CORRELATION ANALYSIS OF SKELETAL MUSCLE REGENERATION AND DIFFERENTIATION OF SATELLITE CELLS

Hongyi Yuan[1], Hairong Du[1], Zi'ao Yuan[1], Xiaoliang Zhi[1], Minghai Zhang[1*], Weijun Guan[2]
[1]College of Wildlife and Protected Area, Northeast Forestry University, Harbin, 150040, China - [2]Institute of Animal Sciences of Chinese Academy of Agricultural Sciences, Beijing, 100193, China

ABSTRACT

Objective*: To establish a method for isolation, culture and identification of skeletal muscle satellite cells (MuSCs) in vitro, that play the main role in skeletal muscle generation after birth, and to investigate its biological characteristics.*

Methods*: In this study, chest muscles of 18-day-old Peking duck embryos were selected as the re-search material, the muscle were cut into minced meat under sterile conditions, by enzyme digestion using 0.1% collagenase I and 0.25% trypsin. After digestion,filtration and resuspended, the isolated cells were cultured in 37°C with 5% CO_2. Skeletal MuSCs were confirmed by immunofluorescence and the RT-PCR of specific markers Pax7, Myf5 and MyoD,*

Result*: High purity satellite cells were obtained by two-step enzyme digestion,the growth kinetics of various MuSC passages were assessed and growth curves were typically sigmoidal. Pax7,Myf5 and MyoD were positive by immunofluorescence and RT-PCR. Differentiation into adipocytes was assessed by oil red O staining, and differentiation into osteoblasts was evaluted by alizarin red staining. Moreover, long multicore muscle fibers and beating cardiomyogenic cells were detected after induction of myogenic differentiation.*

Conclusion*: This study confirmed the multi potent differentiation potential of skeletal muscle satellite cells, which can provide basic data for stem cell research.*

Keywords*: Peking duck, Biological characteristic, Directed differentiation in vitro, Isolation, Skeletal muscle satellite cell.*

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Introduction

Stem cells are rare and specialized cells found in fully differentiated tissue that are essential for organ repair and renewal. Skeletal muscle provides an excellent example of this type of restoration. Its stem cells, which are also called satellite cells, sustain the regeneration of muscles throughout life. The regeneration of skeletal muscle depends mainly on muscle satellite cells (MuSCs).

Skeletal muscle is made up of long polykaryocytes and muscle fibers. It is the main effector of force production and participates in the regulation of metabolism throughout the body[(1)]. MuSCs are typically located between basement and muscle fibrous membranes[(2)]. The first electron microscopy images of frog satellite cells were obtained by Mauro et al. in 1961. Shorthly afterward MuSCs were found in other vertebrates and thought to be the source of myoblasts that could repair muscle fibers[(3)]. Satellite cells have recently been redefined as muscle-derived stem cells that not only have the ability to produce differentiated progeny and self-renew[(4, 5)].

Pivotal aspects of satellite cell biology are found in single-fiber cultures. This includes their self-renewing properties and strong muscle-growing potential[6, 7].

In the 19th century, researchers showed that muscle has a regenerative capacity. From the mid-20th century onward, the underlying cellular mechanisms were elucidated[8, 9]. Flow cytometry was used to analyze the muscle cell population. Highly overlapping populations of mouse MuSCs have been isolated by fluorescence-activated cell sorting. The results showed that 90%-93% of all Pax7-ZsGreen-positive cells were labeled by each of the surface marker schemes.

MuSCs have been isolated and purified by Percoll density gradient centrifugation to analyze cell functions. Hinken and Billin used a technique for prospective isolation of MuSCs by phenotypic screens for modulators of proliferation. This technique increased the purity of MuSCs by up to 98%. In recent years, the efficient and rapid isolation and culture of high purity skeletal muscle satellite cells have been intensively studied. In vitro isolation and culture of skeletal MuSCs have been achieved in chicken[10], mouse[11], sheep[12] and other animals. Similar studies on Peking duck have not been reported.

Previous studies have mainly focused on muscle fiber repair, the self-renewing properties of MuSCs, and the developmental potential of skeletal MuSCs in various organisms. However, to our knowledge there is no related re-search on skeletal MuSCs of Peking duck. Skeletal MuSCs from Peking duck may be helpful to reveal gene expression patterns, regulation of muscle growth and development, and repair mechanisms after muscle injury.

