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chGRP78 is Required for the Activation and Phosphorylation of AKT1 at Serine 478 in Chicken Sells

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ABSTRACT

Background: Chicken serum-mediated proliferation regulates chGRP78 to prevent apoptosis in chicken cells via chGRP78-mediated anti-apoptosis. However, the precise molecular mechanisms underlying the chGRP78-mediated protection against apoptosis remain undefined. In an earlier study, we have shown that chGRP78 is critical for chicken embryo fibroblast (CEF) and DF-1 cell proliferation.

Methods: In this experiment, we highlight AKT1 as a key target of GRP78 during apoptosis. We used 2D gel-based proteomics and bioinformatics prediction analysis for our studies.

Result: Here, we detected chGRP78 binding sites in AKT1-rgulated proteins. chGRP78 promoted AKT1 activation and chGRP78 silencing decreased AKT1 levels. Taken together, we suggest that the AKT1-mediated signaling pathway plays a critical role in GRP78-stimulated fibroblast survival and anti-apoptosis. Our findings have important implications for the maintenance of chicken fibroblast cells via the inhibition of apoptosis.

Key words: AKT1, Chicken embryo fibroblast cells, Chicken GRP78.

INTRODUCTION

Defining the molecular mechanisms of chicken cell proliferation and learning to control these mechanisms may be essential for treating pathological conditions, including normal chicken embryonic development and chicken cell death (Hamidu *et al.,* 2010; Schaefer *et al.,* 2004; Yao *et al.,* 2015). Chicken glucose regulated protein 78 (chGRP78) is typically expressed in mammalian cells and plays a prominent role in the maintenance of cell homeostasis and prevention of apoptosis (Liu and Bowes, 1997). chGRP78 was discovered when proteins that were 78 kDa in size were observed to be strongly upregulated in chicken cells in glucose-free medium (Shiu *et al.,* 1977). Additionally, chGRP78 is a highly conserved protein in most eukaryotes (Stoeckle *et al.,* 1988; Ting and Lee, 1988). Our previous data have demonstrated that chicken chGRP78 silencing promotes chicken cell apoptosis (Jeon *et al.,* 2016). Protein kinase B (AKT) is a human serine-threonine kinase and member of the AGC family of protein kinase. AKT1 is a key regulator of the phosphoinositide 3 (PI3K)/AKT1 signaling cascade that controls cell growth and survival (Toulany *et al*., 2017). Overactive Akt1 is a hallmark of diverse human malignancies (Luo *et al*., 2015). In human cells, the activation of Akt1 occurs in response to growth factor stimulation. Although these important biological roles for chGRP78 have been established, the molecular mechanisms and downstream targets of chGRP78 in the apoptosis process are not completely understood.

To address this lack of understanding, the chGRP78-mediated genes involved in cell proliferation were investigated using two-dimensional (2D) gel electrophoresis and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analyses, which provided a comprehensive profile of protein expression and enabled the identification of candidate pathways and biological processes that were regulated by specific genes. These studies identified the chicken AKT1 protein, which showed increased activation and phosphorylation in chicken cell following GRP78 over-expression. Collectively our data highlight the importance of AKT1 activation in the response of chGRP78-overexpressing cells to chicken cell proliferation. Our results extend the current understanding of the multiple cellular events that are affected by chGRP78 in chicken cells.

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MATERIALS AND METHODS

Cell culture

Primary chicken embryo fibroblast (CEF) cells and the continuous CEF cell line DF-1 (CRL-12203; American

Type Culture Collection, Manassas, VA, USA) were grown in Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 1% penicillin-streptomycin solution (HyClone, Logan, UT, USA) and 2% chicken serum (Sigma, St. Louis, MO, USA). Cells were passaged, cultured and maintained at 37°C in a humidified 5% carbon dioxide incubator. Because CEF cells are difficult to prepare and use for siRNA and overexpression experiments, we used the continuous chicken embryo fibroblast cell line DF-1 (CRL-12203; American Type Culture Collection, Manassas, VA).

