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Determination by Real-Time RT-PCR of Imprinted Expression of the Insulin-Like Growth Factor II (*Igf2*) Gene in Mouse Uniparental Fetuses

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Abstract. Uniparental mouse embryos are generally used in the study of imprinting mechanisms as models to determine parental expression of imprinted genes. In this study, we used the real-time RT-PCR method to carry out a quantitative analysis of the expression of the insulin-like growth factor II (*Igf2*) gene, which is imprinted and expressed solely from the paternal allele, in androgenetic and parthenogenetic fetuses at day 9.5 of gestation. The mean expression, relative to that of control biparental fetuses, was detected to be 319% (90 to 585%) in androgenetic fetuses and 5.9% (3.7 to 9.5%) in parthenogenetic fetuses. The present results confirm that parental-specific expression of the *Igf2* gene is maintained in uniparental fetuses, and also show that the real-time RT-PCR procedure is an effective method for quantitative analysis of gene expression using amounts of mRNA that are too small for other methods to detect.

Key words: Imprinting gene, *Igf2*, Uniparental fetus, Real-time RT-PCR

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A parental genome set is essential for normal development in mammals. Mouse uniparental embryos cannot develop to term, but rather die by day 10 of gestation, even if they have diploid maternal or paternal genomes [1–3]. This phenomenon is responsible for epigenetic gene modification, called genomic imprinting, and creates a functional difference between maternal and paternal genomes. Genomic imprinting regulates the parental gene expression of imprinted genes from either paternal or maternal alleles. Once the genomic imprinting is gained during gametogenesis, the parental expression of imprinted genes is maintained for harmonized gene expression throughout embryogenesis [4–8].

To gain further insight into the relation between

the limited development of uniparental embryos and the expression of imprinted genes, it is important to quantitatively identify the gene expression level in individuals. For this quantitative analysis of a gene expression, Northern blotting analysis [9] is generally used. However, as a large amount of mRNA is required for this analysis, this technique cannot be used to examine gene expression levels in individual embryos in the early developmental stages. There is a RT-PCR method [10, 11] which is able to detect gene expression using only a few copies of mRNA, but it lacks quantification. However, a real-time RT-PCR method that can overcome the drawbacks of both methods [12, 13] was recently developed for the quantitative analysis of gene expression. In the present study, we analyzed the expression of the insulin-like growth factor II (*Igf2*) gene [14], a

paternally expressed imprinted gene, in individual androgenetic and parthenogenetic mouse fetuses at day 9.5 of gestation using the real-time RT-PCR method.

Materials and Methods

Production of uniparental embryos

B6CBF1 (C57BL/6N $\times$ CBA) mice were used as oocyte and sperm donors to produce embryos. They were superovulated with injections of 5 IU of equine chorionic gonadotrophin (eCG; Peamex, Sankyo Ltd., Japan) and 5 IU of human chorionic gonadotrophin (hCG; Puberogen, Sankyo Ltd., Japan) given 48 h apart. Oocytes at metaphase II were released from the oviducts 13–16 h after the hCG injection, and the cumulus cells were removed by digestion with 300 units/ml hyaluronidase in an M2 medium [15].

To produce diploid parthenogenetic embryos by artificial activation, oocytes were cultured for 2 h in a drop of Ca^{2+} -free M16 medium [16] containing 10 mM SrCl_2 (Sr^{2+}) [17, 18]. The oocytes were then cultured in an M16 medium supplemented with 0.4% BSA for 4 days in an atmosphere of 5% CO_2 , 5% O_2 , and 90% N_2 at 37 C. During the culture in the Sr^{2+} medium and following the first 4 h of culture in the M16 medium, 5 $\mu\text{g}/\text{ml}$ cytochalasin B was added to induce diploidy by inhibiting second polar body extrusion. Only those embryos that formed two female pronuclei without polar body extrusion were selected for use. Androgenetic embryos were produced by *in vitro* fertilization of enucleated oocytes, according to the procedure previously reported by Kono *et al.* [19]. This procedure prevents misidentification of male versus female pronuclei during the pronuclear transfer procedure used to construct diploid androgenetic embryos from fertilized pronuclear-stage embryos. When required, pronuclear transfer was performed to produce diploid androgenones in mono-spermic embryos. Control biparental embryos were produced by *in vitro* fertilization.

The obtained blastocysts were transferred to the uterine horns of CD-1 females on day 3 of pseudopregnancy (2.5 days post-coitum), and fetuses were recovered at day 9.5 of gestation. For the following procedures, androgenetic fetuses with 15 to 20 somites and parthenogenetic fetuses with 15 to 25 somites in both of which beating

hearts were detected were introduced to the RNA extraction procedure (Fig. 1).

