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Construction of recombinant plasmid pIRES2-EGFP/CCK and its expression in vivo and in vitro

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Abstract: Objective To construct eukaryotic expression plasmid of porcine *CCK* gene pIRES2-EGFP/CCK and express it in COS-7 cells and hamsters. **Methods** The aimed segments were obtained from intermediate vector pMD18-T/CCK and were inserted into an eukaryotic expression plasmid pIRES2-EGFP to construct a recombinant expression plasmid pIRES2-EGFP/CCK. The recombinant expression plasmid was transfected into COS-7 cells by liposome-mediated gene transfer method and was observed through fluorescence microscope. The plasmid was injected into the skeletal muscle of hamsters directly to detect the expression of the recombinant plasmid in vivo. **Results** A recombinant eukaryotic expression plasmid pIRES2-EGFP/CCK was successfully constructed. Green fluorescent protein could be detected in the transfected COS-7 cells 24, 48, and 72 hours after the transfection. On the 4th day postinjection into the skeletal muscle of hamsters, the protein could be detected at the injection site and the fluorescence intensity became much stronger on the 14th day than that on the 4th day. On the 42nd day the protein level increased. The green fluorescence protein was never expressed in the untransfected cells. **Conclusion** The porcine *CCK* gene eukaryotic expression plasmid pIRES2-EGFP/CCK is constructed successfully, and is expressed in mammal COS-7 cells and hamsters in vivo. The research paves the way for the cross immunity therapy of hamster pancreatic carcinoma.

Key words: cholecystokinin; skeletal muscle; cell transfection; green fluorescent protein; COS-7 cells

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重组质粒 pIRES2-EGFP/CCK 的构建及其在仓鼠体内及 COS-7 细胞中的表达

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[摘要] **目的** 构建猪源性 *CCK* 基因重组真核表达质粒 pIRES2-EGFP/CCK(*CCK* pDNA),研究其在哺乳动物细胞和仓鼠体内的表达。**方法** 以限制性内切酶法从中介载体 pMD18-T/CCK 中切取目的片段 *CCK*,将其克隆入真核表达载体 pIRES2-EGFP 中,以脂质体法转染 COS-7 细胞,同时采用直接肌肉注射法免疫仓鼠,利用荧光显微镜观察绿色荧光蛋白的表达。**结果** 用 *CCK* pDNA 转染 COS-7 细胞后 24,48,72 h 均可检测到绿色荧光蛋白的表达,其中 72 h 组荧光强度达到最强。用 *CCK* pDNA 免疫仓鼠后,第 4 天注射部位可检测到绿色荧光蛋白的表达,第 14 天荧光强度明显增强,第 42 天荧光强度进一步增强,正常对照组始终未检测到绿色荧光蛋白的表达。**结论** 成功构建了猪源性 *CCK* 基因真核表达质粒 pIRES2-EGFP/CCK,并成功地在哺乳动物细胞和仓鼠体内进行了表达,为仓鼠胰腺癌的交叉免疫治疗奠定了基础。

[关键词] 胆囊收缩素; 骨骼肌; 细胞转染; 绿色荧光蛋白; COS-7 细胞
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Cholecystokinin(*CCK*) is a kind of gastrointestinal polypeptide hormone, and has many physiological functions. The structure of *CCK* is of high conservatism. The homology of *CCK* amino acid cloned from cDNA library of house mouse gall bladder, porcine cerebrum, and human gall bladder can reach 80%, some up to 87% ~97%[1]. In recent years, it has been reported that *CCK* relates to the generation and progression of some kinds of tumor in the digestive tract and non-digestive tract, especially in patients with pancreatic carcinoma[2~6]. To research the immunotherapy effect of *CCK* in the treatment of pancreatic cancer, we constructed the porcine *CCK* cDNA eukaryotic expression plasmid pIRES2-EGFP/CCK and expressed it transiently in COS-7 cells. To investigate the expression of recombinant plasmid pIRES2-EGFP/CCK in vivo, the recombinant plasmid was injected into the skeletal muscle of hamster directly and the expression of reporter gene in skeletal muscle in vivo was observed.

1 MATERIAL AND METHODS

1.1 Material T4 DNA ligase was from Promega Corporation. Gel recovery kit and plasmid extraction kit were from Shanghai Bioasia Corporation. Restriction endonucleases *Eco*RI, *Bam*HI and *E. coli* DH5α competent cells were bought from TaKaRa. Lipofectamine™ 2000 Reagent and RPMI 1640 culture medium were products of Gibcol BRL. COS-7 cells were from American Type Culture Collection (ATCC), and pIRES2-EGFP was from BD Biosciences Corporation.