Proteome analysis

The sample lysis solution consisted of 7 M urea and 2 M thiourea containing 4% (w/v) CHAPS, 1% (w/v) DTT, 2% (v/v) pharmalyte and 1 mM benzamidine. The proteins were extracted for 1 hr at room temperature with vortexing. After centrifugation at 15,000 rpm for 1 hr at 15°C, the insoluble fraction was discarded and the soluble fraction was subjected for 2D gel electrophoresis. The protein concent ration was measured using the Bradford method (Bradford, 1976).

IPG dry strips (4-10 NL IPG, 24 cm, Genomine, Korea) were equilibrated for 12-16 hr with a 7 M urea plus 2 M thiourea solution containing 2% CHAPS, 1% DTT and 1% pharmalyte and each strip was loaded with 200 µg of the protein sample. Isoelectric focusing (IEF) was performed at 20°C using a Multiphor™ II electrophoresis unit and an EPS 3500 XL power supply (Amersham Biosciences, UK) according to the manufacturer's instructions. For IEF, the voltage was linearly increased from 150 to 3,500 V over 3 hr to allow for sample entry and subsequently maintained at a constant voltage of 3,500 V, with the focusing complete after 96 kVh. Prior to the second dimension of the 2D electrophoresis, the strips were incubated for 10 min in equilibration buffer (50 mM Tris-Cl pH 6.8 containing 6 M urea, 2% SDS and 30% glycerol), first with 1% DTT and then with 2.5% iodoacetamide. The equilibrated strips were inserted into the SDS-PAGE gels (20 x 24 cm, 10-16%) and SDS-PAGE was performed using a Hoefer DALT 2D system (Amersham Biosciences, UK) per the manufacturer's instructions. The 2D gels were run at 20°C for 1,700 Vh and silver stained as described previously (Oakley *et al.*, 1980), omitting the glutaraldehyde fixation and sensitization steps. The digitized images were quantitatively analyzed using PDQuest (version 7.0, Bio-Rad) per the manufacturer's protocols. Each spot was normalized using the total intensity of the valid spots. We selected the protein spots that deviated more than two-fold from the control or normal samples as showing significant expression levels.

Functional interactome analysis

Experimental data were integrated and analyzed using GeneMANIA (Montojo *et al.,* 2014) Cytoscape plugin. CluGO functional analysis was performed using GO: Biological Process with the default CluGO setting. For the list of genes that were associated with "translation," GO: Biological Process was applied to reconstruct the functional interaction network using the GeneMANIA plugin. The scores and parameters were selected by default. The organic or circular algorithm that determined the node positions based on their connectivity was used to design the network.

siRNA treatment

A total of $1X10^6$ fibroblasts were seeded onto 6-well plates and transfected using RNAiMAX (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Briefly, 7.5 µL of the RNAiMAX reagent was suspended in 100 µL of Opti-MEM (Gibco-BRL/Invitrogen, Carlsbad, CA, USA) and incubated for 5 min at room temperature. The mixture was added to a 100-µL Opti-MEM solution containing 75 nM siRNAs. Then, the RNAiMAX mixture and probes were added dropwise to the fibroblasts in the culture medium lacking antibiotics. The sequence of siRNA for chicken GRP78 was 5′-CACUUCAUCAAACUCUACA-3′.

