Role of prolactin-like protein (PRL-L) in cold-induced increase of muscle mass in chicks

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Abstract

This study examined the hypothesis that a novel prolactin-like protein gene (PRL-L) is involved in cold-induced growth of skeletal muscle in chicks. Six-day-old chicks (*Gallus gallus domesticus*) were exposed to cold at 4 °C or kept warm at 30 °C for 24 hours. Cold exposure induced significant increases in PRL-L expression that coincided with increases in the weight of the sartorius muscle, which comprises both fast- and slow-twitch fibers. Meanwhile, no induction of PRL-L mRNA was observed in the heart, liver, kidney, brain, or fat. Myoblast cells that expressed PRL-L mRNA grew faster than untransduced cells in media containing 2% serum. These results suggested that PRL-L might be involved in in controlling cold-induced muscle growth of chicks.

Keywords: cold adaptation, cold tolerance, muscle growth, prolactin-like protein, thermogenesis

Abbreviations: PRL-L, prolactin-like protein; NT, no treatment; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

1. Introduction

Newborn chicks cannot maintain their body temperature in a cold environment because of their immature thermogenic ability [12,16], but they acquire the capacity for thermogenesis as they post natally develop skeletal muscle. Most chicks (*Gallus gallus domesticus*) older than 6 days tolerate 24 h of cold exposure (4°C) and maintain their body temperature over this time, accompanied by both increasing mass in their leg

muscles (sartorius and quadriceps muscle) and a transformation of muscle fibers from fast- to slow-twitch [5,8]. From the quadriceps of the cold-exposed chicks, 16 independent cold-induced genes were obtained by subtraction and differential display analysis [5]. One of them was in agreement with a novel prolactin like protein (PRL-L) gene [20]. Wang et al. [20] reported that PRL-L was widely expressed at sites outside the pituitary gland of adult chickens. The predicted PRL-like protein precursor is 225 amino acids in length; however, the role of the PRL-L has not yet been reported.

The objective of this study was to examine the role of PRL-L in the skeletal muscle of neonatal chicks. We previously reported that the leg muscle mass of neonatal chicks was increased in response to 24 h of cold exposure, whereas the pectoral muscle mass was not changed [8]. The leg muscles of chicks (i.e., sartorius muscle and gastrocnemius muscle) are composed of both fast- and slow-twitch fibers [11,14], while the pectoralis muscle is composed of only fast-twitch fibers [13]. In this study, the sartorius muscles and gastrocnemius muscles were defined as mixed muscle, and the pectoral muscle was defined as white muscle. Therefore, in the current study, we examined whether PRL-L is involved in cold-induced growth of mixed muscles using this animal model. We herein show that cold exposure induced a marked increase in the expression of PRL-L mRNA in mixed muscle of chicks. Although this gene was increased in white muscle in response to cold exposure, the degree of increase was 50 times higher in mixed muscle compared to that in white muscle. We also observed that myoblast cells transduced with the PRL-L gene showed high proliferation rates under low serum conditions. Finally, our additional study on the localization of PRL-L protein provides evidence that this protein exists in the extracellular matrix in the mixed muscle of chicks.

2. Materials and methods

2.1. Animals and treatment.

Chicks hatched from genetically identical Rhode Island Red (*Gallus gallus domesticus*) eggs were bred at the Agricultural and Forestry Research Center, University of Tsukuba, in accordance with institutional guidelines. All chicks were kept at a thermoneutral temperature (30 °C and 60% relative humidity) until they were exposed to cold temperature (4 °C and 60% relative humidity). All chicks had free access to food and water under constant 24 h lighting. To examine the effects of cold exposure on PRL-L mRNA expression in chicks, two separate experiments were performed. (i) Twelve 6-day-old chicks were randomly divided into two groups (cold exposure and no treatment [NT]). The cold exposure group was exposed to cold temperature for 24 h, and the NT group was kept at a thermoneutral temperature for 24 h as a control. After measurement of body temperature, chicks were killed by cervical dislocation under ether anesthesia after each treatment. Sartorius muscle, pectoral muscle, heart, liver, kidney, brain, and fat were collected and immediately frozen in liquid nitrogen and stored at -80 °C until use. (ii) Thirty-six 6-day-old chicks were randomly divided into five groups. Four of the five groups were exposed to cold temperature for 0.5, 1, 3, and 6 h, respectively, and the remaining group served as controls (0 h). All chicks were killed by cervical dislocation under ether anesthesia after each treatment. Sartorius muscles collected from both legs were weighed, immediately frozen in liquid nitrogen, and stored at -80 °C until use for mRNA expression and protein expression. The gastrocnemius muscle was collected from the NT groups and embedded for

immunofluorescence in OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and rapidly frozen in isopentane chilled in liquid nitrogen. The experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of the University of Tsukuba, Japan.

To examine the effects of cold exposure on PRL-L mRNA expression in pituitary gland of chicks, 12 male broiler chicks (Chunky strain ROS308, provided by Kajiki Kumiai Hina Center, Kagoshima, Japan) were divided into two groups and exposed to cold temperature or thermoneutral temperature for 24 hours. All chicks were killed by cervical dislocation under ether anesthesia after treatment. Pituitary gland was collected, immediately frozen in liquid nitrogen, and stored at -80 °C until use for mRNA expression. The experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of the Kagoshima University, Japan.

2.2. RNA extraction and northern blotting.

Total RNA was purified using TRIzol reagent (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. Northern blot analysis was performed as described previously [7]. Image analysis was performed using a Macintosh computer and the public domain NIH Image program. Grey scale thresholding was used to separate positive staining from background, and no visible band was observed in the negative control lane (background).

