RESEARCH ARTICLE

Cloning and Expression Analysis of *Zygote Arrest 1* (*Zar1*) in New Zealand White Rabbits

Dan Wang, Shu-Yu Xie, Wei Zhang, Cai-Xia Sun, Tao Huang, An-Si Wang, Xue-Lei Han, Gui-Rong Sun, Ming Li

College of Animal Science and Veterinary Medicine, Henan Agricultural University, No. 63 Agricultural Road, Zhengzhou 450002, China.

Running title:Expression of *Zar1* in Rabbits

Corresponding authors: Gui-Rong Sun, College of Animal Science and Veterinary Medicine, Henan Agricultural University, No. 63 Agricultural Road, Zhengzhou 450002, China

E-mail: 3351475136@qq.com

Ming Li, College of Animal Science and Veterinary Medicine, Henan Agricultural University, No. 63 Agricultural Road, Zhengzhou 450002, China

E-mail: 723414196@qq.com, 990891420@qq.com

Keywords: rabbit, *Zar1*, maternal-effect gene, reproductive trait, clone, gene expression

Abstract: *Zygote Arrest 1* (*Zar1*) is an oocyte-specific maternal-effect gene. Previous studies indicate that *Zar1* plays an important role in early embryo development, but little is known about its function in rabbit. The objectives of this study were to clone the New Zealand white rabbit *Zar1* gene and to investigate its expression in various organs in groups of animals with different reproductive traits. We obtained a 709 bp *Zar1* cDNA fragment consisting of an 8 bp exon 1, 161 bp exon 2, 75 bp exon 3, 271 bp exon 4 and 194 bp 3′ sequences. The rabbit *Zar1* nucleotide sequence showed percent identities of 91%, 88%, 88%, 87%, 86%, 87%, 76%, and 82% with *Zar1* orthologues in human, cattle, sheep, pig, mouse, rat, zebrafish and *Xenopus laevis*, respectively, indicating a high homology with other species and evolutionary conservation. Quantitative real-time polymerase chain reaction analyses revealed non-oocyte-specific *Zar1* expression, with expression in spleen, lung, ovary, uterus, heart, liver and kidney. The expression level was highest in the lung. Further analysis of *Zar1* will be required to expand our understanding of its biological functions.

Introduction

A growing number of oocyte-specific genes are being discovered in many vertebrates. These genes, expressed exclusively in oocytes, play important roles after completion of meiosis and fertilization in the control of the embryonic developmental program until zygotic genome activation (Minami *et al.* 2007). Among them are a number of genes, called maternal-effect genes (MEGs), that have a significant role in the success of early embryo development. Known MEGs

include Zygote arrest 1 (*Zar1*) (Wu *et al.* 2003), DNA methyltransferase-1 (*Dnmt1*) (Howell *et al.* 2001), mater (Minami *et al.* 2007), heat-shock factor-1 (*Hsf1*) (Christians *et al.* 2000), formin-2 (*Fmn2*) (Leader *et al.* 2002), nucleoplasmin 2 (*Npm2*) (Burns *et al.* 2003), stella (Payer *et al.* 2003), basonuclin (Ma *et al.* 2006) and Zfp36-like 2 (*Zfp36l2*) (Ramos *et al.* 2003). *Zar1* was the first oocyte-specific MEG identified to play an essential role during the oocyte-to-embryo transition in human and mouse, as elucidated by knockout experiments in mice (Wu *et al.* 2003)**Error! Bookmark not defined.Error! Bookmark not defined.Error! Bookmark not defined.Error! Bookmark not defined.Error! Bookmark not defined.Error! Bookmark not defined.Error! Bookmark not defined.Error! Bookmark not defined.Error! Bookmark not defined.Error! Bookmark not defined.**.

Recently, many reports have indicated that *Zar1* is involved in reproductive traits. In pigs, six single nucleotide polymorphisms (SNPs) in the *ZAR1* gene were identified that were significantly associated with reproductive traits (litter size) (Jian Gao *et al.* 2007). Furthermore, *ZAR1* is also involved with gene transcription and regulation in a variety of neoplastic diseases. *ZAR1* has been suggested as a potential marker to predict the relationship between hepatitis C virus (HCV) and hepatocellular carcinoma (HCC) (Takagi *et al.* 2013). Furthermore, in *Xenopus*, C-terminal zinc finger domains of ZAR1 and ZAR2 bound to the translational control sequence (TCS) in the 3′-UTRs of *Wee1* and *Mos*, thus suppressing translation in oocytes (Yamamoto *et al.* 2013).

