its retention of a native-like structure and composition (McInnes et al., 2022). dECM can be derived from tissue ECM *in vivo* or ECM fabricated by cells cultured *in vitro*. Tissue-derived ECM is similar to native ECM composition, mechanical properties, and microstructure, yet obtaining sufficient tissue-derived ECM from both animal and human sources poses challenges (Ott et al., 2008). However, cell-derived ECM can be procured to serve as an *in vitro* ECM model, which is intricate to identify and isolate from tissue (Hoshiba et al., 2016). Notably, ADSC and fibroblasts (FC) have emerged as potent sources for generating ECM components *in vitro* settings (Tracy et al., 2016; Rosadi et al., 2019). However, due to the limited regenerative potential of fully mature FC, their precursor cells, known as mesenchymal stem cells (MSC), have emerged as promising alternatives owing to their remarkable adaptability (Bianco and Robey, 2001; Blumberg et al., 2012).

The aims of our study were as follows: i) to identify specific markers distinguishing porcine ADSC from FC; ii) to examine the fibrotic response of ADSC to profibrotic factors; iii) to analyze ECM production in fibrotic ADSC; and iv) to evaluate the myogenic effects of SC induced by fibrotic ADSC-derived conditioned medium. In our study, fibrotic responses were induced by treating porcine ADSC with profibrotic factors, such as connective tissue growth factor (CTGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF), followed by the assessment of myogenic effects on SC through exposure to conditioned medium derived from fibrotic ADSC.

Materials and Methods

Chemicals and reagents

Recombinant CTGF was purchased from ProSpecTany TechnoGene (Ness-Ziona, Israel). Recombinant human basic fibroblast growth factor (bFGF) was provided by Bio-Techne (Minneapolis, MN, USA). Recombinant human TGF-β1 and VEGF were obtained from Abcam (Cambridge, UK). Fetal bovine serum (FBS), 0.05% trypsin, and 0.53 mM ethylenediaminetetraacetic (EDTA) solution were obtained from WELGENE Inc. (Gyeongsan, Korea). Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 (DMEM/F-12), Minimum Essential Medium Alpha (α-MEM), Penicillin-Streptomycin-Glutamine (PSG), and phosphate-buffered saline (PBS) were supplied by Gibco™ (Grand Island, NY, USA). Cell culture dishes, 6-well plates, and 24-well plates were purchased from SPL Life Sciences (Pocheon, South Korea).

Isolation, purification, and characterization of adipose tissue-derived mesenchymal stem cells

ADSC were extracted from the back fat of a 1-year-old Berkshire piglet, as previously described (Kim et al., 2023). The Berkshire breed was selected for its meat quality features, characterized by a high percentage of type I fibers, contributing to excellent meat quality (Ryu et al., 2008). The resected tissues were carefully washed and immersed in sterile PBS containing 10% antibiotic-antimycotic (AA; Gibco^{TM}). Fat tissues were separated from muscle tissues using a sterile tweezer and scissors and rinsed with a PBS solution containing 10% AA. For the digestion step, fat tissues were gently transferred into a sterile 50 mL conical tube and centrifuged at 3,000 rpm (MF80, Hanil Science Industrial Co., Ltd., Incheon, Korea) for 5 min. After discarding the supernatant, the remaining fat tissue was digested using a 0.2% digestion solution of 100 mg collagenase type II, 5 mL 10% AA, and 45 mL PBS. Digestion was performed in a shaking incubator at 37°C for 1 h. After incubation, the digested materials were filtered and centrifuged at 5,000 rpm for 5 min. The supernatant was discarded, and the cells were incubated with ammonium-chloride-potassium lysis buffer (Gibco^{TM}) at 4°C for 5 min. Next, 5 mL of neutralization medium was added, and the mixture was centrifuged at 1,500 rpm for 5 min. The isolated cells were resuspended in α-MEM (Gibco^{TM}) supplemented with 10% FBS, 1% AA, and 10 ng/mL of bFGF (Bio-Techne). Finally, the cells were seeded in a new culture flask for further cultivation.

SC were isolated from muscle tissues originating from the femurs of 1-day-old piglets, as previously described (Park et al., 2023). The tissues were washed thrice with Dulbecco's PBS (GibcoTM), which was enriched with 10% penicillin–streptomycin (Gibco^{TM}). The muscle tissues were minced and then dissociated using a digestion solution containing 2 mg/mL of collagenase D (Roche, IN, USA), 1 U/mL of Dispase II (Roche), and 0.25% trypsin-EDTA. The digested tissues were subsequently incubated in DMEM/F-12 supplemented with 10% penicillin–streptomycin at 37°C for 1 h. The homogenate was filtered using a 70 µm cell strainer and neutralized with F12 medium supplemented with 15% FBS. After centrifugation at 1,100 rpm for 5 min, ACK lysis buffer (Gibco^{TM}) was added to the supernatant, which was then incubated on ice for 5 min. The supernatant was again discarded after centrifuging at 1,100 rpm for 5 min. The cell pellet was resuspended in an F12 medium supplemented with 15% FBS, 1% PSG, and 10 ng/mL bFGF. The cells were seeded onto a culture plate and maintained in an incubator at 37°C for 1 h. To isolate SC, the medium containing suspended cells was collected and then transferred to another culture plate coated with 0.1% gelatin.

FC were isolated from the ear margin tissues of 1-day-old piglets, as previously described (Siddiqui et al., 2021). The ear tissues were kept in DMEM/F-12 supplemented with 10% penicillin–streptomycin, minced into 1 mm^3 pieces, and seeded onto a tissue culture flask. The flask was then incubated at 37°C under 5% CO_2 for 3–6 h, allowing the tissue fragments to firmly adhere to the surface of the flask. The adhered cells were maintained in DMEM/F-12 medium containing 10% FBS and 1% PSG. The culture was monitored at 24 h intervals to detect any significant cell outgrowth from the tissue pieces.

Cell culture and treatments

ADSC in passages 3–10, FC in passages 3–8, and SC in passages 3–8 were used for all experiments. All cells were cultured in a humidified incubator at 37°C under 5% CO_2. ADSC were maintained in α-MEM supplemented with 10% FBS, 1% (v/v) PSG, and 10 ng/mL bFGF. FC were maintained in DMEM/F-12 containing 10% FBS and 1% (v/v) PSG. SC were maintained in DMEM/F-12 containing 10% FBS, 1% (v/v) PSG, and 10 ng/mL bFGF. At approximately 80% confluence, the cells were trypsinized and subsequently sub-cultured. The growth medium was refreshed daily to ensure optimal cell growth and maintenance.

Preparation of conditioned medium

To prepare the conditioned medium for CTGF-treated ADSC, the cells were seeded in T-75 flasks with growth medium and cultured until they attained 80% confluency. The cells were treated with CTGF (0, 12.5, 25, and 50 ng/mL) for 72 h. After treatment, the cells were washed with PBS and cultured in serum-free DMEM/F-12 for 48 h at 37°C with 5% CO_2. The medium from ADSC was centrifuged at 2,340 rpm for 10 min, and the supernatant was collected. The collected media were termed CA-CM (0 CA-CM, conditioned media of ADSC treated with 0 ng/mL of CTGF; 12.5 CA-CM, conditioned media of ADSC treated with 12.5 ng/mL of CTGF; 25 CA-CM, conditioned media of ADSC treated with 25 ng/mL of CTGF; 50 CA-CM, conditioned media of ADSC treated with 50 ng/mL of CTGF) and stored at −80°C.

