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Cloning and analysis of IFRG (interferon responsive gene) in rabbit oocytes and preimplantation embryos

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ABSTRACT: Although there is more evidence that shows that IFNs (interferons) plays a very important role in the early development of the embryo, the mechanism of IFNs is still unclear. Our study showed that IFRG is expressed from oocytes- through to the preimplantation embryo in rabbits. This finding provides some clues for better understanding the role of IFNs in the development of the embryo. The full length of rabbit IFRG cDNA (Accession No. AJ584672), with a 2794bp encoding 131 amino acid sequence, was cloned IFRG expression can be detected in 8 different tissues: ovary, heart, lung, liver, kidney, spleen, cerebra, and the 18-day whole-body embryo. Whole-mount *in situ* hybridization showed that IFRG was highly expressed in the inner-cell mass of rabbit blastula. IFRG may play an important role in embryo development and tissue differentiation.

Introduction

IFNs are a family of multifunctional cytokines, which were originally identified because of their ability to confer cellular resistance against viral infection. There is more evidence that indicates that IFNs plays a very important role in early embryo development, especially in implantation. It has been found that, in several mammalian species, IFNs are secreted by the embryonic trophoblast in early pregnancy. In ruminants, interferon-τ (IFN-τ) is an important pregnancy factor and is secreted from the trophoblast during the time of implantation. The major functions of IFN-τ involve maternal and fetal recognition and the establishment of the pregnancy by maintaining the function of corpus luteum Meyer *et al*. (1995) through blocking the release of uterine prostaglandin F_{2} (Thatcher *et al*., 1992). IFN-τ binds to the IFN receptor (IFNR) located at the uterine endometrium (Li and Roberts, 1994). IFNR is thought to be expressed only in the endometrium (Han *et al*., 1997) and not in the conceptuses until at least 15 days of pregnancy. However, a recent report has shown the expression of IFNR at earlier stages in ovine conceptuses (Imakawa *et al*., 2002), which suggests a possible role of IFN-τ via IFNR in an autocrine manner. A recent study has shown that the IFNR subunit is expressed in bovine morula- to blastocyst-stage embryos. These results suggest a novel function for IFNs in promoting embryonic development (Takahashi *et al*., 2003). Interferon-γ (IFNγ) is highly expressed by mammalian trophoblast cells during implantation. Available evidence does not support a role for porcine trophoblast IFN-γ in

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maintaining the function of the corpus luteum (Lefevre *et al*., 1998). However, recent evidence supports a role for porcine trophobalst IFNs in the establishment of pregnancy and implantation (Cencic and Bonnardiere, 2002; Hicks *et al*., 2003). On the other hand, the mRNA expression of the IFN-γ receptor subunits is detected in mouse oocytes and pre-implantation embryos (Truchet *et al*., 2001). In mammalian pre-implantation embryos, the expression of IFN-α gene is observed from oocytes through to the preimplantation embryo in mice (Riego *et al*., 1995). However, the IFN receptor has not been found. A recent study suggests that cross-talk between IFN-γ and α signaling pathways was due to association of IFN-γ receptor (IFNGR2 and IFNGR1) in caveolae (Takaoka *et al*., 2000). Interaction of IFNs with their receptors stimulates the cellular membrane-bound Janus protein kinases (JAKs) Tyk2 and JAK1, which in turn phosphorylate and activate proteins known as signal transducers and activators of transcription (STATs). Activated STAT1 and STAT2 form heterodimers and migrate to the nucleus where they bind to p48, forming the complex ISGF13 that recognizes specific target sequences in the promoters of interferon-α responsive genes (IFRG) (Nicholl *et al*., 2000). The effects of IFNs are mediated by cellular interferon-responsive gene products. This study is the first time that IFRG was observed in rabbit oocytes and preimplantation embryos.

Material and methods

Cloning of full length 3'IFRG cDNA

Our research is based on the previous studies of Li *et al*. (2003). Among the 147 differential displayed fragments, we chose one fragment to further study which is expressed in all the stages of oocytes though to the preimplantation embryo, except for the nuclear transfer (NT) blastocyst embryo. The rabbit cDNA fragment of IFRG was originally isolated as 292bp. A reverse northern blot was undertaken based on our previous report (Li *et al*., 2003). Because IFRG is expressed in oocytes, it is suggested that it may be expressed in ovarian. Total RNA was extracted from the ovarian tissue of a 5-month-old female New Zealand white rabbit, and Northern Blotting was performed. Subsequently, we constructed an ovarian cDNA library with a SMART cDNA Library Construction Kit (Clontech) as protocol described and routine screening were carried out with 3'- IFRG fragment as a probe. using the protocol and routine screening with 3'- IFRG fragment as a probe.