2.3. Quantitative real-time PCR.

Real-time PCR was performed as described previously [9]. In brief, cDNA was synthesized at 40 ng RNA per 10 μL of reaction solution with PrimeScript RT reagent

Kit (Takara, Shiga, Japan), which was set at reverse transcription 37 °C for 15 min, inactivation of reverse transcriptase 85 °C for 5 s, and refrigeration 4 °C for 5 min using PC-320 (ASTEC, Fukuoka, Japan). The primers used in this study are listed in Table 1. Gene expression was measured by real-time PCR using the 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) with SYBR Premix Ex Taq (Takara, Shiga, Japan). The thermal cycle was as follows: 1 cycle at 95 °C for 10 s, and 60 cycles at 95 °C for 5 s, 60 °C for 30 s, and 80 °C for 31 s. Expression of GAPDH mRNA was used as an internal standard and was not significantly different between the cold and control groups. Gene expression results are shown as a percentage of the control value.

2.4. Western blot analysis.

Skeletal muscles from the chicks were homogenized in 2 ml lysis buffer comprising 20 mM Tris-HCl, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid, pH 8.0, and 0.5% Igepal nonionic detergent. The lysate was centrifuged at 14,000g for 30 min at 4 °C, and the supernatant was collected. Total protein concentration was estimated by a protein-dye binding assay [2] using a commercial kit (500-0116; Bio-Rad, Hercules, CA) with mouse IgG as the standard. Aliquots of skeletal muscle were stored at -80 °C until analyzed by western blotting. Western blot analysis was performed as described previously [7].

2.5. Retrovirus preparation.

The GP2-293 packaging cells (purchased from Clontech, Mountain View, CA) were used to generate retroviruses according to the manufacturer's instructions. A total of 6×10^5 cells were placed in a six-well plate and cultured for 24 h. Six micrograms of

retroviral vector pVSVG, pMX GFP, or pMX CTIF-GFP was transfected into the cells by Lipofectamine 2000 (Invitrogen, Tokyo, Japan). After 8 h of incubation under 5% CO_2 at 37 °C, the medium was exchanged with Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Tokyo, Japan) and cultured for another 48 h. The supernatant of the culture medium was taken and filtrated through a filter with a 0.45-μm pore size (Toyo Roshi Kaisha Ltd., Tokyo, Japan).

2.6. Cell culture

C2C12 cells were bought from Riken Bioresource Center. One day prior to transfection, C2C12 cells were subcultured at 2 × 10 cells/well under 5% CO_2 at 37 °C in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The medium was exchanged with DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 4 μg/ml of polybrene, and viral supernatant and cultured for another 24 h. The medium was exchanged every 3 days.

2.7. Cell proliferation assay.

Cell number was assessed using the reagent WST-1 (Roche) according to the manufacturer's instructions. Cells were plated at a density of 4×10^2 cells into 96-well plates with DMEM supplemented with 10% FBS (proliferation media) or 2% horse serum (HS) (differentiation media). After 24, 48, and 72 h of incubation, WST-1 reagent was added and incubated for 4 h, then measured at a wavelength of 450 nm using a microplate reader (Model 680; Bio-Rad, Hercules, CA).

2.8. Immunofluorescence.

To examine the localization the localization of PRL-L protein in skeletal muscle, the gastrocnemius muscles of the NT group chicks were used in this experiment. Serial cross-sections (7 μm thick) were cut with a microtome (CM30503; Leica Microsystems, Wetzlar, Germany) at -28 °C and collected onto slides. The sections were incubated in PBS containing 0.3% (v/v) Triton X-100 (PBS-TX) for 10 min, and then incubated again in PBS-TX for 5 min. They were subsequently rinsed twice in PBS for 5 min and incubated in 100 ml of methanol for 7.5 min, then incubated in PBS containing 0.3% (v/v) H_2O_2 followed by rinsing with PBS. After rinsing three times with PBS-TX for 5 min, the sections were incubated with primary antibody against PRL-L, which was outsourced to Trans Genic Inc. (Kumamoto, Japan), for 12 h at 10 °C. The sections were then rinsed nine times with PBS-TX for 5 min. The sections were subsequently incubated with secondary antibody (Sc-2004; Santa Cruz Biotechnology, Inc.) for 12 h at 10 °C and were then rinsed nine times in PBS-TX. After rinsing with water three times, the sections were mounted with a mounting medium (TA-030-FM; Thermo Fisher Scientific, Waltham, MA). The sections were observed with a fluorescence microscope (BX-51; Olympus, Tokyo, Japan).

2.9. Statistical analysis.

Differences between groups were tested using Fisher's exact probability test for categorical variables, and parametric [if normally distributed, analysis of variance (ANOVA) followed by Tukey's multiple comparisons test or unpaired Student's t-test] or non-parametric (if not normally distributed, Welch's t-test) for continuous variables. P values under 5% were considered to indicate statistical significance. All analyses were performed with a general linear model using SPSS Statistics 17.0 (Statistical Packages

186 for the Social Sciences, released 23 August 2008).

187

188 ## 3. Results

189 ### *3.1. Effects of cold exposure on PRL-L expression in several tissues of chicks.*

190 Body temperature of chicks exposed to cold for 24 h was not different with that
191 of chicks kept at ambient temperature (control, 41.6 ± 0.1 °C; cold, 41.8 ± 0.1 °C). Fig.
192 1A shows the expression levels of PRL-L in a representative northern blot of RNA from
193 several tissues of chicks. A stronger PRL-L mRNA signal was detected in sartorius and
194 pectoral muscle of chicks exposed to cold for 24 h compared to that of the control
195 chicks. The PRL-L mRNA expression in sartorius muscle of cold-exposed chicks was
196 markedly increased (240-fold) compared with that of the control chicks (Fig. 1B), and
197 the increased expression of PRL-L protein was observed in sartorius muscle of
198 cold-exposed chicks compared with control chicks (Fig. 1C). In the pectoral muscle,
199 mRNA expression of PRL-L gene was increased in response to 24 h of cold exposure
200 (Fig. 1D). The basal expression of PRL-L mRNA was higher (38-fold) in sartorius
201 muscle than in pectoral muscle of control chicks at 7 days of age (Fig. 1E). On the other
202 hand, no response of PRL-L mRNA to cold exposure was observed in any other tissues,
203 including brain. Although PRL-L mRNA expression was predominantly expressed in
204 the pituitary gland [20], the PRL-L mRNA expression in the pituitary gland was not
205 changed in response to 24 h of cold exposure (Fig. 1F).

