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-
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 RNA 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).

| 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.
Differential miRNA expression profiles of skeletal muscle at different developmental stages as compared to the 7-week-old chickens with the 14-day-old embryos

| miRNA     | Dwarf chickens                                                                 | Normal-type chickens                                      |
|-----------|---------------------------------------------------------------------------------|----------------------------------------------------------|
| Up-regulated | let-7b, miR-24, miR-30a-5p, miR-30b, miR-30d, miR-99a, miR-100, miR-133a, miR-133b, miR-153c, miR-146b | let-7b, miR-30a-5p, miR-30b, miR-30c, miR-99a, miR-126, miR-133b |
| Down-regulated | miR-15c, miR-16c, miR-17-5p, miR-20a, miR-20b, miR-21, miR-92, miR-106, miR-130b, miR-181b, miR-200b, miR-203, miR-205a, miR-206, miR-451, miR-454, miR-1576, miR-1777 | miR-16, miR-16c, miR-92, miR-106, miR-199*, miR-203, miR-451, miR-454, miR-1579 |

Table 2

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
physiological processes, especially during biological development, 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 by conventional breeding methods until 7 weeks of age. 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 and quickly placed into liquid nitrogen (−196°C) for preservation. 

Total RNA was isolated from 0.2 g of skeletal muscle tissues with TRIzol® (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 μL 6× SSPE buffer (0.90 M NaCl, 60 mM Na2HPO4, 6 mM EDTA, pH 6.8) containing 25% formamide at 34°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 μm for the cDNA expression array and less than 10 μm 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 (log2 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 ≤ 5%; with a fold change ≥ 2; or a fold change ≤ 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 ΔΔCt method was used to measure gene expression with β-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 × 10^5 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 miRNA 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.
References
1. Schwartzbauer G, Menon RK: Regulation of growth hormone receptor gene expression. Mol Genet Metab 1998, 63(4):243–253.
2. Hull K, Harvey S: Growth hormone resistance: clinical states and animal models. J Endocrinol 1999, 163(2):165–172.
3. Porter TE: Regulation of pituitary somatotroph differentiation by hormones of peripheral endocrine glands. Domest Anim Endocrinol 2005, 29(1):52–62.
4. Kühn ER, Geißen SM, Van der Geyten S, Darras VM. The release of growth hormone (GH): relation to the thyrotropic- and corticotropin axis in the chicken. Domest Anim Endocrinol 2005, 29(3):43–51.
5. Pierce AL, Fukuda H, Dickhoff WW: Metabolic hormones modulate the effect of growth hormone (GH) on insulin-like growth factor-I (IGF-I) mRNA level in primary culture of salmon hepatocytes. J Endocrinol 2005, 184(2):341–349.
6. Etherton TD, Bauman DE: Biology of somatotropin in growth and lactation of domestic animals. Physiol Rev 1998, 78(3):745–761.
7. Argetinger LS, Carter-Su C: Mechanism of signaling by growth hormone receptor. Physiol Rev 1996, 76(4):1089–1107.
8. Hocke V, Mett A, Halevy O: Mutual effects of growth hormone and growth factors on avian skeletal muscle satellite cells. Gen Comp Endocrinol 1997, 108(1):161–170.
9. Vasilatos-Younken R, Wang XH, Zhou Y, Day JR, McMurtry JP, Rosebrough NW, Decuypere E, Buys N, Darras V, Beard JL, Tomas F: New insights into the mechanism and actions of growth hormone (GH) in poultry. Domest Anim Endocrinol 1999, 17(2):3181–190.
10. Agarwal SK, Cogburn LA, Burnside J: Overexpression of a truncated growth hormone receptor in the strain of sex-linked dwarf chicken: evidence for a mutation in the intracellular domain. J Endocrinol 1994, 142(3):427–434.
11. Krizetová H: Effects of the sex-linked dwarf gene (dw) on skeletal muscle cellularity in broiler chickens. Br Poult Sci 1993, 34(6):479–485.
12. Huang N, Cogburn LA, Agarwal SK, Marks HL, Burnside J: Overexpression of a truncated growth hormone receptor in the sex-linked dwarf chicken: evidence for a splice mutation. Mol Endocrinol 1993, 7(11):1391–1398.
