The Inhibition on MDFIC and PI3K/AKT Pathway Caused by miR-146b-3p Triggers Suppression of Myoblast Proliferation and Differentiation and Promotion of Apoptosis

Weiling Huang 1,2, Lijin Guo 1,2, Minxing Zhao 1,2, Dexiang Zhang 1,2, Haiping Xu 1,2 and Qinghua Nie 1,2,*

1 Department of Animal Genetics, Breeding and Reproduction, College of Animal Science, South China Agricultural University, Guangzhou 510642, China
2 Guangdong Provincial Key Lab of Agro-Animal Genomics and Molecular Breeding and Key Lab of Chicken Genetics, Breeding and Reproduction, Ministry of Agriculture, Guangzhou 510642, China

* Correspondence: nqinghua@scau.edu.cn; Tel.: +86-20-8528-5759; Fax: +86-20-8528-0740

Received: 26 May 2019; Accepted: 27 June 2019; Published: 29 June 2019

Abstract: Accumulating studies report that microRNAs (miRNAs) are actively involved in skeletal myogenesis. Previously, our study revealed that miR-146b-3p was related to the growth of skeletal muscle. Here, we further report that miR-146b-3p is essential for the proliferation, differentiation, and apoptosis of chicken myoblast. Elevated expression of miR-146b-3p can dramatically suppress proliferation and differentiation, and facilitate apoptosis of chicken myoblast. Besides, we identified two target genes of miR-146b-3p, AKT1 and MDFIC, and found that miR-146b-3p can inhibit the PI3K/AKT pathway. Our study also showed that both AKT1 and MDFIC can promote the proliferation and differentiation while inhibit the apoptosis of myoblast in chicken. Overall, our results demonstrate that miR-146b-3p, directly suppressing PI3K/AKT pathway and MDFIC, acts in the proliferation, differentiation, and apoptosis of myoblast in chicken.

Keywords: miR-146b-3p; AKT1; myoblast; proliferation; differentiation; apoptosis

1. Introduction

Skeletal muscle formation is a vital life process which has always attracted increasing attention from researchers. The development of skeletal muscle goes through the process of myoblast proliferation and differentiation into myotubes [1]. Once the course of myoblast differentiation initiates, the proliferating myoblasts are arrested from the cell cycle, ultimately resulting in the formation of myotubes [2]. In addition to the proliferation and differentiation, apoptosis of myoblast is also recognized as an essential process in skeletal muscle development. Apoptosis is a form of programmed cell death, which is a tightly regulated process [3]. Inhibition of apoptosis can lead to blockage and abnormality in skeletal muscle development, and even worse, cause inflammation and oncogenesis [4,5].

As an intricate physiological process, skeletal muscle development is regulated by a variety of transcription factors, miRNAs are included [6,7]. MiRNAs are a class of endogenous noncoding small RNAs of approximately 18 to 24 nucleotides, usually complementary to the sites in the 3′ untranslated region (3′UTR) of the target messenger RNAs (mRNAs) to repress gene expression post-transcriptionally [8,9]. Emerging studies have revealed that miRNAs are involved in the regulation of multiple life processes such as cell proliferation, cell differentiation, embryonic development, tissue inflammation, and so on [10–13]. In the process of skeletal muscle formation, the role of miRNAs cannot be ignored. So far, a host of miRNAs, particularly miR-1, miR-206, and miR-133, have been...
identified to be capable of mediating the skeletal muscle development [14–19]. For instance, miR-1 and miR-133 played important roles in regulating skeletal muscle proliferation and differentiation in cultured myoblast [14]. MiR-16-5p was able to repress myoblast proliferation and differentiation, and promote myoblast apoptosis [20]. MiR-133a-5p, miR-29b-1-5p, miR-34b-5p, and miR-30a-3p were characterized as important miRNAs in myoblast proliferation and differentiation [21–23].

MiR-146b was primarily established as a regulator in inflammation and cancer [24–26]. To date, only a little research has been done on miR-146b-3p, not to mention the intensive study of its roles in myoblast proliferation, differentiation, and apoptosis in skeletal muscle development. In our previous RNA-seq study (accession number: GSE91060), we found miR-146b-3p was differentially expressed in the leg muscles between the E11 and E16 of Xinghua chicken [27]. Interestingly, according to our another preliminary study (accession number GSE62971), the expression of miR-146b-3p was of higher abundance in chicken with low body weight than those with high body weight in no matter White Recessive Rock or Xinghua chicken, suggesting that miR-146b-3p might be a candidate inhibitor of chicken muscle growth [28]. Therefore, in this assay, we aim to gain insight into how miR-146b-3p acts in the regulation of the skeletal muscle development.

The AKT1 gene, also named protein kinase B α (PKBα), is an important member of the AKT family. More notably, AKT is universally acknowledged as a core factor in the PI3K (phosphatidylinositol 3-kinase)/AKT pathway that plays critical roles in various cellular activities such as cell proliferation, cell differentiation, cell apoptosis, metabolism, protein synthesis, transcription, and so on [29,30]. A study demonstrated that in AKT family, AKT1 primarily promoted and sustained the myoblast differentiation [31]. Absence of AKT1 resulted in growth retardation and apoptosis promotion [32]. Several lines of apoptotic paradigms have also highlighted the principle role of AKT1 in maintaining cell survival and suppressing apoptosis [33–35]. Besides, based on our previous study, AKT1 appears to be one of the candidate target genes of miR-146b-3p [28]. It is of interest to uncover the potential interaction between AKT1 and miR-146b-3p in myoblast development.

The full name of MDFIC is MyoD family inhibitor domain containing. There is a related gene MDFI, also known as I-mfa, which was reported to play a negative role in regulating the transcription of MyoD family in the differentiation of fibroblast [36]. MDFIC contains a cysteine-rich C-terminal region that shares a high degree homology with I-mfa. The construction similarity between MDFIC and I-mfa suggests that MDFIC can be an inhibitor of myoblast differentiation. However, to our surprise, MDFIC tended to promote cell differentiation as well as cell proliferation in our current study. Thus, it is definitely of considerable interest to further determine how myoblast responds when MDFIC is silenced or over-expressed.

In this study, we investigate the function of miR-146b-3p on the proliferation, apoptosis, and differentiation of myoblast. We found that miR-146b-3p can target AKT1 and MDFIC, and suppress PI3K/AKT pathway. In addition, we also verified the effects of AKT1 and MDFIC on myoblast proliferation, apoptosis, and differentiation in chicken.

2. Materials and Methods

2.1. Ethics Statement

All animal experiments in this study were approved by the Animal Care Committee of South China Agricultural University (Guangzhou, People’s Republic of China) (approval number: SCAU#0014).

2.2. Cell Culture

Chicken primary myoblasts (CPMs) were isolated from the leg muscles of 11-embryo-age chicken and cultured in the Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 20% fetal bovine serum (Gibco, Grand Island, NY, USA) and 0.2% penicillin/streptomycin solution (Invitrogen, Carlsbad, CA, USA). The differentiation of myoblasts was induced by RPMI-1640 medium consisting of 2% horse serum (Hyclone, Logan, UT, USA) and
0.2% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). Quail muscle clone 7 (QM-7) cells were cultured in Medium 199 basic (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 10% tryptose phosphate broth solution (Sigma, Louis, MO, USA), and 0.2% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). DF-1 cell lines of chicken embryo fibroblast (DF-1 cells) were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 0.2% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). All cells were cultured in 5% CO₂ at 37 °C.

