Conditioned media from embryonic chick tendon cells promotes C2C12 cell viability in serum-free media

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ABSTRACT

Given the high cost and ethical issues with fetal bovine serum (FBS), the development of serum-free media is essential. Tenocytes, specialized fibroblast cells necessary for tendon structure and signaling, may facilitate serum-free development. This study assessed the ability of embryonic chick tendon-derived cells to adapt to low-serum media and produce conditioned media. An explant isolation method was used, yielding high-purity primary tenocytes, evidenced by the detection of scleraxis through immunocytochemistry and western blotting. Sequential adaptation of tenocytes showed promising results, with positive detection of FGF2, IGF1, TGF-β, and TNF-α in conditioned media from normal and adapted tenocyte cultures. Preliminary applications of conditioned media on C2C12 cell viability showed comparable viability to FBS-based culture media after 24 h. However, longer incubation revealed limitations compared to FBS owing to declining amino acid concentrations and a lack of fatty acids. The findings highlight the potential of tenocyte-derived conditioned media for serum-free media formulation.

Keywords: Embryonic chick; Tenocytes: Tendon; Conditioned media; Serum-free media

1. Introduction

The majority of cultivated meat production costs are attributed to culture media, particularly their components. Fetal bovine serum (FBS) is a widely used growth media component and is considered a vital source of growth factors for optimal cell growth and maintenance in vitro. However, production issues, performance variability, and high costs continue to encourage its replacement, as evidenced by multiple studies showing efforts to reduce its use or replace it with suitable alternatives (Lee et al., 2022; Lee et al., 2023). The development of FBS replacements for cultured meat production could greatly impact its commercialization, leading to improved consumer acceptance and production sustainability.

Given the importance of growth factors and cytokines in cell culture, the production of these components is of significant interest for both biomedical and cultured meat research. One established method for in vitro growth factor production is media conditioning (Peshkova et al., 2023). This technique allows secretory cells to produce growth factors in vitro, transforming basal or culture media into an enriched formulation. Similar to FBS, conditioned media (CM) contain both beneficial and harmful metabolites that can either improve or hinder cell growth and function (Kim et al., 2018). Therefore, optimization of CM use is required. Previous reports on CM production for cultured meat have shown promising results, indicating the effectiveness of monoculture-based CM in

supporting the growth of muscle progenitor cells (Morikura et al., 2024; Harguchi et al., 2022). Although promising, production cost and efficiency remain persistent challenges, particularly for large-scale CM production. Thus, secretory cells that can be cultured at a lower cost while maintaining their secretory function are needed.

Tendons contain a diverse cell population, primarily composed of tenocytes, which are specialized fibroblasts that produce collagen fibrils, growth factors, and cytokines (Gögele et al., 2025, Dex et al., 2017). Although the main function of tendons is often limited to muscle–bone attachment and force dispersion during movement, the potential of tenocytes to produce growth factors and cytokines should not be overlooked (Tsai et al., 2020). These products are essential for the optimal growth and function of neighboring cells and organs, particularly muscles, which are directly attached to tendons (Schweitzer et al., 2010). Considering the hypovascular nature of tendons, tendon-derived cells may hypothetically subsist in low-serum to serum-free culture conditions.

Embryonic chicks are commonly used in biomedical research due to their ease of access and ethical concerns. Sacrificing less than 15-day old embryonic chick would not warrant ethical approval prior to use, which makes them ideal cell donors for cell culture-related research (Sarnella et al., 2024). Furthermore, tendons from embryonic chicks are soft and less fibrous, allowing optimal isolation of cells using explant isolation. Previous studies involving chick tenocyte cells focused on tendon development and repair, highlighting its role in extracellular matrix production,

mechanotransduction, and cell signaling (Waggett et al., 2006; Shukunami et al., 2006). However, no studies have explored the potential of the secretory function of tenocyte cells as sources of growth factors. Although avian cell-derived growth factors may have differences with mammalian counterparts, highly conserved genes among animal species include those that translate into growth factors, which could potentially allow cross-species reactivity.

Given the nature of these cells to subsist in nutrient poor environment, the ability of the cells to adapt into low-serum media during cell expansion and maintenance while maintaining their secretory function could provide a low-cost conditioned media for cell culture. Thus, this study aimed to evaluate the potential of embryonic chick-tendon cells as secretory cells for media conditioning.

