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Research Article

Alterations of Myostatin and Follistatin Gene Expression Profiles in Response to in ovo rhIGF-1 during Quail (*Coturnix coturnix*) Embryonic Development

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Abstract: Myostatin, along with its binding protein follistatin, have been demonstrated to inhibit skeletal muscle development during embryonic and postnatal development, whereas IGF-1 has been reported to stimulate skeletal muscle development and overall growth. The objectives of the current study were to determine the expression profiles of myostatin and follistatin genes, and to evaluate the impact of in ovo administration of recombinant human insulin-like growth factor-1 (rhIGF-1) on these gene expression patterns during quail embryogenesis, with an emphasis on skeletal muscle development. Quail eggs were injected once with 100 ng rhIGF-1 in 10 mM acetic acid, 0.1% BSA per embryo on day 3 of embryonic development. Total RNA was isolated from whole embryos on each of embryonic days (E) 3 to 6 (n = 6 per day/per treatment), from thoracic/abdominal halves of the embryos on E 7 and E 8 (n = 6 per day/per treatment), and from pectoralis muscle tissues on Es 9 to 16 (n= 4 per day/per treatment). Reverse-transcription polymerase chain reaction (RT-PCR) was used to determine the relative expression pattern. Myostatin mRNA was first detected on E 5, while follistatin mRNA was first detected on E 3. Although myostatin mRNA levels from the control group declined from E 10 to E 12, they increased from E 9 to E16; on E 16 it reached its highest point in the rhIGF-1 injected group. In ovo administration of rhIGF-1 on E 3 appeared to delay myostatin expression approximately 1 day as compared to control embryos at E 9, while it enhanced follistatin expression on E 9. Our results suggest that myostatin and follistatin genes may be pivotal regulators of skeletal muscle growth, and that in ovo administration of rhIGF-1on E 3 may alter the expression profiles of myostatin and follistatin genes, which might enhance the skeletal muscle mass of quail embryos.

Key Words: Myostatin, follistatin, quail embryos, in ovo rhIGF-1

Myostatin ve Follistatin Genlerinin Ekspresyon Profilinin Bıldırcın (*Coturnix coturnix*) Embriyolarında in ovo rhIGF-1 Etkisiyle Değişimi

Özet: Myostatin, bağlayıcı proteini Follistatin ile beraber, özel olarak çizgili kas gelişimini hem embriyo ve hemde postnatal dönemde engellediği, buna rağmen, Insulin-like growth factor-1 (IGF-1) büyüme faktörünün çizgili kaslarla beraber tüm embriyo büyümesini stimule ettiği bildirilmiştir. Bu çalışmada büyüme faktörlerinin ayrı ayrı expresyon profilini belirlemek ve embriyonal gelişimin 3'üncü gününde yapılan in ovo recombinant human (rh) IGF-1 (100 ng/embriyo) injeksiyonunun bu genler üzerine etkisi araştırıldı. Bu calışmada, 400 adet fertilize olmuş bıldırcın yumurtası kullanılmıştır. Kontrol ve injekte edilmiş guruplar için ayrı ayrı olmak üzere, embriyonal gelişimin ilk 6 gününde (E 3 ile E 6 arası) embriyonun tamamından, 7. ve 8. günde (E 7 ve E 8) göğüs ve karın kısmından, 9-16. günlerde ise (E 9 ile E 16 arası) sadece göğüs kaslarından RNA'lar elde edildi. Daha sonra, büyüme faktörlerine ait cDNA'lar, Reverse-Transcription Polymerase Chain Reaction (RT-PCR) tekniği kullanılarak ekspresyon profili çıkartıldı. Follistatin gen expresyonu en erken E 3'de görülmesine rağmen, myostatin gen ekspresyonu E 5'de gözlemlendi. Kontrol gurubu bıldırcın embriyolarında, myostatin mRNA miktarı E 10'da başlamak üzere E 12'ye kadar giderek azalmasına rağmen, in ovo rhIGF-1 injekte edilmiş bıldırcın embriyolarında, myostatin mRNA miktarı E 9'da dahada artarak E 16'da en yüksek seviyeye varmıştır. Bu araştırma sonuçları, myostatin ve follistatin genlerinin bıldırcınların embriyonik çizgili kas gelişiminde önemli bir düzenleyici olabileceği ve embriyonal gelişimin 3'üncü gününde yapılan in ovo rhIGF-1 injeksiyonunun bu genlerin ekspresyon profilini değiştirerek daha fazla kas yapımına yol açabileceğini önermektedir.