2.3. RNA Isolation, Complementary DNA (cDNA) Synthesis, and Quantitative Real-Time PCR (q-PCR)

Total RNA from tissues or cells was extracted using Hi Pure Total RNA Mini Kit (Magen, Guangzhou, China) following the manufacturer’s protocol. The Prime Script™ RT reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Otsu, Japan) was used in cDNA synthesis for mRNA. The mRNA expression levels were detected by q-PCR with Bio-rad CFX96 instrument (Bio-Rad, Hercules, CA, USA) using iTAP™ universal SYBR GREEN superMIX (Bio-Rad, Hercules, CA, USA). The reference genes were U6 and GAPDH in the process of quantification of miRNA and mRNA, respectively. All primers were designed by Premier Primer 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by TSINGKE Biotech (Guangzhou, China). The primers of the q-PCR are listed in Table 1. Relative mRNA expression levels were calculated using the 2−ΔΔCT method (ΔCT = CT_target gene − CT_reference gene, ΔΔCT = ΔCT_treat group − ΔCT_control group).

| Gene Name | Primer Sequences (5′→3′) | Size (bp) | Annealing Temperature (°C) | Accession Number |
|-----------|---------------------------|-----------|----------------------------|-----------------|
| AKT1      | F: CACACGCTGACAAAAACCG    | 128       | 60                         | NM_205055.1     |
|           | R: AACAACCTCCCCCTCGTATTGC |           |                            |                 |
| MDFIC     | F: CAAGGCAAGCAAGAAGA      | 126       | 52                         | XM_416018.5     |
|           | R: AGAAGCAATGTTACAGACAGGGT|           |                            |                 |
| Cyclin B2 | F: CACATACGACGAGAAGAG    | 133       | 58                         | NM_001004369.1  |
|           | R: ACACTCATAGGGGACAGGG   |           |                            |                 |
| Cyclin D1 | F: CAGAAAGTGCCAGAGAAGAG  | 188       | 58                         | NM_205381.1     |
|           | R: CAGATGGAGTGTGTCGTTGA  |           |                            |                 |
| Cyclin D2 | F: AATCTGCTTCACAGACGACC  | 150       | 58                         | NM_204213       |
|           | R: TTTGAGACCTCCAACATC    |           |                            |                 |
| PCNA      | F: GTGCTTGAGACCTTGTTT    | 217       | 58                         | NM_204170.2     |
|           | R: CGTATCGAGGATGTTCTCT   |           |                            |                 |
| p21       | F: GAGAGTTCGTCACGATAAGC  | 247       | 58                         | NM_204170.2     |
|           | R: TTTGAGACCTCCAACATC    |           |                            |                 |
| CDKN1B    | F: GCCTGCTGGGCTGAA       | 207       | 58                         | NM_204256.2     |
|           | R: GAGAACCGAAGATGTTGCC   |           |                            |                 |
| Caspase-3 | F: TGGCCCCTGTCAGGCTGAAAG | 106       | 61                         | NM_204725.1     |
|           | R: TCCACTGCTGTGGCTTACCAATC|          |                            |                 |
| Caspase-8 | F: CGCTGACAGAGAAGCCATTT  | 207       | 61                         | NM_204592.2     |
|           | R: GGCTGCTGCTGCTGCTTTATT|           |                            |                 |
| Caspase-9 | F: TCCGCCCGCTGTTCAACCT   | 270       | 61                         | XM_424880.5     |
|           | R: CTTGATCTGGGAGAGCTTTC  |           |                            |                 |
| Fas       | F: TCCACCTGCTCCTTGCTATT  | 78        | 61                         | NM_001199487.1  |
|           | R: GTGCAGTGTGGGGGAAACT    |           |                            |                 |
| Cyp c     | F: CGTCCCAAGAATGTTCCAGATGC|         | 138                        | NM_001079478.1  |
|           | R: CTTTGTCTTATGGTCTTGCTG  |           |                            |                 |
| MYOD      | F: GCTGCTACAGGAAATCCAAAT | 200       | 58                         | NM_204214.2     |
|           | R: CTGGGCTTACAGGTCATCA   |           |                            |                 |
| MYOG      | F: CCGAGGCGTGAAGAGGGTGA  | 320       | 58                         | NM_204184.1     |
|           | R: GCCCTCGTGGGCTGCTGGCAT |           |                            |                 |
| MYHC      | F: CTCCTGACGCTTTCCTTA   | 213       | 58                         | NM_001319304.1  |
|           | R: TGATAGCTGTAGGTCGTTG   |           |                            |                 |
| GAPDH     | F: TCCCTCACCTTTGATGCG    | 146       | 50–62                      | NM_204305.1     |
|           | R: GTCGCTGCGATCTCCCTTCT  |           |                            |                 |
2.4. RNA Oligonucleotides and Plasmids Construction

Gga-miR-146b-3p mimic, miR-146b-3p inhibitor, mimic negative control (NC), inhibitor NC, small interfering RNA (siRNA), and siRNA negative control (si-NC) used in this study were synthesized by Ribobio (Guangzhou, China). The oligonucleotides sequences used in the study are listed in Table 2.

| Sequence Name       | Sequences (5′→3′)                  |
|---------------------|------------------------------------|
| gga-miR-146b-3p mimic | GGGAUACCUAAGUCAAGACG              |
| gga-miR-146b-3p inhibitor | CGUCUUGACUUAGGUAUCCC            |
| si-AKT1             | GCTGAAGAAATGGAAGTGT              |
| si-MDFIC            | ATGGAAGTGGAAATGCACAA            |

The complete CDS sequence of AKT1 and MDFIC were respectively amplified by PCR and were cloned into the expression vector, pcDNA3.1 (Promega, Madison, WI, USA) by using the Xba I and Xho I restriction sites through pJET1.2 cloning vector. The plasmids were named pcDNA3.1-AKT1 and pcDNA3.1-MDFIC, respectively. The segment sequence of the 3′UTR of AKT1 and MDFIC that contained the putative gga-miR-146b-3p binding sequence were amplified, the segment sequence of the 3′UTR of AKT1 was subcloned into Xba I and Xho I sites in the pmirGLO dual-luciferase while the segment sequence of the 3′UTR of MDFIC was subcloned into Hind III and Not I sites reporter vector (Promega, Madison, WI, USA). The 3′UTR mutant plasmids were obtained by converting the binding site of miR-146b-3p from CCATAGG to TTCGCAA. PCR amplification of the mutants and DPNI digestion removed the parental DNA. The primers used to construct the plasmids are listed in Table 3.

| Primer Name           | Primer Sequences (5′→3′)                  | Size (bp) | Annealing Temperature (°C) |
|-----------------------|--------------------------------------------|-----------|---------------------------|
| pcDNA3.1-AKT1         | F: CCCAAGCTTGAACATTCCGCCCATTA                | 1612      | 61                        |
|                       | R: CCCGTGAGAAGAACTGGTAGGCTTCCTC             |           |                           |
| pcDNA3.1-MDFIC        | F: CCCAAGCTTGAACATTCCGCCCATTA                | 1064      | 60                        |
|                       | R: CCCGTGAGAAGAACTGGTAGGCTTCCTC             |           |                           |
| pmirGLO-AKT1-3′UTR-WT | F: CCCAAGCTTGAACATTCCGCCCATTA                | 100       | 55                        |
|                       | R: CCCGTGAGAAGAACTGGTAGGCTTCCTC             |           |                           |
| pmirGLO-AKT1-3′UTR-MT | F: AGAAGTGAACATTCCCGAGATGAGGGAAACT         | 2771      | 68                        |
|                       | R: TCTGAGCTATGACCTAGGATTCCCGCGCTCCTC       |           |                           |
| pmirGLO-MDFIC-3′UTR-WT| F: CCCAAGCTTGAACATTCCGCCCATTA                | 100       | 52                        |
|                       | R: TGTGGGCCGCAATTCGTCTGTTTCAATTCA          |           |                           |
| pmirGLO-MDFIC-3′UTR-MT| F: GCAATTTTCACTCCATAGGCGAATCTTAAAG         | 2771      | 68                        |
|                       | R: GCTTAAGAGATTTGACCCCTTATGTAAATTCA        |           |                           |

Bold sequence represents restriction enzyme recognition sequence.