To prepare the conditioned medium for FC, the cells were cultured until 80% confluency in the growth medium, washed with PBS, and cultured in serum-free DMEM/F-12 for 48 h at 37°C under 5% CO_2. The medium from FC was centrifuged at 2,340 rpm for 10 min, and the supernatant was collected. The collected medium was termed F-CM and stored at −80°C.

Quantitative real-time polymerase chain reaction

To assess the mRNA expression levels of various genes, including MSC-related genes (*CD34*, *CD45*, *CD73*, *CD90*, and *CD105*), fibroblast-related genes (*COL1*, *COL3*, *ACTA2*, *VEGF*, *TGFβR1*, *MMP1*, and *CD144*), stemness-related genes (*OCT4*, *SOX2*, *NANOG*, and *SERPINH1*), and myogenesis-related genes (*Pax7*, *MyoG*, *MyoD*, *Myf6*, and *Myf5*), the cells were cultured in 6-well plates. To determine the fibrotic effect of CTGF, ADSC were treated with CTGF (0, 12.5, 25, and 50 ng/mL) for 72 h. To evaluate the myogenesis in a conditioned medium, SC were treated with a mixture of F-CM and basal DMEM/F-12 (0%, 20%, 40%, 60%, 80%, and 100%) or CA-CM (0%, 12.5%, 25%, and 50%) for 72 h. The differentiation medium (PC; DMEM/F12 supplemented with 5% horse serum and 1% PSG) was used as a positive control. After treatment, RNA extraction and cDNA synthesis were performed using TRIzol reagent (Ambion, Austin, TX, USA) and TOPscript RT DryMIX kit (Enzynomics, Daejeon, Korea), respectively. Real-time polymerase chain reaction (RT-PCR) was performed using a 2X Real-Time PCR Smart mix (BIOFACT Co., Ltd., Daejeon, Korea). PCR thermal cycling consisted of an initial denaturation step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 10 s. Relative quantification of mRNA expression was conducted using the $2^{-\Delta\Delta Cq}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control for normalization.

Primers (BIONICS Co., Ltd., Seoul, Korea) were designed using the AmplifX software, and the primer sequences are shown in Table 1.

Table 1. Primer sequences used in quantitative real-time PCR

Name of gene	Primer sequence (5'−3')
CD34	(F) GCA AAA TCT TTT CGG CTT GAA GG
	(R) CCT TGG CTG CCA CTA ACG T
CD45	(F) TCT TGA CTT CCT GTA AAG AGG
	(R) GAG GGA GCA ATT TCC TTC T
CD73	(F) TGG AGG TAC CTT TGA CCT G
	(R) ATC GTA CGT CAC GTG AAT TC
CD90	(F) ATG AAC CCT ACC ATT GGC A
	(R) TAC TGA ATG GGC AGG TTG G
CD105	(F) CGC TTC AGC TTC CTC CTC CG
	(R) CAC CAC GGG CTC CCG CTT G
COL1	(F) AGA CAT CCC ACC AGT CAC CT
	(R) TCA CGT CAT CGC ACA ACA CA
COL3	(F) TTT TAT GAC GGG CCC GGT GCT
	(R) CCA GGT CCC CTT TTG CAC AAA GC
ACTA2	(F) GTG TGA AGA AGA GGA CAG CAC T
	(R) AAA ACA GCC CTG GGA GCA T
MMP1	(F) TCT AAT GAT TGC TCA GGC T
	(R) AAT CTG ATG GCT CTC ACT T
CD144	(F) TGC AAC GAG CGG GGC GAG TT
	(R) CGC CGC CCT CCT CAT CGT A
OCT4	(F) AGG TGT TCA GCC AAA CGA CC
	(R) TGA TCG TTT GCC CTT CTG GC
SOX2	(F) GCC TGG GCG CCG AGT GGA
	(R) GGG CGA GCC GTT CAT GTA GGT CTG
NANOG	(F) ATC CAG CTT GTC CCC AAA G
	(R) ATT TCA TTC GCT GGT TCT GG
SERPINH1	(F) CCA ACT TTC CAG AAG TTT CTC GG
	(R) CGA GAA CGG ATT CCA CTC TCC
VEGF	(F) ATG AAC TTT CTG CTC TCT TGG G
	(R) TCT CGA TTG GAC GGC AGT A
TGFβR1	(F) AGG CGA CGG CAT TCC AGT GT
	(R) GGC CTG TCT CGC GGA ATT AGG TC
Pax7	(F) GGC ACC GTA CCG AGG ATG AT
	(R) CCG TGG TGG GCC ATC TCT ACT A
MyoG	(F) ATG GAG CTG TAT GAG ACA TC
	(R) GTT TTC CCC GTC ATA GAA GT
MyoD	(F) GAC CTG ATG GAC GGC TGC CAG TT
	(R) TAG GTG CTC GCA CGT GCT CTT CC
Myf5	(F) CGC TCC GCG ACG TAG ATT TG
	(R) GTC CAG GTC CTC GAA GAA GCG CA
Myf6	(F) TGA TGG ACC TTT TTG AAA CTG G
	(R) ATG TTC CTC TCC ACT GCT G
GAPDH	(F) TTT CAC AGA CAG CCG TGT G
	(R) CCC TTT GGA CCA GTC CTC GA

Abbreviations: ACTA2, Alpha smooth muscle actin; CD, Cluster of differentiation; COL1A1, Collagen 1A1; COL3A1, Collagen 3A1; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; MMP1, Matrix metalloproteinase-1; MyoG, Myogenin; MyoD1, Myogenic differentiation 1; Myf5, Myogenic factor 5; Myf6, Myogenic factor 6; NANOG, Nanog homebox; OCT4, Octamer-binding transcription factor 4; Pax7, Paired box protein Pax7; SERPINH1, Serpin family H member 1; SOX2, SRY-box transcription factor 2; TGFβR1, Transforming growth factor beta receptor 1; VEGF, Vascular endothelial growth factor.

Western blot analysis

Cells were treated with CTGF (12.5, 25, and 50 ng/mL) for 72 h, TGF-β (1.25, 2.5, and 5 ng/mL) for 48 h, VEGF (2.5, 5, and 10 ng/mL) for 48 h, or distilled water. Radioimmunoprecipitation assay buffer (Elpis Biotech, Daejeon, Korea) containing a protease inhibitor cocktail (Abbkine, Wuhan, China) was used for cell lysis. Cell lysates were collected and centrifuged at $18{,}000 \times g$ at 4°C for 20 min. The supernatants were analyzed using a bicinchoninic acid protein assay kit.

Protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Subsequently, the membranes were blocked with 3% nonfat milk or bovine serum albumin buffer dissolved in Tris-buffered saline with Tween 20 (TBST) buffer for 1 h at 25°C room temperature and then incubated with anti-α-smooth muscle actin and anti-collagen I (Thermo Fisher Scientific, Pittsburgh, PA), anti-GAPDH (Merck Millipore, Darmstadt, Germany), and anti-α-tubulin (Novus Biologicals, Centennial, CO, USA) primary antibodies at 4°C for 16 h. The membranes were washed 3 times with TBST buffer and subsequently incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Enzo Life Sciences, Lausen, Switzerland) or goat anti-mouse IgG H&L (Abcam) secondary antibodies for 1.5 h at room temperature. The proteins were visualized using an enhanced chemiluminescence detection reagent, and their expression was quantified using the ImageJ software.

Immunofluorescence

For immunofluorescence (IF) staining, ADSC and FC were grown in 24-well plates and exposed to CTGF (12.5, 25, and 50 ng/mL) for 72 h or left untreated. The cells were fixed with 4% paraformaldehyde for 15 min and then permeabilized using 0.1% Triton X-100 in PBS for 10 min. Next, a blocking buffer consisting of 3% bovine serum albumin and 2% normal donkey serum was used to block the cell monolayers. The cells were incubated with anti-α-smooth muscle actin and anti-collagen I antibodies, diluted in blocking buffer, at 4°C for 24 h. After washing with PBS, the cells were incubated with DyLight^{TM}-488-conjugated anti-IgG at room temperature for 1 h and washed thrice with PBS. Subsequently, the cell nuclei were stained with DAPI (1 μg/mL) for 15 min and washed thrice with PBS. Images were captured using an Olympus IX71 fluorescence microscope and digitally recorded using an Olympus DP71 camera and DP controller software (Olympus Optical Co., Ltd., Tokyo, Japan).

Dot blot analysis

To determine the amount of ECM (laminin, fibronectin, and collagen I), CA-CM and basal α-MEM (αM; α-MEM supplemented with 1% PSG) were loaded directly into the wells of a 96-well plate and transferred onto nitrocellulose membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) using the Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were washed with TBST buffer and blocked with 3% nonfat milk buffer dissolved in TBST buffer for 1 h at room temperature. Laminin, fibronectin, and collagen I were detected using anti-laminin (Abcam), anti-fibronectin (Proteintech, Rosemont, IL, USA), and anti-collagen I (Thermo Fisher Scientific) primary antibodies at 4°C for 16 h, respectively. The membranes were then washed 3 times with TBST buffer and subsequently incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase (Enzo Life Sciences) or goat anti-mouse IgG H&L (Abcam) secondary antibodies for 1.5 h at room temperature. The proteins were visualized using an enhanced chemiluminescence detection reagent, and their expression was quantified using the ImageJ software.

Statistical analysis

All experiments were performed at least 3 times, and data are expressed as the mean ± standard error (SE). IBM SPSS Statistics software (version 22.0; IBM SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Statistical significance was set at $P < 0.05$ and estimated using Tukey's post hoc test or independent two-sample *t*-test.

Results

Evaluation of expression of markers for adipose tissue-derived mesenchymal stem cells and fibroblasts

To effectively identify fibrotic differentiation in ADSC, the identification of a reliable marker that can differentiate between ADSC and FC is crucial. Distinguishing ADSC from FC based solely on cell morphology is difficult, prompting the use of various markers identified in previous studies. Consequently, we assessed the mRNA levels of several markers, including MSC-related surface markers (*CD34*, *CD45*, *CD73*, *CD90*, and *CD105*), FC-specific markers (*COL1A1*, *COL3A*1, *ACTA2*, *MMP1*, and *CD144*), and stemness markers (*OCT4*, *SOX2*, *NANOG*, and *SERPHINH1*) in both ADSC and FC. ADSC and FC were seeded in 6-well plates at a density of 1.5×10^5 cells per well and cultured until 80%–90% confluency at 37°C. Regarding MSC surface markers, FC exhibited significantly higher ($P < 0.05$) expression of *CD73* and *CD105* than ADSC (Figure 1A). Among the fibroblast-specific markers, FC showed higher ($P < 0.05$) mRNA expression of *COL1A1*, *COL3A1*, and *ACTA2*, whereas the expression of *MMP1* and *CD144* was lower

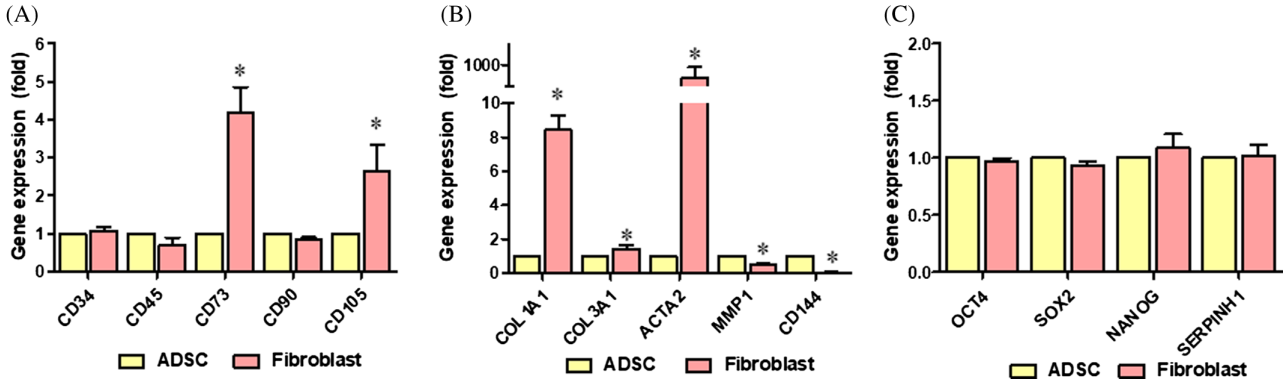

Figure 1. Evaluation of mRNA expression levels of markers to differentiate between ADSC and FC. Cells were grown until 80%–90% confluency at 37°C. Gene expressions of (A) MSC-related surface markers (*CD34*, *CD45*, *CD73*, *CD90*, and *CD105*), (B) fibroblast-specific markers (*COL1A1*, *COL3A1*, *ACTA2*, *MMP1*, and *CD144*), and (C) stemness markers (*OCT4*, *SOX2*, *NANOG*, and *SERPHINH1*) were determined by RT-PCR. GAPDH was used as housekeeping gene. *Indicates a significant difference compared to ADSC for the same gene ($P < 0.05$). Data represent the mean ± standard error. All experiments were performed at least 3 times. ACTA2, Alpha smooth muscle actin; ADSC, Adipocyte-derived stem cell; CD, Cluster of differentiation; COL1A1, Collagen 1A1; COL3, Collagen 3A1; FC, Fibroblast cell; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; MMP1, Matrix metalloproteinase-1; NANOG, Nanog homebox; OCT4, Octamer-binding transcription factor 4; SERPINH1, Serpin family H member 1; SOX2, SRY-box transcription factor 2.

