The final publication is available at

http://dx.doi.org/10.1111/age.12005

IDENTIFICATION OF DIFFERENTIALLY EXPRESSED GENES IN THE OVIDUCT OF TWO RABBIT LINES DIVERGENTLY SELECTED FOR UTERINE CAPACITY USING SSH

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1

Summary

Suppressive subtractive hybridization (SSH) libraries from oviduct at 62h post-mating of two lines of rabbits divergently selected for uterine capacity were generated to identify differentially expressed genes. A total of 438 singletons and 126 contigs were obtained by cluster assembly and sequence alignment of 704 ESTs, from which 54% showed homology to known proteins of the non-redundant NCBI databases. Differential screening by dot-blot validated 71 ESTs, of which 47 showed similarity to known genes. Transcripts of genes were functionally annotated in the molecular function and the biological process gene ontology (GO) categories using the Blast2GO software and were assigned to reproductive developmental process, immune response, amino acid metabolism and degradation, response to stress and apoptosis terms. Finally, three interesting genes, *PGR, HSD17B4* and *ERO1L* were identified as overexpressed in the Low line using RT-qPCR. Our study provides a list of candidate genes that can be useful to understand the molecular mechanisms underlying the phenotypic differences observed on early embryo survival and development traits.

INTRODUCTION

The mammalian oviduct is a dynamic reproductive organ that undergoes several modifications to provide an essential microenvironment required for the establishment of a pregnancy. These modifications are triggered by a network of endocrine, autocrine and paracrine factors during the estrous cycle that serve to facilitate final maturation of gametes, capacitation of spermatozoa, transport of gametes and embryos, fertilization and early cleavage-stage embryonic development (Buhi 2002; Killian 2004; Jeoung *et al*. 2010).

In a divergent selection experiment for uterine capacity (UC) in rabbits, High line (H) showed a higher UC (1.01 kits more) and litter size (2.35 kits more) than Low line (L) (Mocé *et al*. 2005 and Santacreu *et al*. 2005, respectively). These differences were due mainly to the higher embryo mortality before implantation in the L line (Mocé *et al.* 2005). The major part of the pre-implantational embryo mortality was produced before 72 h of gestation, when the embryos are in the oviduct (Mocé *et al*. 2004). Also, differences between lines in embryo development and mortality were found at 62h of gestation (Peiró *et al*. 2007). A reciprocal embryo transfer with embryos recovered at 72h of gestation between H and L lines showed that fetal survival was mainly affected by the maternal genotype (Mocé *et al*. 2004). Furthermore, no differences on ovulation and fertilization rates were found between H and L lines suggesting a direct effect of the oviductal function in the differences in embryo development and survival observed between both lines. As the main differences between lines appeared in the first two generations, the implication of at least one major gene affecting the UC and embryo survival was suggested (Argente *et al*. 2003). Several studies of candidate genes for litter size and its components, such as embryo development or survival, have been performed in the both divergently selected lines (Estelle *et al*. 2006; Merchán *et al*. 2009; Argente *et al*. 2010; García *et al*. 2010; Peiró *et al*. 2010). The main results of these studies

indicated that polymorphisms in *tissue inhibitor of metalloproteinase-1* (*TIMP-1*), *oviductal glycoprotein-1* (*OVGP1*) and *progesterone receptor (PGR)* are associated with the differences in embryo survival between H and L lines. Taking into consideration that interplay between several factors is acting in the oviduct to ensure embryo development and survival, a more extensive functional genomics approach was performed to compare patterns of gene expression in the H and L lines at 62 h of gestation.Thus, the aim of the present work was to identify differential expressed genes in oviduct tissue recovered 62 h after mating in two lines divergently selected for UC using a suppression subtractive hybridization technique (SHH).

MATERIALS AND METHODS

All experimental procedures involving animals were approved by the Polytechnic University of Valencia Research Ethics Committee.

