Effect of Porcine Skeletal Muscle Satellite Cells on Lipid Deposition in Intramuscular Preadipocytes in a Co-culture System: Postprint

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Abstract

This study aimed to investigate the effect of porcine skeletal muscle satellite cells on lipid deposition in intramuscular preadipocytes within a co-culture system. Porcine skeletal muscle satellite cells and intramuscular preadipocytes were isolated, cultured, and characterized. Following identification, the two cell types were seeded onto Transwell cell culture inserts for co-culture, and induced to differentiate separately once cell density exceeded 90%. After 8 days of inducing differentiation in skeletal muscle satellite cells, the differentiation level of intramuscular preadipocytes, expression of differentiation marker genes, and expression of key enzymes in lipid metabolism were examined. The results showed that, compared with intramuscular preadipocytes cultured alone, the number and area of lipid droplets in intramuscular preadipocytes within the co-culture system were extremely significantly reduced ($P<0.01$). Additionally, the gene and protein expression levels of peroxisome proliferator-activated receptor and CCAAT/enhancer-binding protein—transcription factors regulating preadipocyte proliferation and differentiation—were extremely significantly decreased ($P<0.01$), as were the gene and protein expression levels of acetyl-CoA carboxylase and fatty acid synthase in intramuscular preadipocytes ($P<0.01$).

Full Text

Abstract

This study investigated the effects of porcine skeletal muscle satellite cells on lipid deposition in intramuscular preadipocytes within a co-culture system. Porcine skeletal muscle satellite cells and intramuscular preadipocytes were isolated and cultured. Following isolation, culture, and identification, the two cell types were co-cultured in Transwell plates and induced to differentiate

once cell density exceeded 90%. After 8 days of skeletal muscle satellite cell differentiation, the differentiation level of intramuscular preadipocytes, expression of differentiation marker genes, and key enzymes in lipid metabolism were examined. The results demonstrated that compared with intramuscular preadipocytes cultured alone, the number and area of lipid droplets in intramuscular preadipocytes within the co-culture system were significantly reduced (P<0.01). The gene and protein expression levels of peroxisome proliferator-activated receptor (PPAR) and CCAAT/enhancer-binding protein (C/EBP), key transcription factors for preadipocyte proliferation and differentiation, were also significantly decreased (P<0.01). Additionally, the gene and protein expression levels of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in intramuscular preadipocytes were significantly downregulated (P<0.01). These findings indicate that porcine skeletal muscle satellite cells exert an inhibitory effect on lipid deposition in intramuscular preadipocytes within the co-culture system.

Keywords: porcine skeletal muscle satellite cells; intramuscular preadipocytes; co-culture; lipid deposition

Introduction

Vertebrate muscle and adipocytes originate from a common mesodermal lineage and maintain close regulatory connections through autocrine and paracrine signaling during animal growth and development [1-2]. Investigating the interactions between adipose and muscle tissues helps elucidate the mechanisms by which cytokines, nutritional factors, and hormones regulate fat deposition and muscle development. Furthermore, understanding adipose-muscle crosstalk has broad applications in human metabolic disease mechanisms and animal meat quality traits.

To explore the mutual influences between adipose and muscle tissues during organismal development, researchers have established various cell culture systems. Dodson et al. [3] first developed an in vitro co-culture system combining 3T3-L1 preadipocytes with ovine muscle cells at different differentiation stages. Co-culture systems better simulate the physiological environment in vivo compared with monoculture, garnering widespread attention from scholars. In recent years, investigators have established adipose-muscle co-culture systems in sheep, humans, mice, swans, and cattle [4-8]. This study isolated and cultured porcine skeletal muscle satellite cells and intramuscular preadipocytes to establish an indirect co-culture system in vitro, examining the proliferation and differentiation characteristics of both cell types and investigating the regulatory effects of porcine muscle cell differentiation on adipocyte differentiation and intracellular lipid deposition. The findings provide a theoretical basis for further revealing the interactions between adipose and muscle tissues.

Materials and Methods

1.1 Experimental Materials

Reagents: 0.25% trypsin solution, 0.02% ethylenediaminetetraacetic acid (EDTA) (used at 1:1 ratio, Sigma-Aldrich, USA), trypan blue, 0.2% collagenase, cell cryopreservation solution, DMEM/F12 medium (Gibco, USA), fetal bovine serum (Gibco, USA), trypsin, phosphate-buffered saline (PBS) (powder, Beijing Solarbio Science & Technology), insulin, dexamethasone, premium horse serum (Gibco, USA), penicillin-streptomycin solution (100×, Beijing Solarbio Science & Technology), 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich, USA), Oil Red O, Taco™ DNA/RNA extraction kit (Ruiji Marine Biotechnology), PrimeScript™ II 1st Strand cDNA Synthesis Kit (TaKaRa, Japan), All-in-One™ qPCR Mix (GeneCopoeia, USA), and TGX Stain-Free™ FastCast™ Acrylamide Kit (12%, Bio-Rad, USA).

