Refined Mapping of a Quantitative Trait Locus on Chromosome 1 Responsible for Mouse Embryonic Death

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Abstract

Recurrent spontaneous abortion (RSA) is defined as the loss of three or more consecutive pregnancies during the first trimester of embryonic intrauterine development. This kind of human infertility is frequent among the general population since it affects 1 to 5% of women. In half of the cases the etiology remains unelucidated. In the present study, we used interspecific recombinant congenic mouse strains (IRCS) in the aim to identify genes responsible for embryonic lethality. Applying a cartographic approach using a genotype/phenotype association, we identified a minimal QTL region, of about 6 Mb on chromosome 1, responsible for a high rate of embryonic death (~30%). Genetic analysis suggests that the observed phenotype is linked to uterine dysfunction. Transcriptomic analysis of the uterine tissue revealed a preferential deregulation of genes of this region compared to the rest of the genome. Some genes from the QTL region are associated with VEGF signaling, mTOR signaling and ubiquitine/proteasome-protein degradation pathways. This work may contribute to elucidate the molecular basis of a multifactorial and complex disorder as RSA.

Introduction

Embryonic development in mammals begins from the female and male interaction which leads to the oocyte fertilization. After 5 to 6 cell divisions inside the zona pellucida, the blastocyst undergoes its development concurring to the implantation in the uterine tissue. The external cells of the blastocyst develop into the placenta, a pivotal organ which allows immune tolerance, bidirectional foeto-maternal exchanges and crucial synthesis of gestational hormones [1]. All these biological processes are required for the survival of every mammalian species, and logically, they underlie a high level of complexity. Dysfunctions in these processes can lead to infertility. In humans it is a considerable public health problem, affecting up to 15% of couples. Due to the number of factors involved in a successful reproductive process, the mechanisms of infertility are far to being completely understood.

At present, although hundreds of mutant mouse models with reproductive phenotypes have been generated [2] and substantial progress has been made in the identification of genetic causes of human infertility, more than 70% of the cases are still considered as idiopathic [3]. Among these, recurrent spontaneous abortion (RSA) (defined by the occurrence of at least three successive pregnancy losses) affects one to five percent of couples [4]. This pathology can be the result of chromosomal anomalies [5], maternal and fetal structural abnormalities [6,7], thrombophilic disorders [8] and autoimmune disorders such as the antiphospholipid syndrome [9]. However, in fifty percent of the cases the etiology remains unknown [10,11]. Up to now, RSA genetic causes have already been explored with variable degrees of success. For instance, in 2006, Kaare et al. analyzed the entire open reading frame of the Amonialess gene (AMN) in patients affected by RSA but no causal mutations could be identified [12]. More recently, the study of Mercier et al. described a statistical association between the p.Val617Phe mutation of the Janus kinase 2 protein and RSA [13]. All in all, the intrinsic difficulty to genetically dissect mammalian reproductive phenotypes, in which hundreds of genes interact into subtle regulatory networks, has not permitted to identify etiological molecular factors that could explain a significant proportion of infertility cases.

In recent years, in order to overcome these constraints we created an original mouse model of interspecific recombinant congenic strains (IRCS) which permit to localize chromosomal regions associated with complex phenotypes (Quantitative Trait Loci or QTL) [14]. This model is composed of 53 strains of mice which harbor, on average, 2% of Mus spretus SEG/Pas genome fixed at homozygous state on Mus musculus C57Bl6/J (B6) genomic background. Using IRCS animals we have previously shown that 5 QTL of embryonic lethality mapped on a unique segregant fragment in 3 strains, 66H-MMU13, 66H-MMU1 and 135E. The first, Led1 in 66H-MMU13 strain on the MMU13 (~2.6 Mb) comprised between the rs120693734 and D13Mit47 polymorphic markers. The second, Led2 in 66H-MMU1 was analyzed in the present study and the third, Led3 located on MMU19...
Microsatellite Genotyping

The MMU1 strain, which encompasses a unique Spretus chromosomal fragment located on MMU1 is affected by high levels of embryonic death (24.6%). This strain encompasses a QTL of embryonic lethality (named Led2) spreading on 32 Mb and containing 215 genes (143 annotated and 72 predicted) [15].

