



Paracrine effect of fibroblasts on the proliferation and differentiation of bovine satellite cells *in vitro*

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ABSTRACT

The co-culture of fibroblasts and muscle cells is essential for obtaining cultured meat using *in vitro* techniques. While fibroblasts are known to promote myogenesis through cell-cell interactions, their paracrine effects and associated transcriptomic changes remain unknown. This study aimed to investigate the paracrine effect of fibroblasts on myogenesis, focusing on transcriptome profiling. Muscle satellite cells (isolated enzymatically) and fibroblasts (isolated through the explant culture) were co-cultured in 0.4 µm transwell plates for 5 days of proliferation and 24 and 72 h of differentiation. RNA-Seq and protein analysis (Western Blot, ELISA, immunofluorescence) were used to assess changes in myogenic marker expression. RNA-Seq revealed changes in many genes involved in myogenesis, such as upregulation of EGR1, IL6, and SOCS3 and downregulation of ITGA7. ELISA showed significantly higher MyHC levels at the proliferation stage in the co-culture group ($p = 0.0183$), with no significant differences at differentiation stages. To summarize, fibroblasts promote early myogenic differentiation and could modulate the extent of myogenic differentiation.

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Abbreviations: ANK3, ankyrin-3; ANKRD1, Ankyrin repeat domain-containing protein 1; ANKRD3, Ankyrin Repeat Domain 23; ARID5B, AT-Rich Interaction Domain 5B; ATP1B3, ATPase Na⁺/K⁺ - Transporting Subunit Beta 3; bFGF, Basic Fibroblast Growth Factors; BHLHA15, Basic Helix-Loop-Helix Family Member A15; BMP4, Bone morphogenetic protein 4; BMPRI1A, The bone morphogenetic protein receptor, type IA; BMPRI2, Bone Morphogenetic Protein Receptor Type 2; BTG2, BTG Anti-Proliferation Factor 2; CALCRL, Calcitonin receptor-like; CAPZ, F-actin-capping protein subunit alpha-1; CAV1, caveolin-1; CD44, CD44 molecule; CEL2, Cofilin 2; CMKLR1, Chemerin Chemokine-Like Receptor 1; COL6A, collagen VI; COL6A3, Collagen Type VI Alpha 3 Chain; CRYAB, Crystallin Alpha B; CTNBBP1, Catenin Beta Interacting Protein 1; DCN, decorin; DEGs, Differentially Expressed Genes; DMEM, Dulbecco Modified Eagle Medium; ECM, extracellular matrix; EGR1, early growth response 1; ENO1/3, Enolase 1/3; EYA2, Eyes absent homolog 2; FBS, Fetal Bovine Serum; FGF2, Fibroblast Growth Factor; FHOD3, formin Homology 3-Domain Containing 3; FLNC, Filamin C; FOS, Fos Proto-Oncogene; FOXS1, forkhead; GCA, Gracalcin; GDF15, Growth/differentiation factor 15; GREM1, Gremlin 1; DAN Family BMP Antagonist; HDAC4, Histone deacetylase 4; HDAC9, Histone Deacetylase 9; HES1, Hes Family BHLH Transcription Factor 1; HEYL, Hes Related Family BHLH Transcription Factor with YRPW Motif Like; HIF1A, Hypoxia Inducible Factor 1 Subunit Alpha; HMOX1, Heme Oxygenase 1; HOMER1, Homer Scaffold Protein 1; HS, Horse Serum; IGFBP4, Insulin-like growth factor-binding protein 4; IL-6, Interleukin-6; ITGA7, integrin 7 alpha; ITGB, integrin beta; JUN, Jun Proto-Oncogene; KANK1, KN Motif And Ankyrin Repeat Domains 1; LIF, LIF Interleukin 6 Family Cytokine; LMOD1, Leiomodin 1; MET, MET Proto-Oncogene; MIRF, Myogenic Regulator Family; MUSC, muscle satellite cells; MX1, MX Dynamin Like GTPase 1; MyHC, Myosin Heavy Chain; MYL12B, Myosin Light Chain 12B; MYOM1, myomesin 1; MYORC, Myogenesis-Regulating Glycosidase; NES, nestin; NGS, Next Generation Sequencing; NOG, Noggin; NR4A1, Nuclear Receptor Subfamily 4 Group A Member 1; PBS, Phosphate Buffered Saline; Pitx1, Paired Like Homeodomain 1; RB1, RB transcriptional corepressor 1; ROCK1, Rho Associated Coiled-Coil Containing Protein Kinase 1; RUNX1, RUNX Family Transcription Factor 1; SATB2, SATB Homeobox 2; SCUBE2, Signal Peptide CLUB Domain and EGF Like Domain Containing 2; SDC, syndecan; SFRP4, Secreted Frizzled Related Protein 4; SMAD7, SMAD Family Member 7; SOCS3, Suppressor of Cytokine Signaling 3; SOX9, SRY-Box Transcription Factor 9; SPEG, Striated Muscle Enriched Protein Kinase; SPHK1, Sphingosine Kinase 1; SPTBN, β -spectrin; SPP1, Secreted Phosphoprotein 1; TCAP, telethonin; TGF β 1, Transforming Growth Factor Beta 1; TGM2, Transglutaminase 2; TLN, talin-1; WNT10B, Wnt Family Member 10B; WNT2b, Wnt Family Member 2B; XIRP, Xin Actin Binding Repeat Containing 1 (XIRP); ZYX, Zyxin.

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1. Introduction

Fibroblasts, connective tissue cells, synthesize a large amount of extracellular matrix (ECM) enriched in proteins such as collagen types I, III, and IV, fibronectins, laminins, and proteoglycans such as syndecans [1]. The components of the ECM play a crucial role in the regeneration process of muscle cells – e.g., type V and VI collagen are involved in maintaining the satellite cell pool [2]. During muscle regeneration, fibroblasts strongly proliferate, which stimulates the proliferation of muscle satellite cells (MuSCs) and promotes their premature entry into the differentiation stage [3]. Co-culture of fibroblasts with muscle cells is an essential aspect of obtaining cultured meat using *in vitro* techniques. *In vivo*, fibroblasts support muscle regeneration by forming a stabilizing ECM [4]; in turn, *in vitro* studies also show that fibroblasts are essential in regenerating damaged skeletal muscles. Co-culture of MuSCs and fibroblasts has stimulated myocyte differentiation and fusion through cell-cell interactions [5]. However, few studies have focused on the paracrine secretory capacity of fibroblasts and their potential impact on satellite cell proliferation and differentiation. Studies of paracrine signals in cultured fibroblasts and muscle cells showed reduced apoptosis of muscle cells during differentiation. However, Rao et al. [6] suggest that the basis of the stimulating effect on muscle cell differentiation is cell-cell interaction, not paracrine signals. In turn, studies using isotropic muscle sheets confirm the stimulating impact of paracrine factors on muscle differentiation [7].

The inconsistency of literature data highlights the need for a more comprehensive approach in studies on the intercellular communication of MuSCs and fibroblasts, specifically for applications in cultured meat technology. The study aims to examine the effect of fibroblasts on myogenesis at the stage of proliferation and differentiation of MuSCs, based mainly on a transcriptome study. The research hypothesis assumes that MuSCs co-cultured with fibroblasts will present enhanced differentiation, impressed by upregulation of genes involved in the differentiation of MuSCs.

2. Materials and methods

2.1. Cell culture

Fibroblasts were obtained *postmortem* from *Bos taurus* skin tissue from three individual specimens based on the explant culture method. Tissues were rinsed in 70 % ethanol followed by six washes in 1X Phosphate Buffered Saline (PBS) buffer pH 7.2 (Thermo Fisher Scientific), then dissected into 5 mm diameter fragments and cultured in High Glucose DMEM (Dulbecco Modified Eagle Medium) (Thermo Fisher Scientific) with the addition of 10 % Fetal Bovine Serum (FBS) (Thermo Fisher Scientific) and antibiotics 1X Primocin (InvivoGen). Primary fibroblast cultures were grown to approximately 70 % confluency and were passaged with TrypsinEDTA (0.25 %) (Thermo Fisher Scientific) to establish cell lines. Satellite cells were obtained from *musculus longissimus dorsi* from three individual female calf specimens following a previous method [8]. The heterogeneous population of satellite cells was purified from a different cell types (mainly fibroblasts) based on the cell adhesion rate to the plate culture. MACS and FACS methods of purification were not applied. Cells were seeded on a non-coated plate for 1 h; then non-adherent fraction was collected and re-seeded on a non-coated plate for 1 h. The purification efficiency was assessed based on the expression of fibroblasts and pre-adipocytes markers by real-time PCR (Supplementary material 1). The medium was collected and plated on a 0.1 % gelatin-coated plate in Low Glucose DMEM supplemented with 20 % FBS and 10 ng/ml bFGF (Basic Fibroblast Growth Factor). The

entire procedure was repeated twice. Then, three satellite cell pooled lines (3rd passage since isolation) and three fibroblasts pooled lines (10th passage since isolation) were plated in the 6-well multiwell plate and 0.4 µm pore size co-culture plates (Thermo Fisher Scientific). Cell viability were detected by trypan blue staining and counted by Countess 3 Automated Cell Counter (Thermo Fisher Scientific). Satellite cells were plated in lower plates and fibroblasts on the top of micropores in fourth replicates, at density 8000 cells/cm², in High Glucose DMEM with the addition of 20 % FBS. After five days of culture, the differentiation process was initiated by changing the medium to High Glucose DMEM with the addition of 10 % Horse Serum (HS).

2.2. Transcriptome analyses

RNA isolation was performed using PureLink RNA Mini Kit (Thermo Fisher Scientific) and PureLink™ DNase Set (Thermo Fisher Scientific). Quantitative and qualitative assessments of the isolated RNA were carried out using DeNovix DS-1 Spectrophotometer (DeNovix Inc., Wilmington, DE, USA) and TapeStation 2000 (Agilent Technologies, Santa Clara, CA, USA) devices (RNA tapes). Vazyme VAHTS Universal V8 RNA-Seq Library Prep Kit for Illumina™ systems was used to create RNA-seq libraries. The libraries were prepared according to the manufacturer's protocol. Quality and quantity of prepared libraries were performed using TapeStation 2200 (D1000 tapes) and Qubit 2.0 (Thermo Fisher Scientific, Waltham, MA, USA). NGS (Next Generation Sequencing) was carried out as an external service (Admera Health, Inc., South Plainfield, NJ, USA). Sequencing had to utilize conditions as follows: had to be performed on an Illumina device compatible with the library preparation kit, all 24 samples had to pass quality control on every possible step, and the read length is 150 bp pair-end, with 40 million reads per sample.

2.3. Bioinformatics analyses

Raw sequence data were quality checked for the first time with FastQC [9]. Illumina adapters and poly-A sequences were subsequently removed with the fastp program. Sequences that were below 36 bp in length as well as sequences with a quality score below 20 were eliminated [10]. Cleaned sequences were thereafter mapped against the *Bos taurus* (ARS-UCD1.3 - bosTau9) genome using reference annotations containing 23,842 genes in the Ensembl database. Gene-level expression was quantified using the RSEM package, alongside the STAR aligner [11,12]. Differentially expressed gene alignment and statistics were produced using SAMStat and RNA-SeQC [13,14]. To visualize raw sequence data and consolidate results for all samples into one report, we used the MultiQC bioinformatics tool [15]. Differential expression analysis was conducted using DESeq2 software by searching genes with $p_{adj} \leq 0.05$ and $|\log_2 \text{fold change}| \geq 0.5$. Gene Ontology enrichment was carried out using String software v.12.0 referenced to the *Bos taurus* genome. The over-represented pathway analyses were performed in the Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation. The sequence data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE289644.

2.4. Validation of sequencing results

Validation of RNA-Seq results was performed based on real-time PCR. 300 ng samples were reverse transcribed to cDNA using the High-Capacity RNA to cDNA Kit (Thermo Fisher Scientific). The expression levels were estimated for 5 DEGs (ITGA7, EGR1, IL6, SOCS3, and TCAP), for which specific primers were designed using

Primer3web version 4.1.0 software (Table 1). Relative expression levels were determined by real-time PCR using RT HS-PCR MIX SYBR (A&A Biotechnology). The endogenous controls were RPL27 and OAZ1 genes, which were selected based on our previous studies [15]. The RNA-Seq data (normalized counts) and relative expression data were compared based on the Pearson correlation (Statistica software, version 13.1).

¹ [16].

2.5. Effect of fibroblasts on the proliferation of muscle satellite cells

Proliferation rate was assessed using a fluorimetric method based on Cell Titer Blue Assay (Promega, Madison, WI, USA). The reagent was prepared according to the manufacturer's protocol, added to culture plate, and incubated 1 h in 5 % CO₂ in humidified atmosphere at 37 °C. The incubation time and cell density were previously optimized. Fluorescence intensity was measured at 535 nm excitation and 595 nm emission wavelength using PlateReader AF2200 (Eppendorf, Hamburg, Germany). The measurement results were obtained in the RFU unit (relative fluorescence unit). The RFU values obtained at each time point were normalized to day 0 RFU.

2.6. Protein analyses

For ELISA cell lysates were prepared using RIPA Lysis and Extraction Buffer and Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Protein concentrations were determined by the BCA method (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific). The surface of the wells was coated with a protein solution at a concentration of 1 µg/ml in 100 µl of 1 mM carbonate buffer and incubated at room temperature for 16 h. The plate was then washed 4x with Wash Solution (0.02 % Triton X-100 in PBS) and incubated in a blocking buffer (1 % BSA) for 2 h. Then, it was incubated with the primary antibody (myosin - # PA1-28038, Thermo Fisher Scientific) for 1 h, at a concentration of 1:1000 in 0.1 % BSA buffer in PBS, the plate was washed 4x with washing buffer and incubated with the secondary antibody (Donkey anti-Rabbit IgG (H + L) Secondary Antibody, HRP, Thermo Fisher Scientific) at a concentration of 1:4000 in 0.1 % BSA buffer. Washed 4x and incubated with HRP substrate (Sigma) until complete color change, then the reaction was stopped with 1 N HCl (1:1 ratio). Signal detection was performed using absorbance at 410 nm using an Eppendorf Platereader AF 2200 (Eppendorf, Hamburg, Germany).

For Western Blot analyses after determination of the protein concentrations Gel Loading Buffer II (Denaturing PAGE) (Thermo Fisher Scientific) was added to samples and denatured at 100 °C for 10 min. Proteins were separated by SDS-PAGE followed by wet

transfer to a 0.45 µm PVDF membrane for 100 min. The membrane was then blocked in 5 % BSA in TBST Buffer for 1 h at room temperature. The primary antibody for myosin was incubated at the appropriate concentration overnight at 4 °C with gentle shaking. A series of washes in 1x TBST buffer were performed, followed by incubation with the appropriate secondary antibody at the concentration 1:4000, conjugated to horseradish peroxidase (Donkey anti-Rabbit IgG (H + L) Secondary Antibody, HRP, Thermo Fisher Scientific). For visualization, the membrane was incubated with enhanced chemiluminescence reagent (Clarity™ Western ECL Substrate, BioRad) and using ChemiDoc™ Imaging Systems (BioRad, Hercules, CA, USA). Analysis of obtained results was conducted in ImageLab software (BioRad).

For immunofluorescence staining, satellite cells plated on cell imaging chambers coated 0.1 % gelatin were fixed in 4 % paraformaldehyde for 20 min at room temperature (RT) and methanol for 5 min at −20 °C. Cell membrane permeabilization was performed with 0.1 % Tween-20 for 15 min at room temperature and blocking with 3 % BSA (Sigma Aldrich) in PBS for 60 min at room temperature. Then cells were incubated with the antibody (syndecan-4 - sc-12766 AF594, 1:50, Santa Cruz Biotechnology, Inc.) for 90 min, at 37 °C. After washing four times, the slides were prepared using Fluoroshield™ with DAPI (Sigma Aldrich), and the cells were subjected to microscopic analysis using a Zeiss AX100 Imager D.2 microscope (Zeiss, Jena, Germany) and Zeiss Zen 2 Blue Edition Software.

2.7. Statistical analyses

Statistical analyses of gene expression were performed using Statistica software (Statsoft, v.13). The Shapiro-Wilk test was used to assess the normality of data distribution (n = 4). Analyses were conducted using Student's t-test. If the assumptions of Levene's test were not complied, the Mann-Whitney U test was used. Graphs were generated in the GraphPad program (Prism, v. 10.2.3).

