Myogenic differentiation potential of chicken mesenchymal stem cells from bone marrow

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Research

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Abstract

Background: Mesenchymal stem cells (MSCs) have the potential to multilineage differentiation, which can be used for a good model to provide critical insight of chicken muscle development. Differential adhesion method is one of the commonest methods to isolate MSCs based on the ability of plastic adhesion. 5-azacytidine (5-Aza), dexamethasone (DXMS), hydrocortisone (HC) and horse serum had been proved the potential to induce the myogenic differentiation of MSCs. However, the myogenic differentiation of MSCs is still poorly understood in chicken. In present study, we isolated chicken mesenchymal stem cells (cMSCs) from bone using 4-hour differential adhesion method and analyzed the myogenic effect of cMSCs treated with different method based on 5-Aza, DXMS, HC and horse serum.

Results: cMSCs isolated by 4-hour differential adhesion method expressed MSCs special surface markers and presented normal growth characteristic. cMSCs showed great potential of myogenic differentiation by the treatment of 5-Aza and horse serum. RNA-sequence, GO and KGEE enrichment analysis revealed that this effect might be based on demethylation of 5-Aza and ECM-receptor interaction, focal adhesion, PI3K-Akt, p53, TGF-beta signaling pathways. Moreover, DXMS, HC and horse serum also presented potential of myogenic differentiation, but the effect was not as good as 5-Aza and horse serum method.

Conclusions: cMSCs showed potential of myogenic differentiation by the treatment of 5-Aza and horse serum or DXMS, HC and horse serum.

Background

In recent years, the poultry meat marker has been regarded as one of the most important sections of the food industry with an increasing consumption [1–2]. However, there is increasing concern about poultry meat quality and the demand of high-quality poultry meat is rising [3]. It's pretty significant for breeders to regulate muscle development to improve poultry meat quality.

Mesenchymal stem cells (MSCs), one kind of adult stem cells, have a great ability to differentiate into multiple tissue such as muscle, bone and fat [4]. Studying on the multilineage differentiation of chicken mesenchymal stem cells (cMSCs) can provide molecule basis of muscle development, fat deposition, skeletal growth and other physiological process [5]. MSCs can be characterized by detecting the expression of special cell surface marker antigens like CD71, CD90, CD29, CD44 while remaining negative for CD45, CD31, CD34, CD105 [6]. MSCs are relatively easier to isolate from bone marrow due to their adherence to plastic and expansion in culture [7]. Therefore, differential adhesion method is one of the commonest methods to isolate chicken MSCs (cMSCs) from bone marrow, but the time of differential adhesion are usually up to 24 h in previous study [8–10]. This is the first study to shorten the time to 4 h, which greatly improved the efficiency of isolation.

MSCs can differentiate into various cell linages in vitro when being treated with special factors such as cytokines, growth factor and inductive agents [11–14]. Myogenic differentiation is one of the most important applicants in MSCs, which can present critical insight of muscle development [15]. Wakitani et

al. provided support for the suggestion that MSCs treated with 5-azacytidine (5-Aza) provided a source for myoprogenitor cells in vitro [16]. Recently investigators have examined the important of the promoting effect of 5-Aza on the expression of muscle-specific genes and proteins in MSCs in culture [17–18]. Up to now, far too little attention has been paid to use 5-Aza for myogenic differentiation of cMSCs. Adhikari et al. firstly used dexamethasone (DXMS), hydrocortisone (HC) and horse serum for myogenic differentiation of cMSCs, but this work have not treated cMSCs in much detail [5]. The main aim of the present study was to evaluate the effect of myogenic differentiation of cMSCs being treated with two methods based on the use of 5-Aza, DXMS, HC and horse serum. In the first method, 5-Aza and horse serum were used to treat cMSCs for 3 days, 5 days and 7 days. In the second one, 5-Aza were changed to DXMS and HC. The experimental flow is shown below (Fig. 1).

Methods

Ethics statement

The animal experiment performed in this study satisfied the requirements of the Institutional Animal Care and Use Committee at the South China Agricultural University (approval ID: 2021-C018).

Animal and cells

1 to 14-day-old chicken used in cell isolation were purchased from Xufeng Farming Co., Ltd. (Kaiping, China).

Chicken macrophage cell line (HD11) used as positive control in the identification of cMSCs was from Guangdong Provincial Key Laboratory of Animal Health Aquaculture and Environmental Control.

Isolation of cMSCs

Primary cMSCs were isolated from femurs and tibia in 1 to 14-day-old chicken. 4-hour differential adhesion method was used in this study. Chickens were killed and sterilized with 75 % alcohol for 5-10 min. The legs of chicken were dissected in super-clean worktable, soaked in 75 % alcohol for 5 min and kept in serum-free Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher, MA, USA) for dissection. Getting rid of muscles and connective tissues sticked to femurs and tibia bones, and then removing bone epiphysis to expose bone marrow cavity. Marrow cavity was flushed using serum-free DMEM in order to collect total cells existed in marrow cavity. The medium containing bone marrow cells were filtered with 70 μm sterile strainer (Corning, NY, USA) and the filtrate was centrifuged at 1000 rpm for 5 min to get rid of plasma and lipids. Discarding supernatant and using 5 ml growth medium (GM, Dulbecco's Modified Eagle Medium: F-12 (DMEM-F-12) (ThermoFisher, MA, USA) containing 10 % fetal bovine serum (FBS) (ThermoFisher, MA, USA), 100 U/mL penicillin (ThermoFisher, MA, USA) and 100 g/mL streptomycin (ThermoFisher, MA, USA)) to resuspend cell layer. Cells were plated in 60-mm cell culture dishes and were

marked as P0 (passage 0). The medium was changed completely after 4 hours in order to remove the non-adherent cells (mainly non-MSCs cells).

Cell culture and subculture

Cultures were incubated at 37 ℃ in a humidified incubator containing 5 % CO2. When cMSCs at P0 reached 90 % confluently, the cells needed to be subcultured. Cells were washed by PBS (ThermoFisher, MA, USA) firstly, and dissociated with 0.25 % Trypsin-EDTA (ThermoFisher, MA, USA) for 2.5 min. After that, adding 5 ml GM to stop dissociation, and then collecting cell suspension to centrifuge at 1000 rpm for 5 min. Finally, using 10 ml GM to resuspend cell layer, and then subculturing at new cell culture dishes. The sub-culture cMSCs were named as P1 (passage 1), and subsequent passaged cells were named as P2, P3, P4.

