

RESEARCH ARTICLE

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# Let-7b regulates the expression of the growth hormone receptor gene in deletion-type dwarf chickens

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## Abstract

**Background:** A deletion mutation in the growth hormone receptor (*GHR*) gene results in the inhibition of skeletal muscle growth and fat deposition in dwarf chickens. We used microarray techniques to determine microRNA (miRNA) and mRNA expression profiles of *GHR* in the skeletal muscles of 14-day-old embryos as well as 7-week-old deletion-type dwarf and normal-type chickens. Our aim was to elucidate the miRNA regulation of *GHR* expression with respect to growth inhibition and fat deposition.

**Results:** At the same developmental stages, different expression profiles in skeletal muscles of dwarf and normal chickens occurred for four miRNAs (miR-1623, miR-181b, let-7b, and miR-128). At different developmental stages, there was a significant difference in the expression profiles of a greater number of miRNAs. Eleven miRNAs were up-regulated and 18 down-regulated in the 7-week-old dwarf chickens when compared with profiles in 14-day-old embryos. In 7-week-old normal chickens, seven miRNAs were up-regulated and nine down-regulated compared with those in 14-day-old embryos. In skeletal muscles, 22 genes were up-regulated and 33 down-regulated in 14-day-old embryos compared with 7-week-old dwarf chickens. Sixty-five mRNAs were up-regulated and 108 down-regulated in 14-day-old embryos as compared with 7-week-old normal chickens. Thirty-four differentially expressed miRNAs were grouped into 18 categories based on overlapping seed and target sequences. Only let-7b was found to be complementary to its target in the 3' untranslated region of *GHR*, and was able to inhibit its expression. Kyoto Encyclopedia of Genes and Genomes pathway analysis and quantitative polymerase chain reactions indicated there were three main signaling pathways regulating skeletal muscle growth and fat deposition of chickens. These were influenced by let-7b-regulated *GHR*. Suppression of the cytokine signaling 3 (*SOCS3*) gene was found to be involved in the signaling pathway of adipocytokines.

**Conclusions:** There is a critical miRNA, let-7b, involved in the regulation of *GHR*. *SOCS3* plays a critical role in regulating skeletal muscle growth and fat deposition *via* let-7b-mediated *GHR* expression.

## Background

The complete growth and development of chickens is mainly dependent on the "hypothalamus-pituitary-target organ" pathway [1,2]. Depending on the needs of the body, the hypothalamus secretes growth hormone-releasing hormone and

somatostatin. These play dual roles in the modulation and control of pituitary and growth hormone (GH) secretion [3,4]. GH circulates back to the liver *via* the blood and complexes with the GH receptor (GHR) on the liver cell surface to initiate intracellular signaling mechanisms that promote the expression of insulin-like growth factors (IGFs). IGFs circulate to the local tissues of the body through the bloodstream and promote cell growth and differentiation [5].

Skeletal muscle is the major target organ of GH. GH can act directly on the GHRs of skeletal muscle, producing paracrine and autocrine IGF-1 to regulate muscle growth and development [6,7]. Hodik and Vasilatos-

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Younken *et al.* showed that chicken GH can affect skeletal muscle cell proliferation and differentiation, regulates skeletal muscle abundance, and is involved in muscle metabolic regulation [8,9]. GHR is part of the GH-GHR-IGF growth axis, which regulates the expression of IGFs by mediating GH. Thus, it plays a role in regulating skeletal muscle growth and development.

Studies indicate that the sex-linked dwarf chicken (SLD) phenotype is caused by a mutation in the *GHR* gene. Point and deletion mutations, structural gene mutations, and mutations within the *GHR* regulatory region are all thought to be involved in conferring the SLD phenotype [10-13]. Of all these types of mutations, the deletion mutation is believed to be the main cause of this phenotype. Agarwal *et al.* found that the deletion mutation exhibited a 1.7-kb deletion between exon 10 and the 3' untranslated region (3' UTR) of *GHR* [10]. The mutation results in a decrease in the number of muscle fibers and fiber diameter [11]. Dwarf chickens also present with increased carcass lipid content, which could be a result of increased lipogenesis and decreased energy expenditure [14]. Another study suggested that in laying hens, dwarfism reduces the adipose tissue lipid mobilization and likely also reduces *de novo* lipogenesis in the liver [15]. Expression of *GHR* mRNA is significantly up-regulated in dwarf chickens compared with normal chickens [16].

Dwarf phenotypes have been found in humans, mice, cattle, pigs, and other mammals [2,17-19]. Among them, the most studied is Laron syndrome in humans. Laron syndrome is familial dwarfism that was first reported in 1966, in which the serum GH level is normal, but IGF-1 levels are low [17]. Many studies have indicated that most cases of human Laron syndrome are caused by defects in *GHR*. Various types of mutations have been noted in *GHR*, leading to GHR extra-cellular domain inactivation. All of these can affect the binding of GHR and GH, and leads to interruption of GH signal transduction and a subsequent inability for GH to play its normal role [20-29].