3. Results

3.1. Analysis of differentially expressed genes (DEGs) for monoculture and co-culture groups

To study the paracrine effect of fibroblasts on the myogenesis process, 24 cDNA libraries were constructed of muscle satellite cells co-cultured with fibroblasts in four repetitions for three-time points (proliferation, differentiation 24 h, and differentiation 72 h) and were analyzed in comparison with a monoculture of MuSCs. An average 21 million reads per sample were read, from which 93.7 % passed the quality filters. The percentage of aligned sequences was an average 95 % (from 94.10 % to 96 %), which ranged

Table 1
Primer sequence of studied genes.

Gene	Forward	T _m (°C)	Reverse	T _m (°C)
ECR1	5'AGCAACAGCAACAGGAGC'3	50.3	5'CGGGTAACCTGCTCCACTAG'3	56.3
IL6	5'TGAAAGCAGCAAGGAGACAC'3	51.8	5'ACTCCAGAGACAGGAGCAGT'3	53.8
TCAP	5'CTGGGCTGAGTGGAGGATC'3	55.9	5'CTGGCGGTGATAGGTCTCAT'3	53.8
SOCS3 ¹	5'CGGCACTCTCCAACTCTCTGT'3	56.7	5'TCCAGGAACCTCCCAATGG'3	53.2
ITGA7B	5'TCAATCTGGACGTGATGGCG'3	53.8	5'AGAGACCTCCAGTCCGATTTC'3	51.8
RPL27	5'ATAATCACTCATGCCACAA'3	50.5	5'EATGACCTTTGCCCTCTCTC'3	53.8
OAZ1	5'TTCGCCAGAGAGAAGGAAGG'3	53.8	5'GGACCCAGGTACTACAGCA'3	53.8
PAX7	5'AAGCGGACAAGAAGGAGGAG'3	53.8	5'CGGGTTCTGACTCCACATCT'3	53.8
MYF5	5'TGCTTAGGGAACAGGTGGAA'3	51.8	5'AACCTGCTCTCTTTCTGGAC'3	51.8
MYOD1	5'AACACTACAGCGGCACT'3	50.3	5'GTAGTAAGTCGCGTCGTAGE'3	53.8
MYF6	5'CCCTTCAGCTACAGACCCAA'3	53.8	5'CTTGGCAGTTATACAGAGC'3	53.8
MYOG	5'TCCAGTACATAGAGCGCTC'3	53.8	5'CTATCGGAGCTGCAATTCATC'3	54.4

from about 20.28 million reads per sample. Selection of differentially expressed genes (DEGs) was based on $p_{adj} \leq 0.05$ and $|\log_2\text{fold change}| \geq 0.5$.

Differential gene expression analysis of co-cultures compared to MuSCs monocultures showed a significant change in expression ($p \leq 0.05$) of 1133 genes (483 upregulated and 650 downregulated) at the proliferation stage. The gene with the most significant downregulation ($\log_2FC = -1.84$) was GCA (Grancalcin), which binds calcium. In contrast, a gene with significant upregulation ($\log_2FC = 3.49$) was MX1 (MX Dynamin Like GTPase 1), which participates in the cellular antiviral response (Fig. 1A and 2A).

Differential gene expression analysis showed a significant change in expression ($p \leq 0.05$) of 397 genes (158 upregulated and 239 downregulated) at the 24 h differentiation stage. The gene with the most significant downregulation ($\log_2FC = -1.85$ respectively) was SPPI (Secreted Phosphoprotein 1), mainly involved in integrin binding. In contrast, a gene with significant upregulation ($\log_2FC = 3.49$) was EGR1 (early growth response 1), involved in muscle satellite cell differentiation (Fig. 1B and 2B).

Differential gene expression analysis showed a significant change in expression ($p \leq 0.05$) of 1630 genes (922 upregulated and 708 downregulated) at the 72 h differentiation stage. The gene with the most significant downregulation ($\log_2FC = -2.78$) was HDAC9 (Histone Deacetylase 9), mainly involved in the regulation of differentiation of muscle cells in hypoxia conditions. The gene with the most significant upregulation ($\log_2FC = 4.01$) was MX1 (Fig. 1C and 2C) (Supplementary material 2).

3.2. GO and KEGG pathway enrichment analysis for monoculture and co-culture groups

GO enrichment analysis was performed to classify and characterize the biological significance of DEGs. 1133 DEGs at the proliferation stage were classified into 178 biological process (GO: BP) annotations – mainly related to negative regulation of cellular process, negative regulation of biological process and metabolic process (Fig. 3A); 11 molecular function (GO: MF) annotations – mainly related to catalytic activity, binding, protein binding and metal ion binding, and 21 cellular component (GO: CC) mainly related to intracellular membrane-bounded organelle and cytoplasm.

397 DEGs at 24 h differentiation stage were classified into 180 biological process annotations (GO: BP) – mainly related to biological regulation (Fig. 3B); 16 molecular functions (GO:MF) annotations – mainly related to protein binding and ion binding, and 9 cellular components (GO:CC) – mainly related to the extracellular matrix.

1630 DEGs at differentiation 72 h stage were classified into 250 biological process annotations (GO:BP) – mainly related to transcription, cell division, protein phosphorylation, signal transduction and apoptotic process (Fig. 3C); 102 molecular functions (GO:MF) – mainly related to protein binding, ATP binding, metal ion binding, and 11 cellular components (GO:CC) – mainly related to nucleus and cytoplasm. Our study focused on GO terms related to myogenesis such as developmental processes, cell proliferation and differentiation (Supplementary material 3).

Analysis of the over-represented molecular pathways by KEGG pathway classification confirmed the involvement of DEGs at the proliferation and 24 h differentiation stage in the formation of the cytoskeleton of muscle cells (Figs. 4 and 5) – through extracellular matrix proteins (syndecan (SDC), collagen VI (COL6A) at proliferation stage; collagens and decorin (DCN) at differentiation 24 h stage, the formation of cytoskeleton/membrane junctions (integrin beta (ITGB), talin-1 (TLN), desmin (DES), ATP-grasp at the proliferation stage and integrin alpha (ITGA), ATP11A/B, ANKG,

β -spectrin (SPTBN) at the differentiation 24 h stage) and proteins involved in the construction of the sarcomere (telethonin (TCAP), β -actinin (CAPZ), Xin Actin Binding Repeat Containing 1 (XIRP), Zyxin (ZYX), Ankyrin repeat domain-containing protein 1 (ANKRD), Formin, Enolase 1/3 (ENO1/3) at the proliferation stage and TCAP, formin, myomesin (MYOM) at the differentiation 24 h stage).

At the 72 h differentiation stage, a much greater number of genes were involved in the formation of the cytoskeleton – mainly at the stage of the formation of the Z-disc component, M-band component, and myosin thick filament in the sarcomere (Fig. 6). The involvement of the DEGs in the PI3K-Akt, MAPK signaling, FoxO signaling, JAK-STAT signaling, and TNF signaling pathways with an investigated role in myogenesis was also demonstrated. Particularly noteworthy is the overexpression of the HIF-1 signaling pathway – crucial during reducing oxygen consumption conditions (Supplementary material 4).

3.3. The most crucial DEGs involved in the myogenesis process

Among DEGs at the proliferation stage, we selected 15 downregulated and 17 upregulated of the most crucial genes involved in the myogenesis process (Table 2). FGF2 (Fibroblast Growth Factor 2) was the most downregulated gene, which is required for MuSCs proliferation ($\log_2FC = -1.16$). SOCS3 (Suppressor of Cytokine Signaling 3) was the most upregulated gene, promoting myogenic differentiation by inhibiting the JAK1/STAT1/STAT3 pathway [17]. Moreover, the expression of IL-6 (interleukin-6), which induces myogenic differentiation, was upregulated ($\log_2FC = 1.06$) [18]. EGR-1 was also upregulated, which is involved in muscle cell differentiation ($\log_2FC = 1.01$) [19]. Transcription regulators of genes involved in myogenesis like NR4A1 (Nuclear Receptor Subfamily 4 Group A Member 1), HDAC4 (Histone deacetylase 4), PITX1 (Paired Like Homeodomain 1), and EYA2 (Eyes absent homolog 2) were upregulated ($\log_2FC = 0.85$, $\log_2FC = 0.52$, $\log_2FC = 0.50$, $\log_2FC = 0.70$ respectively). Changes in the expression of genes involved in maintaining muscle structure have been observed – downregulation of MYOM1 (Calcitonin receptor-like), and CRYAB (Crystallin Alpha B) ($\log_2FC = -1.07$, $\log_2FC = -0.84$ and $\log_2FC = -0.87$, respectively), and upregulation of DES, XIRP and TCAP ($\log_2FC = 0.79$, $\log_2FC = 0.70$, and $\log_2FC = 0.71$, respectively). The expression of genes involved in crucial pathways like WNT pathway – WNT2B (Wnt Family Member 2B), TGF- β signaling pathway – TGFBI (Transforming Growth Factor Beta 1), BMP pathway – BMPRI1A (The bone morphogenetic protein receptor, type IA) and BMPRI2 (Bone Morphogenetic Protein Receptor Type 2) were changed at the proliferation stage. Changes in the expression of cytoskeletal and structural protein genes were observed – downregulation of CAV1 (caveolin 1), ANK3 (Ankyrin 3) ($\log_2FC = -0.93$ and $\log_2FC = -0.67$) and upregulation of SPHK1 (Sphingosine Kinase 1), CTNBP1 (Catenin Beta Interacting Protein 1), ANKRD3 (Ankyrin Repeat Domain 23), COL6A3 (Collagen Type VI Alpha 3 Chain) and TLN2 (Talin-2). Moreover, downregulation of the other muscle differentiation markers was observed like GDF15 (Growth/differentiation factor 15), HOMER1 (Homer Scaffold Protein 1), ROCK1 (Rho Associated Coiled-Coil Containing Protein Kinase 1), RBL1 (RB transcriptional corepressor 1), CFL2 (Cofilin 2), and HIF1A (Hypoxia Inducible Factor 1 Subunit Alpha).

Among DEGs at the differentiation 24 h stage, we selected 19 downregulated and 18 upregulated of the most crucial genes involved in the myogenesis process (Table 3). ITGA7 (integrin 7 alpha), a marker of muscle satellite cells, showed high downregulation ($\log_2FC = -1.17$). BHLHA15 (Basic Helix-Loop-Helix Family Member A15), HES1 (Hes Family BHLH Transcription

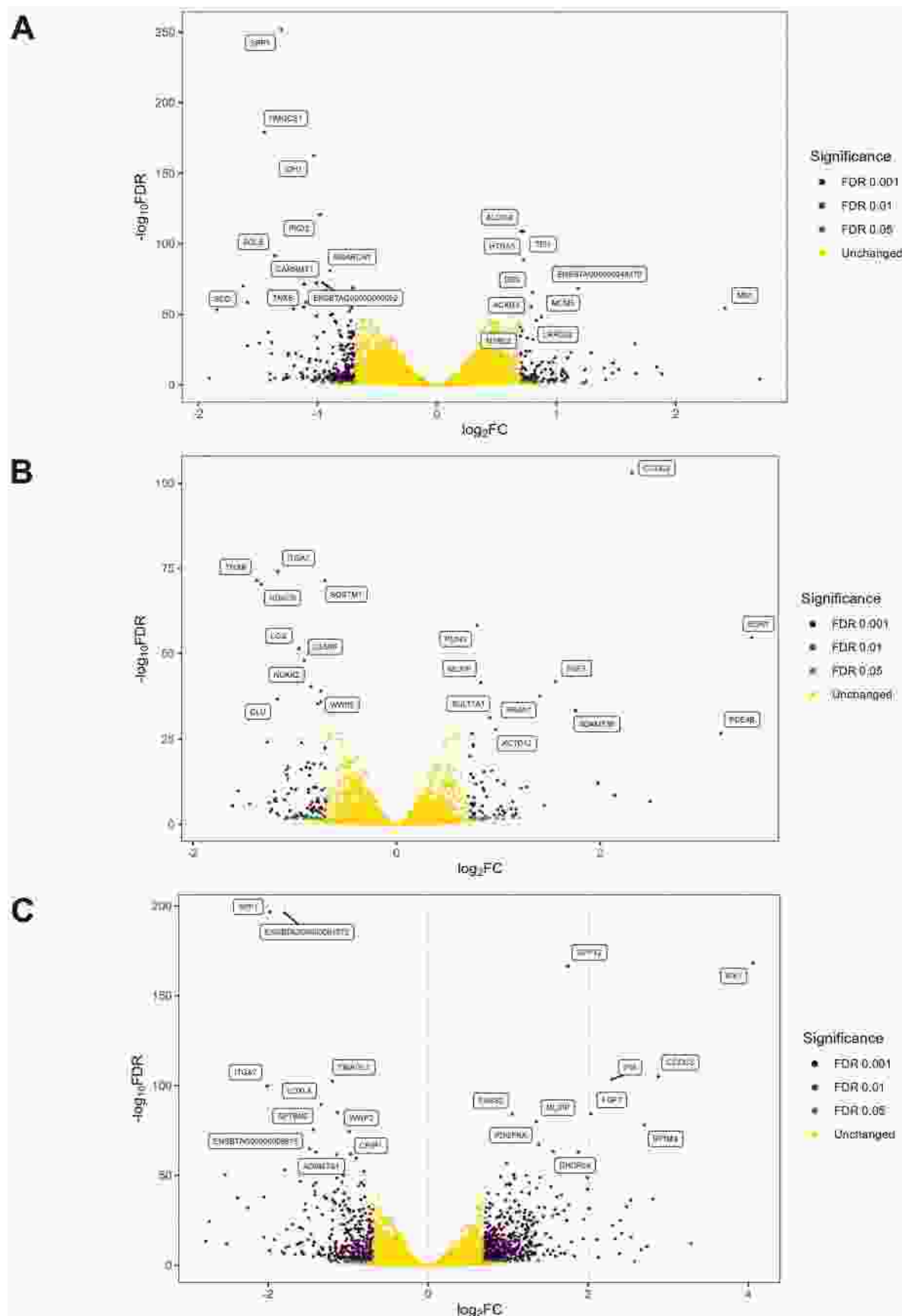


Fig. 1. Volcano Plots of DEGs (A) at the proliferation stage (B) at the differentiation 24 h stage (C) at the differentiation 72 h stage. The yellow dots designate non-significant changes in transcript expression levels. Violet, blue, and green dots correspond to upregulated and downregulated genes, (respectively $FDR \leq 0.001$; $FDR \leq 0.01$; $FDR \leq 0.05$). X-axis relates to $\log_2$ fold change and y-axis to $\log_{10}$ FDR (false discovery rate).

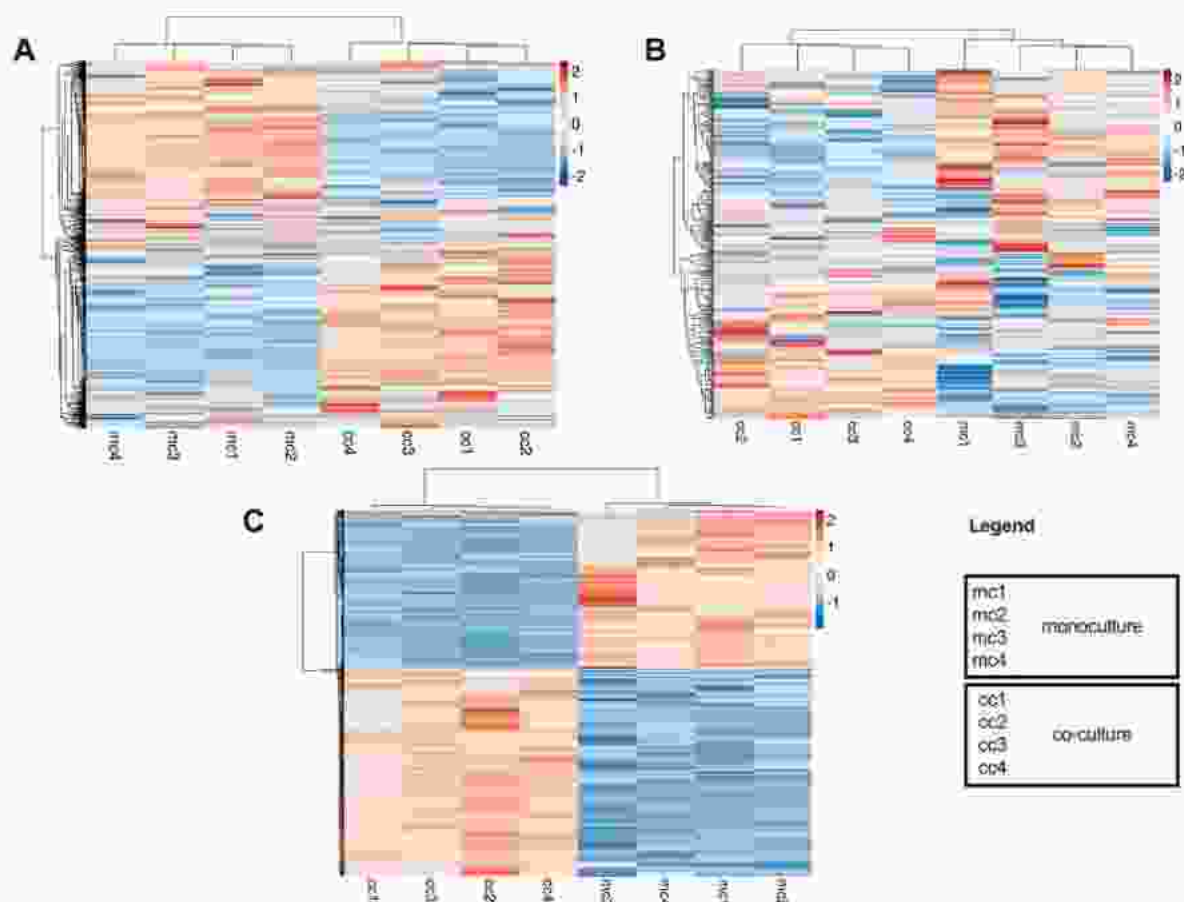


Fig. 2. The heat maps of differential gene expression of monoculture and co-culture (A) at the proliferation stage (B) at the differentiation 24 h (C) at the differentiation 72 h (<https://bit.ly/3u5tvi7>).