Growth curve assay

cMSCs were cultured in 24-well plates with 2 x 10^4 cells/ml per well in advance. In the next 8 days, cells were dissociated by 200 μL 0.25 % trypsin-EDTA for 2.5 min. After that, adding 400 μL GM to stop dissociation and prepare cell suspension. Countstar Automated Cell Counter (ALIT Life Science, Shanghai, China) was used to calculate the cell number of cMSCs on 3 wells daily for 8 days. Every well was counted three times to get the mean value.

RNA extraction and cDNA synthesis

Total RNA of cells was extracted with RNAiso Plus (Takara, Kyoto, Japan) and HiPure Unviersal RNA Mini Kit (Magen, Guangzhou, China), following the manufacturer's protocol. cDNA was synthesized using MonScript™ 5× RTIII All-in-One Mix kits (Monad, Jiangsu, China) for reverse transcription. RNA samples (50 ng-1 μg) were subjected to thermocycling at 37 ℃ for 2 min, followed by 55 ℃ for 15 min, 85 ℃ for 5 min to synthesize cDNA.

Reverse transcription PCR (RT-PCR)

cMSCs were plated in 6-well plates to culture. Cells were harvested to extract total RNA and synthesize cDNA. cDNA sample was subjected to PCR amplification using 2×EasyTaq® PCR SuperMix for PAGE (TransGen Biotech, Beijing, China), and were subjected to thermocycling at 98 ℃ for 3 min, followed by 35 cycles of 98 ℃ for 10 s, 56 ℃ for 10s and 72 ℃ for 10s and followed by 72 ℃ for 2 min.HD11 was employed as positive control, and housekeeper gene *GAPDH* was employed as an internal control. PCR produced were separated by 1.5% agarose gel electrophoresis to visualize the band was detected using GoldView II Nuclear Staining Dyes (Solarbio, Beijing, China). Primers for each marker gene were designed

and checked for target identity using the National Center for Biotechnology Information (NCBI). The information of primers used for RT-PCR assays was presented in **Table 1**.

Immunofluorescence (IF)

cMSCs were plated in 12-well plate for immunofluorescence assay to detect cell surface markers CD44 and CD45. HD11 was employed as positive control in the detection of CD45. Cells were washed with PBS to get rid of medium. Cells were fixed by 20-minute incubation in 4% formaldehyde, washed with PBS three times. The fixed cells were incubated with 0.1% Triton X-100 in PBS for 15 min, washed with PBS three times. After that, cells were blocked with PBS containing 10% goat serum (Beyotime, Shanghai, China) for 30 min and incubated overnight with primary antibody diluted with Immunol staining primary antibody dilution buffer (Beyotime, Shanghai, China). Primary antibodies were directed against cell surface markers CD44 (1:700, Southern Biotech) and CD45 (1:700, Invitrogen). After rinsing with PBS three times, the cells were incubated with the secondary antibody (1:1000, Abcam) for 1 h at room temperature. After rinsing with PBS three times, cells were incubated with PBS containing 10% DAPI staining solution (Beyotime, Shanghai, China) for 5 min and rinsed with PBS three times again. Cells were observed in epifluorescence microscope (Leica DMi8).

Myogenic induction

The first method is that cMSCs were exposed for 3 days to DMEM-F12 medium containing 10 μM 5-Aza (Macklin, Shanghai. China) and 2 % horse serum (Solarbio, Beijing, China) followed by 2-4 days of further culture in DMEM-F12 medium containing 2 % horse serum (Solarbio, Beijing, China).

The second method is that cMSCs were exposed for 3 days to DMEM-F12 medium containing 0.1 μM DXMS (Sigma-Aldrich, MO, USA), 50 μM HC (Solarbio, Beijing, China) and 2 % horse serum followed by 2-4 days of further culture in DMEM-F12 medium containing 2 % horse serum.

Quantitative real-time PCR (qPCR)

cMSCs was plated in 12-well plates. Total RNA extraction and cDNA synthesis were following the method described above. cDNA samples were subjected to ChamQ Universal SYBR qPCR Master Mix (Vazyme Biotech, Nanjing, China) following the manufacturer's protocol. 2^-ΔΔCt method and internal normalization were used to analyze quantification results. GAPDH was employed as housekeeper gene. Primers of muscle-specific genes were designed in NCBI and the information of primers was presented in **Table 1.**

RNA sequencing

Treated cells and control cells were collected to extract total RNA using Trizol reagent kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Three biological replicates were performed for both of groups. RNA quality was assessed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase free agarose gel electrophoresis. After that, eukaryotic mRNA was enriched by Oligo(dT) beads. Then the enriched mRNA was fragmented into short fragments using fragmentation buffer and reverse transcripted into cDNA with random primers. Second-strand cDNA were synthesized by DNA polymerase I, RNase H, dNTP and buffer. Then the cDNA fragments were purified with QiaQuick PCR extraction kit (Qiagen, Venlo, The Netherlands), end repaired, poly(A) added, and ligated to Illumina sequencing adapters. The ligation products were size selected by agarose gel electrophoresis, PCR amplified, and sequenced using Illumina HiSeq2500 by Gene Denovo Biotechnology Co. (Guangzhou, China)

The RNA-sequence data reported in this study was archived in the NCBI SRA database with the accession number PRJNA756416.

Alignment of RNA-sequence reads onto reference genome and expression analysis

RNAs differential expression analysis was performed by DESeq2 software between two different groups. The genes with the parameter of false discovery rate below 0.05 and absolute fold change ≥2 was considered DEGs.

GO and KEGG enrichment analysis

Gene ontology (GO) enrichment analysis provided all GO terms that significantly enriched in DEGs comparing to the genome background, and filtered the DEGs that correspond to biological functions. Firstly, all DEGs were mapped to GO terms in the GO database (http://www.geneontology.org/). Gene numbers were calculated for every term, and significantly enriched GO terms in DEGs were defined by hypergeometric test. The calculated *p*-value were gone through false discovery rate correction, taking false discovery rate ≤ 0.05 as a threshold. GO terms meeting this condition were defined as significantly enriched GO terms.

KEGG enrichment analysis identified significantly enriched metabolic pathways or signal transduction pathways in DEGs comparing with the whole genome background. The calculated *p*-value was gone through false discovery rate correction, taking false discovery rate ≤ 0.05 as a threshold. Pathways meeting this condition were defined as significantly enriched pathways in DEG.

