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Sequence analysis, expression patterns and transcriptional regulation of mouse *Ifrg15* during preimplantation embryonic development

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ABSTRACT

Ifrg15 is a newly identified interferon alpha responsive gene and is implicated in a wide variety of physiological roles in mammals. In the present study, multiple alignments of the deduced amino acids of 10 eutherian mammalian IFRG15/Ifrg15s isolated from open genomic database revealed that they were highly conserved. Real-time PCR showed that mouse *Ifrg15* mRNA was expressed in MII stage oocytes and preimplantation embryos, and its highest value peaked at the stage of mouse blastocysts. To understand the effect of three development-related genes on the promoter activity of mouse *Ifrg15*, promoter analysis using luciferase assays in COS-7 cells were performed. The results showed that the transcription of mouse *Ifrg15* was suppressed by Oct4 and Nanog when transfected with the longest *Ifrg15* promoter reporter gene. After the relatively shorter promoters were co-transfected with Oct4, c-Myc and Nanog, the relative luciferase activities of *Ifrg15* were gradually increased. These *in vitro* results data and expression profiles of *Ifrg15* as revealed by real-time PCR partly indicated that *Ifrg15* transcription might be either potentially regulated or dependent on the post-transcriptional effects of IFN-α mediated by the three genes indirectly. Our data suggested that the mouse Ifrg15 might interact with these key development-related genes and play significant roles on the mouse preimplantation embryos development, especially for the development of mouse blastocysts.

1. Introduction

Interferons (IFNs), belonging to the class of glycoproteins and also known as cytokines, are proteins made and released by host cells in response to the presence of pathogens (van Baarsen et al., 2010). There are 3 major types of IFNs that have been found in human and other mammals, classified as types I, II and III (de Weerd et al., 2007). Functions for IFNs have been found in multiple biological processes including activating immune cells, increasing recognition of infection or tumor cells and the ability of uninfected host cells to resist new infection by virus (Takaoka and Yanai, 2006). Previous studies revealed that IFNs are secreted by the embryonic trophoblast in early pregnancy in several mammalian species (Spencer and Bazer, 2002), indicating that IFNs play a very important role in early embryo development for implantation (Qi et al., 2007). There are three forms of type I IFN, named IFN-α, IFN-β and IFN-ω, in mammals (Liu, 2005). Through binding to specific cell surface receptor complex known as the IFN-α receptor (IFNAR) to activate the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signal pathway (Makawa et al., 2002), type I IFN-α mediates the antiviral and immunomodulatory activities (Takaoka and Yanai, 2006). In mouse, there are two types of newly identified interferon alpha responsive genes, *Ifrg15* and *28*. The isolated complete ORFs of mouse *Ifrg 15* and *28* are 131 and 249 amino acids with molecular masses of 15.3 and 28.4 kDa, respectively. In mouse, *Ifrg15* was detected in the E12.0 mandible by cDNA subtraction assay (Yamaza et al., 2001), and the expression of *Ifrg15* mRNA was coincidently observed in various craniofacial organs as well as in the tooth germ, suggesting that Ifrg15 is closely related to odontogenesis (Akhter et al., 2010). In our previous work, rabbit *Ifrg15* was found to be expressed in the oocytes and preimplantation embryos. Furthermore, the signal of *Ifrg15* was clearly observed in the inner-cell mass of the rabbit blastula by whole-mount *in situ* hybridization (Qi et al., 2007). These results implied the involvement of Ifrg15 in mammalian embryonic and late stage development. Moreover, *Ifrg15* was also cloned from mouse

Abbreviation: IFN, Interferon; IFNAR, IFN-α receptor; Ifrg, Interferon alpha responsive gene; JAK-STAT, Janus kinase-signal transducer and activator of transcription; E12.0, embryonic day 12.0; PCR, polymerase chain reaction; NCBI, National Center for Biotechnology Information; Ct, threshold cycle; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; SE, Standard Error; ANOVA, Analysis of Variance; SD, standard deviation; ORF, open reading frame; aa, amino acid; S, serine; N, asparagines; Ifrg 15-P, Ifrg 15 promoter.