Karen *et al.* found that the lifespan of mice was significantly prolonged after *GHR* was knocked out, but that growth was retarded. While IGF-1, IGFBP-1, IGFBP-3, and IGFBP-4 levels were significantly lower, IGFBP-2 levels were significantly increased, indicating that *GHR* defects led to GH signal transduction obstruction, significantly affecting phenotype [30]. Mavalli *et al.* found that defective skeletal muscle development in both *GHR* and *IGF-1R* mutants was attributable to diminished myoblast fusion and associated with compromised nuclear factor import and activity in activated T cells. Both mutants exhibited impaired skeletal muscle development, characterized by reductions in myofiber number and area as well

as accompanying deficiencies in functional performance [31].

The above studies indicated that mutations in *GHR* could lead to the obstruction of normal human and animal skeletal muscle growth and fat deposition by causing GH signal transduction obstruction. However, the molecular mechanisms underlying the expression of *GHR* and its regulation of chicken skeletal muscle growth and fat deposition remain unclear.

Recently microRNAs (miRNAs) have been reported to be widespread endogenous noncoding RNA molecules involved in the regulation of gene expression [32,33]. In cells, miRNAs pair with a complementary target sequence in target mRNA 3' UTR to mediate the regulation of target gene expression [34]. These miRNAs are thought to be involved in a series of important life processes, including development, neural differentiation, cell proliferation, cell apoptosis and fat metabolism [35]. Using the loss- and gain-of-function method, Kwon *et al.* showed that miRNA-1 of the ancient muscle-specific gene in *Drosophila* regulates functions of the heart and muscle-specific genes *via* their interaction with members of the Notch signaling pathway [36]. Clop *et al.* found that a point mutation within the 3' UTR of *GDF8* in Texel sheep resulted in a target site that allowed miR-1 and miR-206 to act simultaneously. This caused a reduction in the expression of the miRNA-mediated myostatin gene (*MSTN*) post-transcriptionally, leading to muscle hypertrophy [37]. Chen *et al.* demonstrated that miR-1 promotes differentiation of myoblasts into mature muscle cells by acting on *HDAC4*, inhibiting myoblast proliferation. The miRNA miR-133 promotes myoblast proliferation through the *SRF* gene, inhibiting myoblast differentiation [38]. These studies have suggested that there is further scope for understanding molecular mechanisms that regulate *GHR* expression.

In our study, we applied microarray technology to determine the miRNA and mRNA expression profiles in the skeletal muscles of dwarf and normal chickens at different stages of development. Critical miRNAs associated with *GHR* expression and the ways in which they regulate skeletal muscle growth and fat deposition were identified.

## Results

### Differential miRNA expression profiles in skeletal muscle of dwarf and normal chickens

Using signal values greater than 32 as the standard, a total of 124 miRNAs were detected in 22.9% of skeletal muscles of 14-day-old embryos from dwarf chickens. In normal chickens, 125 miRNAs were detected at a rate of 23.1%. At 7 weeks of age, 115 miRNAs were detected in the skeletal muscles of dwarf chickens at a detection rate of 21.2%, with 116 miRNAs detected in the skeletal muscles of normal chickens (21.4%).

Expression profiles were significantly different in four miRNAs between the two different types of chickens (Table 1). In 14-day-old embryos, expression of both miR-1623 and miR-181b was significantly down-regulated in dwarf chickens compared with normal chickens. Expression of let-7b and miR-128 in 7-week-old chickens was significantly up-regulated and down-regulated, respectively.

#### Differential miRNA expression profiles in skeletal muscle at different developmental stages

Expression of 11 miRNAs was up-regulated and 18 miRNAs down-regulated in 7-week-old dwarf chickens as compared with 14-day-old embryos. Expression of seven miRNAs was up-regulated and nine miRNAs down-regulated in normal 7-week-old chickens compared with 14-day-old embryos. Of the differentially expressed miRNAs, expression of let-7b, miR-30a-5p, miR-30b, miR-99a, and miR-133b was commonly up-regulated in both dwarf and normal chickens. The expression of miR-16c, miR-92, miR-106, miR-203, miR-451, and miR-454 was commonly down-regulated (Table 2).

#### Differential mRNA expression profiles in skeletal muscle of dwarf and normal chickens

A total of 38,535 probes were used to detect mRNA, of which the probes displaying hybridization signals represented approximately 42.6–45.6% of the total. Probes lacking hybridization signals represented approximately 52.8–55.7% of the total, with 1.5–1.7% ambiguous hybridization signals. Using the normal chickens as a control group, screening of the differentially expressed genes in skeletal muscles was carried out using Significance Analysis of Microarrays (SAM) software. The screening criteria for signaling pathway analysis were that the q-value (%) was less than 5% and it showed a fold-change less than 2 (Additional file 1: Table S1).

The differential profiles in the skeletal muscle mRNA of the 14-day-old embryos showed that there were 55 genes with a greater than 2-fold change in differential expression between the dwarf and normal chickens. Of these, 33 were up-regulated and 22 were down-regulated. At 7 weeks, 173 genes had a greater than 2-fold change in differential expression between dwarf and normal chickens, with 108 mRNAs up-regulated and 65 down-regulated.