Animal material

Animals used in this study were unilaterally ovariectomized (ULO) rabbits chosen from the 17th generation of the experiment of divergent selection for UC described in Argente et al. (1997). Uterine capacity was estimated as litter size in ULO females. The High (H) and Low (L) lines were selected for 10 generations (Argente *et al.* 1997), and then the selection was relaxed until the 17th generation. Six ULO females from the H line and 12 ULO females from the L line were selected for UC using a BLUP procedure on an animal-repeatability model (details of the model are given by Argente *et al.* 1997) and belong to the parental population of an F2 cross-made to determine the genetic basis of the divergence between these two lines. **Multiparous non lactating females were anesthetized to confirm the ovulation by laparoscopy and then, females were slaughtered by intravenous injection of sodium**

4

thiopental in a dose of 50 mg/kg body weight 62 h after mating. The oviduct was flushed with 5 ml of 150 mM ammonium bicarbonate buffer to remove the embryos and was entirely collected and immediately frozen in liquid nitrogen.

RNA isolation and cDNA synthesis

Total RNA was extracted from 100 mg of oviduct using the RiboPure kit (Ambion, Austin, TX). RNA was quantified with the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the RNA integrity was assessed by Agilent Bioanalyser 2100 and RNA Nano6000 Labchip kit (Agilent Technologies, Palo Alto, CA). For SSH, total RNA pools were generated from 3 H and 3 L lines oviducts and mRNA was further isolated via the MicroPoly(A)Purist Kit (Ambion). For RT-qPCR, 1 μg of total RNA of individual samples was reverse transcribed using the High Capacity cDNA Transcription Kit (Applied Biosystems, Inc., FosterCity, CA) in 20 μl reactions.

Suppression Subtractive Hybridization

SSH was performed using the PCR-Select cDNA Subtraction kit (Clontech, Takara Bio Company, Mountain View, CA). Forward and reverse subtractions were conducted. Briefly, 2 μg of mRNA of the H and L lines were used for double strand cDNA synthesis, and the resulting cDNA was digested with *Rsa*I. For the H-line subtracted library, cDNA from the H line (tester cDNA) was divided in two groups and linked to either adaptor 1 or adaptor 2R. Subtractive hybridization was performed by annealing an excess of the L-line cDNA (driver cDNA) with each sample of adaptor-ligated tester cDNA. The cDNAs were denatured at 98ºC for 1.5 min and incubated at 68ºC for 8 h. Following the first hybridization, the two samples were mixed together and hybridized again with freshly denatured driver cDNA at 68ºC overnight. The two rounds of hybridization would generate a population of molecules,

with different adaptors on each end, representing gene sequences preferentially expressed in the H line (tester).

In order to enrich for the desired differentially expressed cDNAs containing both adaptors, after filling in the ends, two rounds of PCR amplification were done. In the first amplification, PCR primer 1 was used to amplify 1µl of 40 fold diluted subtracted product in a final reaction volume of 25 µl; cycling conditions were: 94ºC for 25s and 30 cycles at 94ºC for 10s, 66ºC for 30s and 72ºC for 1.5 min. Amplified products were diluted 10-fold and 1 µl was added to a 25 µl reaction containing nested primers. Conditions for the second PCR were: 10 cycles at 94ºC for 10s, 68ºC for 30s and 72ºC for 1.5 min. All PCR reactions were carried out using Advantage cDNA polymerase mix (Clontech). The L-line subtracted library was constructed using the same approach but with cDNA from the L line as a Tester and cDNA from the H line as Driver. Hence, gene sequences preferentially expressed in the L line (tester) would be amplified.

Cloning

PCR-amplified subtracted cDNA populations (0.5 µl) were cloned separately into the PCR 2.1-TOPO vector of the TOPO TA cloning kit (Invitrogen, Carlsbad, CA) and transformed into TOP10 competent cells to establish different cDNA subtraction libraries. Bacteria were plated onto Luria Broth (LB) agar plates containing ampicillin and X-gal (5-bromo 4-chloro 3-indoyl-β-D-galactopyranoside) for blue/white screening. A total of 384 white colonies of each subtractive library were randomly selected, overnight grew on LB-ampicillin medium into 96-well plates and plasmid DNA was extracted using NucleoSpin96 Flash kit (Macherey-Nagel, Düren, Germany). All plasmid inserts were analyzed by *EcoR*I digestion and agarose gel electrophoresis.