2.5. MiRNA Targets Prediction and RNA Hybrid Detection

miRDB (http://mirdb.org/miRDB/) was used to predict the target genes of gga-miR-146b-3p. RNAhybrid (http://bibiserv2.ccbio.dbi.uni-bielefeld.de/rnahybrid/) detection was used to calculate the combined minimum free energy (MFE) of gga-miR-146b-3p and the 3′UTR of AKT1 and MDFIC to determine the binding stability of the duplex.

2.6. Cell Transfection

Transfections were performed with Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol with at least three replications. The transfection concentration of the oligonucleotides was 50 nM. The transfection concentration of the plasmids was followed as: 0.1 µg/well for 96-well plate, 0.25 µg/well for 24-well plate, 1 µg/well for 12-well plate, 2.5 µg/well for 6-well plate.
2.7. Dual-Luciferase Reporter Assay

DF-1 cells were seeded in 96-well plates and co-transfected with plasmid of wild-type 3′UTR or mutant 3′UTR with mimic or mimic-NC. After 48 h, the luciferase activity was detected using a Dual-GLO Luciferase Assay System Kit (Promega, Madison, WI, USA) following its instruction. The firefly luciferase and Renilla luminescence activities were detected using multi-function microplate reader (Biotek, Winooski, VT, USA).

2.8. Immunofluorescence

Cells seeded in 12-well plates were fixed in 4% formaldehyde for 20 min and washed three times with PBS for 5 min after 48-h transfection. Subsequently, the cells were treated with 0.1% Triton X-100 for 15 min and blocked with goat serum for 30 min and then incubated with MyHC antibody (B103; DHSB, USA; 0.5 µg/mL) overnight at 4 °C. After that Fluorescein (FITC)-conjugated AffiniPure Goat AntiMouse IgG (H + L) (BS50950; Bioworld, Minneapolis, MN, USA; 1:50) was added to the cells and the cells were incubated at room temperature for 1 h. Besides, the cell nuclei were stained with DAPI for 5 min. And lastly, Leica DMi8 fluorescent microscope (Leica, Wetzlar, Germany) was used to obtain the images, and ImageJ software was used to measure the percentage of the total image area covered by the myotubes.

2.9. 5-Ethynyl-2′-deoxyuridine (EdU) Assay

EdU Assay was performed to test cell proliferation using Cell-Light EdU DNA Cell proliferation Kit (RiboBio, Guangzhou, China). CPMs and QM-7 cells were cultured in 96-well plates for transfection. Briefly, after 48-h transfection, the cells were incubated with 50 nM EdU for 2 h at 37 °C; the cells were then fixed with 4% paraformaldehyde and stained with Apollo dye solution for proliferating cells. Nucleic acids in all cells were stained with Hoechst 33342. Leica DMi8 fluorescent microscope was used to capture five randomly selected fields to visualize the EdU-stained cells. The proliferation rate was the ratio of the number of EdU-stained cells to the number of Hoechst 33342-stained cells.

2.10. Flow Cytometric Analysis of Cell Cycle

CPMs or QM-7 cells were cultured in 12-well plates. After transfection for 48 h, cells were collected and then fixed in 75% ethanol overnight at −20 °C. Subsequently, the fixed cells were stained with propidium iodide (Sigma, Louis, MO, USA) (50 µg/mL) containing 10 µg/mL RNase A (TaKaRa, Otsu, Japan) and 0.2% (v/v) Triton X-100 (Sigma, Louis, MO, USA), and then incubated for 30 min at 37 °C in the dark. Flow cytometric analysis was performed on a flow cytometer (Beckman, Miami, FL, USA) and data was processed using FlowJo7.6 software.

2.11. Flow Cytometric Analysis of Cell Apoptosis

CPMs or QM-7 cells were seeded in 12-well plates and after transfection for 48 h the cells were collected. Then the cells were stained through an Annexin V-FITC apoptosis detection kit (Beyotime, Shanghai, China) and analyzed by a flow cytometer (Beckman, Miami, FL, USA), following the manufacturers protocol.

2.12. Western Blot Assay

Cellular proteins were extracted using ice-cold radio immunoprecipitation (RIPA) lysis buffer (Beyotime, Shanghai, China) with 1 mM phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Beyotime, Shanghai, China). The protein samples were separated by 10% SDS-PAGE at a voltage of 100 volts for 20 minutes and then at 120 volts for 60 minutes. Subsequently, the proteins were transferred onto a nitrocellulose membrane (Whatman, Maidstone, UK) or a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA), and then probed using antibodies according to standard procedures. The antibodies used for Western blots and their dilutions are as follows: Cleaved Caspase-8
(Asp391) (18C8) Rabbit mAb (Cell Signaling Technology, Boston, MA, USA; 1:1000), Anti-Caspase-9 antibody [E23] (Abcam, London, UK; 1:1000), mouse anti-MyHC antibody (Bioss, Beijing, China; 1:1000), rabbit anti-Lamin B antibody (Bioss, Beijing, China; 1:500), rabbit anti-AKT1 (Bioss, Beijing, China; 1:500), rabbit anti-phospho-AKT1 (Bioss, Beijing, China; 1:500), rabbit Anti-phospho-AKT antibody (Bioss, Beijing, China; 1:300), and mouse anti-GAPDH (Boster, Wuhan, China; 1:2000). Finally, the secondary antibody (Boster, Wuhan, China) containing horseradish peroxidase chain reaction (HRP) anti-rabbit/mouse IgG antibody (Boster, Wuhan, China) was incubated in a 1:10,000 dilution.

2.13. Statistical Analysis

All data are derived from at least three replicates of experimental processing. Statistically significant differences were calculated using Student’s t-test. Results are shown as mean ± S.E.M. (standard error of the mean) and the difference was considered as statistically significant when the p-value < 0.05 (*) or p-value < 0.01 (**).

3. Results

3.1. miR-146b-3p Contributes to Cell Cycle Arrest in Myoblast and Suppresses Cell Proliferation

miR-146b-3p was successfully overexpressed with the mimic and silenced with the inhibitor respectively in CPMs and QM-7 cells, indicating that the mimic and inhibitor of miR-146b-3p were available for the following verification experiments (Supplementary Figure S1a–d). We first investigated the effects of miR-146b-3p on myoblast proliferation. Flow cytometry, q-PCR, and EdU assays were performed in CPMs and QM-7 cells, respectively.

After the 48-h transfection in CPMs and QM-7 cells with miR-146b-3p mimic and inhibitor, flow cytometry analysis of the cell cycle was performed. The results showed that overexpression of miR-146b-3p can arrest QM-7 cells and CPMs in the G1 phase, causing the increase of the cell population in the G1 phase while distinct decrease in the S phase (Figure 1a,b). Moreover, the inhibition of miR-146b-3p revealed opposite results (Figure 1a,b). Overall, miR-146b-3p can block the cell cycle progress, thereby inhibiting the myoblast proliferation.