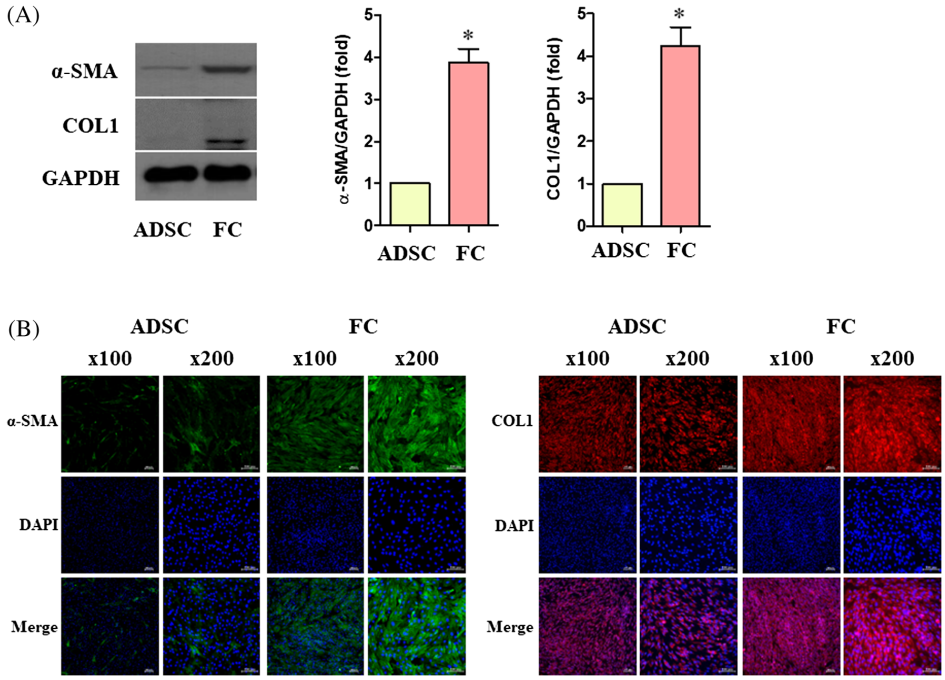

Figure 2. Evaluation of protein expression levels of markers to differentiate between ADSC and FC. The cells were grown until 80%–90% confluency at 37°C. (A) Protein expression of fibroblast-specific markers (α-smooth muscle actin and COL1) were determined using western blotting. GAPDH was used as housekeeping protein. (B) Fluorescence images of α-smooth muscle actin (green), COL1 (red), and DAPI (blue) in ADSC and FC. Magnification: 100× and 200×. *Indicates a significant difference compared to ADSC ($P < 0.05$). Data represent the mean ± standard error. All experiments were performed at least 3 times. ADSC, Adipocyte-derived stem cell; COL1, Collagen I; FC, Fibroblast cell; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; α-smooth muscle actin, Alpha smooth muscle actin.

($P < 0.05$) in FC than in ADSC (Figure 1B). No discernible differences ($P > 0.05$) in stemness markers between ADSC and FC were observed (Figure 1C). The findings suggest that ACTA2 (α-smooth muscle actin) and COL1A1 (COL1) are the most effective markers for differentiating between ADSC and FC.

As mRNA levels of α-smooth muscle actin and COL1 were higher in FC than in ADSC, we assessed the protein levels of α-smooth muscle actin and COL1 through western blotting and IF staining (Figure 2). In western blot analysis, both α-smooth muscle actin and COL1 protein levels were elevated

($P < 0.05$) in FC compared to ADSC (Figure 2A). Furthermore, IF staining revealed a higher expression level of α-smooth muscle actin in FC than in ADSC (Figure 2B). Moreover, increased protein expression of COL1 in FC compared to ADSC was observed. Based on these findings, we recognized and used α-smooth muscle actin and COL1 as markers for fibrotic differentiation in the subsequent studies.

Fibrotic effects of growth factors on adipose tissue-derived mesenchymal stem cells

CTGF has been reported to induce myofibroblast formation by inducing the differentiation of muscle SC (Paradis et al., 2002). Additionally, CTGF modulates the cellular phenotype by interacting with various cytokines and growth factors, such as TGF-β, VEGF, IGF1, and BMP4 (Lipson et al., 2012). Thus, we investigated the fibrotic effects of CTGF, TGF-β, and VEGF on ADSC (Figure 3). To assess the protein expression of fibrotic differentiation markers, cells were seeded in 6-well plates at a density of 1.5×10^5 cells per well and cultured until 80%–90% confluency at 37°C. Subsequently, ADSC were treated with CTGF (0, 12.5, 25, and 50 ng/mL) for 72 h, TGF-β (0, 1.25, 2.5, and 5 ng/mL) for 48 h, and VEGF (0, 2.5, 5, and 10 ng/mL) for 48 h. FC were used as a positive control. The protein expression of α-smooth muscle actin and COL1 in ADSC was examined using western blotting and IF staining. Results demonstrated that 50 ng/mL of CTGF, 5 ng/mL of TGF-β, and 10 ng/mL of VEGF increased ($P < 0.05$) the protein expressions of α-smooth muscle actin and COL1 in ADSC (Figure 3A–C). Among the growth factors tested, CTGF demonstrated the most pronounced fibrotic effect. Furthermore, IF staining revealed the concentration-dependent effect of CTGF in both α-smooth muscle actin and COL1 (Figure 3D). Collectively,