Although many studies have investigated *ZAR1* in various vertebrates and especially in mammalian species such as mice, rats, cattle, sheep and pigs, there is little information available concerning the expression and function of *Zar1* in rabbits. The aim of the present study was therefore to examine the spatial dynamics of *Zar1* expression in rabbits. We cloned a partial *Zar1*

gene sequence and determined its expression in heart, liver, spleen, lung, kidney, uterus and ovary in New Zealand white rabbits to provide a foundation for studying the function of *Zar1* in early embryo development.

Materials and methods

Animals and treatment

New Zealand white rabbits were purchased from Xuchang New Zealand rabbit plant in Yuzhou City, Henan Province, China. The care and management of experimental animals was in concordance with the College of Animal Science and Veterinary Medicine's accepted welfare guidelines.

Experimental groups consisted of high and low reproduction groups according to litter size; animals with a litter size of more than 10 were assigned to the high reproduction group. Five healthy, sexually mature female rabbits of similar weight were assigned to each group. Tissues, including spleen, lung, ovary, uterus, heart, liver and kidney were removed from each rabbit and stored at −80°*C*.

RNA extraction and cDNA synthesis

Total RNA was extracted from tissues using an RNAiso Plus TRIzol kit according to the manufacturer's instructions (TransGen Biotech, Beijing, China). The concentration and purity of

RNA was measured at 260 and 280 nm using a NanoDrop 2000 spectrophotometer and RNA integrity was determined by agarose gel electrophoresis and visualization of the 28S and 18S rRNA. cDNA was synthesised using One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) in a final volume of 20 μl containing 1 μl total RNA, 2×TS 10 μl Reaction Mix, 1 μl TransScript RT/RI Enzyme Mix (2×), 1 μl Anchored Oligo (dT18) Primer (0.5 μg/ μl), 1 μl gDNA Remover, 6 μl RNase Free dH_2O. Reverse transcription was performed at 42°C for 2 min, followed by 37°C for 15 min and 85°C for 5 s. cDNAs were stored at −20°C for later use.

Cloning of the Zar1 gene

Based on predicted *Zar1* mRNA sequences and the whole rabbit genome sequence in NCBI GenBank, primers were designed and synthesized by Shanghai Biological Engineering (Songjiang District, Shanghai, China) and are shown in **Table 1**. *Zar1* cDNA was polymerase chain reaction (PCR) amplified from the reverse-transcription (RT) product template in the following 25 μl reactions: 12.5 μl 2×Taq Master Mix, 1 μl cDNA template, 1 μl forward primer,1 μl reverse primer and 10.5 μl ddH_2O. Amplification conditions were: 94°C for 5 min, 30 cycles of 30 s at 94°C, 30 s at Tm of primer (Table 1), 30s at 72°C, followed by 10 min extension at 72°C.

The PCR products were analysed by agarose gel electrophoresis and then excised and purified using a Gel Extraction Kit (Beijing ComWin Biotech Co.,Ltd.) and then ligated into pMD-18T (Takara). Recombinant plasmids were transformed into competent *E. coli* T10 and transformants subjected to antibiotic selection and isopropyl-beta-D-thiogalactopyranoside/X-gal

blue–white screening. White colonies were cultured and screened by PCR. Positive clones were chosen and sequenced by Shanghai Biological Engineering. DNAMAN was used for sequence alignment and analysis.

Real-time quantitative RT-PCR

Quantitative expression analysis of *Zar1* was performed using the UltraSYBR Mixture (with Rox) following the manufacturer's recommendations（Beijing ComWin Biotech Co.,Ltd.）. Real-time RT-PCR was performed on a Roche Lightcycler 480, in a final reaction volume of 10 µl containing 0.5 µl RT products, 5 µl UltraSYBR Mixture (with Rox), 0.5 µl forward primer (20 pmol/l), 0.5 µl reverse primer (20 pmol/l) and 3.5 µl ddH_2O. The PCR conditions were: 30 s at 95°C, 40 cycles at 95°C for 30 s, 30 s at optimum temperature (61°C), 72°C for 30 s; final elongation at 72°C for 10 min. The housekeeping gene *GAPDH* was used as an internal control. Gene expression in high and low reproduction groups was determined in five independent experiments. For each sample, reactions were repeated in triplicate to ensure the reproducibility of the results. A dissociation curve and the comparative C_T method were used to determine the specificity of the PCR reaction.