Statistical analysis

All experiment in this study were repeated 3 times at least to ensure repeatability and all data are expressed as means ± SEM (standard error of the mean). Independent sample t.test was used to compare differences between two group and P 0.05 was considered as statistically significant between the group. All statistical analyses were performed using SPSS 23.0 for Window (SPSS, Inc., Chicago, IL, USA). Symbol "*" indicates significant difference at $P < 0.05$ and "ns" indicates significant difference at $P > 0.05$.

Results

cMSCs were isolated by 4-hour differential adhesion method.

Isolating $2x10^6$ cMSCs needed 1 chicken and the success rate of this modified method was 80 % overall in this study. Chicken was soaked in alcohol and was dissected in super-clean worktable (Fig. 1a). The legs were soaked in alcohol and serum-free DMEM (Fig. 1b-c). Muscles, connective tissues and bone epiphysis in femurs and tibia were removed, and then using serum-free DMEM to flush the bone marrow cavity (Fig. 1d-f). Finally, the culture was filtered and centrifuging the filtrate to harvest total cell.

The total cell contained cMSCs, hematopoietic stem cells, macrophagocyte, fibroblast, and so on. 4-hour differential adhesion method was used to isolate cMSCs from total cells. After 4-hour differential adhesion, enough cMSCs were isolated and cultured, and the primary cells just needed 10 days to reach the confluence of 90 %. Once the time was reduced to 2 hours, few cMSCs were collected (Fig. 1g). When the time was increased to 24 hours, non-cMSCs were hardly to remove clearly (Fig. 1i).

cMSCs expressed special cell surface markers genes and protein of MSCs.

The RT-PCR analysis revealed that cMSCs expressed *CD71*, *CD90*, *CD29*, *CD44*, and the mRNA of non-MSCs surface marker *CD45*, *CD31*, *CD34* didn't express (Fig. 3a). The immunofluorescence analysis further determined that cMSCs expressed CD44 but not CD45. It suggested that cMSCs isolated by 4-hour differential adhesion method were homogeneous and possessed the biological characteristics of MSCs.

cMSCs showed normal morphological characteristic and growth curve of MSCs

cMSCs had mainly three cell shapes: round cells, spindle cells and polygonal cells during proliferation. After subculture, round cells were in the majority at the beginning. Subsequently, the spindle and polygonal cells increased and round cells almost disappeared. Sub-cultured cMSCs reached 50 % confluence in 4 days, 80 % confluence in 6 days and 100 % confluence in 8 days (Fig. 4a). In growth curve, cMSCs showed a latent phase of 1–4 days, a logarithmic growth phase for 4–7 days. When culture up to 8 days, the number of cells began to decrease due to excessive cell density and insufficient nutrients probably. (Fig. 4b). The results demonstrated that cMSCs isolated by 4-hour differential adhesion method showed normal growth characteristic of MSCs.

cMSCs treated with 5-Aza and horse serum presented great potential of myogenic differentiation

cMSCs treated with 5-Aza and horse serum presented the great promoting effect of the mRNA expression of myogenic genes. The mRNA expression of *MyHC Myomaker*, *MyoD1* and *MyoG* presented the trend of earlier increase and later decrease, and *Desmin* showed increase trend with increase of induced time (Fig. 6a-d). During the induced period, the expression of myogenic marker genes was significantly higher than control cells especially being treated with 5-Aza and horse serum for 5 days. The relative mRNA expression of *MyHC Myomaker*, *Desmin*, *MyoD1* and *MyoG* reached ten-fold, six-fold, twenty-fold, two-fold and four-fold increase when cells were treated for 5 days.

cMSCs treated with DXMS, HC and horse serum showed part potential of myogenic differentiation

cMSCs treated with DXMS, HC and horse serum also showed the effect of myogenic differentiation, but the effect was not as good as cells treated with 5-Aza and horse serum (Fig. 5i). During the induced period, the expression of *MyHC*, *Myomaker* and *Desmin* presented significant increase trend (Fig. 6e-h). It indicated that DXMS, HC and horse serum affected the myogenic differentiation of cMSCs. Another unanticipated finding was that the expression of *MyoD1* and *MyoG* in induced cells were not significantly higher than control cells when treating for 3 and 5 days, and the expression of *MyoG* even presented significantly lower than control cells when treating for 7 days.

The GO and KEGG enrichment analysis of DEGs regulated by the treatment of 5-Aza and horse serum

The result showed that 1244 DEGs were up regulated and 1158 DEGs were down-regulated in the control group versus the 5-Aza-horse serum treated group (Fig. 7a). Significant different were presented in these DEGs (Fig. 7b). The most interesting aspect of the data is that a large number of muscle-specific genes were regulated by the treatment (Fig. 7c). These genes played important roles in myogenic differentiation, and some of them were related to cardiogenic phenotype. In GO enrichment analysis, total 519 GO terms were enriched with 2402 DEGs significantly. 201 terms were cellular component, 253 terms were molecular function and 65 terms were biological process. DEGs mainly enriched in 54 terms which were shown in Fig. 7d. In cellular component, DEGs significantly enriched in the terms of plasma membrane and chromosome (Fig. 7e). In molecular function, most DEGs were enriched in the category of metabolism such as ion and anion binding, adenyl nucleotide/ribonucleotide binding, ATP binding and so on (Fig. 7f). In biological process, chromosome organization was significantly regulated (Fig. 7g). KEGG enrichment analysis demonstrated that 58 pathways enriched 2402 DEGs. ECM-receptor interaction, focal adhesion, PI3K-Akt, p53, TGF-beta signaling pathway and the pathways of cardiomyopathy were significant regulated by the treatment (Fig. 7h).

Discussion

Broiler industry plays an important role in global poultry industry and China is the second largest broiler producting country in the world. It's very important for breeders to improve meat quality traits, which is a hard part for breeders and researchers. The potential multilineage differentiation of MSCs makes it become a good model to study muscle development in critical insights.

Several studies have indicated that differential adhesion method is one of the most convenient protocol to isolate MSCs, which always spent 24–48 h to change culture medium [19–20]. In this study, we first isolated cMSCs from femurs and tibia using 4-hour differential adhesion method to enhance the efficiency. According to the minimal criteria for defining MSCs in International Society for Cellular Therapy [6] and previous work in isolating cMSCs [19–20], cMSCs express MSC specific markers such as CD71, CD90, CD29, CD44 and lack expression of non-MSC markers like CD45, CD34, CD31. Comparison of our findings with the criteria and those previous studies confirms that cMSCs isolated by our method were homogeneous and possessed the biological characteristics of MSCs. In addition, cMSCs isolated by our method showed normal growth characteristic which was consistent with previous studies [21–22].

