Identification of a novel gene K23 over-expressed in fish cross-subfamily cloned embryos

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Abstract A novel gene—K23, differentially expressed in cross-subfamily cloned embryos, was isolated by RACE-PCR technique. It had 2580 base pairs (bp) in length, with a 1,425 bp open reading frame (ORF) encoding a putative protein of 474 amino acids (aa). Bioinformatic analysis indicated that K23 had 22 phosphorylation sites, but it had no signal peptides. Developmental expression analysis in zebrafish showed that K23 transcripts were maternally expressed in ovum and the amount of K23 transcripts increased gradually from zygote to pharyngula period. Subcellular localization analysis revealed that K23 protein was homogeneously distributed both in nuclei and cytoplasm. Taken together, our findings indicate that K23 gene is a novel gene differentially expressed in fish cross-subfamily cloned embryos.

Keywords Nuclear reprogramming · Nuclear transfer · Rare minnow (Gobiocypris rarus) · Zebrafish (Danio rerio)

Introduction

To retain the genetic viability of a certain species near extinction, cross-species nuclear transfer (NT) becomes a potent approach. Utilization of oocytes for recipient cytoplasts from other species that are accessible and abundant is an exciting possibility for endangered species with limited availability of oocytes [1]. However, most embryos produced by cross-species NT are unable to develop to later stages because reproductive NT required many basic biological necessities, such as the compatibility of nuclear-mitochondrial interaction and the reversal of the differentiated state of the transferred nucleus [2, 3]. Efficient nuclear reprogramming needs appropriate gene expression during embryonic development. Thus, differential gene analysis in cross-species cloned embryos can give hint for unveiling the regulatory mechanisms of the feasibility of rescuing genetically endangered animal species.

In the previous study, we have chosen two laboratory fish species, rare minnow (Gobiocypris rarus) and zebrafish (Danio rerio), as a model to study the cross-subfamily NT [4, 5]. By using a suppression subtractive hybridization (SSH) approach, we have totally screened out 50 differentially expressed genes in the cloned embryos at sphere stage [6]. After dot blotting confirmation, real-time RT-PCR analysis showed that a novel gene—K23 was over-expressed in the cloned embryos more 15-folds than that in normally fertilized zebrafish embryos [6]. In this paper, we report the identification of K23 gene and investigate its expression profiles.

Materials and methods

RACE-PCR amplification

The full-length cDNA of K23 gene was isolated by RACE-PCR technique. Using the SMART cDNA as template [6], the combination of universal primer SMART F and K23 R or universal primer SMART R and K23 F was used for 5' or 3' RACE PCR, respectively (Table 1). The generated
PCR products were sequenced, and the full-length cDNA of K23 was composed by both the coding sequence (CDS) and the 5' and 3'UTRs.

Bio-information analyses

Homology search for K23 gene was performed on the sequences listed in EMBL/GenBank/DDBJ databases using EST-BLAST and Protein–protein BLAST (blastp) at the web site of the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Secondary structure analysis of K23 protein sequence was performed by DNAstar software (Lasergene, Madison, Wis.). Phosphorylation sites and signal peptide were predicted by NetPhos 2.0 software (http://www.cbs.dtu.dk/services/NetPhos/) andSignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP/), respectively.

Semi-quantitative RT-PCR assay

Zebrafish embryos were generated by in vitro fertilization and raised as previously described [6]. Embryos were staged according to Kimmel et al. [7]. Total RNA of eight samples (ovum, zygote, 256-cell stage, sphere stage, 50%-epiboly stage, 90%-epiboly stage, 15-somite stage, pharyngula period) was separately isolated using SV™ total RNA kit (Promega). Then, 3 μg total RNA was reverse transcribed (RT) for each sample and 2 μl of the RT product was amplified to analyze the expression patterns of K23 by RT-PCR assay. Primers used for amplifying the cDNA fragments of K23 were K23 F and K23 R (Table 1). As an internal standard, GAPDH F and GAPDH R primers were used to amplify the constitutively expressed gene-GAPDH (Table 1). In order to ensure the reactions remained in log-linear range, 20 PCR cycles were used.

