IGF-1 knockdown inhibits phosphorylation of Akt and ERK in chicken embryonic myotubes

Saneyasu, Takaoki
Nakamura, Tomonori
Honda, Kazuhisa
Kamisoyama, Hiroshi

(Citation)
Growth Hormone & IGF Research,65:101478

(Issue Date)
2022-08

(Resource Type)
journal article

(Version)
Accepted Manuscript

IGF-1 knockdown inhibits phosphorylation of Akt and ERK in chicken embryonic myotubes

Takaoki Saneyasu, Tomonori Nakamura, Kazuhisa Honda, Hiroshi Kamisoyama

Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan

Correspondence: Takaoki Saneyasu, PhD
Department of Bioresource Science,
Graduate School of Agricultural Science, Kobe University
1-1, Rokkodai-cho, Nada-ku, Kobe 657-8501, Japan
Tel: +81 78 803 5808, Fax: +81 78 803 5809
Email: saneyasu@phoenix.kobe-u.ac.jp

TS received JSPS KAKENHI (grant number 18K14568).

Abstract

Objective: We examined whether auto/paracrine insulin-like growth factor-1 (IGF-1) contributes to the phosphorylation of Akt and ERK in chicken myotubes.

Methods: Chicken myotubes were treated with IGF-1 siRNA, and then total RNA and protein were harvested for real-time PCR and western blot analysis.

Results: Treatment with IGF-1 siRNA inhibited the phosphorylation of Akt and ERK, but not of ribosomal protein S6, in chicken myotubes. Interestingly, IGF-1 siRNA downregulated the expression of IGF-2.

Conclusions: The results of this study suggest that auto/paracrine IGF-1 contributes to Akt and ERK phosphorylation in chicken myotubes.

Key words: Akt, chicken myotube, ERK, IGF-1

INTRODUCTION

Skeletal muscle mass is one of the most important traits in meat production. It is controlled by a balance between protein synthesis and degradation; skeletal muscle mass increases when the rate of protein synthesis exceeds that of protein degradation. Therefore, numerous studies have been conducted to clarify the mechanisms of protein metabolism in the skeletal muscles of domestic animals.

The serine/threonine kinase Akt is a key factor in the regulation of protein metabolism in skeletal muscles. For example, mice lacking both skeletal muscle Akt1 (a ubiquitously expressed isoform) and Akt2 (the major isoform in the skeletal muscles) display a significant reduction in body weight and skeletal muscle mass [1]. Akt is activated by phosphorylation in response to extracellular stimuli such as growth factors and insulin [2,3]. Phosphorylated Akt activates the downstream mTOR/S6K1 signaling pathway and stimulates protein synthesis [2,3]. In contrast, phosphorylated Akt inactivates FOXO1, a transcription factor of atrogenes such as atrogin-1 and MuRF-1 [2-5]. Therefore, Akt plays an important role in the regulation of both protein synthesis and degradation in skeletal muscles.

ERK1 and ERK2 are members of a family of mitogen-activated protein kinases. They regulate fundamental cellular processes such as proliferation, survival, growth, metabolism, motility, differentiation, and development [6]. ERK signaling also has important roles in cell growth, although its role is secondary to that of Akt/mTOR signaling [6]. For example, ERK upregulates the transcription of ribosomal DNA by RNA polymerase I (Pol I), of tRNA genes by Pol III, and of ribosomal protein genes by Pol II via various factors [6-9]. Additionally, ERK activates ERK-activated MAPK-interacting kinase, mTOR complex 1, and ERK-activated ribosomal protein S6 kinase [6,10-12], which regulate translation via various factors. Previous studies have suggested that ERK is involved in protein synthesis in chicken skeletal muscle. For example, an ERK inhibitor