Experimental Detail

Experimental animals

Animal experiments were performed following the guidelines established by the Animal Protection and Utilization Committee of the Chinese Academy of Agricultural Sciences. Peking ducks were supplied by the Chinese Academy of Agricultural Sciences.

Skeletal muscle satellite cell isolation and culture

Chest muscle tissues were excised from 18-day-old Peking duck embryos and washed at least five times with phosphate buffered saline (PBS). Ophthalmic scissors were used to cut the tissues into $1mm^3$ pieces. The pulverized muscle tissues were incubated with 0.1% collagenase I (Gibco, USA) for about 30 min, followed by 0.25% trypsin (Gibco) for 1 h with rocking every 5 min at 37°C[10]. The digestion was terminated by the addition of Dulbecco's modified Eagle's medium (DMEM; Gibco) containing fetal bovine serum (FBS; Gibco). The digested muscle tissue was passed through a 100-μm mesh filter. A single cell suspension was obtained by passing the recovered filtrate through a 300-μm mesh filter, and the cells were centrifuged at room temperature for 8 min at 1200 r/min. The cell pellet was resuspended in DMEM/F12 containing 15% FBS[13]. The cell suspension was seeded in a culture flask for differential adherence at 37°C with 5% CO_2 for 2 h. The suspended cells were transferred to a new culture flask coated with gelatin for further culture. The cells typically attached completely after 72 h. On the fourth day, fresh culture medium was added and changed every 2-3 days. When the cell number reached 80% confluency, cells they were washed with PBS three times and then passaged at a split ratio of 1:2 using 0.25% trypsin and 0.01% Ethylene Diamine Tetraacetate Acid (EDTA)[14]. The digestion was terminated by adding DMEM/F12 containing 15% FBS and 2.5 ng/mL basic fibroblast growth factor. The isolated cells were typically pure after three or four passages[15].

Kinetics of cell growth

Passage 2, 4 and 8 cells were used to analyze MuSC growth kinetics. The cells were collected by incubation with 0.25% trypsin and seeded in 24-well plates at 1×10^4 cells/well. Three wells were randomly selected to count cells every day for 7 days[16]. The counting was repeated three times per well. The average cell count and population doubling time were calculated.

Identification of MuSCs

Surface marker detection

Passage 3 cells were fixed at room temperature with 4% (m/v) paraformaldehyde and washed for 15 min with ice-cold PBS three times. The cells were then permeabilized for 10-15 min with 0.25% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) and washed with PBS three times. The cells were blocked for 30 min with 10% normal goat serum (Zhongshan Goldenbridge, Beijing, China). The cells were subsequently incubated with mouse antichicken myogenic differentiation antigen (MyoD; diluted 1:100 in 1% bovine serum albumin (BSA); Abcam, Cambridge, UK), mouse antichicken myo-

genic regulatory factor 5 (Myf5; 1:100; Abcam), or mouse an-ti-chicken Paired Box 7 (Pax7; 1:100; Abcam) antibodies at 4°C overnight[6]. The cells were washed with PBS three times for 5 min each wash. The cells were then incubated with the secondary antibody (rabbit antimouse IgGs,1:100; Abcam) at 37°C for 1 h in the dark. The cells were subsequently washed with PBS three times for 5 min for each wash in the dark. Finally, the cells were incubated for 15-20 min with 1 μg/mL DAPI and then washed with PBS three times. Images were obtained under a laser scanning confocal microscope (TE-2000-E Nikon, Tokyo, Japan)[17].

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated by Trizol (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized with a reverse transcription kit (Takara, Osaka, Japan) and amplified with specific primers by PCR (Table 1). Primers were designed using Primer Premier 5.0 software and synthe-sized by Sangon Biotech (Shanghai, China).PCR products were observed after 2% agarose gel electrophoresis.

