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# Construction of pECFP-C1- CIRP and its effect on cold-induced apoptosis in mouse NIH/3T3 cells

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Cold-inducible RNA-binding protein (CIRP) is suggested to be involved in protecting cells from the damage caused by cold stress. In this study, the recombinant plasmid, pECFP-C1-CIRP, was constructed and transfected into NIH/3T3 cells by lipofectamine™ 2000. The fluorescence microscopy, real-time polymerase chain reaction (RT-PCR) and Western blot detection were done for transient and stable expression assays. CIRP gene was successfully subcloned into eukaryotic expression vector of pECFP-C1, confirmed by restrictive enzyme digestion analysis and DNA sequencing. Fluorescent microscopy study showed that green fluorescence was generated from NIH/3T3 cell. The results of RT-PCR indicated that the CIRP mRNA was expressed in NIH/3T3 cells, while the results of Western blot detection further indicated that the CIRP protein was overexpressed in NIH/3T3 cells. Stable expression in cells transfected with pECFP-C1-CIRP was achieved by selection in a G418-containing medium for 2 weeks. We performed experiments of cold-induced apoptosis under cold treatment of 4, 14 and 24°C. The results of the cell apoptosis detected by fluorescence microscopy indicated that the apoptosis level was reduced in pECFP-C1-CIRP stably expressed group due to overexpression of CIRP. This study suggested that CIRP played a role in protecting cells from cold-induced apoptosis.

**Key words:** Cold inducible RNA-binding protein, NIH/3T3 cell, transfection, cold-induced apoptosis.

## INTRODUCTION

Low temperature is one of the most common stresses for organisms from bacteria to plants and animals. Notably, all organisms from prokaryotes to plants and higher eukaryotes respond to cold shock in a relatively similar manner. Generally, cells respond to cold stress by elite and rapid over-expression of a small group of proteins known as cold-shock proteins (CSPs) (Fujita, 1999). Cold-inducible RNA-binding protein (CIRP) was initially isolated from the BALB/3T3 mouse fibroblasts cells after the cold-induced treatment. CIRP is the first cold shock protein found in mammals. Murine CIRP is a polypeptide

consisting of 172 amino acid residues with molecular weight of 18 kDa. Murine CIRP also contains a consensus of RNA-binding domain (CS-RBD) and a glycine-rich domain (GRD), which plays an essential role in cold-induced growth suppression of mouse fibroblasts (Nishiyama et al., 1997). Subsequently, CIRP from humans, rats, mouse, Mexican axolotls, bull frogs, *Xenopus laevis* and *Anabaena variabilis* have been identified. *Xenopus* CIRP have been reported in three isoforms; XCIRP, XCIRP-1 and XCIRP-2. CIRP is constitutively expressed in many tissues (Lleonart, 2010). And CIRP can be a general stress response protein since it has been induced upon mild cold-shock (Nishiyama et al., 1997), hypoxia (Yang and Carrier, 2001), UV irradiation (Wellmann et al., 2004), osmotic stress (Pan et al., 2004), light signals (Sugimoto et al., 2008) and spaceflight (Baba et al., 2008). However, the cellular localization of the protein seems to be dependent on the type and/or physiological state of the cell in either

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**Abbreviations:** CIRP, Cold-inducible RNA-binding protein; RT-PCR, real-time polymerase chain reaction.

cytoplasm or nucleus.

CIRP is thought to be a modulator of gene expression during mild hypothermic conditions by functioning as RNA chaperones to facilitate translation (Fujita, 1999). In addition, it has been proposed that CIRP binds DNA and possibly serves as a chaperone that assists in the folding/unfolding, assembly/disassembly and transport of various proteins (Fujita, 1999; Gualerzi et al., 2003). Many findings have demonstrated that CIRP was involved in many other physiological and pathological activities, including cold-induced growth suppression (Nishiyama et al., 1997), biological rhythms (Nishiyama et al., 1998), human and animal's reproductive development (Peng et al., 2000, 2006), neural development and regulation (Nishiyama et al., 1998; Bhatia et al., 1999; Van Venrooy et al., 2008). CIRP enhances the product of recombinant protein in cultured mammalian cell (Tan et al., 2008) and bypasses replicative senescence in primary cells through extracellular signal-regulated kinase 1 and 2 activation (Artero-Castro et al., 2009). CIRP also plays a role in male infertility (Nishiyama et al., 1998; Zhou et al., 2009), neuroprotection under hypothermia (Xue et al., 1999; Liu et al., 2010; Saito et al., 2010), embryonic kidney formation (Peng et al., 2000), hibernation (Saito et al., 2000) and tumorigenesis such as endometrial tumorigenesis (Hamid et al., 2003) and breast cancer (Guo et al., 2010).

It has also been reported that CIRP is related to apoptosis, such as scrotal heat stress reduced expression of CIRP in germ cell accompanying with an increase of cell apoptosis (Banks et al., 2005). Mild hypothermia protects cells from TNF- $\alpha$ -induced apoptosis, at least partially, via induction of CIRP, and that CIRP protects cells by activating the extracellular signal-regulated kinase (ERK) pathway (Sakurai et al., 2006). Moderate low temperature preserves the stemness of neural stem cells and suppresses apoptosis of the cells via activation of the CIRP (Saito et al., 2010). Low temperature will cause cell damage and apoptosis; whether or not CIRP can protect cells from such apoptosis caused by cold stress is unknown. Moreover, if it can protect cells at lower temperature, what is the exact mechanism of this protection?

In this study, we constructed a recombinant eukaryotic expression vector of CIRP and explored the feasibility of the CIRP expression in the NIH/3T3 cells. We selected recombinant cells with CIRP gene overexpression and analyzed the effect of CIRP on the cold-induced cell apoptosis. The result of the experiments showed that CIRP played a role in protecting cells from cold-induced apoptosis.

## MATERIALS AND METHODS

*Escherichia coli* strains DH5 $\alpha$ , pECFP-C1 vector, NIH/3T3 cell and anti-CIRP monoclonal antibody were provided by Molecular Biology Room of Heilongjiang Bayi Agricultural University. Lipofectamine™

2000, G418, pGEM-T vector and D-MEM (high glucose) were purchased from Invitrogen Corporation, USA. Gel Extraction Mini Kit and plasmid Extraction Mini Kit were purchased from Omega. RevertAid™ First Strand cDNA Synthesis Kit, Oligo (dT), Taq polymerase, dNTPs, *EcoR* I, *BamH* I, *Xba* I, T4 DNA ligase and DNA molecular weight marker were purchased from TaKaRa, Japan. Fetal calf serum was the product of Hangzhou Sijiqing Biological Engineering Material Co, Ltd, China. Goat anti-rat IgG was purchased from Beijing Bioss Biological Engineering, Ltd, China.

### Polymerase chain reaction (PCR) primers

Pair of primers was designed based on nucleotide sequence of the cold-inducible RNA-binding protein gene cDNA registered in GenBank (Accession: NM\_007705 XM\_986308): P1, forward: 5'-CGGAATTCATGGCATCAGATGAAGG-3', (the *EcoR* I site was underlined). P2, reverse: 5'-CGGGATCCCTTACTCGTTGTGTGTAGCAT-3', (the *BamH* I site was underlined). The primers were synthesized by Sangon Biotech (Shanghai) Co. Ltd.

