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Full Length Research Paper

c-Lysozyme promotes proliferation of chicken embryonic fibroblast through bFGF pathway

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The egg white (EW) contains the majority of bioactive components which maintain embryo growth and differentiation. The discovery of new growth promoting factor in egg white will provide vital clue to understand the developmental regulation of early chicken embryo. The egg white heated with different temperatures (63.5, 70 and 95°C) underwent testing on its growth-promoting effect on chicken fibroblast *in vitro*. The purified c-lysozyme and the expression of related genes in basic fibroblast growth factor (bFGF) pathway were analyzed to ascertain its growth-promoting mechanism. 13 h after egg white treatment, more fibroblast synchronized with serum starvation transited into S phrase from G0/G1 in EW group than in the control group (CM) and reached the phase of peak proliferation at 15 h after treatment. It was found that c-lysozyme had the function of promoting cells growth and was decided by gradient heat inactivation of egg white. The addition of more than 0.25 mg/ml c-lysozyme produced significant increase in the cellular proliferation during 48 to 72 h of culture. At 13 h after c-lysozyme treatment, the bFGF, cyclin D, cyclin A and CDK2 were up-regulated significantly and promoted the transition from G0/G1 into S phrase and the accurate completion of S phrase. C-Lysozyme contains a growth-activating domain to promote the cell proliferation besides its anti-microbe domain.

Key words: c-Lysozyme, fibroblast, fibroblast growth factor receptor (FGFR), cell cycle.

INTRODUCTION

Chicken egg is not only a kind of food but also has other functions such as health-promoting, immuno-regulation, anti-aging and therapeutic property. Eggs contain series of nutrients, vitamins including A, E and K, growth- and differentiation-promoting factors that are essential to the early development from early blastoderm to heart beating stage *in vitro*. The identification of new bioactive factors in egg would expand the application of eggs and further understand the mechanism of blastodermal development and differentiation.

Lysozyme is part of the innate immune system to protect animal from pathogens like *Salmonella*, *Escherichia coli* and *Pseudomonas*. Lysozyme is abundant in many types of secretions, such as tears, saliva, human milk, mucus and egg white. For children fed with infant formula lacking lysozyme in their diet, the risk of suffering diarrheal disease will increase by three times. It is also present in cytoplasmic granules of the polymorphonuclear neutrophils (PMN). The conjunctiva (membrane covering the eye) is protected by secreted enzymes, mainly lysozyme and defensin. Large amounts of lysozyme can be found in egg white as c-lysozyme (Chicken Lysozyme) which plays an important role to maintain early development of chick embryo.

Lysozyme, also known as muramidase or N-acetylmuramide glycanhydrolase, can damage bacterial walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins (found in the cell walls of bacteria, especially Gram-positive bacteria). C-Lysozyme makes up 3 to 5% of total protein in egg, and consists of 129 aas (amino acids). The glutamic acid 35 (Glu35)

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and aspartate 52 (Asp52) have been found to be critical to the activity of c-lysozyme. The Glu35 acts as a proton donor to the glycosidic bond, cleaving the C-O bond in the substrate, while Asp52 acts as a nucleophile to generate a glycosyl enzyme intermediate (Goldman and Smith., 1973). The glycosyl enzyme intermediate then reacts with a water molecule, leading to the hydro-lysis of substrate and leaving the enzyme unchanged.

C-Lysozymes are relatively similar to α-lactalbumin in sequence and structure, making them part of the same family. c-Lysozyme gene gave rise, after gene duplication 300 to 400 million years ago, to α-lactalbumin gene, a protein expressed only in the lactating mammary gland of all but a few mammals and shared only 40% identity in amino acid sequence with c-lysozyme, however it has a similar spatial structure and gene organization (Hashimoto et al., 1996). Although structurally similar, they are quite distinct functionally. Specific amino acid substitutions in alpha-lactalbumin account for the loss of the enzyme activity of lysozyme and the acquisition of the features necessary for its role in lactose synthesis. Human alpha-lactalbumin contains a growth-stimulating peptide of 35 aas from 63 to 97aa that facilitates cell growth. Since the primary and secondary structures of lysozyme have a high homology to those of a-lactalbumin (Qasba and Kumar., 1977), it was confirmed that human lysozyme also contained a growth-activating peptide arising from the digestion by endopeptidase Arg C (Grobler et al., 1994). The c-Lysozyme should have dual functions in both bacteria killing and growth promoting, although not yet confirmed. C-Lysozyme also has an important repairing effect on injured tissue in inflammation.