An extensive literature indicated that 5-Aza has the ability to induce and promote the myogenic differentiation of MSCs by regulating myogenic and cardiac-specific marker genes [23–26]. In present study, the myogenic differentiation potential was firstly shown in cMSCs by the treatment of 5-Aza and horse serum. The result of qPCR showed that mRNA expression level of muscle-specific genes including *MyHc*, *Myomaker*, *Desmin*, *MyoD1* and *MyoG* were significant upregulated in treated cell, which was similar with previous researches. It suggested that 5-Aza and horse serum could activate the myogenic differentiation potential of cMSCs. To further understand the change of biological processes and pathways during treated time, we identified and analyzed DEGs by high-throughput techniques and bioinformatics. First, 1244 up-regulated genes and 1158 down-regulated genes in control and treated cells were identified. The results shown that *MyHc*, *Desmin*, *MyoD1* were in line with the experimental results of qPCR, but the expression of *Myomaker*, *MyoG* did not conform to the experimental result of qPCR, which require further experiment to verify.

GO and KEGG enrichment analysis demonstrated that DEGs between control cells and cells treated with 5-Aza and horse serum were related to chromosome. Previous study had revealed that epigenetic modification played an important role in myogenic differentiation of MSCs [27]. It suggested that 5-Aza and horse serum probably converted cMSCs by demethylation of regulatory loci which leaded to a potential to myogenic differentiate. Li et al. assumed that the myogenic differentiation of MSCs treated with 5-Aza might be related to ECM-receptor interaction, focal adhesion, PI3K-Akt signaling pathway [28]. It was also proved in this study. Besides, we assumed that p53, TGF-beta signaling pathway and cardiomyopathy might also be involved in the processes. Together these results provided important insights that myogenic differentiation in cMSCs treated with 5-Aza and horse serum was possible to differentiate into cardiogenic phenotype via regulating those signaling pathways and biological possesses.

Adhikari et al demonstrated that DXMS, HC and horse serum could make cMSCs show the potential of myogenic differentiation [5]. This finding was also confirmed in this study and we further explored the myogenic effect of DXMS, HC and horse serum in different induced time. The mRNA expression of muscle-specific genes was significantly upregulated when being treated for 7 days, but the effect was not as good as 5-Aza and horse serum method. It's suggested that DXMS, HC and horse serum method had the potential to induce the myogenic differentiation of cMSCs.

Conclusions

To sum up, we first isolated homogeneous cMSCs using 4-hour differential adhesion method. cMSCs was treated with 5-Aza and horse serum could show great potential of myogenic differentiation. This effect might be related to the demethylation of 5-Aza and the pathways of ECM-receptor interaction, focal adhesion, PI3K-Akt, p53, TGF-beta signaling. In addition, we demonstrated that DXMS, HC and horse serum also had the potential to induce the myogenic differentiation of cMSCs.

Abbreviations

5-Aza: 5-Azacytidine

cMSCs: Chicken bone-derived mesenchymal stem cells

DEGs: Differentially expressed genes

DXMS: Dexamethasone

GM: Growth medium

HC: Hydrocortisone

HD11: Chicken macrophage cell line

MSCs: Mesenchymal stem cells

Myh7b: Myosin heavy chain, cardiac muscle isoform X1

MyHC: Myosin heavy chain

Mylk: Myosin light chain kinase

MYO1D: Unconventional myosin-Id isoform X4

MYO1E: Unconventional myosin-Ie isoform X1

MYO5A: Unconventional myosin-Va

MYO5C: Unconventional myosin-Vc isoform X1

MYOC: Myocilin

MyoD1: Myogenic determining factor

MyoG: Myogenin

MYOM1: Myomesin-1

MYOM3: Myomesin-3 isoform X1

MYORG: Myogenesis-regulating glycosidase

MYORG (X1): Myogenesis-regulating glycosidase-like isoform X1

MTMR7: Myosin light chain 1, cardiac muscle isoform X1

Declarations

Ethics approval and consent to participate

All animal experimental protocols were conformed to “The Instructive Notions with Respect to Caring for Laboratory Animals” issued by the Ministry of Science and Technology of the People’s Republic of China, and approved by the Institutional Animal Care and Use Committee at the South China Agricultural University (approval ID: 2021-C018).

Consent for publication

Not applicable.

Availability of data and material

All data generated or analyzed during this study are included in this published article and supplemental material.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

QN and XZ conceived and designed the study. ZZ performed the experiments, interpreted the data and wrote the paper. CZ and BC contributed significantly to analysis and manuscript preparation. SK, MM and JZ helped perform the analysis with constructive discussions. All authors read and approved the final manuscript.

