# CLONING AND EXPRESSION OF WILD BOAR CIRP GENE AFTER COLD-INDUCTION

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**Abstract.** Cold inducible RNA binding protein ( CIRP ) is one of the mammalian cold shock proteins (CSPs) whose expression is up-regulated in response to moderate hypothermia (typically, 25 ~ 33°C). This study was designed to clone the complete coding sequence of the wild boar CIRP gene, and to analyze its expression characteristics under culture conditions of different low temperatures. The cDNA sequence of CIRP, in length of 519 bp was cloned from wild boar fibroblasts under mild cold-treatment. CIRP cDNA encodes a protein of 172 amino acids, which consists of an amino-terminal RNA-binding domain and a carboxyl-terminal glycine-rich domain. The amino acid sequence of CIRP has high homology with other species and is highly conserved during evolution. CIRP cDNA was not isolated from the cells treated at 37°C, 15°C and 4°C. This maybe because the CIRP mRNA abundance in cells was too low without low-temperature-induction, the reverse transcription product is not sufficient as a template to amplify the target gene. Relative quantitation PCR analysis of extract form the northeast boar fibroblasts showed that CIRP mRNA levels was increased markedly in response to mild cold treatment( 32°C and 25°C), but not to severe cold treatment (15°C and 4°C).

**Keywords:** Cold-induced, Wild boar, Fibroblasts, *CIRP* gene, Cloning, Expression.

## Introduction

The general response of cells to cold stress is the elite and rapid over-expression of a small group of proteins, the so-called CSPs. As in the case of prokaryotic and yeast systems, mammalian cells express several CSPs upon exposure to mild hypothermia. The CIRP was the first identified CSP in mammalian cells. CIRP gene has been detected in mouse, rat, and human cells, whose amino acid sequence is highly conserved in these species (Sonna *et al., 2002*). The protein encoded by the CIRP gene constitutively expresses in most tissues of adult mice at a relatively low level. However, it was strongly induced by the mild cold treatment in BALB/3T3 mouse fibroblasts (Al-Fageeh *et al., 2006*). CIRP functions as an RNA chaperone, preventing the formation of mRNA secondary structure at low temperatures (Al-Fageeh *et al.*, 2009). It was also induced to a similar extent in response to light, hypoxia and UV irradiation (Sugimoto et al., 2008; Guo et al., 2010). Beside the predicted role of CIRP as a molecular chaperone upon stress response, emerging evidence strongly suggests that CIRP seems to play major roles in differentiation during embryonic development (Mohamed *et al.*, 2006). In this study, cDNA of the northeast boar CIRP was cloned using RT-PCR and its expression pattern was characterized using Real-time PCR. Such research would be important for better understanding of CIRP gene function in cell growth and degradation at low temperatures.

Upon stress induction, CIRP migrates from the nucleus to the cytoplasm to affect expression of its target mRNAs(Leeuw *et al.*, 2007; Yang *et al.*, 2006). CIRP mediates suppression of mammalian cell growth with prolongation of G1 phase, and contributes to the suppression of apoptosis induced by tumour necrosis factor-a,genotoxic stress or cryptorchidism(Guo *et al.*, 2010; Tomoko *et al.*, 2012).Wild boar (*Sus scrofa*), also known as a wild pig, is a species of the pig genus *Sus*, part of the biological family Suidae. The species includes many subspecies. One species lives in China, which is divided into six subspecies: south China boar (*Sus scrofa chirodontus*), northeast boar (*Sus scrofa ussuricus*), west Sichuan boar (*Sus scrofa moupinensis*), Taiwan boar (*Sus scrofa taivans*), Xinjiang boar (*Sus scrofa nigripes*) and Menggu boar (*Sus scrofa raddeana*).

## Materials and Methods

Wild boar fibroblasts were cultured in D-MEM/F12 (Gibco, Grand Island, USA) supplemented with 10% fetal calf serum (FCS) (Dainippon Pharmaceutical Co., Osaka, Japan) and 100 IU/ml penicillin and 100μg/ml streptomycin in a 5% $CO_2$ humidified incubator at 37°C. For temperature-shift experiments, cells ($5\times10^3$ cells/cm ) were grown for 24h at 38°C and then shifted to 4°C, 15°C, 25°C or 32°C in a 5% $CO_2$ humidified incubator. The cell samples were sacrificed at 2h, 4h, 6h, 8h, 10h or 12h after shifting. Total RNA was extracted from cells using a Trizol reagent (Invitrogen, Grand Island, USA) according to the manufacturer's instructions.