Immunoblotting

The primary antibodies for western blotting used in this study are GRP78 (PA5-22967, Thermo Scientific/ Pierce, Rockford, IL, USA), Cell Signaling Technology, MA, USA), p-AKT1 (9275, Cell Signaling Technology, MA, USA), p-AKT (PAB10323, (Abnova, San Francisco, CA, USA) and β-actin (MA1-91399, Thermo Scientific/Pierce, Rockford, IL, USA). Protein extracts (20 µg) were electrophoresed using a NuPAGE 10% Bis-Tris gel system (Invitrogen, Carlsbad, CA, USA) and transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBST (0.01 M Tris, 0.15 M NaCl and 0.5% (w/v) Tween 20) for 1 hr. The Indicated primary antibodies were incubated at 4°C overnight followed by secondary antibodies were for 2 hr at room temperature. The membranes were washed and incubated with HRP-conjugated secondary antibodies for 1 hr. The ECL (Pierce, Rockford, IL, USA) chromogenic substrate was used to visualize the bands and images were captured on X-ray films. The band densities of scanned X-ray film were analyzed using image J software.

Statistical analysis

All analysis was conducted using the Graph Pad Prism statistical software (Graph Pad Software). An unpaired t test was used for comparisons of the treatment groups and a P-value less than 0.05 was considered statistically significant. The data are presented as the means ± standard errors (SEs) or standard deviations (SDs).

RESULTS AND DISCUSSION

Previous data have shown that chicken chGRP78 silencing promotes chicken cell apoptosis. To characterize the mole--cular mechanisms and biological functions associated with chGRP78 overexpression, we generated stable chGR P78-overexpressing DF1 cell lines and analyzed the protein levels by western blotting. We found that the protein levels of chGRP78 were significantly increased in chGRP78-over expressing in DF1 cell lines compared to the control cells (Fig 1).

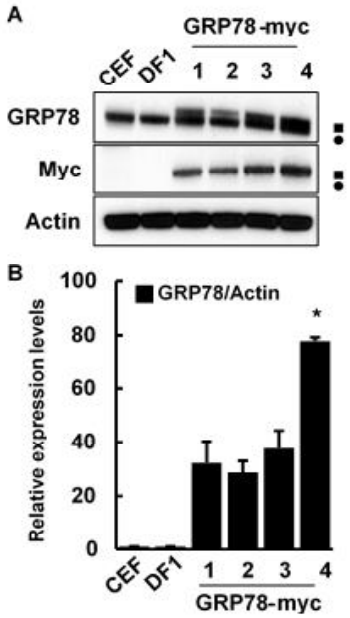


Fig 1: Purification of myc-tagged proteins for the analysis of GRP78 interactions in chicken cells.

(A) Cell lysates from CEF (Chicken embryonic fibroblast) cells, DF1 (Immortal chicken embryonic fibroblast) cells and GRP78-myc transgenic DF1_1, 2, 3 and 4 cell lines were immunoblotted with GRP78 and myc-tag antibodies (GRP78 and GRP78-myc are indicated by filled circles and squares, respectively). (B) Western blot analysis confirmed the changes in protein expression that were initially identified by quantitative bands. Actin was used as the loading control. Error bars indicate the mean ± SD (n=3). *<0.01 compared with the control.

To determine the quantitative variations in protein abundance between the control and chGRP78-over expressing DF1 cells, we performed an LC-MS/MS analysis (Fig S1). Six proteins were identified using peptides that were predicted to be correctly sequenced. To further interpret the functional roles of GOT2, ACTB, ALDOC, ENO1, FABP7 and PDIA3, which were induced by chGRP78, we used KEGG (Fig 2) and the genemania plugin to integrate the interconnected biological networks related to the GOT2, ACTB, ALDOC, ENO1, FABP7 and PDIA3 proteins (Fig 3). Earlier study (Elzaiat *et al.,* 2019) reported that GOT2 and PDIA3 proteins are increased phosphorylation after Akt1 silencing. Such proteins are potential target to kinases inhibited by Akt 1 or of phosphatases activated by Akt1. In addition, ENO1 enhance to glycolysis, migration, growth and Invasion in non- small cell lung cancer through FAK-mediated PKB/Akt pathway (Hong *et al.,* 2016).