Here, we present a thorough genetic dissection of Led2. For this purpose, we created 15 substrains from 66H-MMU1 animals, which encompass distinct overlapping Spretus fragments. Using in vivo high frequency ultrasonography to follow the embryonic development, we used an approach of type “phenotype/genotype association” to refine this QTL of embryonic death. We identified, into the Led2 QTL, one region of approximately 6 Mb, Led2miniB, which has a main effect on the rate of embryonic death. In addition, we pointed out a second region, Led2miniA, which could also have a small effect on the phenotype, although statistically not demonstrated.

Materials and Methods

Ethics Statement

Procedures for handling and experimentation were conducted in accordance with the policies of the Paris Descartes University, the Cochin Institute and the Guidelines for Biomedical Research Involving Animals. The experiments were approved by the departmental veterinary services of Paris (approval number: A75 14-02).

Animals

The 66H-MMU1 strain was created at the Pasteur Institute (Paris) by successive crosses of the two parental species Mus musculus (C57BL6/J) and Mus spretus SEG/Pas (originating initially from Spain). The design of these crosses was reported in a previous work [16]. For this study, 15 new recombinant substrains were generated by backcrosses of 66H-MMU1 with C57BL6/J mice. After weaning, 4 weeks aged mice were maintained in an animal facility of the Cochin Institute (Paris) at normal temperature (21–25°C) and 14 h light/10 h dark photoperiods with free access to water and food.

Microsatellite Genotyping

DNA was extracted from mouse tail fragments by a standard procedure. Eight new microsatellites located on MMU1 (D1Mit439, D1Mit183, D1Mit44, D1Mit383, D1Mit8, D1Mit384, D1Mit255, D1Mit1438) were genotyped in order to precise the boundaries of the Spretus segment present in the 66H-MMU1 genome. Primer microsatellites were retrieved from the Mouse Genome Informatic website (MGI) website of the Jackson Laboratory (www.informatics.jax.org). PCRs were performed using Taq DNA Polymerase (New England Biolabs). PCR products were loaded in a 2% Nusieve, 2% agarose gel (Cambrex and tissues were taken at E12.5. Total RNA of uterine tissue from six mice of the IRC strain of interest was extracted using TRizol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer’s instructions. Similarly, six B6 were used to extract total RNA. In order to duplicate microarray hybridizations of samples from uterine tissues, two pools of three RNA extractions were created for both IRC5 and B6 animals. One microgram of RNA from IRC substrains of interest and the B6 controls was used for hybridization on a NimbleGen expression array. cDNA synthesis, DNA end-labeling, hybridization, scanning, and data normalization were performed at the genomic/transcriptomic platform of the Cochin institute. The average fluorescence values for each transcript were collected chromosome per chromosome for each analyzed strain. To evaluate gene expression modifications in IRC strains, these fluorescence levels (considered as expression values) were divided gene per gene by the corresponding ones from B6 which were taken as a reference. The results of the gene expression were deposited at GEO (NCBI) with the accession reference GSE32460.

cDNA Synthesis by Reverse-transcription and Quantitative RT-PCR

After RNA preparation, the total RNA was treated with DNase I (Invitrogen Life Technologies) for 10 min at room temperature followed by inactivation with EDTA (Sigma-Aldrich). Total uterus RNA was reverse transcribed to obtain cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) following manufacturer’s protocols.

Quantitative PCR was carried out using fast SYBR Green Master Mix (Applied Biosystems) and a real time PCR system (Light Cycler 1.5, Roche Diagnostics, Division Applied Sciences, Meylan, France) according to standard PCR conditions. To validate the primers used in qRT-PCR, four pairs of primers were tested for each gene and four housekeeping genes were also tested to choose the reference gene (Table S1 in supplemental data). For quantitative calculations, values were normalized to mouse Cyclophilin A expression. Primer sequences are listed in Table S1.
Statistical Analysis

Results are expressed as mean±SEM calculated from the variation between individual female. The statistical significance of the differences observed between the mean values of IRCS and the control group (C57BL/6j) samples was evaluated by t-test using the Bonferroni-corrected levels. As we used 7 substrains, a p value <0.007 (0.05/7) was considered as significant. Statistically significant results are labeled as follows in all figures: *: p<0.05; **: p<0.01; ***: p<0.001.