Gene Name	Primer sequences	Circles	Product length (bp)	Tm (°C)
PAX7	F:5'- GCTGGATGAAGGCTCTGATG-3' R:5'-TTGCTGAACCACACCTGAACT-3'	30	207	59
MyoD	F:5'-GCAACCCTATCTACCAAGTGCT-3' R:5'-TCCTATTCTCCAAAGCCCAGA-3'	30	293	59
Myf5	F:5'-AAGGCTCGGATGAAGAGGAG-3' R:5'-GATCTCCACCTTGGGCAGT-3'	30	230	60
MHC	F:5'-TGATGAAGAGGTCCAACCGC-3' R:5'-GTAGAAGCCGTTGTCTGCCT-3'	30	221	60
Desmin	F:5'-CCACCAGATCCAGTCCTACAC-3' R:5'-CTCTACATCCAGGGCCATCTT-3'	30	229	61
RunX2	F:5'-ACAGCCATCTCTCCGTCATC-3' R:5'-GTCCCTTTCCTCCAAACACC-3'	30	201	60
Osteocalcin	F:5'-GGACACCCTAAACTCCATAGCA-3' R:5'-GTCCCTTTCCTCCAAACACC-3'	30	206	60
PPARγ	F:5'- CTCAGGCTACCATTACGGAGT-3' R:5'-TCAGACCTTGGCATTCGTC-3'	30	213	59
LPL	F:5'-GGGAGGAGGATGGAGTCTGT-3' R:5'-GGAACTGATGGCAAGAAACTG-3'	30	292	60
cTNT	F:5'-GCTGAGGGACAAGGCTAAGG-3' R:5'-AAGAGACCAGCAGGGTTTCG-3'	30	239	61
GATA4	F:5'-GTGCATAGACTTGCTTGCCTG-3' R:5'-GCAAGCATCTGTGGAAGGGT-3'	30	288	60
GAPDH	F:5'-CTGGCAAAGTCCAAGTGGTAG-3' R:5'-GACTCCACAACGTATTCAGCA-3'	30	225	59

Table 1: The primer sequences for Satellite cells identification.

Flow cytometry

At 80%-90% confluence, cells were collected, centrifuged for 8 min at 1200 rpm, and resuspended in PBS. The cells were incubated in 70% ethanol at 4°C overnight. After washing with PBS, the cells were blocked at room temperature with goat serum for 1 h, centrifuged for 10 min at 1200 rpm, and incubated with the primary antibody at 4°C overnight. After washing with PBS, the cells were incubated for 1 h in the dark with the secondary antibody. After diluting the cells with PBS, they were filtered through an 80-μm mesh sieve and examined by a FACS Calibur flow cytometer.

In vitro multipotent differentiation of MuSCs

Myogenic differentiation

Cells in the control group were cultured in complete medium and those in the induced group were cultured in differentiation medium. Passage 3 MuSCs were incubated for 10 days in myogenic differentiation medium (DMEM/F12, 2% horse serum). The medium was changed every 3 days. The cells were examined under a microscope for morphological identification and muscle tube formation.

Osteogenic differentiation

To test the osteogenic differentiation potential of MuSCs, cells were cultured in complete culture medium or inducing medium, Passage 3 cells at 50%-60% confluence were transferred to osteogenic differentiation medium consisting of DMEM/F12 with 5% horse serum and 200 ng/mL bone morphogenetic protein 7 (BMP7) for 2 weeks. The medium was replaced every 48 h. After 2 weeks, calcium node formation of cells was measured by alizarin red staining (Sigma, Germany), and expression of osteoblast-specific genes was detected by RT-PCR[18].

Adipogenic differentiation

Control group cells were cultured in complete culture medium and induced group cells were cultured in adipogenic differentiation medium to test the potential adipogenic differentiation of cells. At 50%-60% confluence, passage 3 skeletal MuSCs were incubated in adipogenic differentiation medium (DMEM/F12, 5% FBS, 0.5 mm/L IBMX, 1 /m/L dexamethasone, 100 μm/L indomethacin and 10 μg/L insulin). After 2 weeks of differentiation, oil red O (Sigma) was used to stain the cells for intracellular lipid accumulation. RT-PCR was used to detect the expression of adipocyte specific gene.

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Differentiation of cardiomyocytes

Cells were cultured in complete culture medium or cardiomyogenic inducing medium to assess the cardiomyogenic differentiation potential of MuSCs Passage 3 cells were incubated in differentiation medium consisting of DMEM/F12 ,10% FBS , 9 nM 5-azacytidine (Sigma, St. Louis, MO, USA)(19) for 2 weeks. The cells were harvested and analyzed by RT-PCR.

Results

Isolation, culture and observation of morphology

Primary cells were isolated from skeletal muscle tissues and began to elongate into a spindle shape after 24 h (Figure 1A). Approximately 3 days later, the cells reached 80% -90% confluence and had a parallel arrangement (Figure 1B). The cells were subsequently subcultured using 0.25% trypsin. An hour after passaging, the cells began to adhere. After day 1, the cells had completely adhered to the plate surface (Figure 1C-I). At passage 14, the cells appeared senescent vacuoles were observed and most cells had karyopyknosis.