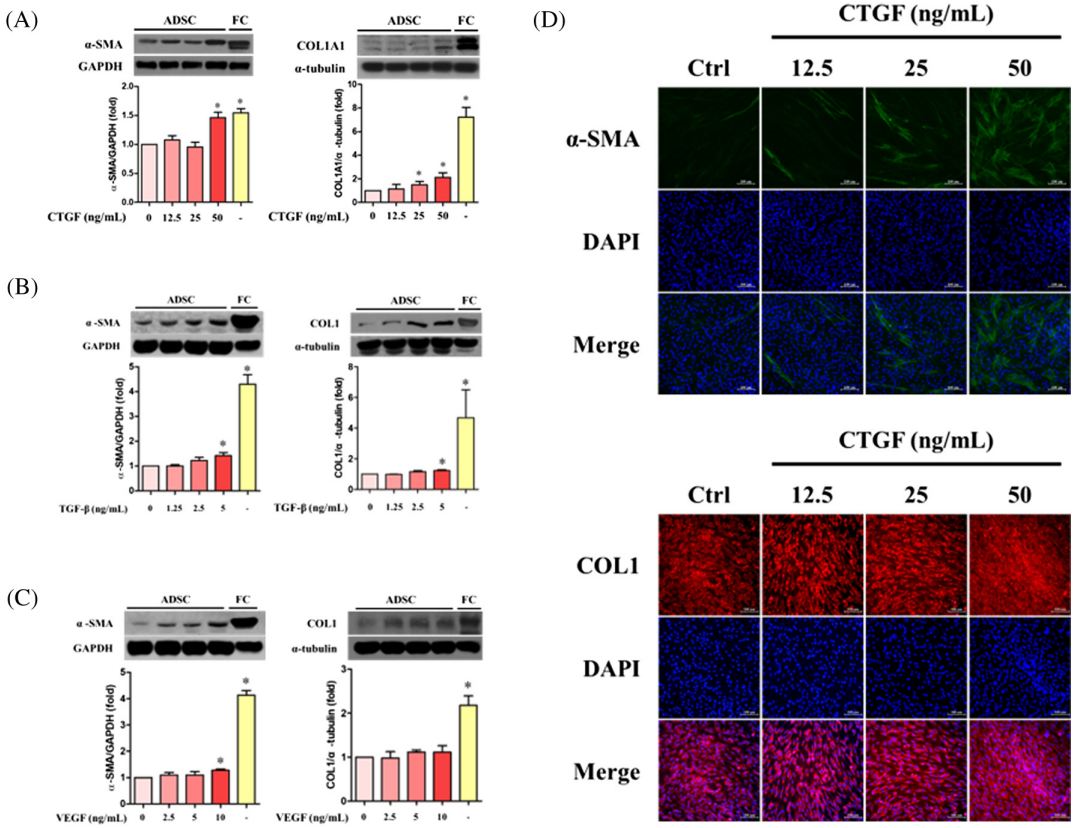

Figure 3. The effect of CTGF, TGF-β, and VEGF on fibrotic differentiation in ADSC. The cells were grown until 80%–90% confluency at 37°C. ADSC were treated with CTGF (0, 12.5, 25, and 50 ng/mL), TGF-β (0, 1.25, 2.5, and 5 ng/mL), or VEGF (0, 2.5, 5, and 10 ng/mL) for 48 h. FC were used as positive control. Protein expression of fibrotic differentiation markers (α-smooth muscle actin and COL1) in ADSC treated with (A) CTGF, (B) TGF-β, and (C) VEGF were determined using western blotting. GAPDH and α-tubulin were used as housekeeping proteins. (D) Fluorescence image of α-smooth muscle actin (green), COL1 (red), and DAPI (blue) in CTGF-treated ADSC. Magnification: 100× and 200×. *Indicates a significant difference compared to untreated ADSC ($P < 0.05$). Data represent the mean ± standard error. All experiments were performed at least 3 times. ADSC, Adipocyte-derived stem cell; COL1, Collagen I; CTGF, Connective tissue growth factor; FC, fibroblast cell; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; TGF-β, Recombinant human TGF beta 1 protein; VEGF, Recombinant vascular endothelial growth factor A; α-smooth muscle actin, Alpha smooth muscle actin.

our data suggest that CTGF, TGF-β, and VEGF can induce fibrotic differentiation, with CTGF being the most effective.

Connective tissue growth factor induced fibrotic markers in adipose tissue-derived mesenchymal stem cells

To investigate the fibrotic effect of CTGF on ADSC, the cells were treated with CTGF at concentrations of 0, 12.5, 25, and 50 ng/mL for 72 h. Subsequently, mRNA expression levels of fibrotic markers (*ACTA2*, *COL1A1*, and *COL3A1*) and fibrotic differentiation-related genes (*VEGF* and *TGFβR1*) were analyzed. The expression levels of *ACTA2*, *COL1A1*, *COL3A1*, *VEGF*, and *TGFβR1* increased in a concentration-dependent manner ($P < 0.05$) when ADSC were exposed to CTGF (Figure 4). These results indicate that CTGF plays a role in mediating fibrotic differentiation by regulating VEGF and TGFβR1 expression.

Enhanced extracellular matrix production by fibrotic adipose tissue-derived mesenchymal stem cells

To assess ECM production, ADSC were treated with CTGF at concentrations of 0, 12.5, 25, and 50 ng/mL for 72 h. Subsequently, the cells were cultured in a serum-free medium for an additional 48 h to produce CA-CM (0, 12.5, 25, and 50 CA-CM). Protein levels of ECM proteins (laminin, fibronectin, and collagen I) in CA-CM were examined using a dot blot assay. Serum-free DMEM/F-12 was used as a negative control. ECM production was increased in the conditioned medium of CTGF-treated ADSC ($P < 0.05$) in a CTGF concentration-dependent manner (Figure 5). Fibronectin and collagen I exhibited a noticeable increase with increasing CTGF concentration, whereas laminin showed an increase to a comparatively lesser extent than that of fibronectin and collagen I, suggesting that CTGF could potentially augment ECM production by ADSC.

Conditioned medium from fibrotic adipose tissue-derived mesenchymal stem cell enhances myogenesis of satellite cells

ECM has been reported to promote myogenic differentiation of porcine muscle stem cells (Wilschut et al., 2010). Therefore, we tested the myogenic potential of conditioned media produced by ADSC (CA-CM) and FC (F-CM). To assess the myogenic effect of F-CM, we generated various concentrations of F-CM by diluting it with basal medium (DMEM/

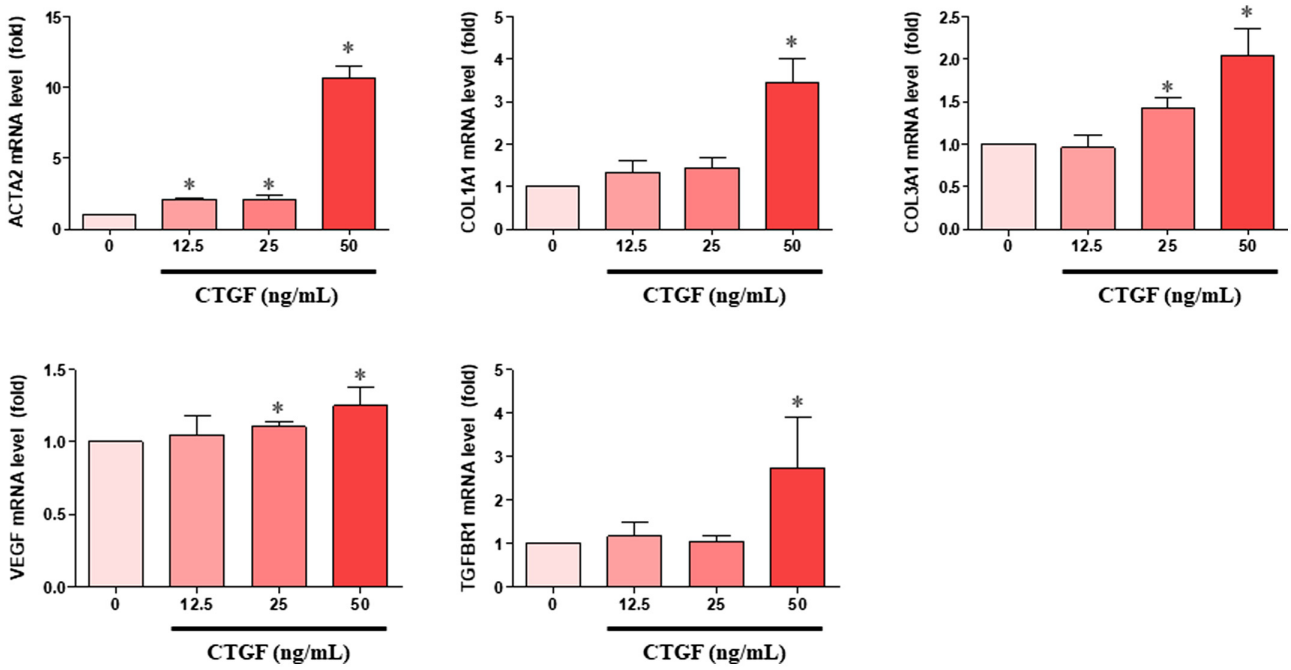

Figure 4. Evaluation of mRNA expression levels of fibrotic markers in CTGF-treated ADSC. ADSC were treated with CTGF (0, 12.5, 25, and 50 ng/mL) for 48 h. Gene expression levels of ACTA2, COL1A1, COL3A1, VEGF, and TGFβR1 were determined by real-time PCR. GAPDH was used as housekeeping gene. *Indicates a significant difference compared to untreated ADSC ($P < 0.05$). Data represent the mean ± standard error. All experiments were performed at least 3 times. ACTA2, Alpha smooth muscle actin; ADSC, Adipocyte-derived stem cell; COL1A1, Collagen 1A1; COL3A1, Collagen 3A1; CTGF, Connective tissue growth factor; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; TGFβR1, Transforming growth factor beta receptor 1.