Results

Creation of 66H-MMU1 Substrains

We started our study using the 66H-MMU1 strain which harbors a high rate of embryonic lethality (24.6%) caused by the Led2 QTL [15]. This QTL of ~32 Mb, which was initially delimited by D1Mit50 (87.0 Mb) and rs6259837 (119.1 Mb) markers located on chromosome 1, corresponds to a spreutus fragment carried by the 66H-MMU1 substrain. However, a uncertainty of ~6.4 Mb existed at the proximal boundary of this QTL since the interval comprised between D1Mit134 (80.6 Mb) and D1Mit50 (87.0 Mb) markers corresponds to this distance and the breakpoint is located somewhere between these two markers. Indeed, D1Mit134 and D1Mit50 allele markers are of B6 and spreutus natures respectively (http://www.pasteur.fr/recherche/unites/Gloos/irсs/ircshome.htm). In an attempt to precise the position of the breakpoint, we genotyped 8 novel markers located on this region that permitted to reduce the recombination region to a 2.5 Mb interval comprised between 84.5 Mb and 87 Mb (markers D1Mit438 and D1Mit50 respectively, see Figure 1). Then, we initiated a fine mapping approach using fifteen recombinant substrain issued from 66H-MMU1 animals. In each of these strains, a crossing-over fragmented the original DNA region of spreutus origin that was initially present in the 66H-MMU1 strain (Figure 1). Among 15 starting strains, seven survived and were available for our study (recombinants, R3, R4, R5, R6, R10, R13 and R14).

In vivo Phenotyping

Females of the different recombinant substrains were crossed with B6 males and their gestation was followed up using in vivo ultrasonography as previously described [15]. Animals obtained from these crosses have a placenta which is heterozygous for all the genes located on the fragment of spreutus origin (B6/SEG), while the uterus was homozygous SEG/SEG for the same spreutus fragment. The control group was obtained by crossing males and females of the B6 strain. A total of 97 gestations (31 and 66 of B6 and IRCS types respectively) were analyzed. For each gestation, we counted the number of implanted and resorbed embryos during three ultrasonographic examinations. There was no correlation between embryonic death and the position inside the womb, which suggests that the death of one embryo did not have deleterious repercussions on the contiguously implanted structures (Figure 2).

We noted a strong variability in the percentage of embryonic death between the different substrains (Table 1), while apparently the number of implanted embryo was not significantly different. The strains R4, R6, R10, R13 and R14 (group 1) presented a percentage of embryonic death that was not statistically different from that of B6 control animals (10±4%, 19.2±5%, 9±3%, 12.5±5% and 16±3% for the five strains, respectively, versus 12.2±2% in the B6 parent). Note that within this group, R6 is distinguished by the highest rate of embryonic death. By contrast, R3 and R5 strains (group 2) presented a percentage of embryonic death (27±5% and 29±6% respectively) significantly higher than B6 control at p = 0.0013 and p = 0.0045 respectively (Table 1). Since the mean number of implanted embryos was not different between the substrains compared to the B6 control (Table 1), we deduced that the increase in embryonic death observed for R3 and R5 IRCS was caused by post-implantation events.

