Growth hormone receptor (GHR) can activate several signaling pathways after binding to growth hormone (GH) to regulate cell growth and development. Sex-linked dwarf (SLD) chickens, normal protein functions are prevented because of exon mutations in the GHR gene, have more severe fat deposition. However, the specific molecular mechanisms responsible for this phenotype remains unclear. We therefore investigated the effect of the GHR gene on adipogenic differentiation of chicken bone marrow mesenchymal stem cells (BMSCs). We found that bone marrow fat deposition was more severe in SLD chickens compared to normal chickens, and the expression of genes related to adipogenic differentiation was enhanced in SLD chicken BMSCs. We also detected enhanced mitochondrial function of BMSCs in SLD chickens. In vitro, overexpression of GHR in chicken BMSCs increased mitochondrial membrane potential but decreased reactive oxygen and ATP contents, oxidative phosphorylation complex enzyme activity, and mitochondrial number. Expression of genes associated with mitochondrial biogenesis and function was repressed during adipogenic differentiation in chicken BMSCs, the adipogenic differentiation capacity of chicken BMSCs was also repressed. With knockdown of GHR, opposite results were observed. We concluded that GHR inhibited adipogenic differentiation of chicken BMSCs by suppressing mitochondrial biogenesis and mitochondrial function.

Keywords: GHR, bone mesenchymal stem cells, mitochondrial biogenesis, mitochondrial function, adipogenic differentiation, sex-linked dwarf chickens
INTRODUCTION

Growth hormone (GH), a peptide hormone regulated by the hypothalamus and secreted by the pituitary gland, binds to cell surface growth hormone receptors to regulate metabolic processes and growth and development (Hu et al., 2021). Growth hormone receptor (GHR), a member of the type I cytokine receptor family, is the key receptor transmembrane protein in the GH–GHR axis. The GHR protein has three primary domains: extracellular, single-pass transmembrane, and cytoplasmic intracellular (Dehkhoda et al., 2018). In sex-linked dwarf (SLD) chickens, normal protein functions are prevented because of exon mutations in the GHR gene, compared with normal chickens (Burnside et al., 1991). Sex-linked dwarf chickens are short, weigh only 60–70% of normal chickens, and have higher feed utilization than normal but also more severe fat deposition (Guillaume, 1976). The molecular mechanisms responsible for the SLD phenotype are not clearly understood.

GH can regulate mitochondrial respiration by binding to the Box1 region of the GHR gene (Perret-Vivancos et al., 2006). In GHR knockout (GHRKO) mice, mitochondrial function and antioxidant capacity increase (Brown-Borg et al., 2009). Mitochondrial biogenesis maintains mitochondrial homeostasis and function by producing new mitochondria (Ploumi et al., 2017), and in GHRKO mice, key regulators of mitochondrial biogenesis increase in liver, kidney, and skeletal muscle (Gesing et al., 2011a; Gesing et al., 2011b). However, those results conflict with those of other reports. Mitochondrial function is severely impaired in osteoblasts and fibroblasts of GHRKO mice (Liu et al., 2019), and deficiency of GHR function impairs mitochondrial function in chicken skeletal muscle and DF-1 cells (Hu et al., 2019). Such conflicting results suggest that the effects of GHR on mitochondria vary by species and tissue.

Bone mesenchymal stem cells (BMSCs), also known as bone marrow-derived mesenchymal stem cells, are multipotent stromal cells with self-renewing ability and multilineage differentiation (Muruganandan et al., 2009). They can differentiate into many different types of cells, including adipocytes, osteoblasts, chondrocytes, myocytes, and neurons (Attia and Mashal, 2020). Most current research on BMSCs focuses on both adipogenesis and osteogenesis. Adipogenic and osteogenic differentiation of BMSCs is regulated by multiple signaling and transcription factors (James, 2013). In general, adipogenic differentiation and osteogenic differentiation in BMSCs are mutually exclusive, with stimulation of osteogenesis suppressing adipogenesis and vice versa (Yang et al., 2014). When expression of peroxisome proliferator-activated receptor-γ (PPARγ) is suppressed, the ability of BMSCs to differentiate toward adipogenesis diminishes, whereas the ability to differentiate toward osteogenesis increases (Li et al., 2017). In GHRKO mice, expression of genes associated with adipogenic differentiation increases in mesenchymal stem cells (MSCs), and the ability to differentiate toward adipogenesis also increases (Olarescu et al., 2015). However, specific mechanisms of the GH–GHR axis and adipogenic differentiation of BMSCs are unclear.