Whole-mount in situ hybridization (WISH) analysis

For WISH, embryos were fixed in MEMPFA (100 mM Mops (Sigma), pH 7.4; 2 mM EGTA (Sigma); 1 mM MgSO4 (Merck); 4% (w/v) paraformaldehyde (Sigma) at different developmental stages. The methods for generation of full-length K23 antisense probes and WISH analysis followed the protocol described by Pei et al. [8]. Images of zebrafish embryos were recorded using an Olympus SZX12 microscope and a digital camera.

Cell culture and sub-cellular localization of K23

Epithelioma papulosum cyprinid (EPC) cells from carp (Cyprinus carpio) were cultured as described by Zhou et al. [9]. For fluorescence microscopy, the coding region of K23 gene was amplified using K23DW primers (Table 1) and cloned into pEGFP-N3 (BD Biosciences) using PstI and BamHI sites. After sequencing validation, the pK23-EGFP and pEGFP-N3 (as negative control) were transfected into EPC cells using Lipofectamine 2000 reagent (Invitrogen), respectively. At 24 h after transfection, cells were removed by trypsin/EDTA and cell nuclei were stained with 5 mg/l Hochest 33342 (Calbiochem). Sub-cellular localization of K23 was judged by the co-localization of GFP protein.

Results

Cloning and characterization of K23 gene

Full-length cDNA of K23 gene was obtained from a SMART cDNA library. It is 2,580 bp in length with an open reading frame (ORF) of 1,425 bp encoding a putative protein of 474 aa, a 5' untranslated region (UTR) of 309 bp, and a 3' UTR of 846 bp. It contains an mRNA instable motif (ATTTA) and a poly(A) signal (AATAAA) followed by a poly(A) tail (Fig. 1).

Homology search of public database found no homologous known gene in other species. The secondary structure analysis by DNAstar software shows that K23 protein has much more β-folds and antigenic regions than α-helices and hydrophilicity regions (Fig. 2a). Predicted by SignalP 3.0 software, K23 protein has no signal peptide. Interestingly, phosphorylation sites analysis by NetPhos 2.0 Server

<table>
<thead>
<tr>
<th>Table 1 Primers used in the present study</th>
<th>Names</th>
<th>Sequences</th>
<th>Length (bp)</th>
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<tr>
<td>SMART F</td>
<td>5-CAACGCAGAGTACGCAGG-3</td>
<td>18</td>
<td></td>
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<tr>
<td>SMART R</td>
<td>5-TCAACGCAGAGTACT(16)-3</td>
<td>30</td>
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<tr>
<td>K23 F</td>
<td>5-AAACAGTGGGATGGGTAAGA-3</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>K23 R</td>
<td>5-CGTTGCAAAGGGACAA-3</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>K23DW F</td>
<td>5-AACTGCAAGTACGCGCATGGAACCTTACAAC-3</td>
<td>29</td>
<td></td>
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<tr>
<td>K23DW R</td>
<td>5-CGGGATCCCTTCAGTCTTTGTAAAGGCGG-3</td>
<td>28</td>
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<tr>
<td>GAPDH F</td>
<td>5-GTGTAGGCGTGGACATGTTGTG-3</td>
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<td></td>
</tr>
<tr>
<td>GAPDH R</td>
<td>5-TGGGAGTCAACCAGGACAAATA-3</td>
<td>22</td>
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</tbody>
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shows that there are 22 phosphorylation sites (12 Ser, 6 Thr and 4 Tyr) (Fig. 2b).

Expression profile of K23 during development

To figure out the expression pattern of K23 gene during embryonic development, semi-quantitative RT-PCR was performed. As shown in Fig. 3a, K23 transcripts were found in all the checked developmental stages and it was expressed maternally in the oocytes. Interestingly, K23 transcripts increased gradually from zygote to pharyngula period (24 hpf) and reached a maximum level at pharyngula period (24 hpf) during embryogenesis in zebrafish. WISH was performed to obtain the temporal-spatial expression pattern of K23 gene and the results showed that K23 expression was detected in all stages (Fig. 3b), which was agreement with semi-quantitative RT-PCR results.
Sub-cellular localization of K23 gene

To study the sub-cellular localization of K23 gene, we constructed pK23-EGFP plasmid with K23 coding sequence fused to EGFP gene. As shown in Fig. 3c, blue signals represented the cell nuclei stained by Hochest 33342, while green signals represented the expression of pEGFP-N3 or pEGFP-K23 fluorescence proteins in EPC cells, respectively. Overlapping of the images of pEGFP-N3 or pEGFP-K23 fluorescent protein with the images of cell nuclei stained by Hochest 33342, the results showed that K23-EGFP fusion protein located both in cytoplasm and nucleus.