Further comparisons between 14-day-old embryos and 7-week-old normal and dwarf chickens indicated consistent up-regulation in the mRNA expression levels of five genes: *ARNT*, *BEAN*, *HSCB*, *LOC770114*, and *RCJMB04\_1j22*. There were three genes, *GHR*, *LOC772190*, and *TMEM70* that presented with consistent down-regulation in normal chickens but consistent up-regulation in dwarf chickens. The mRNA expression of *GHR* in 14-day-old embryos of dwarf chickens was up-regulated 3.57-fold compared with normal chickens, and was up-regulated 5.26-fold in 7-week-old dwarf chickens as compared with normal chickens.

#### Analysis of miRNA target genes and differentially expressed mRNA genes

Among the 34 differentially expressed miRNAs, there were some with the same seed sequence and target genes. This allowed for the classification of the miRNAs into 18 categories. There were another five miRNAs in which the target genes had not yet been discovered. The prediction results of the various types of differentially expressed miRNA target genes are shown in Additional file 2: Table S2.

We compared various differentially expressed miRNA prediction target genes with differentially expressed mRNA of genes from 7-week-old chicken skeletal muscle. In total, corresponding differentially expressed genes were found for 14 types of miRNAs (Additional file 3: Table S3). *GHR* was affected by let-7b, miR-15c, miR-16, and miR-16c.

#### Let-7b-mediated regulation of *GHR* expression

The miRNAs involved in the regulation of *GHR* were let-7b, miR-15c (miR-16, miR-16c), and miR-181b (Additional file 3: Table S3). BLAST analysis indicated that the last 29 bp of the *GHR* 3' UTR exactly coincided with the target site of let-7b. This is consistent with the proposed mechanism of miRNAs as mainly targeting the 3' UTR of target genes. However, the target sites of miR-15c, miR-16, miR-16c and miR-181b were far apart from the *GHR* region. Expression levels of let-7b were significantly up-regulated in both dwarf and normal chickens at both stages of development investigated (Table 2).

**Table 1 The miRNA differential expression profiles of 14-day-old embryos and 7-week-old chickens skeletal muscle of dwarf and normal chickens**

| Developmental stage | miRNA    | Normal chickens | Dwarf chickens | Fold changes | p-value  |
|---------------------|----------|-----------------|----------------|--------------|----------|
| 14-day-old embryos  | miR-1623 | 4,755 ± 837     | 2,184 ± 302    | 2.18         | 8.53E-03 |
|                     | miR-181b | 7,741 ± 900     | 5,284 ± 588    | 1.46         | 2.76E-02 |
| 7-week-old chickens | let-7b   | 6,137 ± 345     | 6,992 ± 299    | 0.88         | 4.77E-02 |
|                     | miR-128  | 1,455 ± 108     | 1,177 ± 99     | 1.24         | 4.87E-02 |

Note: Fold change means the change in let-7b's expression as compared normal with dwarf chickens.

**Table 2 Differential miRNA expression profiles of skeletal muscle at different developmental stages as compared the 7-week-old chickens with the 14-day-old embryos**

| miRNA          | Dwarf chickens   | Normal-type chickens  |
|----------------|--|---|
| Up-regulated   | <b>let-7b</b> , miR-24, <b>miR-30a-5p</b> , <b>miR-30b</b> , miR-30d, miR-99a, miR-100, miR-133a, <b>miR-133b</b> , miR-133c, miR-146b   | <b>let-7b</b> , <b>miR-30a-5p</b> , <b>miR-30b</b> , miR-30c, miR-99a, miR-126, <b>miR-133b</b>                                 |
| Down-regulated | miR-15c, <b>miR-16c</b> , miR-17-5p, miR-20a, miR-20b, miR-21, <b>miR-92</b> , <b>miR-106</b> , miR-130b, miR-181b, miR-200b, <b>miR-203</b> , miR-205a, miR-206, <b>miR-451</b> , <b>miR-454</b> , miR-1576, miR-1777 | miR-16, <b>miR-16c</b> , <b>miR-92</b> , <b>miR-106</b> , miR-199*, <b>miR-203</b> , <b>miR-451</b> , <b>miR-454</b> , miR-1579 |

**Signaling pathway analysis of let-7b-regulated GHR**

Assuming that the dwarf chicken phenotype in this experiment was caused by a deletion mutation in *GHR*, we used the Kyoto Encyclopedia of Genes and Genomes (KEGG) software (<http://www.genome.jp/kegg/>) to conduct a pathway analysis for *GHR*. The results indicated that *GHR* is involved in the JAK-STAT signaling pathway (Additional file 4: Figure S1).