### Amplification of the CIRP gene

The fragment of CIRP gene was amplified from pGEM-T-CIRP (which had been obtained from our laboratory, containing the full-length sequence of CIRP) by PCR with the primers P1/P2. The PCR amplification conditions were: 5 min at 94°C, 35 cycles of 1 min at 94°C, 30 s at 59°C and 1 min at 72°C. At the end of the last cycle, the samples were further incubated at 72°C for 10 min. PCR products were resolved on a 1% agarose gel and then purified according to the Gel Extraction Mini Kit. Finally, the two PCR products obtained were ligated into pGEM-T vector and transformed to *E. coli* DH5 $\alpha$  competent cells. After single resistant clone was formed on the LB-agar plate which had been plated with ampicillin, several clones were randomly selected and identified by PCR and enzyme excision to obtain the positive ones.

### Construction of the pECFP-C1-CIRP

Once the positive clones were determined, recombinant plasmids pGEM-T-CIRP were digested with the enzymes of *Xba* I and *BamH* I, then the small fragments were harvested. Meanwhile, the linear pECFP-C1 fragments were obtained after being digested with the same enzymes. Subsequently, the two digested products were linked with T4 DNA ligase to form the recombinant eukaryotic expression vector pECFP-C1-CIRP. After the construct was transformed to *E. coli* DH5 $\alpha$ , the transformants were identified by cloning PCR, double enzyme digestion and DNA sequencing.

### Extraction and purity of plasmids

Positive clones were inoculated in 300 ml LB medium with additional correspondent resistant drug and cultured at 37°C for 14 h; pECFP-C1 was kanamycin-resistant. The cells were collected by centrifugation at 5000 r/min for 10 min. The sediment obtained was washed with cold STE (0.1 mol/L NaCl, 10 mmol/L Tris-HCl pH 8.0 and 1 mmol/L EDTA) and collected. The subsequent extraction was based on the process of Sambrook and Russell, 2002. Finally, the purity and concentration of the plasmids were assayed with DNA/RNA calculator (Pharmacia Biotech Corporation). Meanwhile, the blank plasmids were also prepared with the same method as above.

### Determination of the optimal G418 concentration

NIH/3T3 cells were cultured in D-MEM supplemented with 10% fetal calf serum and 100 IU/ml penicillin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> in air. Then the cells were digested and seeded in the 6-well cell plates, every well containing 5×10<sup>5</sup> cells. G418 with different concentrations were added into the different wells after the cells were cultured for 24 h. The G418 concentrations were 0, 200, 400, 600, 800 and 1000 mg/L, respectively and the culture was done for 14 days. In this period of time, the selective medium was refreshed every two days. The death situation of the cells was observed to determine the optimal G418 concentrations at lowest level, which can cause death to all the cells in 14 days.

### Transfection of NIH/3T3 Cells

NIH/3T3 cells were seeded in 6-well cell plates to achieve cultures with 80 to 90% confluence after 48 h, and transfections were carried out the instructions of Lipofectamine™ 2000 as follows: Diluted DNA in 250 µL of D-MEM without serum was mixed gently. Appropriate amount of Lipofectamine™ 2000 was diluted in 250 µL D-MEM without serum and incubated for 5 min at room temperature. Then the diluted DNA was combined with diluted Lipofectamine™ 2000, mixed gently and incubated for 20 min at room temperature. Finally, the complex was added to each well containing cells and medium, mixed gently and incubated at 37°C in a CO<sub>2</sub> incubator. The plasmids used for transfection were pECFP-C1-CIRP and pECFP-C1. The ratios of DNA (µg): Lipofectamine™ 2000 varied from 1:0.5 to 1:1.5.

### Selection of transfected cells with stable expression

The cells transfected with pECFP-C1-CIRP were passaged into fresh growth medium 24 h after transfection. Selective medium such that the concentration of G418 was 600 mg/L were added. Two weeks later, the single cell proliferated as clone while others were dead. The single clone was transferred to culture flask in conditioned medium, and the G418 concentration of the selective medium decreased to 300 mg/L. Cell lines expressing CIRP were selected among the Neo<sup>r</sup>-resistant clones.

### Temporal and stable expression assays in transfected cells with fluorescence microscope

The cells transfected with pECFP-C1-CIRP and pECFP-C1 were cultured for 36 h after transfection. Then the expression of green fluorescent protein (GFP) was observed using a Leica fluorescence microscope (Leica, Cambridge, UK) provided with a digital camera. And the selected cells were also assayed with this method.

### RT-PCR identification

Total RNA of transfected cells was extracted using Trizol reagent. Single strand cDNA was performed by the RevertAid™ First Strand cDNA Synthesis Kit. An aliquot of 1 µg RNA was used in each reaction. The PCR was conducted at 94°C for 1 min, 35 cycles of 94°C for 1 min, 59°C for 30 s, 72°C for 1 min, and then a final extension at 72°C for 10 min was performed. Primers were P1/P2 as mentioned above.

### Western-blot detection

The anti-CIRP monoclonal antibody was produced from immunizing

rat by our laboratory. For Western blot detection analysis, 10 µg of proteins were separated by 14% SDS-PAGE. The separated proteins were transferred to polyvinylidene fluoride (PVDF) membrane, and treated with the antiserum (1:20,000 diluted). Bound antibody was detected using a goat anti-rat IgG conjugated with horseradish peroxidase. The target protein was detected by diaminobenzidine (DAB) chromogenic method, and the reactions were terminated with double-distilled water.

### Cold-induced apoptosis analysis

Cells with CIRP stable overexpression and control cells (transfected with empty vector pECFP-C1) were seeded on sterile cover glasses placed in the six-well plates at a density of 1.0×10<sup>4</sup> cells cm<sup>-2</sup> and cultured at 37°C for 24 h. Subsequently, the cells were exposed to the condition of 24, 14 and 4°C for 12 h and then at 37°C for 12 h. Next, the cells were sampled and apoptosis was analyzed by Hoechst 33258 staining, washed twice with phosphate-buffer saline (PBS) and stained with Hoechst 33258 staining solution according to the manufacturer's instructions (Beyotime, Jiangsu, China). Stained nuclei were observed under a confocal microscope (Leica, Germany). The number of cells was determined under the microscope. Apoptosis rate was calculated by dividing the number of the total cells by the number of apoptosis cells. The experiments were repeated three times, and each assay was performed in triplicate. Statistical differences between sample means were calculated by analysis of variance, followed by unpaired Students t test. The results were expressed as the mean ± SEM and P < 0.02 was considered significant.