2. Materials and Methods

2.1. Isolation of embryonic chick tendon cells

2.1.1. Cell isolation

After 15 d of incubation, the chick embryo (Hy-Line 24 Brown) was obtained and sacrificed by a quick severance of the head using a scalpel. The distal digital tendon was identified and isolated using surgical scissors and forceps, then washed with wash buffer containing 1× DPBS with 2% P/S. The tendon sheath was briefly removed using forceps, and the inner tendon was repeatedly washed with wash buffer. The resulting tendon sample was cut into approximately 1 mm × 1 mm

pieces using a scalpel. Following this, the pieces were placed in a 50-mm dish and supplemented with 100 µL FBS per piece. The plate was incubated at 37 °C with 5% CO_2 for 4 h prior to the addition of complete growth medium (Dulbecco's modified Eagle medium (DMEM) + 10% fetal bovine serum (FBS) + 1% P/S) and allowed to incubate undisturbed for 3 d. The growth medium was partially replaced after 3 d. Approximately 5 d after explant preparation, outgrowing cells surrounding the explants were dissociated using TrypLE and centrifuged at 3000 rpm for 5 min to obtain the pellet. The cell suspension was seeded in a non-coated cell culture dish and allowed to expand until reaching 90% confluency, followed by subsequent passaging at a 1:2 split ratio.

2.1.2. Cell characterization

To assess the adaptability of tendon-derived cells to low-serum conditions, passage 3 cells were sequentially adapted from 10% FBS-supplemented growth medium to 5% FBS-supplemented growth medium. Throughout the experiment, the same batch of normal growth medium cells (CTL) and low-serum media-adapted cells (ADPT) was maintained.

2.1.2.1. Immunocytochemistry and western blotting

To confirm tenocyte identity prior to the low-serum adaptation experiment, scleraxis (SCX) expression was evaluated via immunocytochemistry of passage 3 cells. The cells were grown in 48-well plates at 1×10^4 cells/well and incubated at

37 °C with 5% CO_2 for 3 d. The cells were washed three times with 1× DPBS, followed by fixation using 4% paraformaldehyde for 10 min. Fixed cells were permeabilized with 0.2% TritonX in PBS with Tween® 20 (PBST) for 10 min. Following this, the cells were blocked using 2% BSA in PBST for 30 min. Next, the cells were incubated with anti-scx antibody (1:500, SC-518082, Santa Cruz, CA, USA) for 1 h 30 min at room temperature (RT), followed by incubation with secondary antibody (1:1000) and Hoechst 33342 (1:1000) for 1 h. An immunofluorescence microscope was used to detect fluorescent cells.

Cell lysates were loaded at 10 µg/mL per well on 6–10% separating gels for SDS-PAGE. The gels were subsequently transferred for western blotting using Amersham™ Hybond™ 0.2-µm polyvinylidene difluoride membranes from Cytiva™ (Marlborough, MA, USA). The transfer was performed at 100 V for 1.5 h using a wet transfer system. The resulting blots were washed three times with 1× PBST for 5 min, followed by blocking with 2% bovine serum albumin in PBST for 1 h at 4 °C on a shaker. After the blocking step, the membranes were washed three times with PBST for 15 min each. Thereafter, the membranes were incubated with anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5,000, AM4300; Invitrogen, CA, USA) and anti-scleraxis (1:1,000, sc-518082; Santa Cruz, OR, USA) antibodies overnight at 4 °C on a shaker. Subsequently, the membranes were washed three times with PBST for 15 min each prior to incubation with anti-rabbit

immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked (1:2,000, 7076S; Cell Signaling, MA, USA), anti-mouse IgG HRP-linked (1:2,000, 7074S; Cell Signaling, MA, USA), and anti-goat IgG H&L (1:2,000, ab6741; Abcam, Cambridge, UK) antibodies for 1 h at 4 °C on a shaker. The membranes were then washed three times with PBST for 15 min each before brief treatment with Clarity ™ Western ECL substrate (luminol/enhancer and peroxide solutions) (Bio-Rad Laboratories, Inc., CA, USA). The membranes were imaged using an e-Blot Touch Imager (e-BLOT Life Science Co., Ltd., Shanghai, China).