The 111 genes involved in the JAK-STAT signaling pathway have been summarized in Additional file 4: Figure S1. These genes were compared with the differentially expressed mRNAs in 7-week-old skeletal muscles from dwarf and normal chickens. It was found that only one gene, *SOCS3*, appeared in the mRNA expression profiles. The KEGG software was also used to analyze the signaling pathway of *SOCS3* and was found to be involved in the adipocytokine signaling pathway (Additional file 5: Figure S2).

From the adipocytokine signaling pathway, it can be seen that *SOCS3* influences cellular regulation in three ways: inhibiting the *IRS1* gene through inhibition of the phosphorylation of tyrosine in insulin receptor substrate 1 (*IRS1*) and thus inhibiting the insulin signalling pathway; inhibiting the *LEPR* gene; and inhibiting the *JAK* gene.

**Quantitative polymerase chain reaction (qPCR) analysis of the GHR signaling pathway regulation by let-7b**

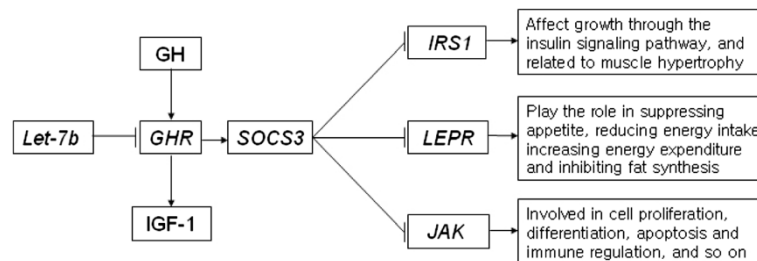
Based on the previously mentioned analyses, a schematic illustration of the *GHR* signaling pathway, as regulated

by let-7b, was developed (Figure 1). To verify the signaling pathway, we carried out qPCR assays to determine mRNA expression levels in the skeletal muscles of 7-week-old dwarf and normal chickens. The qPCR analysis showed that mRNA expression of *GHR* and *SOCS3* was up-regulated 4.24- and 2.95-fold, respectively in dwarf chickens compared with normal chickens. The mRNA expression of these genes was up-regulated 5.16- and 2.67-fold respectively, as shown by microarray analysis. Expression of *IRS1*, *LEPR* and *JAK2* was down-regulated 4.92-, 3.53- and 3.32-fold, respectively. The expression of *MYOD1*, *MyoG* and *Myf5*, which regulate skeletal muscle growth, was down-regulated 3.46-, 3.78- and 4.66-fold, respectively, in dwarf chickens compared with normal chickens. In addition, expression of *IGF1* and *IGF2BP3* in the insulin pathway is also down-regulated 6.73- and 3.97-fold respectively, in dwarf compared with normal chickens (Figure 2).

**Validation of the 3' UTR of GHR as the target site of let-7b**

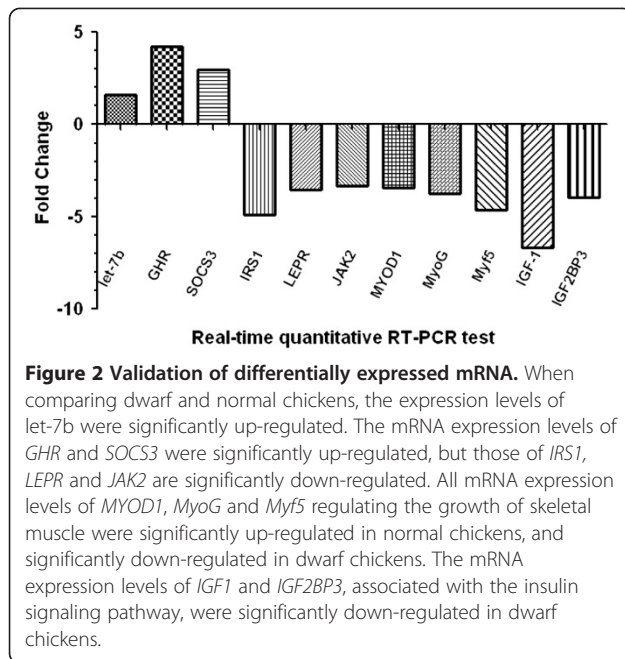
Luciferase activity was decreased in DF-1 cell lines transfected with pmirGLO-let-7b-*GHR* 3' UTR (Figure 3A), but increased in DF-1 cells transfected with pmirGLO-let-7b-*GHR* 3' UTR mutation, and in the control DF-1 cell line (Figure 3 B and C). This confirms that the expression of *GHR* is regulated by let-7b, and that the let-7b target is located in the region of the *GHR* 3' UTR mutation.

In the overexpression assay for let-7b, we observed it was up-regulated 6.41-fold as compared with the control.



**Figure 1 Schematic illustration of the signaling pathway of GHR regulated by let-7b.** miRNA let-7b inhibits the expression of *GHR* and regulates *SOCS3*. *SOCS3* regulates the growth and development of chickens through three signaling pathways: inhibiting the phosphorylation of tyrosine in *IRS1*; inhibiting *LEPR*; and inhibiting *JAK*. *IRS1*, *LEPR* and *JAK* are involved in growth, fat synthesis, and cell proliferation.