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Figures

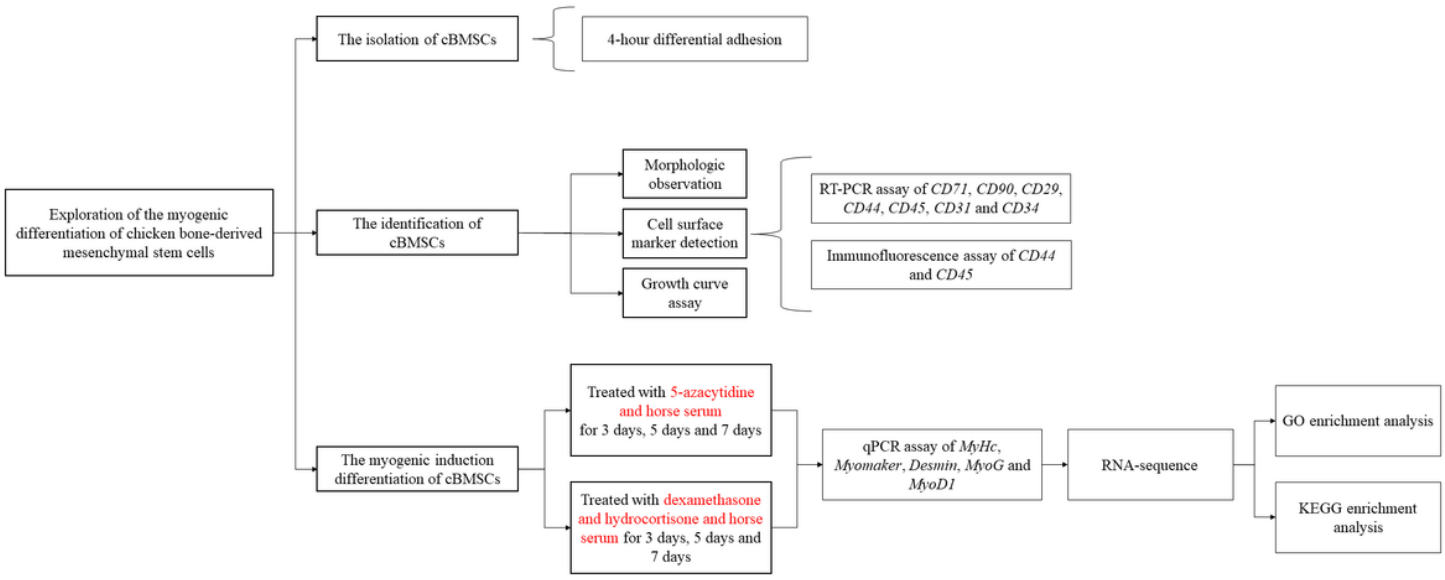

Figure 1

The experimental flow of this study. Experiments in this study can be divided into three major parts, including the isolation of cMSCs, the identification of cMSCs and the myogenic induced differentiation of cMSCs.

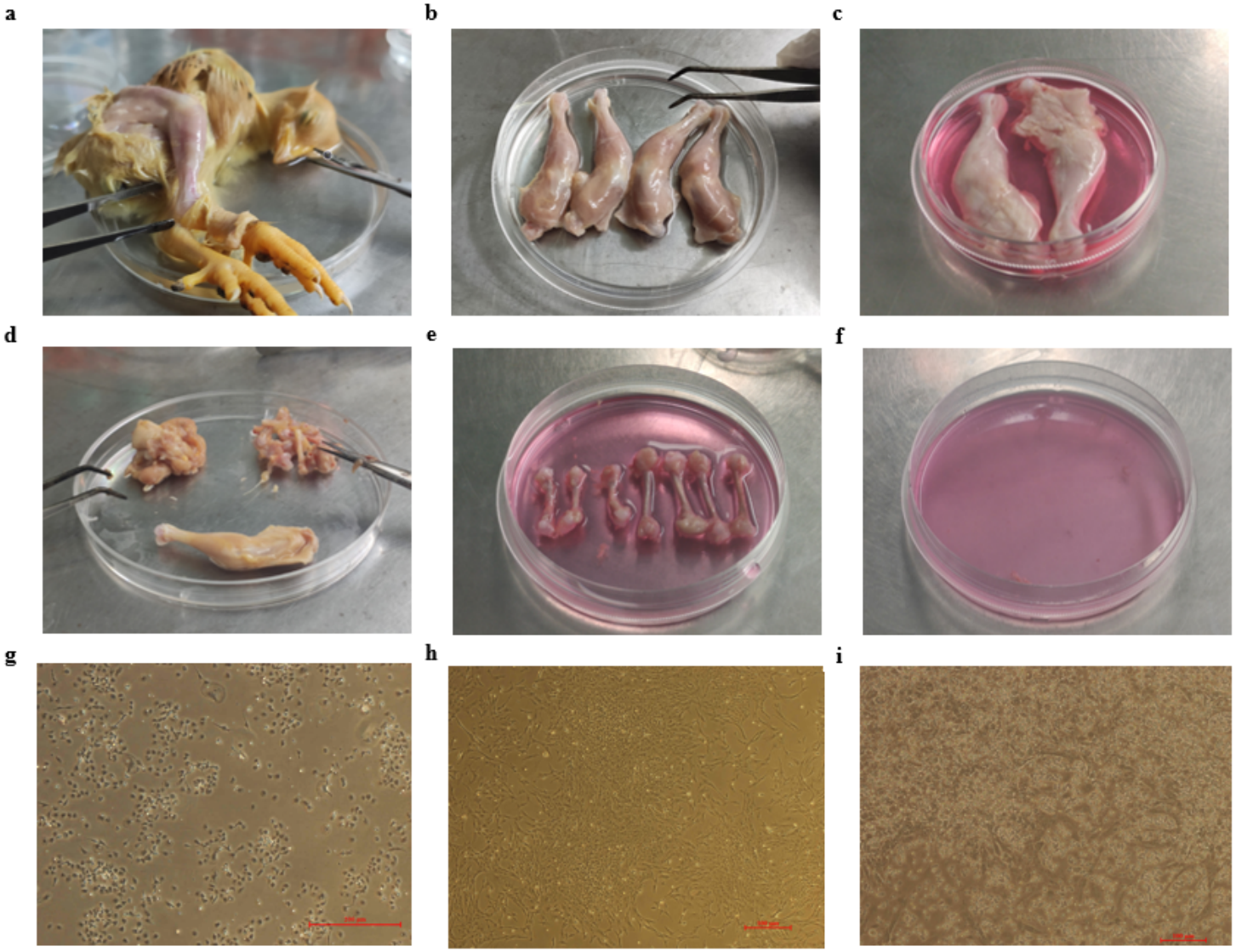

Figure 2

cMSCs isolation. (a) Chicken was soaked in 75 % alcohol for dissection. (b) Legs of chicken were soaked in 75% alcohol again. (c) After disinfection, legs were soaked in serum-free L-DMEM media. (d) Muscles and connective tissues sticked to femurs and tibia were removed by sterile scalpel. (e) Femurs and tibia were soaked in serum-free L-DMEM medium and cut off both side epiphysis to expose the bone marrow cavity. (f) Bone marrow cavity was flushed using serum-free L-DMEM to collect total cells. (g) Cells isolated by 2-hour differential adhesion were cultured for 8 days. (h) Cells isolated by 4-hour differential adhesion were cultured for 8 days. (i) Cells isolated by 24-hour differential adhesion were cultured for 8 days