During proliferation of MSCs, the primary metabolic mode is glycolysis, whereas during their differentiation, the primary mode shifts from glycolysis to mitochondrial-based oxidative phosphorylation (Hsu et al., 2016). Reactive oxygen species (ROS) produced by mitochondrial complex III are required to initiate adipocyte differentiation (Tormos et al., 2011). A key regulator of mitochondrial biogenesis, PGC1α, promotes adipogenic differentiation and inhibits osteogenic differentiation in immortalized human MSCs (Huang et al., 2011). Mitochondria are highly dynamic organelles that can be rapidly restructured to meet metabolic demands in a timely manner (Wang and Hai, 2015). Thus, mitochondria are essential in adipogenic differentiation of BMSCs.

In SLD chickens, liver mitochondrial function declines compared with that in normal chickens (Li et al., 2019). Therefore, SLD and normal chickens were compared in this study, and overexpression and knockdown of GHR in BMSCs were used to determine the effects of GHR on mitochondrial function and adipogenic differentiation of chicken BMSCs.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were performed according to the protocols approved by the South China Agriculture University Institutional Animal Care and Use Committee (approval number SCAU#00017). All animal procedures followed the regulations and guidelines established by this committee and minimized the suffering of animals.

Chickens

For in vivo experiments, the Guangdong Wenshi Southern Poultry Breeding Co., Ltd. (Guangzhou, China), provided 21-day-old yellow-feather chickens. To isolate bone BMSCs, the Yuhe Agriculture and Animal Husbandry Co., Ltd. (Guangzhou, China), provided 3-day-old chickens.

The 21-day-old yellow-feather chickens included 15 SLD chickens and 15 normal chickens. To explore molecular mechanisms of GHR in vivo and determine the cause of fatty deposits in SLD chickens, mitochondrial function, mitochondrial biogenesis, and adipogenic differentiation in chicken BMSCs were examined in those chickens. The 3-day-old normal chickens were only used to isolate BMSCs, and the BMSCs were used to study GHR effects on mitochondria and adipogenic differentiation in vitro.

Paraffin Sections and Hematoxylin and Eosin Staining.

The epiphyses of thighbone from the 21-day-old SLD and normal chickens were fixed with 10% neutral formalin for 5 days, then immersed in 10% hydrochloric acid formaldehyde solution (10 ml hydrochloric acid +10 ml formaldehyde +80 ml water) for 1 h, rinsed in running water for 2 h. The samples were dehydrated in gradient alcohol and transparent with xylene after decalcification, details as follow: 75% alcohol for 2 h; 85%...
alcohol for 2 h; 90% alcohol for 1.5 h; 95% alcohol for 2 h; anhydrous ethanol I for 2 h; anhydrous ethanol II for 2 h; alcohol benzene for 40 min; xylene I for 40 min; xylene II for 40 min; 65°C melted paraffin I for 0.5 h at 65°C; melted paraffin II for 1 h; 65° melted paraffin III for 2 h 45 min. The wax-soaked tissues were embedded in the embedding machine, a paraffin sectioning machine cut 7 to 10-μm-thick sections. After that, hematoxylin and eosin staining was routinely performed. The first step is dewaxing, the steps are as follows: xylene I for 20 min; xylene II for 20 min; 100% ethanol I for 5 min; 100% ethanol II for 5 min; 75% ethanol for 5 min; rinsing with tap water. Then stain sections with hematoxylin solution for 3–5 min, rinse with tap water. Treat the section with hematoxylin differentiation solution, rinse with tap water. Treat the section with Hematoxylin Scott Tap Bluing, rinse with tap water. The sections were then immersed in 85% ethanol for 5 min, 95% ethanol for 5 min, and stained with eosin dye for 5 min. Sections were dehydrated with ethanol and xylene in turn and finally sealed with neutral gum. All the sections were analyzed by microscope (Leica DMI8, Wetzlar, Germany).

**FROZEN SECTIONS AND OIL RED STAINING**

Epiphyseal parts of femurs of the 21-day-old SLD and normal chickens were cut off and soaked in 4% paraformaldehyde for 48 h and then switched to decalcification solution (Servicebio, Wuhan, China) for 30 days, with the solution changed every 2 days. After decalcification, tissues were placed in a 15% sucrose solution in a refrigerator at 4°C to dehydrate and sink and then were transferred to a 30% sucrose solution at 4°C to dehydrate and sink. Dehydrated tissue was placed cut side up on a sample tray and surrounded by drops of OCT embedding agent (Takara, Shiga, Japan) according to the manufacturer’s protocol. RNA was extracted from tissues or cells using RNAiso reagent (Takara, Shiga, Japan) according to the manufacturer’s protocol. RNA extraction was performed using the manufacturer’s protocol. Triglyceride was measured at 510 nm, according to the manufacturer’s protocol. Data were normalized to the control group and expressed as a percentage of the control.