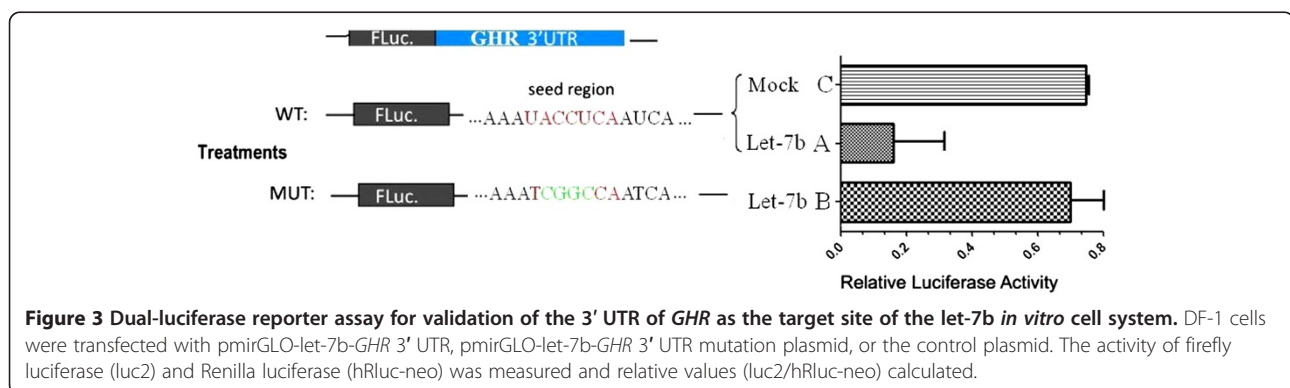
Conversely, expression of *GHR* was down-regulated 1.82-fold. Additionally, expression of *SOCS3*, stimulated by *GHR*, was down-regulated 1.39-fold (Figure 4).

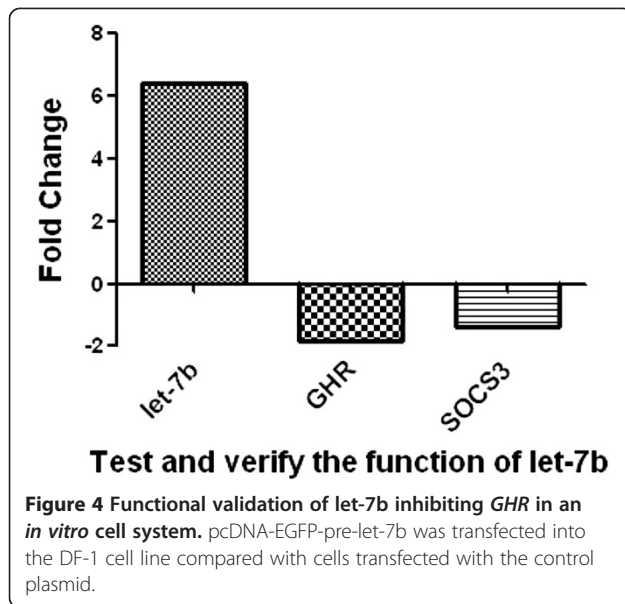
### Discussion

miRNAs are a class of non-coding small RNA molecules with a length of 18–24 nucleotides. They can direct the regulation of the expression levels of certain genes, control cell growth and development, and determine tissue type during cell differentiation by reducing the stability of target genes or inhibiting translation levels to influence cell differentiation, proliferation, and apoptosis. In animal cells, miRNAs, by interacting with a specific sequence of target gene mRNA, inhibit protein synthesis or induce mRNA degradation and post-transcriptionally negatively regulate the expression of target genes [39,40].

In this study, high-throughput microarray technology was used to analyze miRNA and mRNA expression profiles in the skeletal muscles of 14-day-old embryos and 7-week-old dwarf and normal chickens to identify miRNAs related to skeletal muscle growth and development. In chickens, 499 pre-miRNAs and 544 mature-miRNAs have been reported [41,42]. In the present study, 124 and 125 miRNAs were detected in the skeletal muscles of 14-day-old embryos from dwarf and normal chickens, respectively. We also detected 115 and 116 miRNAs in the skeletal muscles of 7-week-old dwarf and normal chickens, respectively. Such tissue-specific miRNA expression has been reported in a few previous studies [41,43-46]. Our data also showed that there is significantly different expression for only a few miRNAs at the same developmental stages in dwarf and normal chickens. However, the expression profiles of a greater number of miRNAs at different developmental stages for dwarf and normal chickens were significantly different. When comparing 7-week-old chickens with 14-day-old embryos, more down-regulated miRNAs than up-regulated miRNAs were detected. This would suggest that down-regulated expression of miRNAs is favorable for muscle growth and development in chickens at 7 weeks. In 7-week-old chickens, as compared with 14-day-old embryos, the expression of *let-7b*, miR-30a-5p, miR-30b, miR-99a and miR-133b was significantly up-regulated, but miR-16c, miR-92, miR-106, miR-203, miR-451 and miR-454 were significantly down-regulated in both dwarf and normal chickens. Considering that *GH* and *GHR* play important roles in chicken growth and development, we focused on observing the miRNAs involved in the regulation of their expression.