Statistical treatment of results

The heart was used as the calibration tissue in the high reproduction group (given a relative expression of 1) and *GAPDH* was used as the reference gene in the *Zar1* gene expression analyses.

Quantification of relative transcript levels was performed using the comparative CT method (Ali-Benali et al. 2005). The Statistical Package for the Social Sciences (SPSS) (version 19.0) was used for all gene expression analyses and all results are expressed as the mean ± SE. Differences among group data were compared using one-way analysis of variance and the Student–Newman–Keuls post hoc test. Differences between groups were compared using the paired-samples T test. Significance was set at $p < 0.05$ and $p < 0.01$ in two-tailed testing.

Results

Cloning of Zar1

The purity, integrity and concentration of total RNA, was determined using 1.2% agarose gel electrophoresis. The results are shown in **supplementary figure 1**: RNA samples showed clear 18S, 28S and 5S bands, which indicated that the extracted total RNA was not degraded. A260 / A280 ratios, measured using a NanoDrop 2000 spectrophotometer, were between 1.8 and 2, indicating that the RNA was free from contamination.

The results of *Zar1* cDNA amplification are shown in **figure 1**. Overlapping cDNA sequences were assembled with DNAMAN to produce a *Zar1* cDNA of 709 bp, including an 8 bp exon 1, 161 bp exon 2, 75 bp exon 3 and 271 bp exon 4 and 194 bp of 3′ sequence. BLAST analyses of the *Zar1* coding sequence indicated that it shared 91%, 88% , 88%, 87%, 86%, 87%, 76%, and 82% nucleotide identity with human (NM_175619.2), cattle (NM_001076203.1), sheep (XM_004010063.1); pig (NM_001129956.1), mouse (NM_174877.3), rat (NM_181385.2),

zebrafish (NM_194381.2) and *Xenopus laevis* (NM_001090489.1), respectively. The predicted amino acid sequence was 100% homologous with the published rabbit sequence (XP_008249752.1), and the identities to human (NP_783318.1), mouse (NP_777366.1), rat (NP_852050.1), Macaque (XP_001103446.1), zebrafish (NP_919362.2) and *X. laevis* (NP_001083958.1) were 84%, 93%, 94%, 84%, 89%, 96%, respectively. High-sequence identity of 97%.was also observed for cattle (NP_001069671.1) and pigs (NP_001123428.1).

MethPrimer software (http://www.urogene.org/methprimer/) was used to predict *Zar1* gene sequences and 5′ upstream (-1000~3907) methylation sites. CpG islands were highly enriched in exon 1 and in the 5′ upstream region (**supplementary figure 2**).

Real-time quantitative RT-PCR analysis of Zar1

To identify functions of *Zar1*, real-time quantitative PCR was performed to assess its expression in different tissues. The results are shown in **figure 2** and **figure 3**.

C_T values of between 14 and 30 (**supplementary figure 3**) indicated that the concentration of the initial cDNA template was appropriate and that reliable amplification was achieved. Dissociation curves for *Zar1* and *GAPDH* (**supplementary figure 4**) showed single product peaks at 84.2°C and 88°C, indicating an absence of non-specific amplified products and primer dimers.

Zar1 mRNA levels in different tissues of the same reproduction groups

As shown in **figure 2**, in the low reproduction group, the expression of *Zar1* in the lungs was

significantly higher than that in the other tissues (P <0.05). *Zar1* expression was not significantly different in the heart, liver, spleen, kidney, ovaries and uterus (P > 0.05). However, the relative expression of *Zar1* in the liver and spleen was higher than heart, kidney, ovaries and uterus, lowest in the heart. In the high reproduction group, the relative expression of *Zar1* in the lungs was also significantly higher than in the other tissues (P <0.05). *Zar1* expression in the spleen and kidneys was also significantly higher than that in the heart, liver, ovaries and uterus (P < 0.05), lowest in the heart. But the level of expression was not significantly different between spleen and kidney (P>0.05).In short, *Zar1* expression were the highest in the lungs, spleen and kidney followed, which the lowest in the heart both in high and low reproduction groups.

3.2.2 *Zar1* mRNA levels in the same tissues of different reproduction groups

The relative *Zar1* mRNA levels in several tissues varied between different reproduction groups, as shown in **figure 3**. *Zar1* expression in the liver and ovaries of the low reproduction group was significantly higher than that of the high reproduction group (P <0.01), but in kidney the opposite expression levels were observed. For the heart and uterus, relative *Zar1* expression was significantly higher in the low reproduction group compared with the high reproduction group (P<0.05). There were significant differences in *Zar1* expression in the spleen and lung between low and high reproduction groups (P> 0.05).