13. Tanaka M, Hayashida Y, Waktla M, Hoshino S, Nakashima K: Expression of aberrantly spliced growth hormone receptor mRNA in the sex-linked dwarf chicken, Gifu 20. Growth Regul. 1995, 5(4):218–223.
14. Touchburn SP, Guillaume J, Ledeboz B, Blum JC: Lipid and energy metabolism in chicks affected by dwarfism (dw) and Naked-neck (Na). Poult Sci 1980, 59(10):2189–2197.
15. Burghelle-Mayeur C, Tixier-Boichard M, Merat P, Démarme Y: De novo lipogenesis and lipolysis activities in normal (Dw) and dwarf (dw) White Leghorn laying hens. Comp Biochem Physiol B 1989, 93(4):773–779.
16. Wu QG, Zheng JK, Yang N: Expression profiling of GH, GHR, and IGF-1 genes in sex-linked dwarf chickens. Yi Chuan. 2007, 29(8):985–994.
17. Laron Z, Pertzalan A, Mannheimer S: Genetic pituitary dwarfishness with high serum concentration of growth hormone—a new inborn error of metabolism? Isr J Med Sci. 1966, 2(15):125–155.
18. Hale CS, Herrin WG, Shibuya H, Lucy MC, Lubahn DB, Keisler DH, Johnson GS: Decreased growth in angus steers with a short TG-microsatellite allele in the P1 promoter of the growth hormone receptor gene. J Anim Sci 2000, 78(8):2099–2104.
19. Aggrey SE, Yao J, Sauber MP, Lin CY, Zadworny D, Hayes JF, Kuhnlein U: Markers within the regulatory region of the growth hormone receptor gene and their association with milk-related traits in Holsteins. J Hered 1999, 90(1):148–151.
20. Amésumé S, Duquesnoy P, Attée O, Novelli G, Gounina S, Postel-Vinay MC, Goossens M: Laron dwarfishness and mutations of the growth hormone receptor gene. N Engl J Med 1989, 321(15):989–995.
21. Edery M, Rozakis-Adcock M, Gouyon L, Finidor J, Lévy-Meyrueis C, Paly J, Dijane J, Postel-Vinay MC, Kelly PA: Lack of hormone binding in COS-7 cells expressing a mutated growth hormone receptor found in Laron dwarfishness. J Clin Invest 1993, 91(5):838–844.
22. Berg MA, Argente J, Chemauze S, Gracia R, Quevago-Aurio J, Hopp M, Pérez-Jurado L, Rosénblom A, Toledo S, Franck U: Diverse growth hormone receptor gene mutations in Laron syndrome. Ann Hum Genet 1993, 57(2):998–1005.
23. Freeth JS, Aylins RM, Whatmore AJ, Townson P, Price DA, Norman MR, Clayton PE: Human skin fibroblasts as a model of growth hormone (GH) action in GH receptor-positive Laron’s syndrome. Endocrinology 1997, 138(5):155–61.
24. Dzin’ ET, Jorge AA, Arnhild I, Rosénblom AL, Bandeira F: Novel nonsense mutation (p.Y113X) in the human growth hormone receptor gene in a Brazilian patient with Laron syndrome. Acta Endocrinol Metab 2008, 58(8):1264–1271.
25. Fassone L, Corneli G, Bellone S, Camacho-Hübner C, Arraetti G, Cappa M, Luteri G, Bona G: Growth hormone receptor gene mutations in two Italian patients with Laron syndrome. J Endocrinol Invest. 2007, 30(5):417–20.
26. Ying YQ, Wei H, Cao LZ, Lu JI, Luo XP: Clinical features and growth hormone receptor gene mutations of patients with Laron syndrome from a Chinese family. Zhongguo Jiaa Er Ke Za Zhi. 2007, 9(4):335–338.
27. Gennero I, Edouard T, Rashad M, Bieth E, Conte-Aurio F, Marin F, Tauber M, Laron syndrome. J Pediatr Endocrinol Metab. 2005, 18(1):91–102.
28. Arman A, Ozon A, Isguven PS, Coker A, Peker I, Yordam N: Overexpression of a truncated growth hormone receptor gene in a patient with Laron dwarfism. J Pediatr Endocrinol Metab. 2008, 21(1):47–58.
29. Arman A, Yılmaz B, Coker A, Sarıöz O, Temiz F, Topaloglu AK: Novel growth hormone receptor gene mutation in a patient with Laron syndrome. J Pediatr Endocrinol Metab. 2010, 23(4):407–414.
30. Coschigano KT, Holland AN, Rinders ME, Litte ED, Flyvbjerg A, Koppich JK: Deletion, but not antagonism, of the mouse growth hormone receptor results in severely decreased body weights, insulin, and insulin-like growth factor I levels and increased life span. Endocrinology 2003, 144(3):979–1010.
31. Mavalli MD, DiGirolamo DJ, Fan Y, Riddle RC, Campbell KS, van Groen T, Frank SJ, Sperling MA, Esser KA, Bamman MM, Clemens TL: Distinct growth hormone receptor signaling modes regulate skeletal muscle development and insulin sensitivity in mice. J Clin Invest 2010, 120(11):4007–4020.
32. Bartel DP: MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004, 116(2):281–297.
33. Filipowicz W, Bhattacharya SN, Sonenberg N: Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet 2008, 9(2):102–114.
34. Doench JG, Sharp PA: Specificity of microRNA target selection in translational repression. Genes Dev 2004, 18(5):504–511.
35. Yao J, Wang Y, Wang W, Wang N, Li H: Solexa sequencing analysis of chicken pre-adipocyte microRNAs. Biosci Biotechnol Biochem 2011, 75(1):54–61.