Anahtar Sözcükler: Myostatin, follistatin, bıldırcın embriyosu, in ovo rhIGF-1

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Introduction

Insulin-like growth factor-1 (IGF-1) is a well known general enhancer of lean tissue growth, which acts both on an endocrine and paracrine mechanisms. Transgenic mice overexpressing IGF-1 have demonstrated general hyperplasia (1), whereas muscular hypertrophy has been observed when overexpression of IGF-1 is restricted to muscles (2). Additionally, in ovo administration of recombinant human IGF-1 (rhIGF-1, 100 ng/per embryo) on embryonic day 3 significantly increased postnatal muscle growth in 42-day-old chickens (3,4).

Myostatin, a member of the TGF-β family of growth factors, is a negative regulator of skeletal muscle growth (5). Mice lacking the myostatin gene have increased muscle mass, and some of the double-muscled cattle breeds possess mutations in the myostatin coding sequence (6,7). In contrast, follistatin has been proposed to be a positive regulator of skeletal muscle growth, as mice lacking the follistatin gene have decreased muscle mass, while mice overexpressing follistatin has resulted in the double-muscled phenotype that is similar to myostatin-null mice (8). Furthermore, follistatin is found to attenuate myostatin binding to its receptors (Act RIIB receptors), suggesting that follistatin binds myostatin to inhibit its function.

Myostatin and follistatin gene expressions have been detected in the very early stage of development in mice and chicken embryos (9,10). Additionally, in ovo administration of rhIGF-1 (100 ng/per embryo) on embryonic day 3 significantly altered the myostatin gene expression pattern during chicken embryonic development (11). Thus, the present study was conducted to determine the effect of in ovo administration of rhIGF-1 (100 ng/per embryo, on embryonic day 3) on temporal expression patterns of myostatin and follistatin genes during quail embryogenesis, with an emphasis on pectoralis muscle development.

Materials and Methods

Injection Procedure

Fertilized quail eggs (Coturnix coturnix) were obtained from the Poultry Facilities of Kafkas University (Kars, Turkey). The injection procedure has been previously described (3). Briefly, 100 ng/100 µl of rhIGF-1 in 10 mM acetic acid, 0.1% BSA was administered, per egg, through the blunt end of the egg on embryonic day 3. Prior to injection, the blunt end of the egg was sterilized with 70% ethanol. A dental drill bit was used to create a single hole, without penetrating the chorioallantoic membrane. Both rhIGF-1 and vehicle were injected into the albumen with a 22-gauge needle. The hole was sealed with an sticker. Eggs were set in a Buckeye incubator/hatcher (temperature: 37 °C ± 0.5 °C; humidity: 86% to 87%).

Tissue collection

Embryos and tissues were harvested in compliance with the approved Kafkas University Animal Care and Use Committee Protocol. The method of tissue collection has been previously described (11). Briefly, all the embryos were isolated and washed free of yolk, albumen, and extra-embryonic membranes with sterile, nuclease-free water. Whole embryos were collected on each of the embryonic days (E) 3 to 6 (n = 6 per day). Cranial halves of embryos, consisting of the lumbosacral level to the neck, without head, were collected on E 7 and E 8 (n = 6 per day). *Pectoralis* muscle was collected on E 9 through E 16 (n = 4 per day). All the tissue collections were performed at consistent times on each sampling day throughout the experimental period, beginning on E 3 and then every 24 h until E 16.

RNA extraction and RT-PCR

Total RNA was extracted from all of the tissues mentioned above using the Tri-Reagent (Sigma, St. Louis, MO, USA) modification of the guanidine isothiocyanate/phenol-chloroform method as described by Chomczynski and Sacci (12). The RNA concentration was estimated using a GeneQuant RNA/DNA Calculator (BioChrom Ltd., Cambridge, UK). Samples of RNA were stored at -20 °C.