Discussion

Early development is controlled by female and zygotic expression. With the development of the embryo, maternal mRNAs are eliminated and zygotic expression is activated (Tadros *et al.* 2009). It is a kind of evolutionary strategy from the meiosis to zygote genome activated by maternal mRNA (Farley *et al.*2008). MEGs play an important role in maternal zygotic transition and early development in mammals. In recent years, many MEGs have been identified in vertebrates. *ZAR1* was one of only a few MEGs considered to be expressed only in ovaries and oocytes. MEGs are transcribed during oocyte development, but most of the mRNAs are translated after the second meiotic division or fertilization of the ovum (Telford et al. 1990). *Zar1* mRNA expression was detected in the early bovine embryo: *Zar1* mRNA was abundant in oocytes and 1-cell embryos, while abundance was low in 2-cell embryos, and absent in five- to eight-cell embryos (Pennetier *et al.* 2006). However, the mechanism of *Zar1* action was not studied in depth.

This study reports the cloning of the rabbit *Zar1* gene and describes the expression pattern of *Zar1* in different tissues. Rabbit *Zar1* consists of four exons and we cloned a 709 bp *Zar1* cDNA, consisting of exons 2–4 and 3′ sequences. The gene is highly conserved at nucleotide and amino acid levels among different species and predicted CpG islands in the 5′ region probably affect *Zar1* transcription.

Wu *et al.* first discovered *Zar1* expression in mouse oocytes and then detected it in human ovaries and testes (Wu X *et al.* 2003). Limited levels of *Zar1* mRNA were observed in pig oocytes and throughout the first division of the embryo, and levels then decreased from morula to blastula (Uzbekova S. *et al.* 2006). However, in cattle, *Zar1* is expressed in many tissues, including the ovaries, testes, skeletal muscles, heart muscles, kidneys (Brevini *et al.* 2004). Similarly, in chicken, *Zar1* is abundantly expressed in gonadal tissues, such as ovaries, fallopian tube, testis and

epididymis and is also expressed at lower levels in non-gonadal tissues such as kidney, spleen, brain and liver (Michailidis *et al.* 2010). In frogs, *Zar1* mRNA is also detected in gonadal and non-gonadal tissues such as ovaries, lung and muscle, but it is not present in testis (Wu X-M *et al.* 2003). These expression patterns indicate that *Zar1* affects a variety of early embryonic developmental processes of vertebrates, and that the location and timing of expression are different among species. Rabbit *Zar1* is expressed in heart, liver, spleen, lung, kidney, ovaries and uterus and is not, therefore, oocyte-specific.

DNA methylation affects chromatin structure, DNA conformation, DNA stability and modulates interactions between DNA and proteins, so as to control gene expression (Roas *et al.* 2008). In both the high and low reproduction groups of New Zealand white rabbits, *Zar1* mRNA was expressed most highly in the lung, moderately in the spleen and kidney, and lowest in the heart. This could be due to different methylation patterns in the various tissues.

Conclusion

We have cloned a 709 bp *Zar1* cDNA from the New Zealand white rabbit and detected its expression in heart, liver, spleen, lung, kidney, uterus and ovary. In the high and low reproduction group rabbits, *Zar1* expression was the highest in lung. This is a preliminary study of *Zar1* expression in different tissues of the rabbit and its mechanism of action warrants further study.

Conflict of interest statement

The authors declare no conflicts of interest.

References

Ali-Benali M. A., Alary R., Joudrier P. and Gautier M. F. 2005 Comparative expression of five *Lea* Genes during wheat seed development and in response to abiotic stresses by real-time quantitative RT-PCR. *BBA- Gene Struct and Expr.* **1730**, 56–65.

Burns K. H., Viveiros M. M., Ren Y., Wang P., DeMayo F. J., Frail D. E. 2003 Roles of *NPM2* in chromatin and nucleolar organization in oocytes and Embryos. *Science.* **300**,633-636.

Brevini T. A. L., Cillo F., Colleoni S., Lazzari G., Galli C. and Gandolfi F. 2004 Expression pattern of the maternal factor *Zygote arrest1(Zar1)* in bovine tissues, oocytes and embryos. *Mol Reprod Dev.* **69**, 375-380.