We predicted interactions using the genemania cytoscape plugin, which enables the construction of a gene-gene functional interactome from a gene list. Therefore, we built an interaction network for the 4 proteins associated with the chGRP78 protein in the apoptosis pathways. Together, these results indicated that the coordinated proteomic changes in chGRP78 overexpression could differentially affect various cellular processes, including apoptosis, to assess AKT1 activation in the presence of the chGRP78 protein in chicken cells. We demonstrated that chGRP78 increases cell proliferation (Fig 4A) and AKT1 phosphorylation (Fig 4B) in DF1 cells. Therefore, the relationship between AKT1 and chGRP78 was investigated further. Knockdown of chGRP78 decreased AKT1 phosphorylation (Fig 4A). These

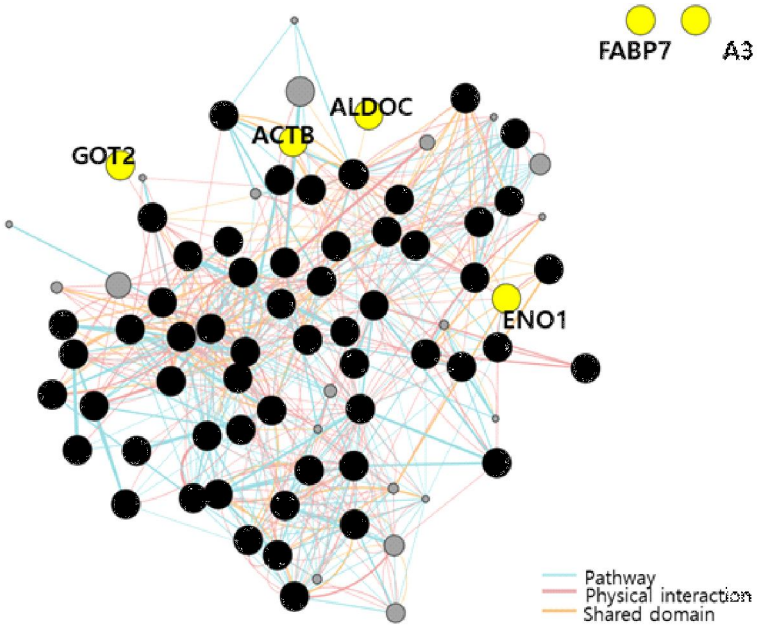


Fig 2: Analysis of the interaction network of apoptosis-related genes from KEGG. Through the interaction analysis, we cropped the network which including targets and neighbor genes from whole interaction network. Yellow color circles indicate identified genes. Black color circles indicate apoptosis related genes. Grey color circles indicate linker genes.

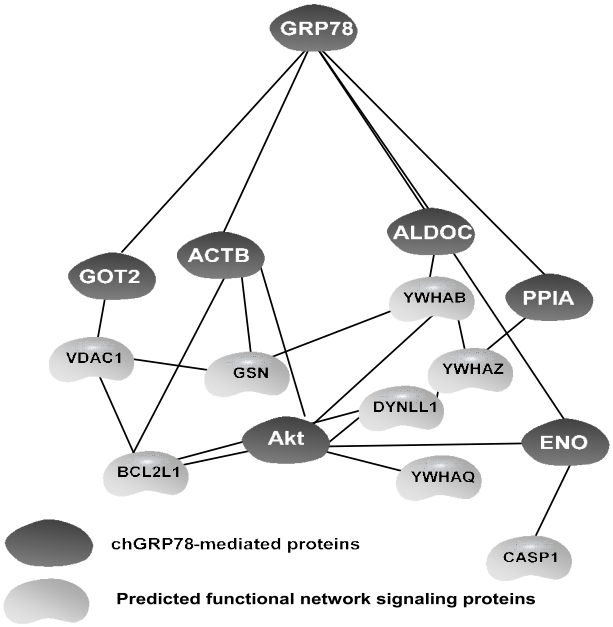


Fig 3: Analysis of the interaction network of apoptosis-related genes using the genemania plugin. Apoptosis-related genes were obtained from KEGG (www.genome.jp/kegg/) and the interaction network was reconstructed with the 6 identified genes (GOT2, ACTB, ALDOC, ENO1, FABP7 and PDIA3) using the genemania cytoscape plugin.