54 abolishes the insulin-induced phosphorylation of S6K1 in chicken myoblasts [13].
55 Fasting significantly decreases the phosphorylation of ERK2 (the only isoform detected
56 in bird tissues) in the leg muscles [14,15]. Therefore, both Akt and ERK play important
57 roles in the regulation of protein metabolism in chicken skeletal muscles.
58 Insulin-like growth factor-1 (IGF-1) is a primary hormone that promotes skeletal growth
59 and stimulates Akt- and ERK-mediated signaling pathways [3,16]. Circulating IGF-1 is
60 mainly produced in the liver, and it acts as an endocrine hormone. Conversely, skeletal
61 muscle IGF-1 acts in an auto/paracrine manner. Previous studies in chickens exhibiting
62 different body weights and growth rates showed that the blood concentration of IGF-1
63 and the levels of IGF-1 mRNA and protein in the skeletal muscle are higher in fast-
64 growing chickens [17,18]. However, previous studies in conditional knockout mice
65 suggest that hepatic IGF-1 is not critical for skeletal muscle growth [19-21]. Another
66 previous study in transgenic mice showed that skeletal muscle-restricted IGF-1 transgene
67 results in hypertrophy [22]. Therefore, these findings suggest that skeletal muscle IGF-1
68 plays a key role in the upregulation of protein synthesis in skeletal muscles. However, it
69 remains unclear whether endogenously produced IGF-1 in the skeletal muscle contributes
70 to the phosphorylation of Akt and ERK.
71 In the present study, we investigated the effects of IGF-1 knockdown on the
72 phosphorylation of Akt and ERK in chicken embryonic myotubes using siRNA.
73
74 **MATERIALS AND METHODS**
75 *Cell culture*
76 The experiments using chicken embryos were approved by the Institutional Animal Care
77 and Use Committee and were performed according to the Kobe University Animal
78 Experimental Regulation.
79 Chicken embryonic myotubes were formed as described previously [23]. Breast muscles

dissected from 10-12 14-days-old chick embryos were minced using surgical scissors and digested with HBSS(+) (Nacalai Tesque, Inc., Kyoto, Japan) containing 0.2% collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) for 20 min at 37°C. The cells were collected by centrifugation and were resuspended in Dulbecco's modified Eagle medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 15% serum (FetalClone III, GE Healthcare Bio-Sciences AB, Uppsala, Sweden), 1× non-essential amino acid solution (Nacalai Tesque, Inc., Kyoto, Japan), and 1× gentamicin/amphotericin solution (Life Technologies, Carlsbad, CA, USA). The cell suspension was passed through a cell strainer to remove tissue debris and was then transferred to an uncoated flask to allow attachment of fibroblasts. After 1 h, the unattached cells were transferred to another uncoated flask and this procedure was repeated 2–3 times. The unattached cells were counted and plated onto collagen I-coated 12-well plates at a density of 1×10^5 cells/well. The cells were incubated in the medium described above at 37°C and 5% CO_2 in humidified air until myotube formation. The myotube formation was confirmed by the observation using a light microscope.

Chicken IGF-1-targeting siRNA (Table 1) was designed using the siDESIGN Center and was synthesized by Horizon Discovery Ltd. (Cambridge, UK). ON-TARGETplus Non-targeting siRNA #1 was purchased from Horizon Discovery Ltd. and used as the control. The transfection of siRNA was performed using Lipofectamine RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA, USA) according to the supplier's protocol. Myotubes were cultured in the above described medium (plus serum, non-essential amino acid, and antibiotic/antimycotic) supplemented with siRNA for 2 days, and further cultured in fresh medium without siRNA for one day.

To confirm the reproducibility, we conducted the same experiments twice independently. The representative results are shown in Figure 1.

106 *Real-time PCR analysis*

107 Real-time PCR analysis was performed as described previously [23]. The cells were first
108 washed with phosphate-buffered saline (PBS). Total RNA was extracted from the cells
109 using Sepazol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan). First-strand cDNA
110 was synthesized from the total RNA using ReverTra Ace® qPCR RT Master Mix with
111 gDNA Remover (Toyobo Co. Ltd, Osaka, Japan). Messenger RNA levels were quantified
112 for each primer (Table 1) using the Thermo Scientific PikoReal PCR System (Thermo
113 Fisher Scientific Oy, Vantaa, Finland), and TB Green Premix Ex Taq II (Tli RNaseH Plus;
114 Takara Bio Inc., Otsu, Japan), according to the supplier's recommendations. The
115 expression levels of target genes were normalized to those of ribosomal protein S17
116 (RPS17). The results are shown relative to those of the control group.