## RESULTS

### Amplification of the CIRP gene

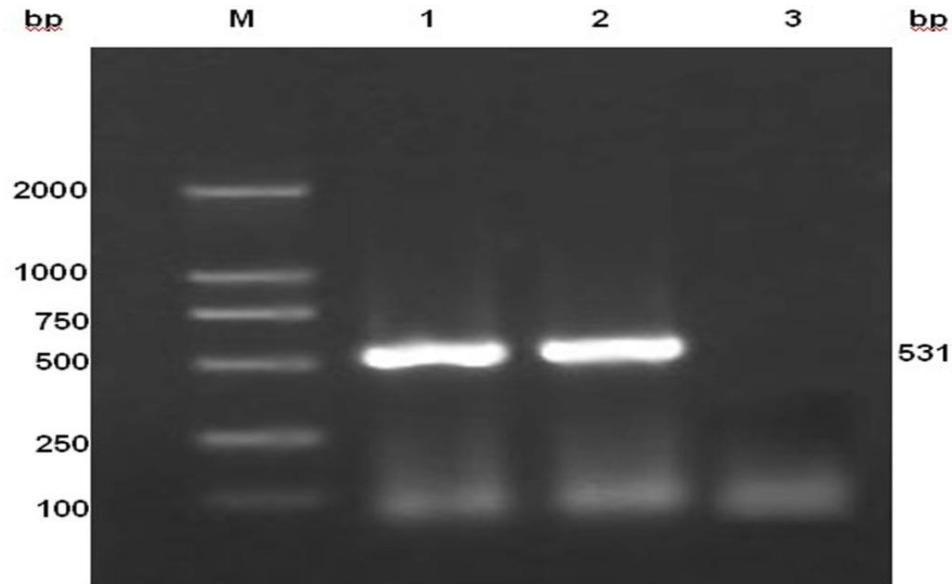
The results of PCR showed that the gene of CIRP was amplified, and the size of the fragment was approximately 530 bp as shown in Figure 1. The PCR products were cloned into pGEM-T vector and also sequenced. The results of DNA sequencing confirmed that the PCR products consisted with the target gene (GenBank: NM\_007705 XM\_986308).

### Construction of the recombinant plasmids

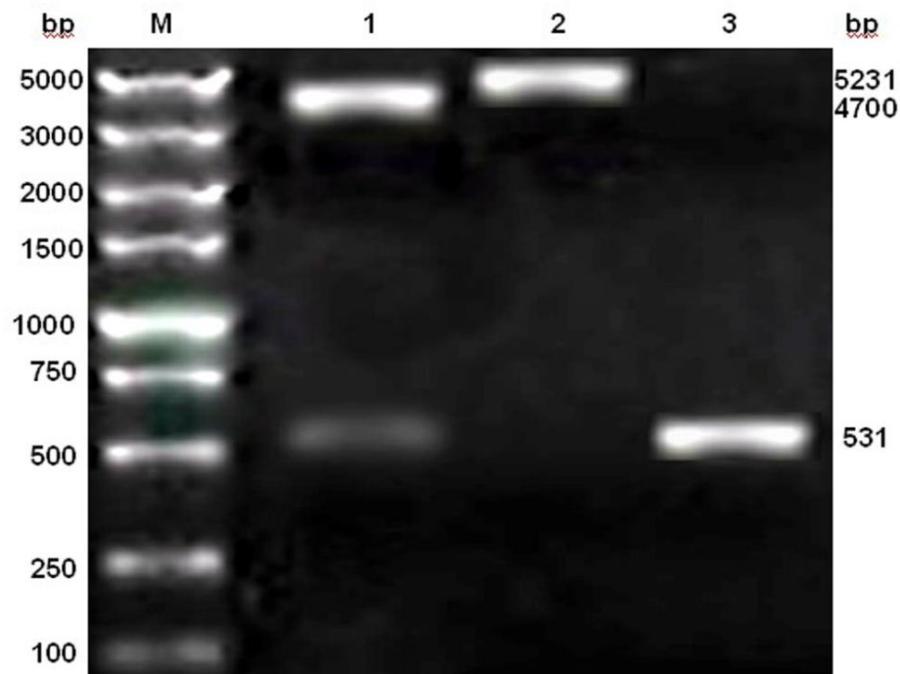
With the accurate result of DNA sequencing, pGEM-T-CIRP was digested by *EcoR* I and *BamH* I. Then the target gene was harvested and further cloned into pECFP-C1 vector. Identification of double enzyme digestion is shown in Figure 2. The plasmid was digested into 4700 and 531 bp fragments (Figure 2), which coincided with the size of pECFP-C1 and target gene. Therefore, the construction of pECFP-C1-CIRP was completed.

### Determination of the optimal G418 concentration

After the cells were cultured in conditioned medium containing different concentration G418 for 14 days, we



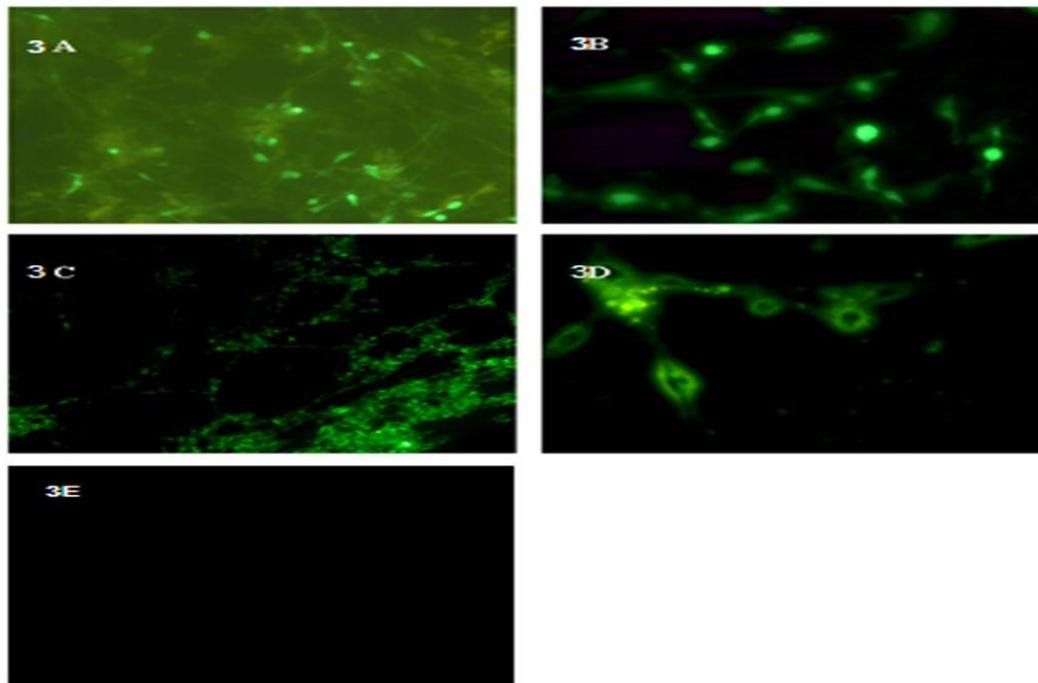
**Figure 1.** Amplification of cold inducible RNA-binding protein (CIRP) by PCR. Lane M, DL2000; Lane 1, the PCR products of the plasmid pGEM-T-CIRP obtained by our laboratory; lane 2, the PCR products of the new recombinant plasmid pGEM-T-CIRP; lane 3, negative control.