Differential Screening

To confirm that the transcripts obtained were in fact increased in representation in the subtracted libraries, 192 transcript clones of each subtraction library were subjected to differential screening. For dot-blot hybridization, 2 identical positively charged nylon membranes (Roche Applied Science, Mannheim, Germany) were prepared using the Bio-Dot apparatus (BioRad, Hercules, CA). Plasmid DNA (1.5 µl) was denatured by adding 200 µl of denaturing solution (0.5 M NaOH -1.5 M NaCl) and incubating for 10 min at room temperature before spotting. Following a neutralization step with 200 µl of neutralization solution (1M Tris-HCl pH8, 1,5M NaCl), the membranes were washed on filter paper saturated in 2X SSC, UV cross-linked and stored at 4ºC. Two digoxigenin (DIG) labeled probes were generated from forward and reverse subtracted amplified products using the DIG High Prime DNA Labeling and Detection Starter kit II (Roche Applied Science). Prior to probe labeling, secondary PCR products were digested with *Rsa*I and purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) to remove adaptors. The membranes were hybridized overnight at 42ºC with 25 ng/ml of probe in DIG Easy Hyb. Replicates of each membrane were hybridized with forward and reverse subtracted labeled probes, respectively. Hybridization signals were captured using the *Luminescent Image Analyzer* LAS-3000 (Fujifilm, Tokyo, Japan) and quantified with Multi Gauge ver.3.0 software (Fujifilm). Over representation of a gene was confirmed when hybridization intensity was at least 1.2 fold greater when probed with labeled subtracted tester cDNA compared to the intensity of subtracted driver cDNA.

Sequencing and annotation

A total of 718 clones, 366 corresponding to the H-line subtracted library and 352 corresponding to the L-line subtracted library, were sequenced using primers M13 forward (-

20) and M13 reverse included in the TOPO TA cloning kit (Invitrogen). Sequencing was performed using the Big Dye Terminator v.3.1 Cycle Sequencing Kit in an ABI 3730 analyser (Applied Biosystems). Sequence chromatogram files were trimmed for quality, vector screened and assembled with Lasergene software (DNASTAR, Inc., Madison, WI). Blast2GO software (Conesa *et al*. 2005) was used to perform BLASTX (high scoring segment pair (HSP) length cutoff 33 and E-value cutoff of 1<e-6) and BLASTN (HSP length cutoff 150 and E-value cutoff of 1<e-15) similarity search against NCBI non-redundant (nr) database with a report of 20 hits.

Functional annotation was then performed by using Blast2GO in BLASTX matched sequences (parameters: E-value hit filter 1.0E-6, annotation cutoff 55, GO weight 5, HSP-hit coverage cutoff 0) (Conesa *et al*. 2005). Additionally, annotation was performed with the InterPro Scan specific tool implemented in the Blast2GO software and the ANNEX dataset to refine the functional annotations (Götz *et al*. 2008). GO terms were summarized according the three principal GO categories: cellular component, biological process and molecular functions. Enzyme mapping of annotated sequences was done by using direct GO to Enzyme mapping and used to query the Kyoto Encyclopedia of Genes and Genomes (KEGG) to define the main metabolic pathways involved (Kanehisa & Goto 2000; Kanehisa *et al*. 2008). GO enrichment analysis was performed by using the Fisher exact test integrated in Blast2GO package (Conesa *et al*. 2005).