Four miRNAs, *let-7b*, miR-16, miR-16c, and miR-181b, are involved in the regulation of *GHR*. BLAST analysis confirmed that the target location of *let-7b* was in the deleted region of *GHR* 3' UTR. But the target locations of miR-16, miR-16c and miR-181b were distant from the deleted region. We concluded that the regulation of *let-7b* could be critical to *GHR* expression. As the





deletion mutation in dwarf chickens results in the loss of the ability of let-7b to pair with sequences in its target gene, the regulation of growth and development is affected.

Skeletal muscle growth and development in chickens is fastest at the 7-week-old stage; conversely, the growth and development of skeletal muscle during the embryonic period is relatively slow. Comparing dwarf with normal chickens, there was significantly different mRNA expression for 173 genes in the 7-week-old chickens; however, there was significantly different mRNA expression for only 55 genes in the 14-day-old embryos. For both 14-day-old embryos and 7-week-old chickens, mRNA expression of *GHR* was up-regulated 3.57- and 5.26-fold, respectively, in dwarf chickens compared with normal chickens. It is suggested that the mRNA corresponding to *GHR* was inhibited in normal chickens as reported by Wu *et al.* [16].

Comparing the different developmental stages (Table 2), expression levels of let-7b were significantly up-regulated in both dwarf and normal chickens. *GHR* expression was up-regulated in dwarf chickens and down-regulated in normal chickens, suggesting that let-7b could play a significant role in inhibiting *GHR* expression, further promoting the growth and development of skeletal muscle.

The let-7b miRNA is a member of the let-7 family. Deletion, or mutation of the function of let-7, may lead to defects in the transformation of nematodes from their larval to adult stage [44]. Methylation, post-translation modifications, and Lin28 genes regulate the let-7 family. Additionally, the family regulates *RAS*, *MYC*, *HMAG2*, *CDC25A*, *CDK2*, and other target genes that influence a variety of biological phenomena and physiological processes, especially during biological development, cell proliferation and differentiation, and

tumor suppression. There are 13 homologs in the let-7 family in the human genome, clustered into eight sites [45]. These gene clusters are located at fragile sites related to lung cancer, breast cancer, urothelial cancer, and cervical cancer, suggesting that they may act as tumor suppressors. Previous studies of the let-7 family have largely focused on tumor suppression mechanisms [46], and studies investigating the family's role in growth and development are rare.

The signaling pathway related to the regulation of the growth and development of skeletal muscle by let-7b-mediated *GHR* has not been previously reported. *GH* plays important roles in regulating animal growth and development, and its action on tissues and cells is mediated through its binding with *GHR* on the cell surface. *GHR* is activated upon binding of *GH* to stimulate the growth and metabolism of muscle, bone, and cartilage cells [3,4]. *GH* also regulates chicken growth through close binding to its receptor and activating expression of *IGF*. The amount and action of *GHR* has direct effect on *GH* physiological function. In the present study, the mRNA expression of *GHR* was significantly up-regulated in dwarf chickens compared with normal chickens. The up-regulated mRNA expression of *GHR* retarded chicken growth, probably owing to a certain compensation mechanism [16]. Our data showed that the retarded growth of dwarf chickens was caused by a deletion in the *GHR* 3' UTR inducing loss of the let-7b target site. Through signaling pathway analysis, we found that let-7b regulates the expression of *GHR*, and further regulates *SOCS3* through the JAK-STAT signaling pathway. Studies have shown that *SOCS3* can inhibit excessive cell growth and induce apoptosis as part of maintaining cell stability [47,48]. *SOCS3* regulates the growth and development of chickens through three adipocytokine signaling pathways. (1) *SOCS3* inhibits the tyrosine in *IRS1*. By inhibiting the phosphorylation of *IRS1*, *SOCS3* inhibits insulin signaling, thus affecting growth. (2) *SOCS3* inhibits *LEPR*, and up-regulated *SOCS3* expression in dwarf chickens may affect the function of leptin. Leptin has a wide range of biological effects, with an important role in the metabolic regulation center of the hypothalamus, which plays a role in suppressing appetite, reducing energy intake, increasing energy expenditure and inhibiting fat synthesis. This helps explain why dwarf chickens are more likely to be obese [14]. (3) *SOCS3* inhibits *JAK*; the JAK-STAT signaling pathway is a recently discovered signal transduction pathway stimulated by cytokines, and is involved in cell proliferation, differentiation, apoptosis, immune regulation, and many other important biological processes.