In addition, we tested the mRNA expression levels of several cell cycle-related genes in CPMs and QM-7 cells by q-PCR. The results showed that miR-146b-3p overexpression can reduce the mRNA expression of cell cycle-promoting genes, such as Cyclin B2, Cyclin D1, Cyclin D2, and PCNA, while increase cell cycle-inhibiting genes like p21 and CDKN1B (Figure 1c,d). The inhibition of miR-146b-3p revealed opposite results to the miR-146b-3p mimic groups (Figure 1c,d). Thus, miR-146b-3p can modulate the cell cycle-related genes to inhibit myoblast proliferation.

To further determine the effects of miR-146b-3p on myoblast proliferation, we also performed EdU staining experiment to observe the cell proliferation status after transfecting miR-146b-3p mimic or inhibitor into CPMs and QM-7 cells respectively. The results showed whether in CPMs or QM-7 cells, the ratio of proliferative cells of the miR-146b-3p mimic group was significantly reduced compared with that of the control group (Figure 1e–h). Conversely, after the inhibition of miR-146b-3p, the ratio of cells in proliferation phase was extremely increased (Figure 1e–h). These results indicate that miR-146b-3p can inhibit the proliferation of myoblast.
QM-7 cells, the ratio of proliferative cells of the miR-146b-3p mimic group was significantly reduced compared with that of the control group (Figure 1e–h). Conversely, after the inhibition of miR-146b-3p, the ratio of cells in proliferation phase was extremely increased (Figure 1e–h). These results indicate that miR-146b-3p can inhibit the proliferation of myoblast.

**Figure 1.** gga-miR-146b-3p inhibits myoblast proliferation. (a,b) Cell cycle analysis of QM-7 cells and chicken primary myoblasts (CPMs) after transfecting miR-146-3p mimic or inhibitor. (c,d) Relative mRNA expression of the cell cycle-related genes after transfection of miR-146-3p mimic or inhibitor in QM-7 cells and CPMs. (e) 5-Ethynyl-2′-deoxyuridine (EdU) staining of QM-7 cells after transfection of miR-146-3p mimic or inhibitor. (f) The fold change of proliferation rates of QM-7 cells with miR-146-3p mimic or inhibitor. (g) EdU staining of CPMs after transfection of miR-146-3p mimic or inhibitor. (h) The fold change of proliferation rates of CPMs with miR-146-3p mimic or inhibitor. Results of all groups are shown as mean ± standard error of the mean (S.E.M.) of three independent assessment methods. Statistical significance of the mean difference was assessed using unpaired two-sample t-tests. *p < 0.05; **p < 0.01. NC, negative control.
3.2. miR-146b-3p Inhibits Myoblast Differentiation

To observe the expression of miR-146b-3p in leg muscle during chicken embryogenesis, we constructed an expression profile of miR-146b-3p from E11 to E18, which showed that the expression of miR-146b-3p declined in fluctuation from E12 to E18 (Figure 2a). Besides, we also induced CPMs to differentiate in vitro (Figure 2b) and found the expression of miR-146b-3p declined when the cells underwent differentiation (Figure 2c). These results indicate the potential inhibitory effects of miR-146b-3p on myoblast differentiation.

![Figure 2](image_url)

**Figure 2.** miR-146b-3p suppresses myoblast differentiation. (a) Relative expression of miR-146b-3p in Xinghua chicken leg muscle from E11 to E18. (b) Morphology of CPMs cultured in growth medium (GM) and differentiation medium (DM) from day 1 to 6. (c) The relative mRNA expression of miR-146b-3p during CPMs-induced differentiation. (d) Relative mRNA expression of the cell differentiation-related genes after transfection of miR-146-3p mimic or inhibitor in CPMs. (e) Immunofluorescence of MyHC after transfection of miR-146-3p mimic or inhibitor in CPMs. (f) Comparison of the area of myotubes described in (e). Results of all groups are shown as mean ± S.E.M. of three independent assessment methods. Statistical significance of the mean difference was assessed using unpaired two-sample t-tests. * p < 0.05; ** p < 0.01. NC, negative control.

Therefore, we moved on to determine the role of miR-146b-3p in myoblast differentiation. It was found that overexpression of miR-146b-3p remarkably decreased the mRNA expression of myoblast differentiation-related genes including *MyoD*, *MyoG*, and *MyHC*, whereas, inhibition of miR-146b-3p...
increased the mRNA expression of MyoD, MyoG, and MyHC (Figure 2d and Supplementary Figure S2). In addition, the protein expression of MyHC was decreased after the overexpression of miR-146b-3p in CPMs, while the protein expression of MyHC was enriched after the inhibition of miR-146b-3p as exhibited (Figure 3e,f).

Immunofluorescence was also performed to track the effects of miR-146b-3p on myoblast differentiation. The results showed that overexpression of miR-146b-3p blocked the formation of myotubes, whereas, inhibition of miR-146b-3p contributed to the formation of myotubes (Figure 2e,f).

All the results above potently state that miR-146b-3p is capable of inhibiting the myoblast differentiation.

**Figure 3.** miR-146b-3p promotes myoblast apoptosis. (a) Flow cytometry of Annexin V-FITC and propidium iodide (PI) dual staining detecting the apoptosis of CPMs after transfection of miR-146-3p mimic or inhibitor. (b) Flow cytometry of Annexin V-FITC and propidium iodide (PI) dual staining detecting the apoptosis of QM-7 cells after transfection of miR-146-3p mimic or inhibitor. (c) Relative mRNA expression of the cell apoptosis-related genes after transfection of miR-146-3p mimic or inhibitor in CPMs. (d) Relative mRNA expression of the cell apoptosis-related genes after transfection of miR-146-3p mimic or inhibitor in QM-7 cells. (e) The protein levels of MyHC, cleaved-caspase 8, and cleaved-caspase 9 after the transfection of mimic or inhibitor in CPMs. (f) Gray value analysis of protein bands in (e). Results of all groups are shown as mean ± S.E.M. of three independent assessment methods. Statistical significance of the mean difference was assessed using unpaired two-sample t-tests. * p < 0.05; ** p < 0.01. NC, negative control.

### 3.3. miR-146b-3p Promotes Myoblast Apoptosis

Apart from proliferation and differentiation of myoblast, the apoptosis of myoblast is also of great significance in the development of skeletal muscle. Therefore, we also paid attention to the potential effects of miR-146b-3p on myoblast apoptosis.

Flow cytometry analysis performed to detect the myoblast apoptosis showed that the expression of miR-146b-3p promoted myoblast apoptosis (Figure 3a,b). The mRNA expression of some well-known apoptosis-related genes, including Caspase 3, Caspase 8, Caspase 9, Cyt c, and Fas, was detected after the overexpression and inhibition of miR-146b-3p in CPMs and QM-7 cells, respectively. The results showed that apoptosis-related genes were all upregulated after the overexpression of miR-146b-3p, while the apoptosis-related genes were downregulated after the inhibition of miR-146b-3p (Figure 3c,d). Furthermore, the protein expression of cleaved-caspase 8 and cleaved-caspase 9 was detected, and the
results showed that miR-146b-3p was able to upregulate the protein expression of cleaved-caspase 8 and cleaved-caspase 9 (Figure 3e,f). Moreover, the expression of miR-146b-3p suppressed the protein level of lamin B (Supplementary Figure S3a,b). These results indicate miR-146b-3p has a positive regulatory effect on myoblast apoptosis.

