Candidate gene analysis for reproductive traits in two lines of rabbits divergently selected for uterine capacity

M. J. Argente, M. Merchán, R. Peiró, M. L. García, M. A. Santacreu, J. M. Folch and A. Blasco

J Anim Sci 2010.88:828-836.
doi: 10.2527/jas.2009-2324 originally published online Nov 20, 2009;

Candidate gene analysis for reproductive traits in two lines of rabbits divergently selected for uterine capacity[1]

M. J. Argente,*[2,3] M. Merchán,†[2] R. Peiró,‡ M. L. García,* M. A. Santacreu,‡ J. M. Folch,† and A. Blasco‡

*Departamento de Tecnología Agroalimentaria, Universidad Miguel Hernández de Elche, 03312, Orihuela, Spain; †Departament de Ciència Animal i dels Aliments, Facultat de Veterinària, Universitat Autònoma de Barcelona, 08193, Bellaterra, Spain; and ‡Instituto de Ciencia y Tecnología Animal, Universidad Politécnica de Valencia, 46071, Valencia, Spain

ABSTRACT: The objective of this work was to analyze 3 functional candidate genes for reproduction in 2 lines of rabbits divergently selected by uterine capacity. Both lines were selected for 10 generations. The selection was then relaxed until the 17th generation, when it was compounded by 61 and 63 does of the High and Low lines, respectively. We sequenced the *SCGB1A1* gene, which encodes the main protein secreted by the rabbit in the uterus and seems to play an important role in implantation. We found 6 SNP in the promoter region cosegregating in 2 haplotypes in both lines with similar frequency. We also analyzed *IGF1* mRNA because of its effects on embryo development, but we did not find any polymorphism between individuals of the 2 lines. The third gene analyzed was the *TIMP1*, which encodes a protein involved in many biological processes related to reproduction. We determined the sequence of its promoter region and found 1 SNP (g.1423A>G) segregating with different frequencies in both lines (0.60 for allele A in the High line and 0.82 for allele G in the Low line). The association study performed in an F_2 population (n = 598) generated by the cross of the 2 lines of rabbits revealed that the AA genotype had 0.88 embryos more than the GG genotype at 72 h of gestation. The difference increased to 2.23 embryos at implantation, but no difference was found between genotypes at birth. These results suggest that *TIMP1* could be a candidate gene for embryo implantation and embryo survival.

Key words: embryo survival and development, insulin-like growth factor I gene, litter size, rabbit, secretoglobin family 1A member 1 gene, tissue inhibitor of metalloproteinases 1 gene

J. Anim. Sci. 2010. 88:828–836
doi:10.2527/jas.2009-2324

INTRODUCTION

Prenatal losses are a limiting factor in the litter size (**LS**) of rabbits, pigs, and mice. Approximately 30 to 40% of the ova shed do not result in fetuses at term (Blasco et al., 1993), and one-third to one-half of these losses occur before implantation (Ford et al., 2002, in pigs; Holt et al., 2004, in mice; Santacreu et al., 2005, in rabbits). Most of these embryonic losses are characterized by an asynchronized development of the embryo with the uterus (see review by Geisert and Schmitt, 2002). Some proteins have an important role in embryo development. Secretoglobin family 1A member 1 (***SCGB1A1***, also known as uteroglobin; Riffo et al., 2007), *IGF1* (Lin et al., 2003), and tissue inhibitor of metalloproteinases 1 (***TIMP1***; Hwang et al., 2000) are involved in regulating the embryogenesis, angiogenesis, morphogenesis, and endometrial tissue remodeling processes that take place during gestation. Therefore, *SCGB1A1*, *IGF1*, and *TIMP1* could explain part of the embryonic losses before implantation in rabbits.

After 10 generations of a divergent selection experiment on uterine capacity (**UC**), the High line showed a greater number of embryos at 72 h of gestation (0.96

[1]This study was funded by projects by the Comisión Interministerial de Ciencia y Tecnología AGL2001-3068 and AGL2005-07624. The authors are grateful to R. Muelas (Departamento de Tecnología Agroalimentaria, Universidad Miguel Hernandez de Elche, Orihuela, Spain) for the excellent assistance provided and to W. Mekkawi (Instituto de Ciencia y Tecnología Animal, Universidad Politécnica de Valencia, Valencia, Spain) for his software. M. Merchán acknowledges an Formación de Personal Investigador (FPI) grant from the Ministerio de Ciencia y Tecnología and R. Peiró an FPI grant from Oficina de Ciencia y Tecnología de la Generalitat Valenciana. Finally, we also acknowledge the detailed comments of B. Kirkpatrick and one referee.

[2]These authors have contributed equally to the studies presented in this manuscript.

[3]Corresponding author: mj.argente@umh.es

Received July 21, 2009.
Accepted November 11, 2009.

embryos), implantation (1.79 embryos), and birth (2.35 kits) than the Low line in intact does (Mocé et al., 2004; Santacreu et al., 2005). These differences appeared mainly in the first 2 generations of selection (Blasco et al., 2005), suggesting that a major gene may be involved in the genetic determination of these traits. In addition, a complex segregation analysis performed by Argente et al. (2003) found evidence of major genes with a moderate effect on UC and a large effect on embryo survival and number of implanted embryos in these rabbit lines. Moreover, Estellé et al. (2006) reported a greater expression of *TIMP1* in the High line. This gene is expressed in the oviduct at 62 h of gestation.

On the basis of the biological function of *SCGB1A1*, *IGF1*, and *TIMP1* and previous results of the abovementioned experiment on divergent selection for UC in rabbits, it is hypothesized that the *SCGB1A1*, *IGF1*, and *TIMP1* genes could play an important role in the reproductive traits in rabbits. The objective of this work was to identify SNP for 3 functional candidate genes, *SCGB1A1*, *IGF1*, and *TIMP1*, and to evaluate associations between these SNP and reproductive traits in an F_2 cross between 2 rabbit lines divergently selected for UC.

MATERIALS AND METHODS

All experimental procedures involving animals were approved by the Research Ethics Committee of Universidad Politécnica de Valencia.