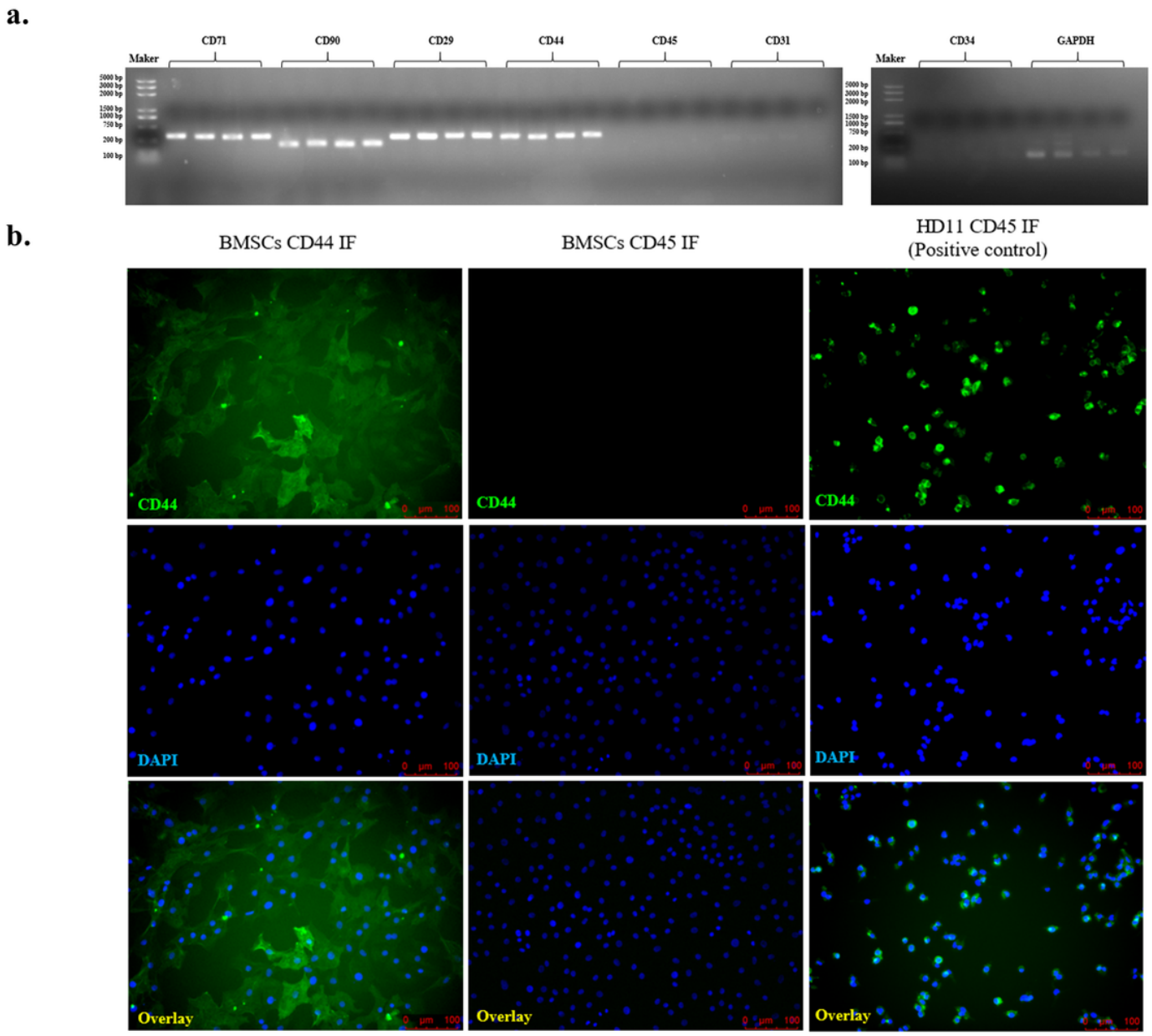

Figure 3

Special cell surface markers of cMSCs were detected by RT-PCR and immunofluorescence. (a) CD71, CD90, CD29, CD44, CD45, CD31, CD34 and GAPDH were detected by RT-RCR. The result showed that cMSCs were tested positive for CD71, CD90, CD29, CD44 and GAPDH as well as cMSCs were tested negative for CD45, CD31 and CD34. Every cell surface marker was detected four times to ensure repeatability. (b) CD44 and CD45 were detected by immunofluorescence. The result showed that cMSCs expressed CD44 and didn't express CD45. HD11 were employed as positive control in CD45 test.

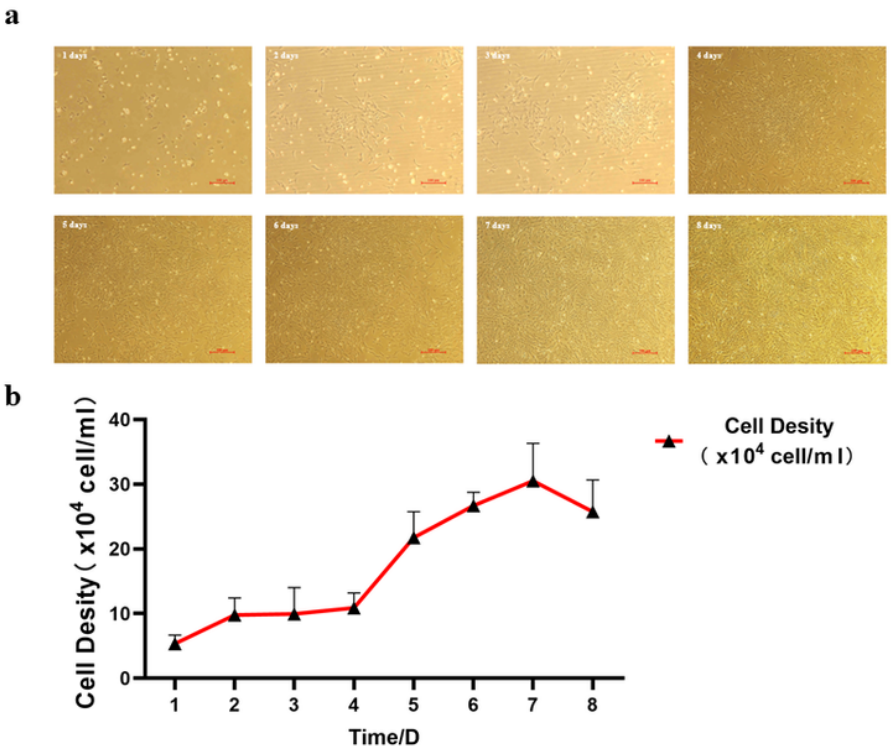

Figure 4

Morphologic observation and growth curve of cMSCs. (a) Morphology observation of cMSCs, which were observed for 8 days. (b) Growth curve of cMSCs.