There are so many pathways to regulate the propagation of eukaryotic cells, such as basic fibroblast growth factor (bFGF) pathway (Kanda et al., 2007), Wnt pathway and so on. The bFGF (FGF-2) is strongly associated with tumor angiogenesis (Fuhrmann-Benzakein et al., 2000), tissue regeneration after injury (Tarnawski et al., 2001), and bFGF binds to cell-surface tyrosine kinase receptors (fibroblast growth factor receptor, FGFR). The presence of heparin-heparan sulfate increases the stability of the FGF-FGFR complex and its efficacy of signaling (Vlodavsky et al., 1996). The bFGF enhances the expressions of cyclin D, CDK2 and cyclin E to promote the transition from G0/G1 to S phrase, and the increase of E2F and cyclin A level to maintain the normal progress of S phrase. The balance expression of genes in this pathway accelerate pass across checkpoint I and efficient synthesis of DNA to hasten cell cycle progress. In this study, we confirmed that the c-lysozymes promote the CEF proliferation and increase the expression of bFGF and FGFR.

MATERIALS AND METHODS

Chicken embryo fibroblasts (CEF) were prepared from 10-day-old pure-breed, healthy white Leghorn chicken embryo (obtained from the Experimental Animal Farm, China Agricultural University). Cell counting kit-8 was purchased from Dojindo Laboratories. Culture plates (96-well) were purchased from Corning Co.; tryptin, and Dulbecco's modified Eagle's medium (DMEM) were supplied from Gibco Co. and fetal bovine serum (FBS) was purchased from Sigma Co. The chicken lysozyme (ChLZ) was purchased from Invitrogen Co. Other reagents were commercial products of special grade.

Culture conditions

CEF cells were cultured with 0.5% FBS-DMEM (Serum starvation, SS) for 24 h. When more than 80% of cells reached cell cycle synchronization, the culture medium was changed to 10% FBS-DMEM (Complete medium, CM) with the addition of different concentrations of egg white or c-lysozyme.

Egg white treatment

Egg white was serially diluted by 10, 20 and 50 times with DMEM, and then stored at -20°C until use. The egg white with different final gradient concentrations (2, 5 and 10%) was added in SS or CM group for 48 h at 37°C in an incubator. In the CM group, the 5% egg white was previously heated at 25, 63.5, 70 and 95°C for 3 min, respectively before the addition.

c-Lysozyme treatment

The c-lysozyme (Invitrogen) was dissolved in DMEM at a concentration of 10 mM, sterilized by filtering through MillexR-GV filter (0.22 µM), and then, immediately prior to use, was diluted 10 times with DMEM. The final gradient concentrations (0.25, 0.5 and 1 mM) were applied to the CEF cells culture that reached cell cycle synchronization respectively in CM.

Measurement of proliferation

Cell proliferation was measured with the cell counting Kit-8 (CCK-8, Dojindo Laboratories) according to manufacturer's protocol. Briefly, 10 µL of solution CCK-8 was added into each well of 96-well plate that contained 5×10^3 treated cells per well, and incubated for 4 h at 37°C. The absorbance was measured at a wavelength of 450 nm using Model 550 Microplate Reader (Bio-Rad).

Cell cycle analysis

Briefly, 5×10^5 CEF cells in 200 µL Ca^{2+}, and Mg^{2+} free phosphate buffered saline (PBS) were fixed in 4 ml 70% ethanol in PBS at 0°C. This was further digested with 1000 U RNase A (Sigma) and stained with 25 µg/ml propidium iodide, then subjected to flow cytometry (FACSCalibur, BD Co.)

Isolation of total RNA and RT-real time PCR

Total cellular RNA was isolated with Trizol (Invitrogen) according to the manufacturer's protocol. Briefly, 1 µg of RNA samples were reverse-transcribed to cDNA and were stored at -20°C until use. Polymerase chain reaction (PCR) was conducted in a 20-µL reaction volume containing 0.2 µL of each primer (10 pmol/µL), 9 µL of SYBR mixture and 2 µL of RT product. The PCR conditions were