Reverse transcription (RT) was performed by adding 2 µg of total RNA to 2 µg of Oligo dT primers and sterile nuclease-free dd H_2O, to make a final volume of 15 µl. The samples were heated at 70 °C for 5 min and then immediately cooled to 4 °C for 2 min. Reverse transcription buffer containing dNTPs (final concentration of each was 10 mM), 25 units of RNase inhibitor, and 200 units of Maloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) were added to each sample. The sample, with a final volume of 40 µl, was incubated at 37 °C for 1 h followed by incubation at

95 °C for 5 min. For the PCR reaction, 1 µl of RT reaction mixture was added to 25 µl of solution containing 2.5 µl of Taq buffer, 0.25 µl Taq DNA polymerase (Ampli-taq Gold, ABI, Foster City, CA, USA), 2.5 µl dNTPs (final concentration of each was 2 mM), 2.5 µl each of forward and reverse primers, and 14.75 µl of sterile nuclease-free H_2O. The PCR reaction began with 1 cycle at 94 °C for 5 min, an annealing step at 60 °C for myostatin or 55 °C for follistatin and β-actin for 1 min and extension at 72 °C for 10 min. The first cycle was followed by another 25 cycles consisting of 30-s intervals at 94 °C, followed by 60 °C for myostatin or 55 °C for follistatin and β-actin, followed by 72 °C. As a control, a PCR reaction without c-DNA was run and no contamination in the reaction mixture was found (data not shown).

PCR primers

All PCR primers were made by Alpha DNA (Montreal, Canada). Primers for myostatin were designed on the basis of published sequences of chicken myostatin (13). The sequence of the forward primer was 5' GACTATCATGCCACAACCGAGACGA 3', while the reverse primer was 5' GTGTACCAGGTGAGTGTGCGGGTATT 3'. Forward and reverse primers predicted a PCR product of 657 base pairs (bp), which corresponds to bases 327-984 of the sequence. Primers for follistatin were designed on the basis of the published sequence of chicken follistatin (14). The sequence of the forward primer was 5' CATCCCGTGCAAAGAAAC 3', while the reverse primer was 5'CTCGTAGGCTAATCCAATG 3'. These primers amplified a PCR product of 445 bp, as previously reported (15), which corresponded to bases 260-705 of the sequence. Primers for β-actin were used to amplify a 285 bp product, as previously published (16), as an internal standard to verify the level of amplification. The sequence of the forward primer was 5' TCATGAAGTGTGACGTTGACATCCGT 3', while the reverse primer was 5' CCTAGAAGCATTTGCGGTGCACGATG 3'.

The amplified PCR products for each gene were visualized on 1.5% agarose gels stained with ethidium bromide. Products were analyzed by densitometry of the stained gels, and data should, therefore, be considered semi-quantitative. The identity of all PCR products was confirmed by sequence analysis. Mean gene values for each sampling day were derived from a minimum of 4 individual sample collections and a minimum of 3 independent cDNA and RT-PCR amplifications per sample.

Results

Figure 1 depicts the patterns of β-actin gene expression in both control and in ovo rhIGF-1 injected quail embryos. β-actin was used as a house-keeping gene and expression levels were stable throughout the experiment in both groups, for each corresponding day.

Myostatin gene expression was first seen in 5-day-old quail embryos and then gradually increased through E 9, in both the control and in ovo rhIGF-1 injected groups (Figure 2). Myostatin mRNA levels from the in ovo rhIGF-1 injected group pectoralis muscles continued to increase on E 10 (~ 3-fold) and then sharply decreased on E 11. Levels then began to increase and reached the highest level on E 16. Myostatin mRNA levels in control pectoralis muscle declined from E 10 to E 12, and then sharply increased on E 13 (~ 5-fold). Levels in control pectoralis muscle remained high until E 16, though there was a sudden decrease on E 14 (Figure 2). Follistatin gene expression was first seen in 3-day-old quail embryos in both the control and in ovo rhIGF-1 injected groups (Figure 3). Follistatin mRNA levels in the control group remained at this level until E 13, on which day there was an approximately 11-fold increase (Figure 3). Follistatin mRNA levels in the control group remained at this level, although it suddenly decreased on E 14 (~ 2-fold). Follistatin mRNA levels from the in ovo rhIGF-1 injected group increased on E 9 (~ 9-fold) and continued to increase, reaching the highest level on E 10. Levels then sharply declined and remained at this level until E 16 (Figure 3).