In the present study, little change in expression of let-7b between dwarf and normal chickens was observed;

however, growth was retarded in dwarf chickens. In dwarf chickens, let-7b could not inhibit the expression of *GHR*. This allows for the gene to be up-regulated as let-7b is unable to pair with *GHR* gene as its target site is deleted. Data from the microarray and qPCR analyses supported that the above pathway, indicating that the expression of *GHR* is inhibited by let-7b, and the expression of *SOCS3* gene is regulated and stimulated by *GHR*. Further qPCR data supported that *SOCS3* could inhibit the expression of *IRS1*, *LEPR* and *JAK*. The expression of *IRS1*, *LEPR* and *JAK* was significantly down-regulated, expression of genes regulating skeletal muscle growth (*MYOD1*, *MyoG* and *Myf5*) and the insulin pathway (*IGF1* and *IGF2BP3*) were also down-regulated significantly.

## Conclusions

A comparison of dwarf chickens with normal chickens at the same developmental stages revealed that expression profiles of only a few miRNAs were significantly different. In 14-day-old embryos, the expression profiles of a greater number of miRNAs were significantly different compared with those in 7-week-old chickens. By combining target gene prediction for differential miRNAs, joint analysis of mRNA expression profiles, and BLAST analysis, the critical role of let-7b in regulating the *GHR* expression was identified. With the aid of KEGG signaling pathway and qPCR analyses, the network through which let-7b-mediated *GHR* regulates growth and development of skeletal muscle as well as fat deposition was established. It was confirmed that *SOCS3* plays a critical role in inhibiting *IRS1*, *LEPR*, and *JAK*.

## Methods

### Animals

Dwarf and normal recessive White Rock chickens, both bred for nearly 10 generations, were used. Dwarf chickens had a 1 773-bp deletion mutation at the end of exon 10 and in the 3' UTR of *GHR*. The two strains were fed under the same conditions to 7 weeks of age. The weight of dwarf chickens was about 30% less than that of normal chickens. Randomly selected embryos of dwarf chickens and normal chickens were incubated for 14 d, dissected, and their sex identified according to gonad development. Nine female embryos for each chicken strain were selected for leg muscle separation. Skin and bones were removed and the muscle divided into three parts. The divided parts were placed into cryopreservation tubes then quickly placed into liquid nitrogen ( $-196^{\circ}\text{C}$ ) for preservation. Nine dwarf and nine normal chickens, fed by conventional breeding methods until 7 weeks of age, were randomly selected, and their leg muscles separated. The central muscle of the gastrocnemius was taken and divided into three parts, placed into cryopreservation tubes and quickly placed into liquid

nitrogen ( $-196^{\circ}\text{C}$ ) for preservation. All animal experiments involved in this study were approved by the Animal Care Committee of South China Agricultural University (Guangzhou, People's Republic of China). Chickens were euthanized as necessary to ameliorate suffering.

### Extraction of total RNA

Total RNA was isolated from 0.2 g of skeletal muscle tissues with TRIzol<sup>®</sup> (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions using an RNeasy MinElute Cleanup Kit. All mRNA was quantified by spectrophotometry (ND-2000, NanoDrop Inc., USA). The purity and yield of RNA was determined using optical density at 260 and 280 nm. RNA integrity was examined by electrophoresis on a 1.2% denaturing formaldehyde gel.

### Microarray analysis

Three pools of RNA were prepared for each chicken strain, with each pool containing RNA from three individuals. The miRNA chips were designed based on miRNAs listed in miRBase Version 15.0 (<http://www.sanger.ac.uk/Software/Rfam/mirna/>), and prepared by LC Sciences (Houston, Texas, USA). The miRNA chips used in the present study contained a total of 542 miRNA sequences. Normalization of chip data was carried out using the Lowess (Locally-weighted Regression) method [49], and t-tests of the data were conducted following normalization. Microarray assays for miRNAs were performed using a service provider (LC Sciences, Houston, Texas, USA). Raw data were provided as ceiling exposure limits or Excel files for subsequent statistical analysis.

Hybridization was performed with 100  $\mu\text{L}$  6 $\times$  SSPE buffer (0.90 M NaCl, 60 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM EDTA, pH 6.8) containing 25% formamide at 34 $^{\circ}\text{C}$ . Hybridization detection was facilitated using fluorescent tag-specific Cy3 and Cy5 dyes. Hybridization images were collected using a laser scanner (GenePix 4000B, Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Twelve microarray data were MIAME compliant, and the raw data were deposited in a database (ArrayExpress, GEO) with the accession number GSE37360, GSE37367 and GSE37368. Data were analyzed by first subtracting the background, and then the signals were normalized using a LOWESS filter.

The raw microarray data set was filtered according to a standard procedure to exclude spots with minimum intensity. It was arbitrarily set to an intensity parameter of P300 for mRNA expression data, and P100 for the miRNA microarray data, on both fluorescence channels. If the fluorescence intensity of one channel was below the cut-off while the other was



above, the lower channel intensity was overridden. Spots with diameters less than 25  $\mu\text{m}$  for the cDNA expression array and less than 10  $\mu\text{m}$  for the miRNA microarray and flagged spots were also excluded from the analyses. For two color experiments, the ratio of the two sets of detected signals (log<sub>2</sub> transformed, balanced) and p-values of the *t*-test were calculated. Differentially detected signals were those with p-values less than 0.01. Any false correction tests were performed for microarray data by qPCR.