Factor 1) and FOXS1 (forkhead box S1) genes, controlling the transcriptional activity of MYOD1 – one of the most important transcription factors regulating the myogenesis process, also showed high downregulation ($\log_2FC = -0.83$, $\log_2FC = -0.64$ and $\log_2FC = -0.51$, respectively). MYORG (Myogenesis-Regulating Glycosidase), involved in muscle fiber development at 24 h differentiation stage, showed downregulation ($\log_2FC = -0.83$). NES (nestin), which is involved in maintaining the population of muscle satellite cells, also showed significant downregulation ($\log_2FC = -0.57$) [20]. In addition, TCAP a scaffold for myofibrils, shows downregulation ($\log_2FC = -0.51$). Moreover, plenty of genes such as CMKLR1 (Chemerin Chemokine-Like Receptor 1), HMOX1 (Heme Oxygenase 1), SMAD7 (SMAD Family Member 7), KANK1 (KN Motif And Ankyrin Repeat Domains 1), TGM2 (Transglutaminase 2), CD44 (CD44 molecule), CRYAB, LIF (LIF Interleukin 6 Family Cytokine), MET (MET Proto-Oncogene), GDF15, WNT10B (Wnt Family Member 10B), ATP1B3 (ATPase Na⁺/K⁺ Transporting Subunit Beta 3), BMP4 (Bone morphogenetic protein 4) mainly involved in the regulation of myogenic proliferation and differentiation showed downregulation at 24h differentiation stage. EGR1 was the most upregulated ($\log_2FC = 3.49$) gene, and it is involved in MuSCs differentiation. DCN enhances myoblast proliferation, and differentiation was upregulated ($\log_2FC = 0.76$). Transcription regulators of genes involved in myogenesis like NR4A1, RUNX1 (RUNX Family Transcription Factor 1), FOS (Fos Proto-Oncogene), JUN (Jun Proto-Oncogene), SOCS3, TGFBR3, SATB2 (SATB Homeobox 2) EYA2 and ARID5B (T-Rich Interaction Domain 5B) also showed upregulation at the 24 h differentiation

stage. Genes involved in important cellular pathways like WNT pathway – WNT2B, SFRP4 (Secreted Frizzled Related Protein 4), IGFBP4 (Insulin-like growth factor-binding protein 4); hedgehog pathway – SCUBE2 (Signal Peptide CUB Domain and EGF Like Domain Containing 2), BMP – NOG (Noggin) were also upregulated at the 24h differentiation stage. Moreover, the expression of the cytokine IL-6, which induces myogenic differentiation, was upregulated ($\log_2FC = 1.04$).

Among DEGs at the 72 h differentiation stage we selected 23 downregulated and 12 upregulated genes most crucial to myogenesis (Table 4). ITGA7 shows the highest downregulation ($\log_2FC = -2.01$). FOXS1 showed higher downregulation than at 24 h differentiation stage ($\log_2FC = 0.99$). Also, HEYL (Hes Related Family BHLH Transcription Factor with YRPW Motif Like) effector gene of Notch pathway shows downregulation at the 72 h differentiation stage ($\log_2FC = -0.93$). Moreover, at the differentiation 72 h stage the following genes were downregulated: DES, BTG2 (BTG Anti-Proliferation Factor 2), SOX9 (SRX-Box Transcription Factor 9), FHOD3 (Formin Homology 2 Domain Containing 3), GREM1 (Gremlin 1 DAN Family BMP Antagonist), LMOD1 (Leiomodin 1), SPEK (Striated Muscle Enriched Protein Kinase), FLNC (filamin C), which are involved in the process of differentiation and cytoskeleton organization. Changes in the expression of structural and cytoskeletal proteins were observed, such as downregulation of MYOM1, CRYAB, GDF15, TLN, NES, ELN and upregulation of SATB2. The highest upregulation was shown by the IL6 gene ($\log_2FC = 2.66$). Higher upregulation of ARID5B ($\log_2FC = 0.88$) and SOCS3 ($\log_2FC = 0.66$) and lower upregulation of EGR1

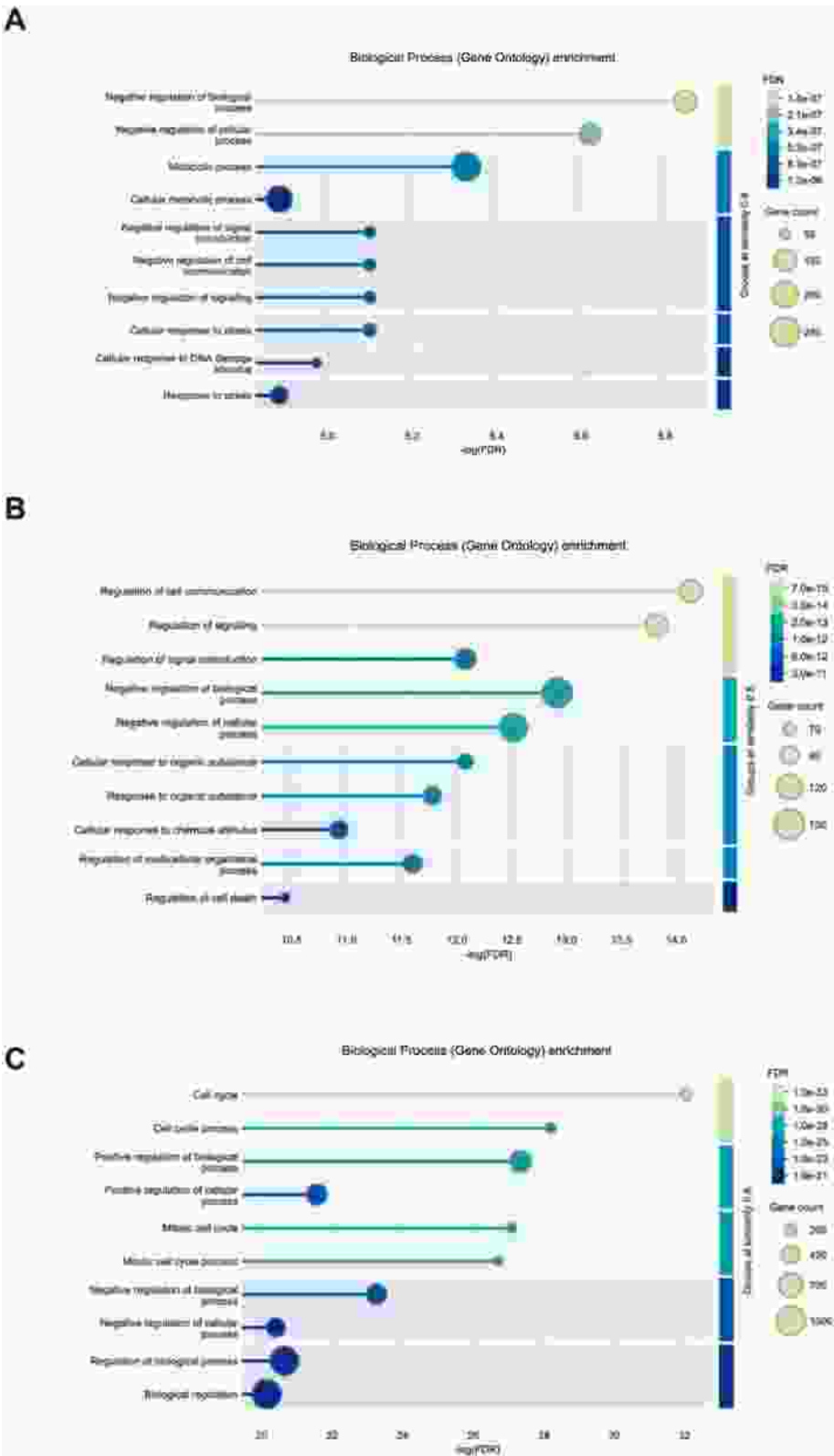


Fig. 3. Biological process (Gene Ontology) enrichment; x-axis represent $-\log(\text{FDR})$ and y-axis represent GO terms categories (A) proliferation stage (B) differentiation 24 h (C) differentiation 72 h (<https://string-db.org/>). Overrepresented enrichment analysis of all differentially expressed genes (DEGs) including up- and down-regulated genes.

($\log_2\text{FC} = 0.62$) genes was demonstrated than at the 24 h differentiation stage. Higher upregulation of HDAC4 ($\log_2\text{FC} = 0.93$) was demonstrated than at the proliferation stage. Downregulation of

KANK1 ($\log_2\text{FC} = -0.99$), SMAD7 ($\log_2\text{FC} = -0.95$), LIF ($\log_2\text{FC} = -0.88$), WNT10B ($\log_2\text{FC} = -0.58$) was demonstrated than at the 24 h differentiation stage. Downregulation of CMKLR1

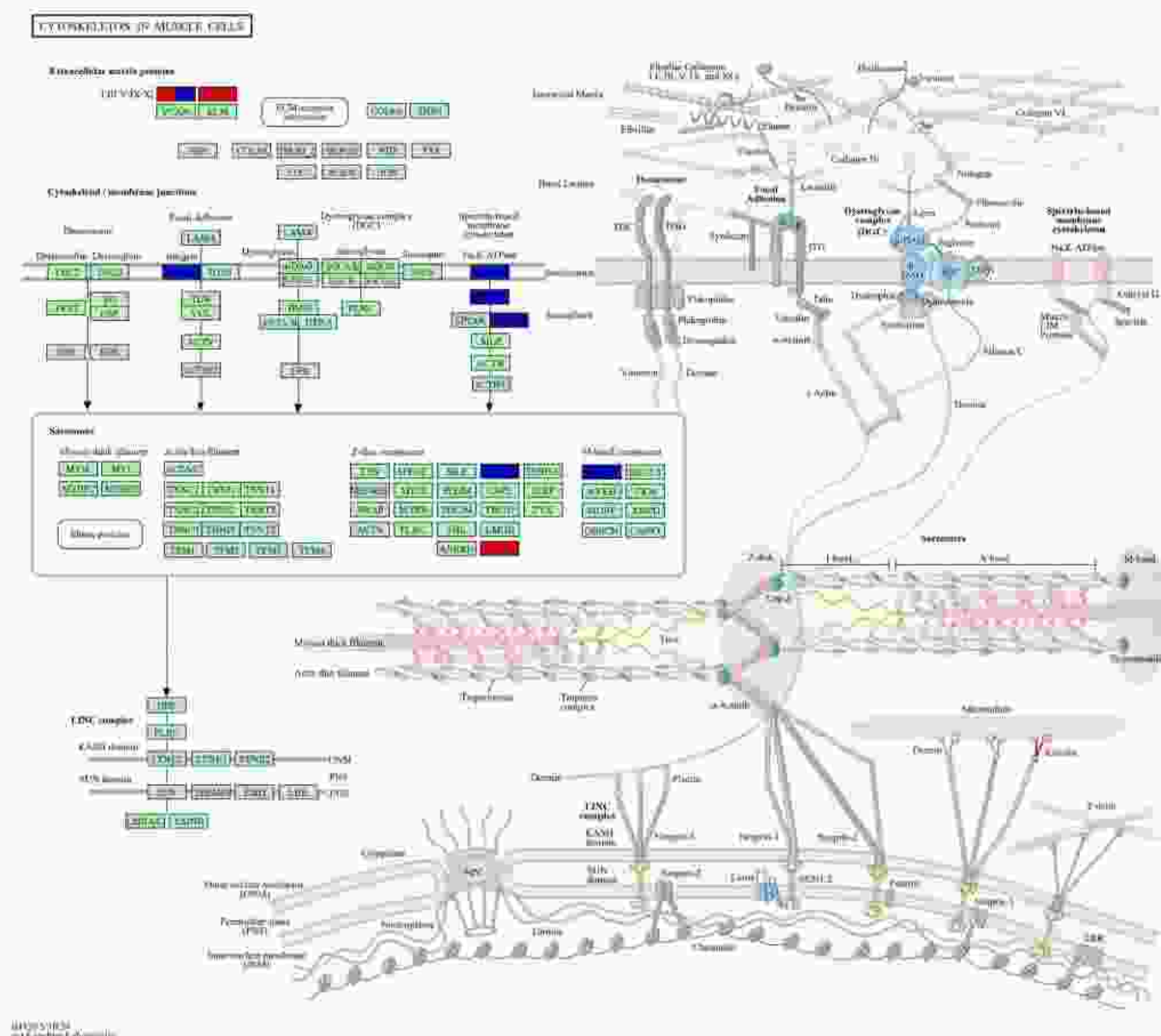


Fig. 5. Cytoskeleton in muscle cells (hsa04820) over-represented molecular pathway in muscle satellite cells cultured in co-culture with fibroblasts and differentiation 24 h compared to monoculture; red color indicates upregulated genes; blue color indicates downregulated genes.

culture group (Fig. 7A) during proliferation stage. Significantly higher expression of MYF5 ($p = 0.0241$) and MYOD ($p = 0.0044$) were observed in the monoculture group during the 24 h differentiation stage (Fig. 7B). No significant differences were observed between the tested group during the 72 h differentiation stage (Fig. 7C).

3.5. Effect of fibroblasts on the proliferation of muscle satellite cells

RNA-Seq analysis did not reveal differential expression of proliferation-related genes such as MKI67 (Marker of Proliferation Ki-67), PCNA (Proliferating cell nuclear antigen), cyclin-dependent kinases (CDK2, CDK4), and cyclins (CCND1, CCNE1) during proliferation and differentiation 24 h stages. However, during 72h differentiation, up-regulation of MKI67, PCNA, and CDK2 was observed, which indicates increased proliferative activity of

muscle cells (Table 6).

Analysis of proliferation rate using the resazurin-based Cell Titer Blue assay (Promega) revealed a significantly higher proliferation rate in muscle cells co-culture with fibroblasts on 3rd ($p = 0.03$), 4th ($p = 0.002$) and 5th ($p = 0.0049$) days under proliferative conditions (Fig. 8).

3.6. Myosin expression at the protein level during differentiation of MuSCs

ELISA results revealed a significantly higher level of skeletal myosin expression at the protein level in the co-culture of fibroblasts and MuSCs at the proliferation stage ($p = 0.0183$). No significant differences were observed after 24 h and 72 h after the initiation of differentiation by horse serum component (Fig. 9A). Western Blot analysis for myosin heavy chains (MyHC) confirmed

Table 2

List of downregulated and upregulated DEGs involved in the myogenesis process at the proliferation stage.