2.1.2.2. Proliferation assay

To determine the difference in proliferation between CTL and ADPT cells, a WST cell counting kit was used. Cells were grown in 96-well plates at 5×10^3/well. Both CTL and ADPT cells were cultured in growth media supplemented with 10% and 5% FBS to determine the response to a sudden increase or decrease in FBS after 24 and 72 h of incubation.

2.1.2.3. Semi-quantitative total collagen assay

Differences in total collagen production across passages 3, 5, and 10 of both CTL and ADPT cells were tested using a Sirius Red-based (SIGMA-ALDRICH, MO, USA) colorimetric semi-quantitative collagen assay, applying the protocol outlined by Szász et al (2023) with modifications. Briefly, CTL and ADPT cells were seeded

in 12-well plates at 5×10^4 cells/well and incubated for 3 d. After washing with 1× PBS, samples were fixed with Kahle's fixative solution for 15 min at RT. Samples were washed three times with 1× PBS, followed by the addition of 1% Direct Red in 1% acetic acid for 1 h at RT. Next, samples were washed with 0.1 M HCl in distilled water (DW) for 5 min. Photographs were captured prior to the removal of HCL solution to document collagen coverage. Stained samples were eluted with 0.1 M NaOH in DW for 5 min at RT. The elute was collected in Eppendorf tubes, vortexed, and 100 µL of the sample was loaded per well. Absorbance was measured at 450 nm using a microplate reader. Results were normalized to the absorbance of non-seeded scaffolds for both treatments.

2.2. Conditioned media production

Passage 3 cells were used from a single donor isolation procedure to produce cells for conditioned media production via sequential adaptation. A set of cells was cultured in 10% and 5% FBS-containing growth media after each passage and labeled as adapted cells (ADPT). Control cells (CTL) were continuously grown in 10% FBS-containing growth media. Upon reaching confluency, cells were seeded separately at 1×10^6 cells per 75 cm^2 T-flask and incubated at 37 ℃ with 5% CO_2. After 3 d, cultures reached >90% confluency and were washed three times with 1× DPBS. DMEM supplemented with 1% PS was added to the pre-washed cultures and incubated for 3 d. After incubation, the conditioned media were collected and

filter-sterilized using a 0.22-μm sterile syringe filter. The filtered CM were stored at −20 °C until further use or analysis (Fig. 1). CM production was repeated at least three times from the starting passage 3 cell stocks after each set of experiments to provide distinct CM batches.

2.2.1. Preliminary characterization of conditioned media

2.2.1.1. pH measurement and color evaluation

The pH of the control and CM samples was measured using a pH meter. Visual evaluation of differences in color among the treatments was also conducted.

2.2.1.2. Amino acid analysis

To determine the amino acid concentration of the unknown sample, standard and test solutions were prepared following the Ninhydrin colorimetric method with HPLC analysis. The standard sample was prepared by diluting the structural amino acids 25-fold and the urea amino acids 20-fold using the appropriate solvent. Two test tubes were prepared: one containing 100 μL of the standard sample and another containing 100 μL of the unknown sample. A total of 1 mL of Ninhydrin reagent and 1 mL of buffer solution (PF-1) were added to each tube. The mixtures were then heated at 100 °C for 3 min under identical conditions to allow the color development reaction to proceed. After cooling, absorbance was measured at 570 nm to quantify the Ninhydrin-amino acid complex. The dilution ratio of the unknown sample was determined by comparing its absorbance value with that of

the standard sample. For precise quantification, amino acid analysis was conducted using a HITACHI L-8900 Amino Acid Analyzer equipped with a HITACHI HPLC Packed Column (#2622PF, 4.6 × 60 mm, ion-exchange column). The elution buffer system consisted of KANTO HITACHI High-Speed Amino Acid Analyzer Buffers PF-1, PF-2, PF-3, PF-4, and RG. The Wako Ninhydrin Coloring Solution Kit for HITACHI was used as the coloring reagent. Detection was performed using dual visible detectors at 570 nm (VIS1) and 440 nm (VIS2) with a UV detector, and an injection volume of 20 µL was used. Data acquisition and analysis were performed using EZChrom Elite software (version 3.1.5b). Standard references for urea amino acid samples included Wako PF Standard (Type B, 016-08641) and AN-II (015-14461).