**Reverse-Transcription Quantitative PCR**

RNA was extracted from tissues or cells using RNAiso reagent (Takara, Shiga, Japan) according to the manufacturer’s protocol. Concentration of RNA samples and optical density (OD) value of 260/280 were detected using a Nanodrop 2000c spectrophotometer (Thermo, Waltham, United States). Samples were stored at ~80°C for later use. For reverse-transcription quantitative PCR (RT-qPCR), cDNA was synthesized using MonScript™ RTIII All-in-One Mix with dsDNase (Monad Co., Ltd., Guangzhou, China). ChamQ Universal SYBR qPCR Master Mix (Vazyme, Guangzhou, China) was used in RT-qPCRs run on a Bio-Rad CFX96 Real-Time Detection instrument (Bio-Rad, Hercules, United States) according to the manufacturer’s protocol. The reaction procedure included initial denaturation at 95°C for 3 min, followed by denaturation at 95°C for 10 s and annealing at 60°C for 30 s, for a total of 40 cycles. At the end of the cycle, the dissolution curve was analyzed, and the detection temperature was 65–95°C. Relative gene expression was measured using RT-qPCR twice for each reaction, and β-actin was used as the control. The primers used in RT-qPCR are listed in Table 1.

**Extraction of Chicken Bone Mesenchymal Stem Cells and Cell Culture.**

Bone mesenchymal stem cells were extracted using the appropriate separation kits (TBD science, Tianjin, China) following the manufacturer’s protocol.

Bone mesenchymal stem cells from 21-day-old SLD and normal chickens were extracted by cell separation kits and cultured in vitro to the appropriate density (the first generation). Then, assays were conducted on mitochondrial function and related gene expression and protein levels.

Bone mesenchymal stem cells from 3-day-old normal chickens were extracted by cell separation kits and were cultured in vitro and passed to the third generation. Overexpression and knockdown of GHR were to explore the effects of GHR on mitochondrial and adipogenic differentiation in chicken BMSCs.

Bone mesenchymal stem cells were cultured in Gibco Dulbecco’s Modified Eagle Medium (DMEM): F-12 (Gibco, Waltham, United States) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco). All cells were cultured at 37°C in a 5% CO₂ humidified atmosphere.

**Induction of Adipogenic Differentiation**

Bone mesenchymal stem cells were seeded into 6-well plates at 1.25 × 10³ cells per cm². Bone mesenchymal stem cells were induced with adipogenic medium containing DMEM/F12 (10% FBS), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, Darmstadt, Germany), 1 μM dexamethasone (Sigma-Aldrich), 10 μg/ml insulin (Sigma-Aldrich), and 200 μM indomethacin (Sigma-Aldrich). The medium was replaced every 2 days for 6 days.
Plasmid Construction, Small Interfering RNA, and Transfection.

Third generation BMSCs were plated onto 6-well plates, and transfection began when the density reached approximately 80%. After 6 h of transfection, the DMEM/F12 medium was changed to adipogenic induction medium to induce adipogenic differentiation of BMSCs. GeneCreate (Wuhan, China) synthesized the plasmid pcDNA3.1-\textit{GHR}. Plasmid transfection was performed using Lipofectamine 3,000 reagent (Invitrogen, Waltham, United States) following the manufacturer’s protocol, and nucleic acids were diluted in OPTI-MEM (Gibco). All cells were analyzed 72 h after transfection.

Guangzhou RiboBio (Guangzhou, China) synthesized small interfering RNAs (siRNA) used for \textit{GHR} knockdown. In preliminary experiments, four siRNAs were designed to interfere with \textit{GHR}, and the si-GHR with the highest interference efficiency was used. The siRNA sequence is provided in Table 2. The si-GHR sequence was transfected in BMSCs to a final concentration of 100 nM using Lipofectamine 3,000 reagent (Invitrogen, United States) according to the manufacturer’s protocol. Cells were analyzed at 72 h after transfection.

Detection of Reactive Oxygen Species

Production of ROS in mitochondria was measured using an ROS assay kit (Beyotime, Shanghai, China) according to the manufacturer’s protocol. Dichlorofluorescein (DCF) fluorescence was determined using a Fluorescence/Multi-Detection Microplate Reader (Bio-Tek). Data were normalized to the control group and are expressed as a percentage of the control.