Sequences have been deposited in the EST database of NCBI (accession numbers: JK749866 - JK750313)

Reverse Transcription Quantitative Real Time PCR (RT-qPCR)

RT-qPCR was used to verify the differential expression of 2 selected genes (*ANXA* and *OVGP1*) of the H-line subtracted library and of 3 selected genes (*ERO1L*, *HSD17B4* and

PGR) of the L-line subtracted library. Expression was analyzed in oviduct from 18 ULO female rabbits, 6 from the H and 12 from the L lines. We used the ABI PRISM 7900 Sequence Detection System in combination with SYBR Green chemistry (Applied Biosystems). Rabbit GAPDH mRNA was used as endogenous control (Estellé *et al.* 2006). Primers were designed using the primer Express 2.0 software (Applied Biosystems) and are shown in Table S1. The PCR amplicons were between 94 and 197 bp-long. We used the $2^{-\Delta\Delta CT}$ method for relative quantification (RQ) of gene expression (Livak & Schmittgen 2001), a comparative technique in which a target gene is normalized to an endogenous control and relative to a calibrator sample. This method requires the target and endogenous PCR efficiencies to be nearly equal. Thus, we performed validation experiments for each gene. We plotted the log input amount of cDNA (dilutions of 1:20, 1:200, 1:2,000, 1:20,000 and also 1:2 and 1:200,000 for some genes of an oviduct cDNA sample) versus the ΔCt, obtaining absolute slopes of: 0.0074, 0.0240, 0.0313, 0.0172 and 0.0344 for *ANXA*, *OVGP1*, *ERO1L*, *HSD17B4* and *PGR* genes, respectively. Absolute slopes < 0.1 in all cases hold the assumption that $2^{-\Delta\Delta CT}$ method may be used for relative quantification.

PCR amplifications were performed in a total volume of 20 μl containing 5 μl of cDNA sample diluted 1:1,000. FastStart Universal Sybr green master (Rox) (Roche Applied Science) was used in all reactions except in *PGR* amplification, where SYBR Green PCR Core reagents (Applied Biosystems) with 2.5 mM $MgCl_2$ were utilized. Primers were used at 300 nM each in all cases, apart from *PGR* amplification where primer concentration was 900 nM. Each sample was analyzed by triplicate. Thermal cycle was: 10 min at 95°C and 40 cycles of 15s at 95°C and 1 min at 60°C. A dissociation curve was drawn for each primer pair in order to assess that there were not primer dimer formation. The sample of lowest expression level in the first plate of each assay was used as calibrator.

Statistical Methods

The RT-qPCR data was analyzed across lines using the GLM procedure of SAS. Subsequent analyses were done using the test of Student-Newman-Keuls. Differences were considered statistically significant at $p < 0.01$.

RESULTS AND DISCUSSION

SSH libraries

In order to gain insight into the differences in embryo development and survival between two divergent rabbit lines selected for UC (the H and L), a SSH approach was performed to characterize the differential expression pattern in the oviduct at 62h of gestation. Oviductal mRNA from the L line was subtracted from the oviductal mRNA from the H line and *vice versa.* Three hundred sixty-six clones and 352 clones from the H and L-line subtracted libraries, respectively, were isolated and sequenced (most of them in forward and reverse directions) (Table 1). After sequence assembly, 67 contigs and 220 singletons from the H line and 59 contigs and 218 singletons from the L line were obtained with an average size of 600 bp. From both libraries, the majority of contigs (95.8%) contained 5 or fewer ESTs, whereas only two contigs from the H line were made from 8 and 12 ESTs and three contigs from the L line were made from 6 to 8 ESTs, indicating a high degree of normalization and subtraction efficiency.

Homologies were found in 95.1% and 95.7% of the transcripts from H and L-line subtracted libraries, respectively, when automatic sequence similarity search was done with Blast2GO software (BLASTN or BLASTX).The sequence redundancy within each library was low, 244 different sequences of the GenBank were represented in the H-line subtracted library (non-redundancy of 63.5%; Table S2) and 236 in the L-line subtracted library (non-redundancy of 66.5%; Table S3). When both libraries were analysed jointly, there were only 25 unigenes

repeated, indicating a very low level of redundancy between both libraries. Table 1 summarizes the global analysis performed in both SSH libraries.