3.4. miR-146b-3p Targets AKT1 and MDFIC and Downregulates PI3K/AKT Pathway Activity

It is well established that miRNAs function mainly by targeting their target genes. To further explore how miR-146b-3p works in the regulation of skeletal muscle development, we tried to predict its possible target genes on the miRDB website. We found that miR-146b-3p was predicted to be able to target AKT1 and MDFIC. The seed sequences of miR-146b-3p were perfectly complementary to the 3′UTR region of AKT1 and MDFIC (Supplementary Figure S4a). Besides, the MFE between miR-146b-3p and AKT1 3′UTR was approximately −21.9 kcal/mol (Supplementary Figure S4b) and MDFIC 3′UTR was −24.2 kcal/mol (Supplementary Figure S4c), indicating they possess a stable combination. The mRNA expression of AKT1 and MDFIC can be suppressed after overexpression of miR-146b-3p while enhanced after inhibition of miR-146b-3p (Figure 4a). As a core factor in PI3K/AKT pathway, the phosphorylation of AKT can affect PI3K/AKT pathway activity. Subsequently, we tested the expression and phosphorylation of AKT1 protein and phosphorylation of AKT, it was found that miR-146b-3p was able to negatively regulate PI3K/AKT pathway activity (Figure 4b,c).

Moreover, the dual-luciferase report experiments were performed and showed that the matching of miR-146b-3p and the 3′UTR region of AKT1 or MDFIC led to a significant suppression of luciferase activity, whereas mutation of target sites failed to weaken the luciferase activity (Figure 4d,e), coinciding with the mRNA expression analysis results above.

Finally, we conducted a series of recovery validation experiments on the function of myoblast proliferation, differentiation, and apoptosis. The effects of miR-146b-3p on myoblast proliferation, differentiation, and apoptosis were all restored or even reversed by the co-expression assays (Figure 4f–h).

These results sufficiently indicate the direct target relationships between miR-146b-3p and AKT1 or MDFIC. To further learn about how miR-146b-3p works by interacting with AKT1 and MDFIC, we explored the effects of AKT1 and MDFIC on myoblast proliferation, differentiation, and apoptosis.

3.5. Both AKT1 and MDFIC Can Facilitate the Proliferation of Myoblast

To determine how AKT1 and MDFIC exert biological effects on myoblast proliferation, AKT1 and MDFIC were successfully overexpressed and silenced in CPMs and QM-7 cells, respectively (Supplementary Figure S1e–l). The flow cytometry, q-PCR, and EdU assays were then performed.

Flow cytometry analysis of the cell cycle revealed that in both CPMs and QM-7 cells, overexpression of AKT1 or MDFIC led to a significant promotion of cell transition from G1 phase to S phase, while knockdown of AKT1 or MDFIC significantly blocked cell transition from G1 phase to S phase (Figure 5a,b and Supplementary Figure S5a,b). These results illustrate that both AKT1 and MDFIC contribute to the cell cycle progress.

We also detected the mRNA expression levels of some cell cycle-related genes after overexpressing or silencing AKT1 or MDFIC in CPMs and QM-7 cells by q-PCR, respectively. The results showed that both AKT1 and MDFIC overexpression can upregulate the cell cycle promoting genes including Cyclin B2, Cyclin D1, Cyclin D2, and PCNA, while downregulate cell cycle inhibiting genes like p21 and CDKN1B (Figure 5c,d and Supplementary Figure S5c,d). It turned out to be an opposite result after silencing AKT1 or MDFIC (Figure 5c,d and Supplementary Figure S5c,d). These results indicate that both AKT1 and MDFIC can regulate the cell cycle-related genes and participate in promoting myoblast proliferation.

In addition, the EdU assay showed that in both CPMs and QM-7 cells, the proportion of proliferative cells remarkably increased, whereas the knockdown of AKT1 decreased the proportion of proliferative cells (Figure 5e,f and Supplementary Figure S5e,f); and, MDFIC showed the same effect (Figure 5g,h and Supplementary Figure S5g,h).
Taken together, we draw a conclusion that both AKT1 and MDFIC can promote myoblast proliferation in chicken.

Figure 4. miR-146b-3p targets AKT1 and MDFIC and downregulates PI3K/AKT pathway activity. (a) Relative mRNA expression of AKT1 and MDFIC in QM-7 cells after overexpression or inhibition of miR-146b-3p. (b) The phosphorylation levels of AKT and AKT1 and the protein expression of AKT1 after transfecting miR-146b-3p mimic or inhibitor in CPMs. (c) Gray value analysis of protein bands in (b). (d) Dual-luciferase report assay performed after co-transfecting the wild type or mutant 3′UTR of AKT1 with miR-146b-3p mimic or mimic NC in DF-1 cells. (e) Dual-luciferase report assay performed after co-transfecting the wild type or mutant 3′UTR of MDFIC with miR-146b-3p mimic or mimic NC in DF-1 cells. (f) Relative mRNA expression of the cell differentiation-related genes after co-transfection. (g) Relative mRNA expression of the cell apoptosis-related genes after co-transfection. (h) Cell cycle analysis of QM-7 cells after co-transfection. Results of all groups are shown as mean ± S.E.M. of three independent assessment methods. Statistical significance of the mean difference was assessed using unpaired two-sample t-tests. *p < 0.05; **p < 0.01. NC, negative control.
Figure 5. AKT1 and MDFIC promote myoblast proliferation. (a) Cell cycle analysis of CPMs after overexpression or inhibition of AKT1. (b) Cell cycle analysis of CPMs after overexpression or inhibition of MDFIC. (c) Relative mRNA expression of the cell cycle-related genes after overexpression or inhibition of AKT1 in CPMs. (d) Relative mRNA expression of the cell cycle-related genes after overexpression or inhibition of MDFIC in CPMs. (e) EdU staining of CPMs after overexpression or inhibition of AKT1. (f) The fold change of proliferation rates of CPMs with pcDNA3.1-AKT1 or si-AKT1. (g) EdU staining of CPMs after overexpression or inhibition of MDFIC. (h) The fold change of proliferation rates of CPMs with pcDNA3.1-MDFIC or si-MDFIC. Results of all groups are shown as mean ± S.E.M. of three independent assessment methods. Statistical significance of the mean difference was assessed using unpaired two-sample t-tests. * \( p < 0.05; \) ** \( p < 0.01. \) NC, negative control.
3.6. In Terms of Myoblast Differentiation, Both AKT1 and MDFIC Exhibit Positive Impacts

During the leg muscle development of Xinghua chicken in the embryonic period, the expression of AKT1 and MDFIC was gradually upregulated from E11 to E18 (Figure 6a,b), suggesting that AKT1 and MDFIC may be involved in the regulation of the differentiation of skeletal myoblast.