Christians E., Davis A. A., Thomas S. D. and Benjamin I. J. 2000 Embryonic development: maternal effect of *Hsf1* on reproductive success. *Nature.* **407**, 693-694.

Farley B.M. and Ryder S. P. 2008 Regulation of maternal mRNAs in early development. *Crit rev biochem mol*. 4**3**, 135 -162.

Gao J., Wang Z. h., Gong Y. C., Yang L. G., Peng X. L., Liu J. 2007 Mutations of Porcine *Zygote Arrest*（*Zar1)*Gene and Its Effects on Litter Siz. *J Agric Biotech.* **15**, 735-740.

Howell C. Y., Bestor T. H., Ding F., Latham K. E.,Mertineit C., Trasler J. M. 2001 Genomic

imprinting disrupted by a maternal effect mutation in the *Dnmt1* gene. *Cell*. **104**, 829-838.

Leader B., Lim H., Carabatsos M. J., Harrington A., Ecsedy J., Pellman D. 2002 *Formin-2*, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes. *Nat Cell Biol.* **4**, 921-928.

Ma J., Zeng F. Y., Schultz R. M., and Tseng H. 2006 *Basonuclin*: a novel mammalian maternal -effect gene. *Development.* **133**，2053-2062.

Minami N., Suzuki T. and Tsukamoto S. 2007 *Zygotic* gene activation and maternal factors in mammals. *J Reprod Develop.* **53**, 707-715.

Michailidis G., Argiriou A. and Avdi M.2010 Expression of chicken zygote arrest 1 (Zar1) and Zar1-like genes during sexual maturation and embryogenesis. *Vet Res Commun.* **34,** 173-184.

Payer B., Saitou M., Barton S. C., Thresher R., Dixon J. P., Zahn D. 2003 Stella is a maternal-effect gene required for normal early development in mice. *Curr Biol.* **13**,2110-2117.

Ramo S. B., Stumpo D. J., Kennington E. A., Phillips R. S., Bock C. B., Ribeiro-Neto F. 2004 The CCCH tandem zinc-finger protein *Zfp36l2* is crucial for female fertility and early embryonic development. *Development.* **131**,4883-4893.

Roa S. J. C.,Garcfa M. P., Melo A .A., Tapia E. O., Villaseca H. M., Araya O. J. C. 2008 Gene methylation patterns in digestive tumors. *Rev Med Chil*. **136** , 451-458.

Telford N. A.,Watson A .J. and Schultz G. A. 1990 Transition from maternal to embryonic control in early mammalian development: a comparison of several species. *Mol Reprod Dev*. **26**, 90-100.

Tadros W. and Lipshitz H. D.2009 The maternal-to-zygotic transition:A play in two acts. *Development*. **136**, 3033-3042.

Takagi K., Fujiwara K., Takayama T., Mamiya T., Soma M. and Nagase H. 2013 DNA hypermethylation of *zygote arrest 1 (Zar1*) in hepatitis C virus positive related hepatocellular carcinoma. *SpringerPlus.* **2**, 150.

Wu X., Viveiros M. M., Eppig J. J., Bai Y., Fitzpatrick S. L. and Matzuk M. M. 2003 *Zygote arrest 1 (Zar1)* is a novel maternal-effect gene critical for the oocyte-to-embryo transition. *Nat Genet.* **33**, 187- 191.

Wu X. M., Wang P., Brown C. A., Zilinski C. A.and Matzuk M. M. 2003 *Zygote Arrest 1 (Zar1*) is an evolutionarily conserved gene expressed in vertebrate ovaries. *Biol Reprod.* **69**, 861–867.

Uzbekova S., Roy-Sabau M., Dalbies-Tran R., Perreau C., Papillier P., Mompart F. 2006 *Zygote arrest 1* gene in pig, cattle and human: evidence of different transcript variants in male and female germ cells. *Reprod Biol Endocrin.* **4**, 1-14.

Yamamoto T. M., Cook J. M., Kotter C. V., Khat T., Silva K. D., Ferreyros M. 2013 *Zar1* represses translation in Xenopus oocytes and binds to the TCS in maternal mRNAs with different characteristics than *Zar2*. *BBA - Gene Regul Mech*. **1829**, 1034 - 1046.