Next, a functional annotation of both libraries was performed. BLASTX analysis of the 287 contigs and singletons of H line revealed that 150 ESTs (52.3%) displayed significant similarity to existing protein sequences. The Blast top-hits were from proteins of *Oryctolagus cuniculus* (48.7%). Furthermore, among these sequences, 139 unigenes were functionally annotated with 7 unigenes with only one GO term (5.0%). Similarly, 153 sequences (55.2%) from the 277 contigs and singletons of L line matched with known protein sequences (top hit species *Oryctolagus cuniculus*, 44.4%), of which 141 sequences were functionally annotated. Only 6 unigenes presented one GO term (4.2%).

Sequences from both libraries were assigned into the three principal GO categories: molecular function, biological process and cellular component (Fig.1). At the second GO level, the most dominant biological terms represented were "cellular process" (18% of sequences) and "metabolic process" (15%). Concerning the molecular function, "binding" (62%) and "catalytic activity" (31%) were the most represented terms. In the cellular compartments category, "cell" (36%) was the most represented term, followed by "organelle" (30%) and "macromolecular complex" (18%).

When a GO term Enrichment Analysis was performed using the list of genes annotated from both libraries, neither overrepresented nor underrepresented GO terms were identified between them. Different reasons could account for this result, such as the low percentage of matched sequences with the existing proteins available in the NCBI database, the high stringency used during the analysis or the existence of false-positive differentially expressed genes in the SSH libraries.

Differential screening

Next, in order to validate these differentially expressed transcripts, 192 clones of each subtraction library (the H and L lines) were subjected to differential screening by dot blot. With determination of greater signal intensity when probed with the labeled subtracted tester cDNA, 29 and 42 clones from the H and L-line subtracted libraries were identified, respectively. In the H-line subtracted library, the 29 clones matched to a total of 16 known genes (Table 2) and *OVGP1* and *MT-CO1* transcripts were represented 3 and 6 times, respectively. In the L line, the 42 clones matched to a total of 31 known genes (Table 3). *TCP1, CAT* and *ERO1L* transcripts were detected twice and *B2M* cDNA was detected five times.

For further interpretation of the results, a GO classification using Blast2GO software was performed with the differentially expressed genes. According to the molecular function, ESTs from both libraries were predominantly involved in "binding" (58%) and "catalytic activity" (31%; Fig. 2A). Concerning the biological process, the most represented categories were "cellular" (16%) and "metabolic processes" (14%; Fig. 2B). Reproduction related ESTs were found in 4% of the combined sequences (Fig. 2B), although the L line presented more differentially expressed genes (*UBC*, *SEP15*, *TCP1*, *CLU*, *HSD17B4* and *PGR*) than the H line (*RPS20*, *RPS4X*, *ENV* and *OVGP1*). Interestingly, the *OVGP1* and the *PGR* genes has been previously selected as candidate genes to explain the differences in early embryo survival and development observed between the divergent rabbit lines selected for UC using an F2 population (García *et al*. 2010; Peiró *et al*. 2010).

Furthermore, in the L line, differentially expressed genes related with the "immune response" term were also identified (*CLU*, *EPHX2, MX1* and *B2M*). It is well accepted that MHC class I molecules and B2M are involved in discrimination of self from non-self during pregnancy and a down-regulation of their expression is beneficial to prevent fetus rejection (Ramsoondar