Figure 6. Both AKT1 and MDFIC promote myoblast differentiation, while suppress myoblast apoptosis. (a) Relative expression of AKT1 in Xinghua chicken leg muscle from E11 to E18. (b) Relative expression of MDFIC in Xinghua chicken leg muscle from E11 to E18. (c) Relative mRNA expression of AKT1 during CPMs induced differentiation. (d) Relative mRNA expression of MDFIC during CPMs-induced differentiation. (e,f) Relative mRNA expression of the cell differentiation-related genes after overexpression or inhibition of AKT1 or MDFIC in CPMs. (g,i) Immunofluorescence of MyHC after overexpression or knockdown of AKT1 and MDFIC in CPMs. (h,j) Comparison of the area of myotubes described in (g,i). Results of all groups are shown as mean ± S.E.M. of three independent assessment methods. Statistical significance of the mean difference was assessed using unpaired two-sample t-tests. * p < 0.05; ** p < 0.01. NC, negative control.
With the CPMs differentiating, the mRNA expression levels of AKT1 and MDFIC were getting higher (Figure 6c,d). The overexpression of AKT1 or MDFIC significantly upregulated the mRNA expression of the myogenic marker genes, and it showed an opposite effect after the interference of AKT1 or MDFIC (Figure 6e,f and Supplementary Figure S6a,b). Moreover, the protein expression of MyHC was also upregulated after the overexpression of AKT1 or MDFIC in CPMs, and it was downregulated after the knockdown of AKT1 or MDFIC in CPMs (Figure 7e,f).

**Figure 7.** AKT1 shows an inhibitory effect on myoblast apoptosis and so does MDFIC. (a,b) Flow cytometry analysis of Annexin V-FITC and PI dual staining detecting the apoptosis of CPMs after transfection of pcDNA3.1-AKT1 or si-AKT1. (c) Relative mRNA expression of the cell apoptosis-related genes after transfection of pcDNA3.1-AKT1 or si-AKT1 in CPMs. (d) Relative mRNA expression of the cell apoptosis-related genes after transfection of pcDNA3.1-MDFIC or si-MDFIC in CPMs. (e,f) The protein levels of MyHC, cleaved-caspase 8, and cleaved-caspase 9 after the overexpression or silence of AKT1 and MDFIC in CPMs. (g,h) Gray value analysis of protein bands in (e,f). Results of all groups are shown as mean ± S.E.M. of three independent assessment methods. Statistical significance of the mean difference was assessed using unpaired two-sample t-tests. *p < 0.05; **p < 0.01. NC, negative control.
Immunofluorescence staining was also performed and the results showed that overexpression of AKT1 or MDFIC facilitated the formation of myotubes, while it showed opposite results after silencing AKT1 or MDFIC (Figure 6g–j).

All these results demonstrate that both AKT1 and MDFIC can promote the differentiation of myoblast.

3.7. AKT1 Shows an Inhibitory Effect on Myoblast Apoptosis and so Does MDFIC

As described earlier, in the development of myoblast, cell apoptosis is also an important process. We wondered if miR-146b-3p can interact with AKT1 or MDFIC in regulating myoblast apoptosis.

Flow cytometry results showed that myoblast apoptosis was suppressed after the overexpression of AKT1 in QM-7 cells and CPMs (Figure 7a,b). Besides, the mRNA expression levels of several well-known apoptosis-related genes including Caspase 3, Caspase 8, Caspase 9, Cyt c, and Fas were detected. The results showed that the apoptosis-related genes were downregulated after the overexpression of AKT1 or MDFIC, while apoptosis-related genes were upregulated with the knockdown of AKT1 or MDFIC (Figure 7c,d and Supplementary Figure S6c,d). In addition, the protein levels of cleaved-caspase 8 and cleaved-caspase 9 were downregulated by the expression of AKT1 and MDFIC (Figure 7e–h). Moreover, both AKT1 and MDFIC can promote the protein expression of lamin B (Supplementary Figure S3c–f). The regulatory effects of AKT1 and MDFIC on apoptosis-related genes were contrasting to those of miR-146b-3p, suggesting that miR-146b-3p can promote the chicken myoblast apoptosis by inhibiting AKT1 and MDFIC.

4. Discussion

In this study, we characterized the role of miR-146b-3p in the regulation of skeletal muscle development. We confirmed that miR-146b-3p can regulate myoblast proliferation, differentiation, and apoptosis via negatively regulating the activities of the PI3K/AKT1 pathway and another target gene MDFIC (Figure 8).

It has been widely reported that miR-146b-3p mainly associates with glioma and thyroid tumor [26,37], while there is no research revealing its significant participation in skeletal muscle development. In our previous study, miR-146b-3p was proposed as a promising candidate gene related to the muscle development of chicken [28]. Here, we have performed a series of experiments in vitro to further determine the biological function of miR-146b-3p in skeletal muscle development. In verification, both mimic and inhibitor can cause large biological changes, some changes may be small, but they also have statistically significant differences. For example, compared with the control, inhibiting miR-146b-3p resulted in a transition of the cell cycle from G1 to S and the change of cell percentage in S is not particularly large. But the values in the group are so similar, resulting in a small S.E.M. in the group and a significant difference between the groups. Experiments such as EdU assay, Immunofluorescence assay, and the caspase cleavage assay have directly shown that these differences can cause large biological changes in myoblast proliferation, differentiation, and apoptosis. In fact, the expression change of one gene is often accompanied by the expression changes of multiple genes, which all together can lead to a specific phenotype. For instance, miR-16 was able to simultaneously target SENE1, Bcl6, and FOXO1, which altogether resulted in promoting chicken myoblast proliferation [19,20]. Therefore, we concluded that miR-146b-3p inhibited chicken myoblast proliferation and differentiation and promoted apoptosis.

To well grasp the biological effects of miRNAs, their target genes are supposed to be taken into consideration. In this study, we found miR-146b-3p can target and inhibit the expression of AKT1 and MDFIC and downregulate the activity of the PI3K/AKT pathway simultaneously.

AKT1, present in a variety of cell types, is widely distinguished as a key factor mediating numerous cellular activities including cell proliferation and differentiation [38–40]. In our present study, AKT1 showed positive effects on myoblast proliferation and differentiation, coincidently contrary to those of miR-146b-3p. Besides, a variety of cell death paradigms have identified AKT1 as a vital
anti-apoptotic gene [41–43]. It is well established that AKT1 is a vital gene in the PI3K/AKT pathway. An important function of activated PI3K in cells is the inhibition of programmed cell death, and AKT, a perfect candidate of PI3K, can serve in favor of PI3K to mediate cell-survival and cell-apoptosis responses [35,44,45]. Here, our results provide another piece of important evidence that AKT1 is an essential suppresser of the myoblast apoptosis. Of note, the Western blot showed not only the protein abundance and the phosphorylation level of AKT1, but also the phosphorylation level of AKT that can lead to the inhibition of myoblast proliferation and differentiation and the promotion of myoblast apoptosis. Thus, our results collectively suggest that miR-146b-3p can suppress PI3K/AKT pathway by directly targeting AKT1.

![Figure 8. Model of miR-146b-3p mediated regulatory mechanism in myoblast proliferation, differentiation, and apoptosis. In simple terms, miR-146b-3p downregulates the expression of AKT1 and MDFIC by targeting the 3'UTR of their mRNA. Both the phosphorylation of AKT1 and AKT can trigger the activation of PI3K/AKT pathway, thereby promoting the expression of cell cycle-related genes and myoblast differentiation-related genes and suppress cell apoptosis-related genes, which in turn facilitate cell proliferation and differentiation and inhibit apoptosis. In addition, the expression of MDFIC can also lead to the same effect.