1.2 Construction of *CCK* gene eukaryotic expression plasmid The intermediate vector pMD18-T/CCK and the expression vector pIRES2-EGFP were digested with *Eco*RI and *Bam*HI, and then electrophoresed with 1% agarose gel. The gel containing the aimed segment *CCK* and vector pIRES2-EGFP were cut and recovered respectively (following the instructions of the kit). The *CCK* was inserted into pIRES2-EGFP vector using T4DNA ligase, according to the instructions of the kit. The linked product was transfected into the *E. coli* DH5α competent cells and then the cells were incubated on the LB agar plates with 50 μg/mL of kanamycin at 37 ℃ over night. The single colony was chosen and inoculated in liquid LB medium, and was put on the shaker at 37 ℃ over night. The recombined plasmid was extracted, and identified by double digesting. The DH5α strains with pIRES2-EGFP/CCK were incubated in large quantity to extract plasmid pIRES2-EGFP/CCK. After identified by digesting, the plasmid was stored for the later experiment.

1.3 Transfection of COS-7 cells COS-7 cells were transfected following the instructions of the lipofectamine™ 2000 reagent, as it follows: COS-7 cells were incubated in RPMI 1640 medium containing 100 mL/L fetal calf serum (FCS), digested in logarithm increase period with 2.5 g/L trypsin 24 hours before the transfection, and plated in 24-well plate with 8×10^4 cells per well. At 90% ~95% confluency, the cells were washed with serum-free RPMI 1640 medium. In every well, 0.2 mL lipofectamine™2000: DNA complexes prepared in advance and 0.8 mL serum-free RPMI 1640 medium were added to the wells and mixed by gentle rocking. The cells were incubated in 37 ℃ constant temperature incubator under 5% CO_2. The expression of green fluorescent protein in the cells was observed with fluorescence microscopy after 24, 48, and 72 hours of the transfection, and the transfect ratio was measured. The transfected cells were collected to extract total RNA of them. The extracted total RNA was applied as the template to amplify the aimed segment by using RT-PCR method.

1.4 In vivo gene transfer using direct intramuscular injection approach Female Syrian golden hamsters of 4 ~5 weeks old (40 ~50 g) were anesthetized generally through intraperitoneal injection of 60 mg/kg mebumal sodium to prevent DNA from being squeezed out in muscle contracting. When the hamsters were in coma and the muscles were relaxed, they were shaved to remove hair from the lateral surface of the hind limb, and then injected with 100 μL 25 g/100mL hypertonic sucrose into the quadriceps muscles of the thighs to create osmotic pressure. The osmotic pressure caused muscle fiber to atrophy and loose temporarily and further facilitate the diffusion of DNA[7]. Fifteen minutes later, at the previous injection sites, 100 μL recombinant plasmid (50 μg/100 μL) was injected slowly. The injection lasted no less than 10 seconds. When it was finished, the syringe needle remained unmoved for 5 ~10 seconds before it was pulled out lest the plasmid was leaked from the pinholes. After 4, 14, and 42

days, hamsters were killed and the quadriceps muscles were removed to make frozen sections. Fluorescence microscopy was used to observe the expression of green fluorescent protein in the skeletal muscle tissues.

2 RESULTS

2.1 Assessment of recombinant plasmid pIRES2-EGFP/CCK The intermediate vector pMD18-T/CCK and the expression of vector pIRES2-EGFP were digested with *Eco*RI and *Bam*HI respectively, getting the aimed segments *CCK* from the former (Figure 1). The aimed segments were inserted into pIRES2-EGFP to obtain a recombinant plasmid pIRES2-EGFP/CCK. The plasmid was digested with *Eco*RI and *Bam*HI, obtaining two bands. The larger is the vector and the smaller is measured about 430 bp, identical to the gene of porcine *CCK* (Figure 2).