et al. 1999; Joyce *et al*. 2008). A higher expression of this gene may be related with the reduction of embryo survival observed in the rabbits of the L line; however, further investigation is required to verify this hypothesis. In order to confirm the differential expression profiles observed between both lines, a RT-qPCR analysis was performed with five genes (*ANXA* and *OVGP1* of the H-line subtracted library and *ERO1L, HSD17B4* and *PGR* of the L-line subtracted library) and 18 ULO female rabbits (6 from the H and 12 from the L lines). Genes were selected according their reproductive function (*OVGP1*, *HSD17B4* and *PGR*) and the highest signal intensity by dot-blot (*ANXA* and *ERO1L*). While the 3 selected SSH-enriched genes from L line, *ERO1L, HSD17B4* and *PGR,* were confirmed by RT-qPCR as overexpressed (p<0.01; Fig.3), *ANXA* and *OVGP1* were not confirmed as differentially expressed in the oviduct of H line compared to the L line, supporting results obtained in a previous study for the *OVGP1* gene (Merchán *et al*. 2009). However, when only RT-qPCR expression values from the females used for SSH construction (3 from each line) were analyzed, both genes exhibited higher levels of expression (1.5 fold and 2-fold increase in *OVGP1* and *ANXA1* expression, respectively) in the H line compared with the L line (data not shown). Several factors may explain this result including: 1) a sampling effect in the selection of the animals for SSH, 2) the inclusion in the RT-qPCR analysis of animals with less extreme phenotypes or 3) divergent selection for UC was more effective in the L line than in the H line (Argente *et al*. 1997; Santacreu *et al*. 2005; Mocé *et al*. 2005) and hence, animals from the H line showed more variability in gene expression because they may be less genetically uniform than L animals. Of the 3 validated genes by RT-qPCR from L line, *HSD17B4* and *PGR* are involved in "reproductive development process". HSD17B4 is a multifunctional protein, ubiquitously expressed, implicated in steroid and fatty acid metabolism (Breitling *et al*. 2001). In pigs, progesterone has been identified as a positive regulator of this enzyme in the uterus, where the HSD17B4 catalyses the oxidation of 17β-

estradiol to estrone (Adamski *et al*. 1992; Kaufmann *et al*. 1995) providing a good endometrium environment for embryo implantation. However, to our knowledge, this is the first study in which the expression of HSD17B4 has been described in the rabbit oviduct albeit the expression of other proteins of the same family has been identified (Krusche *et al*. 2001). Taking into account the steroid inactivation role of the HSD17B4 protein, we could hypothesize that overexpression of *HSD17B4* in the oviduct of L-line rabbits could modify the estrogen concentration, changing the oviduct fluid composition and subsequently affecting the early embryonic development (Murray 1993; Killian 2004; Gad *et al*. 2011). Similarly, changes in steroid concentration could also modify the expression of *PGR*, causing the desynchronization of oviductal and uterine environments and affecting the posterior development and implantation of embryos (Gad *et al*. 2011; Waclawik 2011). *ERO1L* gene is involved in the "oxidation-reduction process". It is essential for maintain redox homeostasis within endoplasmic reticulum (ER) and this is necessary for the proper folding of many secreted proteins through disulphide bond formation (Sevier & Kaiser 2008). An overexpression of *ERO1L* can produce an increase of reactive oxygen species in the ER (Gross *et al*. 2006), generating oxidative stress and cell death (Haynes *et al*. 2004). Taking into account that proteins constitute a major component of oviduct secretions, the maintenance of redox conditions in oviduct cells would be relevant for the proper folding and secretion of those oviductal proteins which are required for reproductive function. The overexpression of *ERO1L* in the L line could be associated with the reduction of embryo survival and development observed in the rabbits of the L line. In fact, transcripts of genes involved in "amino acid metabolism and degradation" (*HSD17B4*, *CRYL1* and *CAT*), "response to stress" (*SEP15*, *EPHX2*, *ERO1L*, *HSP90B1…*) and "apoptosis" (*MX1*, *CLU*, *GHITM*, *UBC…*) were also identified in the L line as differentially expressed. In conclusion, the present study provides a list of candidate genes differentially expressed in the oviduct of

two lines of rabbits divergently selected for UC. The genes overexpressed in the L line seem to be related to a decrease of early embryo development and survival, in agreement with previous results found in this line (Blasco *et al*. 2005; Mocé *et al*. 2005; Santacreu *et al*. 2005; Peiró *et al*. 2007). However, further studies are necessary to elucidate the relevance of those genes and proteins on rabbit reproductive traits.