When we were searching the targets for miR-146b-3p, the gene named MDFIC caught our eyes. As its name implies, MDFIC is an inhibitor of the MyoD family, which suppresses the myoblast differentiation. However, according to our preliminary reply verification, the overexpression of MDFIC can reverse the inhibitory effects of miR-146b-3p on chicken myoblast differentiation (Figure 4f), which aroused our interest to further detect how exactly MDFIC acts in the development of chicken myoblast. It was unexpected but our data did show that MDFIC promoted not only myoblast differentiation but also proliferation in chicken.
MyoD and MyoG, two essential members of the MyoD family, are upregulated to promote and maintain the process of myoblast differentiation [2,46]. Here, we are pleasantly surprised that MDFIC exhibited positive impacts on the mRNA expression of MyoD and MyoG in chicken myoblast (Figure 6). Moreover, immunofluorescence also provided a convincing demonstration that MDFIC can promote myotubes formation in chicken (Figure 6i,j). The article that originally identified I-mfa as a myogenic repressor interacting with members of the MyoD family described their animal material was mouse and the transfected cells were NIH3T3 cells, a kind of fibroblasts [36]. While in our study, the animal material was chicken and the transfected cells were QM-7 cells and CPMs, both are myoblasts. Our experiment materials are completely different from those of the above study. The function of MDFIC can be different, depending on species and cell types.

Here, we identified MDFIC as a novel factor promoting chicken myoblast proliferation and differentiation and inhibiting chicken myoblast apoptosis. As a matter of fact, to date, there are no reports about the role of MDFIC in myoblast development of chicken. Therefore, our study is the first to uncover the exact function of MDFIC in this field.

Collectively, our study sheds light on a mechanism by which miR-146b-3p inhibits myoblast proliferation and differentiation and promotes myoblast apoptosis via suppressing PI3K/AKT pathway activity and MDFIC expression. These discoveries reveal a novel model of miR-146b-3p on regulating skeletal muscle development.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2073-4409/8/7/656/s1, Figure S1: The transfection efficiency of plasmids and RNA oligonucleotides. Figure S2: The relative mRNA expression levels of cell differentiation-related genes after transfection of miR-146-3p mimic or inhibitor in QM-7 cells. Figure S3: The effects of miR-146b-3p, AKT1 and MDFIC on the protein levels of lamin B. Figure S4: The target relationship between miR-146b-3p and AKT1 or MDFIC. Figure S5: The effects of AKT1 or MDFIC on proliferation in QM-7 cells. Figure S6: The effects of AKT1 or MDFIC on differentiation and apoptosis in QM-7 cells.

**Author Contributions:** Weiling Huang was responsible for research designing and for most of the experiments and manuscript writing; Lijin Guo participated in the experiments and data analysis; Minxing Zhao conducted part of the experiments; Dexiang Zhang participated in the data analysis; Haiping Xu reviewed and modified the manuscript; Qinghua Nie carried out the design of the whole research and guided the research progress.

**Funding:** This research was funded by the grants from Science and Technology Planning Project of Guangdong Province (2018B020203001) and Guangzhou city (201504010017), and the Ten Thousand Talents Program of China (W03020593).

**Conflicts of Interest:** The authors declare that they have no conflict of interest.

**References**

1. Buckingham, M. Skeletal muscle formation in vertebrates. *Curr. Opin. Genet. Dev.* 2001, 11, 440–448. [CrossRef]
2. Braun, T.; Gautel, M. Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis. *Nat. Rev. Mol. Cell Biol.* 2011, 12, 349–361. [CrossRef] [PubMed]
3. Fuchs, Y.; Steller, H. Programmed cell death in animal development and disease. *Cell* 2011, 147, 742–758. [CrossRef] [PubMed]
4. Kaczanowski, S. Apoptosis: Its origin, history, maintenance and the medical implications for cancer and aging. *Phys. Biol.* 2016, 13, 31001. [CrossRef] [PubMed]
5. Fulda, S. Tumor resistance to apoptosis. *Int. J. Cancer* 2009, 124, 511–515. [CrossRef]
6. Dahmane, G.R.; Erzen, I.; Holeman, A.; Skorjanc, D. Effects of divergent selection for 8-week body weight on postnatal enzyme activity pattern of 3 fiber types in fast muscles of male broilers (Gallus gallus domesticus). *Poult. Sci.* 2010, 89, 2651–2659. [CrossRef]
7. Perry, R.L.; Rudnick, M.A. Molecular mechanisms regulating myogenic determination and differentiation. *Front. Biosci.* 2000, 5, D750–D767. [CrossRef]
8. Bartel, D.P. MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* 2004, 116, 281–297. [CrossRef]
9. Ambros, V. The functions of animal microRNAs. *Nature* 2004, 431, 350–355. [CrossRef]
10. Lai, E.C. Micro RNAs are complementary to 3’ UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat. Genet.* 2002, 30, 363–364. [CrossRef]
11. Lewis, B.P.; Shih, I.H.; Jones-Rhoades, M.W.; Bartel, D.P.; Burge, C.B. Prediction of mammalian microRNA targets. *Cell* **2003**, *115*, 787–798. [CrossRef]  
12. Stefani, G.; Slack, F.J. Small non-coding RNAs in animal development. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 219–230. [CrossRef] [PubMed]  
13. Felekkis, K.; Touvana, E.; Stefanou, C.; Deltas, C. microRNAs: A newly described class of encoded molecules that play a role in health and disease. *Hippokratia* **2010**, *14*, 236–240. [PubMed]  
14. Chen, J.F.; Mandel, E.M.; Thomson, J.M.; Wu, Q.; Callis, T.E.; Hammond, S.M.; Conlon, F.L.; Wang, D.Z. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat. Genet.* **2006**, *38*, 228–233. [CrossRef] [PubMed]  
15. Kim, H.K.; Lee, Y.S.; Sivaprasad, U.; Malhotra, A.; Dutta, A. Muscle-specific microRNA miR-206 promotes muscle differentiation. *J. Cell Biol.* **2006**, *174*, 677–687. [CrossRef] [PubMed]  
16. Chen, J.F.; Tao, Y.; Li, J.; Deng, Z.; Yan, Z.; Xiao, X.; Wang, D.Z. microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation and repressing Pax7. *J. Cell Biol.* **2010**, *190*, 867–879. [CrossRef] [PubMed]  
17. Ge, Y.; Chen, J. MicroRNAs in skeletal myogenesis. *Cell Cycle* **2011**, *10*, 441–448. [CrossRef] [PubMed]  
18. Luo, W.; Wu, H.; Ye, Y.; Li, Z.; Hao, S.; Kong, L.; Zheng, X.; Lin, S.; Nie, Q.; Zhang, X. The transient expression of miR-203 and its inhibiting effects on skeletal muscle cell proliferation and differentiation. *Cell Death Dis.* **2014**, *5*, e1347. [CrossRef] [PubMed]  
19. Luo, W.; Wu, H.; Ye, Y.; Li, Z.; Hao, S.; Kong, L.; Zheng, X.; Lin, S.; Nie, Q.; Zhang, X. The transient expression of miR-203 and its inhibiting effects on skeletal muscle cell proliferation and differentiation. *Cell Death Dis.* **2014**, *5*, e1347. [CrossRef] [PubMed]  
20. Cai, B.; Ma, M.; Chen, B.; Li, Z.; Abdalla, B.A.; Nie, Q.; Zhang, X. MiR-16-5p targets SESN1 to regulate the p53 signaling pathway, affecting myoblast proliferation and apoptosis, and is involved in myoblast differentiation. *Cell Death Dis.* **2018**, *9*, 367. [CrossRef]  
21. Chen, B.; Yu, J.; Guo, L.; Byers, M.; Wang, Z.; Chen, X.; Xu, H.; Nie, Q. Circular RNA circHIPK3 Promotes the Proliferation and Differentiation of Chicken Myoblast Cells by Sponging miR-30a-3p. *Cells* **2019**, *8*, 177. [CrossRef] [PubMed]  
22. Wang, Z.; Zhang, X.; Li, Z.; Abdalla, B.A.; Chen, Y.; Nie, Q. MiR-34b-5p Mediates the Proliferation and Differentiation of Myoblasts by Targeting IGFBP2. *Cells* **2019**, *8*, 360. [CrossRef] [PubMed]  
23. Chen, X.; Ouyang, H.; Wang, Z.; Chen, B.; Nie, Q. A Novel Circular RNA Generated by FGFR2 Gene Promotes Myoblast Proliferation and Differentiation by Sponging miR-133a-5p and miR-29b-1-5p. *Cells* **2018**, *7*, 199. [CrossRef] [PubMed]  
24. Sonkoly, E.; Stahle, M.; Pivarcsi, A. MicroRNAs and immunity: Novel players in the regulation of normal immune function and inflammation. *Semin. Cancer Biol.* **2008**, *18*, 131–140. [CrossRef] [PubMed]  
25. Hurst, D.R.; Edmonds, M.D.; Scott, G.K.; Benz, C.C.; Vaidya, K.S.; Welch, D.R. Breast Cancer Metastasis Suppressor 1 Up-regulates miR-146, Which Suppresses Breast Cancer Metastasis. *Cancer Res.* **2009**, *69*, 1279–1283. [CrossRef] [PubMed]  
26. Katakowski, M.; Buller, B.; Zheng, X.; Lu, Y.; Rogers, T.; Osobamiro, O.; Shu, W.; Jiang, F.; Chopp, M. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett.* **2013**, *335*, 201–204. [CrossRef]  
27. Jebessa, E.; Ouyang, H.; Abdalla, B.A.; Li, Z.; Abdullahi, A.Y.; Liu, Q.; Nie, Q.; Zhang, X. Characterization of miRNA and their target gene during chicken embryo skeletal muscle development. *Oncotarget* **2018**, *9*, 17309–17324. [CrossRef]  
28. Ouyang, H.; He, X.; Li, G.; Xu, H.; Jia, X.; Nie, Q.; Zhang, X. Deep Sequencing Analysis of miRNA Expression in Breast Muscle of Fast-Growing and Slow-Growing Broilers. *Int. J. Mol. Sci.* **2015**, *16*, 16242–16262. [CrossRef]  
29. Brazil, D.P.; Yang, Z.Z.; Hemmings, B.A. Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem. Sci.* **2004**, *29*, 233–242. [CrossRef]  
30. Wu, M.; Falasca, M.; Blough, E.R. Akt/protein kinase B in skeletal muscle physiology and pathology. *J. Cell. Physiol.* **2011**, *226*, 29–36. [CrossRef]  
31. Wilson, E.M.; Rotwein, P. Selective control of skeletal muscle differentiation by Akt1. *J. Biol. Chem.* **2007**, *282*, 5106–5110. [CrossRef] [PubMed]
32. Chen, W.S.; Xu, P.Z.; Gottlob, K.; Chen, M.L.; Sokol, K.; Shiyanova, T.; Roninson, I.; Weng, W.; Suzuki, R.; Tohe, K.; et al. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. *Genes Dev.* 2001, 15, 2203–2208. [CrossRef] [PubMed]