Animal Material

A total of 15 animals from the High line and 21 animals from the Low line from the 17th generation of a divergent selection experiment on UC were used to analyze polymorphisms in the *SCGB1A1*, *IGF1*, and *TIMP1* genes. Both lines were selected for 10 generations (Blasco et al., 2005); the selection was then relaxed until the 17th generation, when it was compounded by 61 and 63 does of the High and Low lines, respectively. These lines were derived from a synthetic line selected for 12 generations by LS at weaning (Estany et al., 1989). The synthetic line was founded in 1981 as a synthetic line, crossing animals of 4 specialized maternal lines of Californian and New Zealand White breeds. An F_1 population was generated by the reciprocal cross of the High and Low lines from the 17th generation of the divergent selection experiment on UC. Parental animals were selected for UC by using a BLUP procedure on an animal-repeatability model with year-season and parity-lactation stage effects (with 5 levels: nulliparous does, lactating and nonlactating does of second parity, and lactating and nonlactating does with more than 2 parities). Groups of full-sib families were generated by mating 70 F_1 intact does to 10 F_1 bucks, obtaining a total of 598 F_2, which were crossed to 127 F_2 males. All animals were reared in individual cages and were fed a commercial diet. The photoperiod used was 16 h light:8 h dark. Does were mated first at 18 wk of age and thereafter at 10 d after each parturition.

A laparoscopy was performed on all F_2 does during their second gestation, 12 d after mating, and the numbers of corpora lutea and implanted embryos were recorded. The laparoscopic technique is described in detail by Santacreu et al. (1990).

The F_2 does that completed their fourth lactation (n = 331) were mated and slaughtered at 48 or 72 h of gestation by intravenous injection of sodium thiopental in a dose of 50 mg/kg of BW (thiobarbital, B. Braun Medical S.A., Barcelona, Spain). The total reproductive tract was removed, and oviducts and uterine horns were separated and flushed once with 5 and 10 mL of 150 m*M* ammonium bicarbonate solution, respectively.

Traits

Litter size and number born alive (**NBA**) were measured in does up to 4 parities. At d 12 of the second gestation, ovulation rate (**OR**; estimated as the number of corpora lutea) and number of implanted embryos (**IE**; estimated as the number of implantation sites) were recorded by laparoscopy on all does. Embryo survival (**ES**) was analyzed as IE fitting OR as a covariate, fetal survival (**FS**) as LS fitting IE as a covariate, and prenatal survival (**PS**) as LS fitting OR as a covariate.

The females were slaughtered during the fifth gestation, and the following traits were recorded: OR, total number of embryos (**TE**), number of normal embryos (**NNE**), and number of oocytes (**OO**) recovered. Embryos were classified according to morphological criteria (Hafez, 2000) using a binocular stereoscopic microscope. The following traits were analyzed: fertilization rate [% **FR** = 100 × TE/(TE + OO)], percentage of normal embryos (% NNE = 100 × NNE/TE); percentage of early morulae (% **EM** = 100 × EM/NNE); percentage of compacted morulae (% **CM** = 100 × CM/NNE); and percentage of blastocysts (% **B** = 100 × B/NNE). Early ES (**EES**) was analyzed as NNE fitting OR as a covariate.

All embryos were recovered in the oviduct at 48 h of gestation. However at 72 h of gestation, the embryos were recovered both in the oviduct and in the uterine horn, and some embryos may have been lost when separating the oviduct and uterine horn before embryo recovery.

Amplification and Sequencing of the SCGB1A1, IGF1, and TIMP1 Genes

DNA Isolation. Ear tissue was recovered from 15 High and 21 Low line animals. At least 3 mL of venous blood from the marginal ear vein of 80 F_1 and 598 F_2 animals was collected in K_3EDTA plastic tubes with a concentration of 1.8 mg of EDTA per 1 mL of blood. Genomic DNA from 300 mg of ear tissue was purified by standard procedures using proteinase K digestion followed by phenol/chloroform extraction and precipi-

Table 1. Nucleotide sequences of primers used for amplifying and sequencing the rabbit *SCGB1A1* and *TIMP1* DNA and the *IGF1* cDNA

Primer[1]	Use[2]	Sequence	Amplified length, bp
UTEP-F	PCR, Seq	5′-AGCAATGCTCTGAACCCAGTGAC-3′	3,703
UTE3F-R	PCR, Seq	5′-AAGGACTTTGGAAGGCGTCAGAG-3′	
UTEI1-F	Seq	5′-AGAGCACCAGCAGAGCTGATTTC-3′	
IGF1-IF	RT-PCR, Seq	5′-ATGATTACACCTACAGTAAAGATGCGC-3′	409
IGF1-IaR	RT-PCR, Seq	5′-CCTGTAGTTCTTGTTTCCTGCACTC-3′	
TIMP1-mma-F	PCR, Seq	5′-CCATATTGTGGCCTTTGATCACG-3′	1,370
TIMP1-mma-R	PCR, Seq	5′-ACCCGCTTACCTCTAGCGTCTG-3′	
TIMP1-mma-F2	Seq	5′-GGCCATTTGCCTGTGATTCT-3′	
TIMP1-mma-R2	Seq	5′-GGTGAAGACAGGCTGAGGCATA-3′	
TIMP1-Pyro-F-bio	PCR	5′-AAGTAGCAAGGGAGTAATGGAGAG-3′	142
TIMP1-Pyro-R	PCR	5′-TGGTTAATTCAGGGAGGACAGA-3′	
TIMP1-Pyro-R-seq	Geno	5′-TCCTGACGATAGGGAT-3′	

[1]UTEP, UTE3F, and UTEI1 are primers used for amplification and sequencing of the *SCGB1A1* gene. IGF1-I and IGF-Ia are primers used for reverse transcription and sequencing of the *IGF1* gene. TIMP1-mma and TIMP1-Pyro are primers used for amplification and sequencing of the *TIMP1* gene. F: forward primer. R: reverse primer. F2 and R2: alternative forward and reverse primers.
[2]RT-PCR = reverse transcription-PCR; Seq = sequencing reaction; Geno = genotyping by pyrosequencing.

tation with ethanol (Ausubel et al., 1987). Genomic DNA from 80 μL of venous blood collected in EDTA sample tubes was purified with the DNA Isolation from Fresh and Frozen Blood, Tissue Culture Cells, and Buccal Swabs protocol described by Applied Biosystems (http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041387.pdf).