set as follows: 1 cycle of 94°C for 5 min, 40 cycles of 95°C for 30 s, 60°C for 30 s, 68°C for 20 s. All real-time qPCR tests were run in triplicate. The following PCR primers were used: β-actin (forward,5′-GAGAAATTGTGCGTGACATCA-3′; reverse, 5′-CCTGAACCT-CTCATTG CCA-3′); cdk2 (forward,5′-GCTCTACCTGGTCTT-TGAGTTCCT-3′; reverse, 5′-GCAGCT GGAACAGGTAGCTCTT-3′); bFGF (forward, 5′-CTTCCGTGACCGGTAAGTGT-3′; reverse, 5′-TGCAGCTTCAAGCAGAAGAA-3′); FGFR2 (forward, 5′-GTGCC-AGCAGCAAACACTGT-3′; reverse, 5′-AGCCGCCAATAC-GATGTTCT-3′); E2F1 (forward, 5′-TTCCTGGCGGATGAGT TCAT-3′; reverse, 5′-TCCCCAAAGTCACAGTCAAAGA-3′); and cyclinA2 (forward, 5′- TTGCTCATGGACCTTCACA-3′; reverse, 5′-GCGTCAATAAGCGATACTGCAT-3′). The results are expressed as means ±SEM. The significance of difference between mean values was evaluated by two tailed Student's test and $P < 0.05$ was considered statistically significant.

RESULTS

Effect of EW on proliferation of chicken embryo fibroblast cells

First, we measured the number of fibroblast cells after 24 and 48 h of treatment with EW at concentrations of 2, 5 and 10%, respectively. After 24 h of EW treatment, the numbers of fibroblast cells with all of concentrations supplemented EW were compared to control (CM, without EW treatment). After 48 h, the numbers of fibroblast cells cultured with 2 and 5% EW were significantly increased compared to those of CM ($p<0.05$). However, 10% EW did not show any effects on cell proliferation (Figure 1A). In starvation with 0.5% FCS, EW did not show any growth stimulation from 24 to 48 h post-treatment, which indicated that some certain growth-regulating factors exist in EW (Figure 1B).

Regulation of active components in EW to cell cycle

After serum starvation with 0.5% FBS in DMEM medium for 24 h, 79.08% chicken fibroblast cell were arrested at G0/G1 phrase and then, the medium was removed and CM medium with or without 5% EW was added. At 15 h, the fibroblast cells that had passed Checkpoint I and entered into G2/M phrase in CM culture with EW were significantly more than that in CM without EW. At 21 h, more fibroblast cells in CM+EW had entered S phrase to restart the next cell cycle. The 8.5% difference in fibroblast cell percentage of S phrase between CM and CM+EW was significant (Figure 2). This indicates that the EW accelerated the progress of cell cycle if FBS is sufficient in the culture.

Temperature sensitivity of active components in EW

The growth-facilitating component in EW was temperature-sensitive. Heating at 98 and 70°C for 10 min can inactivate their growth promoting activity on cell division completely (Figure 3). After being heated at 63.5°C for denaturing, the promotion effect was lowered compared with the untreated EW, but still maintained the strong stimulatory activity compared with the CM ($p<0.01$). In view of this result, we presumed that c-lysozyme would be a candidate factor to promote cell growth.

c-Lysozyme enhanced CEF proliferation

According to the concentration of lysozyme in EW, the serial amount of purified c-lysozyme was added to the culture medium to test its effect on cell proliferation in different periods. After 24 h of culture with 0.25 mg/ml c-lysozyme, the proliferation potential of fibroblast increased significantly compared with the CM culture ($p<0.05$). This growth-promoting effect was the same to that previously in EW. From 48 to 72 h of co-incubation, c-lysozyme promoted the proliferation of CEFs in a dose-dependent manner (Figure 4). Compared to the control, the difference were significant ($p<0.01$) but in SS condition, the addition of c-lysozyme did not show any growth promoting effects regardless of dose (data not showed).

Molecular regulation of c-lysozyme on gene expression related with cell cycle

We monitored the expression levels of different gene at different time point after EW or c-lysozyme treatment by quantitative PCR (qPCR). 13 h after addition of EW or c-lysozyme was the key time point for preparing CEFs to boost the DNA synthesis and cell division. The expression of fibroblast growth factor receptor (FGFR) were up-regulated both in the addition of EW or c-lysozyme and bFGF pathway was related with cell cycle. The E2F that was related with S progress through Cyclin A and CDK2 that associate with G0/S transition through Cyclin E were up-regulated significantly.

Moreover, at the 15th hour, most cells had entered division phrase and the genes acting during G0/G1 phrase did not show significant changes except for CDK2 in EW treatment, of which the mechanism was unclear (Figure 5). At the 21st hour, endogenous bFGF, and cyclin A were increased. After c-lysozyme addition, all genes were associated with the DNA synthesis in S phrase. However, in the EW treatment group, FGFR and CDK2 were up-regulated to accumulate the proteins for the next cell cycle and pass the checkpoint I.