AAGCTTGCATGCCTGCAGGTCGACGATTCCGGGATCCTGTTTTCTTCTGCCTCCTCTGAGTCAGGAGGTAGCTTCCAGTGGCGCTGACCAAGGC
GGGGTCTTCTAGGAGGTATATTCATACTCCTCTGCACTGCGCCGGCCAAAATCCATCCAGCCCATGTAGTCCCGGTCACTTATTCTGTGGCTGG
GGTCCAGGCTCTGCAGATTCTTAATCATAGATACTCGGCCAGAAGGTGCTTTTCGAGCCTGCTGGATGTATCTGGCCAGCAGCGCGCCCGGGT
GCGCTCGGGACTCGCCGTCTACCTTTTGCACCGCCCTCAGCTGCCGCCGGTGCGCCTCCTCCTCCTGTGCCCCGGGGACCGCGGAGTCCGCGG
GAGGCACCGGCTGCGCCAGGGTGCCTGCCGCCAGGACCGCCATCAGCACACACAGGCACAAGCCGCCGTTCATGAATTCGTAATCATGGTCA
TAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGA
GTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGGCCGCTTTTCCAGTCGGGGAAANCTGTCGTGGCCAGCTGCATTAAATGGATCNGCCA
ACGCGCGGGGAGAAGCGGTTTTGCGGTAATTGGGGNGCTCTTTCGGCTTCCTCGCNCAATGANTCNGCTTGCGCTCCGGTCCGTTCGGGCTGG
CGGNNGAAGGGGNAATAAGTTCACTTCNAAGGGCGGNNAATAACGGTTTATCCNCCAGAAATCCAGGGGGNNTAACGCCANGGAAAAAAAA
ATGNNGAAACNAAAAGGCCCNCNAA

Fig. 1 Sequence of aimed segments of porcine CCK The underlined parts are digestion sites

2.2 Expression and assessment of recombined plasmid pIRES2-EGFP/CCK in COS-7 cells COS-7 cells were transfected with pIRES2-EGFP/CCK while the untransfected ones functioned as negative control. The results showed that 24 hours posttransfection, the green fluorescent protein could be detected in the transfected COS-7 cells (Figure 3A), while 48 hours posttransfection, the number of positive cells increased tremendously. In each field of vision, there were several to dozens of positive cells and much brighter green fluorescence centered in the cytoplasm (Figure 3B). And 72 hours posttransfection, the green fluorescence of positive cells became even stronger (Figure 3C). These cells appeared in pairs or piles. In comparison, no green fluorescence could be seen in the untransfected cells (Figure 3D). RT-PCR method was used to detect *CCK* mRNA level in the cells. The result showed that a high expression of *CCK* mRNA could be detected in COS-7 cells transfected with pIRES2-EGFP/CCK while no expression of porcine *CCK* mRNA was found in the untransfected ones (Figure 4).

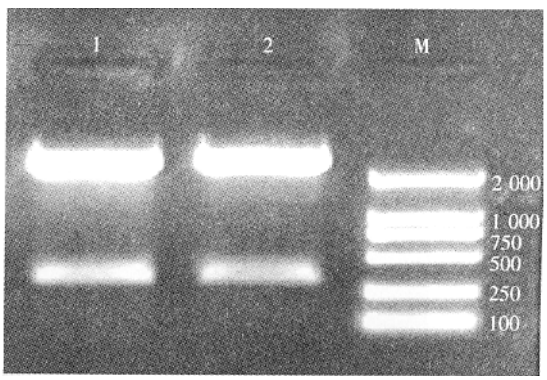

Fig. 2 Restriction analysis of recombinant plasmid pIRES2-EGFP/CCK 1, 2: pIRES2-EGFP/CCK was digested with *Eco*RI and *Bam*HI, obtaining 2 bands. The smaller is the aimed segments, measured about 430 bp, and the larger is the vector. M: DNA marker