Ensembl ID	Log2Fold Change	padj	Gene name
ENSBTAG00000005691	-1.16	8.74E-56	FCF2
ENSBTAG00000019585	-1.07	1.28E-09	MYOM1
ENSBTAG000000062162	-0.93	1.47E-67	CAV1
ENSBTAG00000000434	-0.87	4.72E-23	CRYAB
ENSBTAG00000008483	-0.84	2.42E-15	CALCRL
ENSBTAG00000015618	-0.73	3.90E-12	GDF15
ENSBTAG00000025853	-0.71	4.99E-05	HOMER1
ENSBTAG00000019052	-0.67	8.00E-03	ANK3
ENSBTAG00000008403	-0.63	3.70E-42	ROCK1
ENSBTAG00000006420	-0.56	1.06E-22	BMPR2
ENSBTAG00000006640	-0.53	2.00E-11	RB1
ENSBTAG00000015053	-0.52	5.91E-37	CHL2
ENSBTAG00000020935	-0.52	1.93E-55	HIF1A
ENSBTAG00000000231	-0.52	1.62E-14	BMPK1A
ENSBTAG00000004602	0.50	5.61E-05	ITX1
ENSBTAG00000005933	0.52	4.00E-04	CTNNB1P1
ENSBTAG00000017764	0.52	1.12E-05	HDAC4
ENSBTAG00000008507	0.55	5.48E-20	SPHK1
ENSBTAG000000030190	0.68	6.87E-45	COL6A3
ENSBTAG00000020457	0.69	9.38E-31	TGFB1
ENSBTAG000000046512	0.70	2.16E-17	XIRP1
ENSBTAG00000011730	0.71	7.00E-03	TCAP
ENSBTAG00000013336	0.70	2.54E-16	EYA2
ENSBTAG00000010849	0.74	3.09E-02	ANKRD23
ENSBTAG00000005353	0.79	4.74E-66	DES
ENSBTAG00000000507	0.85	6.92E-15	NR4A1
ENSBTAG00000003667	0.86	8.00E-02	TLN2
ENSBTAG00000014291	0.93	1.56E-16	WNT2B
ENSBTAG00000010069	1.01	2.06E-10	EGR1
ENSBTAG00000014921	1.06	1.01E-04	IL6
ENSBTAG00000008441	1.09	8.28E-20	SOC3

and 24 h differentiation stages. Transforming growth factor- β (TGF- β) inhibits the activation of satellite cells, keeping the cells in the quiescent phase [22,23]. FOS is involved in activating proliferation under the influence of external factors.

Quiescent satellite cells indicate expression of Pax7, and as a result of cell activation, begin to express genes from the MRF family (Myogenic Regulator Factors) – Myf5 and MyoD [24]. The role of Pax7 is to regulate genes that promote entry into the proliferative cycle and inhibit myogenic differentiation [25]. MyoD stimulates chromatin reorganization so that other transcription factors can act. In our study, RNA-Seq analysis showed no significant changes in the expression of genes from the PAX and MRF families, however, qPCR due to greater sensitivity indicates changes in gene expression. Significantly higher expression of PAX7 and MYF5 were observed in the co-culture group at the proliferation stage. At the 24 h differentiation stage higher expression of Myf5 and MyoD genes were noted in the mono-culture group. These results are consistent with RNA-Seq analysis which revealed that at the 24 h differentiation stage, downregulation of HES1, BHLHA15, and FOXS1 was observed – genes controlling the transcriptional activity of the MYOD1 gene. HEYL, an effector of the Notch signaling pathway, inhibits MYOD expression, which increases muscle satellite cell proliferation [26]. Our RNA-Seq analysis revealed the downregulation of HEYL and FOXS1 at the 72 h differentiation stage in co-culture. Lahmann et al. [27] reported oscillatory expression of HES1 in activated muscle cells, which induces oscillatory expression of MyoD. Moreover, upregulation of the EYA2 gene was observed (at proliferation and differentiation stages), which activates genes such as MyoD, Myf6, or MyoG [28]. Downregulation of MyoD gene repressors – mainly at the 24h differentiation stage indicates the promotion of differentiation in co-culture of muscle satellite cells and fibroblasts through increased MyoD expression.

Table 3

List of downregulated and upregulated DEGs involved in the myogenesis process at the differentiation 24 h stage.

Ensembl ID	Log2FChange	padj	Gene name
ENSBTAG00000012897	1.17	9.50E-75	ITGA7
ENSBTAG00000015618	1.07	1.23E-17	GDF15
ENSBTAG000000046010	0.83	8.11E-03	BHLHA15
ENSBTAG00000008376	0.83	1.42E-04	MYOG
ENSBTAG00000015582	0.69	3.65E-11	HMOX1
ENSBTAG00000012441	0.67	3.90E-14	SMAD7
ENSBTAG00000010976	0.66	6.44E-30	KANK1
ENSBTAG00000016208	0.65	1.76E-09	TGM2
ENSBTAG00000000569	0.64	2.53E-14	HES1
ENSBTAG00000011578	0.61	2.22E-32	CD44
ENSBTAG00000000434	0.60	2.11E-33	CRYAB
ENSBTAG00000015794	0.57	2.45E-27	NES
ENSBTAG00000007424	0.56	3.61E-02	LIF
ENSBTAG00000015347	0.55	5.58E-04	WNT10B
ENSBTAG00000014140	0.53	8.52E-25	ATP1B3
ENSBTAG00000006161	0.54	2.52E-05	MET
ENSBTAG00000003835	0.52	1.11E-04	BMP4
ENSBTAG00000011730	-0.51	1.46E-02	TCAP
ENSBTAG00000009206	-0.51	1.23E-15	FOXS1
ENSBTAG00000010179	0.50	3.64E-12	COL5A3
ENSBTAG00000013336	0.50	2.89E-07	EYA2
ENSBTAG00000016334	0.52	3.43E-02	SATB2
ENSBTAG00000014291	0.54	1.69E-05	WNT2B
ENSBTAG000000024269	0.55	2.00E-03	TGFB1
ENSBTAG00000008441	0.56	2.60E-12	SOC3
ENSBTAG00000008611	0.56	1.45E-43	IGFBP4
ENSBTAG00000006835	0.57	1.61E-16	JUN
ENSBTAG00000004322	0.58	5.49E-13	FOS
ENSBTAG0000001333	0.58	7.20E-13	ARID5B
ENSBTAG00000015366	0.59	1.7E-02	SERP4
ENSBTAG00000003505	0.76	6.36E-07	DCN
ENSBTAG00000020573	0.76	1.42E-08	SCUBE2
ENSBTAG00000040282	0.68	8.37E-08	NOG
ENSBTAG00000004742	1.02	5.30E-06	RUNX1
ENSBTAG00000014921	1.04	1.21E-04	IL6
ENSBTAG00000000507	1.41	2.49E-38	NR4A1
ENSBTAG00000010069	3.49	2.19E-55	EGR1

Plenty of other factors are also involved in the proliferation process, such as ITGA7, IL-6, BMP4 and NOG. ITGA7, which is the main receptor of the adhesion protein laminin in skeletal muscle cells, is involved in determining the polarity of satellite cells, and its high expression is observed mainly in quiescent satellite cells of adults. However, the role of ITGA7 is not limited to the early stages of myogenesis – ITGA7 is involved in the formation of connections between cells and the extracellular matrix and in fully formed muscles [29,30]. In our study, RNA-Seq analysis showed downregulation of ITGA7 at all stages, progressing with differentiation, with a high correlation with qPCR validation analysis ($R^2 = 0.77$). Another important factor is interleukin-6 (IL-6), which supports the proliferation process by activating the JAK/STAT (Janus kinase/Signal Transducer and Activator of Transcription) pathway and which upregulation was observed with differentiation in RNA-Seq analysis (with high correlation to qPCR validation – $R^2 = 0.97$). High levels of IL-6 increased the expression of MyoD and MyoG and shifted cells to the pro-differentiation G_0/G_1 phase [18]. At the 24 h differentiation stage, downregulation of BMP4 was observed, along with simultaneous upregulation of its antagonist – NOG, which increases the expression of cell cycle inhibitors such as p21 and p57 [31].

4.2. Differentiation of muscle satellite cells and fusion of myotubes

In differentiating myoblasts, the expression of Pax7 and MyoD genes is decreased, and the expression of MyoG and Myf6 increases successively, and the myocytes resulting from

Table 4

List of downregulated and upregulated DEGs involved in the myogenesis process at the differentiation 72 h stage.

Ensembl ID	Log2FC	padj	Gene name
ENSBTAG00000012897	−2.01	2.09E−100	ITGA7
ENSBTAG00000019585	−1.92	8.11E−15	MYOM1
ENSBTAG00000000434	−1.66	1.60E−06	CRYAB
ENSBTAG000000063257	−1.35	7.97E−07	BTG2
ENSBTAG00000015618	−1.21	1.13E−17	GDF15
ENSBTAG000000045824	−1.06	1.78E−11	SOX9
ENSBTAG00000010976	−0.99	7.06E−38	KANK1
ENSBTAG00000009206	−0.99	2.91E−20	FOXS1
ENSBTAG00000012441	−0.95	2.55E−26	SMAD7
ENSBTAG00000000688	−0.93	9.34E−08	HEY1
ENSBTAG00000007424	−0.88	1.50E−08	LIF
ENSBTAG000000021581	−0.88	5.12E−12	PHOD3
ENSBTAG00000005353	−0.78	1.24E−29	DES
ENSBTAG000000050495	−0.76	3.20E−17	GREM1
ENSBTAG00000003667	−0.68	8.66E−03	TLN
ENSBTAG000000068434	−0.65	9.75E−06	CMKLR1
ENSBTAG000000015794	−0.62	0.0008766	NES
ENSBTAG000000021576	−0.6	2.59E−19	LMOD1
ENSBTAG000000016208	−0.59	0.02	TGM2
ENSBTAG000000046176	−0.59	2.97E−13	SPEG
ENSBTAG000000015347	−0.58	2.33E−05	WNT10B
ENSBTAG000000006253	−0.58	1.89E−20	FLNC
ENSBTAG000000008376	−0.52	7.11E−03	MYORG
ENSBTAG000000016334	0.59	0.010	SATB2
ENSBTAG000000010069	0.62	1.00E−15	EGR1
ENSBTAG000000008441	0.66	2.99E−05	SOC3
ENSBTAG000000051333	0.88	7.40E−23	ARID5B
ENSBTAG000000020573	0.90	6.37E−31	SCUBE2
ENSBTAG000000013336	0.91	2.60E−20	EYA2
ENSBTAG000000017764	0.93	5.84E−15	HDAC4
ENSBTAG000000024269	0.93	3.00E−13	TGFBR3
ENSBTAG000000026266	1.00	2.01E−08	MYL12B
ENSBTAG000000018025	1.13	5.64E−33	TGFBR1
ENSBTAG000000004742	1.22	1.15E−50	RLN1
ENSBTAG000000014921	2.66	2.15E−09	IL6

differentiation are finally fused into myotubes. EGR1 promotes myogenic differentiation by positively regulating the expression of MyoC gene [19]. In our RNA-Seq analysis, we observed upregulation of EGR1 mainly at the proliferation stage and at 24 h and 72 h differentiation stages, high correlation to qPCR validation results ($R^2 = 0.97$). Moreover, qPCR analysis indicates a statistically higher expression of MYOG in the co-culture group at the proliferation stage. Reduced NES levels translated into increased early myogenesis – expressed in the expression of myogenic markers such as MyHC or MYOG at the protein level. NES showed an inhibitory effect on differentiation through long-term inhibition of Cdk5 activation by p25 [32]. RNA-Seq analysis showed progressive downregulation of NES in co-culture during subsequent differentiation stages. Several genes involved in myogenesis in muscle fiber regeneration conditions showed changes in expression, such as downregulation of GDF15, HMOX1, LIF, or upregulation of genes such as Runx1, GDF15, a member of the TGF- β family, is co-expressed with other growth factors important for muscle regeneration, and its main role is to downregulate muscle micro-RNAs. At the proliferation stage, GDF15 stimulates myoblasts by activating regenerative pathways through macrophages [33]. HMOX1 expression is induced by oxidative stress and has an inhibitory effect on myotube formation during myogenic differentiation. Moreover, the influence of HMOX1 on enzymes processing miRNAs such as miR-1, miR-133a, and b, miR-206 has been demonstrated [34]. RNA-Seq analysis showed downregulation of this gene at the 24 h differentiation stage, which may indicate a transition from a regeneration program to a differentiation program. LIF supports the regeneration of damaged muscles – it has a

pro-proliferative effect while at the same time inhibiting it at the early myogenesis stage by activating the ERK signaling pathway [35]. RNA-Seq analysis showed increasing downregulation of LIF with differentiation in co-culture. Runx1 is strongly expressed in regenerating muscles, cooperating with MyoD and c-Jun to control the balance of proliferation and differentiation of primary myoblasts [36]. RNA-Seq analysis showed upregulation of Runx1 in the co-culture during the 72 h differentiation stage.

At the proliferation stage, upregulation of genes such as SOCS3, HDAC4, NR4A1 – important during myogenic differentiation and muscle fiber regeneration – was also demonstrated. SOCS3 promotes myogenic differentiation by inhibiting the LIF-induced signaling pathway [17]. HDAC4 is involved in the regulation of proliferation by inhibiting p21 expression and regulation of myogenic differentiation by inhibiting Sharp1 expression, and its knockout resulted in the inhibition of the differentiation process [37]. NR4A1 is involved in myogenic differentiation, and overexpression of NR4A1 results in increased myoblast fusion and faster myogenic differentiation [38]. Moreover, RNA-Seq analysis revealed upregulation of cytoskeletal and structural proteins such as XIRP1, TCAP, ANKRD23, DES, TLN2 and extracellular matrix proteins such as COL6A3 important for myogenic differentiation at the proliferation stage. Structural protein XIRP1 together with XIRP2 play key roles in muscle fiber regeneration [39]. TCAP regulates the myogenic differentiation process and fulfills a structural function by linking the N terminus of titin with other Z-disk proteins [40]. The presence of DES – a muscle-specific member of the intermediate filament is crucial for maintaining the structure and integrity of myofibrils and maintaining the correct structure of organelles in the cell [41]. Moreover, RNA-Seq analysis revealed upregulation of PITX1, WNT2B and CTNNB1 – related to the WNT pathway – during proliferation. Pitx1 (and Pitx2, Pitx3) inhibits satellite cell proliferation and induces myogenic differentiation [42]. WNT/ β -catenin signaling is crucial for myogenic differentiation, and WNT2B expression increases with differentiation [43].

Moreover, proteomic analysis showed a significantly higher expression of MyHC in the co-culture group at the proliferation stage ($p = 0.0183$); however, no statistical differences were revealed between groups at the differentiation stages. Myosin is a contractile protein specific to skeletal muscles. Moreover, immunofluorescence staining was performed for differentiation marker – syndecan-4, which is mainly involved in the activation, proliferation, and self-renewal of the satellite cell population, and also affects the expression of genes from the MRF family [44]. Ablation of syndecan 4 (*Sdc4*−/−) inhibits the process of activation, proliferation and differentiation of satellite cells, and also the lack of expression of syndecan genes results in decreased expression of MyoD at the protein level [45]. In our study, the expression of syndecan-4 was observed at all stages of the experiment – in monoculture and co-culture (Fig. 8). At the 72 h differentiation stage, internalization of syndecan-4 from the cytoplasm to nuclear localization were observed. Literature data indicate that syndecan-4 during differentiation shows expression in the nuclear region, including the nuclear envelope [21]. The above observations confirm the stimulating effect of fibroblast paracrine signaling on myogenic differentiation.