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genomic DNA and expressed in prokaryotic expression system (Ding et al., 2010).

It is well known that many development-related genes, such as octamer-binding transcription factor 4 (Oct4), Nanog and c-Myc, play important roles during mouse preimplantation development in a temporal and spatial manner (Hamatani et al., 2004; Kageyama et al., 2007). The expression of mouse *Oct4* was detected in *in vivo* and *in vitro*-developed embryos from zygote to blastocyst stages by real-time polymerase chain reaction (PCR) and no *Nanog* mRNA expression was detected in the pronuclear to 4-cell stage embryos (Li et al., 2005), while c-Myc signal was detected in mouse oocytes as well as in preimplantation embryos by immunocytochemistry (Suzuki et al., 2009). These results indicate that the expression of these development-related genes mentioned above, as well as *Ifrg15*, correlated with the potential of preimplantation development. Although there are few reports in mammals till now, the existence of mouse *Ifrg15* counterparts in other vertebrates, the expression patterns and potential roles of *Ifrg15* in mouse preimplantation embryo and its transcription regulation manner are still unknown. Therefore, the present study intends to 1) explore whether there are *Ifrg15*-like sequences of in other non-mammalian vertebrates in the open genome database; 2) examine the expression pattern of mouse *Ifrg15* in mouse preimplantation embryo by real-time PCR; 3) examine whether the development-related genes could regulate mouse *Ifrg15* expression to form a pathway for the early stage of mammalian embryonic development.

2. Materials and methods

2.1. Animals

Kunming white mice were purchased from the Experiment Animal Center of Anhui Medical University and maintained in a 14-h light and 10-h dark photoperiod at 20–25 °C for at least 2 weeks before use. All animals were maintained in accordance with the Animal Experiment Standard of Fuyang Teachers College.

2.2. Data mining and sequence analysis of vertebrate Ifrg15s

Alignment of vertebrate IFRG15/Ifrg15s was performed by the progressive, neighborhood-joining alignment method, ClustalX. To determine whether there are Ifrg15-like sequences in other vertebrates, genomic contigs corresponding to the mammalian IFRG15/Ifrg15s genes were identified by searching in the NCBI database at: http://www.ncbi.nlm.nih.gov/ and other genome databases. The multiple sequence alignments are presented in Boxshade 3.2. For the construction of phylogenetic trees, Human, sumatran orangutan, monkey, mouse, pig, rabbit, cat, cattle, dog, horse IFRG15/Ifrg15s were included. An unrooted tree, using human IFRG28 (AJ251832) as an outgroup, was constructed from the Phylip distance matrix output of the alignment in ClustalX and presented by treeview 3.2. The values on the tree represent bootstrap scores of 1000 trials, indicating the credibility of each branch. All IFRG15/Ifrg15 protein sequences were obtained from the open genome database. The accession numbers of these IFRG15/Ifrg15 sequences are as follows: Human (NP_071742), Sumatran orangutan (NP_001125531), monkey (XP_001115205), mouse (XP_002726240), rabbit (NP_001075601), cattle (NP_001099117), pig (ENSSSCP00000016469), cat (ENSFCAP00000001014), dog (ENS CAFP00000018633), horse (ENSECAP00000001232).

2.3. Collection and culture of oocytes and embryos

Collection and culture of oocytes and embryos were performed according to the methods described previously (Wu et al., 2012). MII stage oocytes, zygotes, 2-cell, 4-cell, 8- to 16-cell embryos, morulae, and blastocysts were collected for study as described previously (Wu et al., 2012).