Equipment: Sterile culture dishes, CO cell incubator, inverted fluorescence microscope, Transwell cell co-culture plates, SW-CJ-1D superclean bench, high-speed centrifuge, DT5-2 low-speed centrifuge, and real-time quantitative PCR instrument.

1.3.1 Isolation and Culture of Porcine Skeletal Muscle Satellite Cells and Intramuscular Preadipocytes

Three-day-old healthy Large White piglets were obtained from Tianjin Wuqing Nongkang Pig Farm for primary cell isolation. Under aseptic conditions, subcutaneous longissimus dorsi muscle was dissected from piglets. Connective tissue and blood vessels were removed, and the muscle was minced into 1-2 mm^3 fragments. The tissue was digested with 0.2% type II collagenase at 37°C for 2 hours, filtered through a 200-mesh sieve, and centrifuged at 1,500 rpm for 5 minutes. The supernatant was discarded, and the pellet was washed with DMEM/F12 medium, filtered through a 400-mesh sieve, and centrifuged at 1,500 rpm for 10 minutes. The resulting pellet was resuspended in DMEM/F12 complete medium containing 10% fetal bovine serum and 1% penicillin-streptomycin, then seeded into 10 cm sterile culture dishes and incubated at 37°C with 5% CO . Preadipocytes were purified using differential adhesion methods: after 2 hours of culture, the medium was removed and adherent cells were washed twice with PBS to obtain purified intramuscular preadipocytes [9]. Skeletal muscle satellite cell isolation followed the protocols described by Yang et al. [10] and Yan et al. [11]. Both cell types were seeded into separate 10 cm sterile culture dishes and maintained at 37°C with 5% CO , with medium changes every 2 days. Cell numbers were determined using a hemocytometer.

1.3.2 Cell Identification

Skeletal muscle satellite cell identification: Sterile coverslips were placed in culture dishes for cell climbing. When cells reached 70-80% confluence, differentiation medium (2% horse serum + 1% penicillin-streptomycin +

DMEM/F12) was applied for 3 days. The differentiation medium was removed, cells were washed three times with PBS, and fixed with acetone at room temperature for 20 minutes. After three PBS washes, cells were treated with Triton-X 100 (1:1,000) for 15 minutes, washed three times with PBS, and blocked with bovine serum albumin (BSA) for 2 hours. Desmin primary antibody (1:50) was added and incubated overnight at 4°C. Following three PBS washes, fluorescently labeled secondary antibody (1:2,000) was added and incubated in the dark for 1 hour. After three PBS washes, nuclei were stained with 1 g/mL 4' ,6-diamidino-2-phenylindole (DAPI) for 10 minutes (protected from light), washed three times with PBS, mounted, and observed under a fluorescence microscope.

Intramuscular preadipocyte identification: When cells in the culture dish reached full confluence and contact inhibition was maintained for 2 days, differentiation was induced using complete medium containing 10 g/mL insulin for 2 days. The medium was then replaced with complete medium for an additional 4 days of culture. When numerous lipid droplet-like round cells were observed under an inverted microscope, Oil Red O staining was performed to confirm intramuscular preadipocyte identity [12].

1.3.3 Co-Culture Setup

When the two primary cell cultures reached 80-90% confluence, they were digested with 0.25% trypsin at 37°C and resuspended in DMEM/F12 medium. After counting, cells were seeded into Transwell co-culture plates. Intramuscular preadipocytes were seeded in the upper chamber at a density of 2.0×10 cells/cm², while skeletal muscle satellite cells were seeded in the lower chamber at 1.0×10 cells/cm², using complete medium containing 4% fetal bovine serum. When skeletal muscle satellite cells reached 80% confluence, the medium was replaced with DMEM/F12 containing 2% horse serum to induce differentiation, with medium changes every 48 hours. After intramuscular preadipocytes underwent 2 days of contact inhibition, differentiation was induced using complete medium containing 10 g/mL insulin for 2 days, followed by complete medium until visible lipid droplets appeared.