206 Increased PRL-L mRNA expression was observed in sartorius muscle after
207 exposure of chicks to cold for 3 h (Fig. 2A, B). The PRL-L mRNA expression was
208 further increased at 6 of cold exposure. The overall body weight of chicks was not
209 affected by cold exposure (data not shown), in agreement with our previous studies [7].

The ratio of sartorius muscle to body weight, which is calculated as a ratio between the sartorius muscle weight (mg) and the body weight (g) and expressed in mg/g body weight, was significantly increased in chicks exposed to cold for 6 h (Fig. 2C). On the other hand, the ratio of pectoral muscle to body weight was not different among treatments (Fig .2D). In addition, Fig 3 shows that time course of Caspase-3 gene expression in sartorius muscle of cold-exposed chicks. The Caspase-3 mRNA expression was tended to increase after 1h of cold exposure and then be decreasing, while there was no statistical difference among treatments.

The 5'-flanking region of the PRL-L gene includes the putative binding sites for nuclear factor of activated T cells (NFAT) and cAMP response element-binding protein 1 (CREB) [20]. And either NFAT or CREB interacts with peroxisome proliferator-activated receptor-gamma coactivator-1 (PGC-1α) [4,12], whose expression was acutely increased in the sartorius muscle of cold-exposed chicks [8]. Therefore, mRNA expression of these genes was examined in the sartorius muscle of chicks exposed to cold for 1 h (Fig. 4). PGC-1α and NFATc1 mRNA expression in sartorius muscle were elevated at 1 h of cold exposure. On the other hand, NFATc3 and CREB1 mRNA expression were not changed in sartorius muscle of chicks in response to 1 h of cold exposure.

3.2. Exogenous expression of PRL-L gene in C2C12 cells.

PRL-L was introduced into the cell line C2C12 by retroviral gene transfer (Fig. 5). Stable PRL-L mRNA expression was confirmed. Cell proliferation rates did not differ among three cell lines when cultured in DMEM with 10% FBS. On the other hand, when cultured in DMEM with 2% HS, C2C12-PRL-L-GFP cells showed higher

234 proliferation rates than those of untransduced C2C12 cells. There was no difference
235 between C2C12-GFP cells and untransduced C2C12 cells. Caspase-3 mRNA expression
236 was lower in C2C12-PRL-L-GFP cells than in C2C12-GFP cells at both time points (0
237 and 24 h) in DMEM with 2% HS.
238
239 *3.3. In vivo localization of PRL-L protein.*
240 *In vivo* localization of PRL-L protein in cross-sections of gastrocnemius
241 muscle of chicks was detected by fluorescence microscopy (Fig. 6). PRL-L was
242 observed to present in the extracellular matrix of skeletal muscle, while this protein was
243 not observed in myofibers.
244
245 **4. Discussion**
246 PRL-L has been cloned from chicken brain and is widely expressed in chicken
247 tissue including the brain, heart, kidney, lung, skeletal muscle, ovary, testis, and spinal
248 cord [20]. In this study, weak expression of PRL-L mRNA was confirmed by northern
249 blotting in all tissues examined in this study in chicks kept at a thermoneutral
250 temperature. Exposure of chicks to cold for 24 h markedly induced expression of PRL-L
251 mRNA both in the sartorius muscle and the pectoral muscle. PRL-L mRNA expression
252 in other tissues examined in this study, including pituitary gland, was not elevated in
253 response to 24 h of cold exposure. Therefore, PRL-L might have a role not so much in
254 pituitary gland but in the skeletal muscle of cold-exposed neonatal chicks.
255 Although PRL-L mRNA expression was increased in both the mixed sartorius
256 muscle and the white pectoral muscle, the degree of increase was higher in the mixed
257 sartorius muscle than that of the white pectoral muscle. In addition, the degree of

increase was 50 times higher in sartorius muscle compared to pectoral muscle. Because little information is available for differences in intracellular signaling pathways between slow- and fast-twitch fibers of skeletal muscle in birds, the reason for the higher responsiveness of PRL-L gene expression to cold exposure in the mixed sartorius muscle compared with the white pectoral muscle remains unclear. In humans, treadmill running induces different intracellular signaling responses in slow- and fast-twitch fibers of skeletal muscle [19]. Therefore, disparity in the responsiveness of this gene between the mixed and white muscles may be caused by the differences in the intracellular signaling pathways between slow- and fast-twitch fibers. The basal expression level of PRL-L mRNA in the mixed sartorius muscle was higher than that in the white pectoral muscle. These results indicate that the change in PRL-L expression induced by cold exposure might occur predominantly in slow-twitch fibers in mixed muscle. Moreover, PRL-L mRNA expression was increased preceding the increase in sartorius muscle weight. This is in agreement with our previous study that revealed that neonatal chicks exposed to cold show increased sartorius muscle weight in association with decreased myostatin mRNA expression [7,8]. These findings suggest that changes in the gene expression of PRL-L that occur early in cold exposure might be related to the increase in the mixed sartorius muscle weight of cold-exposed neonatal chicks.