Surprisingly, RNA-Seq analysis showed downregulation of many genes crucial during myogenic differentiation (24 h and 72 h differentiation stages) in the co-culture, such as MYORG, CMKLR1, SMAD7, KANK1, TGM2, CD44, MET, TCAP, GREM1, LMOD1, SPEG, and FLNC. MYORG (alternatively called NET37) is crucial for myogenic differentiation and is involved in muscle fiber development. In C2C12 myoblasts, its regulation during differentiation and its potential role in the IGF-II pathway during differentiation have been shown. The lack of NET37 resulted in lower activation of Akt

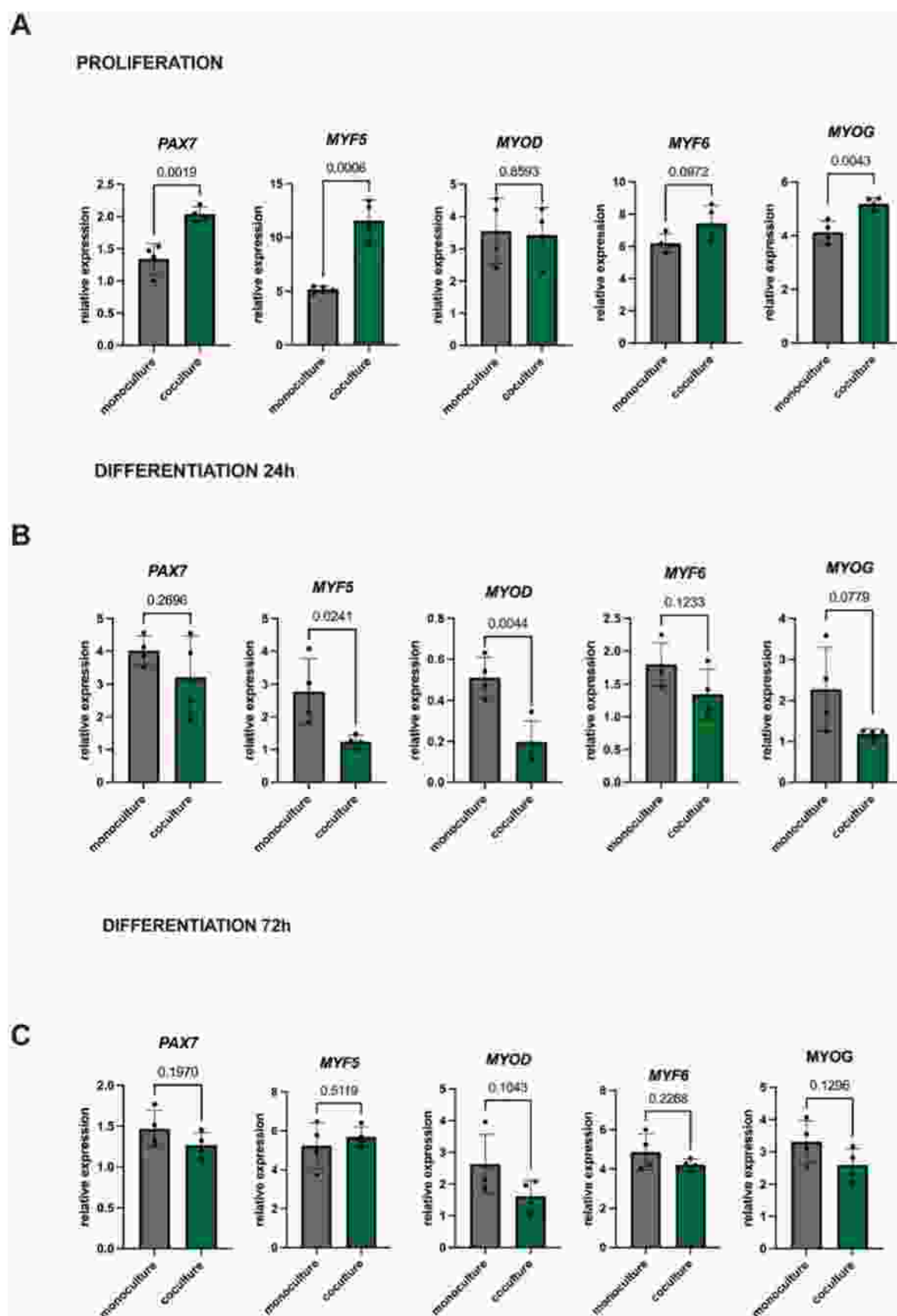


Fig. 7. Relative gene expression of MuSCs marker genes from PAX and MRF families (A) at the proliferation stage (B) at the differentiation 24 h (C) at the differentiation 72 h; $n = 4$.

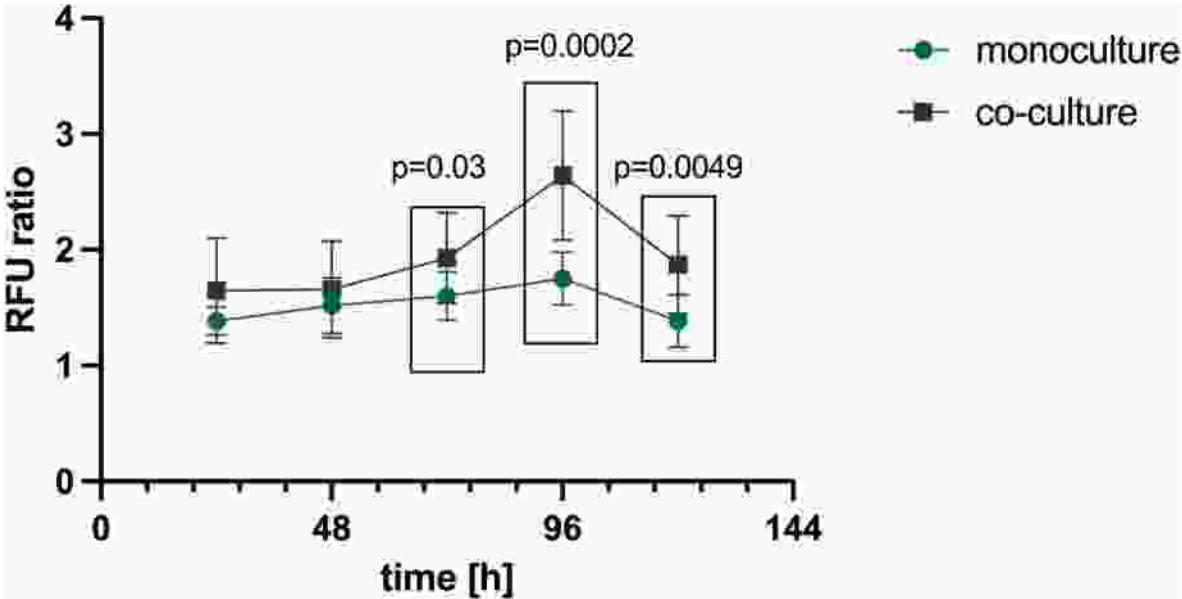


Fig. 8. Proliferation rate of muscle satellite cells co-cultured with fibroblasts, in comparison to monoculture, revealed by Cell Titer Blue Assay (Promega).

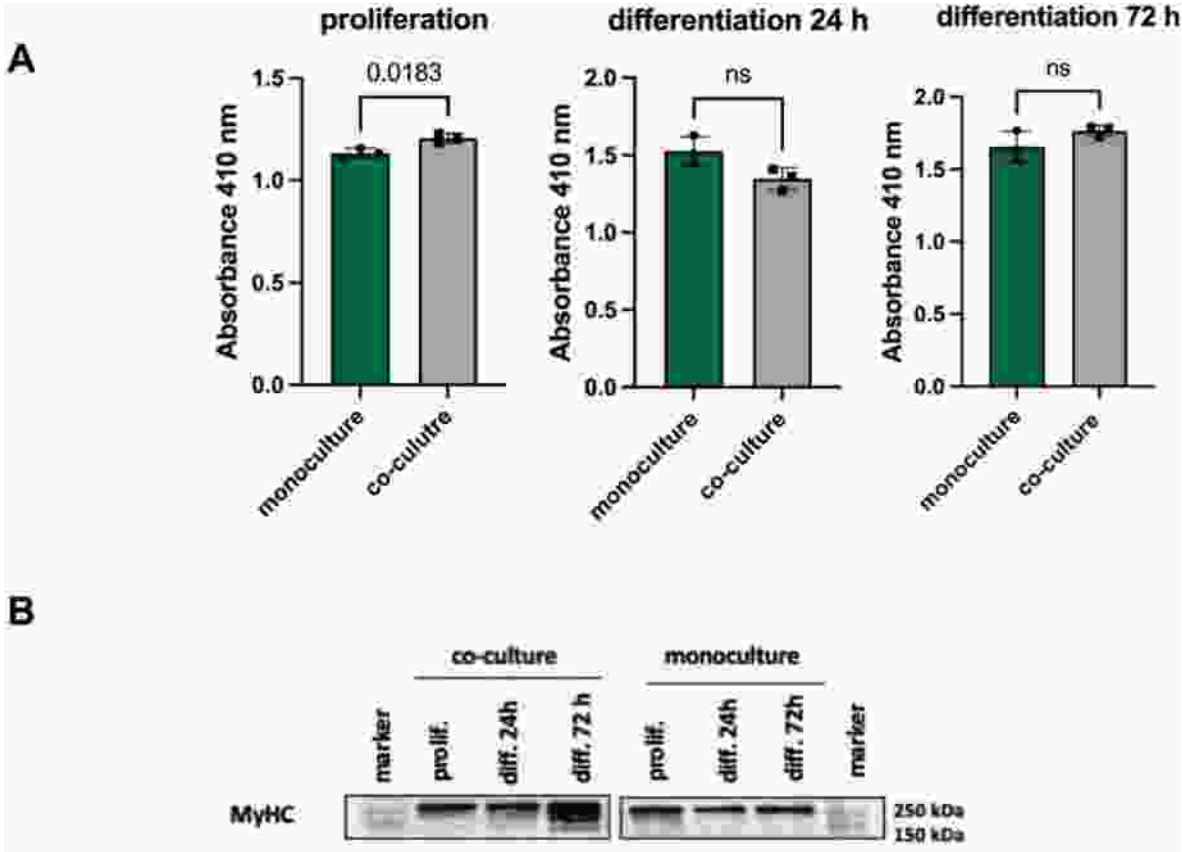


Fig. 9. (A) Skeletal myosin protein level in muscle satellite cells cultured in monoculture or co-culture with fibroblasts (n = 3) determined by ELISA (B) Protein levels of myosin heavy chain (MyHC) – 228 kDa in muscle satellite cells cultured monoculture and co-culture with fibroblasts determined by Western Blot.

and non – expression of IGF-II – both involved in the Akt-mTOR pathway, which controls mitochondrial metabolism [46]. CMKLR1 gene, which was downregulated during differentiation and which plays a role in myogenic differentiation. It was observed that reduced expression of CMKLR1 resulted in lower expression of MyoD and MyoG genes, with concomitant higher expression of Myf5 and Myf6 genes [47]. SMAD7 enhances myoblast differentiation and also interacts with MyoD to enhance its transcriptional

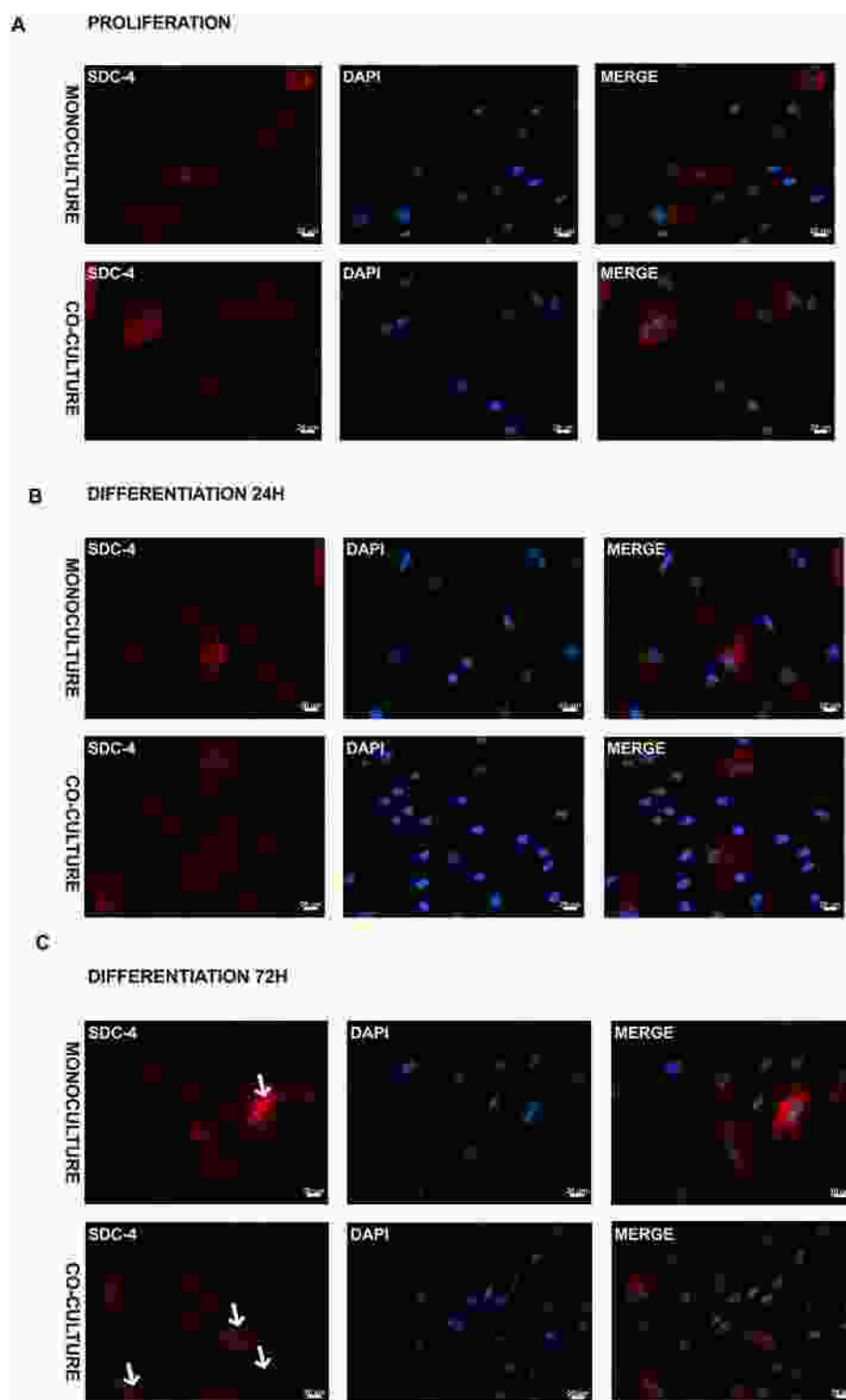


Fig. 10. Localization of syndecan-4 (SDC-4) during muscle differentiation in monoculture and co-culture with fibroblasts. During differentiation syndecan-4 internalizes from cytoplasm to nucleus. (A) proliferation (B) differentiation 24 h (C) differentiation 72 h. Nucleus were stained by DAPI; scale bar 20 μ m.

activity [48]. KANK1 significantly regulates the process of muscle cell proliferation and differentiation, and inhibition of KANK1 expression resulted in the inhibition of MyoD and MyoG gene expression and myotube formation during myogenic differentiation. RNA-Seq analysis showed increasing downregulation of

KANK1 during differentiation in co-culture. TGM2 stimulates muscle cell differentiation by enhancing the activation of mTOR signaling [49]. CD44 is involved in muscle cell differentiation and migration and plays a key role in early myogenesis. Mylona et al. [50] showed that CD44 knockout resulted in delayed myotube

Table 5
The Pearson correlation (R^2) coefficient for validated genes.

Gene	R^2	p value
EGR1	0.9658	<0.001
IL6	0.9744	<0.001
ITGA7	0.7741	<0.001
TCAP	0.3230	0.0038
SOCS3	0.1790	0.0394

formation and progression of differentiation, along with slower muscle cell migration. RNA-Seq analysis at the 24 h differentiation stage revealed downregulation of CD44 in co-culture. LMOD1 expression increases at the early differentiation stage – promoting myogenic differentiation, including myotube formation with increasing length. Reduction of LMOD1 expression resulted in a change in the muscle cell phenotype to a spherical shape and a decrease in the number of cell nuclei [51]. In our study, RNA-Seq analysis indicated downregulation of LMOD1 during 24 h differentiation. SPEG deficiency causes decreased proliferation and differentiation of myogenic cells, and SPEG knockout mice showed abnormalities in contraction, calcium management and abnormalities in the formation of triads related to muscle contraction [52]. RNA-Seq analysis showed downregulation of SPEG at the 72 h differentiation stage in co-culture. FLNC belongs to the family of actin cross-linking proteins and is found in the Z-discs of sarcomeres and muscle sarcolemma. FLNC plays an important role in muscle cell differentiation and maintaining the elongated structure of muscle fibers. FLNC deficiency resulted in abnormalities during myoblast differentiation and fusion, forming multinucleated spherical muscle cells [53]. RNA-Seq analysis revealed downregulation of FLNC at the 72 h differentiation stage in co-culture. Downregulation of many genes involved in myogenic differentiation indicates that at stages of differentiation (24 h and 72 h), components of medium – specifically the type of serum have a prevailing effect on the course of myogenesis over the paracrine effect of fibroblasts. According to our previous studies, horse serum increases MuSCs differentiation, which may be related to a lower content of growth factors than in fetal bovine serum. Moreover, culturing fibroblasts in the condition of horse serum supplementation may reduce the rate of cell proliferation and division and affect cell morphology [54] (Supplementary 7), which may affect the paracrine secretory capacity of fibroblasts.