117

118 *Western blot analysis*

119 Western blot analysis was performed as described previously [23]. Briefly, the cells were
120 rinsed with ice-cold PBS. The cells were then scraped into 100 μL of lysis buffer. The
121 cell lysates were ultrasonicated and centrifuged at 17,900 × g for 15 min at 4°C. The
122 supernatants were stored at -80°C. Protein concentration was determined using the Pierce
123 BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). The lysates (3 μg of
124 protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis
125 (SDS-PAGE) and western blotting using HorizeBlot (ATTO Co., Tokyo, Japan),
126 according to the manufacturer's instructions. Bands were detected using Chemi-Lumi one
127 Super (Nacalai Tesque, Inc., Kyoto, Japan), visualized with LumiCube (Liponics Inc.,
128 Tokyo, Japan), and quantified using the CS Analyzer software (ATTO Co., Tokyo, Japan).
129 Anti-Akt (#9272), anti-phospho-Akt (Thr308) (#13038), anti-phospho-Akt (pAkt)
130 (Ser473) (#9271), anti-p44/42 MAPK (Erk1/2) (#4695), anti-phospho- p44/42 MAPK
131 (Erk1/2) (Thr202/Tyr204) (#9101), anti-ribosomal protein S6 (#2217), anti-phospho-

ribosomal protein S6 (Ser240/244) (#5364), anti-β-actin (#8457), anti-α-tubulin (#2125), horseradish peroxidase (HRP)-linked anti-rabbit IgG (#7074), and HRP-linked anti-mouse IgG (#7076) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-IGF-1 (#05-172) was purchased from EMD Millipore Corp. (Temecula, CA, USA). Anti-β-actin and anti-α-tubulin antibodies were used as loading controls. The results are shown relative to those of the control group.

Statistical analysis

Data were analyzed using the two-tailed *t*-test performed using Excel 2013 (Microsoft, USA).

RESULTS

IGF-1 siRNA treatment significantly decreased IGF-1 expression at 2 days after siRNA addition in the myotubes, and siRNA treatment tended to decrease IGF-1 expression at 3 days (Fig. 1A). Unexpectedly, IGF-2 expression was significantly decreased by IGF-1 siRNA treatment (Fig. 1B). Akt phosphorylation at Thr308 and Ser473 tended to be inhibited at 2 days, and it was significantly inhibited at 3 days (Fig. 1C,D). Similarly, siRNA treatment significantly inhibited ERK phosphorylation (Fig. 1F). However, no significant change was observed in the phosphorylation of ribosomal protein S6 (RPS6, Fig. 1H), and total protein level of Akt, ERK, and RPS6 (Figure 1E,G,I). IGF-1 protein was not detected in any of the myotube samples.

DISCUSSION

Fast-growing chickens have higher levels of IGF-1 in both the blood and skeletal muscles [17,18], suggesting that both endocrine and auto/paracrine IGF-1 play important roles in the growth of skeletal muscles. According to the manufacturer's publication [24],

158 FetalClone III contains 136 ng/mL IGF-1. In our previous study, medium without
159 FetalClone III significantly decreased the phosphorylation rate of Akt in chicken
160 myotubes, compared to medium containing 15% FetalClone III [23]. Therefore, it seems
161 that the medium used in the present study contained enough IGF-1 to phosphorylate Akt
162 in chicken myotubes. Under these conditions, the present study showed that IGF-1
163 knockdown inhibited Akt and ERK phosphorylation in the myotubes. Therefore, the
164 findings of the present results suggest that auto/paracrine IGF-1 contributes to the
165 upregulation of Akt and ERK signaling pathways in chicken myotubes, even though
166 endocrine IGF-1 regulates Akt and ERK signaling.
167 In the present study, IGF-1 siRNA significantly downregulated the expression of both
168 IGF-1 and IGF-2 expression. The BLAST results revealed that there was no significant
169 similarity between the IGF-2 and IGF-1 siRNA sequences used in the present study,
170 suggesting that IGF-1 siRNA did not directly suppress IGF-2 expression in the myotubes.
171 A previous study on C2C12 myoblasts reported that IGF-2 also induces Akt and ERK
172 phosphorylation [25]. Another study in C2C12 cells showed that the inhibitor of
173 phosphatidylinositol 3-kinase, but not ERK, significantly inhibits IGF-2 expression,
174 whereas transfection with a constitutively active form of Akt significantly upregulates the
175 mRNA levels of IGF-1 and IGF-2 [26]. These findings raise the possibility that IGF-1
176 knockdown inhibits Akt and ERK phosphorylation, inhibition of Akt phosphorylation
177 leads to suppression of IGF-2 expression, and suppression of IGF-2 enhances the
178 inhibition of Akt and ERK phosphorylation in chicken myotubes. Further studies are
179 required to clarify how IGF-1 knockdown inhibits IGF-2 expression in chicken myotubes
180 and whether endogenous IGF-2 contributes the regulation of Akt and ERK signaling.
181 IGF-1 activates the Akt/mTOR/S6K1 pathway in C2C12 myotubes [16]. Phosphorylation
182 of RPS6 at Ser 240/244 can be used as a specific biomarker for the mTOR/S6K pathway
183 [27]. In the present study, no significant difference was observed in RPS6