Detection of ATP Content

ATP levels were measured using an ATP assay kit (Beyotime) according to the manufacturer’s protocol. A Fluorescence/Multi-Detection Microplate Reader (BioTek) was used to determine ATP levels. Data were normalized to the control group and are expressed as a percentage of the control.

Detection of Mitochondrial Membrane Potential

Mitochondrial membrane potential (\(\Delta \Psi_m\)) was measured using a JC-1 kit (Beyotime) according to the manufacturer’s protocol. Mitochondria were fixed with JC-1, and after cells were incubated with JC-1 for 20 min at 37°C, fluorescence was determined using a Fluorescence/Multi-Detection Microplate Reader (Bio-Tek). Rotenone, 10 \(\mu\)mol/L, was used as a standard inhibitor of \(\Delta \Psi_m\). Data (the ratio of aggregated and monomeric JC-1) were normalized to the control group and are expressed as a percentage of the control.

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### TABLE 1 | Primer sequences in reverse-transcription quantitative PCR.

| Gene   | Primer sequences (5′–3′)                        | Temperature (°C) | Size (bp) |
|--------|-------------------------------------------------|------------------|-----------|
| GHR    | F-GCAAGTGCAAGGTCACCTGAG R-CGCGACATTTCTTCCAGCT  | 56               | 153       |
| ND1    | F-AACCCAAGGACCTCCTCCT  R-GTGCGGCGCATAATTCACCA  | 56               | 154       |
| ND2    | F-GCGAGCGATTGAAACCCAATA R-TCATTGCTCGGTGATAGAGG | 56               | 103       |
| CYTB   | F-CAGCAGACATCTCCTACC  R-GAAAGAGGCGCGCTTGG    | 56               | 104       |
| COX1   | F-CTGGGGTAAACACAGAACA R-ACTCTGCTGAGTGTTAGAGT  | 56               | 132       |
| COX2   | F-TGGGGGTAAACAGAACA  R-ACTCTGCTGAGTGTTAGAGT  | 56               | 70        |
| ATP6   | F-TCAGGCGCAACTGCTCTAC  R-AAGAACCAAGGCGCTTGG  | 56               | 123       |
| ATP8   | F-AACCCGAAACCCAATTTCTCA  R-AAGGTCTGGGGGCTTCTT | 56               | 139       |
| PGC1α  | F-TCTCTTCTCTCAAGGCGATC  R-TCTTGCAGTGAGGAGGAC  | 56               | 153       |
| NRF1   | F-AAGGAGACACTCCCTTCTCA  R-TGTTGCTGCTTCGGCTTCTT | 56               | 163       |
| TFAM   | F-GACCTCAAGGTTGGCTACAC  | 56               | 144       |
| PPARγ  | F-CCAGCAGCACTGAGCGAGTT  R-AAGGACCAAAACACATGCC  | 60               | 275       |
| CEBPα  | F-GACAGGAGGGCAACGAGAACC R-CCTGAAATGTGCCCGAGAGT | 60               | 195       |
| CEBPβ  | F-AACTGCTGACCTCGCTCT    R-CCAGAGCTGCTTGCGCTTC  | 60               | 241       |
| β-actin| F-GATATTGCTGGCGCTGGTTG  R-TCAGCGGCTGACGATCTACCTC  | 56               | 178       |

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TABLE 2 | Oligonucleotide sequence in this study.

| Fragment name | Sequence (5’–3’) |
|---------------|-----------------|
| si-GHR        | CCUCAUUGGAUACCAUA |

Detection of Enzymatic Activity of Mitochondrial Oxidative Phosphorylation Complexes

Commercial assay kits (Solarbio, Beijing, China) were used to measure enzyme activity of mitochondrial oxidative phosphorylation (OXPHOS) complexes in BMSCs according to the manufacturer’s protocol. Complex I enzyme activity was determined by the change in absorbance of NADH at 340 nm. Complex II enzyme activity was determined by the change in absorbance of 2,6-dichlorophenol indophenol at 600 nm. Enzyme activity of complex III and complex IV was determined by the change in absorbance of reduced cytochrome c at 550 nm. Absorbance was determined using a Fluorescence/Multi-Detection Microplate Reader (Bio-Tek). Data were normalized to the control group and are expressed as a percentage of the control.