DISCUSSION

EW not only contains rich nutrients, regulators for embryonic differentiation, development and microbe-clearance, but also has multiple functions on reparation

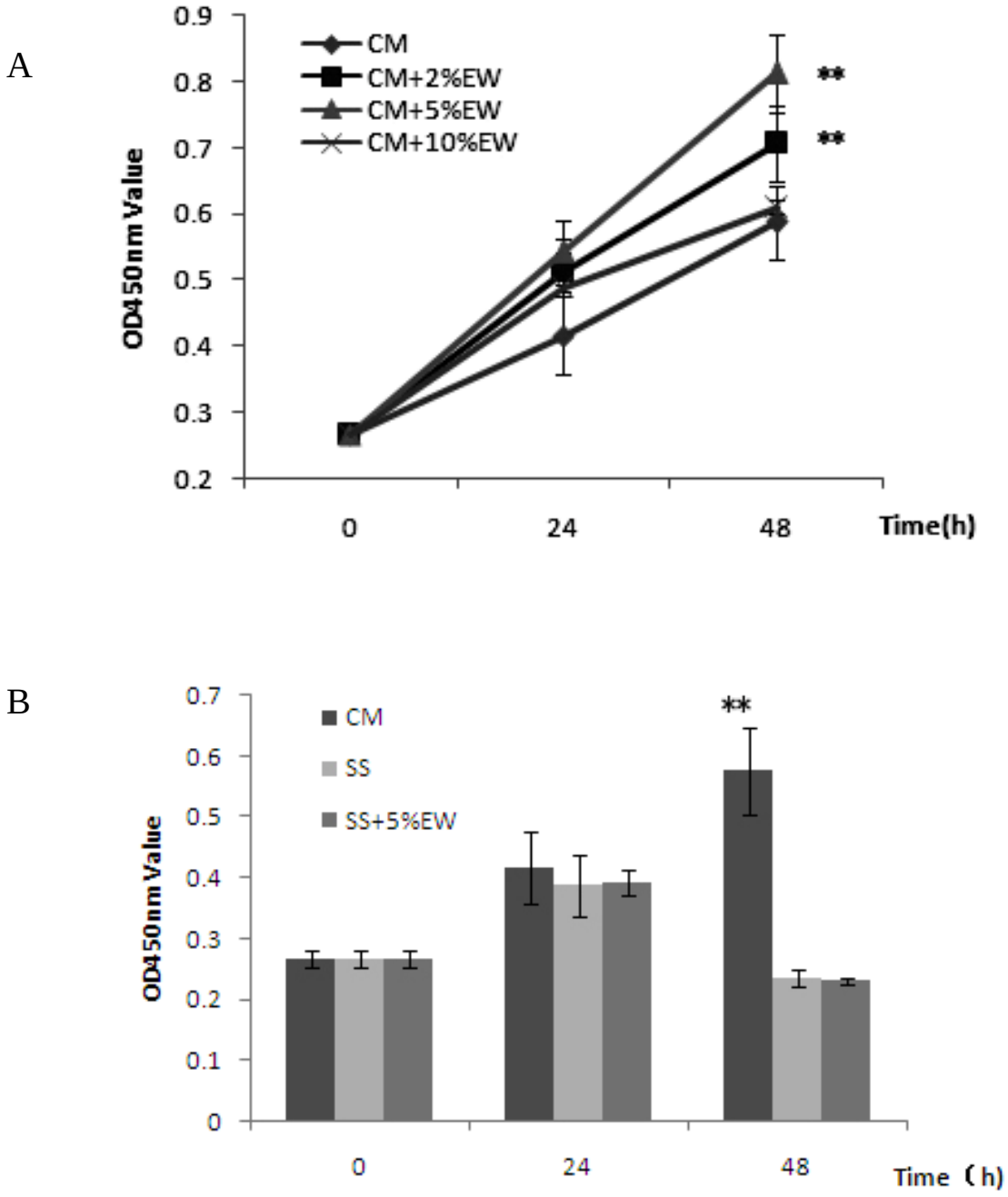

Figure 1. Effects of EW addition on fibroblast proliferation in CM or serum starvation. A, Fibroblast cells were cultured in CM with 0.2, 5 and 10% EW and the number of cells were measured at 24 and 48 h; B, fibroblast cells were treated with 10% FBS-DMEM (CM), 0.5% FBS-DMEM (SS) and 0.5% FBS-DMEM with 5% EW (SS+5%EW), respectively. At 24 and 48 h after treatment, number of fibroblast cells was measured. CM, Complete medium; SS, serum starvation; **significant difference ($p<0.01$).