RNA Preparation. Oviduct and testes tissues were collected from High and Low line does and bucks. Total RNA was extracted from 100 mg of each tissue using Trizol reagent (Invitrogen, Barcelona, Spain). Synthesis of cDNA was performed with a Thermoscript reverse transcription-PCR kit (Invitrogen).

SCGB1A1. We designed the primers UTEP-F, UTE3F-R, and UTEI1-F based on the sequence of the rabbit *SCGB1A1* gene (GenBank accession number X01423; Table 1) to analyze the promoter and coding DNA sequence (**CDS**) region. We amplified a 3,703-bp fragment comprising the 5′ and 3′ flanking regions that included the 3 exons and 2 introns of the gene (Figure 1). Amplification was performed in a PCR mixture that contained 2 m*M* $MgCl_2$, 0.2 m*M* deoxynucleotide 5′-triphosphate, 0.3 μ*M* of each primer, 50 ng of genomic DNA, 1× buffer, and 1.3 U of Expand High Fidelity PCR System Polymerase (Roche, Mannheim, Germany) in a final volume of 25 μL. The thermal cycling profile was 95°C for 5 min, 30 cycles of 95°C for 30 s, 65°C for 1 min, 72°C for 110 s from cycle 10 to 30, and a final extension step of 72°C for 7 min. The amplified product was purified with kit ExoSAP-IT (Amersham Biosciences Europe GmbH, Freiburg, Germany) and sequenced with the BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The promoter and exon 1 were sequenced with primer UTEP-F, exon 2 was sequenced with an internal primer complementary to the end of intron 1 (**UTEI1-F**), and exon 3 was sequenced with the UTE3F-R primer. Sequences were analyzed in a capillary electrophoresis device (ABI Prism 3100 Avant, Applied Biosystems) and subsequently aligned with SeqScape version 2.1 software (Applied Biosystems).

IGF1. The rabbit *IGF1* gene structure is unknown, but it is expected to be as long in other species as in the human (with 95 kb and 6 exons). Its mRNA is also expected to be submitted to alternative splicing, as happens in other species.

We designed the pair of primers IGF1-IF (forward) and IGF1-IaR (reverse) based on the sequence of the rabbit *IGF1* cDNA corresponding to the form A (GenBank accession number U75390; Table 1) to amplify and sequence the CDS region (Figure 1). The PCR reaction was performed in a final volume of 25 μL containing 1.5 m*M* $MgCl_2$, 0.2 m*M* deoxynucleotide 5′-triphosphate, 0.5 μ*M* each primer, 1× buffer, 0.6 U of Taq DNA Polymerase (Invitrogen), and 2 μL of the reverse transcription reaction. Thermocycling was 95°C for 5 min, 35 cycles of 95°C for 30 s, 65°C for 1 min, 72°C for 90 s, and a final extension of 72°C for 7 min. Polymerase chain reaction products were purified with a QIAquick Gel Extraction Kit Protocol (Qiagen, Valencia, CA) and sequenced and analyzed as described previously.

TIMP1. In a previous study (Estellé et al., 2006), we sequenced and described the rabbit *TIMP1* gene structure, which extended 4.5 kb and contained 6 exons. We also analyzed the proximal promoter region (56 bp), exon 1, intron 1, and the 5′ end of exon 2 and the CDS region of the *TIMP1* gene in the High and Low lines, but we did not find polymorphisms. In addition, the expression of the *TIMP1* gene was analyzed by real-time PCR quantification in the oviducts and uteruses of both lines at 62 h of gestation, and the Low line presented 35% less expression in oviducts than the High line. For these reasons, we looked for polymorphisms in the promoter region that could explain the differences between lines for mRNA expression. We designed the pair of primers TIMP1-mma-F (forward) and TIMP1-mma-R (reverse; Table 1) to amplify and sequence the promoter region of the rabbit *TIMP1* gene (Figure 1) that had been partially sequenced before in the draft