Cloning of Full Length 5'IFRG cDNA

The full length of IFRG was not obtained by screening the ovary cDNA library. The 5' RACE ready cDNA was synthesized according to the user manual of SMART RACE cDNA Amplification Kit, GSP1 (5'-GTGTATGTGGGCCACGGAAGCAGAT-3') and NGSP1 (5'-AGCTGGTGCAGTTCCCCAA AGTAC-3') primer were designed, and 5'-RACE was performed.

RT-PCR Analysis

RT-PCR was conducted to detect IFRG expression in different tissues. We extracted total RNA using an RNeasy Mini Kit (Qiagen) according to the handbook. The total RNA then was reverse transcribed into cDNA. The PCR primer was designed according to ORF (IFRG-1:5'-CCATGTTCTCAGATAATTCGCA-3', and IFRG-2: 5'-TTACTTGCTTTGACCAAGTTTT-3'). PCR was performed as follows: 94ºC∞5 min, followed by 34 cycles of 94ºC∞30s, 57ºC∞30s, 72ºC∞50s, and 72ºC∞7 min.

Whole mount in situ hybridization

RT-PCR products were cloned into pGEM-T vector (Promega). The cDNA-containing plasmids were linearized with Nco I or Sal I, depending on the orientation, to generate either the antisense or sense probe. The resulting fragments were extracted by phenol-chloro-

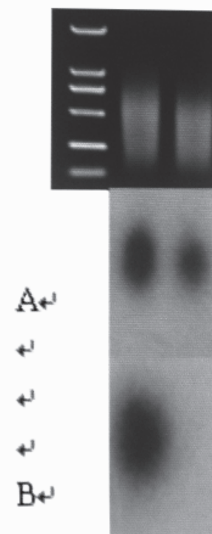

FIGURE 1. Reverse Northern hybridization. A: β-actin probe. B: IFRG probe. M: DL2000 marker. 1: blastula cDNA pool. 2: NT blastula cDNA pool.

form, precipitated by ethanol, and used as plasmid templates for riboprobe synthesis. *In vitro* transcription was performed with 1 mg of plasmid template in a final volume of 20 µl that contained the digoxigenin RNA labeling mix, transcription buffer, 10 mM dithiothreitol, RNase inhibitor (1 unit), and SP6 RNA polymerase or T7 RNA polymerase.The final volume was incubated for 2 hr at 37ºC. The template cDNAs were digested with RNase-free DNase (5 units) for 15 min at 37ºC, and the reaction was stopped by adding 0.2 M EDTA, pH 8.0 (2 µl). The riboprobes were precipitated in ethanol in the presence of 4 M LiCl and quantified with a series of digoxigenin-labeled control RNAs according to the manufacturer's instructions (Roche Diagnostics).

White New Zealand female rabbits were induced to superovulate as described by Cristians *et al*. (1994). The rabbits received five subcutaneous injections of FSH. Twelve hours after the last injection of FSH, the rabbits mated and were injected intravenously with 100 IU hCG. Blasula embryos were collected from the uteruses of the rabbits at 84hr post-coitum. The blastula embryos were fixed overnight in 4% paraformaldehyde, then washed in PBS containing 0.1% Tween-20 (PBST), then dehydrated by an ascending methanol concentration series, and then immediately rehydrated on ice in the reverse order. Rehydrated embryos were washed 3 times with PBST at room temperature. Embryos were permeabilized by 3 incubations in a cocktail of ionic and nonionic detergents (RIPA: 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris, pH 8.0), with each incubation lasting 10 min. Embryos were then postfixed with 4% paraformaldehyde/0.2% electron microscopy grade glutaraldehyde in PBS that was performed at 65ºC. Embryos were washed 5 times in PBST and incubated for 15 min with a 1:1 ratio of hybridization mixture (HB): 50% deionized formamide, 5∞saline-sodium citrate [SSC], pH 7.0, 50 mg/ml heparin, 0.1%Tween 20 and PBST that was followed by a brief wash with HB at room temperature. Embryos were incubated for 1–3 hr in prehybridization mixture (HB) that contained 100 mg/ml tRNA and 100 mg/ml sheared denatured herring sperm DNA. The probes were denatured at 58ºC for 10 min and added to the HB mixture. Hybridization was carried out overnight in a box saturated with 50% formamide/5∞ SSC to prevent evaporation at the temperatures used for each probe. Posthybridization washes included 50% formamide in 2∞ SSCT for 30 min, 2∞ SSCT containing 0.5% SDS (2∞ 15 min), 0.2∞ SSCT containing 1% SDS (2 ∞ 15 min), and 0.1∞ SSCT containing 2% SDS (2 ∞ 20 min). Finally, the DIG color detection was performed using NBT/BCIP (Huamei), and sections were dehydrated, mounted, and photographed.