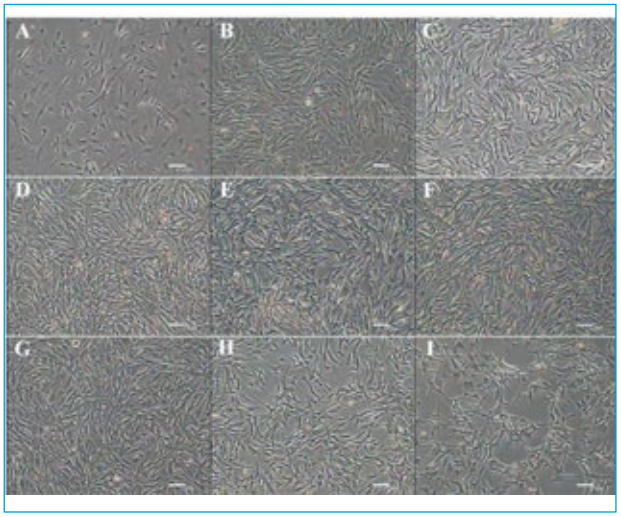

Fig. 1: Muscle satellite cells (MuSCs) morphology of the skeleton in vitro 40×. Morphology of cultured MuSCs of the skeleton in vitro. Satellite cells were circular and highly refractive before being attached (A); After 3 days, adherent cells showed fusiform or spindle-shaped (B); As the cell consistency increased, cells were ranked parallelly in a regular arrangement (C); 2nd passage (D); 4th passage (E); 6th passage (F); 8th passage (G); 10th passage (H) and the 12th passage (I) displayed karyopyknosis, vacuolization and other senescent appearance.

Growth kinetics

The growth kinetics of various MuSC passages were assessed and growth curves were typically sigmoidal (Figure 2). Cell proliferation of passages 3, 6 and 9 was similar. The cell growth entered the logarithmic phase after 1-3 days of incubation and was stable at around 7 days (Figure 2).

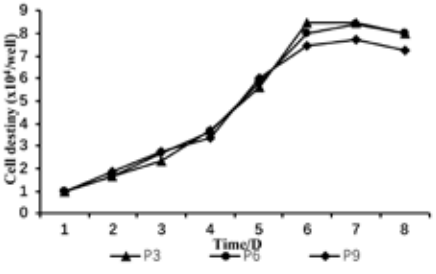

Fig. 2:Passages 3, 6 and 9 typical sigmoidal growth curves included incubation, exponential growth, and plateau periods. The horizontal axis represents the number of days.

Characterization of MuSCs

Immunofluorescence of MuSCs

Pax7,Myf5 and MyoD are specific markers of MuSCs. These markers were expressed in Peking Duck MuSCs. Furthermore, they were all localized in the nucleus (Figure 3).

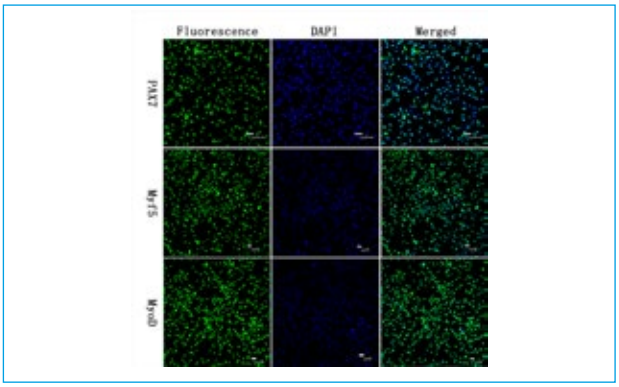

Fig. 3: Skeletal muscle satellite cells identification by immunofluorescent labeling. Pax7+, myf5+ and MyoD+ expression in muscle satellite cells of the skeleton. They are all shown in the left panel (green) in the nucleus. Nuclei in the middle panel were stained with DAPI and images were merged on the right panel.

RT-PCR

mRNA expression of MyoD, Pax7, and Myf5 was detected in MuSCs. The internal ontrol was GADPH (Figure 4).