QTL Fine Mapping

In order to refine Led2 localization, we realized an analysis by genotype/phenotype segregation. R3 and R5 strains (which exhibit the phenotype) shared a large spreutus region (>84.5 Mb to 90.5 Mb), with the R6 strain (which does not display the embryonic resorption phenotype) and the rest (until <100.3 Mb) is also shared with the other strains (R4, R10, R13 and R14) which are not affected. This configuration suggests that two spreutus regions, shared by R3 and R5 strains and not present together in the other strains, seem to be necessary to explain the apparition of the phenotype in R3 and R5. We defined a first spreutus subfragment called Led2minA which encompasses D1Mit50 to...
D1Mit305 region (>84.5 Mb to <90.5 Mb) and a second region called Led2minB located at the rs3692309 marker (>92.5 Mb to <100.3 Mb) (see gray boxes in Figure 1). When these two spretus regions (Led2min) are separated as in R6 (that contains Led2minA only) or in R4, R10, R13 or R14 (Led2minB only) the phenotype of embryonic death is absent. The presence of the two spretus regions seems indispensable to permit the manifestation of the phenotype, it’s the case for R3 and R5 (gray hatched boxes in Figure 1). To statistically prove the presence of these two QTLs, Led2minA and Led2minB each one responsible for a part of the effect on the phenotype, and an eventual epistatic interaction between these two QTLs able to increase the embryonic death, we compared several recombinants among themselves, R6 bearing Led2minA, R4 bearing Led2minB and R3 (or R5) bearing these two spretus regions.

The results of statistical t-tests are shown in Figure 3. When we compared the mean rate of embryonic death between R4 and R3 (or R5), the statistical result (significant difference at $P \leq 0.01$) proved the presence of Led2minA QTL. By contrast, the comparison between R6 and R3 (or R5), did not statistically indicate the presence of Led2minB QTL. However the embryonic death rates of R3 and R5 both have a tendency to be higher than that of R6 (Figure 1) suggesting a possible very small effect of Led2minB on the phenotype. In the same way, the difference in embryonic death rate between R4 (Led2minB only) and R3 (or R5) was 17%–19% which is comparable to 15%–17% difference between B6 (with no spretus regions) and R3 (or R5) also suggesting a nil or very small effect of Led2minB. In consequence this result did not support the presence of an epistatic interaction

**Figure 2. Ultrasound biomicroscopic in vivo observation of the embryonic development.** During the gestation of B6 and IRC mice the embryonic development was followed up by an in vivo ultrasonic method. The viability of developing embryo (A) was assessed by the presence of heartbeats and a positive umbilical cord Doppler. Dead embryos (B) displayed a central highly echogenic zone (C) corresponding to the embryonic resorption.

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**Table 1.** Statistical analysis of the embryonic resorption phenotype.

| Strains | Number of gestations | Implanted embryos; Mean ± SEM (p value) | Embryonic resorption rate; Mean ± SEM (p value) |
|---------|----------------------|------------------------------------------|-------------------------------------------------|
| B6      | 31                   | 7.90 ± 0.41 (p = 0.041)                  | 12% ± 2 (p = 0.2848) |
| R3      | 13                   | 7.46 ± 0.50 (p = 0.2717; NS)             | 27% ± 5 (p = 0.0013) |
| R4      | 15                   | 7.20 ± 0.71 (p = 0.1845; NS)             | 10% ± 4 (p = 0.3021; NS) |
| R5      | 4                    | 9.50 ± 0.50 (p = 0.0932; NS)             | 29% ± 6 (p = 0.0045) |
| R6      | 6                    | 8.50 ± 0.99 (p = 0.2848; NS)             | 19% ± 5 (p = 0.1158; NS) |
| R10     | 8                    | 9.12 ± 0.51 (p = 0.0828; NS)             | 9% ± 3 (p = 0.2188; NS) |
| R13     | 12                   | 8.83 ± 0.45 (p = 0.1045; NS)             | 12% ± 5 (p = 0.4349; NS) |
| R14     | 8                    | 8.75 ± 0.70 (p = 0.1759; NS)             | 16% ± 8 (p = 0.2351; NS) |

Comparison between IRCs and B6 control using t-test with Bonferroni-corrected level. (NS: non-significant).
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between Led2minA and Led2minB regions, but a Led2minB additive effect could be revealed by increasing sample size of this “QTL” representative strains. For this reason, the genes present in these two regions, Led2minA and Led2minB are listed in Table 2.

Is Placenta or Uterus Responsible for the Led2min Effect?