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ATTGCTACTTGTGCTGTCTTTGTAGGTCCAG
ATG TTC TCA GAT AAT TCG CAT TGC CCT GAT TGC GGA CAG CAG TGG
 M   F   S   D   N   S   H   C   P   D   C   G   Q   Q   W
TTC CCT AGT CTA GAA CTA GGC CAC TGG TTG TAC CAA ACT GAG CTT
 F   P   S   L   E   L   G   H   W   L   Y   Q   T   E   L
GTT GAA AAT GAG TGT TAC CAA GTG TTC TTA GAC CGC ATC AAC AGG
 V   E   N   E   C   Y   Q   V   F   L   D   R   I   N   R
GCT GAT TAT TGC CCC GAG TGT TAC CCT GAT AAT CCT GCT AAT AGA
 A   D   Y   C   P   E   C   Y   P   D   N   P   A   N   R
AGC CTT GTT CTT CCG TGG TCT TTC CCC CTT GAG TGG GCT CCA CAA
 S   L   V   L   P   W   S   F   P   L   E   W   A   P   Q
AAC CTC ACC AGG TGG ACC TTT GAA AAA GCT TGT CAC CCA TTT CTT
 N   L   T   R   W   T   F   E   K   A   C   H   P   F   L
CTG GGT CCT CCG CTG GTT AGG AAA AGG ATC CAT GAC TCC AGA ATT
 L   G   P   P   L   V   R   K   R   I   H   D   S   R   I
GCT GGT TTT AAC CCT GCT TTA CAG TTG ATC TTG ACC AGA ACC GAC
 A   G   F   N   P   A   L   Q   L   I   L   T   R   T   D
AAA ACT TTA AAC AAA AAA CTT GGT CAA AGC AAG TAA
 K   T   L   N   K   K   L   G   Q   S   K
TTTCTGTGACAGTCAAGATCATGGAGACTCTAGAGTCTCCACAAATATG
GAAAAATAACTTTTTTAAATCTGACCCAGGTTCTCAGTAC
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FIGURE 2. Open reading frame of IFRG cDNA (GenBank accession: AJ584672) and its encoding amino acid sequence.

Results

DNA sequence analysis and reverse Northern Blot Differential display was performed as Li described Li *et al*. (2003). Briefly, single oocyte and preimplantation embryos were added to lysis buffer, then reverse transcription was performed used HT(15)C 5'-AAGCTTTTTTTTTTTTTTTC-3' as primer. PCR was performed used HT(15)C as anchor primer and HAP1(5'-AAGCTTGATTGCC-3') as arbitrary primer. The differential displayed fragment recovered from differential display (DD) gel was successfully subcloned and sequenced. Modified reverse Northern Blot was performed to further identify this fragment. IFRG was expressed in the normal blastocyst embryo, but not in the NT blastocyst embryo (Fig.1).

The cDNA sequence of rabbit IFRG and its encoded amino acid sequence

By screening rabbit ovarian cDNA library t 1822bp fragment of IFRG was obtained, which was not the full length cDNA of IFRG, but contains the intact 3'end. Therefore, 5'-RACE was performed. Gene special primer and nested gene special primer were designed using the DNAman software. The 1843bp cDNA fragment was obtained by 5' RACE and which contains the intact 5' end. The full length cDNA of IFRG is 2794bp which encoded 131 amino acids. The cDNA sequence of rabbit IFRG was submitted to GenBank and the accession No. AJ584672 was given. The intact ORF of the gene was shown in Figure 2. The NCBI search indicated that the gene has 98% homology to Homo sapiens and Mus musculus IFRG.

PCR amplification

PCR amplifications were performed according to the procedure in the section of material and method. The products of PCR using IFR-1 and IFR-2 primers for different tissues were shown in Figure 3.