and stimulation after injury. The identification of proliferation factors in EW will be helpful to understand the mechanism of development of early stage embryo in chicken and can be used to develop a new medicine or additives for improving animal health and growth performance. There are many kinds of protein in EW, such as ovalbumin, transferrin, lysozyme, immunoglobins, ovomucin, and so on. Ovalbumin is the main protein found in egg white, making up 60 to 65% of the total protein (Huntington and Stein, 2001). Its function is still unclear, although it is presumed to be a storage protein (Peter, 2002). It was reported that ovalbumin have no growth promoting function. After denaturing EW at 70°C, EW lost all functions on cell proliferation that

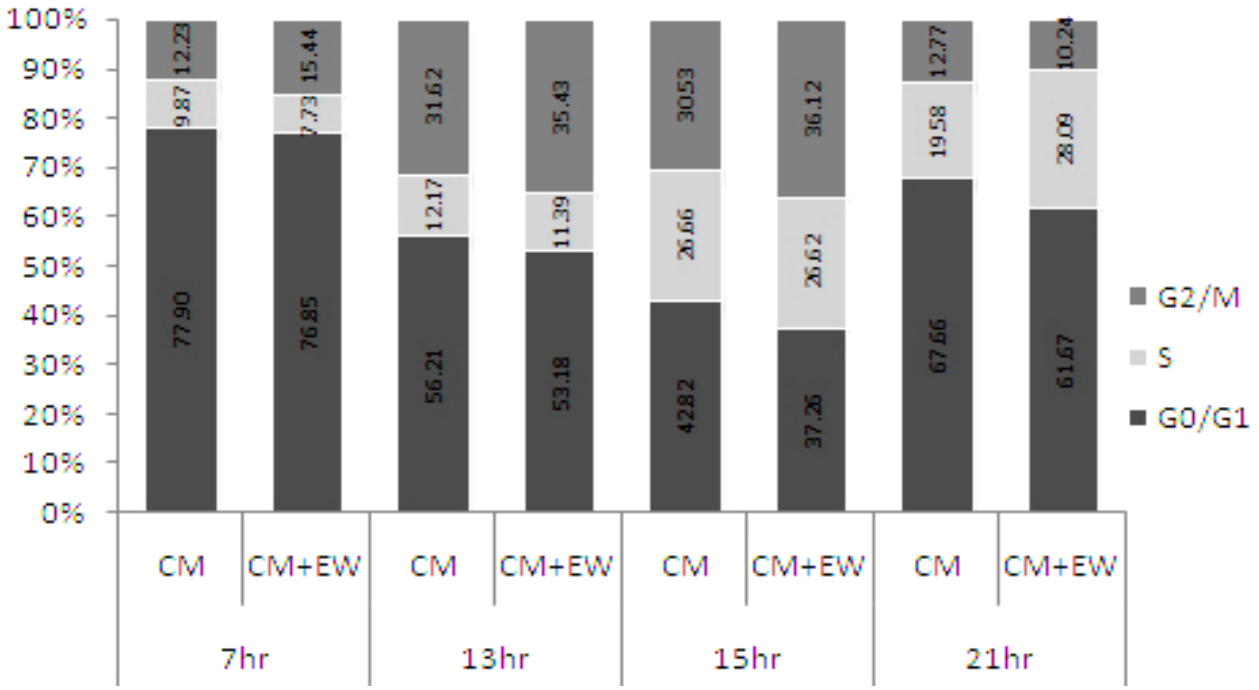

Figure 2. Changes of cell cycle of fibroblast cells after EW addition. Fibroblast cells were subjected to serum starvation with 0.5% FBS-DMEM for 24 h and cultured in CM medium with or without 5% EW. Flow cytometry was used to investigate cell cycle distribution at 7, 13, 15 and 21 h after treatment. CM, complete medium; CM+EW, 5% EW in CM; G0/G1, percentage of G0/G1 phrase; S, percentage of S phrase; G2/M, percentage of G2/M phrase.