184 phosphorylation at Ser 240/244 in chicken myotubes, even though Akt phosphorylation
185 was inhibited by IGF-1 siRNA. Previous *in vitro* [28] and *in vivo* [29] studies have shown
186 that leucine induces phosphorylation of mTOR and S6K1, but not Akt. Other essential
187 amino acids such as lysine, methionine, phenylalanine, tryptophan, and threonine
188 phosphorylate S6K1, but not Akt and mTOR, in C2C12 myotubes to a lesser extent than
189 leucine [28]. Another study in C2C12 myotubes showed that the absence of glucose in
190 the culture medium inhibits phosphorylation of S6K, but not Akt and ERK [30]. It is
191 therefore likely that essential amino acids and glucose in the culture medium upregulate
192 the mTOR/S6K1 signaling pathway in an IGF-1-independent manner, at least under the
193 present experimental conditions.
194
195 ## CONCLUSIONS
196 The results of this study suggest that auto/paracrine IGF-1 contributes to Akt and ERK
197 phosphorylation in chicken myotubes, even though a sufficient amount of IGF-1 exists in
198 the medium.
199
200 **ACKNOWLEDGMENT**
201 This work was supported by JSPS KAKENHI (grant number 18K14568).
202
203 **REFERENCES**
204 [1] Jalswal N, Gavin MG, Qulnn III WJ, Luongo TS, Gelfer RG, Baur JA, Tltchenell PM.
205 The role of skeletal muscle Akt in the regulation of muscle mass and glucose homeostasis.
206 Mol Metab. 28 (2019) 1-13.
207 [2] Manning BD, Toker A. AKT/PKB signaling: Navigation the network. Cell. 169 (2017)
208 381-405.
209 [3] Sandri M. Signaling in muscle atrophy and hypertrophy. Physiology. 23 (2008) 160-

170.

[4] Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, Walsh K, Schiaffino S, Lecker SH, Goldberg AL. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell. 117 (2004) 399-412.

[5] Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, Gonzalez M, Yancopoulos GD, Glass DJ. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell. 14 (2004) 395-403.

[6] Lavoie H, Gagnon J, Therrien M. ERK signaling: a master regulator of cell behaviour, life and fate. Nat Rev Mol Cell Biol 21 (2020) 607-632.

[7] Felton-Edkins ZA, Fairley JA, Graham EL, Johnston IM, White RJ, Scott PH. The mitogen-activated protein (MAP) kinase ERK induces tRNA synthesis by phosphorylating TFIIIB. EMBO J. 22 (2003) 2422-2432.

[8] Grandori C, Gomez-Roman N, Felton-Edkins ZA, Ngouenet C, Galloway DA, Eisenman RN, White RJ. c-Myc binds to human ribosomal DNA and stimulates transcription of rRNA genes by RNA polymerase I. Nat Cell Biol. 7 (2005) 311-318.

[9] Zhao J, Yuan X, Frödin M, Grummt I. ERK-dependent phosphorylation of the transcription initiation factor TIF-IA is required for RNA polymerase I transcription and cell growth. Mol Cell. 11 (2003) 405-413.

[10] Ma L, Chen Z, Erdjument-Bromage H, Tempst P, Pandolfi PP. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell. 121 (2005) 179-193.