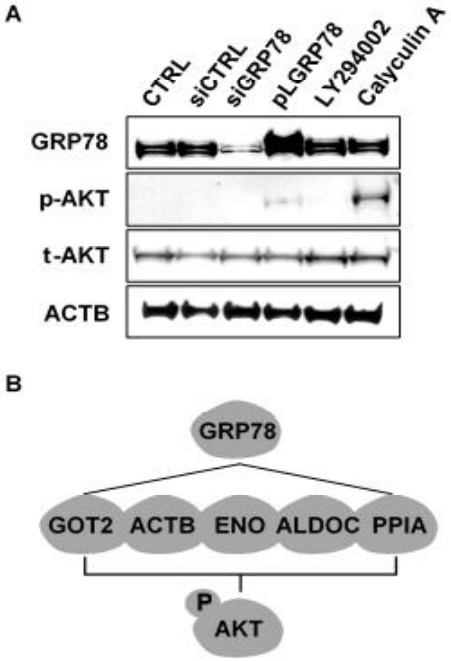
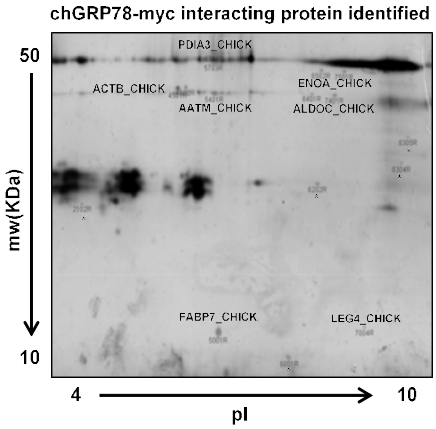


Fig 4: chGRP78 play to role in the phosphorylation and activation of AKT1.

The number of viable cells was determined by the MTT assay (A). DF-1 cells were transfected with non-target siRNA (siCTRL), siRNA-GRP78 (siGRP78) and GRP78 p1lasmid (pLGRP78) and LY294002 and CalyculinA treatment, harvested at 48 h post-transfection and subjected to western blotting analyses of GRP78 expression (B). Schematic presentation of the chGRP78 related proteins in chicken cells (C).



UniProt accession[a]	Protein names	Gene names
PDIA3_CHICK	Protein disulfide-isomerase A3	PDIA3
ACTB_CHICK	Beta-actin	ACTB
FABP7_CHICK	Fatty acid-binding protein, brain	FABP7
AATM_CHICK	Aspartate aminotransferase, mitochondrial	GOT2
ENOA_CHICK	Alpha-enolase	ENO1
LEG4_CHICK	Beta-galactoside-binding lectin	
ALDOC_CHICK	Fructose-bisphosphate aldolase C	ALDOC

Fig S1: chGRP78-myc interacting protein identified Identification of DEPs in DF-1 cell exhibiting stable chGRP78 protein.

data suggest that down-regulation of chGRP78 increases AKT1 phosphorylation at Ser478. In support of these findings, previous studies have suggested that GRP78 silencing reduces AKT1 activity (Gray *et al.,* 2013; Xia *et al.,* 2016). Additionally, GRP78 protects cells against apoptosis through multiple pathways (Jolly and Morimoto, 2000).

CONCLUSION

Thus, we presume that the phosphorylation and activation of AKT1 promotes anti-apoptosis in chicken cells. Therefore, chGRP78-mediated AKT1 activation may be strongly involved in the activation of CEF cell proliferation. Together, our findings suggest that the AKT1-mediated proliferative and anti-apoptotic phenotypes in chicken cells are stimulated through chGRP78 expression.

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