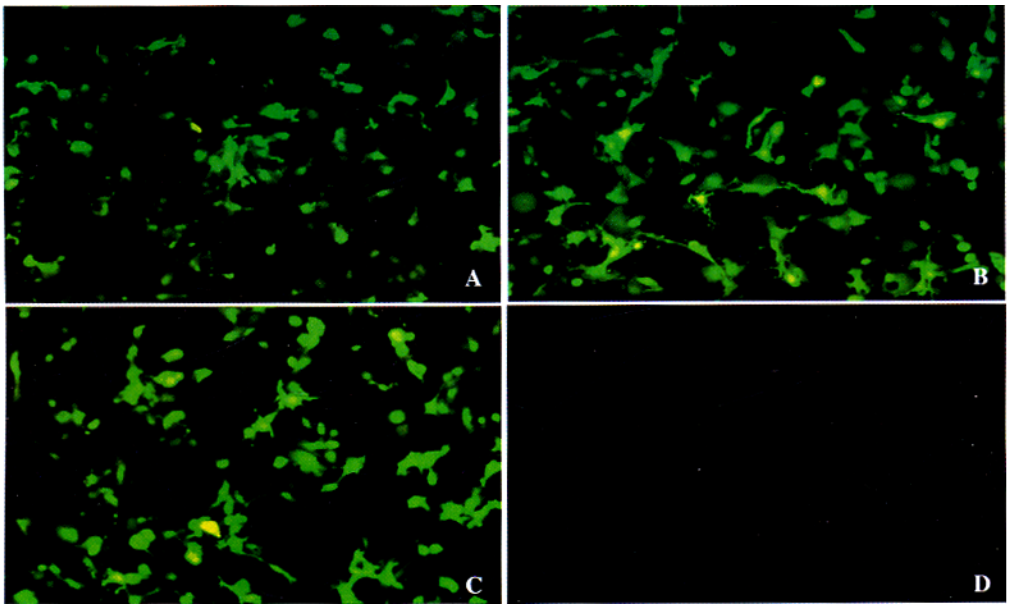

Fig. 3 Expression of green fluorescent protein in COS-7 cells transfected with recombinant plasmid pIRES2-EGFP/CCK (×100) A: Expression in COS-7 cells 24 hours posttransfection; B: Expression in COS-7 cells 48 hours posttransfection; C: Expression in COS-7 cells 72 hours posttransfection; D: Negative control

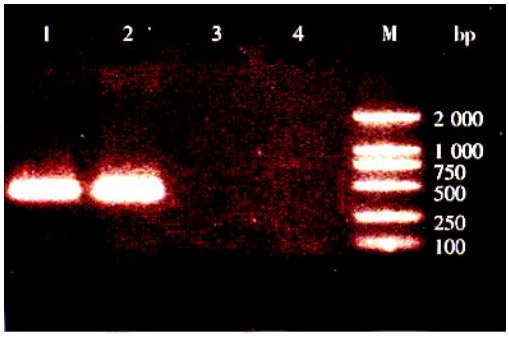

Fig. 4 Agarose gel electrophoresis of RT-PCR products 1: COS-7 cells transfected with pIRES2-EGFP/CCK; 2: Muscle tissue injected with pIRES2-EGFP/CCK; 3: COS-7 cells transfected with null plasmid; 4: Muscle tissue injected with null plasmid; M: DNA marker

2.3 **Expression of green fluorescent protein in skeletal muscle in vivo** The green fluorescent protein could be detected at the injection site on the 4th day after the injection (Figure 5A), and the fluorescence intensity became stronger on the 14th day (Figure 5B). On the 42nd day, the protein level was much more than before (Figure 5C). The expression of green fluorescence was never detected in the control group (Figure 5D). The *CCK* mRNA level in the tissues of skeleton muscle was detected with RT-PCR method. We found an obvious expression of porcine *CCK* mRNA in muscular tissues injected with pIRES2-EGFP/CCK, while no expression of porcine *CCK* mRNA was found in the muscular tissues injected with null plasmid (Figure 4).