Since R3 and R5 mice did not harbor any obvious developmental anomaly or pathology, excepting for some embryonic death, it was reasonable to suspect that placental and/or uterine dysfunctions could be responsible for the embryonic lethality increase. Thus, we initiated a genetic approach in order to identify in which of these two organs dysfunction could be related with the phenotype. In the previous set of experimental crosses, IRCs females were mated with B6 males. Genetically, this permitted the co-existence of heterozygous foeto-placental alleles (B6/SEG) and homozygous uterine alleles (SEG/SEG) within the same genomic region (spretus fragment). Conversely, we performed reverse crosses (B6 × IRCs), giving a heterozygous placenta for the genes of the fragment, but a B6 homozygous uterus. In this situation, the would-be disorders ought to find their origin exclusively from a placental-fetal/embryonic defect, caused by the spretus state of the MMU1 fragment, but not from a B6 womb defect. In this optic we realized the cross B6 with × IRCs (IRCS group2). We observed that the mean of embryonic resorption rate (±SEM) was 0.97±0.04 and not significantly different from the control (B6 × IRCs: 0.12±0.02; p = 0.118) whereas the inverse crossing, leading also to a heterozygous foeto-placental complex implanted in homozygous spretus uterus (R3), produced a significantly higher embryonic resorption rate (0.27±0.05, p = 0.001) (Figure 4). From this last observation, we concluded that a uterine dysfunction is very likely at the basis of the observed phenotype.

Assessing of Differentially Expressed Genes in Uterus

We analyzed the expression level of uterine genes in pregnant R3 females compared to those from B6 control animals at E12.5, an important time point when most resorption occurred during our study. For this purpose, we hybridized cDNA synthesized from RNA uterine tissue to Nimblegen mouse microarrays. The transcriptome was interrogated a total of 25,631 mouse transcripts. Gene expression levels were quantified by fluorescence intensity assessing and ranged from 20 to >50,000 arbitrary units of fluorescence (AUF) (mean value ~5,250 AUF). These results were highly reproducible since they showed strong correlations between experimental duplicates (r = 0.967 for B6 and 0.983 for R3). Thus, for subsequent analysis, we took the average of both values for each transcript. We first focused on transcripts with fluorescence levels higher than 100 AUF, we assumed that values under this threshold were very close to background signals and, with this threshold, we selected 18,085 transcripts (70.6% of the total). We considered a gene as differentially expressed if a two-fold difference of expression (up or down) was observed. Consequently, 3,436 (19% of the expressed uterine genes) transcripts were modified in R3 uterus when compared to those expressed in B6 (Table 3). A similar number of repressed and induced genes was observed (10.9% and 8.1%, respectively). This deregulation was identified over all the genome. However a significantly higher proportion of genes were deregulated when only the MMU1 spretus fragment or the two Led2min regions were considered (Table 3).

In order to validate the differential gene expression obtained by the microarray analysis, we checked 7 genes of the Led2minA QTL region by quantitative RT-PCR. As shown in Table 4 we obtained a very good agreement between microarray and qPCR results.

Considering that the uterine dysfunction can take its origin from a deregulation of the gene expression or/and non-synonymous coding polymorphisms accumulated during independent evolutive processes of Mus musculus and Mus spretus species, we listed the genes of the Led2min QTL corresponding to these criteria and thus potentially involved in embryonic resorption. Finally, we considered the transcripts with an expression level >500 AUF and either exhibiting a deregulation (R3/B6 expressive ratio >2 or <0.5) and the presence of non synonymous polymorphisms between Mus musculus and Mus spretus (provided by SANGER database: http://www.sanger.ac.uk/) (Table 5).