[11] Waskiewicz AJ, Flynn A, Proud CG, Cooper JA. 1997 Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO J. 16 (1997) 1909-1920.

[12] Zhao Y, Bjorbaek C, Moller DE. Regulation and interaction of pp90(rsk) isoforms

236 with mitogen-activated protein kinases. J Biol Chem. 271 (1996) 29773-29779.
237 [13] Duchêne S, Audouin E, Crochet S, Duclos MJ, Dupont J, Tesseraud S. Involvement
238 of the ERK1/2 MAPK pathway in insulin-induced S6K1 activation in avian cells. Domest
239 Anim Endocrinol 34 (2008) 63-73.
240 [14] Dupont J, Tesseraud S, Simon J. Insulin signaling in chicken liver and muscle. Gen
241 Comp Endocrinol. 163 (2009) 52-57.
242 [15] Dupont J, Tesseraud S, Derouet M, Collin A, Rideau N, Crochet S, Godet E, Cailleau-
243 Audouin E, Métayer-Coustard S, Duclos MJ, Gespach C, Porter TE, Cogburn LA, Simon
244 J. Insulin immuno-neutralization in chicken: effects on insulin signaling and gene
245 expression in liver and muscle. J Endocrinol. 197 (2008) 531-542.
246 [16] Rommel C, Bodine SC, Clarke BA, Rossman R, Nunez L, Stitt TN, Yancopoulos
247 GD, Glass DJ. Mediation of IGF-1-induced skeletal myotube hypertrophy by
248 PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. Int Rev Cell Mol Biol. 320 (2015)
249 41-73.
250 [17] Jia J, Ahmed I, Liu L, Liu Y, Xu Z, Duan X, Li Q, Dou T, Gu D, Rong H, Wang K,
251 Li Z, Talpur MZ, Huang Y, Wang S, Yan S, Tong H, Zhao S, Zhao G, Te Pas MFW, Su Z,
252 Ge C. Selection for growth rate and body size have altered the expression profiles of
253 somatotropic axis genes in chickens. PLoS One. 13 (2018) e0195378.
254 [18] Xiao Y, Wu C, Gui G, Zhang G, Yang H. Association of growth rate with hormone
255 levels and myogenic gene expression profile in broilers. J Anim Sci Biotech. 8 (2017) 43.
256 [19] Iresjö BM, Svensson J, Ohlsson C, Lundholm K. Liver-derived endocrine IGF-I is
257 not critical for activation of skeletal muscle protein synthesis following oral feeding.
258 BMC Physiol. 13 (2013) 7.
259 [20] Sjögren K, Liu JL, Blad K, Skrtic S, Vidal O, Wallenius V, LeRoith D, Törnell J,
260 Isaksson OGP, Jansson JO, Ohlsson C. Liver-derived insulin-like growth factor I (IGF-I)
261 is the principal source of IGF-I in blood but is not required for postnatal body growth in

mice. Proc Natl Acad Sci USA. 96 (1999) 7088-7092.

[21] Yakar S, Liu JL, Stannard B, Butler A, Accili D, Sauer B, LeRoith D. Normal growth and development in the absence of hepatic insulin-like growth factor I. Proc Natl Acad Sci USA. 96 (1999) 7324-7329.

[22] Musarò A, McCullagh K, Paul A, Houghton L, Dobrowolny G, Molinaro M, Barton ER, Sweeney HL, Rosenthal N. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet. 28 (2001) 195-200.

[23] Saneyasu T, Honda K, Kamisoyama H. Myostatin increases Smad2 phosphorylation and atrogin-1 expression in chick embryonic myotubes. J Poult Sci. 56 (2019) 224-230.

[24] Cytiva, Tokyo, Japan, Growth factors in serum products. https://cdn.cytivalifesciences.com/dmm3bwsv3/AssetStream.aspx?mediaformatid=10061&destinationid=10016&assetid=18092, 2020 (accessed 15 January 2021)

[25] Mu X, Qi W, Liu Y, Zhou J, Li Y, Rong X, Lu L. IGF-II-mediated downregulation of peroxisome proliferator-activated receptor-γ coactivator-1α in myoblast cells involves PI3K/Akt/FoxO1 signaling pathway. Mol Cell Biochem. 432 (2017) 199-208.