Analysis of gene expression

Total RNA was isolated from each fetus at day 9.5 of gestation using an SV Total RNA Isolation System (Promega Co., Ltd., USA). For semi-quantitative RT-PCR, 10 fetuses were pooled before RNA extraction. After quantitative measurement of extracted total RNA from each embryo by GeneQuant Pro (Pharmacia Biotech, Sweden), fetuses from which more than 1 μg RNA was extracted were subjected to cDNA synthesis. The first strand of cDNA was synthesized from 1 μg of the total RNA from each embryo by SuperScript reverse transcriptase II (Gibco-BRL, Ltd., USA) according to the manufacturer's instructions. To

Fig. 1. Androgenetic (A-1 to A-3), parthenogenetic (P-1 to P-3), and control biparental (C-1 to C-3) fetuses at day 9.5 of gestation. Fetuses such as A-1 and P-1 were not introduced in the RNA extraction procedure.

determine the biological activity of the examined samples, the expression of the beta-actin gene [20] was analyzed first in each strand. For the semi-quantitative analysis, PCR was carried out with cDNA transcribed from 0.1 to 100 ng total RNA. The cDNA was subjected to PCR, which was carried out in a 50- μ l reaction buffer containing 1.25 U of Taq DNA polymerase (Takara Shuzo Co., Ltd., Japan), 1 pmol of each primer, 1.0 mM of $MgCl_2$ for the beta-actin gene and 2.0 mM for the *Igf2* gene, and 0.2 mM of dNTPs. The amplification consisted of a total of 28 cycles at 94 C, 54 C, and 72 C for 60 sec each for the beta-actin gene and a total of 32 cycles at 94 C, 60 C, and 72 C for 60 sec each for the *Igf2* gene in a GeneAmp PCR system (Perkin-Elmer Co., Ltd., USA). Primer sets used were 5'-AGGCACCAAGGTGTGATGGT-3' and 5'-GGTCTCAAACATGATCTGGG-3' for the beta-actin gene and 5'-CATGTCTGGGCCTTTGAA-3' and 5'-TTGGCTCCAGGATGATGT-3' for the *Igf2* gene.

Quantitative analysis of *Igf2* gene expression was performed by means of real-time RT-PCR with the LightCycler™ (Roche Diagnostics Ltd., Germany). By adding a fluorescent reagent to the PCR, the fluorimetric analysis of the PCR products formed was conducted as a real-time measurement during each PCR cycle. Based on the resulting indexes, an initial quantity of analyzing cDNA/RNA was estimated. A hundredth of each cDNA was subjected to PCR, which was carried out in a 20- μ l LightCycler™ FastStart DNA Master SYBER Green I (Roche Diagnostics Ltd., Germany) containing 0.2 pmol of each primer, and 2.0 mM $MgCl_2$. The amplification consisted of a total of 35 cycles at 95 C for 1 sec, at 57 C for 10 sec, and at 72 C for 22 sec. The primer set used was the same as used for semi-quantitative analysis.

Results

To obtain total RNA samples from mouse fetuses at day 9.5 of gestation, androgenetic and parthenogenetic embryos were constructed by standard manipulation procedures. After *in vitro* culture for 4 days, 58.0% (105/181) of androgenetic embryos and 90.0% (416/462) of parthenogenetic embryos developed to the blastocyst stage, 22.9% (24/105) and 6.3% (26/416), respectively, of blastocyst-stage embryos yielded fetuses at day 9.5

of gestation. As these results show, the frequencies of fetus production at day 9.5 of gestation of both types of uniparental embryos (13.3% in androgenones and 5.6% in parthenogenones) were apparently lower than both the frequency of fetus production of control fertilized embryos (70%) and the frequency noted in our previous reports [21, 22]. Of the live fetuses, which were judged by the detection of a heartbeat, fetuses from which more than 1.0 μ g of total RNA was extracted were used for gene expression analysis. The frequencies of such analyzable fetuses were 54.2% (13/24) and 46.2% (12/26) in androgenones and parthenogenones, respectively. The expression of the beta-actin gene was detected in all samples analyzed, indicating that they were all biologically active.