Received 29 December 2015, in revised form 14 April 2016; accepted 20 April 2016

Unedited version published online: 25 April 2016

Table 1. PCR primers for *Zar1* and *Gapdh*

Name	Primer Sequence	Tm (°C)	Length (bp)
exon2-exon4	F1: 5'GCGTGTCTTGTTGGTAACCTGTGGA3' R1: 5'TGCACTGTCTCAGATGATGTACTTG 3'	63	357
exon4 and 3' flanking region	F2:5'CAAGTACATCATCTGAGACAGTGCA 3' R2: 5'TTCCATTTTCCCCACGAGGTTTTTG 3'	60	338
QPCR	F3:5'CAAGGGCGGAGATTATCTGTGTTAG 3' R3:5'ATAGGCACTCTCCCAGCGGATGTTA 3'	61	141
GAPDH	F4:5'ATGGTGAAGGTCGGAGTGAAC3' R4: 5' CTCGCTCCTGGAAGATGGT3'	59	235

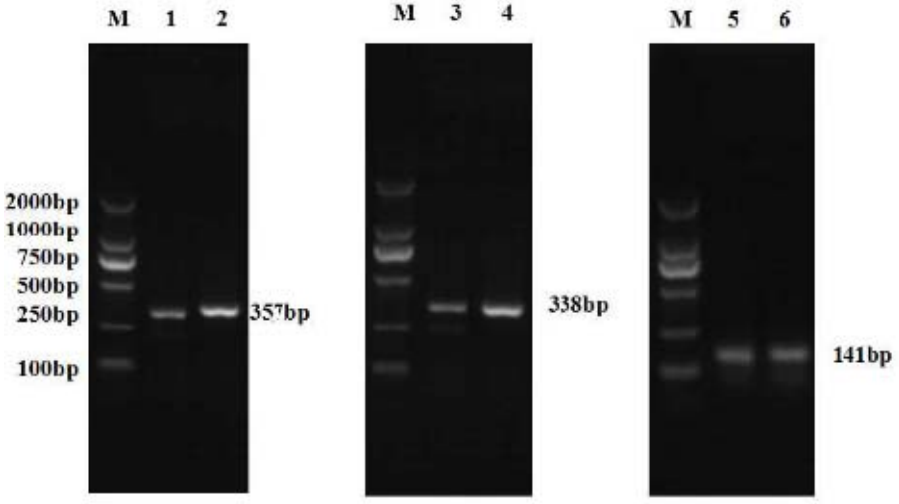


Fig 1. Electrophoresis analysis of ZAR1 RT-PCR product. M. DM2000 DNA Marker；1-2.: 357bp product of ZAR1; 3-4: 338bp product of ZAR1; 5-6: 141bp product of ZAR1.

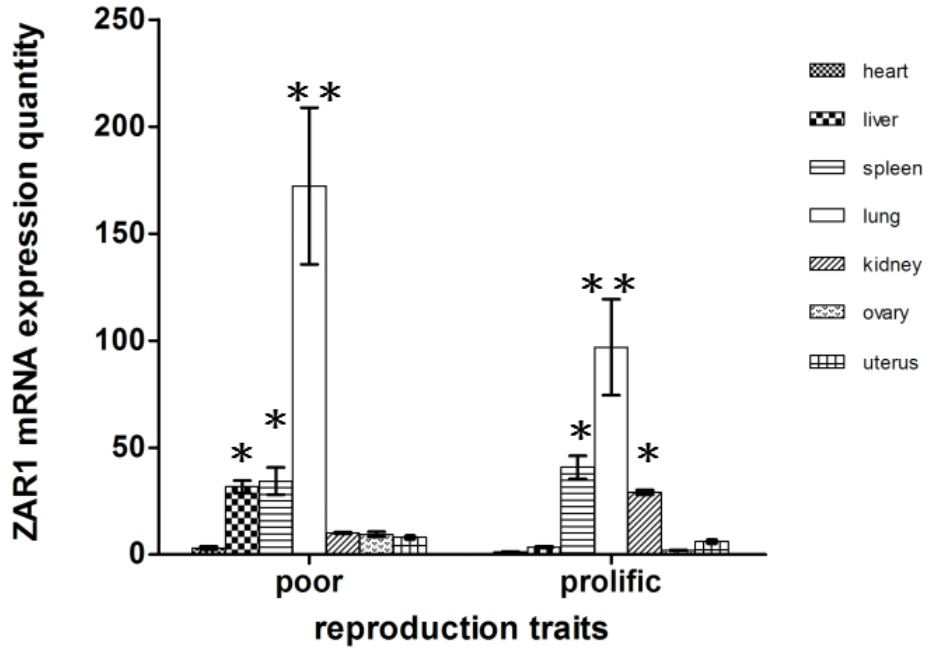


Fig 2. Real-time PCR analysis of Zar1gene in rabbits different tissuesof same reproductive traits. The values are the mean±SE of three independent experients. The significant levels of data are all P <0.05. The different number of "*" are significantly different (p<0.05）; the same number of "*" are not significantly different(p>0.05).