At the stage of differentiation induced by the change of medium to differentiation medium, upregulation of genes involved in myogenesis was demonstrated, such as SOCS3 (24 h differentiation and 72 h), HDAC4 (24 h differentiation and 72 h differentiation), SATB2 (24 h differentiation and 72 h differentiation), ARID5B (differentiation 24 h and 72 h), NR4A1 (24 h differentiation), SOCS3, NR4A1, and HDAC4 promote myogenic differentiation. Upregulation of SATB2 gene, a transcription factor of HDAC4, was demonstrated [55]. ARID5B regulates the differentiation process by regulating the production of prostaglandins – PGI₂, and ARID5B

knockout inhibited the differentiation and fusion process of myoblasts [56]. Moreover, expression analysis showed increasing downregulation of CRYAB with differentiation in co-culture. Overexpression of CRYAB inhibited differentiation-induced apoptosis and reduced the expression of MyoD and MyoG in the differentiation medium-deprived muscle cell culture conditions [57]. Increased expression of genes such as SOCS3, HDAC4, SATB2, ARID5B, NR4A1 or CRYAB are associated with regenerative processes and the transition of cells from the proliferation stage to the differentiation stage.

5. Conclusions

To sum up, the co-culture of muscle satellite cells with fibroblasts at the proliferation stage promotes early myogenic differentiation by upregulating the expression of genes like EGR1, IL6, SOCS3, and plenty of cytoskeleton and structural genes. At the 24 h stage of differentiation, a downregulation of transcription factors such as BHLHA15 and FOX1 controlling the transcriptional activity of MyoD and upregulation of genes such as IL6, SOCS3, EGR1, NR4A1 were observed, which influences the promotion of myogenic differentiation. However, several genes involved in differentiation, such as MYORG, TCAP showed downregulation at the 24 h and 72 h differentiation stage, which indicates that fibroblast paracrine factors can regulate the extent of myogenic differentiation, and cell culture conditions, effects like compounds of the medium may have a prevailing impact on the course of myogenesis over the paracrine secretory capacity of fibroblasts. Moreover, a higher ratio of fibroblasts due to faster proliferation rate could have a suppressive effect on terminal differentiation (from example, via TGF- β signalling).

CRediT authorship contribution statement

Karolina Zygmunt: Writing – original draft, Visualization, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Kacper Zukowski:** Writing – review & editing, Software, Methodology, Formal analysis. **Katarzyna Piórkowska:** Writing – review & editing, Supervision. **Grzegorz Smolucha:** Writing – review & editing, Formal analysis. **Alicja Wierzbicka:** Writing – review & editing, Validation. **Wojciech WitarSKI:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors have declared no conflict of interest.

Table 6
List of genes involved in proliferation during all of experiment stages.

Gene	proliferation		Differentiation 24 h		Differentiation 72 h	
	log2Fold change	P adj	log2Fold change	P adj	log2Fold change	P adj
MKI67	0.38	2.15e-08	−0.03	0.81	1.35	9.46e-44
PCNA	0.09	0.19	0.19	0.11	0.59	1.55e-10
CDK2	0.3	0.01	0.25	0.12	0.71	2.22e-06
CDK4	0.21	0.0002	−0.05	0.70	−0.07	0.39
CCND1	0.37	3.54e-11	−0.33	0.01	−0.32	5.61e-05
CCNE1	−0.28	0.05	0.11	0.68	0.41	0.03

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biomim.2025.110065>.

Data availability

The sequence data have been deposited in the Gene Expression Omnibus (GEO) under accession number GSE289644.

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Modulation of bovine muscle satellite cells myogenesis by paracrine fibroblast-derived signaling in three-dimensional spinner flask culture system

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ABSTRACT

Studies examining the paracrine effect of fibroblasts on the myogenesis of bovine muscle satellite cells (MuSCs) have confirmed their stimulating effect on proliferation and early differentiation. However, traditional two-dimensional (2D) cell culture models fail to accurately represent the complexity of *in vivo* muscle tissue. This study aims to investigate the paracrine effect of fibroblasts on myogenesis in a three-dimensional (3D) cell culture model. Cells were cultured in monoculture and co-culture with fibroblasts in a spinner flask using gelatin microcarriers, Cultishper, which maximizes the growth surface area for adherent cells. Then, muscle cells from the co-culture were sorted by the FACS method based on negative expression of CD90, a fibroblast-associated marker. The progress of myogenesis was assessed based on qPCR analysis for selected muscle markers and at the protein level for skeletal myosin. Fluorescent staining and luminescent metabolic assays were performed to control culture conditions. The obtained results revealed up-regulation of genes involved in cell activation and proliferation (*PAX7*, *MYF5*, and *MYOD*) and differentiation (*MYOG*, *EGR1*, and *MYH*). Metabolism analyses did not show changes between mono- and co-culture conditions. To summarize, fibroblasts through paracrine signaling promote early differentiation of MuSCs and potentially proliferation, which provides valuable insights for the advancement of cultured meat production.

1. Introduction

Cultured meat - a promising alternative to conventional meat husbandry - relies on the cultivation of cells with the potential for self-renewal and differentiation into muscle fibers. Cultured meat technology currently is primarily based on embryonic stem cells with the potential for myogenic differentiation, but it is also a known possibility to use postnatal muscle satellite cells (MuSCs) – precursors of myoblasts, which are located between the basement membrane of muscle fibers and the sarcolemma, remaining in a quiescent state under homeostatic conditions. Upon activation, such as in response to mechanical injury, MuSCs upregulate the expression of myogenic genes associated with self-renewal, proliferation, differentiation, and fusion of muscle fibers. As unipotent stem cells, MuSCs can differentiate into one specific cell type – myoblasts – making them a valuable research material in the context of cultured meat production (Nunes et al., 2025). Ensuring optimal culture conditions, including temperature, gas concentrations

(O₂, CO₂), sufficient aeration, low shear stress mixing, osmolality, pH, and the appropriate composition of the growth medium, which properly stimulates muscle growth, is crucial for the successful formation of muscle tissue *in vitro* in complex systems such as bioreactors. Numerous bioactive compounds, including growth factors (e.g., IGF-1, TGFβ) and hormones such as testosterone, insulin, and dexamethasone, have been shown to enhance myogenesis; however, their application is associated with high costs (Ahmad et al., 2023). In addition, functional proteins such as VEGF, FGF, along with heme proteins like myoglobin (Mb), can also modulate myogenic processes (Syverud et al., 2016; Simsa et al., 2019). One of the major directions in the development of cultured meat technology is the replacement of animal-derived components, the acquisition of which requires animal sacrifice – most notably serum, a source of growth factors and hormones. Several serum-free and animal-free media formulations have been developed, including bovine cell culture media originated by Mosa Meat (Hong et al., 2021). Instead of relying only on biological and chemical compounds such as growth

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factors, hormones, or small molecules, a promising strategy would be exploiting the potential of cells forming the niche of muscle cells, such as fibroblasts, preadipocytes, and fibroadipogenic progenitors (FAPs). Fibroblasts, connective tissue cells, play a crucial role in muscle fiber organization by synthesizing extracellular matrix (ECM) components and secreting cytokines and growth factors in response to mechanical stimuli (Hicks et al., 2014; Thummarati and Kino-oka, 2020). The potential application of fibroblasts in cultured meat technology is significant – recent studies report the possibility of transdifferentiating fibroblasts into muscle-like cells, although current methods for obtaining cells capable of myogenic differentiation are still primarily based on genetic manipulation like lentiviral transduction (Jeong et al., 2022; Zhao et al., 2025). The potential of fibroblasts can also be exploited indirectly, through their paracrine effects on muscle cells, further stimulating myogenic differentiation.

The use of two-dimensional cell cultures is associated with limited representativeness of *in vitro* conditions and simplified intercellular interactions compared to *in vivo* conditions. The monolayer formed by cultured cells does not reflect their natural growth and organization. An alternative is three-dimensional (3D) culture, which, depending on research requirements, offers significantly more opportunities to model the cellular environment. Mesenchymal stem cells (MSC) cultured as spheroids have been shown to exhibit distinct gene expression patterns associated with crucial cellular processes, as well as improved paracrine signaling (Jensen and Teng, 2020). The use of 3D cell cultures allows for more robust investigations of mechanisms such as cell viability, density, proliferation, differentiation, intercellular communication, and the expression of cell type-specific markers, both at the gene and protein levels (Antoni et al., 2015).

This study aims to assess the influence of fibroblast-derived paracrine signaling on the myogenesis of bovine MuSCs co-cultured with bovine fibroblasts on gelatin microcarriers, enabling the cultivation of attachment-dependent cells, in a spinner flask system in a 3D cell culture system. Based on previous results obtained using a 2D Transwell co-culture model, we hypothesized that fibroblasts may stimulate early myogenic differentiation by upregulating the expression of MuSCs' specific markers.

2. Materials and methods

2.1. Bovine fibroblasts and muscle satellite cell lines establishment

Fibroblasts were isolated *postmortem* from *Bos taurus* skin tissue (two individuals) using the explant culture method, as previously described (Zygmunt et al., 2025). The primary fibroblast line was purified by the MACS method for CD90 positive selection (Supplementary 1). Muscle satellite cells were obtained from the *musculus longissimus dorsi* (two individuals) using a previously described method (Zygmunt et al., 2023). For enrichment of a homogeneous MuSCs population, fluorescence-activated cell sorting (FACS) was conducted. Cell suspension was resuspended in a FACS buffer composed of Phosphate Buffer Saline (PBS, pH 7.4), 5% Fetal Bovine Serum (FBS), 0.5 µM Ethylenediaminetetraacetic acid (EDTA), and antibiotic Primocin (InvivoGen, San Diego, CA, USA). Cells were incubated on ice for 45 min with primary antibody against CD31 (1 µg/mL, #MA3100, Thermo Fisher Scientific, Waltham, NY, USA) and CD45 (1 µg/mL, #80297–1-RR100UL, Thermo Fisher Scientific) to eliminate endothelial and hematopoietic cells, respectively. Following three PBS washes, cells were incubated with fluorophore-conjugated secondary antibodies - Alexa Fluor 488 (1:200, #A2441, Thermo Fisher) and Alexa Fluor 594 (1:200, #A21201, Thermo Fisher) — for 20 min on ice. CD31/CD45 negative cells were sorted by FACS using CytoFlex SRT (Beckman Coulter, Brea, CA, USA) and seeded in cell culture flasks pre-coated with 1% gelatin in Low Glucose Dulbecco Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific) with the addition of 20% FBS (Thermo Fisher Scientific) and Primocin. A second FACS-based enrichment was

performed to isolate myogenic progenitors. Cell suspensions were labelled against CD56 (1 µg/mL, #14255–1-AP, Thermo Fisher Scientific) and CD29 (1 µg/mL, cat. #14–0299–82, Thermo Fisher Scientific), followed by incubation with fluorophore-conjugated secondary antibodies. MuSCs were defined as a CD29/CD56 positive cell population (Supplementary 2) (Xu et al., 2015).

2.2. Three-dimensional spinner flask culture system

The proliferation stage was performed in a growth medium composed of High Glucose DMEM supplemented with 20% FBS (Thermo Fisher Scientific), MEM Non-Essential Amino Acids (Thermo Fisher Scientific), 25 mM HEPES (Thermo Fisher Scientific) and Primocin. At the differentiation stage, cells were cultured in High Glucose DMEM, supplemented with 10% horse serum (Thermo Fisher Scientific), 25 mM HEPES and Primocin. Cultispher G microcarriers with standard porosity (#M9418, Sigma Aldrich) were prepared according to the manufacturer's protocol. Briefly, for preparation, microcarriers were washed twice with PBS free of calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions, then sterilized by autoclaving (121°C, 20 min). After sterilization, microcarriers were resuspended in growth medium (at a concentration of 0.01 g/mL). MuSCs and fibroblasts (in a ratio of 2:1) were seeded separately onto prepared microcarriers at a density of 7200 cells/mL (8500 cells/cm²) in non-adherent 100 mm culture dishes (total number of cells in monoculture and co-culture was equal). Before seeding, cell viability was assessed using trypan blue dye and quantified using the Countess Automated Cell Counter 3 (Thermo Fisher Scientific). Following an 18-hour attachment period, cell-seeded microcarriers were transferred to 250 mL WHEATON (CELSTIR®) spinner flasks. Agitation was initially set at 30 RPM on the 1st day, increased to 50 RPM during the proliferation stage (4 days in the growth medium), and further increased to 70 RPM during the differentiation stage (3 days in the differentiation medium). Agitation speeds were previously optimized, considering factors such as: cell type, media composition and mechanical resistance of microcarriers. A fed-batch feeding strategy was used, ensuring a continuous supply of fresh nutrients and thus exponential cell growth - 50% of the medium was exchanged every day. All experiments were performed with three biological replicates (with two technical repetitions).

For visualization, cells were stained with CellTracker CMDFDA (1:100, #C2925, Thermo Fisher Scientific). Then, cells were fixed in 4% PFA for 20 min and stained with DAPI (1:1000, #10236276001, Sigma Aldrich) for 20 min at room temperature. Microscopic observations were conducted daily using a Zeiss AX100 imager D.2 microscope (Zeiss, Germany) and Zeiss Zen 2 Blue Edition Software.

To isolate muscle cells from the co-culture with fibroblasts, negative selection for CD90, a fibroblast-associated marker, was conducted using the FACS method. Cells cultured on microcarriers were incubated in a FACS buffer with a primary antibody against CD90 (1 µg/mL, #MA135307, Thermo Fisher Scientific), for 45 min on ice, followed by three washing steps. Then, cells were incubated with Alexa Fluor 594-conjugated secondary antibody for 20 min on ice. Then, CD90-negative muscle cells were sorted by the FACS method and selected for further analysis.

2.3. Quantitative analysis of metabolic activity of cells cultured in monoculture and co-culture

Culture media samples were collected at both proliferation and differentiation stages from monoculture and co-culture conditions, and subsequently centrifuged at 1000x g to remove cells and cellular debris. Dedicated bioluminescent assays (Promega) were used to assess the metabolism of four key metabolites. Glucose metabolism was measured using the Glucose-Glo™ Assay, lactic acid using the Lactate-Glo™ Assay, and glutamine/glutamate using the Glutamine/Glutamate-Glo™ Assay. All analyses were performed according to the manufacturer's protocols.

Luminescent signals were detected and quantified using the BioTek Cytation 1 Cell Imaging Multimode Reader (Agilent Technologies).

2.4. RNA isolation and gene expression analysis

Cells were resuspended in lysis buffer, and RNA was isolated by PureLink RNA Mini Kit (Thermo Fisher Scientific) and PureLink™ DNase Set (Thermo Fisher Scientific). Quantitative and qualitative assessments of the isolated RNA were carried out using a DeNovix DS-1 Spectrophotometer (DeNovix Inc.), and the integrity of the isolated RNA was checked electrophoretically on a 1.5% agarose gel. 250 ng samples were reverse transcribed to cDNA using the High-Capacity RNA to cDNA Kit (Thermo Fisher Scientific). Relative expression levels were determined by qPCR using RT HS-PCR MIX SYBR (A&A Biotechnology). The endogenous controls were *ACTB* and *YWHAZ* genes. (Table 1).

2.5. Protein analysis

Cell lysates were prepared using RIPA Lysis and Extraction Buffer and Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific). Protein concentrations were determined by the BCA method using Micro BCA™ Protein Assay (Thermo Fisher Scientific). Plates were coated with a protein solution at a concentration of 0.1 µg/mL in 100 µl of 1 mM carbonate buffer and incubated at 4°C for 16 h. The plate was then washed 4x with Wash Solution (0.02% Triton X-100 in PBS) and incubated in a blocking buffer (1% BSA) for 1 h. Then it was incubated with the primary antibody –myosin (cat. number #PA1–28038, Thermo Fisher Scientific) for 1 h, at a concentration of 1:1000 in 0.1% BSA buffer in PBS, the plate was washed 4x with washing buffer and incubated with the secondary antibody (Donkey anti-Rabbit IgG (H+L) Secondary Antibody, HRP, Thermo Fisher Scientific) at a concentration of 1:4000 in 0.1% BSA buffer). Washed 4x and incubated with HRP substrate (Sigma) until complete color change, then the reaction was stopped with 1 N HCl (1:1 ratio). Signal detection was performed using absorbance at 410 nm using an Eppendorf Platereader AF 2200 (Eppendorf).