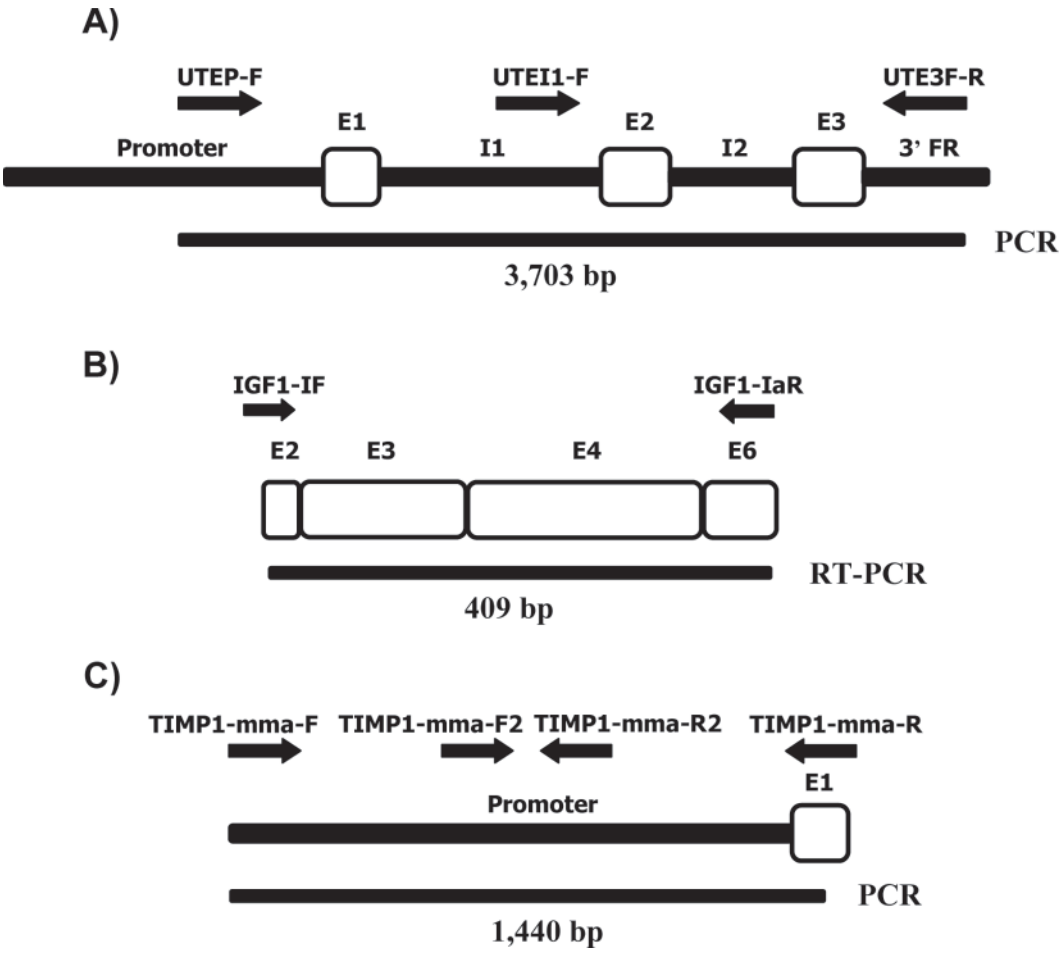

Figure 1. Schematic diagram showing the strategy used to amplify and sequence the A) *SCGB1A1*, B) *IGF1*, and C) *TIMP1* genes. Arrows indicate the approximate position of primers. Exons (E1, E2, E3, E4, E6) are depicted as boxes. Sizes of the amplicons are shown under the solid bars. RT = reverse transcription; F = forward primer. R = reverse primer. F2 and R2 = alternative forward and reverse primers.

genome sequence of the rabbit (Broad Institute, Cambridge, MA). The PCR conditions are exactly as described previously for *IGF1*, but using 50 ng of genomic DNA, 0.6 U of Eco Taq (Ecogen, Barcelona, Spain), and an annealing temperature of 62°C. Sequencing was performed as described previously. Sequence analysis was performed with Lasergene software (DNASTAR, Madison, WI) to predict if the SNP found in the promoter region could alter a transcription factor binding site.

Genotyping of TIMP1 Gene

The implementation of a pyrosequencing protocol to genotype animals was carried out for the SNP g.1423A>G. The PCR of a 25-μL final volume containing 1.5 m*M* $MgCl_2$, 0.2 m*M* deoxynucleotide 5′-triphosphate, 0.5 μ*M* each primer (the biotinylated forward primer TIMP1-Pyro-F-bio and the reverse primer TIMP1-Pyro-R; Table 1), 1× buffer, 0.6 U of AmpliTaq Gold (Applied Biosystems), and 50 ng of DNA was used. Thermocycling was 95°C for 10 min, 45 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 90 s, and a final extension of 72°C for 15 min. Subsequently, the sequencing reaction was done in a PSQ HS 96 system (Pyrosequencing AB, Uppsala, Sweden) with the reverse primer TIMP1-Pyro-R-seq (Table 1). The animals genotyped as AA_{1423} were named AA, the animals genotyped as AG_{1423} were named AG, and the animals genotyped as GG_{1423} were named GG.

Statistical Analysis

Allele Frequency in High and Low Lines and Reproductive Traits for the F_2 Population. A chi-square test with the Fisher correction was used to test the association between the frequency of alleles and the High and Low lines.

An association study for the *TIMP1* gene was performed using Bayesian methodology. Litter size and NBA were analyzed from a total of 2,066 records of 598 does, and the following animal-repeatability model was used:

$$y_{ijklm} = YS_i + PL_j + G_k + a_{ijkl} + p_{ijkl} + e_{ijklm}, \quad [1]$$

where YS_i was the effect of year-season (with 8 levels), PL_j was the effect of parity-lactation stage of the doe (with 5 levels: nulliparous does, lactating and nonlactating does of second parity, and lactating and non-

lactating does with more than 2 parities), G_k was the effect of the *TIMP1* gene genotype (with 3 levels: AA, AG, and GG), a_{ijkl} was the breeding value of the animal, p_{ijkl} was the permanent environmental effect, and e_{ijklm} was the error.

For all univariant analyses, bounded uniform priors were used for all environmental variables and **a** and **p** were assumed to be a priori independent and normally distributed:

$$\mathbf{a} \mid \sigma_a^2 \sim N\left(\mathbf{0}, \mathbf{A}\sigma_a^2\right), \text{ and}$$

$$\mathbf{p} \mid \sigma_p^2 \sim N\left(\mathbf{0}, \mathbf{I}\sigma_p^2\right),$$

where **A** is the known additive genetic relationship matrix and **I** is the identity matrix. The pedigree file included 702 animals.

Information on the last 4 generations, in which no selection pressure was applied, was used to establish the genetic parameters. Marginal posterior distributions of all unknowns, conditional to the variance components, were estimated using Gibbs sampling. The heritability of 0.10 and repeatability of 0.15 were estimated for NBA and LS. The heritabilities of 0.30, 0.20, 0.10, 0.05, and 0.15 were estimated for OR, IE, ES, FS, and PS, respectively.

A total of 561 records were used to analyze OR, IE, and ES, and a total of 477 records were used to analyze FS and PS. An animal model was used, including the same environmental effects as in the former model (Eq. 1).

A total of 172 records were used to analyze the % FR and embryonic stage of development at 48 h of gestation. The model included the effects of YS and G, the presence of a hemorrhagica follicle (with 3 levels: zero, between 1 to 5, and 6 or more follicles), the interval between weaning of the last litter and the slaughter of does (with 2 levels: 1 mo or more), and operator. The EES also included OR as a covariate. A total of 159 records were used to analyze the % FR, embryonic stage of development, and EES at 72 h of gestation using the same model as before, including the effect of the presence or absence of embryos in the uterus.

For all analyses, a chain of 200,000 samples was used, with a burn-in period of 40,000 for each trait. Convergence was tested using the Z criterion of Geweke (Sorensen and Gianola, 2002), and Monte Carlo sampling errors were computed using time-series procedures as described by Geyer (1992).