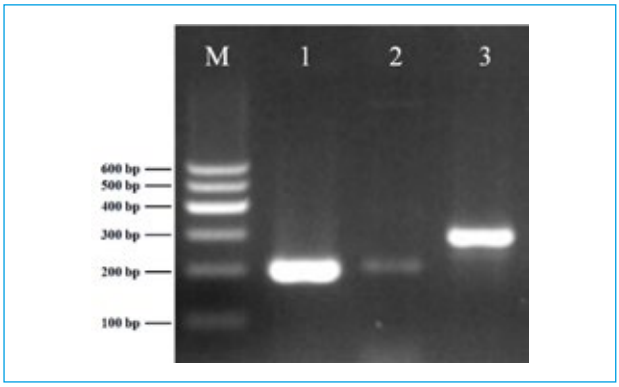

Fig. 4: Detection of MyoD, Myf5 and Pax7 in duck muscle satellite cells of the skeleton by RT-PCR. 1. Pax7; 2. Myf5; 3. MyoD.

Flow cytometry

The specific markers were also analyzed by flow cytometry. This analysis confirmed that these genes were expressed in Peking Duck MuSCs (Figure 5). In the negative control the primary antibody was replaced with PBS.

Skeletal MuSCs differentiation

Myogenic differentiation

Cell proliferation became slow and anastomosis of the cells began when the cells were culture in medium containing 2% horse serum. From day 6 post-induction, some cells began to differentiate into multinucleated myotubes (Figure 6A). Furthermore, immunofluorescence showed that differentiated cells expressed myosin heavy chain (MHC) (Figure 6B).

The control group cultured in complete medium showed no multinucleated myotubes (Figure 6C). RT-PCR showed that Desmin and MHC genes were expressed whereas the control group did not show expression of these genes (Figure 6D).

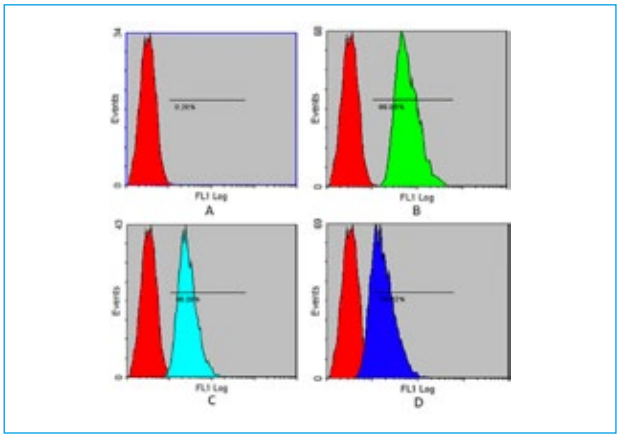

Fig. 5: Positive rate analysisof flow cytometry in expression of the skeletal MuSCs. (A) Negative control's primary antibody was replaced with PBS. The rate of Myf5+(B), MyoD+(C), Pax7+(D). Skeletal muscle satellite cells was 99.88%, 98.28%, 74.82%.

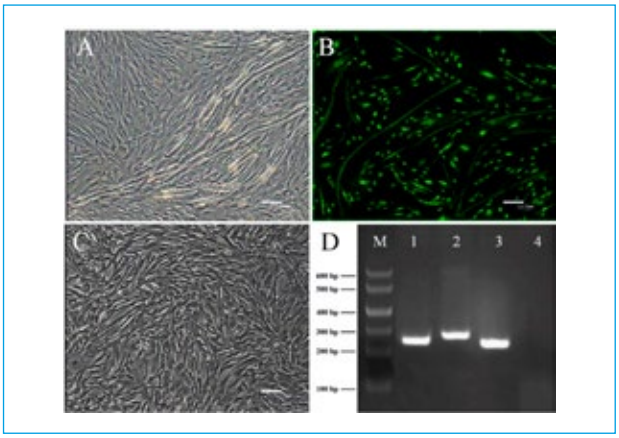

Fig. 6: Myogenic differentiation of MuSCs. Multinucleated myotubes form by inducing terminal differentiation of the skeletal muscle satellite cells. (**A**) Induction about 6 days, there much formed myotubes were observed. (**B**) Immunofluorescence staining of myoblasts, green indicates MHC. (**C**) Control group. (**D**) RT-PCR analysis , lane 1 and 2 show that myoblasts positively expressed MHC and Desmin, group 2. Lane3 and 4 show GAPDH and negative controls; Marker=100bp DNA ladder.