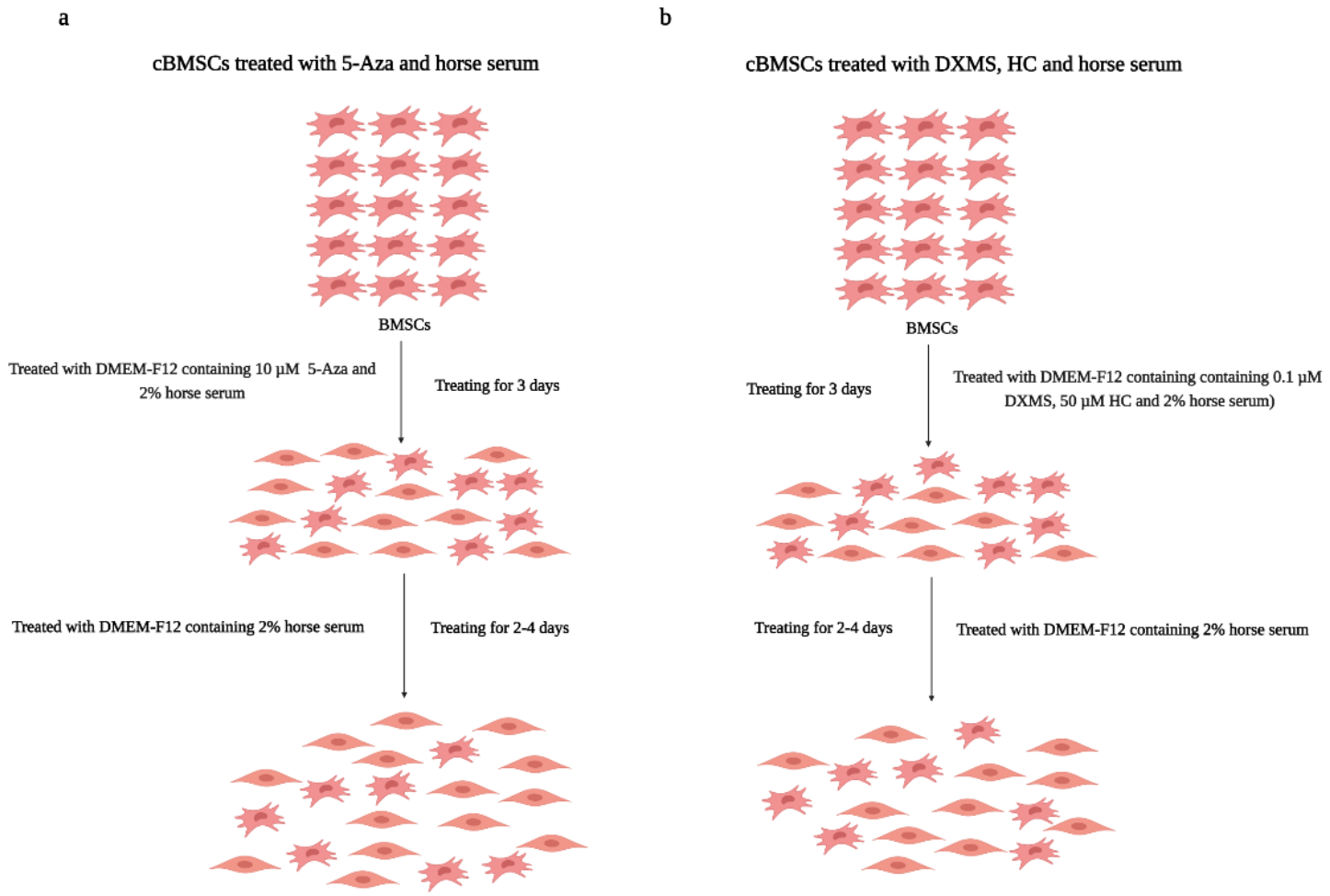

Figure 5

The methods were used in myogenic differentiation of cMSCs. The figure was created with BioRender.com (a) Using 10 μM 5-Aza and 2 % horse serum to induce the myogenic differentiation of cMSCs. (b) Using 0.1 μM DXMS, 50 μM HC and 2 % horse serum to induce the myogenic differentiation of cMSCs..