Then, we searched whether deregulated transcripts could be grouped into functional clusters using DAVID database [17], considering 1758 transcripts with a threshold of >500 AUF in the expression level. This analysis led to the identification of five functional groups of genes and signaling pathways that were deregulated, such as ribosome protein genes (p value: 0.00054), endocytosis process (p value: 0.0027), VEGF (vascular endothelial growth factor) (p value: 0.00054), TGF-β signaling pathway (p value: 0.0027), and the presence of non synonymous polymorphisms between Mus musculus and Mus spretus.
Table 2. Gene list in the minimal Led2min region.

| Start of the gene | End of the gene | Gene symbol | Gene name |
|-------------------|----------------|-------------|-----------|
| Led2minA          | 84600220       | Trip12      | thyroid hormone receptor interactor 12 |
|                   | 84618314       | Fbxo36      | F-box protein 36 |
|                   | 84785729       | Scl16a14    | solute carrier family 16 (monocarboxylic acid transporters), member 14 |
|                   | 84906589       | LOC665317   | LOC665317 |
|                   | 84967431       | A3S0021D15Rik | A3S0021D15Rik |
|                   | 84990836       | A3S0040E14Rik | A3S0040E14Rik |
|                   | 85035923       | LOC624074   | LOC624074 |
|                   | 85075992       | LOC665378   | LOC665378 |
|                   | 85125792       | LOC620078   | LOC620078 |
|                   | 87313813       | LOC665338   | LOC665338 |
|                   | 87327496       | LOC665408   | LOC665408 |
|                   | 87408069       | Sp110       | nuclear body protein |
|                   | 87431873       | LOC434484   | LOC434484 |
|                   | 87481184       | Sp100       | nuclear antigen Sp100 Gene |
|                   | 87548251       | A630001G21Rik | A630001G21Rik |
|                   | 87568115       | LOC651657   | LOC651657 |
|                   | 87626533       | Cab39       | calcium binding protein 39 Gene |
|                   | 87725735       | Itm2c       | integral membrane protein 2C Gene |
|                   | 87759653       | 4933407L21Rik | 4933407L21Rik |
|                   | 87770707       | Gpr55       | G protein-coupled receptor 55 Gene |
|                   | 87852883       | Spata3      | spermatogenesis associated 3 Gene |
|                   | 87877133       | 2810459M11Rik | 2810459M11Rik |
|                   | 87895789       | Psmd1       | proteasome (prosome, macropain) 26S subunit, non-ATPase, 1 |
|                   | 87930441       | Htr2b       | 5-hydroxytryptamine (serotonin) receptor 2B |
|                   | 87985962       | Armc9       | armadillo repeat containing 9 Gene |
|                   | 88134638       | B3gn17      | UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7 |
|                   | 88154437       | LOC383538   | LOC383538 |
|                   | 88175889       | Ncl         | nucleolin |
|                   | 88217495       | Nmur1       | neuromedin U receptor 1 |
|                   | 88257499       | 1700019O17Rik | 1700019O17Rik |
|                   | 88357906       | Ptma        | prothymosin alpha |
|                   | 88374185       | Pde6d       | phosphodiesterase 6D, cGMP-specific, rod, delta |
|                   | 88418270       | Cops7b      | COP9 (constitutive photomorphogenic) homolog, subunit 7b (Arabidopsis thaliana) |
|                   | 88497461       | Nppc        | natriuretic peptide type C |
|                   | 88535013       | 4930429A22Rik | 4930429A22Rik |
|                   | 88681263       | Dis3I2 : DIS3 mitotic control homolog (S. cerevisiae)-like 2 |
|                   | 88739906       | LOC620213   | LOC620213 |
|                   | 88917860       | Akp5        | Alpl2 alkaline phosphatase, placental-like 2 |
|                   | 88929174       | Alpi        | alkaline phosphatase, intestinal |
|                   | 88956178       | Akp3        | alkaline phosphatase 3, intestine |
|                   | 88978825       | Ecel1       | endothelin converting enzyme-like 1 |
|                   | 89014484       | 1700027L20Rik | 1700027L20Rik |
|                   | 89021807       | Chrm        | cholinergic receptor, nicotinic, delta polypeptide |
|                   | 89036980       | Chrm        | cholinergic receptor, nicotinic, gamma polypeptide |