**Figure 2.** Identification of the recombinant plasmid. Lane M, DL5000; lane 1, the products of pGEM-T-CIRP enzyme excision by *EcoR* I and *Bam*H I; lane 2, the plasmid pGEM-T-CIRP; lane 3, products of PCR amplified from the new recombinant plasmid pGEM-T-CIRP.

found that the concentration which was the lowest (600 mg/L) could cause death to the untransfected cells. This

concentration was thus used as the optimal G418 concentration for selection.



**Figure 3.** The fluorescence light in NIH/3T3 cells as viewed under fluorescence microscope. (A) Effects of pECFP-C1-CIRP transfected into NIH/3T3 cells 36 h later under fluorescence microscope ( $\times 100$ ). (B) Effects of pECFP-C1 group under fluorescence microscope ( $\times 200$ ). (C) Effects of pECFP-C1-CIRP transfected into NIH3T3 cells after 3 weeks selection by G418 under fluorescence microscope ( $\times 200$ ). (D) Effects of pECFP-C1-CIRP group after 5 weeks selection by G418 observed under fluorescence microscope ( $\times 400$ ). (E) Wild-type cells under fluorescence microscope.

### Transfection of NIH/3T3 Cells

Thirty-six hours after transfection, the expression of green fluorescent protein (GFP) was detected with fluorescence microscope. As described in Figure 3A and B, the cells transfected with pECFP-C1-CIRP and pECFP-C1 generated green fluorescence intensively. Furthermore, green fluorescence was distributed uniformly in the cell. However, the wild-type cell did not generate fluorescence relatively (Figure 3E), indirectly demonstrating that construction of pECFP-C1-CIRP was right and the target gene CIRP was expressed in NIH/3T3 cells. Thereafter, the concentration of G418 in the selective medium was 600 mg/L.

Two weeks later, the single cell proliferated to form the clone, while others were dead. The single clone was transferred to culture flask in conditional medium, and the concentration of serum was increased to 15%, while the selective medium was decreased to 300 mg/L. After 3 weeks of selection, most of the selected cells generated the fluorescence (Figure 3C), showing that stable CIRP expression was achieved in the NIH/3T3 cells in the appropriate drugs. The green fluorescence was mainly distributed in the cytoplasm. Meanwhile, several days after the G418 screening, some fusion proteins were released outside the cytomembrane (Figure 3D).

### RT-PCR detection

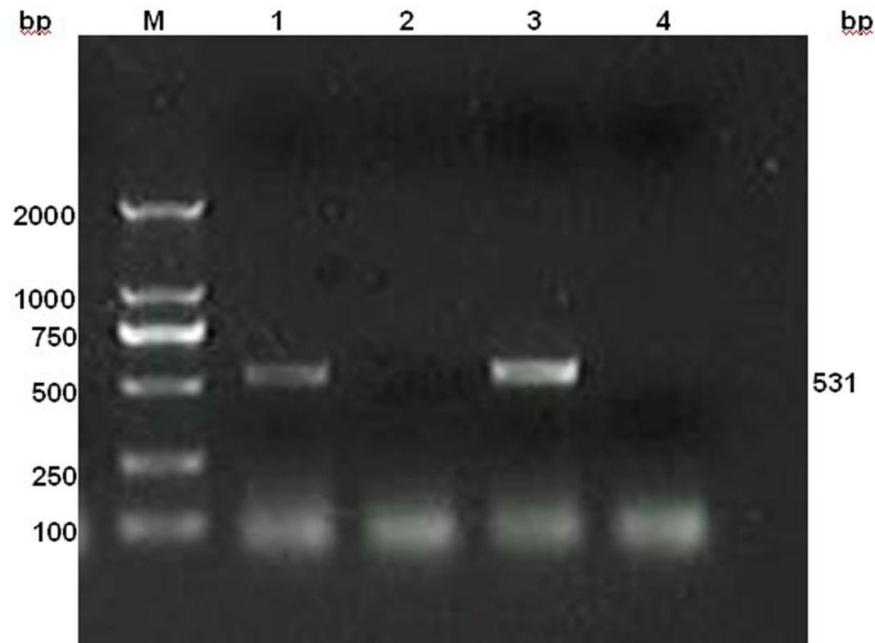
RT-PCR was performed at 36 h after the transfection. The nucleotide sequence of the bands showed that the 531 bp band corresponded to CIRP sequences, whereas the fragment was not obtained in the negative control (PCR reaction mixture without sample) or the cells transfected with pECFP-C1 as shown in Figure 4, thus indicating that CIRP mRNAs were correctly processed in the corresponding NIH/3T3 cells.

### Western blot detection

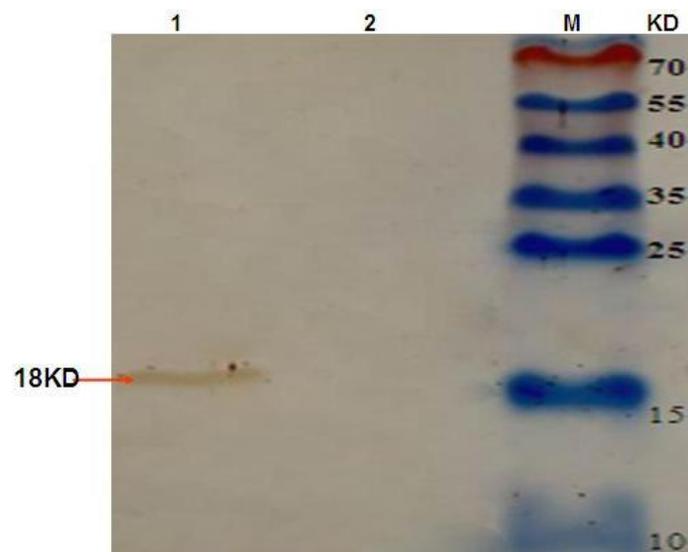
Proteins about 18 kDa were only obtained from the cells that stably expressed pECFP-C1-CIRP, but not the cells that stably expressed pECFP-C1 (Figure 5).

### Apoptosis analysis

Hoechst 33258 staining results showed that there were significant morphological changes in the nuclear chromatin at 14°C. The nuclei of the control cells (no apoptosis) were stained in a less bright blue and the color was homogeneous, while the blue emission light in