Mito-Tracker Green Staining and Hoechst 33,342 Staining

Mito-tracker green staining and Hoechst 33,342 staining were used to label mitochondria and nuclei in BMSCs, respectively. At 72 h after transfection, cells were washed twice with phosphate buffered saline (PBS) and incubated with Mito-tracker green (Beyotime) for 30 min. Cells were then suspended in PBS, and 10 µL of Hoechst 33,342 dye was added (Beyotime). After washing twice with PBS, a fluorescence microscope (Nikon TE2000-U, Tokyo, Japan) was used to capture five randomly selected fields that were analyzed with NIS-Elements software.

Oil Red O Staining and Quantification

Bone mesenchymal stem cells were seeded into 6-well culture plates. After transfection and differentiation for 5 days, differentiated BMSCs were washed with PBS and then fixed with 4% formaldehyde for 30 min. Differentiated BMSCs were dyed with oil red O working solution (BBI, Shanghai, China) for 60 min at room temperature and then washed three times with PBS, according to the manufacturer’s specification. After washing, a fluorescence inverted light microscope (Leica DMi8) was used to capture images. At the end, stain in cells was extracted by isopropanol and absorbance was measured at 510 nm with a Fluorescence/Multi-Detection Microplate Reader (Bio-Tek).

Western Blot Analysis

Radio-immune precipitation assay buffer (Beyotime) with phenylmethyl sulfonyl fluoride protease inhibitor (Beyotime) was used to lyse tissue and cellular proteins. The homogenate was centrifuged at 13,000 g for 10 min at 4°C. The supernatant was collected, and protein concentration was determined immediately using a bichinchoninic acid assay protein quantification kit (Beyotime). Proteins were separated in 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and then probed with antibodies following standard procedures.

The following antibodies and their dilutions were used in western blot: mouse anti-PGC1 alpha antibody (1C1B2, 1:5,000; Proteintech, Rosemont, United States), rabbit anti-NRF1 antibody (AF7620, 1:1,000; Beyotime), rabbit anti-TOMM20 antibody (AF1717, 1:1,000; Beyotime), rabbit anti-PPAR gamma polyclonal antibody (bs-0530R, 1:1,000; Bioss, Beijing, China), rabbit anti-C/EBP alpha polyclonal antibody (bs-24540R, 1:1,000; Bioss), rabbit anti-beta-actin antibody (bs-0061R, 1:5,000; Bioss), goat anti-rabbit IgG-HRP (BS13278, 1:10,000; Bioworld, Minnesota, United States), and goat anti-mouse IgG-HRP (BS12478, 1:10,000; Bioworld).

Statistical Analyses

All experiments were performed at least three times. Data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using Student’s t-test, with statistical significance indicated as *p < 0.05, **p < 0.01, and ***p < 0.001.

RESULTS

More Severe Fat Deposition in Sex-Linked Dwarf Chicken Bone Marrow Tissue

To compare the differences in fat deposition of bone marrow tissues between SLD and normal chickens, we did pathological sections on bone marrow tissues of 21-day-old SLD and normal chickens. Hematoxilin and eosin staining of bone marrow tissues from SLD and normal chickens showed the percentage of fat in bone marrow tissues of SLD chickens was significantly higher than that of normal chickens (Figures 1A–C). Oil red O staining on bone marrow tissues of SLD and normal chickens showed the percentage of lipid droplets in bone marrow tissues of SLD chickens was higher than that of normal chickens (Figures 1D–F). Furthermore, triglyceride content in the bone marrow tissue of SLD chickens was highly significantly higher than that of normal chickens (Figure 1G). To investigate whether the cause of this phenotype in SLD chickens was due to differentiation of BMSCs, expression of marker genes of adipogenic differentiation in BMSCs of SLD and normal chickens was examined. Expression of the marker genes PPARγ, C/EBPα, and C/EBPβ increased significantly in SLD chickens (Figure 1H). The results were similar in protein level assays (Figure 1I). Thus, fat deposition in the bone marrow tissue of SLD chickens was much more severe than that of normal chickens. This result might be due to differentiation of BMSCs in SLD chickens.

Mitochondrial Function and Mitochondrial Biogenesis Were Strengthened in Sex-Linked Dwarf Chicken Bone Mesenchymal Stem Cells