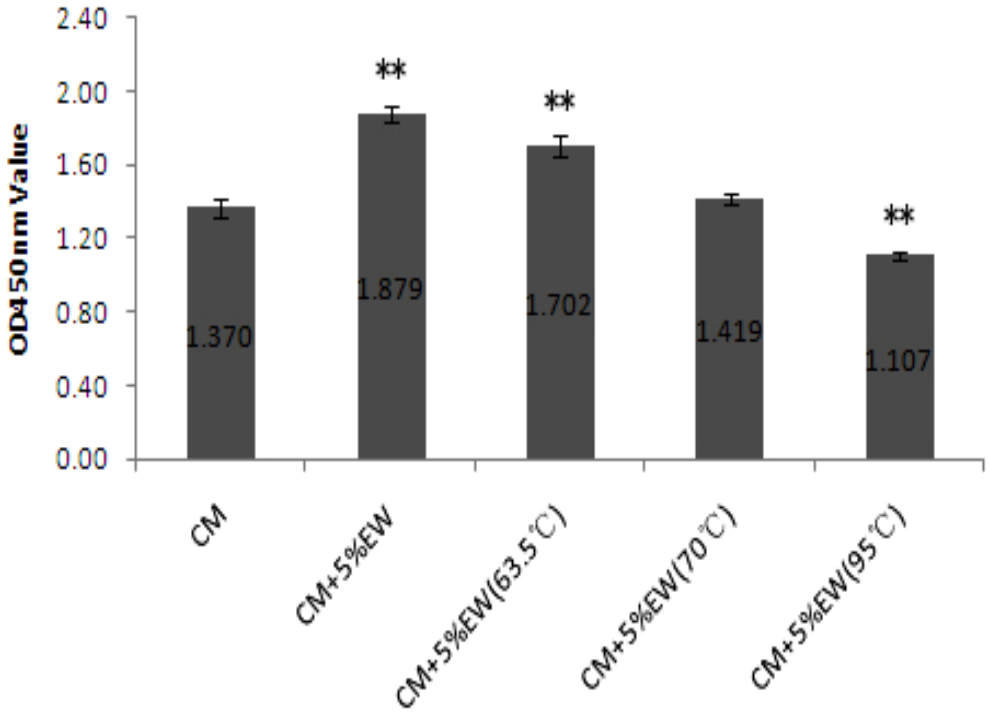

Figure 3. Temperature sensitivity of active component in EW for growth-promoting. EW was treated at 25, 63.5, 70 and 95°C for 3 min and added into CM respectively with 5% concentration. Cell proliferation was measured with cell counting Kit-8 and the absorbance was measured at 450 nm. CM or CM+5%EW were the medium composition; the temperature for deactivation were showed in parenthesis; **, very significantly different ($p<0.01$); *significantly different ($p<0.05$).