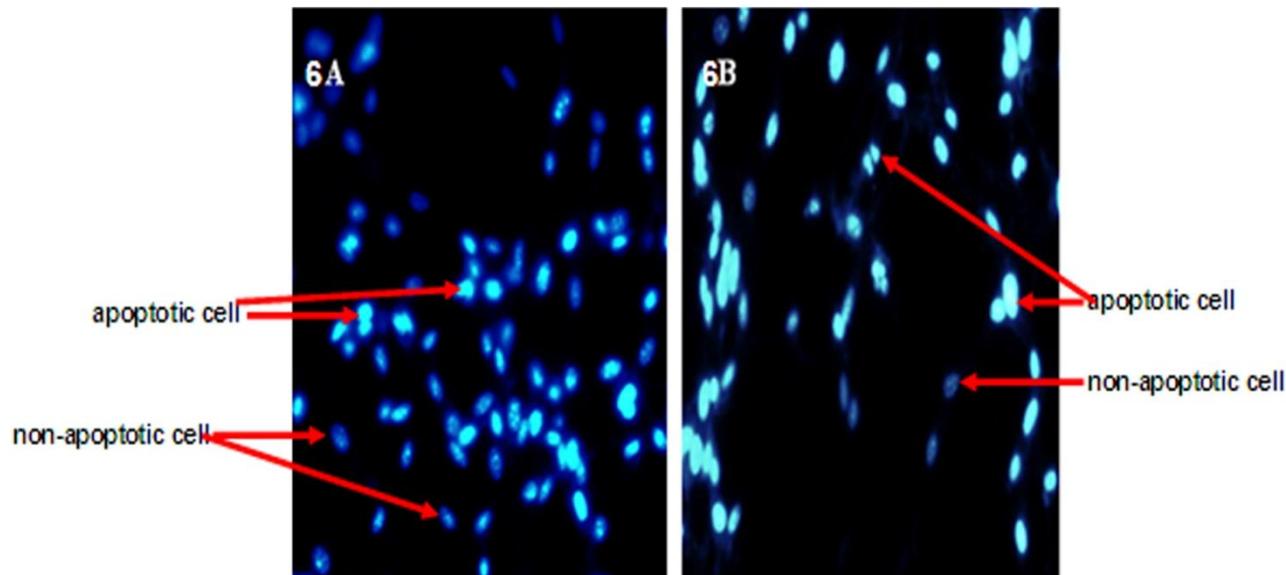


**Figure 4.** Identification of pECFP-C1-CIRP transfected into NIH/3T3 cells after 36 h by RT-PCR. Lane M, DL2000; lane 1, products of PCR amplified from the new recombinant plasmid pGEM-T-CIRP; lane 2, pECFP-C1 transfected group; lane 3, pECFP-C1-CIRP transfected group; lane 4, negative control (PCR reaction mixture with no sample).

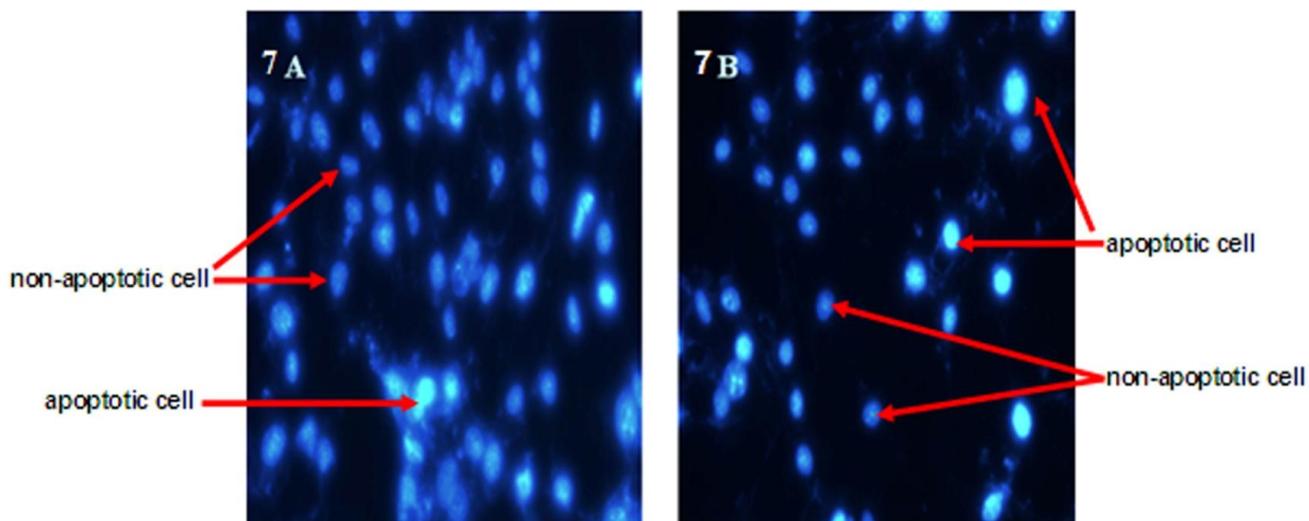


**Figure 5.** Western blot detection analysis of NIH/3T3 cell lysates using an anti-CIRP monoclonal antibody. NIH/3T3 cell were harvested after the transfection, and 10 ug of samples was separated by 14% SDS-PAGE. Note special recognition of an 18-KD protein by the anti-CIRP antibody. Markers are indicated on the right. Lane 1, Cells that can stably express pECFP-C1-CIRP; lane 2, cells that can stably express pECFP-C1.

apoptotic cells was much brighter than the control cells. Condensed chromatin could also be found in many



**Figure 6.** Apoptosis detected by fluorescence microscopy after 12 h cold induction at 14°C (×200). (A) pECFP-C1-CIRP transfected group, the apoptotic degree was  $60.6 \pm 0.1\%$ ; (B) control group, the apoptotic degree was  $89.2 \pm 0.1\%$ .



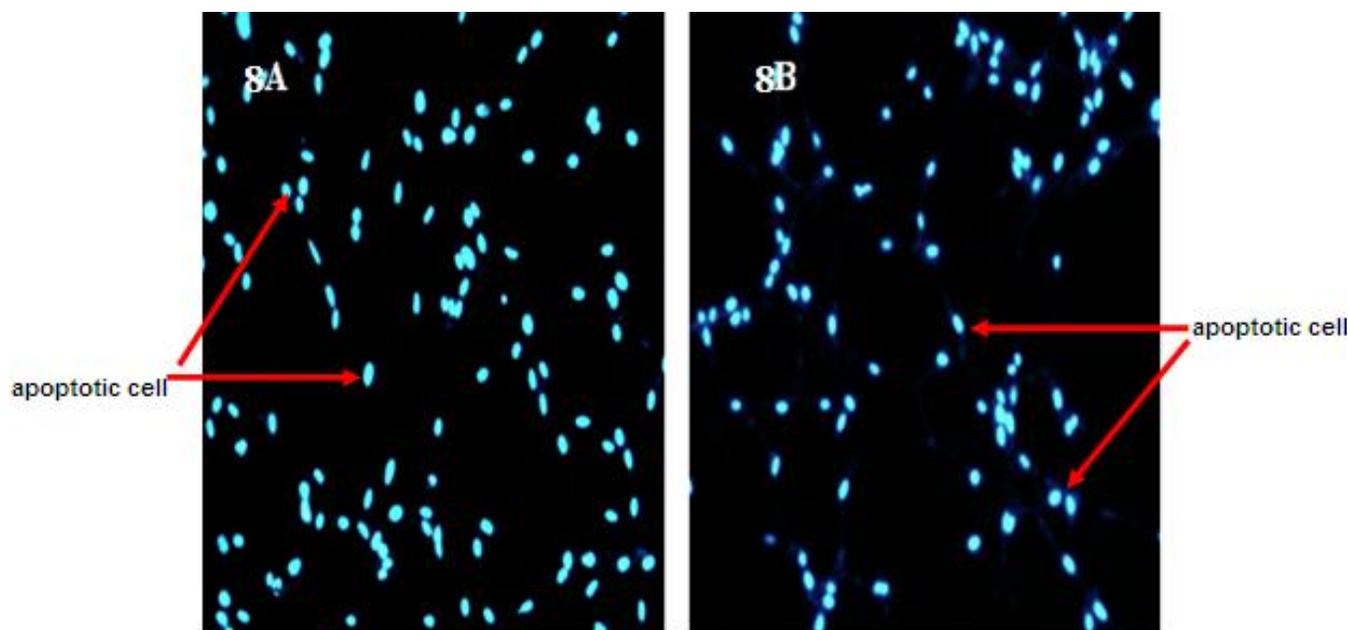
**Figure 7.** Apoptosis detected by fluorescence microscopy after 12 h cold induction at 24°C (×200). (A) pECFP-C1-CIRP transfected group, the apoptotic degree was  $19.7 \pm 0.1\%$ ; (B) control group, the apoptotic degree was  $30.3 \pm 0.1\%$ .