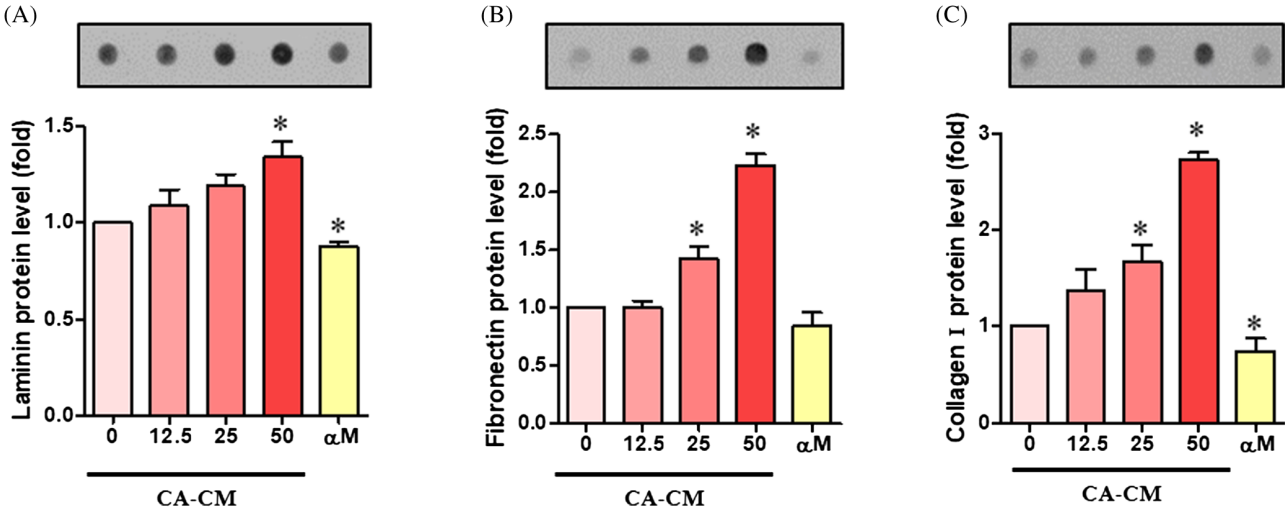

Figure 5. ECM production by CTGF-treated ADSC. The cells were grown until 80%–90% confluency at 37°C. ADSC were treated with CTGF (0, 12.5, 25, and 50 ng/mL) for 72 h. After treatment, the cells were cultured with serum-free α-MEM for 48 h, and the medium (termed as CA-CM) was collected. Serum-free medium was used as positive control. Protein expression levels of ECM components (laminin, fibronectin, and collagen I) in ADSC treated with (A) CTGF, (B) TGF-β, and (C) VEGF were determined using western blotting. GAPDH and α-tubulin were used as housekeeping proteins. (D) Fluorescence image of α-smooth muscle actin (green), COL1 (red), and DAPI (blue) in CTGF-treated ADSC. Magnification: 100× and 200×. *Indicates a significant difference compared to untreated ADSC ($P < 0.05$). Data represent the mean ± standard error. All experiments were performed at least 3 times. ADSC, Adipocyte-derived stem cell; COL1, Collagen I; CTGF, Connective tissue growth factor; FC, Fibroblast cell; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; TGF-β, Recombinant human TGF beta 1 protein; VEGF, Recombinant vascular endothelial growth factor A; α-smooth muscle actin, Alpha smooth muscle actin.

F-12 + 1% PSG) to achieve final concentrations of 0%, 20%, 40%, 60%, 80%, and 100%. Additionally, CA-CM (0 CA-CM, 12.5 CA-CM, 25 CA-CM, and 50 CA-CM) were generated by treating ADSC with varying concentrations of CTGF (0, 12.5, 25, and 50 ng/mL). mm DMEM/F-12 medium supplemented with 5% horse serum and 1% PSG (PC) was used as positive control for myogenesis of SC. This study aimed to explore the potential impact of conditioned media from ADSC, specifically those undergoing fibrotic differentiation, on the myogenic differentiation of SC. SC were seeded in 6-well plates at a density of 3×10^5 cells per well and cultured until 80%–90% confluency. Subsequently, SC were exposed to PC, F-CM, or CA-CM for 72 h. DMEM/F-12 medium containing 5% horse serum and 1% PSG was used as a positive control for myogenesis. mRNA expression levels of myogenic markers (*Pax7*, *MyoG*, *MyoD*, *Myf5*, and *Myf6*) were examined in the treated cells. The data demonstrated that mRNA expression of *Pax7* significantly decreased ($P < 0.05$), whereas that of *MyoG*, *MyoD*, *Myf5*, and *Myf6* increased ($P < 0.05$) in SC with increasing concentrations of F-CM (Figure 6A). SC treated with CA-CM showed a significant decreased expression of *Pax7* ($P < 0.05$) but elevated expression levels of *MyoG*, *MyoD*, *Myf5*, and *Myf6* ($P < 0.05$) with increasing concentrations of CTGF (Figure 6B). These data imply that the ECM produced by CTGF-stimulated ADSC has the potential to enhance the myogenic differentiation of SC.

Discussion

The major findings of this study are as follows. First, α-smooth muscle actin and COL1 are identified as the most effective fibrotic differentiation markers for differentiating between ADSC and FC. Second, CTGF, TGF-β, and VEGF exert fibrotic effects on ADSC, with CTGF exhibiting the most potent effect. Furthermore, CTGF induces fibrotic differentiation in ADSC by modulating VEGF and TGFβR1 expression. Third, we generated F-CM and CA-CM and demonstrated the effect of CA-CM on the myogenic differentiation of SC. Collectively, these findings indicate that the ECM components present in CA-CM enhance the myogenic potential of SC and potentially contribute to improvements in cultivated meat production.