Acknowledgments

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Table 1. Global analysis of the clones generated by Suppression Subtractive
Hybridization

SSH collections	H library	Llibrary
Number of clones sequenced	366	352
Number of clones sequenced forward and reverse	356	346
Number of clones sequenced only forward or reverse	10	6
Number of contigs	67	59
Singletons	220	218
Number of contigs and singletons matching sequences of the GenBank (BLASTN or BLASTX)	273 (95.1%)	265(95.7%)
Number of different sequences of the GenBank represented	244	236
Number o sequences of the GenBank represented by more than one clone	36.5%	33.5%

Table 2. Differential expressed genes identified in the oviduct of H rabbit line by SSH and dot-blot (differential screening).

Nº of clones	Transcript	Gene Symbol	Species	AccessionNumber	E-Value
1	*annexin A1*	*ANXA1*	Oryctolagus cuniculus	NP_001164623	7.53E-160
1	*armadillo repeat-containing protein 4*	*ARMC4*	Oryctolagus cuniculus	XP_002710027	9.20E-124
1	*Cancer susceptibility candidate 3*	*CASC3*	Nomascus leucogenys	XM_003278263	7.53E-89
6[a]	*cytochrome c oxidase subunit I*	*MT-CO1*	Lingulodinium polyedrum	AEF98135.1	2.81E-104[b]
1	*elongation factor 1-gamma*	*EEF1G*	Macaca mulatta	XP_001095725	9.31E-36
1	*Envelope protein*	*ENV*	Feline leukemia virus	BAK41631	2.39E-11
1	*Formin binding protein 4*	*FNBP4*	Pan troglodytes	XM_508418	2.95E-103
1	*integral membrane protein 2B*	*ITM2B*	Oryctolagus cuniculus	XP_002720768	1.05E-115
1	*mitogen-activated protein kinase 10*	*MAPK10*	Ailuropoda melanoleuca	XM_002913591	0.0
1	*NADH dehydrogenase subunit 1*	*MT-ND1*	Oryctolagus cuniculus	NP_007549	2.45E-73
1	*NADH dehydrogenase subunit 4*	*MT-ND4*	Oryctolagus cuniculus	NP_007558	3.78E-99
3[a]	*oviduct-specific glycoprotein*	*OVGP1*	Oryctolagus cuniculus	NP_001075574	2.29E-164[b]
1	*40S ribosomal protein S20 isoform 2*	*RPS20*	Pan troglodytes	XP_001154691	6.44E-61
1	*40S ribosomal protein S4*	*RPS4X*	Meleagris gallopavo	XP_003205701	8.90E-73
1	*succinate dehydrogenase cytochrome b560 subunit*	*SDHC*	Macaca mulatta	XP_001093391	7.95E-84
1	*Signal recognition particle 72 kda*	*SRP72*	Callithrix jacchus	XM_002745814	8.66E-66

[a]Clones corresponding to two different contigs or singletons
[b]Best value

Table 3. Differential expressed genes identified in the oviduct of L rabbit line by SSH and dot-blot (differential screening).

