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## MOLECULAR CLONING AND mRNA EXPRESSION ANALYSIS OF GDF9 GENE IN NEW ZEALAND WHITE RABBITS

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### ABSTRACT

Growth differentiation factor 9 (GDF9) has been shown to be involved in regulating follicular development and reproduction in many mammalian species. However, the related information about the effect of GDF9 gene on reproductive traits of New Zealand white rabbit is rarely reported. In this study, New Zealand white rabbits were distributed into the poor group (6-8 pups) and the prolific group (8-12 pups). Molecular cloning and expression analyses were employed to characterize the rabbit GDF9 gene. We obtained a total of 2515bp genomic DNA sequence and 1359bp cDNA sequence. Aligning the cDNA sequence of the poor and the prolific, three potential mutation sites (C.539C>T, C.562G>C, C.718C>G) were found in exon 2 of GDF9 gene, which resulted in the corresponding amino acid changing (P.183T>M, P.188E>Q, P.240L>V). By the results of real-time quantitative PCR, GDF9 gene had no tissue-specific, which expressed in all collected tissues (heart, liver, spleen, lung, kidney, uterus and ovary). In general, the expression level of GDF9 gene in ovary was the highest, it increased significantly than other tissues ( $P<0.05$ ), the expression quantity on liver was the next, and heart and spleen were the lowest in New Zealand white rabbits. Comparing the two groups, the expression level of GDF9 gene was very different in liver and uterus ( $P<0.01$ ), and also in heart, spleen and ovary ( $P<0.05$ ). However, it had no significant difference in lung and kidney. The three potential mutation sites of GDF9 gene we found might be a potential genetic marker for improving litter size in rabbits.

**Keywords:** New Zealand white rabbits, GDF9, cloning, RQ-PCR.

### INTRODUCTION

Growth differentiation factor 9 (GDF9) is an oocyte-derived factor which belongs to the TGF- $\beta$  superfamily and exerts positive effects on granulosa cells proliferation in many species, such as mice, rat, human and hen (Juengel et al, 2004; Johnson et al, 2005). Besides, GDF9 gene was first identified as an oocyte-derived growth factor required for ovarian somatic cell function (Dong et al, 1996), and it is a growth factor secreted by oocytes in growing ovarian follicles (Mcpherron et al, 1993). Reproductive traits have a major impact on the ovulation rate and litter size of rabbit. There are some reports showing that GDF9 gene was an obvious candidate gene with a major effect on litter size in sheep (Davis et al, 2005). Some mutations in GDF9 gene have different effects on ovulation rate in each estrus and even they can cause infertility in some cases (Souza et al, 2001), such as, a mutation in the autosomal GDF9 gene that causes increased ovulation rates in heterozygous ewes (Hanrahan et al, 2004). Moreover, some studies have indicated that the ovulation rate and litter size can be genetically regulated by a set of different genes, called fecundity (Fec) genes (Davis et al, 1982). Heterozygous ewe carriers of the FecG<sup>H</sup> (G, GDF9; <sup>H</sup>, high fertility) allele exhibit one to two additional ovulations compared with non-carriers, whereas homozygous mutant ewes are sterile (Hanrahan et al, 2004). Based on the important role of GDF9 gene in reproduction, GDF9 gene was considered as a potential candidate gene for the prolificacy of mammals. The structure, expression and function of GDF9 gene in many mammals are extensively characterized (Johnson et al, 2005; Sendai et al, 2001). However, little is known about the physiological role and reproductive function of GDF9 in rabbits. For this reason, the present study performed a series of experiments to clone GDF9 DNA and cDNA, and analyzed its expression patterns.

## MATERIALS AND METHODS

### Animals and sampling

New Zealand white rabbits were divided into two groups according to their prolificacy records: the prolific group (>8-10) and the poor group (6-8), and from each group were randomly selected 5 female rabbits as samples. Seven tissues including heart, liver, spleen, lung, kidney, uterus and ovaries were collected and stored in liquid nitrogen firstly, then stored at -80°C until RNA and DNA extraction.

### Cloning, sequencing and real-time fluorescent quantitative PCR

GDF9 gene was amplified and three specific primers were synthesized by Shanghai Biological Engineering Co.LTD (China) (Table1). The PCR products were analysed by electrophoresis and then excised and purified from agarose gel using Rapid DNA agarose gel recovery kit (Kangwei, China) and ligated to the vector pMD18-T vector (TaKaRa Co., Ltd., Japan). Transformants using DH5 $\alpha$  (TaKaRa) were subjected to antibiotic selection and isopropyl- $\beta$ -D-thiogalactopyranoside(IPTG)/X-gal blue-white screening. White colonies were cultured and identified by PCR amplification. Positive clones were chosen and sent to Shanghai Biological Engineering Co.LTD for sequencing.

RT-qPCR was used to measure the mRNA expression pattern of GDF9 transcript in different reproductive traits of different tissues (heart, liver, spleen, lung, kidney, uterus and ovary) with GAPDH gene as the reference gene. Reverse transcription was performed with equal quantities of total RNA (1 $\mu$ g). The obtained cDNA was diluted to 1:5 with DEPC (diethyl pyrocarbonate) and used as a template for qRT-PCR. Two specific primers (Table 1) were used to amplify a PCR product of 172 bp for GDF9 and a PCR product of 235 bp for GAPDH. Negative controls without cDNA template were contained in this experiment. Real-time RT-PCR was carried out in a volume of 10 $\mu$ l reaction mixture. The qRT-PCR amplifications were performed in triplicate to ensure the reproducibility of the results.