[26] Jiao S, Ren H, Li Y, Zhou J, Duan C, Lu L. Differential regulation of IGF-I and IGF-II gene expression in skeletal muscle cells. Mol Cell Biochem. 373 (2013) 107-113.

[27] Meyuhas O. Ribosomal protein S6 phosphorylation: Four decades of research. Int Rev Cell Mol Biol . 320 (2015) 41-73.

[28] Atherton PJ, Smith K, Etheridge T, Rankin D, Rennie MJ. Distinct anabolic signaling responses to amino acids in C2C12 skeletal muscle cells. Amino Acids. 38 (2010) 1533-1539.

[29] Le Plénier S, Walrand S, Noirt R, Cynober L, Moinard C. Effects of leucine and citrulline versus non-essential amino acids on muscle protein synthesis in fasted rat: a common activation pathway? Amino acid 43 (2012) 1171-1178.

[30] Kelly SC, Patel NN, Eccardt AM, Fisher JS. Glucose-dependent trans-plasma membrane electron transport and p70S6K phosphorylation in skeletal muscle cells. Redox Biol 27 (2019) 101075.

Figure legend

Figure 1. Effects of IGF-1 siRNA on the mRNA levels of IGF-1 and IGF-2 and phosphorylation of Akt, Erk, and ribosomal protein S6 in chicken myotubes. (A) IGF-1 mRNA level. (B) IGF-2 mRNA level. (C, D) Phosphorylation rate of Akt. (E) Total Akt protein level. (F) Phosphorylation rate pf ERK. (G) Total ERK protein level. (H) Phosphorylation rate of ribosomal protein S6. (I) Total ribosomal protein S6 protein level. Data are expressed as mean ± SEM of six wells in each group. The two-tailed *t*-test was used to analyze the differences between groups. **,*Significance with respect to the control group ($P < 0.01$, 0.05, respectively). RPS17, ribosomal protein S17; RPS6, ribosomal protein S6.

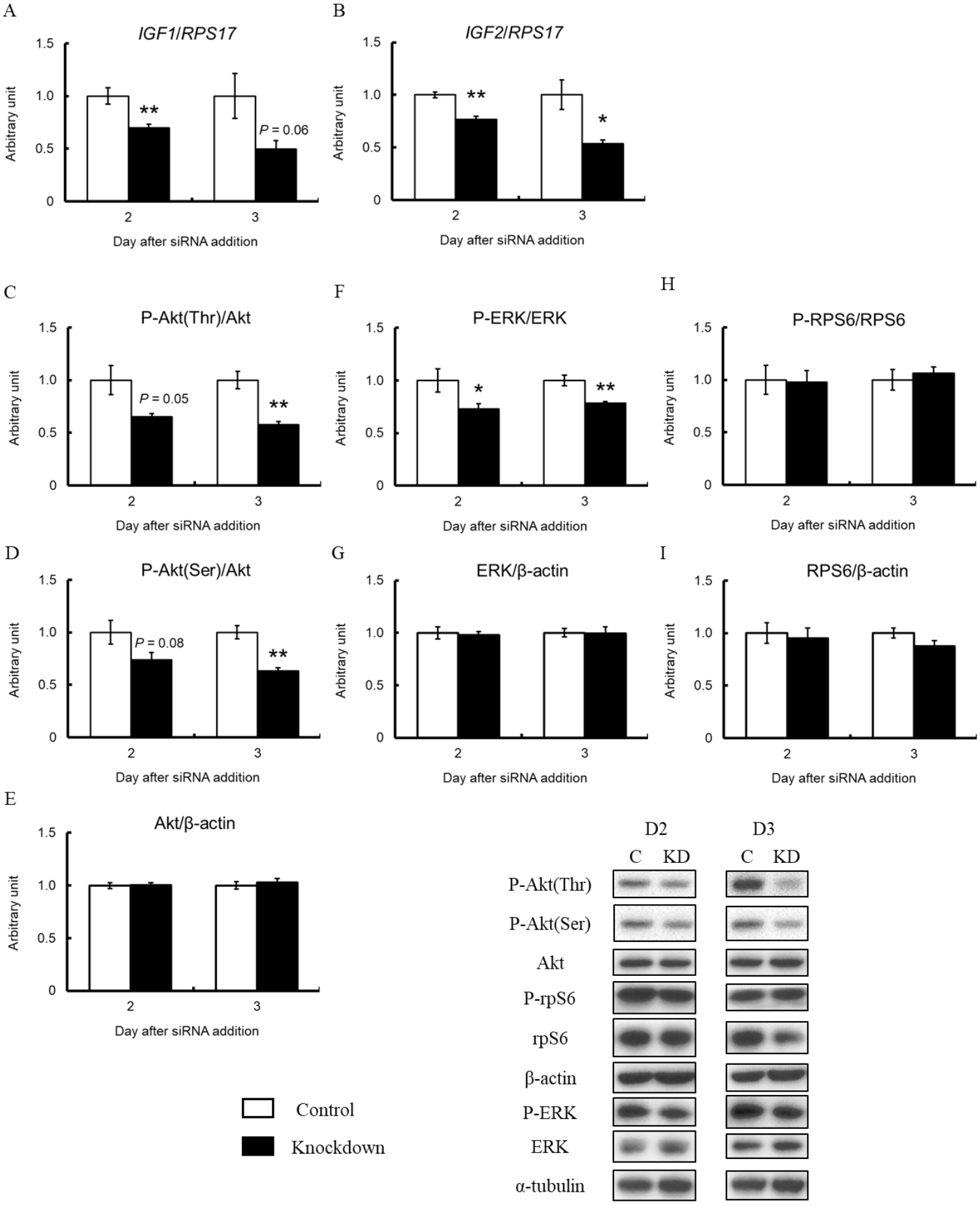
303 Table 1 Sequences of chicken IGF-1-targeting siRNA and primers used for real-time PCR