Inferences were made from the estimated marginal posterior distributions of the differences between genotypes as in Peiró et al. (2008). We proposed the relevant values for these differences (**Rv**). In classical statistics, the size of the experiment is usually established for finding a significant difference between 2 treatments when this difference is considered to be relevant. An Rv is the quantity below which this difference has no biological or economic meaning. When analyzing traits different from the one used to design the experiment, there is no relationship between the relevance of the difference and its significance. In these cases, we can find nonsignificant differences that are relevant and significant differences that are irrelevant. In a Bayesian framework, we can propose an Rv for each trait and estimate the probability of a difference between treatments being relevant for each case ($\mathbf{P_r}$). Finally, in classical statistics, nonsignificance does not imply that 2 treatments are equal. In Bayesian statistics, we can estimate the probability of a difference being, in absolute value, less than an Rv [i.e., the probability of both treatments being similar in biological or economic terms ($\mathbf{P_s}$)]. These probabilities allow us to distinguish between 2 genotypes having the same effect (large P_s) or not finding differences because of poor precision of the experiment (small P_s and P_r).

RESULTS

Polymorphism Analysis

SCGB1A1 Gene. A 3,703-bp fragment of the rabbit *SCGB1A1* gene, including the promoter region (485 bp); exons 1, 2, and 3 and introns 1 and 2 (3,073 bp); and the 3′ flanking region (145 bp), were amplified and partially sequenced in a total of 36 animals of the High and Low lines. Six polymorphic sites were found in the promoter region segregating in both lines (Table 2). We found 29 homozygotes for the 6 polymorphic sites with the nucleotide composition defined as haplotype B, 1 homozygote with the nucleotide composition defined as haplotype A, and 6 heterozygotes. Both lines showed similar frequencies ($P > 0.10$) at these haplotypes.

IGF1 Gene. Two isoforms of the rabbit *IGF1* mRNA were amplified and sequenced: isoform A (409 bp) and isoform B (461 bp), which included an insertion of 52 bp corresponding to exon 5. Isoform B of the

Table 2. Polymorphisms and haplotypes of the rabbit *SCGB1A1* gene in the High and Low lines of the selection experiment on uterine capacity

	Promoter region[1]						Frequency	
Haplotype	2886	3099	3217	3221	3248	3252	High line (n = 15)	Low line (n = 21)
A	A	T	G	A	C	T	0.16	0.07
B	G	C	A	G	T	C	0.84	0.93

[1]Nucleotide position in reference to GenBank accession number X01423.

Table 3. Mean, SD, and number (N) of does and litters (within parentheses) in the F_2 population

Trait[1]	Mean	SD	N
OR	14.7	2.8	561
48 h[2]			
% FR	98.1	4.9	172
NNE	11.9	2.3	172
% EM	13.3	25.7	172
% CM	86.7	25.7	172
72 h[3]			
% FR	97.6	6.3	159
NNE	11.8	2.7	159
% EM	12.2	25.8	159
% CM	72.9	24.3	159
% B	14.9	24.7	159
IE	11.1	3.8	561
LS	8.2	3.2	598 (2,066)
NBA	7.3	3.5	598 (2,066)

[1]OR = ovulation rate; % FR = fertilization rate; NNE = number of normal embryos; % EM = percentage of early morulae; % CM = percentage of compacted morulae; % B = percentage of blastocysts; IE = number of implanted embryos; LS = litter size; NBA = number born alive.
[2]48 h of gestation.
[3]72 h of gestation.

IGF1 mRNA was purified and sequenced in High and Low line animals; however, we did not find any polymorphic site.

TIMP1 Gene. A 1,440-bp fragment that included part of the promoter region and exon 1 of the rabbit *TIMP1* gene was amplified and sequenced in High and Low line animals (GenBank accession number AY829731). We found 1 SNP in the promoter region, g.1423A>G, segregating in both lines with different frequencies. Allele A was more frequent in the High line (0.60), whereas allele G was more frequent in the Low line (0.82). This SNP was genotyped in our 598 F_2 does by pyrosequencing, and a total of 25 AA, 300 AG, and 273 GG genotypes were identified. We analyzed whether the polymorphic site could alter a transcription factor binding site, but the predictive result was negative.

Association Study with the TIMP1 Gene

Table 3 shows raw means and SD for LS, NBA, OR, IE, % FR, NNE, % EM, % CM, and % B. Features of the estimated marginal posterior distributions of the differences (**D**) between the AA and GG genotypes and between the AA and AG genotypes are presented in Tables 4 and 5, respectively. All Monte Carlo SE were very small and a lack of convergence was not detected by the Geweke test. Marginal posterior distributions were approximately normal; thus mode, mean, and median were similar, and only the posterior mean of the difference is shown.

Tables 4 and 5 also display the values that we assume to be relevant for each trait (Rv). We consider 0.5 kits to be a relevant difference for LS and OR as described by Peiró et al. (2008), and also for IE and for the survivals ES, FS, and PS. We consider one-half of this amount (i.e., 0.25 embryos) as a relevant value for EES because Mocé et al. (2004) found that one-half of the difference in the number of IE in the lines that originated our population occurred before 72 h of gestation. The relevant value for all the embryonic stages of development was established as one-third of the phenotypic SD of the trait, 8%.

The AA genotype showed greater OR than the GG genotype (P = 85%), although this difference may be irrelevant (P_r = 64%). No relevant differences (P_r = 8% and P_r = 50%, respectively) between homozygote genotypes were found in % FR at 48 and 72 h of gestation. We did not find (P_r = 29%) any relevant difference between the AA and GG genotypes for EES at 48 h of gestation, but at 72 h of gestation, there was a relevant difference between genotypes (D = 0.88 embryos, P_r = 83%). At 48 h of gestation, both homozygote genotypes also showed a similar embryonic stage of development, but at 72 h the AA genotype had a more advanced embryonic stage of development, showing a smaller % EM (D = −16.33, P_r = 76%), a greater % CM (D = 19.85, P_r = 81%), and a % B (P_s = 25% and P_r = 43%) similar to the GG genotype (Table 4). The AA genotype had a substantially greater number of IE than the GG genotype (D = 2.23 embryos, P_r = 96%) and also had greater ES (D = 1.66 embryos, P_r = 93%). Prenatal survival of the AA genotype may be also greater, although we cannot be sure about the relevance of this difference. The differences between genotypes in ES were not transferred to differences in LS. We did not find differences in LS and NBA between genotypes (P_s approximately 70%).