A swine EST sequence (GenBank: AK234205) was searched in the NCBI database by comparison with

human CIRP sequence (GenBank: NM_001280) in BLAST. One pair of primers was designed according to the swine EST sequence. F: 5'-ATGGCATCAGATGAAG GC-3', R: 5'-TTACTCGTTGTGTGTAGCAT-3'. For Real-time PCR measurement of CIRP mRNA in different low temperature culture conditions, F: 5'-GGTGAAGGT CGGAGTGAACG-3, R: 5'-CTCGCTCCTGGAAGATG GTG-3'. For the housekeeping gene, GAPDH, was designed as followed, F: 5'-GGTGAAGGTCGGAGTGA ACG-3', R: 5'-CTCGCTCCTGGAAGATGGTG-3' (GenBank: NM_001206359). The first-strand cDNAs were prepared from the total RNAs of cells (at 37°C, 32°C, 25°C, 15°C and 4°C incubator for 8h) by using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Fost City, CA, USA) with random primers, according to the manufacturer's instructions. The PCR reaction was repeated for 35cycles at 94°C for 30s, 52°C for 30s and 72°C for 1min.The product of RT-PCR was purified by Gel Extraction Mini Kit (Watson, Shanghai, China), then cloned to pMD18-T (TaKaRa) vector and sequenced forward and reverse by the ABI 3730 sequencer (Bioasia, Shanghai, China).

Fragments amplified by RT-PCR were assembled by DNAMAN package Version 5.2.2. The conserved domain was analyzed in Prosite Database (http://cn.expasy.org/prosite/). The homologous nucleotide sequence was searched in BLAST (Blastp, TBlastX, http://www.ncbi.nlm.nih.gov /BLAST) and Pfam (http://www.sanger.ac.uk/Software/Pfam/) respectively. Using the boar CIRP gene sequence as a source gene, we compared CIRP genes from different species with on-line BLAST (http://www.ncbi.nlm.nih.gov /BLAST) and analyzed results using DNAStar software.

Quantitative Real-time PCR was performed with an ABI Prism 7000 sequence detection system (Applied Biosystems). The gene expression reaction was performed in 20µl reactions containing 10µl of 2×SYBR Gene PCR Master Mix (Applied Biosystems, Fost City, CA, USA), 0.8µl of RT product, 0.8µl of forward primer, 0.8µl of reverse primer and 7.6µl of autoclaved distilled water. The reaction mixtures were incubated at 95°C for 10 min, followed by 40 amplification cycles of 95°C for 30s and 60°C for 1min. The Ct values of CIRP in different groups were normalized by GAPDH and the differences were analyzed by relative quantification $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

**Results**

From the fibroblasts of wild boar treated at 32°C and 25°C for 8h rather than treated at 37, 15°C and 4°C for 8h, a fragment of about 519 bp by RT-PCR was amplified. After assembling the sequence of RT-PCR products by DNAMAN (version 5.2.2), CIRP of the north-eastern wild boar was obtained. It encoded 172 amino acids. The predicted amino acid sequence displayed two main features: the presence of an amino-terminal consensus RNA-binding domain (CS-RBD) and a carboxyl-terminal glycine-rich domain. The CS-RBD of CIRP contained consensus sequences of RNP1, RNP2, and a number of others which are mostly hydrophobic conserved amino acids interspersed throughout the motif (Fig. 1).

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1       ATGGCATCAGATGAAGGCAAACTTTTCGTTGGAGGGCTGAGCTTTGACACCAATGAGCAG
1        M  A  S  D  E  G  K  L  F  V  G  G  L  S  F  D  T  N  E  Q
61      TCGCTGGAGCAGGTCTTCTCAAAATATGGGCAGATCTCAGAAGTGGTAGTAGTGAAGGAC
21       S  L  E  Q  V  F  S  K  Y  G  Q  I  S  E  V  V  V  V  K  D
121     AGGGAGACCCAGCGATCGAGGGGCTTTGGGTTTGTCACCTTTGAGAACATTGATGACGCC
41       R  E  T  Q  R  S  R  G  F  G  F  V  T  F  E  N  I  D  D  A
181     AAGGACGCTATGATGGCCATGAACGGGAAGTCTGTGGATGGGCGGCAGATCCGGGTTGAC
61       K  D  A  M  M  A  M  N  G  K  S  V  D  G  R  Q  I  R  V  D
241     CAGGCCGGCAAGTCATCTGATAACCGATCCCGTGGGTACCGAGGTGGCTCCTCGGGGGGC
81       Q  A  G  K  S  S  D  N  R  S  R  G  Y  R  G  G  S  S  G  G
301     CGGGGCTTCTTCCGTGGGGGCCGAGGCCGGGGCCGTGGGTTCTCCAGAGGAGGAGGGGAT
101      R  G  F  F  R  G  G  R  G  R  G  R  G  F  S  R  G  G  G  D
361     CGAGGCTATGGTGGTAGCCGGTTCGAGTCCAGGAGTGGGGGCTATGGCGGCTCCAGGGAC
121      R  G  Y  G  G  S  R  F  E  S  R  S  G  G  Y  G  G  S  R  D
421     TACTACAGCAGCCGGAGTCAGGGTGGCGGCTATGGTGACCGGAGCTCAGGCGGGTCCTAC
141      Y  Y  S  S  R  S  Q  G  G  G  Y  G  D  R  S  S  G  G  S  Y
481     AGAGACAGCTACGACAGTTATGCTACACACAACGAGTAA
161      R  D  S  Y  D  S  Y  A  T  H  N  E  *
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Fig.1. **Cloned CDS and deduced amino acid sequence of CIRP. The amino acid sequence is shown in single-letter code below the nucleotide sequence. The putative RNP1 motif** (FVGGL) **and RNP2** (RGFGFV) **are underlined. GGYGG and GGGYG, well conserved among GRPs, are also underlined. Terminal codon is indicated by an asterisk**