33. Kennedy, S.G.; Wagner, A.J.; Conzen, S.D.; Jordan, J.; Bellacosa, A.; Tsichlis, P.N.; Hay, N. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev.* 1997, 11, 701–713. [CrossRef]

34. Romashkova, J.A.; Makarov, S.S. NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 1999, 401, 86–90. [CrossRef] [PubMed]

35. Chang, F.; Lee, J.T.; Navolanic, P.M.; Steelman, L.S.; Shelton, J.G.; Blalock, W.L.; Franklin, R.A.; McCubrey, J.A. Involvement of PI3K/Akt signaling in cell cycle progression, apoptosis, and neoplastic transformation: A target for cancer chemotherapy. *Leukemia* 2003, 17, 590–603. [CrossRef]

36. Chen, C.M.; Kraut, N.; Groudine, M.; Weintraub, H. I-mf, a novel myogenic repressor, interacts with members of the MyoD family. *Cell* 1996, 86, 731–741. [CrossRef]

37. Chou, C.; Chen, R.; Chou, F.; Chang, H.; Chen, Y.; Lee, Y.; Yang, K.D.; Cheng, J.; Huang, C.; Liu, R. miR-146b is Highly Expressed in Adult Papillary Thyroid Carcinomas with High Risk Features Including Extrathyroidal Invasion and the BRAFV600E Mutation. *Thyroid* 2010, 20, 489–494. [CrossRef] [PubMed]

38. Lin, Z.; Zhou, P.; von Gise, A.; Gu, F.; Ma, Q.; Chen, J.; Guo, H.; van Gorp, P.R.; Wang, D.Z.; Pu, W.T. PI3kb links Hippo-YAP and PI3K-AKT signaling pathways to promote cardiomyocyte proliferation and survival. *Circ. Res.* 2015, 116, 35–45. [CrossRef] [PubMed]

39. Carpten, J.D.; Faber, A.L.; Horn, C.; Donoho, G.P.; Briggs, S.L.; Robbins, C.M.; Hostetter, G.; Boguslawski, S.; Moses, T.Y.; Savage, S.; et al. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature* 2007, 448, 439–444. [CrossRef]

40. Vivanco, I.; Sawyer, C.L. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer* 2002, 2, 489–501. [CrossRef]

41. Larson-Casey, J.L.; Deshane, J.S.; Ryan, A.J.; Thannickal, V.J.; Carter, A.B. Macrophage Akt1 Kinase-Mediated Mitophagy Modulates Apoptosis Resistance and Pulmonary Fibrosis. *Immunity* 2016, 44, 582–596. [CrossRef] [PubMed]

42. Atsaves, V.; Zhang, R.; Ruder, D.; Pan, Y.; Leventaki, V.; Rassidakis, G.Z.; Claret, F.X. Constitutive control of AKT1 gene expression by JUNB/CJUN in ALK+ anaplastic large-cell lymphoma: A novel crossstalk mechanism. *Leukemia* 2015, 29, 2162–2172. [CrossRef] [PubMed]

43. Leszczynska, K.B.; Foskolou, I.P.; Abraham, A.G.; Anbalagan, S.; Tellier, C.; Haider, S.; Span, P.N.; O’Neill, E.E.; Buffa, F.M.; Hammond, E.M. Hypoxia-induced p53 modulates both apoptosis and radiosensitivity via AKT. *J. Clin. Investig.* 2015, 125, 2385–2398. [CrossRef] [PubMed]

44. Das, T.P.; Suman, S.; Alatassi, H.; Ankem, M.K.; Damodaran, C. Inhibition of AKT promotes FOXO3a-dependent apoptosis in prostate cancer. *Cell Death Dis.* 2016, 7, e2111. [CrossRef] [PubMed]

45. Rana, C.; Piplani, H.; Vaish, V.; Nehru, B.; Sanyal, S.N. Downregulation of PI3-K/Akt/PTEN pathway and activation of mitochondrial intrinsic apoptosis by Diclofenac and Curcumin in colon cancer. *Mol. Cell. Biochem.* 2015, 402, 225–241. [CrossRef] [PubMed]

46. Berkes, C.A.; Tapscott, S.J. MyoD and the transcriptional control of myogenesis. *Semin. Cell Dev. Biol.* 2005, 16, 585–595. [CrossRef] [PubMed]

© 2019 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (http://creativecommons.org/licenses/by/4.0/).