In situ localization of transcripts for IFRG in rabbit blastula embryo

The synthesis of sense and antisense probes is shown in Figure 4. The whole mount *in situ* hybridization is shown in Figure 5. In the inner-cell mass, IFRG is highly expressed. The synthesis of sense and antisense probes (Fig. 4) and the whole mount *in situ* hybridization (Fig. 5) indicated that the IFRG is highly expressed in the inner-cell mass.

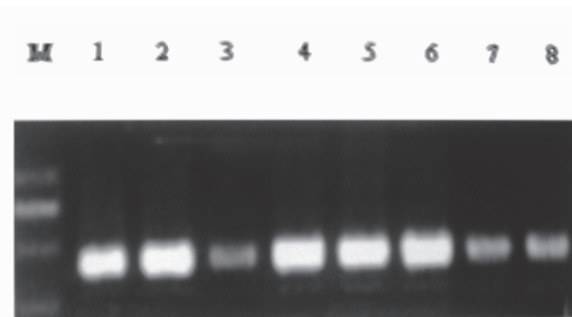

FIGURE 3. RT-PCR of rabbit multi-tissues
M: DNA marker (DL2000); 1: rabbit 18-day embryo; 2: ovary; 3: liver; 4: kidney; 5: spleen; 6: lung; 7: cerebra; 8: heart. Arrow points to the PCR products.

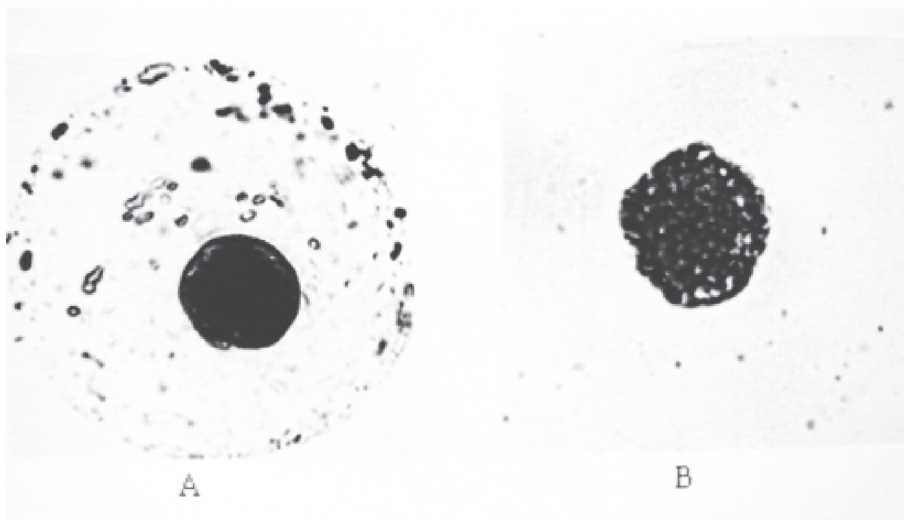

FIGURE 4. The expressions of IFRG in blastula embryo by *in situ* hybridization.
A represents anti-sense results and B is the sense hybridization results.

Discussion

Recent experiments have shown that interferon may play a very important role in early embryo development and implantation, but the physiological role of these IFNs in early embryonic development is not clear. As we know, the effects of IFNs are mediated by cellular interferon-responsive gene products. In this study, we found for the first time that IFRG exists in oocytes and preimplantation embryos. This finding may provided some clops inetter understand the role of IFNs in embryo development.

Blastocyst is a very important stage which is prepared for implantation. Daniels *et al*. (2000) compared the transcription pattern of normal preimplantation bovine embryos and NT- embryos. Six important developmental genes were detected: Oct4, IL6, FGF2, FGF4,FGFr2, and gp130. Three of these, Oct4, FGF2, and gp130, shared a similar transcription pattern in all preimplantation stage embryos. However, a number of morula- and blastocyst- stage embryos that were derived from NT showed abnormal transcription of FGF4,FGFr2, and IL6. Previous evidence shows that these three genes play a very important role in implantation, post- implantation development, or both in mice. In our studies, IFRG is also not expressed in the NT blastocyst stage embryo. This finding may provide some indirect evidence regarding the implantation function of IFNs.

Our results of whole mount *in situ* hybridization indicated that IFRG is highly expressed in inner-cell mass. As we know, inner-cell mass develops into epiblast and hypoblast and finally into embryonic tissue. Multi-tissue RT-PCR also verified that IFRG was expressed. IFRG may play important roles in embryo development and also participate formation of embryo tissue. Further study of the function of IFRG in preimplantation development is necessary for understanding embryonic development and differentiation in mammals.

Acknowledgments

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