2.2.1.3. SDS-PAGE and western blotting

To preliminarily characterize the CM in terms of its protein profile, SDS-PAGE and western blot methods were employed. A protein precipitation technique was used to concentrate the proteins present in the conditioned media. Briefly, 1 mL of CM was aliquoted into 1.5 mL Eppendorf tubes. Then, 250 µL of 100% TCA was added to each tube, followed by brief agitation. The tubes were centrifuged at 14000 rpm for 20 min at 4 °C. Subsequently, samples were washed twice with ice-cold 100% acetone. The remaining liquid was allowed to evaporate under a fume hood for 30 min at RT. A 20 µL sample buffer (4× Laemmli buffer with mercaptoethanol) was

used to dilute the precipitated protein prior to loading onto 12% polyacrylamide gels. SDS-PAGE was run at 100 V for 80 min. For visualization of protein bands, the gel was stained using a staining buffer for 4 h and destained using a destaining buffer for 6 h.

To detect the secretion of growth factors and cytokines, a qualitative western blot analysis was conducted. After gel electrophoresis, the protein bands were transferred to a PVDF membrane via the wet transfer sandwich method using 100 V for 90 min at 4 °C. The membranes were washed with 1× PBST three times and blocked using 2% BSA in PBST. After blocking, the membranes were washed three times and incubated with primary antibodies FGF2 (1:1000, NBP1-47749, Novus, CO, USA), IGF1 (1:1000, LS-C68994, LSBio, CA, USA), TNF-α (1:1000, LS-C70664, LSBio, CA, USA), and TGF-β (1:1000, AF-101-NA, MN, USA) overnight at 4 °C on a shaker. The membranes were washed again prior to incubation with the secondary antibody such as anti-rabbit immunoglobulin G (IgG) horseradish peroxidase (HRP)-linked (1:2,000, 7076S; Cell Signaling, MA, USA), anti-mouse IgG HRP-linked (1:2,000, 7074S; Cell Signaling, MA, USA), and anti-goat IgG H&L (1:2,000, ab6741; Abcam, Cambridge, UK) antibodies for 90 min at 4 °C on a shaker. After secondary antibody hybridization, the membranes were washed three times and briefly immersed in ECL substrate prior to chemiluminescent imaging using e-Blot.

2.4. Preliminary application of conditioned media

2.4.1 Cell viability

The effect of CM on cell viability was assessed using a WST-8 assay kit. C2C12 cells were suspended in growth media and seeded at 5×10^3 per well, followed by incubation at 37 °C with 5% CO_2 for 24 h as a pre-attachment period. The wells were then washed three times using 1× DPBS prior to treatment supplementation and incubated for 24 h. After incubation, 10 µL of WST-8 reagent was added per well and incubated for 2 h prior to measuring absorbance at 450 nm using a microplate reader.

2.4.2. Cell proliferation assay

To quantitatively determine cell proliferation, 96-well plates were seeded with 5×10^3 cells per well, directly suspended in experimental media and incubated at 37 °C with 5% CO_2 for 0, 24, 48, and 72 h. A total of 10 µL of WST-8 reagent was added per well, followed by incubation for 2 h prior to measurement of absorbance at 450 nm using a microplate reader. All absorbance values were normalized to the absorbance of the CTL at 0 h to obtain percent viability for all subsequent readings. Percent viability was calculated using the following equation:

Percent cell viability = (Treatment absorbance at time x / CTL absorbance at 0 h) × 100%

2.4.3. Brightfield microscopy

Changes in cell growth were assessed by descriptive comparison of 24 h and 72 h microscopy images of cells cultured in CM. Cells were seeded at 1×10^5 cells/mL in 6-well plates and incubated under standard conditions.

2.4.4. Cell differentiation

Cells were seeded in 6-well plates at 1×10^5 cells/mL. Upon reaching full confluency in CTL (regardless of confluency in other treatments), the cells were washed three times with 1× PBS and supplemented with differentiation media containing DMEM with 2% HS and 1% P/S. Brightfield microscopy images were generated after 24 h and 72 h of incubation.

2.5. Statistical analysis

This research used tendon-derived cells from a single cell donor via explant isolation. Three CM production batches were treated as sources of 3 biological replicates, with at least 5 technical replicates per treatment. One-way analysis of variance was conducted to determine significant differences in tests with three or more independent variables. Tukey's honestly significant difference test was used for post-hoc analysis. Significant differences were denoted using letter notation.