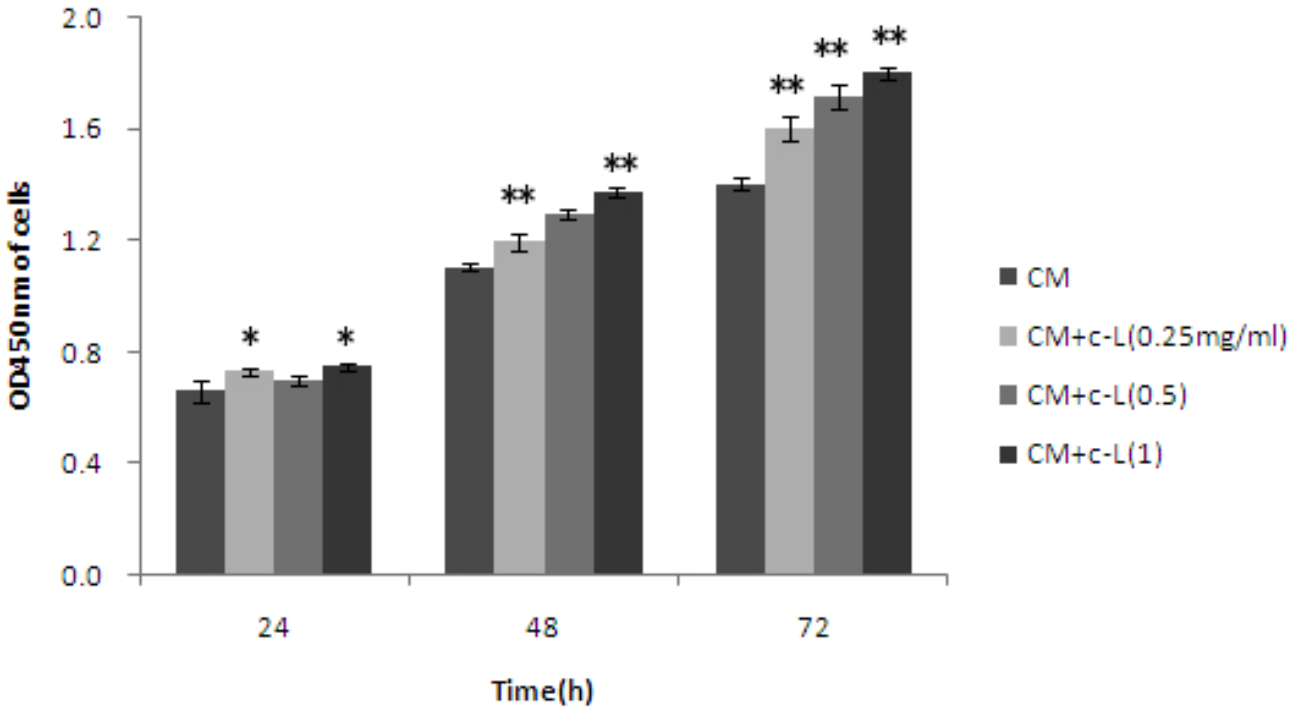

Figure 4. Effect of c-lysozyme on cell proliferation. Fibroblast cells were cultured in CM with 0, 0.25, 0.5, 1 mg/ml c-Lysozyme. Absorbance was detected at 450 nm at 24, 48 and 72 h after culture. CM was the medium composition; **very significantly different ($p<0.01$); *significantly different ($p<0.05$).

excluded ovalbumin and also showed the growth promoting component in EW as a regulation factor, not a nutrient.

Seed et al. (1988) found that basic fibroblast growth factor (bFGF) existed in EW and at the embryo of different stages, which had a broad spectrum of activities on many kinds of cells, including increased growth, migration, and induction of plasminogen activator and type IV collagenase. The intriguing function of bFGF is to induce embryonic development, limb regeneration and differentiation of mesenchymal cells (Rifkin and Moscatelli., 1989). The denatured temperature for bFGF is 46 to 65°C and from pH 4 to 8 (Vemuri et al., 1994). After the denaturing of EW at 63.5°C for 10 min, the bFGF was destroyed, but the denatured EW still had growth promoting effect. This report therefore indicates that another kind of protein played important function in the cell proliferation. When the denaturing temperature of EW reached 70°C, all growth promoting function were inactivated.

The c-lysozyme became the first related candidate bioactive protein. c-Lysozyme was the first enzyme to have its three-dimensional structure determined by X-ray diffraction techniques and showed strong killing activity on Gram-positive bacteria by hydrolyzing the peptide-glycan, and produced Gram-negative bacteria killing function after the change of its conformation by heating at pH 6.0 condition, but the catalytic activity would be abolished after the Asp53 was changed into serine (Ibrahim., 1998). Lysozyme is a globular protein with a deep cleft across part of its surface where the substrate fit into, which is very important for catalytic activity (Ibrahim et al., 2001). Three antibacterial peptides including fractioned residues 1-38, 18-38 and 39-56 amino acids (AA) were produced after digestion of c-lysozyme by pepsin. These peptides share structural motifs commonly found in different innate immune defenses (Ibrahim et al., 2005; Thammasirirak et al., 2010). C-Lysozyme could induce a growth-promoting activity in serum free medium. Compared with the human growth active peptides of lysozyme (Grobler et al., 1994), c-lysozyme owns a conserved growth promoting peptide. We hypothesized that there are 3 domains in c-lysozyme, including residues 35-52 AA for antibacterial activity, and 60-94 AA for growth promoting ability which was decided by the conformation at different denaturing temperature and pH (Ibrahim et al., 2001), and not inhibited by the serum addition.

There are many pathways controlling the cell cycle, such as Wnt, FGF and p53 pathways and so on. The bFGF is an important growth factor with strong activity to facilitate proliferation and differentiation of cells. After binding with FGF receptor, bFGF could up-regulate E2F, which speeds up S phrase completion by increasing expression of cyclin A, and CDK2, thus promoting G/S transition by enhancing expression of cyclin E. bFGF exert its proliferation promoting function by boosting G0 to S phrase through passing checkpoint I and guarantee