Discussion

Cross-species NT in fish has been successfully manipulated for over 40 years [10]. Recently, cross-genus cloned fish derived from common carp nuclei and goldfish enucleated eggs was given birth [11]. The result showed that the somitogenesis progress and vertebral numbers of the cloned fish were consistent to the egg-providing species, but not to the donor cell species. It suggested that the maternal factors deposited in matured eggs contributed largely to early pattern formation and had a long-lasting effect on the development of vertebrates. Interestingly, another study showed that the maternal nucleolus was essential for early embryonic development and nucleoplasmic components were essential for the successful development of zygote-somatic cell NT embryos [12]. In the previous study, K23 transcripts were expressed maternally in ovum and over-expressed (about 15-fold) in cross-subfamily cloned embryos. Sub-cellular localization of K23 protein indicated that K23 protein could penetrate the nuclei, and might be participate in signal transduction by its twenty-two putative phosphorylation sites. Moreover, major breakthroughs were reported whereby expression of four transcription factors Oct4/Sox2/c-myc/Klf4 or OCT4/SOX2/NANOG/LIN28 could reprogram mouse or human somatic cells into induced pluripotent stem (iPS) cells [13–18]. Those transcription factors induced the genome of differentiated cells to be reprogrammed into an embryonic state. Therefore, whether K23 may function as transcription factor deserves us for further study.

To date, the molecular mechanisms of nuclear reprogramming remain elusive. Gene expression analysis of individual embryos will undoubtedly yield new insights into the regulatory mechanisms involved in cross-species NT in a direct way [8]. In our previous study, we have totally screened out 50 differentially expressed genes in the cloned embryos at sphere stage by using a SSH approach [6]. Among them, about 10% are related to redox
Fig. 3 Expression pattern analysis and sub-cellular localization of K23 gene.

(a) Reverse transcriptase-PCR analysis of K23 transcription during development. 1-ovum; 2-zygote; 3-256-cell stage (2.5 hpf); 4-sphere stage (3.8 hpf); 5-50%-epiboly stage (5.25 hpf); 6-90%-epiboly stage (9 hpf); 7-15-somite stage (16.5 hpf); 8-pharyngula period (24 hpf). GAPDH was used as endogenous reference.

(b) Expression of K23 transcripts as detected by WISH during embryogenesis in zebrafish. (A) high stage (3.3hpf, hours post-fertilization), (B)dome stage (4.3hpf), (C) 50%-epiboly stage (5.25hpf), (D) bud stage (10hpf), (E) 4-somite stage (11.3hpf), (F) 8-somite stage (13hpf), (G) 17-somite stage (17.5hpf), (H) 25-somite stage (17.5hpf), (I) 2 dpf (day post-fertilization). Embryos in (A)–(G) and (I) are lateral views, (A)–(C) with the animal pole to the top; embryo in (H) is dorsal view, with anterior to the top. Bar 200 μm is for (A)–(H); bar 600 μm is for (I).

(c) Sub-cellular localization of pEGFP-K23 expressed in EPC cells. The sub-cellular localization of control (pEGFP-N3) and pEGFP-K23 expressed GFP signals in EPC cells was in the upper and lower rows, respectively. All three panels had the same view field at 24 h after transfection.
function, such as selenoprotein W1, 5-lipoxygenase and glutaryl-coenzyme dehydrogenase etc; about 6% are responsible for cell growth and division, including geminin, daz-like gene and cofactor of BRCA2, etc. In addition, nuclear-mitochondrial compatibility played an important role for the success of cross-species NT, which was agreement with recent review [2]. Other studies indicated that epigenetic modification such as DNA methylation and histone deacetylation might contribute to cross-species nuclear reprogramming [19–21]. Remodeling of donor cell centrosomes and the centrosome-associated cytoskeleton was also found to be crucially important for nuclear cloning [22]. Currently nuclear reprogramming in cross-species cell constructs was frequently associated with inappropriate gene expression during embryonic development [23, 24]. Although we found no solid evidences that over-expression of K23 gene obviously affected the nuclear reprogramming in such cross-subfamily cloned embryos, further studies on other differentially expressed genes will give more data in a comprehensive way.

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References