PGC-1α mRNA expression in murine muscles differs in terms of predominant fiber types; i.e., PGC-1α mRNA expression in slow-fiber-enriched soleus muscle is higher than that in fast-fiber-enriched muscles [10]. Similarly, in neonatal chicks, PGC-1α mRNA expression was higher in mixed sartorius muscle than white pectoral muscle [7,8]. PGC-1α interacts with NFAT, myocyte enhancer factor 2, and CREB [4,12]. The 5'-flanking region of the PRL-L gene includes the putative binding sites for

some transcriptional factors, including NFAT and CREB [20]. In this study, both PGC-1α and NFATc1 mRNA expression in mixed sartorius muscle of chicks were increased preceding the cold-induced increase in PRL-L mRNA expression. Therefore, PGC-1α and NFATc1 may be involved in the transcriptional regulation of the PRL-L gene in mixed muscles of cold-exposed chicks. However, because little information is available for the interaction between PGC-1α and NFATc1 in birds, further studies are needed to gain more information about the interactive role of these proteins in enhanced growth of mixed muscles of cold-exposed chicks.

Although there was no effect of exogenous expression of PRL-L in C2C12 cells incubated in proliferation conditions, a proliferative effect of PRL-L was observed in differentiation conditions. Meanwhile, exogenous expression of PRL-L affected neither slow- nor fast-type skeletal muscle troponin I expression in differentiated C2C12 cells (supplemental Figure 1). In this study, C2C12-PRL-L-GFP cells showed lower expression of caspase-3 mRNA under the differentiation conditions, suggesting that PRL-L might affect caspase-3 gene expression. Fernando et al. [3] reported that inhibition of caspase-3 activity leads to dramatic reduction in both myotube formation and expression of muscle-specific proteins; hence, it was suggested that caspase-3 activity is required for progression of skeletal muscle differentiation [3,6]. While the degree of decrease was weak, caspase-3 mRNA expression in the sartorius muscle of chicks was observed to be decreased after 6 h of cold exposure. Although it is unclear whether caspase-3 activity is influenced by exogenous PRL-L expression, it is possible that the enhanced growth observed in C2C12-PRL-GFP cells under differentiation conditions occurred because of the lower expression of the capase-3 gene affected by exogenous expression of the PRL-L gene.

306 Because PRL-L was predicted to contain signal peptides [20], this protein
307 might be secreted outside the cells. We observed that PRL-L tended to be present in the
308 extracellular matrix of the mixed gastrocnemius muscle *in vivo*. In bovines, PRL-related
309 protein is located in and anchored to the extracellular matrix through interactions with
310 type IV collagen [18]. In humans, PRL is an autocrine or paracrine growth factor for
311 both myometrial and leiomyoma cells [15]. These reports and our findings showing
312 localization of the PRL-L protein allow to us to postulate that PRL-L acts as autocrine
313 and/or paracrine factor in mixed muscle growth of cold-exposed chicks. Furthermore,
314 we confirmed that molecular weight of the recombinant PRL-L protein (25 kDa) was
315 similar to the predicted molecular weight of PRL-L protein [20], while PRL-L antibody
316 recognized 36 kDa of protein in the sartorius muscle (supplemental Figure 2). Although
317 the reason for the discordance of the molecular weight remains unclear, N-linked
318 glycosylation is well known to occur on either secreted protein or membrane bound
319 protein [1]. This result may support the hypothesis that PRL-L might act as secreted
320 protein and raise the possibility that the PRL-L protein might be regulated by
321 post-translational modification such as N-glycosylation.

322 In conclusion, PRL-L is specifically induced in mixed muscle of cold-exposed
323 chicks, suggesting that PRL-L could play a role in mixed muscle growth of chicks
324 induced by cold exposure as a secreted protein.

325

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331 Technology of Japan.

332

333 **Figure legends**

334 Fig. 1. Effects of cold exposure on PRL-L expression in chicks. (A) Representative
335 northern blot data of PRL-L mRNA in various tissues of chicks. (B) Quantitative
336 real-time PCR analysis of PRL-L gene expression in sartorius muscle. (C)
337 Representative western blot results for PRL-L and α-tubulin protein in sartorius muscle
338 (upper, PRL-L; lower, α-tubulin). (D) Quantitative real-time PCR analysis of PRL-L
339 gene expression in pectoral muscle. (E) Comparison of PRL-L gene expression level
340 between sartorius and pectoral muscles of NT chicks. (F) Quantitative real-time PCR
341 analysis of PRL-L gene expression in pituitary gland. Results are normalized by
342 GAPDH mRNA and expressed as % of respective control value. Values are expressed as
343 means ± SE (n = 6). NT, nontreatment. $*P < 0.05$ (vs. control).

344

345 Fig. 2. Time course of PRL-L gene expression in sartorius muscle of cold-exposed
346 chicks. (A) Representative northern blot data of PRL-L mRNA in sartorius muscle of
347 chicks. (B) Expression of PRL-L mRNA in sartorius muscle of chicks. Results are
348 normalized by β-actin mRNA and expressed as % of respective control value. (C) Ratio
349 of sartorius muscle to body weight of chicks. (D) Ratio of pectoral muscle to body
350 weight of chicks. Values are expressed as means ± SE (n = 6). $*P < 0.05$ (vs. control).

351

352 Fig. 3. Time course of Caspase-3 gene expression in sartorius muscle of cold-exposed
353 chicks. Results are normalized by GAPDH mRNA and expressed as % of respective

354 control value. Values are expressed as means ± SE (n = 6).

355

356 Fig. 4. Quantitative real-time PCR analysis of gene expression in sartorius muscle of
357 chicks exposed to cold for 1 h. (A) Expression of PGC-1 α mRNA in sartorius muscle
358 of chicks. (B) Expression of NFATc1 mRNA in sartorius muscle of chicks. (C)
359 Expression of NFATc3 mRNA in sartorius muscle of chicks. (D) Expression of CREB1
360 mRNA in sartorius muscle of chicks. Results are expressed as % of respective control
361 value, and values are expressed as means ± SE (n = 6). $*P < 0.05$ (vs. control).