The predicted amino acid sequence of CIRP was compared with known sequences in GenBank using the Genetics Computer Group software package. Alignment of the amino acid sequence revealed that it was 99.4%, 94.6%, 99.4%, 97.6%, 97.6%, 99.4%, 97.0% , 93.8%, 97.6% and 96.4% identical to that of the CIRP in cattle, chicken, dog, human, rhesus monkey, giant panda, chimpanzee, African savanna elephant, Sumatran orangutan, and white-tufted-ear marmoset, respectively. Therefore, the coding region of CIRP was correctly cloned from the wild boar fibroblasts by mild cold treatment (32°C and 25°C).

The nucleotide sequence and amino acid sequence of wild boar *CIRP* gene comparing with other species were listed in Table 1.

Table.1 **Nucleotide and amino acid sequence analysis results of different species on *CIRP* gene**

| species | nucleotide sequence (%) | amino acid sequence (%) | GenBank accession |
|---|---|---|---|
| cattle | 87.86 | 99.4 | NM_001034278 |
| chicken | 79.50 | 94.6 | DQ126684 |
| dog | 87.28 | 99.4 | XM_863509 |
| human | 85.93 | 97.6 | AB451225 |
| rhesus monkey | 85.93 | 97.6 | NM_001261316 |
| giant panda | 89.21 | 99.4 | XM_002923513 |
| chimpanzee | 86.90 | 97.0 | AK306265 |
| African savanna elephant | 80.92 | 93.8 | XM_003422526 |
| Sumatran orangutan | 85.16 | 97.6 | NM_001131220 |
| white-tufted-ear marmoset | 86.90 | 96.4 | XM_002761530 |

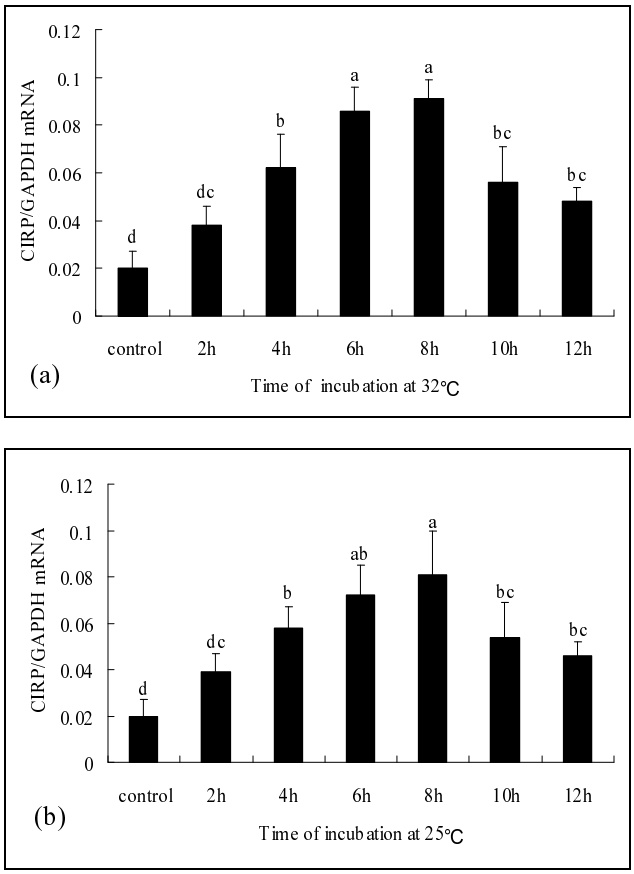


Fig. 2 **Real-time PCR of *CIRP* and the relative levels of each message compared with the 37°C control determined**