The differences between the AA and AG genotypes in OR, IE, ES, and PS were similar to the differences found between homozygote genotypes. The AG and GG genotypes had similar OR, IE, ES, and PS. No relevant differences were found for % FR. The AA genotype showed greater EES than the AG genotype at both 48 and 72 h of gestation, and these differences were relevant; however, the embryonic stage of development differed at only 72 h of gestation, with the AA genotype having a smaller (D = −13.61 and P_r = 69%) % EM, a greater (D = 16.08 and P_r = 73%) % CM, and % B similar (P_s = 27% and P_r = 40%) to the AG genotype. As before, the differences in genotypes are lost at birth; both genotypes had similar LS and NBA (P_s approximately 70%).

DISCUSSION

Previous results suggested the presence of a major gene affecting ES, IE, and UC segregating in the lines that originated our population (Argente et al., 2003; Blasco et al., 2005). In rabbits, the first genetic map of its genome recently became available, but this map is based on microsatellites constituting only 111 markers (Chantry-Darmon et al., 2006). This fact has made the genome scanning approach difficult for QTL detec-

Table 4. Features of the estimated marginal posterior distributions of the differences (D) between the AA and GG genotypes of the SNP g.1423A>G for the promoter region of the *TIMP1* gene[1]

Trait[2]	D	$HPD_{95\%}$	P, %	Rv	P_s, %	P_r, %
OR	0.73	−0.71, 2.19	85	0.5	31	64
48 h[3]						
% FR	0.94	−2.82, 4.39	70	3.5	91	8
EES	−0.01	−0.92, 0.89	51	0.25	42	29
% EM	−3.28	−22.73, 17.66	62	8	53	33
72 h[4]						
% FR	3.40	−2.51, 8.73	88	3.5	49	50
EES	0.88	−0.55, 2.21	90	0.25	12	83
% EM	−16.33	−39.40, 5.96	92	8	22	76
% CM	19.85	−7.68, 46.44	92	8	17	81
% B	−3.52	−16.78, 24.01	56	8	25	43
IE	2.23	0.31, 4.09	98	0.5	4	96
ES	1.66	0.19, 3.09	100	0.5	7	93
FS	−0.42	−1.66, 0.70	76	0.5	48	45
PS	0.74	−0.73, 2.23	83	0.5	33	60
LS	−0.05	−1.00, 0.86	54	0.5	70	12
NBA	0.01	−1.03, 0.99	51	0.5	66	18

[1]D = posterior mean of the difference (AA − GG); $HPD_{95\%}$ = greatest posterior density region at 95%; P = P(D > 0) when D > 0 and P(D < 0) when D < 0; Rv = proposed relevant difference between genotypes; P_s = probability of similarity (probability of the absolute value of D being less than Rv); P_r = probability of relevance [P(D > Rv) when D > 0 and P(D < Rv) when D < 0].

[2]OR = ovulation rate; % FR = fertilization rate; EES = early embryo survival; % EM = percentage of early morulae; % CM = percentage of compacted morulae; % B = percentage of blastocysts; IE = number of implanted embryos; ES = embryo survival; FS = fetal survival; PS = prenatal survival; LS = litter size; NBA = number born alive.

[3]48 h of gestation.

[4]72 h of gestation.

Table 5. Features of the estimated marginal posterior distributions of the differences (D) between the AA and AG genotypes of the SNP g.1423A>G for the promoter region of the *TIMP1* gene[1]

Trait[2]	D	$HPD_{95\%}$	P, %	Rv	P_s, %	P_r, %
OR	0.88	−0.35, 2.21	91	0.5	23	76
48 h[3]						
% FR	0.77	−2.92, 4.03	33	3.5	93	6
EES	0.70	−0.17, 1.58	94	0.25	13	85
% EM	−1.29	−20.90, 18.57	54	8	58	25
72 h[4]						
% FR	3.00	−2.70, 8.45	85	3.5	55	34
EES	0.67	−0.75, 1.92	85	0.25	18	74
% EM	−13.61	−36.02, −8.15	89	8	28	69
% CM	16.08	−10.65, 42.05	88	8	23	73
% B	−2.43	−48.00, 48.00	52	8	27	40
IE	2.10	0.45, 3.91	100	0.5	4	96
ES	1.42	0.08, 2.81	99	0.5	12	88
FS	−0.26	−1.51, 0.76	68	0.5	55	35
PS	0.77	−0.73, 2.10	86	0.5	31	62
LS	0.01	−0.96, 1.07	51	0.5	67	18
NBA	−0.05	−0.94, 0.91	54	0.5	71	13

[1]D = posterior mean of the difference (AA − AG); $HPD_{95\%}$ = greatest posterior density region at 95%; P = P(D > 0) when D > 0 and P(D < 0) when D < 0; Rv = proposed relevant difference between genotypes; P_s = probability of similarity (probability of the absolute value of D being less than Rv); P_r = probability of relevance [P(D > Rv) when D > 0 and P(D < Rv) when D < 0].

[2]OR = ovulation rate; % FR = fertilization rate; EES = early embryo survival; % EM = percentage of early morulae; % CM = percentage of compacted morulae; % B = percentage of blastocysts; IE = number of implanted embryos; ES = embryo survival; FS = fetal survival; PS = prenatal survival; LS = litter size; NBA = number born alive.

[3]48 h of gestation.

[4]72 h of gestation.

tion in this species, making the physiological candidate gene approach more interesting. Among the available genes, we examined *SCGB1A1* and *IGF1* because they are 2 important growth factors for oviductal fluid (Hill, 1992; Schultz and Heyner, 1993). Both proteins could play an important role in LS by means of controlling cellular proliferation, differentiation, and morphogenesis during mammalian embryogenesis (Riffo et al., 2007, for *SCGB1A1*; Herrler et al., 1998, and Lin et al., 2003, for *IGF1*); moreover, Gibson et al. (2001), in mice, and Korwin-Kossakowska et al. (2004), in pigs, have reported an important effect of the *IGF1* gene on LS. For these reasons, the *SCGB1A1* and *IGF1* genes were chosen as candidate genes for LS and its components. However, in our study we found that the High and Low lines showed similar frequencies for the 6 identified SNP of the *SCGB1A1* gene, and no polymorphic sites were detected for the *IGF1* gene. Thus, selection for UC has not changed the allelic frequencies for the analyzed *SCGB1A1* gene, and the *IGF1* sequence has not been modified.