2.6. Statistical analyses

Statistical analyses were performed using Statistica software (Statsoft, v.13) and GraphPad program (Prism, v. 10.2.3). Normality of data distribution was evaluated by the Shapiro-Wilk test, and analyses were performed using Student's *t*-test for gene expression analysis and two-way ANOVA for metabolic analysis.

Table 1
Primer sequence of the studied genes.

Gene	Forward	T _m (°C)	Reverse	T _m (°C)
<i>EGR1</i>	5'AGCAACAGCAACAGCAGC '3	50.3	5'GGGGTAACCTCGTCTCCACTAG '3	56.3
<i>IL6</i>	5'TGAAAGCAGCAAGGAGACAC '3	51.8	5'ACTCCAGAAGACCAGCAGTG '3	53.8
<i>TCAP</i>	5'CTGGGCTGAGTGGAAGGATC '3	55.9	5'CTGCCGGTGATAGGTCTCAT '3	53.8
<i>SOC3</i> ¹	5'GGCCACTCTCCAACATCTCTGT '3	56.7	5'TCCAGGAACCTCCGAATGG '3	53.2
<i>ITGA7B</i>	5'TCAATCTGGACGTGATGGGC '3	53.8	5'AGAGACCTCCAGTGCGATTT '3	51.8
<i>TGFB1</i>	5'CCAGAGCGGACTACTACG '3	55.4	5'GCTCGGACGTGTTGAAGAAC '3	53.8
<i>ACTB</i>	5'AAGATCTGGCACCACACCTT '3	51.8	5'CTGGGTCATCTTCTCACGGT '3	53.8
<i>YWHAZ</i>	5'TCATACAAAGACAGCACGCT '3	49.7	5'TTTTCCCCTCCTTCTCTGC '3	53.8
<i>PAX7</i>	5'AAGCGGACAAGAAGGAGGAG '3	53.8	5'CGGGTCTGACTCCACATCT '3	53.8
<i>MYF5</i>	5'TGCTTAGGGAACAGGTGGAA '3	51.8	5'AACTGCTGCTCTTCTGGAC '3	51.8
<i>MYOD1</i>	5'AACACTACAGCGGCGACT '3	50.3	5'GTAGTAAGTGCAGGTCGTAGC '3	53.8
<i>MYFF6</i>	5'CCCTCAGCTACAGACCCAA '3	53.8	5'CCTTGGCAGTTATCACGAGC '3	53.8
<i>MYOG</i>	5'TCCAGTACATAGAGCGCCTG '3	53.8	5'CTATGGGAGCTGCATTCACTG '3	54.4

¹ (Wall et al., 2005)

3. Results

3.1. Fluorescence visualization of cells cultured on gelatin microcarriers

Muscle cells and fibroblasts cultured on microcarriers (Fig. 1 A) were stained with CellTracker CMDFDA (green fluorescence) and DAPI (blue fluorescence) to visualize the cytoplasm and nuclei, respectively. Microscopic observations revealed the typical morphology of adherent cells with oval-shaped nuclei and confirmed the effective attachment of cells to microcarriers (Figs. 1B, 1E, Supplementary 3). Notably, in the co-culture group following horse serum treatment during differentiation, no microcarriers aggregation or clustering was observed, in contrast to the monoculture condition, where mild microcarrier aggregation was detected (Fig. 1 C). Moreover, the ability of muscle cells to form aggregates was observed in both study groups, which may provide valuable insights for future 3D cell culture models (Fig. 1D).

3.2. Separation of muscle cells from fibroblasts in the co-culture group

For further analyses, muscle cells cultured in co-culture with fibroblasts were sorted by negative selection for CD90, a surface marker associated with fibroblasts. The representative histogram of co-culture (Fig. 2B) confirms the presence of two distinct populations of cells - CD90-negative (expected muscle cells) and CD90-positive (fibroblasts) compared to monoculture (Fig. 2A). To confirm the correctness of FACS analysis the expression of PDGFRα, a fibroblast marker, was quantified by qPCR analysis (Supplementary 4).

3.3. Quantitative analysis of metabolic activity of cells cultured in co-culture and in monoculture

Glucose consumption decreased in both monoculture and co-culture conditions following differentiation induction (Fig. 3A). Moreover, lactate secretion decreased during differentiation, indicating potential downregulation of the glycolytic pathway (Fig. 3B). Specifically, in co-culture conditions, a significantly lower lactate secretion was observed during differentiation compared to proliferation conditions ($p = 0.0113$). Similarly, glutamate secretion decreased during differentiation in both cell culture conditions. In contrast, glutamine consumption increased following induction of differentiation (Fig. 3C and D). In co-culture and monoculture conditions, significantly lower glutamate secretion was observed during differentiation compared with proliferation ($p = 0.0175$ and $p = 0.0421$, respectively). Importantly, no significant differences were observed between monoculture and co-culture in the levels of consumed or secreted metabolites at a particular stage.

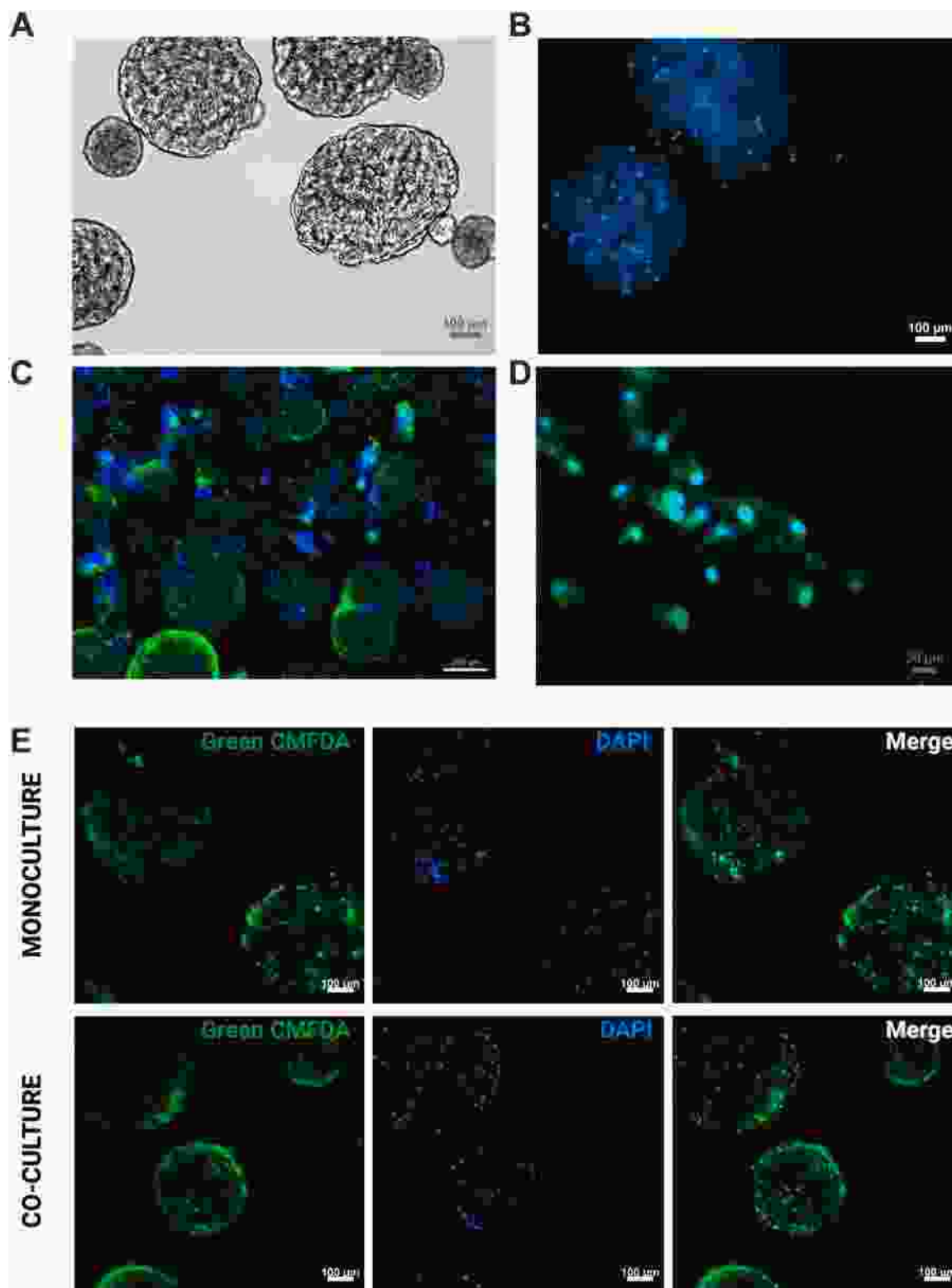


Fig. 1. (A) Cultispher G microcarriers after preparation according to the manufacturer's protocol, before seeding cells. Scale bar represents 100 µm (B) Muscle cells cultured on Cultispher G microcarriers after an 18-hour attachment period, nucleus stained with DAPI. Scale bar represents 100 µm (C) Microcarriers aggregation in the monoculture group at the 3rd day of differentiation, stained with Cell Tracker CMFDA and DAPI. Microscopic observations were conducted by BioTek Cytation 1 Cell Imaging Multimode Reader (Agilent Technologies). Scale bar represents 200 µm (D) Representative image of muscle cells, which formed aggregates on the 3rd day of differentiation. Scale bars represent 20 µm. (E). Cells cultured on Cultispher G microcarriers stained with Cell Tracker CMFDA and DAPI on the 3rd day of differentiation. Microscopic observations were conducted using a Zeiss AX100 imager D.2 microscope (Zeiss, Germany) and Zeiss Zen 2 Blue Edition Software. Scale bars represent 100 µm.

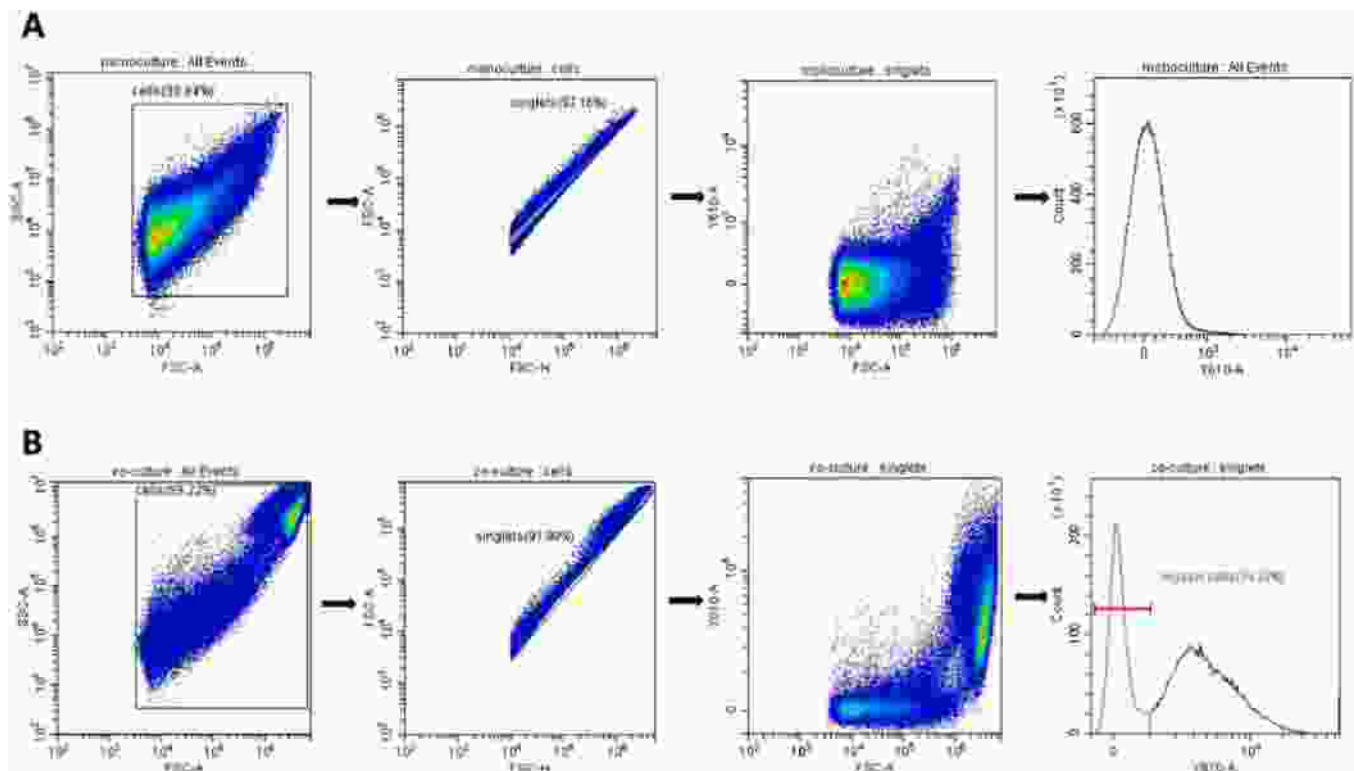


Fig. 2. Enrichment of bovine muscle cells through negative selection for CD90, a fibroblast-associated marker, using the FACS technique. (A) muscle cells mono-culture (B) muscle cells and fibroblasts co-culture. Firstly, cells were gated to exclude debris and doublet cells. Subsequently, CD90 negative muscle cells were gated for sorting for further analysis in the co-culture group. The representative histogram shows fluorescence intensity in channel Y610, on a logarithmic scale. Data were acquired using the CytoFlex SRT sorter and analyzed with CytExpert Software (Beckman).

3.4. Changes in the gene expression of muscle satellite cells markers

Significantly higher expression of *PAX7* was observed in the co-culture group ($p = 0.0160$), which is involved in maintaining the quiescent state and proliferation of muscle cells. Significantly higher expression of *Myf5*, *MyoD* and *MyoG* was observed in the co-culture group ($p = 0.0310$, $p = 0.0045$, $p = 0.0036$, respectively), genes involved in proliferation and myogenic differentiation. No statistical differences were observed in *Myf6* expression between the tested groups. Significantly higher expression of *EGR1* was observed in the co-culture group ($p = 0.0009$), which is involved in the differentiation of muscle satellite cells. No statistical differences were observed in *SOCS3* (promoting MuSCs differentiation), *IL6* (inducing myogenic differentiation by JAK2-STAT3 signalling) and *TCAP* (sarcomere protein), but the tendency to higher expression were observed in the co-culture group. No statistical differences were observed in *ITGA7* (determining the polarity of MuSCs) and *TGFβ1* (involved in TGFβ1 signaling pathway) expression between the tested groups (Fig. 4 A, 4B). Statistically higher expression of skeletal myosin ($p = 0.0019$) was observed in the co-culture group, determined by ELISA (Fig. 4 C). To confirm that the observed effects are derived from fibroblast paracrine signaling, we perform two types of validation. Firstly, MuSCs were cultured in fibroblast-derived conditioned medium, which partly recapitulated the myogenic responses observed in the co-culture group. Secondly, analysis of the expression of crucial growth factors (FN1, TGFβ1, IGF1, PDGFα, and FGF6) from fibroblasts was conducted by qPCR (Supplementary 5).

4. Discussion

Multiple studies, including two-dimensional (2D) cell culture models, have confirmed the stimulating paracrine effect of fibroblasts on myogenesis, revealing their ability to modulate myogenic

differentiation. For instance, using the transwell system, a reduction in myoblast apoptosis during differentiation was demonstrated (Zhang et al., 2010). Conditioned medium derived from fibroblast culture significantly enhanced C2C12 myotube formation, confirming the supportive role of fibroblasts' paracrine signals in myogenesis (Hinds et al., 2013). In contrast, the co-culture of C2C12 myoblasts with 3T3 fibroblasts using a microfabricated, reconfigurable device revealed the inhibitory effect of paracrine fibroblast signals on myogenic differentiation, attributed to the FGF-2 signaling pathway (Rao et al., 2013). Co-culture of Jeju Black Pig muscle cells and fibroblasts for 24 and 48 h resulted in changes in the expression of key genes from the PAX and MRF families. Specifically, MYF5 expression was upregulated, while PAX3, PAX7, MYOD, and MYOG (after 24 h) were downregulated (Siddiqui et al., 2022). In our recent studies, RNA-Seq analysis of bovine MuSCs co-cultured with fibroblasts using a transwell system revealed that fibroblasts promote early myogenic differentiation (through up-regulation genes like *EGR1*, *IL6*, *SOCS3*). Moreover, paracrine signaling of fibroblasts could modulate the extent of myogenesis, but a higher ratio of paracrine signals to muscle cells could have a suppressive effect on terminal differentiation (e.g. via TGFβ signaling). At the differentiation stage (24 h and 72 h after initiation of differentiation), downregulation of late myogenesis genes like *TCAP* or *MYOM* were observed (Zygmunt et al., 2025).