2.4. Real-time PCR

Five zygotes or embryos were used for each time point and 3 samples (replicates) were collected from each stage. Total RNA was extracted from all the samples and cDNAs were synthesized according to the protocols of RNeasy Plus Micro Kit and Sensiscript RT Kit (QIAGEN, Germany), respectively. Real-time PCR was carried out according to the manufacturer's instructions of FastStart Universal SYBR Green Master (Roche, Sciences, Maryland, USA). *Gapdh* was used as an internal control. The threshold cycle (Ct) was defined as the fractional cycle number by the method of global minimum. The ratio change in the *Ifrg15* gene relative to *Gapdh* control gene was determined by the $2^{-\triangle\triangle Ct}$ method. Data were expressed as the mean ± SE for the 3 replicates. A Kruskal-Wallis test was used to determine significant difference ($P<0.05$) with GraphPad Prism 5 software (GraphPad Software, San Diego, CA). All primer sequences used for the present study are listed in Table 1.

2.5. Plasmid constructs

Different 5′-flanking regions of mouse *Ifrg15* gene (1.6, 1.2, 0.6 and 0.12 kb, ligation into *Mlu* I and *Hin*d III sites) were generated by PCR using primers listed in Table 1, and subcloned into the pGL3-Basic Vector (Promega Corp., Madison, WI). All development-related genes used in luciferase assays, including mouse *Oct4*, *Nanog* and *c-Myc* were amplified and cloned into the pcDNA3.1 expression vector (Invitrogen, Carlsbad, CA) using gene-specific open reading frame (ORF) primers. Plasmids used in transfection experiments were purified using a QIAfilter Plasmid Midi Kit (QIAGEN), and the purity was verified by spectrophotometry and agarose gel electrophoresis. The constructs and orientation of the insert were confirmed by direct sequencing.

2.6. Cell culture, transient transfections and luciferase assay

Cell culture, transient transfections and luciferase assays were performed as reported previously (Li et al., 2012). Briefly, COS-7 cells were transfected using Lipofectamine (Invitrogen) with the following plasmids: 1) 0.5 μg of normal or truncated constructs of the *Ifrg15* promoter cloned into the pGL3-Basic luciferase reporter vector; 2) 0.01–0.02 μg of pcDNA3.1 expression plasmid (Invitrogen), containing the ORF of *Oct4*, *Nanog* and *c-Myc*; and 3) pRL-TK (Promega), at 100 ng/well. *Renilla* luciferase from pRL-TK was employed as an internal control for transfection efficiency. Firefly luciferase and *Renilla* luciferase readings were obtained using the Dual-Luciferase Reporter Assay System (Promega) and Veritas luminometer (Turner Biosystems). Relative luciferase activity was calculated by dividing the firefly luciferase activity with the *Renilla luciferase* activity. Results are presented as the mean ±

Table 1
List of primer sequences used in real-time PCR and promoter analyses.

Primer	Sequence	Purpose
Ifrg15-F	GCCTTGGTCTTTCCCACTTGAG	For real-time PCR
Ifrg15-R	CATCCATTTCTTCTGGGTCCTCC	
Gapdh-F	ATTCAACGGCACAGTCAAGG	
Gapdh-R	GGTCCTCAGTGTAGCCCAAGA	
Ifrg15-PF1	CGACGCGTCCGTAAACACCAAGAGCAT	For promoter assay
Ifrg15-PF2	CGACGCGTCATACTGGTGGTACAATCC	
Ifrg15-PF3	CGACGCGTTGGTGCCAGTCGGCTCTAC	
Ifrg15-PF4	CGACGCGTAGGTGCTCATAACAACTTC	
Ifrg15-PR	CCCAAGCTT CTGACGATAGTGGCAAGCA	
Oct4-F	CGGAATTCGCCACCATGGCTGGACACCTGG	For pcDNA3.1 expression plasmid
Oct4-R	CCGCTCGAGTCAGTTTGAATGCATG	
c-Myc-F	CGGGATCCGCCACCATGAGTGTGGGTCTT	
c-Myc-R	CCGCTCGAGTCATATTTCACCTGG	
Nanog-F	CGGAATTCGCCACCATGCCCCTCAACGTG	
Nanog-R	CCGCTCGAGTTATGCACCAGAGTT	

Bases underlined show the restriction sites.