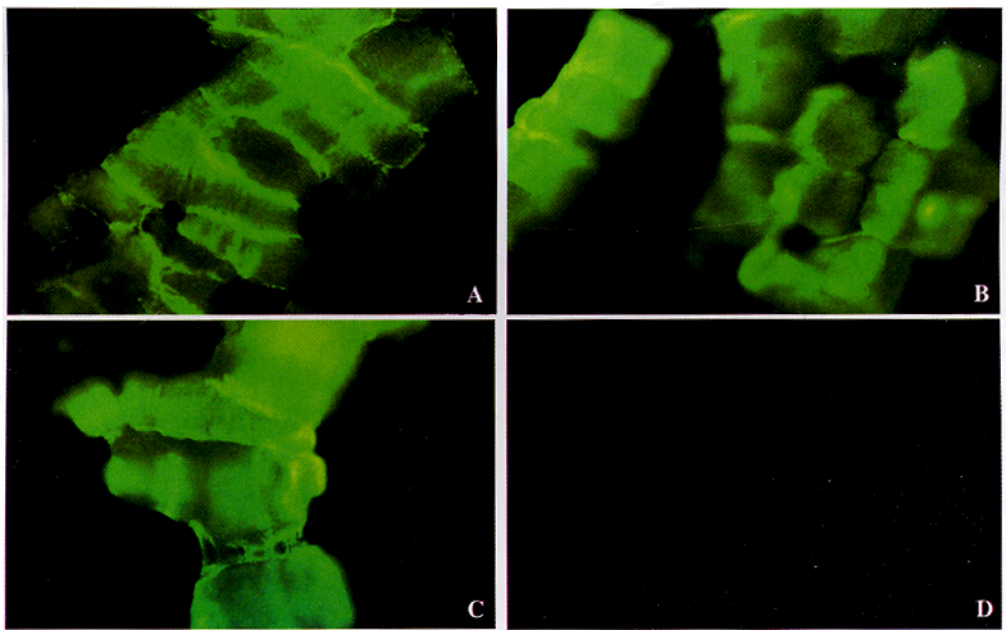

Fig. 5 Expression of green fluorescent protein in skeletal muscle (×200) A: Expression in muscle 4 days postinjection with pIRES2-EGFP/CCK; B: Expression in muscle 14 days postinjection with pIRES2-EGFP/CCK; C: Expression in muscle 42 days postinjection with pIRES2-EGFP/CCK; D: Negative control

3 DISCUSSION

Pancreatic cancer is of high malignant degree and unfavorable prognosis. In recent years, the incidence of pancreatic cancer is remarkably increasing on the worldwide scale[8]. In the past dozens of years, the diagnosis level, surgery skills, and systemic chemotherapy have been improved greatly. However, the prognosis of pancreatic cancer remains poor, because the pancreatic carcinoma is hard to diagnose at its early stage. Even if it is diagnosed at the early stage, many patients have liver metastasis, peritoneal dissemination, and local recurrence[9]. At present, there is still no efficient way to inhibit the occurrence of these issues. Therefore, how to apply molecular biological and molecular genetic approaches to diagnose and treat cancer is quite a hotspot in the field.

Nucleic acid vaccine is also called gene vaccine or DNA vaccine. It is a genetic treatment method arising in recent years. That is to say, exogenous gene (DNA or RNA) that encodes a certain antigen protein is led into body cells, to compose antigen protein in host cells. The antigen protein induces the host 's immune response to the protein. In this way, the disease can be prevented and cured[10]. Recently, a new tendency is that DNA encoded antigen is the variation or xenogeneic homologue of target antigen, with the purpose of breaking through immunotolerance [11~13]. The encoded expression protein provokes the antitumor immune response of body and autoimmune response. This reaction can produce long-lasting and strong tumor rejection. Weber, et al. [12] found if a mouse is vaccinated with DNA plasmid (hgp75) constructed with human melanoma cellular membrane glycoprotein *gp75* gene, the metastasis of lung will decrease by 86%. The mouse plasmid(mgp75) and DNA plasmid of uncoded antigen gene have a similar effect, suggesting that DNA plasmid of homologous variation gene can inhibit the metastasis of tumor.

In this study, eukaryotic expression plasmid of porcine *CCK* gene pIRES2-EGFP/CCK was constructed and transfected into mammal cells COS-7. The expression of green fluorescent protein could be detected in COS-7 cells 24, 48, and 72 hours after the transfection, suggesting that the recombinant plasmid could be expressed transiently in vitro. After the recombinant plasmid was injected directly into the skeletal muscle of hamsters, the green fluorescent protein could be detected at the injection sites on the 4 th day; the fluorescence intensity became stronger on the 14 th day; the level of fluorescence became ever stronger than before on the 42nd day. The result is identical to the records of Bartlett, et al. [14] and Davis, et al. [15], demonstrating that the recombinant plasmid pIRES2-EGFP/CCK can have a long-term expression in vivo. In the further experiment, the aimed segments will be inserted into the eukaryotic expression plasmid pcDNA3. 1, which has the same promoter human cytomegalovirus (CMV) with pIRES2-EGFP, to construct nucleic acid vaccine pcDNA3. 1/CCK. And then, nucleic acid vaccine is used to vaccinate the models of hamster pancreatic carcinoma. It is anticipated to produce lasting specific humor immunity by the means of a cross immune reaction. Specific antibody may prevent the combination of CCK with its receptor, and further inhibit the progression of pancreatic carcinoma.

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