1.3.4 Real-Time Quantitative PCR

Total RNA was extracted using the TaKaRa RNA kit according to the manufacturer' s protocol. First-strand cDNA synthesis was performed using a kit from Fermentas (USA). Gene-specific primers were as follows: PPAR (5 -ACCACTCGCATTCCTTTGAC-3 , 5 -CCACAGACTCGGCACTCAAT-3), C/EBP (5 -ATGGAGCAAGCCA ACTTCTAC-3 , 5 -GCCAGGAACTCGTCGTTGAA-3), ACC (5 -CTCCTAACTGCTGAGCTGTCTCTCT-3 , 5 -AGTCTTTCTCTTCAATTCTTGCCT-3), FAS (5 -AAGGAGGAGTCAACGGG-3 , 5 -GATGGTGAGGAGTCGGAT-3), LPL (5 -CGAAGTATTGGCATCCAGAAAC-3 , 5 -TTGATCTCATAGCCCAAGTTGTT-3), and -actin (5 -ACCACAGCCGAGAGAGAAAT-3 , 5 -GACCTGACCATCAGGGAGTT-3). All primers were synthesized by Shanghai Bioengineering Technology

Service. PCR amplification conditions were: 95°C for 5 minutes, followed by 30 cycles of 94°C for 60 seconds, 55-60°C for 40 seconds, and 72°C for 40 seconds, with a final extension at 72°C for 10 minutes and cooling to 4°C. Melting curves were generated, and relative quantification was performed using the 2^(-ΔΔCt) method.

1.3.5 Western Blot Analysis

Cultured cells were lysed on ice using lysis buffer and centrifuged at 10,000×g for 10 minutes at 4°C. Supernatant protein concentrations were measured. Total proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with primary antibodies against -actin, C/EBP , PPAR , FAS, and ACC (Abcam, UK), followed by blocking with 5% skim milk for 1 hour. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at 37°C for 1 hour. Protein bands were detected using ChemiDoc XRS chemiluminescent reagent (Millipore, USA) and a Bio-Rad gel imaging system. Gray values were analyzed for relative quantification.

1.4 Statistical Analysis

Data were analyzed using SPSS 17.0 software. Significant differences were determined by one-way ANOVA, with $P<0.05$ considered significant and $P<0.01$ considered highly significant. Results are presented as mean ± standard deviation (mean±SD).

Results

2.1 Cell Morphology and Identification

2.1.1 Morphology and Identification of Skeletal Muscle Satellite Cells

Under an inverted microscope, primary satellite cells gradually formed spindle or fusiform shapes after adherence (Figure 1 [Figure 1: see original paper]-A). Upon mutual fusion, cells arranged in an orderly orientation (Figure 1-B). After 2 days of differentiation, multinucleated myotubes formed with centrally located nuclei, appearing as elongated parallel structures (Figure 1-C). Immunofluorescence identification using the specific porcine skeletal muscle satellite cell marker Desmin revealed positive cytoplasmic expression (Figure 1-D), confirming the cultured cells as porcine skeletal muscle satellite cells (Figure 1-E, Figure 1-F).

Figure 1. Culture and identification of porcine skeletal muscle satellite cells (100×). A, B, C: Porcine skeletal muscle satellite cell culture; D: Desmin immunofluorescence staining in cytoplasm; E: DAPI staining; F: Merge of D and E.

2.1.2 Morphology and Identification of Intramuscular Preadipocytes

Intramuscular preadipocytes grew adherently. After 2 days of contact inhibition (Figure 2 [Figure 2: see original paper]-A), differentiation was induced with induction solution I for 48 hours, causing cells to gradually become round (Figure 2-B). Subsequent induction with solution II for 48 hours, followed by 4 days in complete medium containing 10% fetal bovine serum, resulted in numerous lipid droplet-like round cells. Oil Red O staining confirmed abundant intracellular lipid droplets (Figure 2-C), verifying the cells as intramuscular preadipocytes.

Figure 2. Morphology and identification of porcine intramuscular preadipocytes (100×).

2.2 Effects of Skeletal Muscle Satellite Cells on Lipid Deposition in Intramuscular Preadipocytes

On day 8 of intramuscular preadipocyte differentiation, numerous lipid droplets appeared within cells. Oil Red O staining revealed abundant, large lipid droplets in control group cells (Figure 3 [Figure 3: see original paper]-A), whereas co-cultured cells showed fewer lipid droplets with markedly lighter and duller staining (Figure 3-B). Quantitative analysis demonstrated that both lipid droplet number and area were significantly lower in the co-culture group compared with the control group ($P<0.01$) (Figure 3-C, Figure 3-D). These results indicate that co-culture with skeletal muscle satellite cells inhibits intramuscular preadipocyte differentiation and lipid deposition.

Figure 3. Effects of skeletal muscle satellite cells on lipid deposition in intramuscular preadipocytes (100×). A: Control group; B: Co-culture group; C: Lipid droplet number; D: Lipid droplet area. ** indicates highly significant difference ($P<0.01$).