362

363 Fig. 5. Effect of exogenous expression of PRL-L gene in C2C12 cells by retrovirus gene
364 transfer. (A) PRL-L mRNA was expressed only in C2C12-PRL-L-GFP cells. Cells were
365 plated in (B) DMEM with 10% FBS or (C) DMEM with 2% HS. Cell proliferation was
366 determined by the absorbance of the WST-1 reagent and measured at the indicated time
367 periods. Results are expressed as % of respective control value (0 h), and values are
368 expressed as means ± SE (n = 12). $*P < 0.05$ (vs. control). (D) Effect of exogenous
369 expression of PRL-L gene on caspase-3 mRNA expression in C2C12 cells. Cells were
370 grown for the indicated time periods in differentiation medium. Results are normalized
371 by GAPDH mRNA and expressed as % of respective control value (0 h of C2C12-GFP
372 cells). Values are expressed as means ± SE (n = 3).

373

374 Fig. 6. Immunofluorescence staining of PRL-L in cross-sections of gastrocnemius
375 muscle of 7-day-old chicks. (A) Bright-field micrograph of cross-section of
376 gastrocnemius muscle of chicks. (B) PRL-L immunofluorescence in the same
377 microscopic field of A. (C) Merging of the images shown in A and B. Note the presence

of PRL-L, the location of which corresponds to spaces between myofibers (arrowheads). Bar, 20 μm.

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441 [20] Y. Wang, J. Li, A.H.Y. Kwok, W. Ge, F.C. Leung, A novel prolactin-like protein
442 (PRL-L) gene in chickens and zebrafish: cloning and characterization of its tissue
443 expression. Gen. Comp. Endocrinol. 166 (2010) 200-210.

Tables

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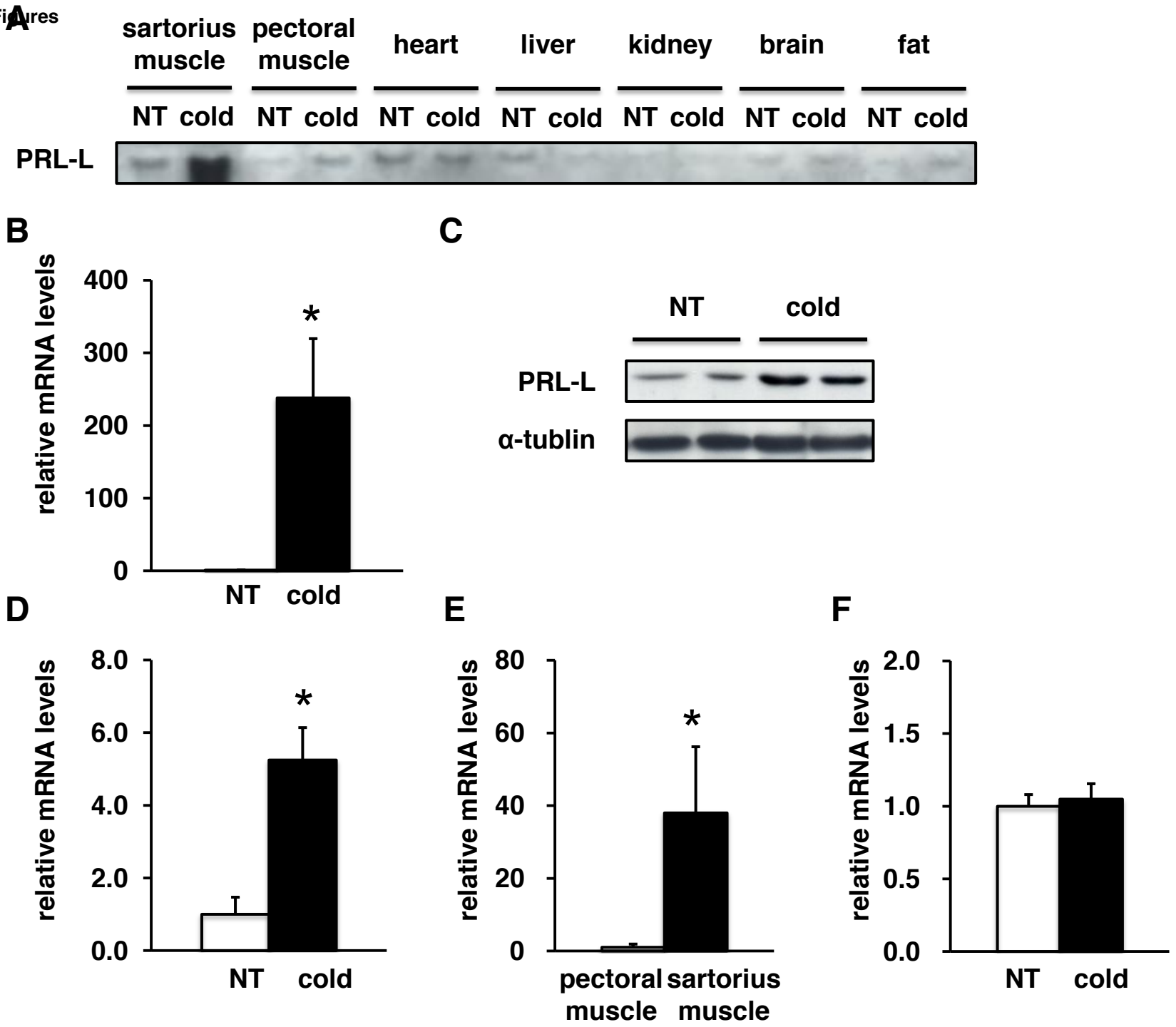
Table 1 List of primers sequence used for quantitative real time polymerase chain reaction