Nº of clones	Transcript	Gene Symbol	Species	AccessionNumber	E-Value
1	*15 kda seleno protein*	*SEP15*	Homo sapiens	CAC04186	3.24E-64
1	*actin, alpha cardiac muscle 1*	*ACTC1*	Homo sapiens	EAW92317	1.76E-49
1	*actin gamma 1*	*ACTG1*	Pan troglodytes	BAF62404	1.49E-17
1	*Arsenate reductase*	*ARSC*	Bacillus sp. Rice-C	CAQ48291	7.56E-06
1	*ATP H+ mitochondrial F1 gamma polypeptide 1*	*ATP5C1*	Oryctolagus cuniculus	XP_002717372	7.39E-126
5[a]	*beta-2-microglobulin precursor*	*B2M*	Oryctolagus cuniculus	XP_002717967	1.18E-56[b]
2[a]	*catalase*	*CAT*	Campylobacter jejuni	CAA59444	1.31E-10
1	*Kynurenine aminotransferase III*	*KATIII*	Oryctolagus cuniculus	XP_002715920	5.66E-155
1	*clathrin heavy chain 1*	*CLTC*	Oryctolagus cuniculus	XM_002719087	0.0
1	*clusterin*	*CLU*	Oryctolagus cuniculus	NP_001075518	8.65E-19
1	*Component of oligomeric Golgi complex 3*	*COG3*	Homo sapiens	NM_031431.3	3.45E-59
1	*lambda-crystallin*	*CRYL1*	Oryctolagus cuniculus	NP_001075747	1.90E-49
1	*EFR3 homolog A*	*EFR3A*	Pan troglodytes	XM_003311924	2.43E-123
1	*eukaryotic translation initiation factor 3 subunit I*	*EIF3I*	Synthetic construct	AAX41399	3.82E-152
1	*Epoxide hydrolase 2*	*EPHX2*	Oryctolagus cuniculus	XP_002709497	1.74E-126
2[a]	*ERO1-like*	*ERO1L*	Oryctolagus cuniculus	XP_002718351	4.10E-155[b]
1	*fibrinogen like-2*	*FGL2*	Homo sapiens	NM_006682	1.70E-49
1	*fus-interacting serine-arginine-rich protein 1*	*FUSIP1*	Pan troglodytes	BAK62032	2.32E-10
1	*growth hormone inducible transmembrane protein*	*GHITM*	Nomascus leucogenys	XM_003274525.1	1.94E-33
1	*hydroxysteroid (17-beta) dehydrogenase 4*	*HSD17B4*	Oryctolagus cuniculus	XP_002710444	1.33E-90
1	*Heat shock protein 90kDa beta (Grp94), member 1*	*HSP90B1*	Oryctolagus cuniculus	AF001631.1	0.0
1	*Lysosomal protein transmembrane 4 alpha*	*LAPTM4A*	Oryctolagus cuniculus	NM_001082639	0.0
1	*leucine zipper transcription factor-like 1*	*LZTFL1*	Oryctolagus cuniculus	XP_002713363	9.56E-88
1	*MIR143 host gene*	*MIR143HG*	Homo sapiens	NR_027180	1.38E-12
1	*Myxovirus resistance protein 1*	*MX1*	Oryctolaguscuniculus	XP_002723805	7.01E-41
1	*progesterone receptor*	*PGR*	Homo sapiens	X06624	0.0
1	*ras-related protein Rab-6A*	*RAB6A*	Nomascus leucogenys	XM_003254741	2.86E-62
2[a]	*t-complex 1*	*TCP1*	Homo sapiens	EAW47617	2.03E-53[b]
1	*ubiquitin C*	*UBC*	Bos taurus	BAC56573	1.68E-88
1	*vacuolar sorting protein 35*	*VPS35*	Homo sapiens	AAG01989	7.57E-108
1	*zinc finger, AN1-type domain 5*	*ZFAND5*	Nomascus leucogenys	XM_003267410	0.0

[a]Clones corresponding to two different contigs or singletons
[b]Best value

Figure legends

Figure 1. GO terms distribution of sequences from both H and L-line subtracted libraries according to molecular function, biological process and cellular component.

Figure 2. GO terms distribution in the biological process (A) and molecular function (B) of ESTs differentially expressed by dot blot from H and L-line subtracted libraries.

Figure 3. Relative abundance of 3 oviductal transcripts identified as overexpressed in the L rabbit line after SSH and differential screening (*ERO1L, HSD17B4,* and *PGR*). Open bars denote Low (L) rabbit line samples, while solid bars denote High (H) rabbit line samples. Mean + SEM is represented for each group with **p<0.01 and ***p<0.001 indicating statistical differences. Relative transcript levels were normalized to *GADPH*.