The *TIMP1* gene has been recognized as a multifunctional protein that is implicated in many reproductive processes, such as embryonic development, angiogenesis, and implantation (Takigawa et al., 1990; Satoh et al., 1994; Zhao et al., 2002). Nothnick (2001a) reported in mice that knockout females had a smaller LS after their second parity. Therefore, the *TIMP1* gene was chosen as the third candidate gene for LS and its components. In a previous study (Estellé et al., 2006), the rabbit *TIMP-1* gene sequence was screened for polymorphism between the High and Low lines. However, no polymorphisms were found in the analyzed regions (CDS, proximal promoter, exon 1, intron 1, and exon 2). In the same study, significant differences between the High and Low lines were reported for *TIMP-1* mRNA abundance in the oviduct at 62 h of gestation. These results motivated us to sequence the promoter region of the rabbit *TIMP1* gene to find genetic variants that might explain the differential expression observed between lines. After sequencing the promoter region in animals from the High and Low lines, we found a diallelic SNP, g.1423A>G. The A allele was more frequent in the High line and the G allele was more frequent in the Low line.

In the present study, we did not find any relevant difference for OR between the AA and GG genotypes. Similarly, Nothnick et al. (1997) did not find differences in mice for folliculogenesis, steroidogenesis, and OR between control and *TIMP1* knockout females, females incapable of expressing the functional *TIMP1* gene product. Thus, taking all these results into account, it seems that the *TIMP1* gene does not affect OR.

There were no relevant differences in % FR among genotypes at 48 and 72 h of gestation. A similar % FR was also found in previous results in our lines (Mocé et al., 2004; Peiró et al., 2007) and by other authors using other lines with different ES (Torres et al., 1987; García-Ximénez and Vicente, 1992; Bolet and Theau-Clement, 1994).

Embryonic stage of development at 48 h of gestation was similar among the 3 genotypes. At 72 h of gestation, the AA genotype had a more advanced embryonic stage of development than the GG and AG genotypes. These differences in early embryo development seem to be associated with greater ES in the AA genotype than in the GG and AG genotypes at this stage of gestation, representing 32 and 25% of the SD for EES, and 80 and 65% of the SD for % CM, respectively. A disruption of the *TIMP1* gene product is associated with a significant increase in uterine matrix metalloproteinase activity, leading to a negative effect on the development of embryos (Hwang et al., 2000; Nothnick, 2000, 2001a,b). Estellé et al. (2006) reported greater expression of the *TIMP1* gene in the oviduct at 62 h of gestation in the High line. These results agree with a more advanced embryonic stage of development and greater ES reported by Mocé et al. (2004) in the High line at 72 h of gestation. Other authors also found that a more advanced embryonic stage of development in early gestation is accompanied by greater ES (Moler et al., 1980, in mice; Torres et al., 1987, in rabbits).

The differences in ES between the AA genotype and the GG and AG genotypes increased at implantation, but these differences were not found at birth. Nothnick (2001a) observed that the effect of the absence of *TIMP1* on LS did not occur until the third litter, indicating that prolonged absence of the *TIMP1* gene is required for these deficiencies to be manifested.

In summary, the High and Low lines showed similar frequencies for the 6 identified SNP of the *SCGB1A1* gene, and no polymorphic sites were detected for the *IGF1* gene. The *TIMP1* gene is mainly expressed in the oviduct and seems to have an important role in early embryo development and survival. The results of the association study showed that the allele A_{1423} located in the promoter region had a positive effect on EES at 72 h of gestation and also on IE and ES. The action of this gene on these traits seems to be dominant. A genetic validation in commercial lines or breeds would be required to use these genetic variants in marker-assisted selection or gene-assisted selection to improve the response to selection for reproductive traits.

LITERATURE CITED

Argente, M. J., A. Blasco, J. A. Ortega, C. S. Haley, and P. M. Visscher. 2003. Analyses for the presence of a major gene affecting uterine capacity in unilaterally ovariectomized rabbits. Genetics 163:1061–1068.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley-Interscience.

Blasco, A., J. Bidanel, G. Bolet, C. S. Haley, and M. A. Santacreu. 1993. The genetics survival of pigs and rabbits: A review. Livest. Prod. Sci. 37:1–21.

Blasco, A., J. A. Ortega, M. A. Santacreu, and A. Climent. 2005. Divergent selection for uterine capacity in rabbits. I. Genetic parameters and response to selection. J. Anim. Sci. 83:2297–2302.

Bolet, G., and A. Theau-Clement. 1994. Fertilisation rate and preimplantation embryonic development in two rabbit strains of different fecundity, in purebreeding and crossbreeding. Anim. Reprod. Sci. 36:153–162.

Chantry-Darmon, C., C. Urien, H. de Rochambeau, D. Allain, and B. Pena. 2006. A first-generation microsatellite-based integrated genetic and cytogenetic map for the European rabbit (*Oryctolagus cuniculus*) and localization of angora and albino. Anim. Genet. 37:335–341.

Estany, J., M. Baselga, A. Blasco, and J. Camacho. 1989. Mixed model methodology for the estimation of genetic response to selection in litter size of rabbits. Livest. Prod. Sci. 21:67–75.

Estellé, J., Y. Sastre, M. Merchán, R. Peiró, M. A. Santacreu, and J. M. Folch. 2006. TIMP-1 as candidate gene for embryo survival in two divergent lines selected for uterine capacity in rabbits. Mol. Reprod. Dev. 73:678–684.

Ford, S. P., K. A. Vonnahme, and M. E. Wilson. 2002. Uterine capacity in the pig reflects a combination of uterine environment and conceptus genotype effects . J. Anim. Sci. 80(E-Suppl.):E66–E73.

García-Ximénez, F., and J. S. Vicente. 1992. Effect of ovarian cystic or haemorrhagic follicles on embryo recovery and survival after transfer in hCG-ovulated rabbits. Reprod. Nutr. Dev. 32:143–149.

Geisert, R. D., and R. A. M. Schmitt. 2002. Early embryonic survival in the pig: Can it be improved? J. Anim. Sci. 80(E-Suppl.):E54–E65.