Animal	Gene		Sequence (5'-3')	Position (5'-3')	Size (BP)	Accession No.
chicken	PRL-L	Forward	GAAGTGCCACACGGCCACCA	266 - 441	76	NM_001001461.1
		Reverse	TGCTGAGGCTCTCACCAGGTAGC			
chicken	GAPDH	Forward	CCTCTCTGGCAAAGTCCAAG	120 - 319	200	NM_204305.1
		Reverse	CATCTGCCCATTTGATGTTG			
chicken	caspase-3	Forward	TGGCGATGAAGGACTCTTCT	557-729	173	NM_204725.1
		Reverse	CTGGTCCACTGTCTGCTTCA			
chicken	PGC-1α	Forward	GCAATTGAAGAGCGTCGTGT	2005 - 2114	110	NM_001006457.1
		Reverse	CCATAGCTGTCTCCATCATC			
chicken	NFATc1	Forward	AAGGCATCTGCTGGGGGCCA	1525 - 1623	99	XM_418906.3
		Reverse	GCGGTCGTCTGCAGTCCCAA			
chicken	NFATc3	Forward	GGGGCCTGGAGCATCTCCCAT	4 - 73	70	XM_414078.3
		Reverse	CCAGCCGCACGCACACAATG			
chicken	CREB1	Forward	GACGCGGTGTGTTACGTGGGG	123 - 367	245	NM_204450.2
		Reverse	TGCAGAAGACGTGGCGTGAGC			
mouse	capase-3	Forward	CTGCCGGAGTCTGACTGGAA	504-600	97	NM_009810.2
		Reverse	ATCAGTCCCACTGTCTGTCTCAATG			
mouse	GAPDH	Forward	AAATGGTGAAGGTCGGTGTG	65-172	108	XM_001479371.3
		Reverse	TGAAGGGGTCGTTGATGG			


A
sartorius muscle
pectoral muscle
heart
liver
kidney
brain
fat
NT cold NT cold NT cold NT cold NT cold NT cold NT cold
PRL-L
B
relative mRNA levels
400
300
200
100
0
*
NT cold
C
NT
cold
PRL-L
α-tublin
D
relative mRNA levels
8.0
6.0
4.0
2.0
0.0
*
NT cold
E
relative mRNA levels
80
60
40
20
0
*
pectoral muscle
sartorius muscle
F
relative mRNA levels
2.0
1.5
1.0
0.5
0.0
NT cold

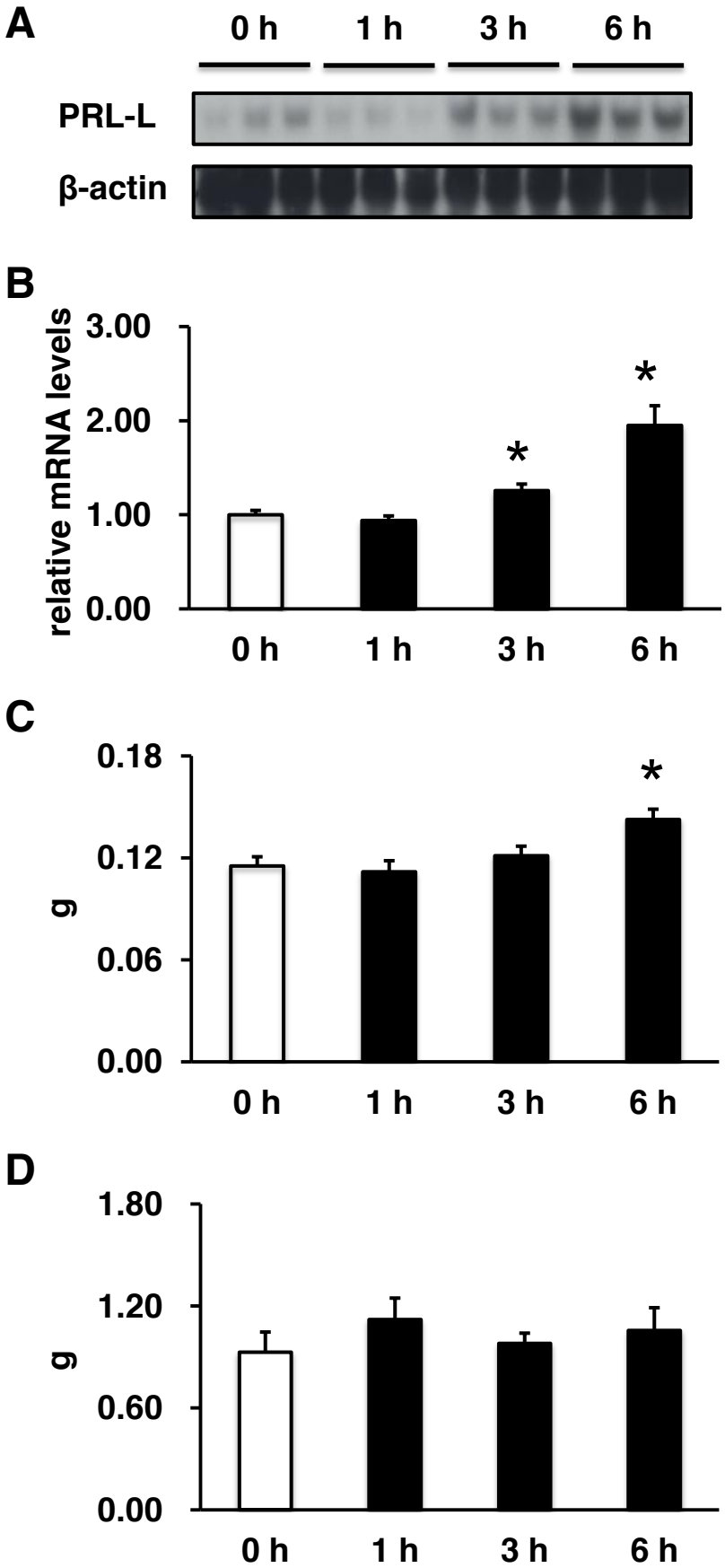

A
0 h
1 h
3 h
6 h
PRL-L
β-actin
B
relative mRNA levels
3.00
2.00
1.00
0.00
*
*
0 h
1 h
3 h
6 h
C
g
0.18
0.12
0.06
0.00
*
0 h
1 h
3 h
6 h
D
g
1.80
1.20
0.60
0.00
0 h
1 h
3 h
6 h