To confirm that the paternal expression of *Igf2* is maintained in uniparental fetuses, we first examined the expression of the *Igf2* gene using pooled samples of androgenetic and parthenogenetic fetuses by semi-quantitative RT-PCR. The expression levels in androgenetic and parthenogenetic fetuses were roughly determined to be 100% and 1% of that in control fetuses, respectively (Fig. 2). For the quantitative analysis, we used the real-time RT-PCR method, which is able to detect the precise expression level in an individual fetus. In setting the standard curve, the regression coefficient was -1.00 and the mean squared error was less than 0.2% (Fig. 3). Each measurement was performed in duplicate, and the coefficient of variation in each sample ranged from 2.3 to 32.6%. The expression level in individual uniparental fetuses was shown as a relative percentage against that of the controls (100 ± 33

Fig. 2. Semi-quantitative analysis of the expressions of beta-actin and *Igf2* genes in androgenetic (A), parthenogenetic (P), and control biparental (C) fetuses by RT-PCR. PCR products amplified from cDNA samples that were transcribed from 100 ng (lanes 1, 5, and 9), 10 ng (lanes 2, 6, and 10), 1 ng (lanes 3, 7, and 11) and 0.1 ng (lanes 4, 8, and 12) of total RNA.

Fig. 3. The standard curve was given by the PCR amplification initiated from standard samples in a dilution series. Crossing points of the standards were used to plot a graph of cycle number of PCR reaction versus log concentration of the standards. Detailed cycle number and percent concentration are also indicated in each standard. The regression coefficient (r) was -1.00 and the mean squared error (error) was 0.168 .

(means $\pm$ SD)%, ranging from 43 to 146%). The quantitative analyses of 12 fetuses per group showed that the expression of the *Igf2* gene was $318 \pm 181\%$ in androgenones, ranging from 90 to 591%, and $5.9 \pm 1.7\%$ in parthenogenones, ranging from 3.7 to 9.5% (Fig. 4). This result confirms that the imprinted expression of *Igf2* from maternal and paternal alleles is maintained in individual uniparental fetuses.

Discussion

In this study, we have shown that the precise level of expression of the *Igf2* gene can be detected in individual embryos in an early stage of implantation using the real-time RT-PCR method [12, 13], and that parental-specific regulation is maintained in mouse uniparental fetuses; that is, the gene is expressed and repressed in both androgenones and parthenogenones.

Uniparental embryos are often used for the study of imprinting mechanisms as models for parental expression of imprinted genes [2, 5, 23–25] since

Fig. 4. Quantitative analysis of insulin-like growth factor II (*Igf2*) gene expression in androgenetic (A), parthenogenetic (P), and control biparental (C) fetuses by real-time RT-PCR. Twelve samples were examined for each of these three groups. The expression level of each fetus is indicated with a dot. The average of the expression levels in the control fetuses was prescribed as a standard (100%). Shaded boxes indicate the average of the expression level in each group (androgenetic fetuses: 319%; parthenogenetic fetuses: 5.9%).

they are constructed solely from maternal or paternal genomes. The quantity of *Igf2* gene expression in androgenetic fetuses is expected to be approximately two times that found in control fertilized fetuses, since the latter have two active alleles. Interestingly, the expression level of the *Igf2* gene in androgenones was detected to be

approximately three times (mean 319%) that of the controls in this study, suggesting that expression of the *Igf2* gene from the alleles is accelerated in androgenones. On the other hand, parthenogenetic fetuses, which consist of two suppressed maternal alleles of the *Igf2* gene, express the *Igf2* gene at 5.9% of the level of control fetuses. This result confirms a previous report by Sasaki *et al.* [26], in which the expression of the *Igf2* gene was detected to be approximately 4% that of the controls using a sensitive RT-PCR protocol to specifically quantify primary transcripts. Here, we have shown that the repressed status in the maternal alleles is strictly maintained in parthenogenones, as the expression in individuals is less than 10% that of the controls, with a variation of within 2.5 times. It remains unclear, however, whether or not this expression in parthenogenones is coincidental with that from maternal alleles in fertilized fetuses, since the expression level has not yet been determined in biparental fetuses. It is well known that the *Igf2* gene regulates fetal development and growth [27, 28]. The molecular mechanism underlying the limited development of uniparental embryos is still unknown, but the repression of the *Igf2* gene may perhaps be related to the small body size of

parthenogenones. This possibility is under consideration in a gene-targeting study in mice, in which the body weight is reduced to 60% that of wild-type mice when the mutation is inherited from the father [27].

The fact that the gene expression level was significant among the individual androgenones compared with those of parthenogenones and controls, although we used only living fetuses with similar body sizes and in the same developmental stage suggests that viability of fetuses, which affects transcription of genes, is significantly reduced in some of the androgenones at 9.5 days post-coitum because at that stage they had already reached the limit of their life [1].

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