However, all the above-mentioned studies were conducted under 2D culture conditions, which are fundamentally limited in their ability to replicate the complex microenvironment conditions of *in vivo* muscle tissue. In contrast, three-dimensional (3D) cell culture systems more accurately mimic *in vivo* tissue architecture, cell morphology, and function, particularly in the context of cultured meat production technology. In the present study, gelatin-based microcarriers (Cultishper G) were used to maximize the available surface area of cell attachment. Microcarriers increase cell yield and ensure efficient gas and nutrient

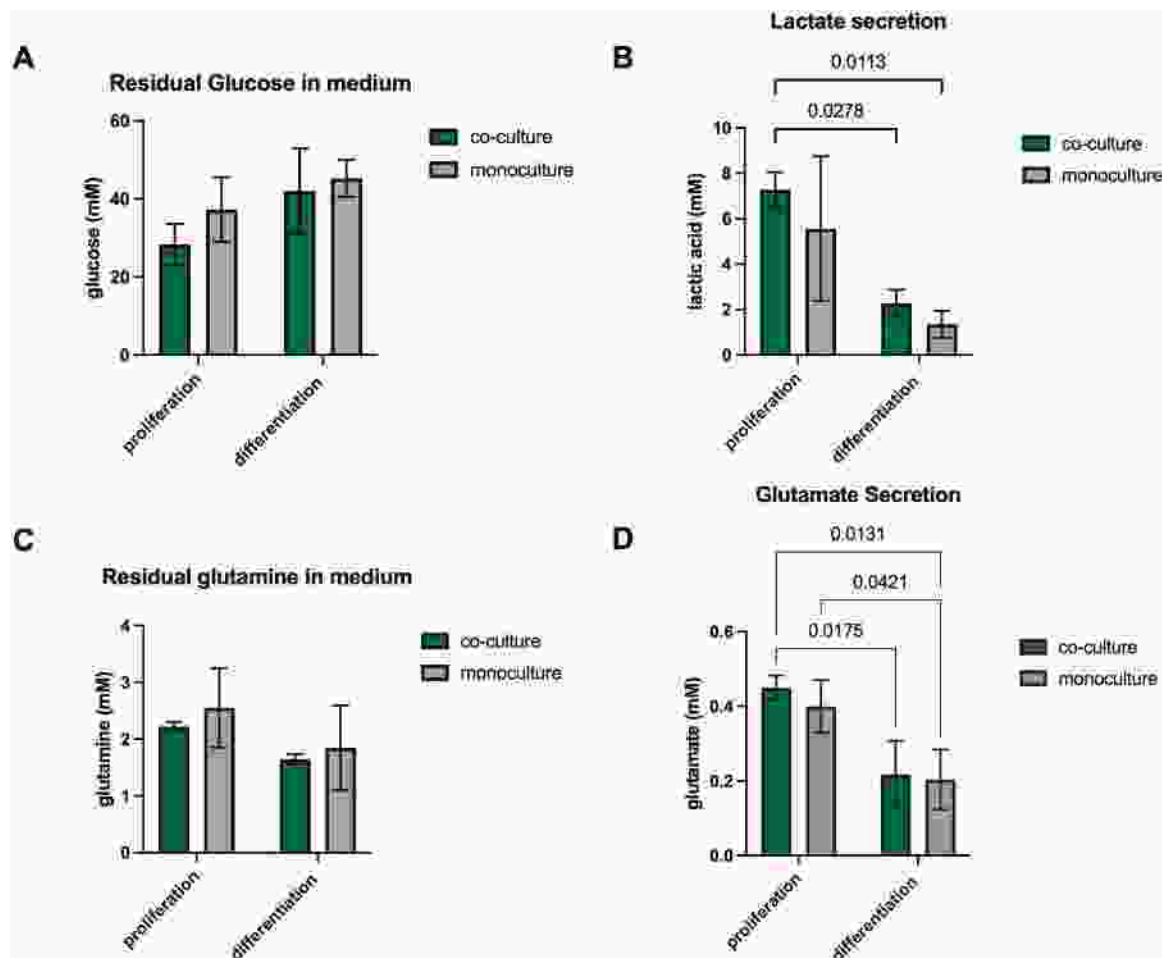


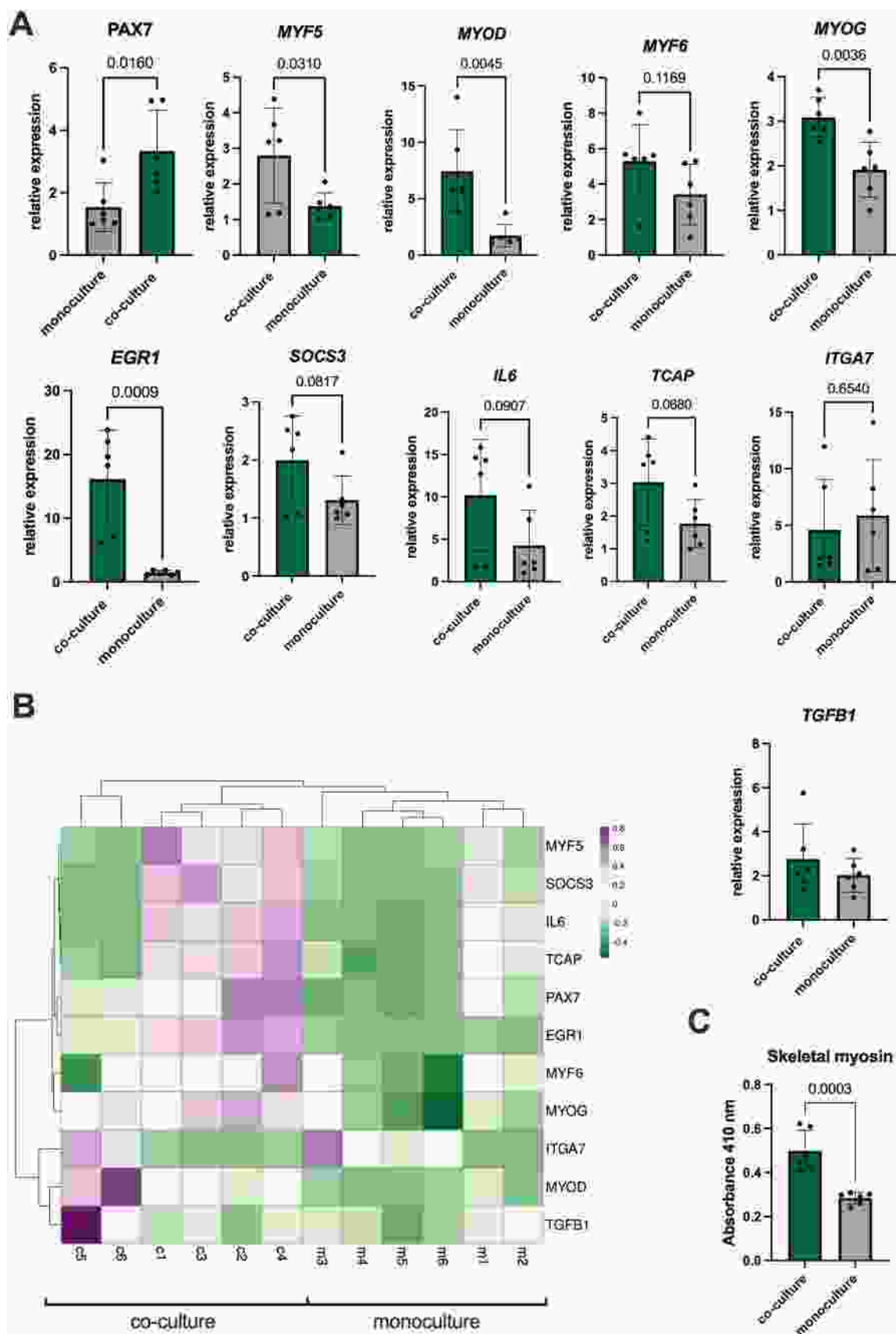
Fig. 3. Quantitative analysis of metabolic activity: (A) Residual glucose amount in medium (mM), (B) Lactate secretion (mM), (C) Residual glutamine amount in medium (mM), (D) Glutamate secretion (mM) in the co-culture of MuSCs with fibroblasts and monoculture of MuSCs ($n = 3$), measurement by luminescence-based assays (Promega) and analyzed by two-way ANOVA (Statistica software).

exchange. In consequence to the highly porous structure of microcarriers, cells settle within the pores and subsequently proliferate, effectively mimicking *in vivo* conditions and ensuring the maintenance of intercellular interactions (Yiik Swan et al., 2024). Spinner flask provides a homogenous suspension culture environment through controlled agitation, promoting equable microcarriers distribution and nutrient mass transfer (Yang et al., 2022).

Our studies on the impact of paracrine signalling from fibroblasts on myogenesis indicate significantly higher expression of myogenic genes, including those from the PAX family (PAX7), the MRF family (Myf5, MyoD, and MyoG), as well as EGR1, in muscle cells co-cultured with fibroblasts. Pax7, a paired-box transcription factor, is expressed in quiescent satellite cells and, in co-expression with Pax3, plays a critical role in promoting myoblast proliferation (Collins et al., 2009). In the G_0 phase, approximately 90% of the cells are myogenic precursor cells (Pax7⁺/Myf5⁺) and undergo symmetric division, leading to myogenic differentiation. In contrast, the remaining 10% represent myogenic stem cells (Pax7⁺/Myf5⁻), which undergo asymmetric division to self-renewal stem cell population (Shirakawa et al., 2022). Recent studies (Olguin and Olwin, 2004) suggest that increased Pax7 expression suppresses MyoD expression, thereby inhibiting myogenic differentiation. In contrast, Zammit et al (Zammit et al., 2006). revealed that Pax7 and MyoD are co-expressed in proliferating MuSCs, which is consistent with our results. Members of the MRF family, specifically Myf5 and MyoD, are crucially involved in the early stages of MuSCs myogenesis (Gayraud-Morel et al., 2007). Approximately 30% of MyoD binding sites in myoblasts overlap with Myf5 binding sites, but with different

functions – Myf5 remodels chromatin structure, whereas MyoD maintains the muscle phenotypes by initiating transcription of genes crucial for myogenic differentiation (Hernández-Hernández et al., 2017). In our studies, significantly higher expression of Myf5 and MyoD was observed, suggesting cooperation between the proliferation and activation of transcription factors crucial for myogenic differentiation in muscle cells co-cultured with fibroblasts. Moreover, activation of terminal differentiation relies on another MRF family member, such as Myf6 and MyoG (Hernández-Hernández et al., 2017). Importantly, MyoG plays a key role in regulating cell cycle exit. EGR1 promotes myogenic differentiation by positively regulating MyoG expression, likely through a direct interaction with one predicted binding site in the MyoG promoter region. Overexpression of EGR1 has been shown to enhance myotube formation, fusion, and elongation. Moreover, during the differentiation process, EGR1 translocates to the nucleus, depending on serine phosphorylation at position 533 in the C-terminal region (Zhang et al., 2018). Our results show significantly increased EGR1 expression in co-cultures (with simultaneous higher MyoG expression), which contributes to the enhanced myogenic differentiation.

However, in the 3D cell culture, no significant differences in the expression of IL6, SOCS3, and TCAP were observed, although a tendency toward higher expression in co-culture was observed. Interleukin-6 (IL-6), a myokine secreted by skeletal muscle during physical activity, has been indicated as a critical regulator of myogenic differentiation and myotube formation. *In vitro* studies using C2C12 myoblasts have demonstrated that IL-6 expression increases during differentiation, and genetic ablation resulted in abnormal expression of myogenic markers



(Hoene et al., 2013). SOCS3 (suppressor of cytokine signaling 3) is the most important regulator of the JAK1/STAT3 signaling pathway, essential for myogenic differentiation and inflammatory response. Its expression is noted in mature muscle fibers, as well as in muscle stem cells (Swiderski et al., 2016). Notably, overexpression of SOCS3 induced significantly higher expression of alpha-actin, myogenin, and MHC, which are essential for muscle cell differentiation (Caldow et al., 2011). TCAP (telethonin) is a Z-disc-associated protein integral to the structure of mature sarcomeres. It is expressed during myogenic differentiation, and its activation is dependent on MyoD and MyoG (Zhang et al., 2011).

Comparing the 2D and 3D culture results, a key conclusion is that fibroblasts, through paracrine signaling, promote the early differentiation of MuSCs. This is evidenced by the upregulation of key myogenic markers, such as *MYOG* and *EGR1*, observed across both culture systems, as well as *IL6* and *SOCS3* in 2D cell culture conditions, accompanied by the concomitant downregulation of terminal differentiation genes. Notably, in 2D cultures, mainly changes in marker expression were observed in proliferative conditions. Following the shift to differentiation medium, this support effect was reduced, with the simultaneous upregulation of the TGF- β 1 signaling pathway (Zygmunt et al., 2025).

Myosin is a contractile protein specific to skeletal muscle in adults, and four isoforms of myosin heavy chain (MyHC) are classified: Myh1, Myh2, and Myh4 expressed in fast-twitch muscle fibers – type II (IIX, IIA, and IIB, respectively) and Myh7 expressed in slow-twitch muscle fibers (type I) (Ojima et al., 2022). Analysis of skeletal muscle expression at the protein level in our study showed a significantly increased expression of myosin in the co-culture group ($p = 0.0019$), indicating enhanced myogenic differentiation of muscle cells co-cultured with fibroblasts.

Moreover, as the 3D spinner flask system and gelatin microcarriers significantly maximize the available surface area for high-density cell growth, it was essential to monitor cellular metabolism through the analysis of four key metabolites: glucose, lactate, glutamine, and glutamate. Reduced glucose consumption during the differentiation stage was observed in both study groups, along with a concomitant reduction in lactate secretion. It was proven that proliferating MuSCs increase glycolysis but differentiating MuSCs increase glucose utilization for mitochondrial respiration (oxidative metabolism) (Yucel et al., 2019). Furthermore, a decrease in extracellular glutamate levels was observed, alongside a concomitant increase in glutamine consumption during differentiation. This metabolic profile is likely associated with reduced glutamine anaplerosis in the TCA cycle and decreased activity of mitochondrial GLUD1 during differentiation (Soro-Arnáiz et al., 2024). Moreover, glutamine supports the progression of myogenesis by promoting myotube formation and upregulating the expression of MyoG and MYH1 – the crucial markers of myogenic differentiation (Takatoya et al., 2025).

5. Conclusion

In summary, the co-culture of muscle satellite cells (MuSCs) with fibroblasts under three-dimensional culture conditions confirmed our previous observations from the 2D system that fibroblasts stimulate myogenesis, which was observed by up-regulation of genes involved in proliferation (*PAX7*, *MYF5*, *MYOD*) and early differentiation (*MYOG*, *EGR1*). Nevertheless, markers of mature skeletal muscle like *TCAP*, *SOCS3*, or *IL6* did not reveal significant changes in expression. These results indicate that paracrine signals from fibroblasts can support myogenesis, providing valuable insights for optimizing the microenvironmental conditions for efficient *in vitro* muscle formation, towards cultured meat production.

CRediT authorship contribution statement

Karolina Zygmunt: Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration,

Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Katarzyna Piórkowska:** Writing – review & editing, Supervision. **Julia Adamiak:** Writing – review & editing, Formal analysis. **Wojciech Witarowski:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors have declared no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biocel.2026.106970.

Data availability

Data will be made available on request.

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opracowaniu założeń koncepcyjnych i metodologii, analizach z zakresu hodowli komórkowych, biologii molekularnej i proteomiki, walidacji metod, analizie statystycznej wyników, wizualizacji i gromadzenia danych, przygotowaniu oryginalnej wersji manuskryptu.

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Opracowaniu założeń koncepcyjnych i metodologii, pozyskaniu funduszy na badania, kierowaniu projektem badawczym, zapewnieniu narzędzi analitycznych, opiece merytorycznej oraz korekcie i edycji manuskryptu.



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