treated cells and some of them formed the structure of apoptotic bodies, which is one of the classic characteristics of apoptotic cells. Based on this character, we found that the apoptotic degree of control groups ( $89.2 \pm 0.1\%$ ) was greater than the transfected ones ( $60.6 \pm 0.1\%$ ) at 14°C (Figure 6), demonstrating that anti-apoptotic activity was probably mediated by CIRP. However, there was no significant difference in cell viability under 24°C, and the apoptotic degree of control groups ( $30.3 \pm 0.1\%$ ) was greater than the transfected ones ( $19.7 \pm 0.1\%$ ) as shown in Figure 7. Moreover, when treated under 4°C, nearly all the cells were

apoptotic and the apoptotic degree of control groups ( $100.0 \pm 0.1\%$ ) was greater than the transfected ones ( $99.8 \pm 0.1\%$ ) (Figure 8). Having compared the transfected group and control, it was revealed that there was significant difference at 14°C, but little difference at 4 and 24°C.

## DISCUSSION

Since the discovery of CIRP (cold-inducible RNA-binding protein), it has been rarely reported in domestic research.



**Figure 8.** Apoptosis detected by fluorescence microscopy after 12 h cold induction at 4°C ( $\times 100$ ). (A) pECFP-C1-CIRP transfected group apoptotic degree is  $99.8 \pm 0.1\%$ ; (B) the control group apoptotic degree is  $100.0 \pm 0.1\%$ .

Structurally, CIRP contains ribonucleoprotein (RNP) motif, which is one of the major RNA-binding motifs and has been the most widely found and best characterized. It is known that this type of proteins containing RNP motif participate in the post-transcriptional regulation, including mRNA processing, masking, translational activation, turnover, localization and polyadenylation (Burd and Dreyfuss, 1994). In plants, several proteins belonging to the same RNP-binding family are expressed predominantly in meristematic and growing tissues, and are likely to be related in growth regulation and response to external stimuli. In *E. coli*, CsdA, one of the major cold-shock proteins, is related to cell growth and protein synthesis at low temperatures. Similarly, the expression of CIRP in animal cells was induced in response to the lower temperature, resulting in the growth suppression that was mainly featured with the prolongation of G1 phase of the cell cycle. CIRP participates in regulating the synthesis of the related protein for the cell protection (Nishiyama et al., 1997). These activities were the adaptive response to low-temperature environment.

In addition, by analyzing the effects of low culture temperature (32°C) and cold-inducible RNA-binding protein (CIRP) expression on apoptosis *in vitro*, Sakurai et al. (2006) found that CIRP protected cells by activating the ERK pathway from TNF- $\alpha$ -induced apoptosis. This result would help to elucidate the molecular mechanisms of the mild hypothermia showing protective effects on patients with brain damage and cardiac arrest. However, CIRP can be induced by mild cold stress (32°C) in many cell lines and not only by temperatures below 20°C. Cold stress is one of the apoptosis factors, and CIRP plays an

important role in the cold-induced process, which is due to an active response by the cell rather than passive effects. On the basis of this viewpoint, we speculated that this protein probably possessed protective effects in the cold-induced apoptosis. Therefore, we purposed to construct a recombinant eukaryotic expression vector of CIRP, transfect into NIH/3T3 cells and selected the recombinant cells with stable expression, as well as to develop a model of cold-induced apoptosis and verify whether it is resistant to cold-induced apoptosis.

The pECFP-C1 vector is widely used in eukaryotic transfection, and exogenous gene cloned into the vectors can be highly expressed in many cell lines. pECFP-C1 encodes an enhanced cyan fluorescent variant of the *Aequorea victoria* green fluorescent protein gene (GFP). The ECFP gene contains six amino acid substitutions. The Tyr-66 to Trp substitution gives ECFP fluorescence excitation (major peak at 433 nm) and emission (major peak at 475 nm). The other five substitutions (Phe-64 to Leu; Ser-65 to Thr; Asn-146 to Ile; Met-153 to Thr; and Val-163 to Ala) enhance the brightness and solubility of the protein, primarily due to improved protein-folding properties and efficiency of chromophore formation. Furthermore, upstream sequences flanking ECFP have been converted to a Kozak consensus translation initiation site, increasing the translational efficiency of the ECFP mRNA. GFP was expressed as the fusion with target protein, so target protein and its cellular localization can be verified by observing green fluorescence generated by GFP in living cells.

In this study, the CIRP gene was directly amplified using the template (pGEM-T-CIRP), although gene

mutation did not appear. A recombinant eukaryotic expression vector of CIRP was constructed with a pair of primers. To optimize the transfection efficiency, DNA purity was examined by detection with DNA/RNA Calculator. The ratios of DNA ( $\mu\text{g}$ ): Lipofectamine<sup>TM</sup> 2000 varied from 1:0.5 to 1:1.5, and 36 h after transfection and the target gene was detected by RT-PCR. Moreover, green fluorescence was observed with the fluorescence microscope, and the result of Western blot detection demonstrated that the construction of pECFP-C1-CIRP was successful; CIRP gene was expressed in NIH/3T3 cells. The green fluorescence mainly distributed around of the nucleus, being consistent with the report in Nishiyama et al. (1997), and we also observed that some fusion proteins secreted to outside the cytomembrane.

At present, the biological function of CIRP is unclear, although several investigations suggest that CIRP play an important role in cold stress response and in other physiological settings as well. Maternal CIRP is required for embryonic kidney formation in *X. laevis* (Peng et al., 2000, 2006). In the mouse brain, CIRP shows a relationship to the regulation of circadian rhythms (Nishiyama et al., 1998). Some researchers infer that CIRP also plays diverse tissue-specific roles, including maintenance of normal cellular function and morphogenesis. Hamid et al. (2003) have reported the diversity of CIRP expression in endometrial carcinomas, endometrial hyperplasias and normal endometria cells. The results suggest that CIRP may participate in the cell cycle regulation of normal endometrium and the loss of its expression may be involved in endometrial carcinogenesis. Summarily, CIRP was related to many physiological processes, playing an important role in organism. Therefore, deep investigation of biological function is meaningful in the future.