Mitochondria can synthesize ATP through oxidative phosphorylation to provide a major source of energy for
adipogenic differentiation of MSCs (Zhang et al., 2013). Therefore, we speculated that mitochondria have an important role in the production of lipids in bone marrow tissue. We investigated the differences in mitochondrial function in the bone marrow tissue of SLD chickens and normal chickens. The mRNA levels of mtDNA-encoded OXPHOS-related and mitochondrial biogenesis-related genes in BMSCs of SLD and normal chickens were measured by RT-qPCR. Genes included ND1, ND2, CYTB, COX1, COX2, ATP6, ATP8, PGC1α, NRF1, and TFAM. Protein levels of PGC1α, NRF1, and TOMM20 in BMSCs of SLD and normal chickens were measured by western blot. The mRNA expression of genes was elevated significantly in BMSCs of SLD chickens (Figures 2A,B). Protein levels of PGC1α, NRF1, and TOMM20 also increased in SLD chickens (Figure 2C). Furthermore, to indicate mitochondrial function, ΔΨm, ROS production, and ATP content were examined. Compared with normal chickens, ΔΨm decreased and ROS production and ATP content increased in SLD chickens (Figure 2D–F). Therefore, in SLD chickens, changes in mitochondrial function might affect adipogenic differentiation of BMSCs and thus adipogenesis.

**Growth Hormone Receptor Represses Mitochondrial Function and Mitochondrial Biogenesis in Chicken Bone Mesenchymal Stem Cells**

To investigate the effect of GHR on mitochondrial function, BMSCs were isolated and cultured in vitro, and then, GHR was overexpressed or knocked down. Efficiency of GHR overexpression and knockdown efficiency in chicken BMSCs differentiated for 72 h was examined by RT-qPCR. Compared with the control group, GHR expression was significantly upregulated after transfection with the vector of GHR (Figure 3A) and significantly downregulated after
transfection with si-GHR (Figure 3B). Similar to in vivo experiments, mitochondrial function was examined after overexpression and knockdown of GHR in differentiated BMSCs. After overexpression of GHR in BMSCs, mRNA expression of mtDNA-encoded OXPHOS-related and mitochondrial biogenesis-related genes decreased (Figures 3C,E). Protein levels of PGC1α, NRF1, and TOMM20 showed similar results (Figure 3G). In addition, ΔΨm increased, and ROS production and ATP content decreased (Figures 3I, K, M). Opposite results were observed after knockdown of GHR (Figures 3D, F, H, J, L, N). These results suggested that GHR repressed mitochondrial function during adipogenic differentiation in chicken BMSCs.

Growth Hormone Receptor Represses the Enzymatic Activity of Oxidative Phosphorylation Complexes in Chicken Bone Mesenchymal Stem Cells

To further investigate the effects of GHR on mitochondrial function in chicken BMSCs during adipogenic differentiation, enzyme activity of mitochondrial OXPHOS complexes was examined in differentiated BMSCs after overexpression and knockdown of GHR. Enzymatic activities of OXPHOS complexes I, II, III, and IV decreased significantly in chicken BMSCs after overexpression of GHR (Figures 4A–G), whereas after GHR knockdown, enzymatic activities increased significantly (Figures 4E–H). Overall, the results indicated that GHR repressed mitochondrial function by suppressing enzymatic activity of OXPHOS complexes in chicken BMSCs during adipogenic differentiation.

Growth Hormone Receptor Reduces Mitochondrial Number by Mitochondrial Biogenesis in Chicken Bone Mesenchymal Stem Cells

The previous results showed that GHR suppressed mRNA and protein levels of critical genes for mitochondrial biogenesis, and we further explored the effects of GHR on mitochondrial biogenesis in chicken BMSCs. Mitochondrial biogenesis increases the number of mitochondria to meet intracellular energy requirements. Therefore, the effect of GHR on mitochondrial function was explored by assaying the number of mitochondria. Mito-tracker staining was used to label mitochondria, with fluorescence intensity representing mitochondrial quantity. After overexpression of GHR in BMSCs, fluorescence intensity weakened, and the number of mitochondria decreased (Figures 5A, C). After knockdown of GHR, fluorescence intensity strengthened, and the number of mitochondria increased (Figures 5B, D). Thus, in addition to repressing mitochondrial function, GHR reduced mitochondrial number and quality by repressing mitochondrial biogenesis during adipogenic differentiation of chicken BMSCs.

Growth Hormone Receptor Represses Chicken Bone Mesenchymal Stem Cells’ Adipogenic Differentiation

Finally, GHR was overexpressed and knocked down in chicken BMSCs to investigate the effects of GHR on adipogenic differentiation. Adipogenic differentiation was induced in
BMSCs, and expression of associated genes was detected by RT-qPCR. The genes were PPARy, C/EBPα, and C/EBPβ. Protein levels of PPARy and C/EBPα in chicken BMSCs differentiated for 72 h were measured simultaneously by western blot. Expression of adipogenic differentiation-related genes was significantly downregulated after overexpression of GHR (Figure 6A), whereas after knockdown of GHR, expression was significantly upregulated (Figure 6C). Protein levels of PPARγ and C/EBPα in chicken BMSCs showed similar results (Figures 6B,D). Furthermore, the oil red O test was used to measure lipid droplet content in chicken BMSCs differentiated for 5 days after overexpression and knockdown of GHR. Overexpression of GHR depressed the lipid droplet depot in BMSCs, whereas knockdown had the opposite effect (Figures 6E–H). In addition, overexpression of GHR repressed triglyceride production in BMSCs (Figure 6I), whereas knockdown of GHR (Figure 6J) produced opposite results. Thus, GHR repressed fat deposition in chickens by inhibiting adipogenic differentiation of chicken BMSCs.