Osteogenic differentiation

Satellite cells displayed significant changes in appearance after induction with osteogenic medium containing horse serum and BMP7. By day 3 some cells had a polygonal shape (Figure 7A). Triangular or polygonal cells increased in number over time and showed multilayered growth and crystal grains. At

day 15, nodules were observed by alizarin red staining (Figure 7B). The morphology of cells cultured in complete medium showed no significant changes, and alizarin red staining was negative (Figure 7C). Osteogenic differentiation of the satellite cells was also analyzed by RT-PCR. Osteocalcin and Runtrelated transcription factor 2 (RunX2), which are specific osteogenic markers, were detected (Figure 7D).

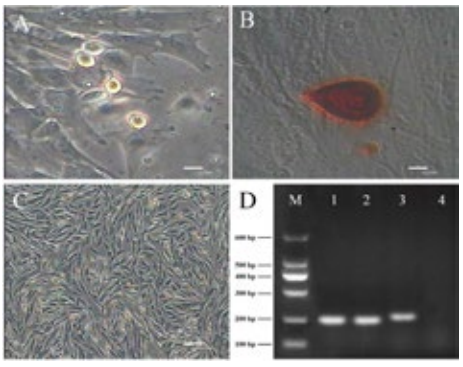

Fig. 7: Skeletal muscle osteogenic differentiation. (**A**) The third day some cells changed to more polygonal shape. (**B**) At 15 days affect, the nodules were stained with alizarin red. (**C**) Negative controls. (**D**) Satellite cells osteogenic differentiation were analyzed through RT-PCR assay, lane 1and 2 show the specific markers Osteocalcin and RunX2, lane3 and 4 show GAPDH and negative controls; Marker=100bp DNA ladder.

Adipogenic differentiation

Adipogenic differentiation of skeletal MuSCs was shown by positive staining with oil red O. Three days after induction, a few lipid droplets appeared in cells cultured in adipogenic medium.

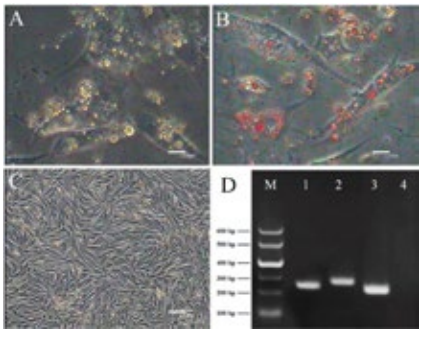

Fig. 7: Adipogenic differentiation of the skeletal muscle. (**A**) Plenty of lipid droplets appeared on day 14 in differentiated cells; (**B**) Oil Red O was used to stain the lipid droplets. (**C**) Negative controls. (**D**) Analysis for RT-PCR, lane 1and 2 show the specific markers of adipogenic cell expressing markers PPARγ and LPL, lane3 and 4 show GAPDH and negative controls; Marker=100bp DNA ladder.

After 2 weeks, the morphology of skeletal muscle satellite cells changed from fibroblast-like to oblate with many lipid droplets (Figure 8A). As differentiation progressed, a large amount of lipid droplets in differentiated cells was stained by oil red O (Figure 8B). The morphology of cells cultured in the complete medium did not change and oil red O staining was negative (Figure 8C). Moreover, expression of adipocyte specific genes, peroxisome lipoprotein lipase (LPL), and proliferator-activated receptor-γ(PPARγ) was detected by RT-PCR (Figure 8D).

Cardiomyogenic differentiation

Afterday 10 of differentiation, beating cardiomyogenic cells were clearly detected (Figure 9A) with rhythmic beating of about 70 beats/min.Cells cul-tured in complete medium showed no alteration in morphology (Figure 9B) Furthermore, two specific markers, GATA binding protein 4 (GATA4) and cardiac troponin (cTNT), were detected by RT-PCR (Figure 9C).