3. Results and Discussion

3.1. Characterization of embryonic chick tenocytes

3.1.1. Explant isolation yields SCX-positive tendon-derived cells

The explant isolation technique has been shown to be an effective method of isolating primary tendon-derived cells (Fig. 2A). Cells obtained using this technique showed positive expression of scleraxis (SCX), an early differentiation marker for tenocytes (Fig. 1B) (Li et al., 2015; Shukunami et al., 2018). Furthermore, western blot analysis of cell lysates from passage 3 and passage 5 cells also detected SCX, indicating the effectiveness of the explant isolation technique in producing tenocyte cultures (Fig. 2C). However, limitations remain regarding the long-term in vitro culture of tendon cells due to their susceptibility to phenotypic drift, reduced proliferative capacity, and decreased expression of related biomarkers (e.g., scleraxis, collagen, mohawk, tenomodulin). Therefore, early-passage cells are recommended for in vitro tendon studies (Nichols et al., 2021). In this study, these effects were observed after passage 10, leading to the use of earlier passages.

3.1.2. Sequential adaptation allowed low-serum proliferation

The proliferation assay demonstrated the potential of sequential adaptation to reduce FBS levels for cell maintenance and proliferation (Fig. 2D). No significant differences were observed among treatment groups after 24 h of incubation. After

72 h, ADPT cells cultured in 5% FBS showed no significant difference compared to CTL cells cultured in 10% FBS. In contrast, ADPT cells cultured in 10% FBS showed the highest proliferation among all treatment groups. CTL cells cultured in 5% FBS showed the lowest proliferation, indicating the negative effect of a sudden decrease in FBS supplementation on cell proliferation. The non-significant difference between CTL cells in 10% FBS and ADPT cells in 5% FBS confirms the effectiveness of sequential adaptation in reducing the FBS requirement for proliferation. Furthermore, the increase in ADPT cell proliferation in 10% FBS showed that these cells can actively respond to the increase in FBS concentrations, highlighting their ability to subsist in both low and high FBS conditions without adversely affecting their proliferation ability. Normal tendons are relatively hypovascular compared to tendons with underlying pathologies and those undergoing healing (Riggin et al., 2020). This suggests that in vivo, tenocytes can maintain physiological functions even with limited nutrient availability from the blood. This ability inspired the possibility of low-serum or serum-free media culture conditions.

3.1.3. ADPT cells yield similar intracellular collagen levels with CTL cells

As an important extracellular matrix protein among animal cells, collagen production was assessed to determine the effect of sequential adaptation on tenocytes (Fig. 2E, F). Total collagen production of primary tenocyte cells at

passage 3 was higher compared to the succeeding passages, regardless of whether the cells were normal or adapted. Immediately following sequential adaptation at passage 5, total collagen production in ADPT cells was significantly reduced compared to CTL cells (Fig. 2E). However, by passage 10, no significant difference was observed, suggesting further adaptation of ADPT cells to low-serum conditions. Microscopy images of Sirius Red-stained cells also showed comparable stain distribution, suggesting similar collagen production in both CTL and ADPT cells in 10% and 5% FBS, respectively. As previously mentioned, in vitro culture can affect protein expression in tenocyte cells (Qiu et al., 2012). Although a significant decrease in total collagen production was observed from passage 3 to passage 5, the increase and stabilization observed by passage 10 indicates the ability of tenocytes to adapt to in vitro conditions, regardless of normal or reduced FBS supplementation (Fig. 2E). Several studies have explored low-serum or serum-free cultivation of human tenocyte cells (Qiu et al., 2012; Wang et al., 2011; van Vijven et al., 2020). Vijven et al. (2020) demonstrated the dynamic response of tenocytes to serum deprivation, reporting a shift in gene expression levels towards native levels after 1 week. These findings indicate the potential of tenocytes cells for the production of CM, given their ability to maintain cell physiology and functionality under serum-free media conditions.