**DISCUSSION**

Since their discovery in 1940, research on SLD chickens has been uninterrupted. Mutation of the GHR gene in SLD chickens interferes with binding of GH to GHR (Lin et al., 2012), and therefore, SLD chickens are a specific animal model for mutation of the GHR gene (Agarwal et al., 1995). Fat deposition is more severe in SLD chickens than in normal chickens (Guillaume, 1976). In a previous study, compared with normal chickens, red bone marrow was severely depleted and replaced by yellow bone marrow in 7-week-old SLD chickens (Li et al., 2021). It was hypothesized that the SLD phenotype was due to a functional deletion of the GHR gene. Therefore, in this study, the relation between GHR and adipogenic differentiation of BMSCs was explored.

In this study, fat deposition in bone marrow tissue of 21-day-old SLD chickens was greater than that in normal chickens, consistent with previous findings (Li et al., 2021). In addition, triglyceride content of bone marrow tissue in SLD chickens was twice as high as that in normal chickens, consistent with fat deposition. Fat in bone marrow tissue is primarily derived from adipogenic differentiation of MSCs (Hardouin et al., 2014). The balance between adipose and bone tissues in bone marrow tissue is maintained primarily by two types of MSC differentiation: adipogenic and osteogenic (Duque, 2008). When adipogenic differentiation of MSCs increases, osteogenic differentiation is relatively weakened, resulting in fat deposition (Nuttall et al., 2014). Therefore, it was hypothesized that the more severe fat deposition in SLD bone marrow tissue was due to increased adipogenic differentiation capacity of BMSCs in SLD chickens because of deficiency in normal GHR gene function. To test the hypothesis, BMSCs were extracted from SLD and normal chickens, and differences in expression of genes associated with adipogenic differentiation in the two groups of cells were examined. Expression of PPARγ, C/EBPα, and C/EBPβ in BMSCs was significantly higher in SLD chickens than in normal chickens. PPARγ, as the predominant transcription factor in adipocyte differentiation, also plays an important role in adipogenic differentiation of BMSCs. In one study, addition of the PPARγ
agonist rosiglitazone activated adipogenic differentiation of mouse BMSCs (Gimble et al., 1996). During adipogenic differentiation of MSCs, C/EBPβ, PPARγ, and C/EBPa are sequentially activated (Zhao et al., 2015).

An increasing number of studies show that regulation of mitochondrial dynamics and function is critical for successful differentiation of MSCs. Adipogenic differentiation of MSCs is accompanied by changes in the mitochondrial phenotype, including increased mitochondrial biogenesis and abundance of OXPHOS complexes (Hofmann et al., 2012). Therefore, mitochondrial function of BMSCs from SLD and normal chickens was examined by using ATP content, ROS, and ΔΨm assays. The ATP content was higher in the BMSCs of SLD chickens, indicating that deficiency in GHR function led to an increase in mitochondrial oxidative phosphorylation capacity and therefore production of ATP. Changes in mitochondrial function of BMSCs may affect adipogenic differentiation of BMSCs and ultimately increase fat deposition in SLD chickens.

To further confirm the conjecture on the mechanism of fat deposition in bone marrow tissue of SLD chickens, BMSCs were extracted from chickens for cellular verification. Expression of mitochondrial genes encoding OXPHOS and genes associated with mitochondrial biogenesis was examined after overexpression and knockdown of GHR in BMSCs. The results were consistent with those obtained in vivo. When GHR was knocked down in chicken BMSCs, ΔΨm decreased, ROS production and ATP content increased, and protein levels of PGC1α, NRF1, and TOMM20 was enhanced. The opposite result after overexpression of GHR.