2.3 Effects of Skeletal Muscle Satellite Cells on Proliferation and Differentiation Transcription Factor Expression

To verify the effects of skeletal muscle satellite cells on intramuscular preadipocyte proliferation and differentiation, expression levels of the transcription factors C/EBP and PPAR were examined. As shown in Figure 4 [Figure 4: see original paper], both gene and protein expression levels of PPAR and C/EBP were significantly lower in co-cultured intramuscular preadipocytes compared with the control group ($P<0.01$). These findings demonstrate that skeletal muscle satellite cells suppress the expression of adipocyte differentiation marker genes.

Figure 4. Effects of skeletal muscle satellite cells on proliferation and differentiation transcription factor expression in intramuscular preadipocytes. A: Expression levels of intramuscular preadipocyte proliferation and differentiation marker genes; B: Expression levels of related proteins. PPAR : peroxisome proliferator-activated receptor ; C/EBP : CCAAT/enhancer-binding protein ; -actin: -actin.

2.4 Effects of Skeletal Muscle Satellite Cells on Key Lipid Metabolism Enzyme Expression

To further investigate the effects of porcine skeletal muscle satellite cells on lipid metabolism in intramuscular preadipocytes, expression levels of key enzymes FAS, ACC, and LPL were examined. As shown in Figure 5 [Figure 5: see original paper], gene and protein expression levels of FAS and ACC were significantly lower in co-cultured intramuscular preadipocytes ($P<0.01$), while LPL expression was significantly higher ($P<0.01$). These results indicate that skeletal muscle satellite cells inhibit lipid metabolism in intramuscular preadipocytes while promoting lipolysis.

Figure 5. Effects of skeletal muscle satellite cells on key lipid metabolism enzyme expression in intramuscular preadipocytes. A: Expression of lipid metabolism genes in intramuscular preadipocytes; B: Expression of related proteins. FAS: fatty acid synthase; ACC: acetyl-CoA carboxylase; LPL: lipoprotein lipase; -actin: -actin.

Discussion

Co-culture methods for muscle and adipose cells have been widely used to study cell growth characteristics and secretory functions. In vitro co-culture of skeletal muscle satellite cells and intramuscular preadipocytes provides an important model for investigating interactions between muscle and adipose tissues. Ailhaud et al. [13] reported that muscle cells and preadipocytes maintain close contact during embryonic development. Hausman et al. [6] first attempted direct mixed culture of intramuscular preadipocytes and skeletal muscle satellite cells. Although this approach approximates the in vivo environment, it yields few adipocytes and contaminates the culture with other cell types, making it unsuitable for quantitative and qualitative analysis. Dodson et al. [3-4] demonstrated that co-culture of 3T3-L1 adipocytes and muscle cells promotes adipocyte survival, growth, and proliferation while inhibiting differentiation. Yan et al. [11] examined direct co-culture of porcine preadipocytes and muscle satellite cells, finding that the co-culture system promoted adipocyte growth and proliferation while suppressing differentiation.

The present study investigated the differentiation characteristics of intramuscular preadipocytes in an indirect co-culture system with porcine skeletal muscle satellite cells. Compared with monoculture, intramuscular preadipocytes in co-culture showed fewer and smaller lipid droplets, with quantitative analysis revealing highly significant reductions in both lipid droplet number and area. These findings indicate that muscle cells inhibit intramuscular preadipocyte differentiation and fat deposition, consistent with the results of Yan et al. [11]. Previous studies have shown that FAS is a key enzyme converting acetyl-CoA and malonyl-CoA to triglycerides, regulating long-chain fatty acid synthesis in mammals. ACC is a critical enzyme in lipid metabolism, while LPL plays a key role in triglyceride catabolism [14]. PPAR and C/EBP are highly expressed in

adipose tissue and play important roles in adipogenesis and lipid storage [8,15-16]. This study found that co-culture significantly reduced the gene and protein expression of PPAR and C/EBP in porcine intramuscular preadipocytes, significantly decreased FAS and ACC expression, and significantly increased LPL expression. These results demonstrate that porcine skeletal muscle satellite cells in the co-culture system inhibit intramuscular preadipocyte differentiation while promoting lipolysis, confirming the important regulatory role of muscle cells in adipocyte development [17-18].

Conclusion

This study successfully isolated and cultured porcine skeletal muscle satellite cells and intramuscular preadipocytes, establishing an indirect co-culture system. The results confirmed that skeletal muscle satellite cells in the co-culture system inhibit lipid deposition in intramuscular preadipocytes.

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Note: Figure translations are in progress. See original paper for figures.