The detection of mRNA expression profiles using Affymetrix's Chicken Genechip was completed by the Beijing Capital Bio Corporation (Beijing, China). The mRNA chip used in the present study contained a total of 38,535 probes. Each sample had three biological replicates, and SAM software was used for the analysis of differentially expressed genes. The screening criteria were as follows: q-value  $\leq$  5%; with a fold change  $\geq$  2; or a fold change  $\leq$  0.5.

#### Correlation analysis between miRNA and mRNA expression profiles

We combined our miRNA expression data and mRNA expression data to generate a miRNA-mRNA interaction database using target gene mapping methods and MAS software. The miRNA and mRNA expression chip profile-associated analyses combined with network predictions, estimates the target genes of differentially expressed miRNAs. To further validate microarray results, we performed qPCR experiments for representative genes. The target genes of these miRNAs were identified by qPCR.

#### Target gene prediction

TargetScan 5.1 (<http://www.targetscan.org/>) was used to carry out target gene prediction for the differentially expressed miRNA. TargetScan 5.1 proposed the concept of the 'seed region,' increasing prediction accuracy, was the software with the lowest false positive rate for predicting miRNA targets.

#### miRNA target gene and mRNA differential expression profiles

Differentially expressed miRNA prediction target gene sets were compared with the mRNA differential expression profiles from 7-week-old chicken skeletal muscle, and the target genes affected by miRNA were selected.

#### Signaling pathway analysis

KEGG is a bioinformatics database established by the Kanehisa Laboratory of the Japan Kyoto University Bioinformatics Centre [50,51]. KEGG links genome information with gene function, thereby linking genomic and functional information. In this study, the KEGG PATHWAY database (<http://www.genome.jp/kegg/>) software

platform was used for signaling pathway analysis of *GHR* regulatory networks.

#### qPCR analysis

Quantitative PCR was used to detect mRNA expression levels of the major genes in the signaling pathway. Using published genome sequences, the Primer Premier 5 software was used for primer design (Additional file 6: Table S4). In the present study, the Ct value was applied to detect the mRNA expression of the samples, and three replicates were set for each sample. The thermal cycling protocol was: 95°C for 1 min, then 40 cycles of 95°C for 15 s, appropriate annealing temperature for 45 s, and 72°C for 45 s. The final step after cycling was an extension at 72°C for 40 s. Melting curve analysis was carried out to determine the specificity of PCR products. The  $\Delta\Delta\text{Ct}$  method was used to measure gene expression with  $\beta$ -actin as the reference gene.

#### Luciferase reporter assays

Based on the data in the miRBase bank (<http://www.mirbase.org/>), primers for amplifying pre-let-7b were designed (Additional file 7: Table S5). PCR products including pre-let-7b were ligated and transformed using the pEASY-T1 Simple Clone Kit (Trans Gen Biotech, Beijing, China). Two plasmids, pcDNA3.1-EGFP (Invitrogen) and pEASY-T1-pre-let-7b, were used for constructing the let-7b expression plasmid pcDNA3.1-EGFP-pre-let-7b. Based on the data in GenBank, primers for amplifying the *GHR* 3' UTR region were designed (Additional file 7: Table S5). The plasmid pmirGLO-let-7b-*GHR* 3' UTR was prepared for verification of *GHR* mRNA expression. Two types of plasmids, the wild-type, and a mutant with *GHR* deleted were prepared. Plasmids pcDNA-EGFP-pre-let-7b and pmirGLO-let-7b-*GHR* 3' UTR were co-transfected into DF-1 cells ( $3 \times 10^4$  cells). Validation of *GHR* as the target of let-7b, and luciferase reporter assays for functional validation *in vitro* were conducted. Expression levels of *GHR* and other correlated genes were measured using qPCR analysis *in vitro*.

#### Additional files

**Additional file 1: Table S1.** The skeletal muscle mRNA differential profile of 14-day-old embryos and 7-week-old chickens of normal chickens and dwarf chickens.

**Additional file 2: Table S2.** The summary table of prediction results of the differentially expressed.

**Additional file 3: Table S3.** The intersection genes of the differentially expressed genes of miRNA target genes and mRNA genes.

**Additional file 4: Figure S1.** The JAK-STAT signaling pathway with *GHR* gene involved in KEGG links the genome information with gene function. The pathway includes 111 genes in total.



**Additional file 5: Figure S2.** Adipocytokine signaling pathway with the SOCS3 gene involved in.

**Additional file 6: Table S4.** Sequences of primers used for qRT-PCR.

**Additional file 7: Table S5.** Sequences of primers used for vectors construction.

#### Competing interests

The authors declare that they have no competing interests.

#### Authors' contributions

SL, HM, WL, YL, SW and XJ carried out experiments. SL, HL and XJ analysed data. SL, WL and XZ wrote the paper. SL, HL, QN, YL and XZ designed experiments. All authors have read and approved the final manuscript.

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