FIGURE 4 | GHR represses the enzymatic activity of OXPHOS complexes in chicken BMSCs. After overexpression of GHR, enzymatic activity of complex I (A), complex II (B), complex III (C), and complex IV (D) in bone mesenchymal stem cells. After knockdown of GHR, enzymatic activity of complex I (E), complex II (F), complex III (G), and complex IV (H) in bone mesenchymal stem cells. In all panels, data are presented as the mean ± S.E.M. of three biological replicates. *p < 0.05, **p < 0.01, ***p < 0.001.
Collectively, the results suggest that GHR inhibits mitochondrial function and mitochondrial biogenesis during adipogenic differentiation in chicken BMSCs. This conclusion is also supported by enzymatic activity of complexes I, II, III, and IV after overexpression and knockdown of GHR. Enzymatic activity of complexes I, II, III, and IV was enhanced after knockdown of GHR. The opposite result after overexpression of GHR. Complexes I, II, III, and IV are important components of the mitochondrial electron transport chain and are involved in the adipogenic differentiation of BMSCs through mitochondrial oxidative phosphorylation. In one study, inhibition of the mitochondrial electron transport chain suppressed adipogenic differentiation of MSCs (Zhang et al., 2013). Mitochondrial biogenesis is regulation of the number of mitochondria through mitochondrial self-renewal in response to energy demands triggered by developmental signals and environmental stressors (Popov, 2020). Mitochondrial biogenesis increases during adipogenic differentiation of MSCs (Zhang et al., 2013). In immortalized human MSCs, overexpression of PGC-1α increases mitochondrial function and biogenesis and promotes adipogenic differentiation of MSCs (Hao et al., 2020). TFAM can bind to the mitochondrial light strand promoter and functions in mitochondrial transcription regulation (Hao et al., 2020), and knockdown of TFAM in MSCs inhibits adipogenic differentiation (Zhang et al., 2013). Furthermore, Mito-tracker staining validated the effect of GHR on mitochondrial biogenesis. The number of mitochondria decreased after overexpression of GHR, indicating that GHR inhibited mitochondrial biogenesis. The opposite result was observed after knockdown of GHR.
FIGURE 6 | GHR represses chicken BMSCs’ adipogenic differentiation. (A,B) Expression of genes \((n = 3)\) and protein levels \((n = 2)\) associated with adipogenic differentiation after overexpression of GHR. (C,D) Expression of genes \((n = 3)\) and protein levels \((n = 2)\) associated with adipogenic differentiation after knockdown of GHR. (E,F) Oil red O test and lipid droplet quantification in BMSCs differentiated for 5 days after overexpression of GHR \((n = 3)\). Scale bars = 100 µm. (G,H) Oil red O test and lipid droplet quantification in BMSCs differentiated for 5 days after knockdown of GHR \((n = 3)\). Scale bars = 100 µm. (I,J) The triglyceride (TG) contents in BMSCs after overexpression and knockdown of GHR \((n = 3)\). In all panels, data are presented as the mean ± S.E.M. of three biological replicates. *\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\).
In addition, whether GHR inhibited adipogenic differentiation of chicken BMSCs in vitro was investigated. After overexpression of GHR in chicken BMSCs, expression of differentiation-related genes, including PPARγ, C/EBPα, and C/EBPβ, was repressed and lipid droplet production and triglyceride levels decreased. Notably, PPARγ determines the direction of adipogenic differentiation of MSCs (Zhuang et al., 2016). With knockdown of GHR, opposite results were obtained. Thus, GHR can inhibit adipogenic differentiation of chicken BMSCs.

In conclusion, sex-linked dwarf chickens had severe fat deposition in bone marrow tissue than normal chickens. Increased adipogenic differentiation of BMSCs in SLD chickens was associated with increases in mitochondrial biogenesis and function and expression of genes related to differentiation. After overexpression of GHR in chicken BMSCs, mitochondrial function, mitochondrial biogenesis and adipogenic differentiation of BMSCs were repressed. The opposite results were observed after knockdown of GHR. Therefore, GHR inhibits excessive adipogenic differentiation of chicken BMSCs by repressing mitochondrial biogenesis and mitochondrial function. This suppression might explain the clinical manifestation of severe fat deposition in SLD chickens.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

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**ETHICS STATEMENT**

The animal study was reviewed and approved by the South China Agriculture University Institutional Animal Care and Use Committee. Written informed consent was obtained from the owners for the participation of their animals in this study.

**AUTHOR CONTRIBUTIONS**

CZ designed the study, wrote the manuscript, conducted the experiments, and analyzed the data. BH participated in the design of the study and wrote the manuscript. ZL, HW, YZ, and JL participated in data collection and interpretation and helped perform some of the experiments. WL and QN engaged in useful discussion and revised the manuscript. DZ and QL provided experimental animals. HL and XZ developed concepts, designed and supervised the study, and wrote the manuscript.

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