**Table 1:** Description of primers used in this study

Name	Sequences(5'-3')	Tm(°C)	Length(bp)
E1	F1: TTGAAGGTCATTCTCTGTGTGTCT	60	796
	R1: TTCTAACAGGTTTCCGCTACACACG		
E2	F2: GCACATACTACACGGCACCA	58	1216
	R2: CAGCACTCACTCTGCCAA		
I	F1: TGTGGTTTTGCTGTCTTGCC	58	2280
	R1: GTCCCCCTTGCAGTATCGAG		
Y	F3: ATTCAGGAAGAAACACAAGTGGGCT	62	172
	R3: GGACGCTAGCACAGTTAGGTTCAAG		
GAPDH	F4: ATGGTGAAGGTCGGAGTGAAC	62	235
	R4: CTCGCTCCTGGAAGATGGT		

### Statistical Analysis

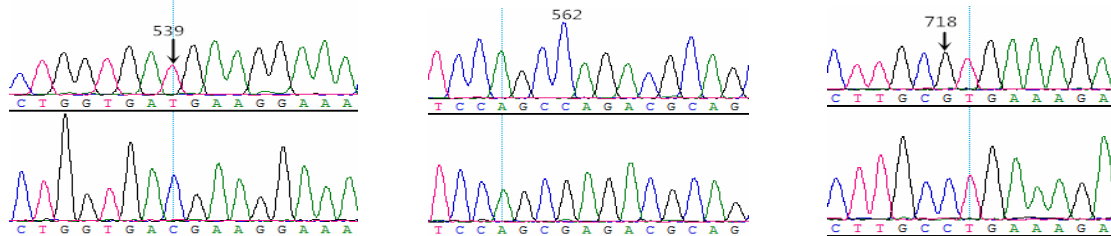
The expression level of different tissues in different reproductive traits were analyzed by the  $2^{-\Delta\Delta CT}$ . The data of qRT-PCR were expressed as the mean $\pm$ SE, analyzed by one-way analysis of variance (ANOVA), and then followed by the Fishers least significance difference (LSD) test. Differences were considered significant when  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Cloning and sequencing of GDF9 gene

Based on the predicted sequence, we amplified the GDF9 DNA and cDNA. Splicing the fragments, we obtained a 2515 bp DNA sequence and a 1359 bp cDNA sequence. Rabbit GDF9 spanned 2515 bp and contained two exons and one intron, in which exon 1, exon 2 and the intron spanned 397, 962 and 1156 bp, respectively. High amino acids identity between the amplified sequence and *Ochotona princeps* (75%) was

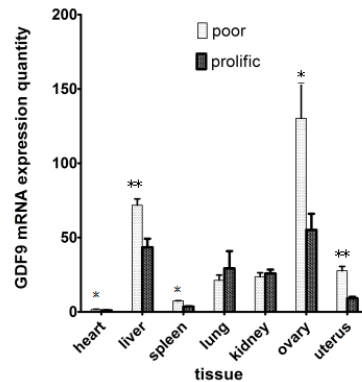
found. The identification to *Ovis aries* (CC187994.1) and human (NP 005251.1) were 68% and 72%, respectively; the identification to those of mouse (AAH52667.1) and rat (CA A57488.1) were both 66%. Aligning the cDNA sequence of the prolific and the poor rabbits, three different bases in exon 2 were found. The different bases were C539T, G562C, C718G in exon 2 (Figure1). And the corresponding amino acid sequence also changed (Thr > Met, Glu > Gln, Leu > Val). GDF9 gene as the candidate genes or main gene of controlling reproductive traits of many species has been reported in the domestic and foreign (Hanrahan et al, 2004; Barzegari et al, 2010; Palmer et al, 2006). In this study, we found three potential mutations sites in exon 2 of GDF9 which resulted in corresponding amino acid changing. We speculated the differences were due to the different reproduction traits, and these mutations might contribute to the greater litter size of rabbit.



**Fig.1:** Sequence comparison of the poor and the prolific in exon 2 of GDF9 gene in New Zealand white rabbits

### Expression analysis of GDF9 gene

GDF9 had no tissue specificity; it was expressed in all of the collected tissues (heart, liver, spleen, lung, kidney, ovary and uterus) of New Zealand white rabbits. Both poor and prolific rabbits had apparently higher expression level of GDF9 gene in ovary and liver than in other tissues. The lowest expression was in heart and spleen (Figure 2). Comparing with the prolific group, the level expression of GDF9 mRNA in the liver and uterus was significantly higher ( $P < 0.01$ ), as well as the expression in the heart, spleen and ovary ( $P < 0.05$ ) in the poor rabbit; but it had no significantly different in lung and kidney between the two groups ( $P > 0.05$ ) (Figure 2).



**Fig.2:** The expression levels of GDF9 gene in New Zealand white rabbit.

Notes: \* means  $P < 0.05$ , \*\* means  $P < 0.01$ .

These results were consistent with other results. The study about bony fish of zebrafish and rainbow trout demonstrated GDF9 gene only existed in gonads (Halm, 2008; Bobe, 2008). Liu et al (2007) found the level expression of GDF9 gene was the highest in carp ovarian and testicular tissue, the liver and muscle were the second and the heart, kidney, spleen were comparatively low with the real-time fluorescent quantitative PCR methods, which is similar to the expression of GDF9 in our study. Besides, the expression pattern of GDF9 gene shows that it not only plays an important role in the reproductive organs of the rabbit, but also has a universally significant role in other organs.

### CONCLUSIONS

Our analysis of the cDNA of GDF9 gene showed that it may have an important effect on different reproductive traits of New Zealand white rabbit. DNA sequence was 2515bp in length and cDNA sequence was 1359bp. Three different bases in exon 2 were found and the qRT-PCR analysis showed that the levels of GDF9

expression were higher in ovary than in other tissues and different between the prolific and the poor prolific rabbits.

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