(a) Fibroblasts were incubated at 32°C, (b) Fibroblasts were incubated at 25°C. All data shown are the means of triplicate experiments ±SD

Note: a-d means in a group with no common superscript differ significantly (P<0.05)

The effect of cold stress on the CIRP gene expression of the north-eastern wild boar fibroblasts in vitro at temperatures of 32°C and 25°C is shown in Fig. 2. The levels of the CIRP transcript changes were identified by comparing with the control incubation at 37°C. The levels of CIRP mRNA increased 2–12h after a temperature shift from 37°C to 32°C and 25°C, reaching maximum level at 8h post-temperature downshift, respectively, 4.51 fold and 4.05 fold in the level of CIRP transcript. 10h post cold-shock, the levels of the CIRP transcript had decreased and were approximately two fold increase in the level of CIRP transcript, and this level was maintained until 12h after temperature downshift.

**Discussion**

It is well established that the general response of mammalian cells to cold stress involves the attenuation of transcription and translation, leading to a generalized reduction in protein synthesis (Murata *et al.*, 2005; Underhill, 2007; Lleonart, 2010). However, there is a select number of cold shock proteins whose synthesis continues or is up-regulated during the period of exposure to moderate hypothermia (Sonna *et al.*, 2002; Artero-Castro *et al.*, 2009). The best characterized cold shock protein is CIRP in mammalian cells. Previous reports have shown that both CIRP transcription and translation are induced under sub-physiological temperature conditions of 25–33°C in mouse, rat, and human cells (Sonna *et al.*, 2002). CIRP expression can be induced by a variety of stressors other than cold, such as ultraviolet irradiation and hypoxia. Our results showed that the CIRP cDNA was cloned from the north-eastern wild boar fibroblasts treated at 32°C and 25°C for 8h rather than treated at 37, 15°C and 4°C. This may be because that CIRP mRNA transcription level in cells was too low without low-temperature-induction or under too low temperature, the reverse transcription

product is not sufficient as a template to amplify the target gene.

CIRP belongs to the ribonucleic acid-(RNA)-binding proteins (RBPs) family, RBPs diverse affinity and specificity towards target RNAs that play a crucial role in almost every aspect of RNA metabolism (biogenesis, maturation, transport, cellular localization, and turnover) (Kishore *et al.*, 2010). RBPs contain well-conserved RNA binding domains (RBDs) mediating RNA contact but also auxiliary domains involved in protein-protein interactions, making RBPs versatile regulators of gene expression. Amino acid sequence analysis showed that the north-eastern wild boar CIRP consisted of an amino-terminal CS-RBD and a carboxyl-terminal domain rich in glycine. The CS-RBD contains two highly conserved sequences, RNP1 and RNP2. RNP consensus sequence is one of the major RNA-binding motifs (Fujita J. 1999). The CS-RBD possesses the ability to bind RNA, to protect and restore native RNA conformations during stress (Nishiyama, 1997). The carboxyl-terminal part was rich in glcine, serine, arginine, and tyrosine. The glycine-rich domain enhances RNA-binding via protein/protein and/or protein/RNA interactions (Jin *et al.*, 2009). Multiple alignment of CIRP shows that CIRP is a type of conserved protein among species. This means that CIRP may participate in many aspects of biological processes in their evolutionary courses. CIRP has been reported to activate transcription during the period of exposure to moderate hypothermia in BALB/3T3 mouse embryonic fibroblast cells, CHO Chinese hamster ovary cells, MCF-7 breast cancer cells and T47D human breast cancer cells and other cells ( Tan *et al.*, 2008; Guo *et al.*, 2010). It was shown that similarly, transcription of CIRP was increased in the wild boar fibroblasts under sub-physiological temperature conditions ( 25°C and 32°C), and that it expressed in a time-dependent manner.

**Conclusions**

The cDNA of CIRP was firstly cloned from the north-east boar fibroblasts treated by the lowered culture temperature (32°C). The predicted amino acid sequence displayed two main features: the presence of an amino-terminal consensus RNA-binding domain (CS-RBD) and a carboxyl-terminal glycine-rich domain. The amino acid sequence of CIRP has high homology with other species and is highly conserved during evolution. CIRP, the expression of which was increased markedly in response to mild cold treatment( 32°C and 25°C), but not to severe cold treatment(15°C and 4°C).

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