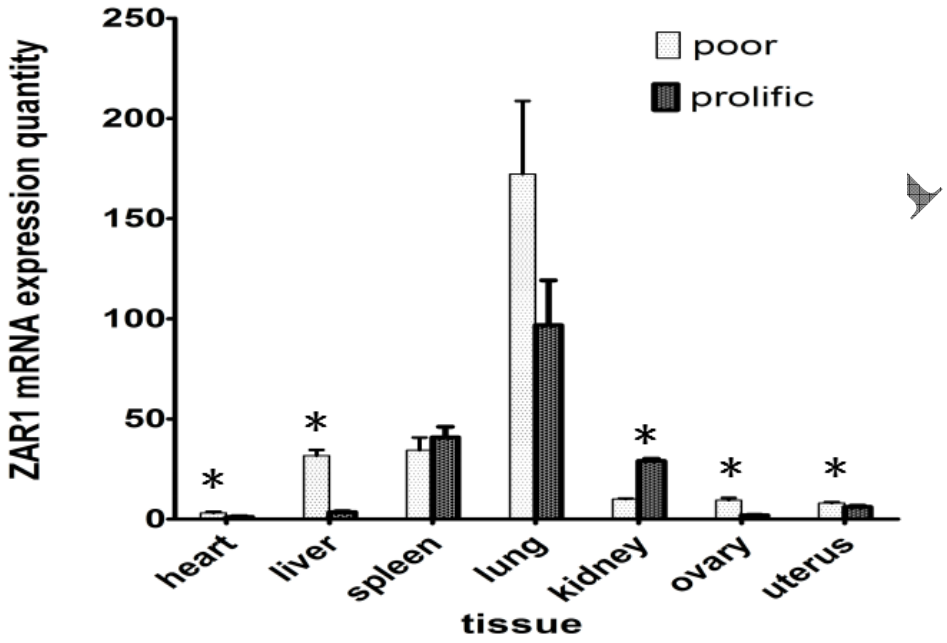
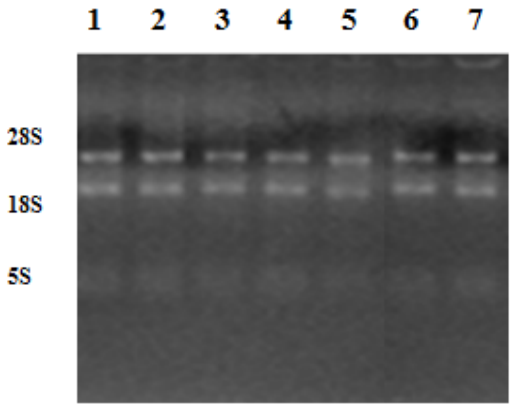


Fig 3. Real-time PCR analysis of Zar1gene in rabbits same tissues of different reproductive traits. The values are the mean±SE of three independent experients. The significant levels of data are all P <0.05. The different number of "*" are significantly different (p<0.05）; the same number of "*" are not significantly different(p>0.05).