In this paper, we found that at 14°C, the apoptosis ratio of the treated group was much lower than that of the control group. However, there was not much difference between the groups that stably expressed pECFP-C1-CIRP and the control group at 24°C; neither of the two groups was damaged seriously and not many apoptosis appeared. While at 4°C, both of the two groups were damaged seriously and almost all of the cells showed apoptosis. Meanwhile, the apoptosis ratio of the group that stably expressed pECFP-C1-CIRP at all the three temperatures was lower than that of the control group. We therefore speculated that: (1) the damage from the environment was relatively light at 24°C and the expression of CIRP can be induced at 24°C, hence both the cells that stably expressed pECFP-C1-CIRP and the control group have enough CIRP to protect the cells from damages caused by the cold. However, the CIRP level of the control group was lower than that of the treated group that stably expressed pECFP-C1-CIRP. Therefore, neither of groups was seriously apoptosized at 24°C, and the apoptosis ratio of the treated group that stably express pECFP-C1-CIRP was slightly lower than the

control group; (2) At 14°C, the temperature was too low to induce CIRP, as CIRP activation is one of the earliest responses to conditions of moderate, but not severe, cold shock (Lleonart et al., 2010). Consequently, the control group of the cells almost contained no CIRP. However, the cells that can stably express pECFP-C1-CIRP have enough CIRP to resist the apoptosis caused by the cold. As a result, the differences of the apoptosis rate between the two groups were great, and apoptosis rate of the group that stably expressed pECFP-C1-CIRP was much lower than that of the control group. At 4°C, the environment was too severe for cells to survive, even though there was much CIRP in the group that can stably express pECFP-C1-CIRP, so that almost all the cells of both groups were totally apoptosized. From the result, we can conclude that CIRP can protect calls from apoptosis caused by cold.

Meanwhile, RBM3 is another well-characterized cold-shock protein, which is very similar with CIRP in structure and function (Lleonart et al., 2010). They are both activated upon moderate cold-shock, hypoxia (Wellmann et al., 2010), space fly (Lebsack et al., 2010) and other environment stress. They both belong to glycine-rich RNA-binding protein family and both of them are thought to be modulators of gene expression during mild hypothermic conditions by functioning as RNA chaperones that facilitate translation (Al-Fageeh and Smalesl., 2006). Furthermore, RBM3 also takes part in protein synthesis (Dresios et al., 2005), neural development (Pilotte et al., 2009), hypothermia induced neuroprotection (Chip et al., 2011), hibernation (Fedorov et al., 2011) and cancer (Sureban et al., 2008; Jögi et al., 2009; Zeng et al., 2009; Ehlén et al., 2010). RBM3 was also found to have apoptotic modulatory capabilities (Sutherland et al., 2005), and the most important is that RBM3 overexpression reduced apoptosis of muscle cells and the collapse of the membrane potential in response to staurosporine. Moreover, the increase in caspase-3, -8, and -9 activities in response to staurosporine was returned to control levels with RBM3 overexpression in muscle cells. These results also indicate that increasing the expression of RBM3 will decrease muscle cell necrosis as well as apoptosis (Ferry et al., 2011). Besides, does CIRP decrease cell apoptosis induced by cold by the same process? Does CIRP also decrease muscle cell necrosis as well as apoptosis induced by cold? These questions remain to be clarified.