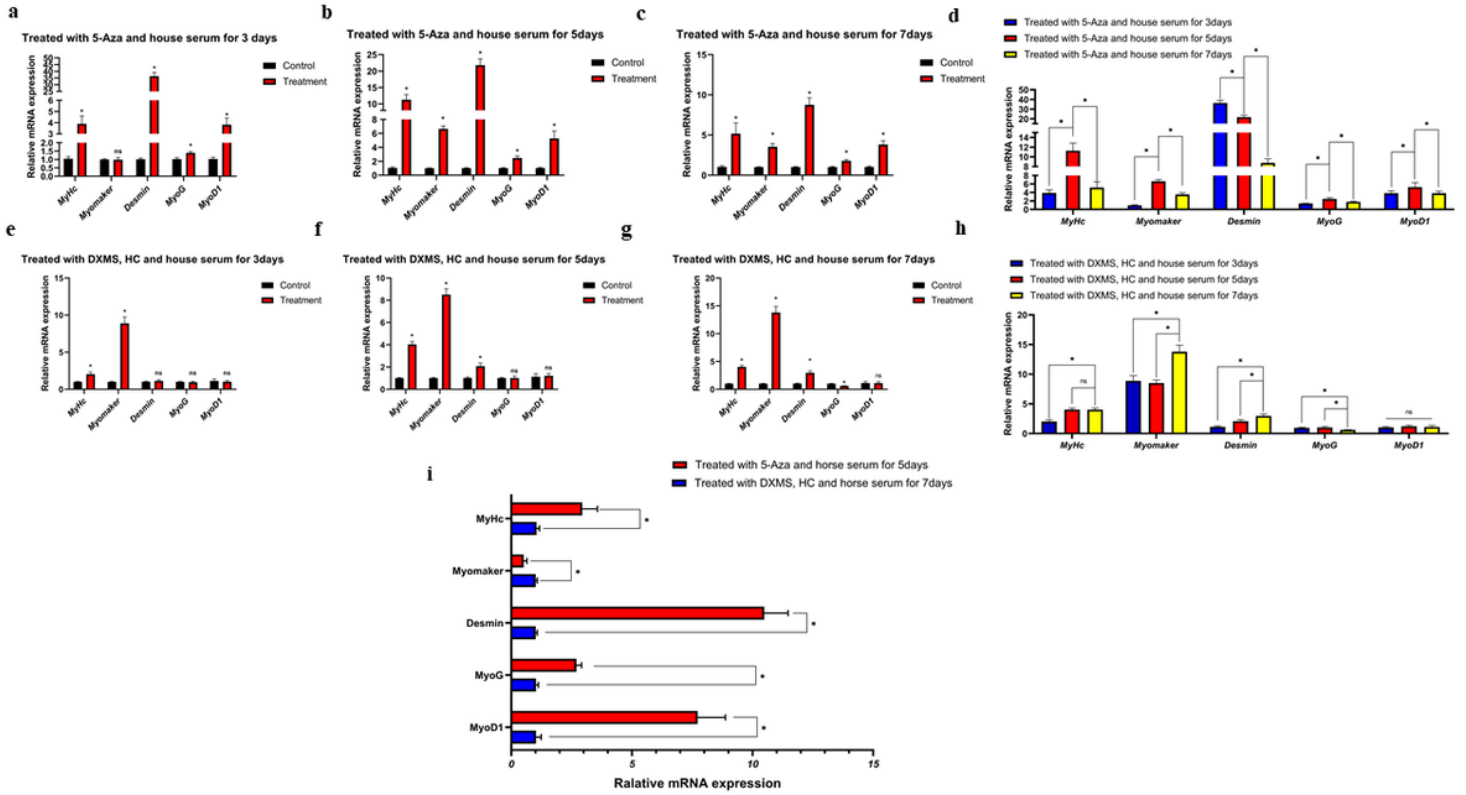

Figure 6

Relative myogenic genes expression was analyzed between myogenic induced cells and control cells. GAPDH was employed as housekeeping gene. Symbol “*” indicates significant difference at $P < 0.05$ and “ns” indicates significant difference at $P > 0.05$. (a-d) Comparative analysis of relative myogenic gene expression among control cells and cells treated with 5-Aza and horse serum for 3 days, 5 days and 7 days. (e-h) Comparative analysis of relative myogenic gene expression among control cells and cells treated with DXMS, HC and horse serum for 3 days, 5 days and 7 days. (i) Comparative analysis of relative myogenic gene expression between cells treated with 5-Aza and horse serum for 5 days and cells treated with DXMS, HC and horse serum for 7 days.

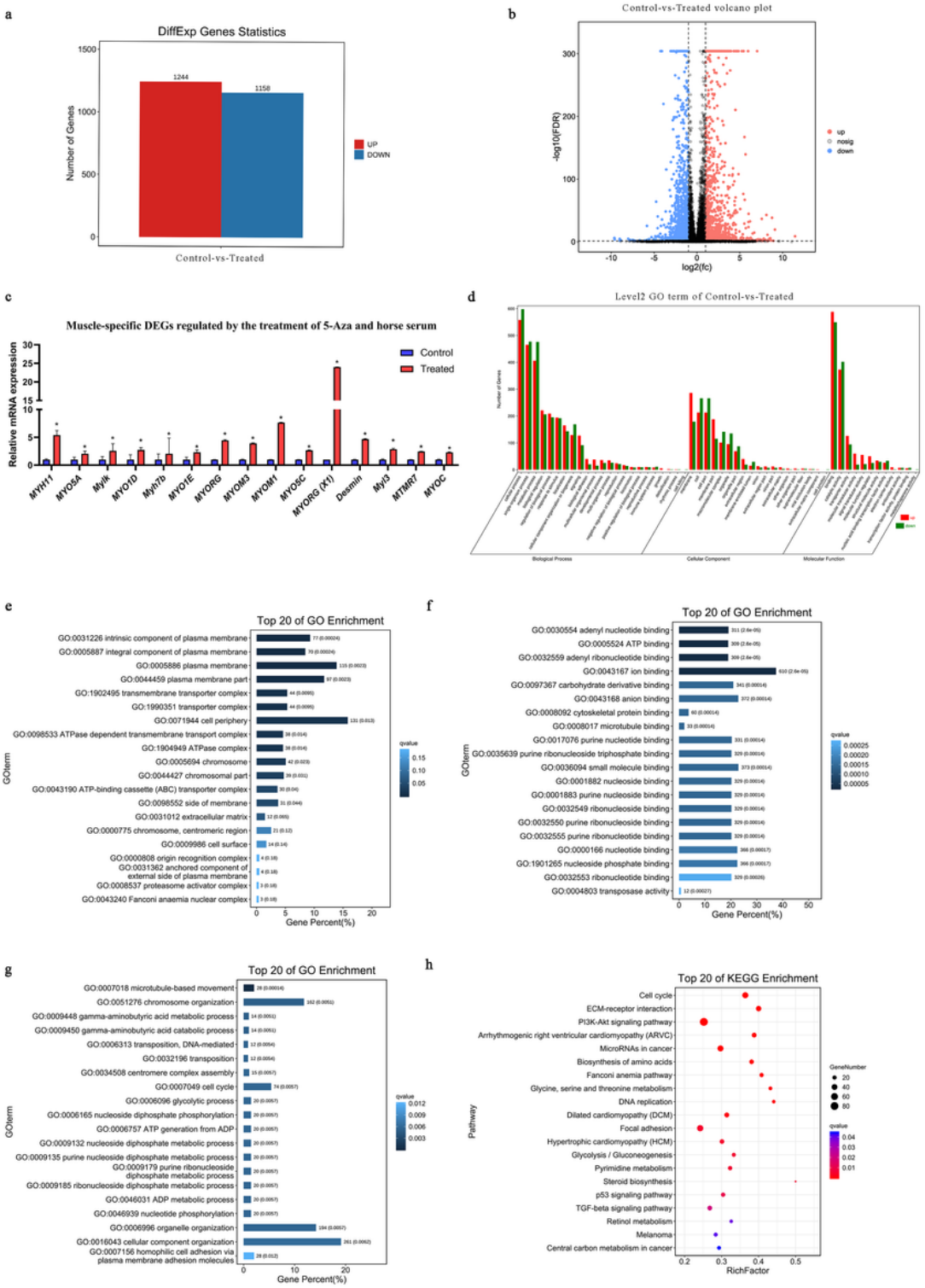

Figure 7

GO and KEGG analysis of differentially expressed cells (DEGs) selected by RNA-sequence. (a) The number of up-regulated and down-regulated DEGs in control cells vs. treated cells. (b) The volcano plots map of all DEGs in control cells vs. treated cells. (c) Muscle-specific DEGs regulated by the treatment of 5-Aza and horse serum. (d) GO enrichment analysis of up-regulated and down-regulated DEGs. (e) GO enrichment analysis of DEGs in cellular component. (f) GO enrichment analysis of DEGs in molecular

function (g) GO enrichment analysis of DEGs in biological process. (h) Pathway enrichment analysis of DEGs using KEGG database.

Supplementary Files

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- Supplementalmaterials.docx