MSC are stem cells known for their ability to adhere to culture surfaces, form colony-like structures resembling FC, and undergo multiple passages during proliferation (Friedenstein et al., 1970; Horwitz et al., 2005). In this study, we utilized ADSC, a type of MSC. To assess fibrotic differentiation in ADSC, distinguishing markers from FC is necessary. MSC and FC share similar morphological characteristics, express certain mesenchymal cell surface markers, and exhibit comparable gene expression profiles. For instance, a previous study demonstrated that MSC and FC display similar morphologies, share the same cell surface markers (positive for CD73, CD90, and CD105, and negative

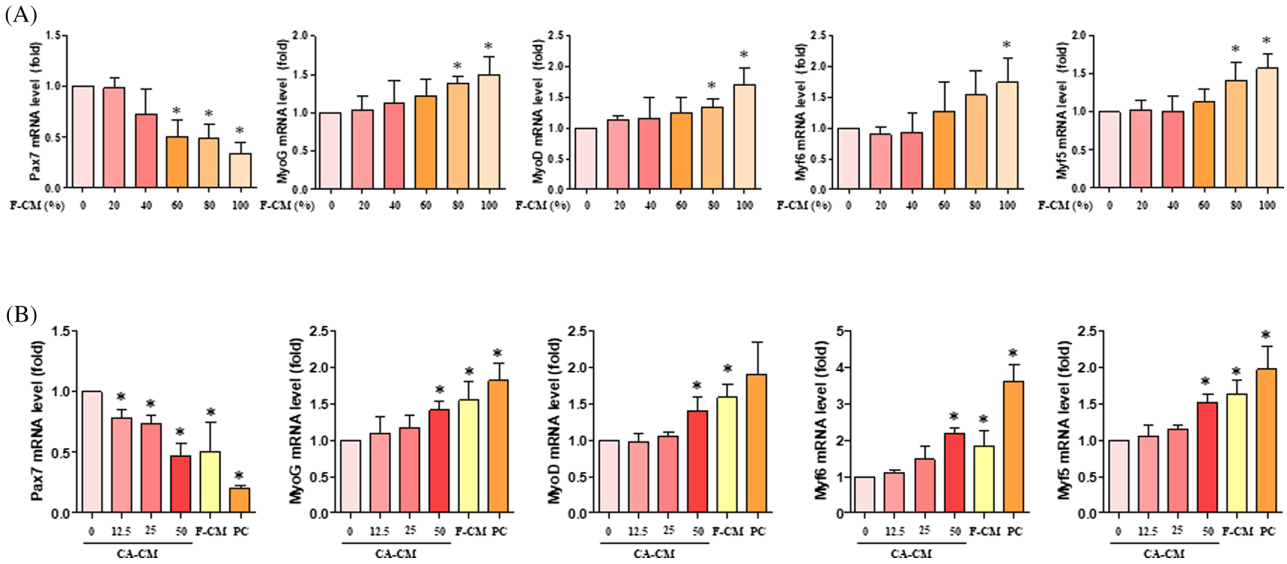

Figure 6. Myogenic effect of F-CM and CA-CM on SC. SC were grown until 80%–90% confluency at 37°C. The cells were treated with F-CM (0%, 20%, 40%, 60%, 80%, and 100%) and CA-CM (0%, 12.5%, 25%, and 50%) for 72 h. DMEM/F-12 medium supplemented with 5% horse serum and 1% PSG (PC) was used as positive control for myogenesis. Protein expression levels of myogenesis markers (Pax7, MyoG, MyoD1, Myf5, and Myf6) in SC treated with (A) F-CM and (B) CA-CM and PC were determined by real-time PCR. GAPDH was used as housekeeping gene. *Indicates a significant difference compared to untreated SC ($P < 0.05$). Data represent the mean ± standard error. All experiments were performed at least 3 times. ADSC, Adipocyte-derived stem cell; CA-CM, Conditioned medium of adipocyte-derived CTGF-treated stem cells; CTGF, Connective tissue growth factor; F-CM, Fibroblast-conditioned medium; FC, Fibroblast cell; GAPDH, Glyceraldehyde-3-phosphate dehydrogenase; MyoG, Myogenin; MyoD, Myogenic differentiation 1; Myf5, Myogenic factor 5; Myf6, Myogenic factor 6; Pax7, Paired box protein Pax7; SC, Satellite cell.

for CD14, CD34, and CD45), and have the potential to differentiate into adipocytes, chondrocytes, and osteoblasts (Denu et al., 2016). Thus, well-defined molecular markers that can definitively distinguish MSC from FC are lacking.

In the current study, we determined the mRNA expression of cell surface markers (*CD34*, *CD45*, *CD73*, *CD90*, and *CD105*), FC-specific markers (*COL1A1*, *COL3A*1, *ACTA2*, *MMP1*, and *CD144*), and stemness markers (*OCT4*, *SOX2*, *NANOG*, and *SERPHINH1*) in both ADSC and FC. Our findings revealed that FC expressed *CD73*, *CD105*, *COL1A1*, *COL3A1*, and *ACTA2* to a higher extent ($P < 0.05$) and *MMP1* and *CD144* to a lesser extent ($P < 0.05$). Previous studies have shown that *CD73* and *CD105* are highly expressed in both MSC and FC; however, quantitative differences may exist. For example, human skin-derived fibroblasts and adipose tissue-derived stem cells displayed elevated surface antigen levels for conventional MSC markers (CD44, CD73, and CD105) (Alt et al., 2011). Furthermore, FC exhibited significantly higher protein expression of α-smooth muscle actin and Collagen I according to western blotting and IF staining assays. Therefore, we selected α-smooth muscle actin (*ACTA2*) and Collagen I (*COL1A1*), the FC markers that showed the most evident difference, as the optimal markers for assessing fibrotic differentiation.

Fibrosis is characterized by the excessive accumulation of fibrous connective tissue, including the ECM, in wounded tissue (Wynn, 2011). FC play a key role in fibrotic differentiation as they are responsible for producing essential components of the ECM. These components include fibrous proteins (collagen and elastin), adhesive proteins (laminin and fibronectin), and ground substances (hyaluronan and glycoproteins) (Kendall and Feghali-Bostwick, 2014). These ECM components are assumed to be associated with the quality of cultivated meat. For example, Collagen I forms collagen fibers in the skeletal muscle and promotes myoblast proliferation (Gillies and Lieber, 2011). Furthermore, fibronectin and laminin enhance the proliferation, adhesion, and differentiation of porcine muscle stem cells (Kjaer, 2004; Zhang et al., 2021). Therefore, our objective was to enhance ECM production by inducing fibrotic differentiation in ADSC.

CTGF is a member of the protein family that regulates cell signaling pathways, resulting in deposition and remodeling of the ECM and activation of myofibroblasts, leading to fibrotic differentiation (Lipson et al., 2012). CTGF interacts with various cytokines and growth factors, such as TGF-β, VEGF, IGF1, and BMP4. TGF-β is a central mediator of fibrotic differentiation due to its ability to induce the synthesis and deposition of the ECM (Xu et al., 2018). TGF-β ligands activate a cell membrane receptor complex composed

of TGF-β receptor I (TGFβRI) and TGF-β receptor II (TGFβRII). This complex phosphorylates small mothers against decapentaplegic (SMAD)2 and small mothers against decapentaplegic (SMAD)3, leading to the subsequent formation of a transcriptional complex with small mothers against decapentaplegic (SMAD)4. This assembled complex then translocates into the cell nucleus, binding to the promoter regions of downstream target genes, including *ACTA2* and *CTGF* (Shi and Massagué, 2003). Additionally, VEGF plays a role in ECM metabolism by increasing the expression of matrix metalloproteins, which are crucial for ECM remodeling (Zhang and Chu, 2019). To enhance ECM production, we treated ADSC with fibrotic differentiation-related cytokines and growth factors (CTGF, TGF-β, or VEGF). All cytokines and growth factors exhibited a concentration-dependent effect on fibrotic differentiation in ADSC. Among them, CTGF had the most potent fibrotic effect, directly inducing fibrotic differentiation, whereas the others stimulated fibrotic differentiation via signaling pathways or in combination with CTGF. Subsequently, we assessed the mRNA expression of fibrotic differentiation-related markers (*ACTA2*, *COL1A1*, *COL3A1*, *VEGF*, and *TGFβR1*) in CTGF-treated ADSC. Our study revealed that CTGF significantly increased ($P < 0.05$) the expression of these fibrotic differentiation-related markers in a concentration-dependent manner. The TGF-β signaling pathway via TGFβRI has a significant impact on fibrotic differentiation. For example, knockdown of *TGFβR1* slightly decreased the expression of *CTGF* and *COL1A1* in primary mouse myoblasts (Hillege et al., 2020). Therefore, our results indicate that CTGF induces fibrotic differentiation in ADSC by interacting with *VEGF* and *TGFβR1*.