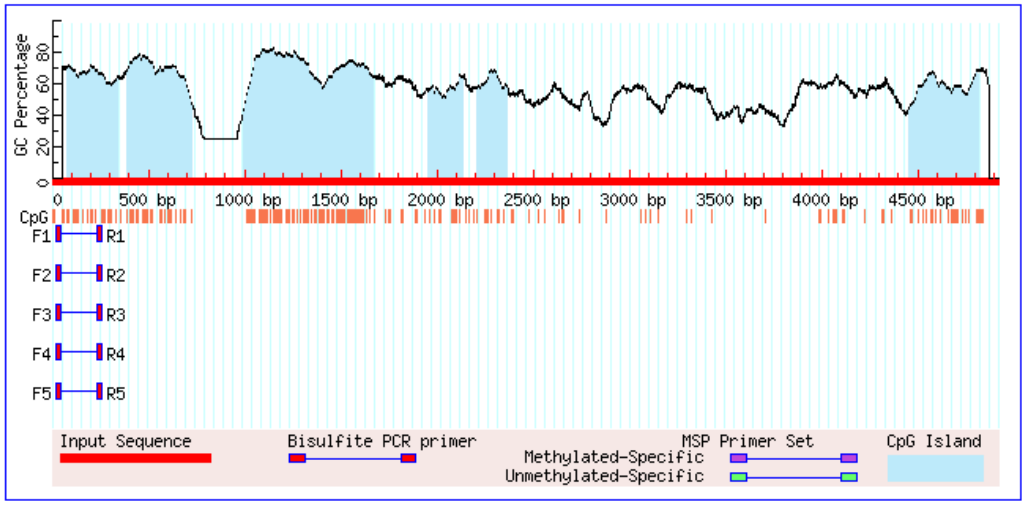


Supplement Fig 1. Examination of total RNA

1 heart; 2 liver; 3 spleen; 4 lung; 5 kidney; 6 ovary; 7 uterus;

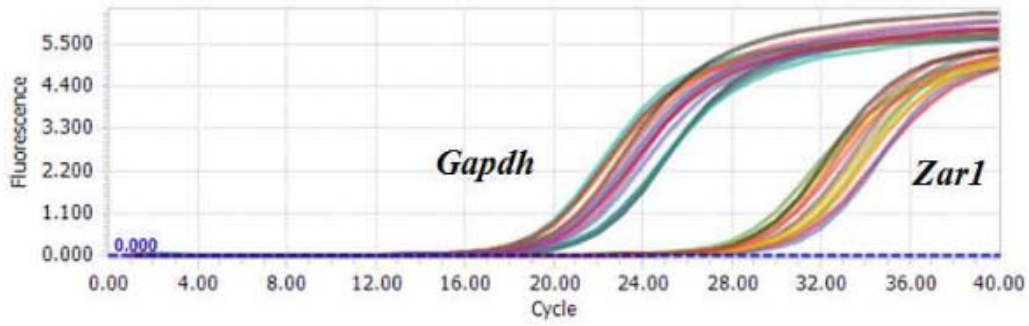
MethPrimer result

Please cite MethPrimer: Li LC and Dahiya R. MethPrimer: designing primers for meth
PMID: 12424112

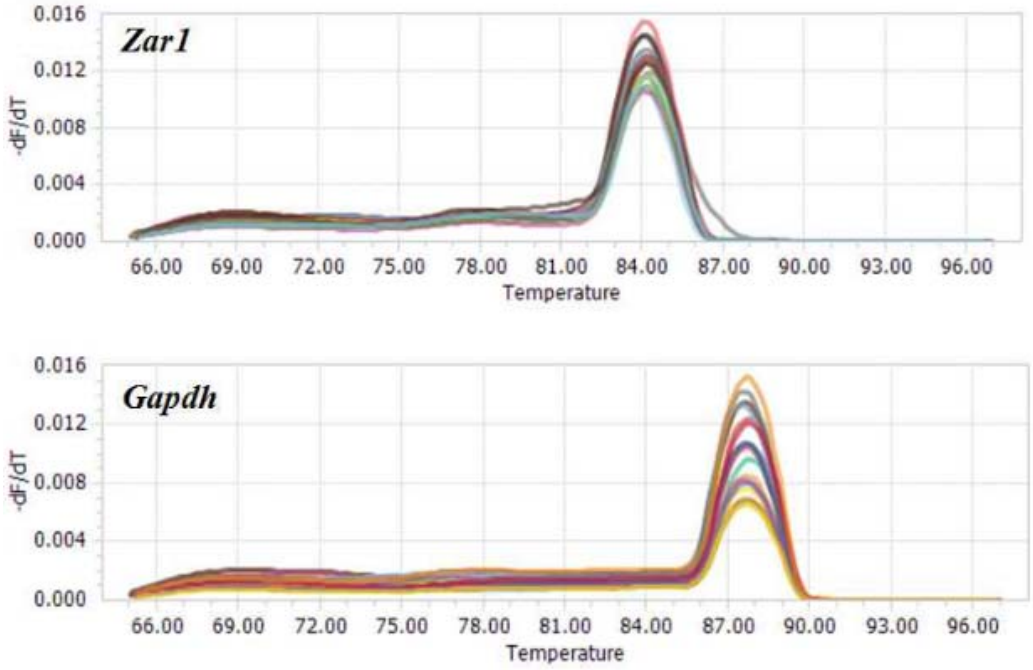


Sequence Name:
Sequence Length: 4917

Supplementary Fig 2. ZAR1 gene methylation site predicted



Supplementary Fig 3. The amplification curve of ZAR1 gene



Supplementary Fig 4. The dissolution curve of ZAR1 gene