Discussion

Myostatin and follistatin are both secreted proteins involved in the control of muscle mass during embryonic development. They have opposite effects on muscle growth; as documented by genetic models, the potential function of follistatin is as a positive regulator of muscle differentiation, whereas myostatin is a negative regulator of skeletal muscle growth (8). Endocrine studies have demonstrated that IGFs, particularly IGF-1, are involved in controlling muscle growth, as well as overall growth (2). In the light of these findings, it is expected to see that myostatin and follistatin gene expression patterns should initially overlap in control groups. In ovo rhIGF-1 administration on E 3 enhances follistatin gene expression while depressing myostatin gene expression. However, as

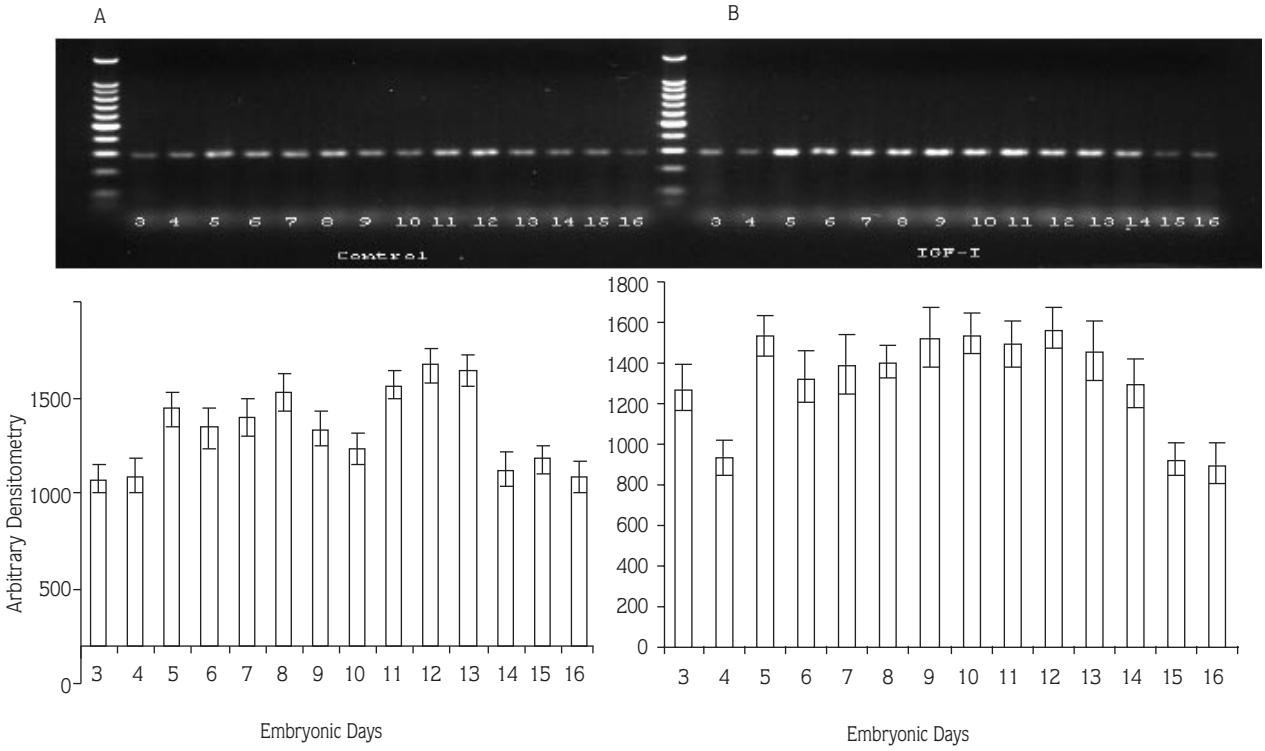

Figure 1. Representative steady-state levels of the control (A) and rhIGF-1 treated (B) groups' β-actin mRNA in whole embryo and pectoralis muscle during quail embryonic development (n = 6 or 4 per day, respectively). The bands for β-actin mRNA were analyzed by densitometry and the integration values (mean ± SD) were expressed in arbitrary densitometric units on each sampling day.