3.2. Conditioned media characterization

3.2.1. CMA had higher pH

pH is an important consideration in cell culture media development. Differences in pH among treatment groups were observed. FBS supplementation in CTL resulted in a significant change compared to the original pH of the basal media (NCTL) (Fig. 3A). CM from CTL and ADPT cells showed increased pH, similar to the pH of FBS-supplemented media. This increase may be attributed to the continuous production of cellular metabolites such as ammonia and the utilization of other basal media components during CM production (Synoground et al., 2021). A darker red color was observed in CTL, CMC, and CMA treatments, corresponding to their observed pH values (Fig. 3B). Phenol red, the pH indicator used in DMEM basal media, develops into a darker red color at higher pH and yellow at lower pH.

3.2.2. Tenocytes synthesized non-essential amino acids in serum-free media

To briefly compare the changes in the composition of basal media after incubation with tenocyte cells, the amino acid profiles of NCTL, CMC, and CMA were determined (Fig. 3C, D). For essential amino acids, a significant reduction in all amino acid levels was observed, indicating continuous cellular metabolic activity of both CTL and ADPT cells during the 72-h incubation for conditioned media production (Fig. 3C). A previous study reported a significant decrease in glutamic acid concentrations corresponding to the decrease in glutamine as a result of cell

utilization (Yoo et al., 2020). Moreover, the detection of asparagine, alanine, and proline indicates active production of other non-essential amino acids, which are not present in DMEM basal media (Fig. 3D). The high synthesis of alanine can be attributed to the involvement of tenocytes in the Cahill cycle, which promotes the breakdown of amino acids (Sarabhai et al., 2019). Additionally, tenocytes, notable for their collagen production, synthesize glycine, proline, and alanine to support tendon repair and maintenance, primarily because these amino acids are used in collagen formation 9Vieira et al., 2018). The decrease in serine, cystine, tyrosine, and arginine can be attributed to their active role in cell proliferation and maintenance (Lee et al., 2024).

3.2.3. Tenocytes secreted growth factors in conditioned media

To determine the protein profile of conditioned media, CM proteins were precipitated using TCA precipitation and visualized via SDS-PAGE. Prominent bands were more visible in the first collection of CMC and CMA compared to the faint bands in the second collection, indicating a decrease in overall protein production after 72 h of CM production (Fig. 4A). This suggests that tenocyte cultures are currently limited in their ability to produce CM, and continuous CM production may require further supplementation to support cellular activity. Important growth factors and cytokines were detected using qualitative western blot analysis only (Fig. 4B–E). FGF2 and TNF-α were positively detected in both CMC

and CMA (Fig. 4B, E). Faint bands within the 25–35 kDa range indicate the presence of IGF1 and TGF-β, with CMA showing more prominent bands for TGF than CMC (Fig. 4C, D). Although several growth factors were detected, the quantity of growth factors were not ascertained given their high dilution. Thus, a more sensitive quantification method will be required to establish tenocyte growth factor secretion efficiency for conditioned media production. In addition to extracellular matrix production, tenocytes secrete growth factors and cytokines involved in cell signaling within the tendon and to neighboring tissues such as bone and muscle (James et al., 2008; Sakai et al., 2025). FGF2, TGF-β, and IGF-1 are important for cell proliferation, differentiation, and collagen synthesis in tenocytes (James et al., 2008). TNF-α is associated with inflammatory responses but may support proliferation when bound to TGF-β (Backman et al., 2013). However, these mechanisms have only been reported in human tenocytes, and reports from chicken tenocytes are lacking. Further research is required to elucidate the effects of growth factors derived from chicken tenocytes on intra- and interspecies cell lines. During embryonic chick tendon development, collagen fibril formation begins from embryonic day 7 onward, indicating that tenocytes at this stage can secrete relevant growth factors, providing insight into the rapid maturation of tendons at later stages of embryonic development (Edom-Vovard et al., 2004). This highlights chick embryonic tenocytes as potential sources of growth factors for media conditioning. To date, media conditioning using chick tenocytes has not been reported.

Furthermore, using chick embryos up to 17 d in the EU and 14 d in the USA and UK exempts the requirement for ethical approval for experimentation, making them an ethical source of animal-derived growth factors (Ribatti et al., 2023). However, there remains limitations toward large-scale production and use of conditioned media. The passaging limitation of tenocyte cells in vitro is a major consideration for continuous growth factor production. Recent advances in cell immortalization techniques, whether spontaneous or via cell engineering, can be a viable solution for this problem (Soice and Johnston, 2021). Furthermore, the establishment of low-serum or serum-free culture media for sufficient cell proliferation and maintenance of tenocyte cells could impact the overall production cost, highlighting the significant costs incurred in FBS usage (Yu et al., 2023).