Geyer, C. M. 1992. Practical Markov chain Monte Carlo (with discussion). Stat. Sci. 7:467–511.

Gibson, J. M., J. D. Aplind, A. White, and M. Westwood. 2001. Regulation of IGF bioavailability in pregnancy. Mol. Hum. Reprod. 7:79–87.

Hafez, E. S. E. 2000. Preservation and cryoperservation of gametes and embryos. Pages 431–442 in Reproduction in Farm Animals. E. S. E. Hafez and B. Hafez, ed. Lippincott Williams and Wilkins, Baltimore, MD.

Herrler, A., C. A. Krusche, and H. M. Beier. 1998. Insulin and insulin-like growth factor-I promote rabbit blastocyst development and prevent apoptosis. Biol. Reprod. 59:1302–1310.

Hill, D. J. 1992. Peptide growth factor interactions in embryonic and fetal growth. Horm. Res. 38:197–202.

Holt, M., O. Vangen, and W. Farstad. 2004. Components of litter size in mice after 110 generations of selection. Reproduction 127:587–592.

Hwang, W., H. Kim, E. Lee, J. Lim, S. Roh, T. Shin, K. Hwang, and B. Lee. 2000. Purification and embryotrophic roles of tissue inhibitor of metalloproteinase-1 in development of "HanWoo" (*Bos taurus coreanae*) oocytes co-cultured with bovine oviduct epithelial cells. J. Vet. Med. Sci. 62:1–5.

Korwin-Kossakowska, A., G. Sender, and J. Kuryl. 2004. Associations between the microsatellite DNA sequence in the IGF1 gene, polymorphism in the ESR gene and selected reproduction traits in F_1 (Zlotnicka Spotted × Polish Large White) sows. Anim. Sci. Pap. Rep. 22:215–226.

Lin, T. C., J. M. Yen, K. B. Gong, T. T. Hsu, and L. R. Chen. 2003. IGF-1/IGFBP-1 increases blastocyst formation and total blastocyst cell number in mouse embryo culture and facilitates the establishment of a stem-cell line. BMC Cell Biol. 4:14–30.

Mocé, M. L., M. A. Santacreu, A. Climent, and A. Blasco. 2004. The effect of divergent selection for uterine capacity on prenatal survival in rabbits: Maternal and embryonic genetic effects. J. Anim. Sci. 82:68–73.

Moler, T. L., S. E. Donahue, G. B. Anderson, and G. E. Bradford. 1980. Effects of maternal and embryonic genotype on prenatal survival in two selected mouse lines. J. Anim. Sci. 51:300–303.

Nothnick, W. B. 2000. Disruption of the tissue inhibitor of metalloproteinase-1 gene results in altered reproductive cyclicity and uterine morphology in reproductive-age female mice. Biol. Reprod. 63:905–912.

Nothnick, W. B. 2001a. Disruption of the tissue inhibitor of metalloproteinase-1 gene in reproductive-age female mice is associated with estrous cycle stage-specific increases in stromelysin messenger RNA expression and activity. Biol. Reprod. 65:1780–1788.

Nothnick, W. B. 2001b. Reduction in reproductive lifespan of tissue inhibitor of metalloproteinase 1 (TIMP-1)-deficient female mice. Reproduction 122:923–927.

Nothnick, W. B., P. Soloway, and T. E. J. R. Curry. 1997. Assessment of the role of tissue inhibitor of metalloproteinase 1 (TIMP-1) during the periovulatory period in female mice lacking a functional TIMP-1 gene. Biol. Reprod. 56:1181–1188.

Peiró, R., M. Merchán, M. A. Santacreu, M. J. Argente, M. L. García, J. M. Folch, and A. Blasco. 2008. Identification of single-nucleotide polymorphism in the progesterone receptor gene and its association with reproductive traits in rabbits. Genetics 180:1699–1705.

Peiró, R., M. A. Santacreu, A. Climent, and A. Blasco. 2007. Early embryonic survival and embryo development in two divergent lines selected for uterine capacity. J. Anim. Sci. 85:1634–1639.

Riffo, M., K. D. González, and A. Nieto. 2007. Uteroglobin induces the development and cellular proliferation of the mouse early embryo. J. Exp. Zool. A, Ecol. Genet. Physiol. 307:28–34.

Santacreu, M. A., M. A. Mocé, A. Climent, and A. Blasco. 2005. Divergent selection for uterine capacity in rabbits. II. Correlated response in litter size and its components estimated with a cryopreserved control population. J. Anim. Sci. 83:2303–2307.

Santacreu, M. A., M. P. Viudes, and A. Blasco. 1990. Evaluation par coelioscopie des corps jaunes et des embryons. Influence sur la taillé de portée chez la lapine. Reprod. Nutr. Dev. 30:583–588.

Satoh, T., K. Kobayashi, S. Yamashita, M. Kikuchi, Y. Sendai, and H. Hoshi. 1994. Tissue inhibitor of metalloproteinases (TIMP-1) produced by granulosa and oviduct cells enhances in vitro development of bovine embryo. Biol. Reprod. 50:835–844.

Schultz, G. A., and S. Heyner. 1993. Growth factors in preimplantation mammalian embryos. Oxf. Rev. Reprod. Biol. 15:43–81.

Sorensen, D., and D. Gianola. 2002. Likelihood, Bayesian, and MCMC Methods in Quantitative Genetics. Springer, New York, NY.

Takigawa, M., Y. Nishida, F. Suzuki, J. Kishi, K. Yamashita, and T. Hayakawa. 1990. Induction of angiogenesis in chick yolk-sac membrane by polyamines and its inhibition by tissue inhibitors of metalloprotinases (TIMP-1 and TIMP-2). Biochem. Biophys. Res. Commun. 171:1264–1271.

Torres, S., F. Hulot, and C. Sevellec. 1987. Early stages of embryonic development in two rabbit genotypes. Reprod. Nutr. Dev. 27:715–719.

Zhao, Y. G., A. Z. Xiao, and X. M. Cao. 2002. Expression of matrix metalloproteinase -2, -9 and tissue inhibitors of metalloproteinase -1, -2, -3 mRNAs in rat uterus during early pregnancy. Mol. Reprod. Dev. 62:149–158.