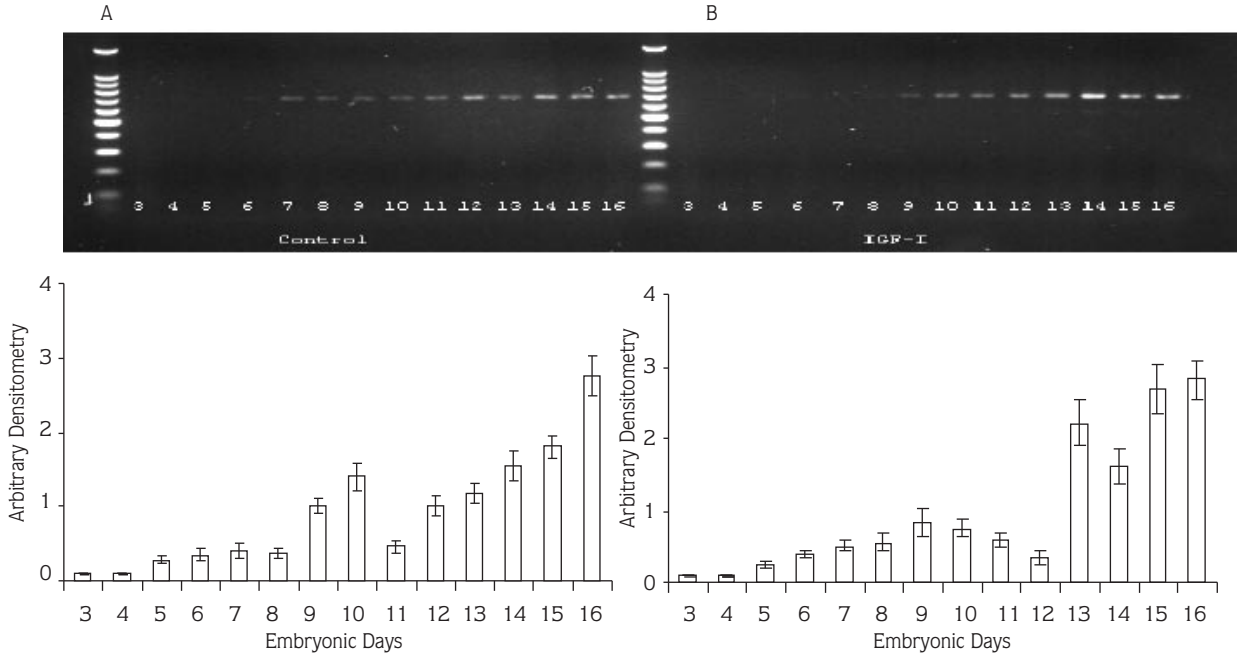

Figure 2. Representative steady-state levels of the control (A) and rhIGF-1 treated (B) groups' myostatin mRNAs in whole embryo and pectoralis muscle during quail embryonic development (n = 6 or 4 per day, respectively). The bands for myostatin mRNA were analyzed by densitometry and the integration values (mean ± SD), after normalization to β-actin, were expressed in arbitrary densitometric units on each sampling day.