3.3 Preliminary conditioned media application

3.3.1 CMC and CMA improved cell viability of C2C12 cells in serum-free condition

To preliminarily determine the effectiveness of CMC and CMA in cell culture, C2C12 cells were used as a model cell line for myogenic cells. In terms of cell viability, all treatments containing CMC or CMA showed significantly higher cell viability compared to basal media alone; however, this performance was inferior to that observed for FBS-supplemented culture media (Fig. 5A). Given the serum-free nature of the treatments containing either CMC or CMA, the observed viability after

24 h of incubation suggests that the conditioned media contain essential growth factor levels comparable to FBS-supplemented media. Brightfield microscopy images after 24 h of incubation showed elongated C2C12 cells in all treatments containing CMC or CMA, whereas CTL exhibited a mix of globular and elongated cells (Fig. 5B). This suggests that CM supported the faster attachment and elongation of C2C12 cells compared to NCTL, which showed globular cells only. This effect may be explained by the presence of FGF2 and soluble collagen in CM, allowing the attachment of cells even on uncoated culture dishes (Caliari et al., 2012). Similarly, CM derived from HepG2 and NIH/3T3 cells supported the adhesion of bovine myogenic cells on uncoated dishes (Morikura et al., 2024). Considering the difference in source species between C2C12 cells (mouse) and CM (chicken), the compatibility of conditioned media derived from CTL and ADPT tenocyte cells with C2C12 cells provides insights into the potential use utilizing chicken-derived growth factors for mammalian cell culture. This is consistent with the ability of FBS to support the growth of nearly all cell lines, regardless of source species (Lee et al., 2022).

3.3.2 CMC and CMA did not support the proliferation of C2C12 cells

The proliferation of C2C12 cells in CM treatments was significantly lower than in the FBS-supplemented group (Fig. 5C). This result is expected, given differences in composition and the decrease in amino acids during CM production

(Fig. 3C). Considering the available nutrients across all treatments, it is reasonable to conclude that fresh FBS-supplemented growth media contains non-depleted levels of amino acids from the basal media and other essential factors from FBS, making it predictably more effective compared to serum-free media. Furthermore, the volume of conditioned media used in each treatment directly affects nutrient availability, corresponding to the amount of fresh basal media included. Therefore, optimization of serum-free media with either CMC or CMA must account for this factor to improve its ability to support cell proliferation. Microscopy images suggest that C2C12 cells reached full confluence after 48 h, with an observable smaller size compared to cells in CM-containing treatments (Fig. 5D). This finding is similar to the observations of Jang et al. (20220, who reported notable size differences in C2C12 cells between serum-based and serum-free media. Furthermore, both CMC- and CMA-treated cultures showed early myotube formation after 48 h. This may be explained by the early attachment and elongation of cells within 24 h in CM-supplemented treatments (Fig. 5A, D). Detection of proliferation-related growth factors (e.g., FGF2, IGF-1, TGF-β) demonstrates the potential of tenocyte-derived conditioned media for cell culture applications (Fig. 4B–D). However, the amount of available growth factors required to effectively support cell proliferation, particularly under serum-free media conditions, remains to be undetermined (Stout et al., 2022).

3.3.3 Difference in growth factor-levels during proliferation affected the differentiation potential of C2C12 cells

To determine whether serum-free media affected the differentiation capacity of C2C12 cells, all cells were subjected to standard differentiation media supplemented with 2% HS. After 24 h of differentiation induction, all treatments, except NCTL, reached full confluence, with longer and larger cells observed in CM treatments compared to CTL (Fig. 5E). Formation of myotubes was observed in all treatments, with CTL showing the highest level of differentiation, followed by CMC-supplemented treatments. Differences in differentiation levels between CMC- and CMA-supplemented treatments may be attributed to variations in the production of growth factors and cytokines by CTL and ADPT tenocyte cells. Specifically, the thicker TNF-α band detected in CMA may explain the difference in the observed differentiation rate between CMC and CMA-treated cultures (Fig. 4E). High levels of TNF-α can inhibit differentiation and promote proliferation instead (Chen et al., 2006; Shirakawa et al., 2021). Therefore, optimization of serum-free formulation using CMA must consider this factor. However, quantification of growth factors in the conditioned media is still needed to provide a more reliable interpretation.