36. Kwon C, Han Z, Olson EN, Srivastava D: MicroRNA1 influences cardiac differentiation in Drosophila and regulates Notch signaling. Proc Natl Acad Sci U S A 2005, 102(52):18986–18991.

37. Clop A, Marcq F, Takeda H, Pictrot D, Tordo X, Bibé B, Boux J, Caiment F, Elsen JM, Eychenne F, Larzul C, Laville E, Meish F, Milekovic D, Tobin J, Charlier C, Georges M: A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nat Genet 2006, 38(7):813–818.

38. Chen JF, Mandel EM, Thomson JM, Wu Q, Callis TE, Hammond SM, Conlon FL, Wang DZ: The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet 2006, 38(2):228–233.

39. Ichimura A, Ruike Y, Terasawa K, Tsujimoto G: miRNAs and regulation of cell signaling. FEBS J 2011, 278(10):1610–1618.

40. Huntzinger E, Izaurralde E: Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet 2011, 12(2):99–110.

41. Glazov EA, Cottee PA, Barris WC, Moore RJ, Dalrymple BP, Tizard ML: A microRNA catalog of the developing chicken embryo identified by a deep sequencing approach. Genome Res 2008, 18(6):957–964.

42. Kozomara A, Griffiths-Jones S: miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res 2011, 39(Database issue):D152–D157.

43. Roush S, Slack FJ: The let-7 family of microRNAs. Trends Cell Biol 2008, 18(10):505–516.

44. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G: The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 2000, 403(6772):901–906.

45. Ruby JG, Jan C, Player C, Axtell MJ, Lee W, Nusbaum C, Ge H, Bartel DP: Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in C. elegans. Cell 2006, 127(6):1193–1207.

46. Boyerinas B, Park SM, Hau A, Murmann AE, Peter ME: The role of let-7 in cell differentiation and cancer. Endocr Relat Cancer. 2010, 17(1):F19–F36.

47. Yang SJ, Xu CJ, Wu JW, Yang GS: SOCS5 inhibits insulin signaling in porcine primary adipocytes. Mol Cell Biochem 2010, 345(1–2):45–52.

48. Sabio G, Das M, Mora A, Zhang Z, Jun JY, Ko HJ, Barrett T, Kim JK, Davis RJ: A stress signaling pathway in adipose tissue regulates hepatic insulin resistance. Science 2008, 322(5907):1539–1543.

49. Bolstad BM, Irizarry RA, Astrand M, Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003, 19(2):185–193.

50. Kanehisa M: The KEGG database. Ncovatis Found Symp 2002, 247:91–101.

51. Kanehisa M, Goto S, Hattori M, Akid-Kinoshita KF, Itoh M, Kawashima S, Katayama T, Araki M, Hirakawa M: From genomics to chemical genomics: new developments in KEGG. Nucleic Acids Res 2006, 34(Database issue):D354–D357.