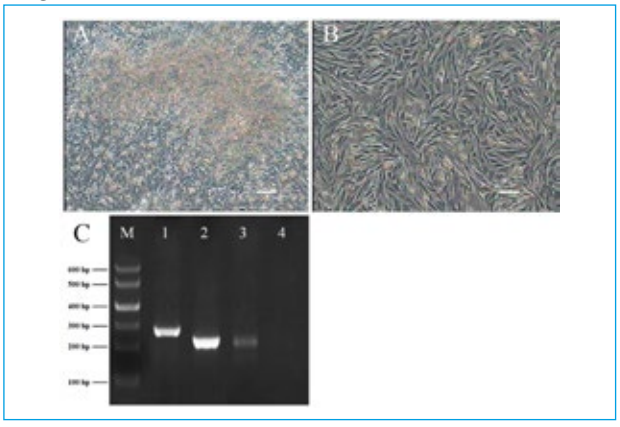

Fig. 9: Cardiomyogenic differentiation of the skeletal muscle. (**A**) Differentiation with azacytidine for ten days. (**B**) Negative controls(**C**) Analysis for RT-PCR, lane 1and 2 show the specific markers of cardiomyocytes cell expressing markers GATA4 and cTNT,l ane3 and 4 show GAPDH and negative controls; Mark-er=100bp DNA ladder.

Discussion

Primary cells have been widely used in molecular breeding, pathology,and physiology as an important cellular model system for in vitro research. Skeletal MuSCs are abundant in the muscle tissue of young animals, but their proportion decreases with age. In previous studies on the isolation of primary skeletal muscle satellite cells from poultry, 15-day-old chicken embryos, chicks at the 4th day after exhumation,or 16-day-old pigeon embryos

are generally used. In this study, 18-day-old duck embryos were selected, mainly because the amount of thoracic muscle tissue of duck embryos before 17 days old is small, whereas the connective tissue of 19-day-old duck embryos is increased,which introduces more heterogenous cells and results in a decreased proportion of isolated skeletal muscle satellite cells.

The objective of this study was to determine culture conditions to enable the growth and characterization of putative poultry MuSCs. MuSCs are difficult to isolate because they are located between basement and muscle cell membranes[20].

In this study, we used 0.1% collagenase I and 0.25% trypsin to digest the muscle tissue and isolate the cells. We purified MuSCs of Peking duck through differential adhesion because fibroblasts are more likely to adhere to culture dishes coated with gelatin. Previous studies have shown that myoblasts, such as C2C12 myoblasts or cloned myocytes isolated from adult skeletal muscle, show osteoblastic and adipogenic differentiation capacities after treatment with BMPs or fat inducers[21]. Furthermore, blocking the Wnt signaling pathway induces the C2C12 cells[22]. However, whether duck skeletal MuSCs possess similar differentiation capabilities remained unknown.

In this study, we showed that MuSCs of the duck skeleton had the potential to differentiate into osteocytes, adipocytes, and skeletal myocytes. Currently, evidence can does not support the claim that satellite cells can differentiate terminally to non-muscle lineages[23]. Our study confirmed that satellite cells ex-posed to adipogenic induction medium expressed terminal markers of adipogenic differentiation, while maintaining myogenic regulator expression, which is the same as reported in previous studies[24, 25].

Studies have shown that after 48 hours of induction, a small number of muscle tubes and a large number of multinucleated muscle tubes can be seen in porcine skeletal MuSCs. After 6 days of induction, the cytoplasm gradually became clear. A small amount of lipid droplets appeared 9 days later and the number of lipid droplets formed 12 days later and intercellular calcification was observed 7 days afer induction. A large amount of muscle tube formation was observed in skeletal muscle satellite cells of sheep on the 4th day of induction. A small amount of fat drops appeared 3days after induction and a large number of fat drops appeared 2 weeks later. Many muscle tubes were formed 10 days after induction. In this study, some of the Peking duck skeletal MuSCs differentiated into multinucleated muscle tubes after 6 days of induction. After 10 days of culture under conditions of osteogenic induction some cells became ovoids and aggregated to form nodules. At 14 days, the number of nodular cells was significantly increased and the amount of calcium salt was also increased. After 21 days of alizarin red staining, the nodules were bright red. After 2 days of culture under conditions of adipogenesis induction, the cell density increased and the cell body also became larger. After 7 days of induction, the formation of small lipid droplets were observed. With the increase of induction time, the small lipid droplets increased and fused with each other to form large and round lipid droplets.

Conclusion

In this study, we found an optimal method to isolate and culture duck MuSCs, We confirming the identity of these cells by cell morphology and specific marker expression. Furthermore, we induced these satellite cells into adipogenic muscle, and osteogenic cells, proving that they have the capacity to differentiate into multiple lineages. We have therefore developed a method that makes it possible to establish a Peking duck MuSCs cell bank. This type of cell bank should serve as a valuable resource for the research community.

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Corresponding Author:
Dr. Minghai Zhang
Email: zhangminghai2004@126.com
(China)