4. Conclusion

Tenocytes are an underappreciated source of secretory factors that can be used as potential animal-derived growth factors for cell culture applications. In this study, CM derived from normal and low-serum-adapted cells demonstrated the ability to promote cell adhesion, elongation, proliferation, and early differentiation of C2C12 cells. These effects can be attributed to the presence of important growth factors (e.g., FGF2, IGF-1, TGF-β), whereas TNF-α appeared to inhibit differentiation in CMA-treated cultures. The effectiveness of both CMC and CMA in supporting cell adhesion and proliferation makes them promising candidates for serum-free media formulations. Given their ability to survive under low-serum or serum-free media conditions, optimizing CMC or CMA production could significantly reduce culture media costs. The low-cost production system demonstrated in this study can facilitate the development of serum-free media for cell culture applications, particularly cultured meat production. Cyclic culture maintenance of low-serum-adapted cells could completely eliminate the use of FBS in CM production.

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Declaration of interests

The authors declare no conflicts of interest.

Data statement

Data are available upon reasonable request

Ethics statement

No IACUC approval was needed in this study, according to the European Directive 2010/63/EU

CRedIT author statement

Ermie Jr. Buen Mariano: Conceptualization, data curation, formal analysis, methodology, software, interpretation of results, writing and editing of manuscript. **Colin Venter, Da Young Lee, Yewon Shin, Chaehyeon Bok, Woojin Lee, Soyoung Choi** : Investigation, writing and editing of manuscript. **Sun Jin Hur**: Conceptualization, interpretation of results, writing and editing of manuscript, and supervision.

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Figure Legends

Figure 1. Schematic diagram of sequential tenocyte adaptation and conditioned media production

Figure 2. Characterization of embryonic chick-derived tenocyte cells. A: Microscopy images of explants with visible migration of cells; B: Detection of scleraxis via ICC (10X scale bar: 100 µm, 20X scale bar: 50 µm); C: Detection of scleraxis via qualitative western blotting. D: Cell viability assay of CTL and ADPT tendon cells after 24 and 72 h of incubation; E: Semi-quantitative collagen assay of tendon cells across selected passages; F: Microscopy images of tendon cells for collagen detection via Sirius Red staining. Data are presented as the mean ± standard deviation (SD).

Figure 3. Characterization of conditioned media. A: pH measurements of conditioned media; B: Representative photograph of control samples and conditioned media; C: Bar graph representing essential amino acid levels; D: Bar graph representing non-essential amino acid levels. CTL: 10% FBS-supplemented growth media; NCTL: DMEM media only; CMC: Conditioned media from CTL tenocyte cells; CMA: conditioned media from ADPT tenocyte cells. Data are presented as the mean ± standard deviation (SD).

Figure 4. Detection of growth factors in conditioned media. A: SDS-PAGE of TCA-precipitated proteins; B–E: Qualitative western blots of FGF2, IGF1, TGF-beta, and TNF-alpha. CMC: conditioned media from CTL tenocyte cells; CMA: conditioned media from ADPT tenocyte cells; FGF2: basic fibroblast growth factor; IGF: insulin-like growth factor; TGF: transforming growth factor; TNF: tumor necrosis factor.

Figure 5. Preliminary application of conditioned media to C2C12 cells. A: Cell viability assay of conditioned media using C2C12 cells; B: Brightfield microscopy of C2C12 cells after 24 h of incubation; C: Proliferation assay of C2C12 cells; D: Brightfield microcopy of C2C12 cell proliferation after 24 and 48 h; E: Brightfield microscopy of C2C12 cells after 24 and 72 h of incubation in differentiation media; CTL: 10% FBS; NCTL: DMEM only; CMC: pure conditioned media from unadapted tenocyte cells; CMC1: 50% CMC + 50% DMEM; CMA2: 25% conditioned media from ADPT cells + 75% DMEM; CMA: pure conditioned media from adapted tenocyte cells; CMA1: 50% conditioned media from ADPT cells + 50% DMEM; CMA2: 25% conditioned media from ADPT cells + 75% DMEM.