Figure 2

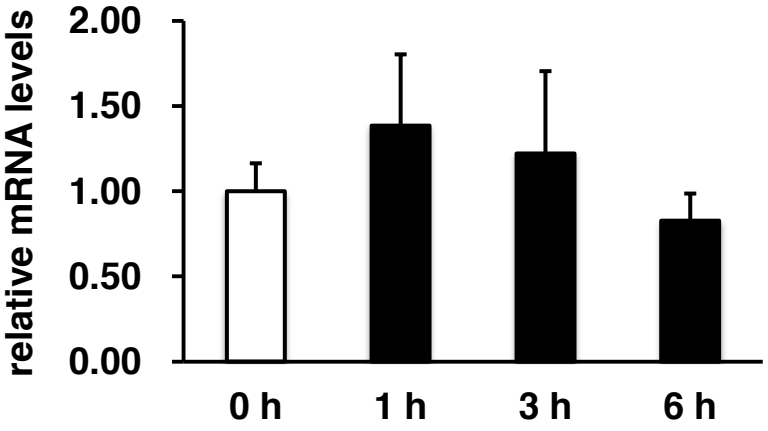


Figure 3

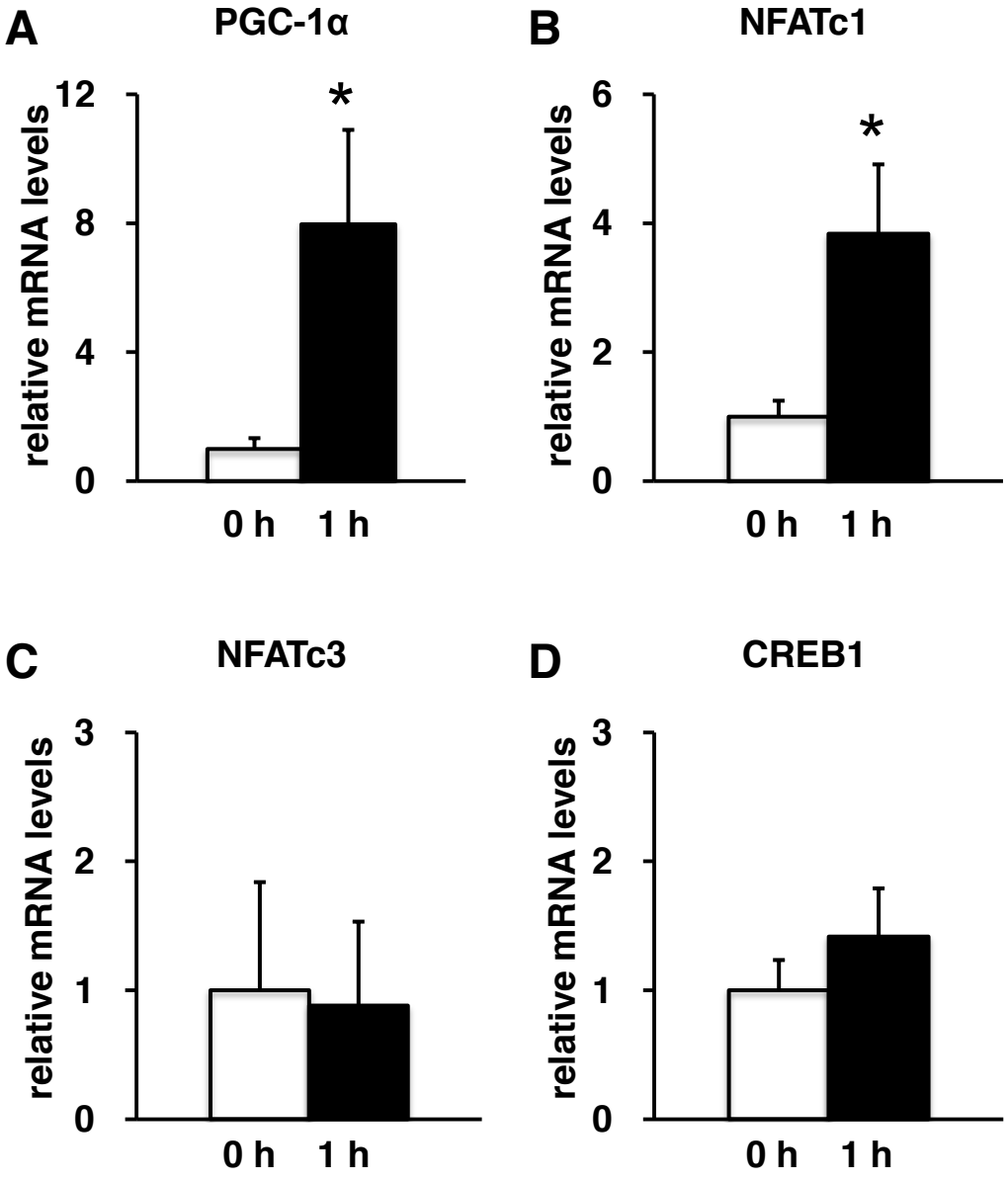

A
PGC-1α
relative mRNA levels
12
8
4
0
*
0 h
1 h
B
NFATc1
relative mRNA levels
6
4
2
0
*
0 h
1 h
C
NFATc3
relative mRNA levels
3
2
1
0
0 h
1 h
D
CREB1
relative mRNA levels
3
2
1
0
0 h
1 h