304 analysis

	Sense	Antisense
IGF-1 siRNA	5'-CAGUAAGCCUACAGGGUAUUU-3'	5'-AUACCCUGUAGGCUUACUGUU-3'
IGF-1 primers	5'-GGCTTCTACTTCAGTAAGCCT-3'	5'-GCCTCCTCAGGTCACAACTC-3'
IGF-2 primers	5'-CCTGGCTCTGCTGGAAACC-3'	5'-GAGAGGTCACGCTCTGACTTGA-3'

305 siRNA and primers for chicken IGF-1 (NM_001004384) were designed using siDESIGN
306 Center (Horizon Discovery Ltd., Cambridge, UK) and Primer-BLAST (National Center
307 for Biotechnology Information, Bethesda, MD, USA), respectively. IGF-2
308 (NM_001030342) primers were designed using Primer Express 3.0 (Applied Biosystems,
309 Foster City, CA, USA).

Figure


A
IGF1/RPS17
Arbitrary unit
1.5
1.0
0.5
0
**
P = 0.06
2
3
Day after siRNA addition
B
IGF2/RPS17
Arbitrary unit
1.5
1.0
0.5
0
**
*
2
3
Day after siRNA addition
C
P-Akt(Thr)/Akt
Arbitrary unit
1.5
1.0
0.5
0
P = 0.05
**
2
3
Day after siRNA addition
F
P-ERK/ERK
Arbitrary unit
1.5
1.0
0.5
0
*
**
2
3
Day after siRNA addition
H
P-RPS6/RPS6
Arbitrary unit
1.5
1.0
0.5
0
2
3
Day after siRNA addition
D
P-Akt(Ser)/Akt
Arbitrary unit
1.5
1.0
0.5
0
P = 0.08
**
2
3
Day after siRNA addition
G
ERK/β-actin
Arbitrary unit
1.5
1.0
0.5
0
2
3
Day after siRNA addition
I
RPS6/β-actin
Arbitrary unit
1.5
1.0
0.5
0
2
3
Day after siRNA addition
E
Akt/β-actin
Arbitrary unit
1.5
1.0
0.5
0
2
3
Day after siRNA addition
Control
Knockdown
D2
D3
C
KD
C
KD
P-Akt(Thr)
P-Akt(Ser)
Akt
P-rpS6
rpS6
β-actin
P-ERK
ERK
α-tubulin