ECM proteins are essential for myotube development and myogenic differentiation (Ahmad et al., 2021). Thus, we assessed ECM production in CTGF-treated ADSC. Our results demonstrated that CTGF increased ($P < 0.05$) the secretion of laminin, fibronectin, and Collagen I by ADSC in a concentration-dependent manner. Although the precise mechanisms by which CTGF synthesizes these proteins are still being investigated, a previous study showed that primary osteoblasts transfected with CTGF small interfering RNA exhibited a significant decrease in collagen I and fibronectin mRNA expression (Arnott et al., 2007). Furthermore, C57BL10 mice transfected with *CTGF/CCN2* gene (*Ctgf/Ccn2*$^{+/-}$), which reduces CTGF protein levels, exhibited decreased accumulation of muscular fibronectin and collagen compared to *Ctgf/Ccn2*$^{+/+}$ mice (Rebolledo et al., 2019). Our results are consistent with these findings, suggesting that CTGF plays a pivotal role in increasing ECM production in ADSC.

For cultivated meat production, the myogenic differentiation of SC is a critical step in myofiber formation and muscle development (Kumar et al., 2023). Myogenesis is regulated by various factors such as Pax7, Myf5, Myf6, MyoD, and MyoG (Le Grand and Rudnicki, 2007; Bentzinger et al., 2012; Eng et al., 2013). We determined the myogenic effect of conditioned media from FC and CTGF-treated ADSC. Our data showed that the media obtained from FC and CTGF-treated ADSC significantly decreased ($P < 0.05$) the mRNA expression of *Pax7* and increased the expression of *MyoG*, *MyoD*, *Myf6*, and *Myf5*. Downregulation of *Pax-7* is a reliable indicator of myogenesis in SC. Consistent with our findings, a previous study reported that upregulation of *Pax-7* inhibited both myogenesis and cell cycle progression in SC (Olguin and Olwin, 2004). Additionally, the MRF family of transcription factors consisting of *MyoD, Myf5, Mrf4, MyoG,* and *Myf6* plays a central role in regulating gene expression during vertebrate muscle development, both in early and adult myogenesis and myotube differentiation (Ropka-Molik et al., 2011; Hernández-Hernández et al., 2017; Zammit, 2017). Therefore, our data suggest that inducing fibrotic differentiation in ADSC can enhance myogenesis in SC and potentially contribute to cultivated meat production (Figure 7).

Co-culture systems are widely employed in tissue engineering because they promote the formation of

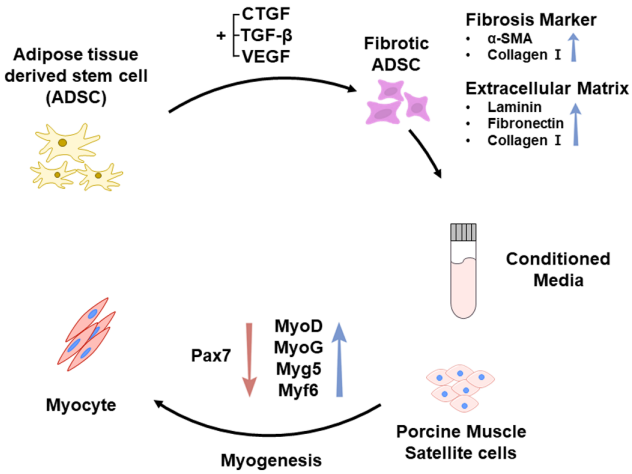

Figure 7. Schematic representation of enhanced myogenesis in porcine muscle satellite cells induced by conditioned medium from fibrotic adipose-derived stem cells (ADSC). ADSC, Adipocyte-derived stem cell; CTGF, Connective tissue growth factor; MyoG, Myogenin; MyoD, Myogenic differentiation 1; Myf5, Myogenic factor 5; Myf6, Myogenic factor 6; Pax7, Paired box protein Pax7; TGF-β, Recombinant human TGF beta 1 protein; VEGF, Recombinant vascular endothelial growth factor A; α-smooth muscle actin, Alpha smooth muscle actin.

tissues involving multiple cell types and enhance cell proliferation and differentiation (Paschos et al., 2015; Kovina et al., 2021). A co-culture system encompasses 2 distinct methods: indirect and direct. In an indirect co-culture system, cells are cultivated in a shared environment, and cell interactions occur through soluble factors, excluding direct physical contact (David et al., 2023). One representative method involves utilizing a conditioned medium produced by one type of cell to culture other cells, promoting interaction between water-soluble components and cells (Bogdanowicz and Lu, 2013). For instance, conditioned media from human bone marrow-derived MSC enhanced bone regeneration by increasing the expression of osteogenic marker genes, such as osteocalcin and *Runx2*, in rat MSC (Osugi et al., 2012). Therefore, a co-culture system using a conditioned medium is a valuable technology for controlling cell growth and differentiation through cells that secrete growth factors and cytokines essential for cultivated meat production. Our data demonstrated that a conditioned medium containing the ECM from fibrotic ADSC can induce the myogenesis of SC. Paracrine communication through the ECM and other signaling molecules between cells is likely crucial for myogenic differentiation. A previous study also showed similar data wherein co-culture of FC and muscle SC derived from Jeju black pig increased the gene expressions of *MyoD* and *Myf5* (Siddiqui et al., 2022). These data indicate that FC may regulate the myogenesis of SC. Furthermore, muscle stem cells cultured on ECM (collagen type I, fibronectin, and gelatin) and Matrigel coatings showed increased proliferation and myogenic differentiation capacity (Wilschut et al., 2010). Taken together, CA-CM not only promotes the myogenesis of SC but also induces ECM accumulation, potentially improving the texture of cultivated meat and enhancing muscle differentiation. Our research showed that treating ADSC with CTGF increased the production of ECM. This ECM, along with other signaling molecules it contains, might create an environment that promotes cell-to-cell communication. This paracrine communication between cells could play a role in the development of muscle cells from SC. Although further studies are necessary, our findings may provide fundamental data that contribute to the advancement of cultivated meat production.

Conclusions

In summary, our findings demonstrate that CTGF induces fibrotic differentiation in porcine ADSC, resulting in the production of ECM components such as Collagen I, laminin, and fibronectin. Furthermore, conditioned medium from fibrotic ADSC promotes myogenesis in porcine SC. Taken together, conditioned media from fibrotic ADSC could be a valuable tool for promoting myogenic differentiation of SC, contributing to the advancement of cultivated meat production.

Acknowledgments

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