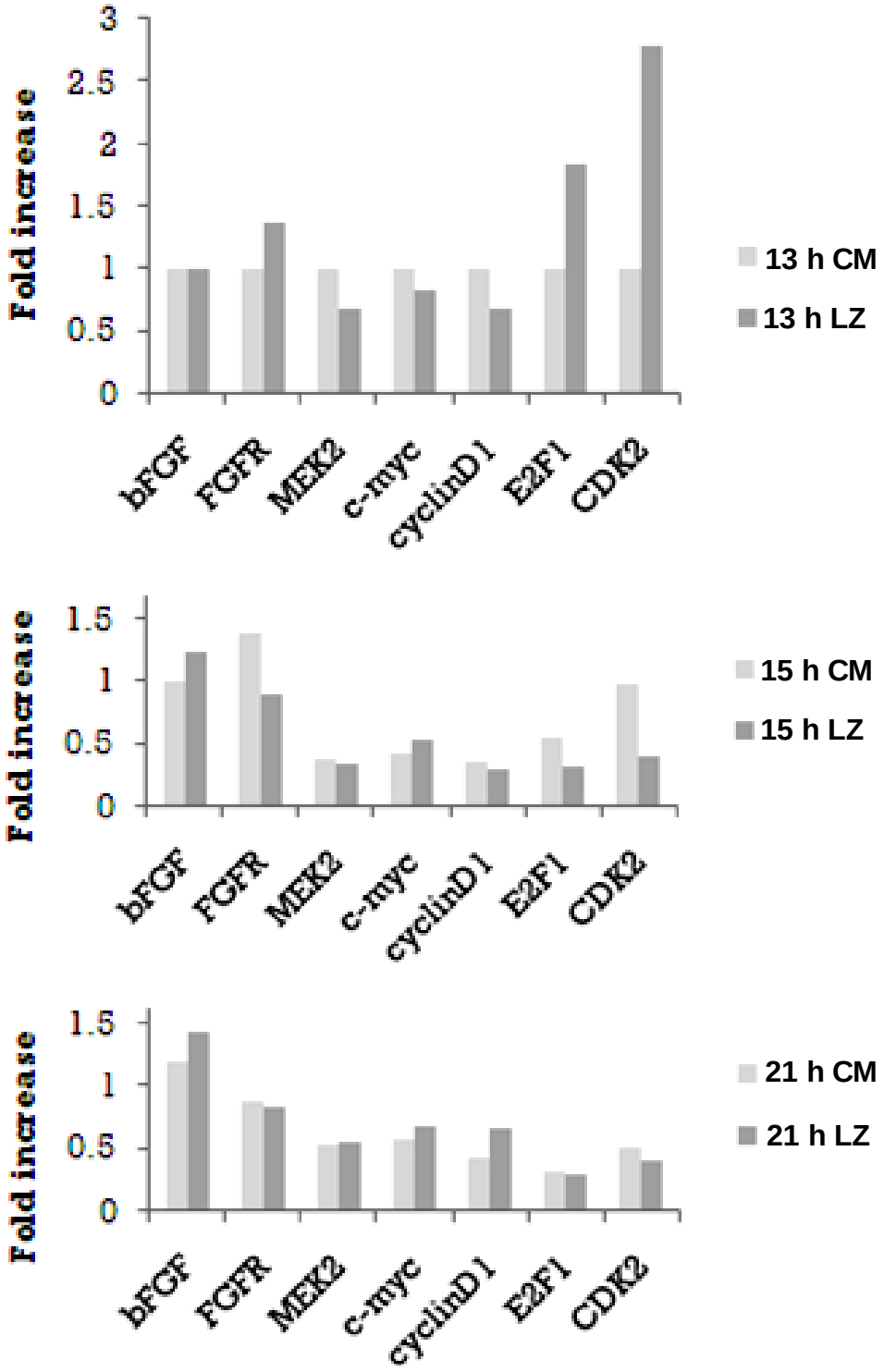

Figure 5. Gene expression in different phrase of cell cycle after c-lysozyme treatment. Expression levels of different gene at different time after c-Lysozyme treatment were measured by q-PCR.

the normal progress of S phrase. C-Lysozyme treatment increased the expressions of FGFR, E2F and CDK2 at the 13th hour after addition to prepare the protein accumulation for passing the checkpoint I, but the cell cycle did not show any significant changes. Until the 15th hour, most CEFs began the DNA synthesis and cell division (the number of G0/G1 in CM+EW was significantly lower than in CM); the expressions of genes

related with cell cycle startup, such as FGFR, E2F, CDK2 and so on, returned back to basic level. Meanwhile, no significant difference was found at this time point. At the 21st hour of addition of EW, 28.09% CEFs had entered the S phrase and cyclin A1 was up-regulated significantly which was the main factor to maintain S phrase progress. At the same time, the CEFs in CM was prepared to enter the next cell cycle and FGFR and CDK2 were up-regulated. It was concluded that c-lysozyme could initiate cell cycle through FGF pathway.

We first ascertained that c-lysozyme facilitated proliferation of CEFs through FGF pathway and expanded c-lysozyme functional mechanism by predicting three structure domains. Multifunction discovery would expand the application of c-lysozyme in medicine, nutriology and molecular genetics.

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