Figure 4

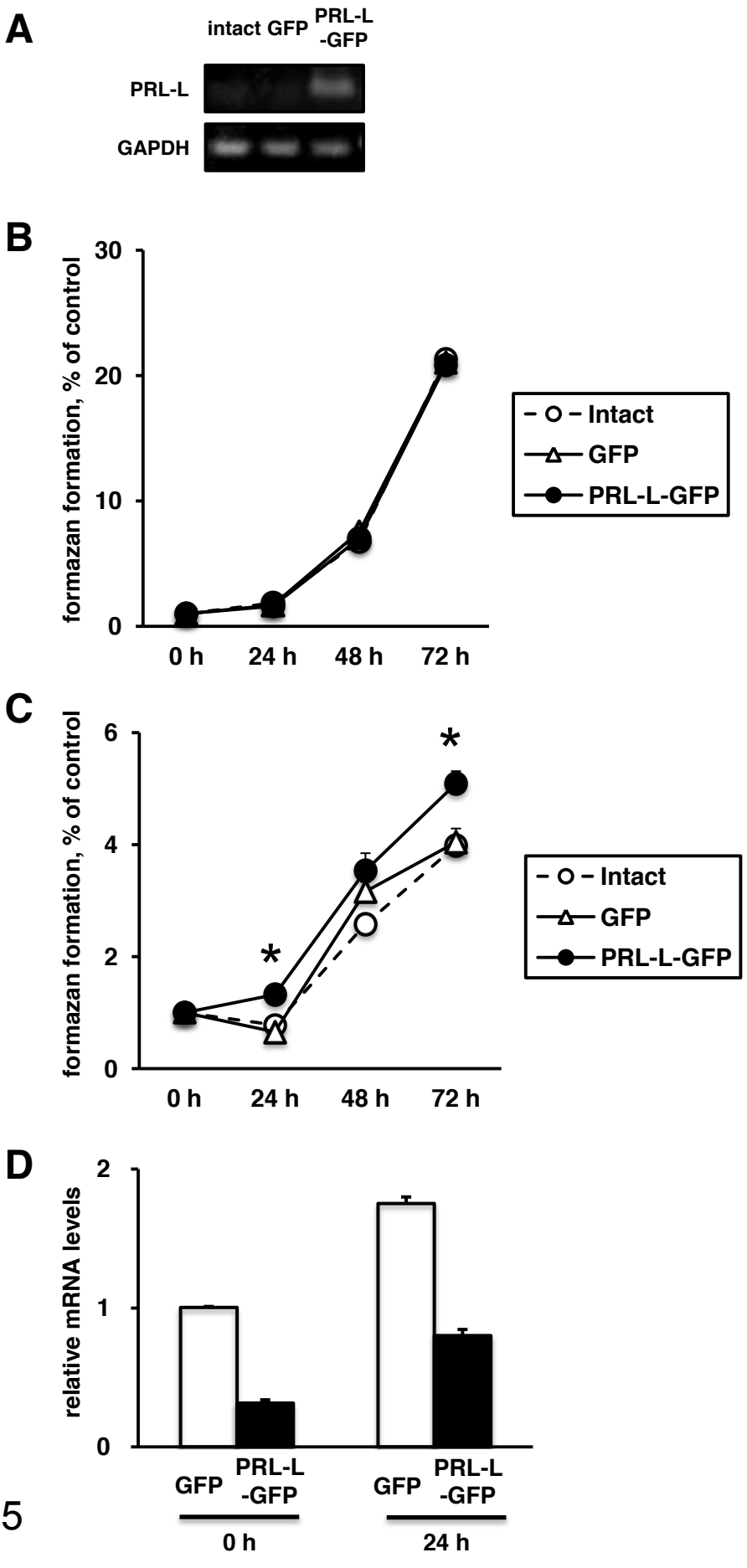

A
intact GFP
PRL-L -GFP
PRL-L
GAPDH
B
formazan formation, % of control
30
20
10
0
0 h
24 h
48 h
72 h
Intact
GFP
PRL-L-GFP
C
formazan formation, % of control
6
4
2
0
*
*
0 h
24 h
48 h
72 h
Intact
GFP
PRL-L-GFP
D
relative mRNA levels
2
1
0
GFP
PRL-L -GFP
GFP
PRL-L -GFP
0 h
24 h

Figure 5

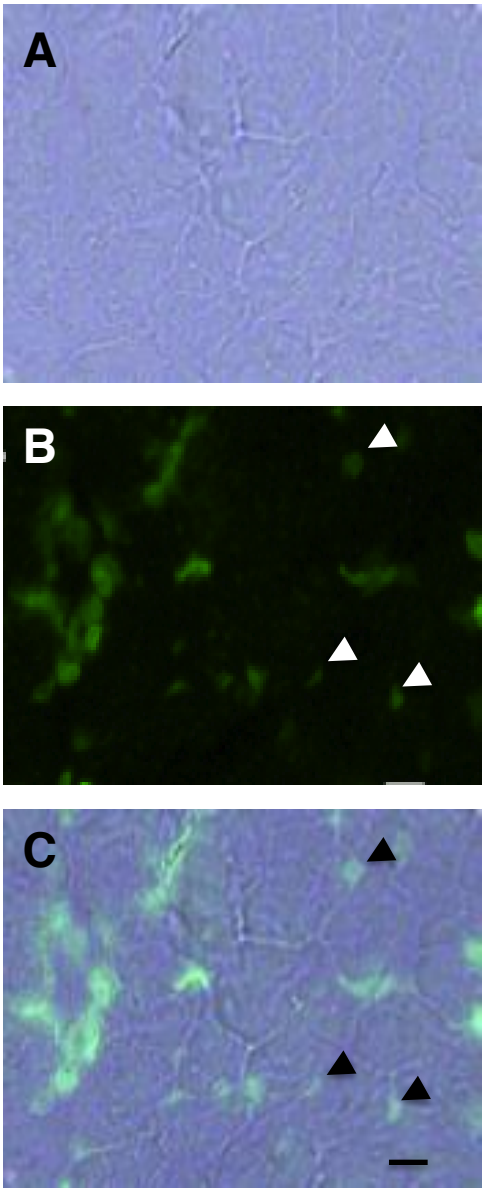


Figure 6