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## REFERENCES

- AL-FAGEEH M B, Smales C Mark (2006). Control and regulation of the cellular responses to cold shock: the responses in yeast and mammalian system. *Biochem. J.* 397: 247-259.
- Artero-Castro A, Callejas FB, Castellvi J, Kondoh H, Carnero A, Fernández-Marcos PJ, Serrano M, Ramón y Cajal S, Leonart ME (2009). Cold-inducible RNA-binding protein bypasses replicative senescence in primary cells through extracellular signal-regulated kinase 1 and 2 activation. *Mol. Cell Biol.* 29(7): 1855-1868.
- Baba T, Nishimura M, Kuwahara Y, Ueda N, Naitoh S, Kume M, Yamamoto Y, Fujita J, Funae Y, Fukumoto M (2008). Analysis of gene and protein expression of cytochrome P450 and stress-associated molecules in rat liver after spaceflight. *Pathol. Int.* 58(9): 589-595.
- Banks S, King SA, Irvine DS, Saunders PT (2005). Impact of a mild scrotal heat stress on DNA integrity in murine spermatozoa. *129(4):* 505-514.
- Bhatia R, Dube DK, Gaur A, Robertson DR, Lemanski SL, McLean MD, Lemanski LF (1999). Expression of axolotl RNA-binding protein during development of the Mexican axolotl. *Cell Tissue Res.* 297(2): 283-290.
- Burd CG, Dreyfuss G (1994). Conserved structures and diversity of functions of RNA-binding proteins. *Science (Wash. DC).* 265: 615-621.
- Chip S, Zelmer A, Ogunshola OO, Felderhoff-Mueser U, Nitsch C, Bührer C, Wellmann S (2011). The RNA-binding protein RBM3 is involved in hypothermia induced neuroprotection. *Neurobiol Dis.* [Epub ahead of print]
- Dresios J, Aschrafi A, Owens GC, Vanderklish PW, Edelman GM, Mauro VP (2005). Cold stress-induced protein Rbm3 binds 60S ribosomal subunits, alters microRNA levels, and enhances global protein synthesis. *Proc. Natl. Acad. Sci. USA.* 102(6): 1865-1870.
- Ehlén A, Brennan DJ, Nodin B, O'Connor DP, Eberhard J, Alvarado-Kristensson M, Jeffrey IB, Manjer J, Brändstedt J, Uhlén M, Pontén F, Jirstrom K (2010). Expression of the RNA-binding protein RBM3 is associated with a favourable prognosis and cisplatin sensitivity in epithelial ovarian cancer. *J. Transl. Med.* 8: 78.
- Fedorov VB, Goropashnaya AV, Tøien O, Stewart NC, Chang C, Wang H, Yan J, Showe LC, Showe MK, Barnes BM (2011). Modulation of gene expression in heart and liver of hibernating black bears (*Ursus americanus*). *BMC Genomics.* 12: 171.
- Ferry AL, Vanderklish PW, Dupont-Versteegden EE (2011). Enhanced survival of skeletal muscle myoblasts in response to overexpression of cold shock protein RBM3. *Am. J. Physiol. Cell Physiol.* 301(2): C392-402.
- Fujita J (1999). Cold shock response in mammalian cells. *J Mol Microbiol Biotechnol.* 1(2): 243-255.
- Gualerzi CO, Giuliodori AM, Pon CL (2003). Transcriptional and post-transcriptional control of cold-shock genes. *J. Mol. Biol.* 331(3): 527-539.
- Guo X, Wu Y, Hartley RS (2010) Cold-inducible RNA-binding protein contributes to human antigen R and cyclin E1 deregulation in breast cancer. *Mol. Carcinog.* 49(2): 130-140.
- Hamid AA, Mandai M, Fujita J, Nanbu K, Kariya M, Kusakari T, Fukuhara K, Fujii S (2003). Expression of cold-inducible RNA-binding protein in the normal endometrium, endometrial hyperplasia, and endometrial carcinoma. *Int. J. Gynecol. Pathol.* 22: 240-247.
- Jögi A, Brennan DJ, Rydén L, Magnusson K, Fernö M, Stål O, Borgquist S, Uhlen M, Landberg G, Pålman S, Pontén F, Jirstrom K (2009). Nuclear expression of the RNA-binding protein RBM3 is associated with an improved clinical outcome in breast cancer. *Mod. Pathol.* 22(12): 1564-1574.
- Lebsack TW, Fa V, Woods CC, Gruener R, Manziello AM, Pecaut MJ, Gridley DS, Stodieck LS, Ferguson VL, Deluca D (2010). Microarray analysis of space flown murine thymus tissue reveals changes in gene expression regulating stress and glucocorticoid receptors. *J. Cell Biochem.* 110(2): 372-381.
- Liu A, Zhang Z, Li A, Xue J (2010). Effects of hypothermia and cerebral ischemia on cold-inducible RNA-binding protein mRNA expression in rat brain. *Brain Res.* 1347: 104-110.
- Leonart ME (2010). A new generation of proto-oncogenes: cold-inducible RNA binding proteins. *Biochim. Biophys. Acta.* 1805(1): 43-52.
- Nishiyama H, Itoh K, Kaneko Y, Kishishita M, Yoshida O, Fujita J (1997). A Glycine-rich RNA-binding Protein Mediating Cold-inducible Suppression of Mammalian Cell Growth. *J. Cell Bio.* 137: 899-908.
- Nishiyama H, Xue J-H, Sato T, Fukuyama H, Mizuno N, Houtani T, Sugimoto T, Fujita J (1998). Diurnal change of the cold inducible RNA-binding protein (CIRP) expression in mouse brain. *Biochem. Biophys. Res. Commun.* 245: 534-538.
- Pan F, Zarate J, Choudhury A, Rupperecht R, Bradley TM (2004). Osmotic stress of salmon stimulates upregulation of a cold inducible RNA binding protein (CIRP) similar to that of mammals and amphibians. *Biochemistry.* 43(7): 451-461.
- Peng Y, Kok KH, Xu RH, Kwok KH, Tay D, Fung PC, Kung HF, Lin M-C (2000). Maternal cold inducible RNA binding protein is required for embryonic kidney formation in *Xenopus laevis*. *FEBS Lett.* 482: 37-43.
- Peng Y, Yang P-H, Tanner JA, Huang J-D, Li M, Lee H-F, Xu R-H, Kung H-F, Lin M-C (2006). Cold-inducible RNA binding protein is required for the expression of adhesion molecules and embryonic cell movement in *Xenopus laevis*. *Biochem. Biophys. Res. Commun.* 344: 416-424.
- Pilote J, Cunningham BA, Edelman GM, Vanderklish PW (2009). Developmentally regulated expression of the cold-inducible RNA-binding motif protein 3 in eutherian rat brain. *Brain Res.* 1258: 12-24.
- Sakurai T, Itoh K, Higashitsuji H, Nonoguchi K, Liu Y, Watanabe H, Nakano T, Fukumoto M, Chiba T, Fujita J (2006). Cirp protects against tumor necrosis factor- $\alpha$ -induced apoptosis via activation of extracellular signal-regulated kinase. *Biochem. Biophys. Acta.* 1763: 290-295.
- Saito T, Adachi Y, Wu Q, Mori KJ, Sugimoto K (2000). Cloning and characterization of amphibian cold inducible RNA-binding protein. *Comp. Biochem Physiol. B. Biochem. Mol.* 125: 237-245.
- Saito K, Fukuda N, Matsumoto T, Iribe Y, Tsunemi A, Kazama T, Yoshida-Noro C, Hayashi N (2010). Moderate low temperature preserves the stemness of neural stem cells and suppresses apoptosis of the cells via activation of the cold-inducible RNA binding protein. *Brain Res.* 1358: 20-29.
- Siomi H, Dreyfuss G (1997). RNA binding proteins as regulators of gene expression. *Curr. Opin. Genet. Dev.* 7: 345-353.
- Sambrook J, Russell W (2002). *Molecular Cloning: A Laboratory manual*. 3rd ed. Beijing: Science Press, (in Chinese).
- Sugimoto K, Jiang H (2008). Cold stress and light signals induce the expression of cold-inducible RNA binding protein (cirp) in the brain and eye of the Japanese treefrog (*Hyla japonica*). *Comp. Biochem. Physiol. A Mol. Integr Physiol.* 151(4): 628-636.
- Sureban SM, Ramalingam S, Natarajan G, May R, Subramaniam D, Bishnupuri KS, Morrison AR, Dieckgraefe BK, Brackett DJ, Postier RG, Houchen CW, Anant S (2008). Translation regulatory factor RBM3 is a proto-oncogene that prevents mitotic catastrophe. *Oncogene.* 27(33): 4544-4556.
- Sutherland LC, Rintala-Maki ND, White RD, Morin CD (2005). RNA binding motif (RBM) proteins: a novel family of apoptosis modulators? *J. Cell Biochem.* 94(1): 5-24.
- Tan H-K, Lee M-M, Yap MG, Wang D-I (2008). Overexpression of cold-inducible RNA-binding protein increases interferon-gamma production in Chinese-hamster ovary cells. *Biotechnol. Appl. Biochem.* 49: 247-257.
- Van Venrooy S, Fichtner D, Kunz M, Wedlich D, Gradl D (2008). Cold-inducible RNA binding protein (CIRP), a novel XTcf-3 specific target

- gene regulates neural development in *Xenopus*. *BMC Dev. Biol.* 7(8): 77.
- Wellmann S, Bührer C, Moderegger E, Zelmer A, Kirschner R, Koehne P, Fujita J, Seeger K (2004). Oxygen-regulated expression of the RNA-binding proteins RBM3 and CIRP by a HIF-1-independent mechanism. *J. Cell Sci.* 117: 1785-1794.
- Wellmann S, Truss M, Bruder E, Tornillo L, Zelmer A, Seeger K, Bührer C (2010). The RNA-binding protein RBM3 is required for cell proliferation and protects against serum deprivation-induced cell death. *Pediatr. Res.* 67(1): 35-41.
- Xue J-H, Nonoguchi K, Fukumoto M, Sato T, Nishiyama H, Higashitsuji H, Itoh K, Fujita J (1999). Effects of ischemia and H<sub>2</sub>O<sub>2</sub> on the cold stress protein CIRP expression in rat neuronal cells. *J. Free Radic. Biol. Med.* 27(11-12): 1238-1244.
- Yang C, Carrier F (2001). The UV-inducible RNA-binding protein A18 (A18 hnRNP) plays a protective role in the genotoxic stress response. *J. Biol. Chem.* 276: 47277-47284.
- Zeng Y, Kulkarni P, Inoue T, Getzenberg RH (2009). Down-regulating cold shock protein genes impairs cancer cell survival and enhances chemosensitivity. *J. Cell Biochem.* 107(1): 179-188.
- Zhou K-W, Zheng X-M, Yang Z-W, Zhang L, Chen H-D (2009). Overexpression of CIRP may reduce testicular damage induced by cryptorchidism. *Clin. Invest. Med.* 32(2): E103-111.