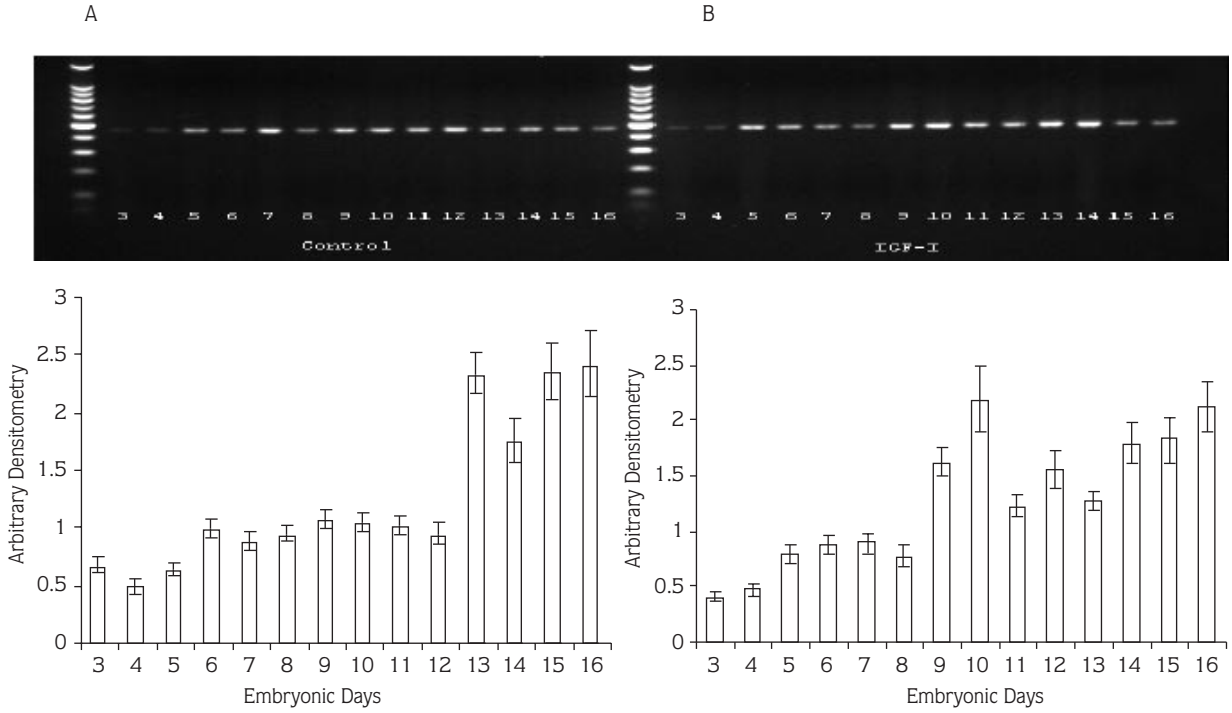

Figure 3. Representative steady-state levels of the control (A) and rhIGF-1 treated (B) groups' follistatin mRNAs in whole embryo and pectoralis muscle during quail embryonic development (n = 6 or 4 per day, respectively). The bands for follistatin mRNA were analyzed by densitometry and the integration values (mean ± SD), after normalization to β-actin, were expressed in arbitrary densitometric units on each sampling day.

shown in Figures 2 and 3, myostatin and follistatin gene expression patterns did not exactly overlap in the control group. These results are consistent with findings of transgenic animal studies. For example, follistatin transgenic animals overexpressing follistatin demonstrated increased muscle mass, which was much greater than the increase in myostatin null mice (8), suggesting that the effects of follistatin may be the result of the inhibition of a ligand other than myostatin.

Secondly, although overall myostatin expression patterns in both the control and in ovo rhIGF-1 injected groups were similar during both whole embryo and embryonic pectoralis muscle development, in ovo administration of rhIGF-1 on E 3 appeared to delay myostatin expression approximately 1 day compared to control embryos at E 9 (Figure 2), while enhancing follistatin expression on E 9 (Figure 3). A delay of myostatin expression would allow myoblasts additional time in the replicative phase of the cell cycle, which would ultimately lead to increased muscle fiber formation. Thus, delayed expression of myostatin may cause muscle hyperplasia in quail embryos, as seen in postnatal chicken skeletal muscle increase due to in ovo rhIGF-1 administration (3,4).

We have detected myostatin mRNA in quail embryos as young as 5 days old in both the control and rhIGF-1 injected groups, in contrast to our previous findings in chicken embryos (10). This could be due to the differences between quail and chicken embryonic development. On the other hand, we assume that early myostatin expression (E 5) in the present study originated from presumptive myoblasts, given the fact that skeletal muscle tissue is the main source of myostatin (5). However, this assumption does not preclude the possibility of myostatin expression generated from other early embryonic tissues, because myostatin mRNA was detected in adipose tissues and in chicken embryos, before the myogenic lineage was established (10,17). Thus, further studies using in situ hybridization should be carried out to clarify the exact origin of myostatin gene expression in early quail embryonic development.

In vitro studies have shown that IGF-1 up-regulates myogenic regulatory factors (MRF, MyoD, Myogenin, and

Myf-5), which are thought to play important roles in the growth of muscle fibers (18). MRFs bind to an E-box (19). Given the fact that myostatin gene has an E-box in its upstream region (20), IGF-1 may down-regulate myostatin if MRFs compete with other transcriptional factors that up-regulate it. However, considering the complexity of the IGF system, such as the secretion of binding proteins and the presence of 2 surface receptors, it is difficult to pin-point the exact mechanism by which a single in ovo rhIGF-1 injection altered developmental expression patterns of myostatin and follistatin genes during chicken embryonic development.

In conclusion, our results demonstrate that in ovo administration of rhIGF-1 on E 3 altered developmental expression patterns of myostatin and follistatin genes. A complete understanding of the interaction between IGF-1 and the myostatin and follistatin genes during embryonic development could be beneficial to food animal agriculture.

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