|                   | 89045084       | Eif4e2      | eukaryotic translation initiation factor 4E member 2 |
|                   | 89095534       | Ethd1       | EF hand domain containing 1 |
|                   | 89158223       | Tmcl15      | Giggf2 GRB10 Interacting YIF protein 2 |
|                   | 89301371       | LOC600653   | LOC600653 |
|                   | 89308004       | Ngf         | Ngf |
|                   | 89308004       | Ngef        | Ngef |
| Start of the gene | End of the gene | Gene symbol | Gene name |
|------------------|-----------------|-------------|-----------|
| 89425720         | 89428998        | Neu2        | neuraminidase 2 |
| 89451549         | 8951673         | Inpp5d      | inositol polyphosphate-5-phosphatase D |
| 89587241         | 89635393        | Atg16l1     | autophagy-related 16-like 1 (yeast) |
| 89634850         | 89676328        | Sag         | retinal S-antigen |
| 89776291         | 89839722        | Usp40       | ubiquitin specific peptidase 40 |
| 89926245         | 90050168        | Ugt1a7c     | UDP glucuronosyltransferase 1 family, polypeptide A7C |
| 89965979         | 90050174        | Ugt1a6a     | UDP glucuronosyltransferase 1 family, polypeptide A6A |
| 90031781         | 90050168        | Ugt1a2      | UDP glucuronosyltransferase 1 family, polypeptide A2 |
| 90035905         | 90036919        | Dnajb3      | DnaJ (Hsp40) homolog, subfamily B, member 3 |
| 90094279         | 90108691        | 643076222Rik | |
| 90134452         | 90220022        | Trpm8       | transient receptor potential cation channel, subfamily M, member B |
| 90238189         | 90257609        | Spp2        | secreted phosphoprotein 2 |
| 90331041         | 90341237        | Glr1p       | glutamine repeat protein 1 |
| 90529783         | 90533314        | Arh4c       | ADP-ribosylation factor-like 4C |
| **Led2minB**     |                 |             |           |
| 92505564         | 92510197        | LOC433332   |           |
| 92597263         | 92674343        | Col6a3      | collagen, type VI, alpha 3 |
| 92745512         | 92780165        | Mlh1        | melanophilin |
| 92783513         | 92784418        | LOC623503   |           |
| 92788540         | 92800026        | Rab17       | RAB17, member RAS oncogene family |
| 92883912         | 92959324        | Lrflp1      | leucine rich repeat (in FLII) interacting protein 1 |
| 92975508         | 93001201        | Gm817       | RNA binding motif protein 44 |
| 93010446         | 93054085        | Ramp1       | receptor (calcitonin) activity modifying protein 1 |
| 93080775         | 93082727        | LOC623550   |           |
| 93080971         | 93116330        | Ube2f       | ubiquitin-conjugating enzyme E2F (putative) |
| 93128743         | 93151480        | Scy         | selenocysteine lyase |
| 93152480         | 93178709        | Gm556       | espin-like |
| 93181478         | 93192810        | 4631423J02Rik | (Klhl30) kelch-like 30 (Drosophila) |
| 93196865         | 93204619        | BC056923    | (Fam132b) family with sequence similarity 132, member B |
| 93206236         | 93291819        | Ilkap       | integrin-linked kinase-associated serine/threonine phosphatase 2C |
| 93235288         | 93238577        | 1700020N18Rik |           |
| 93241888         | 93243628        | Hes6        | hairy and enhancer of split 6 (Drosophila) |
| 93246387         | 93289702        | Per2        | period homolog 2 (Drosophila) |
| 93325082         | 93358670        | Traf3p1     | TRAF3 interacting protein 1 |
| 93370970         | 93390034        | Aob1        | ankyrin repeat and SOCS box-containing 1 |
| 93631882         | 93678433        | Twist2      | twist homolog 2 (Drosophila) |
| 93763139         | 93978799        | Hdac4       | histone deacetylase 4 |
| 94270113         | 94301416        | Ndufa10     | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 10 |
| 94310086         | 94311025        | Olfr1416    | olfactory receptor 1416 |
| 94321223         | 94322159        | Olfr1415    | olfactory receptor 1415 |
| 94341493         | 94342432        | Olfr1414    | olfactory receptor 1414 |
| 94405378         | 94405550        | Olfr1413    | olfactory receptor 1413 |
| 94418737         | 94419703        | Olfr1412    | olfactory receptor 1412 |
| 94426926         | 94427898        | Olfr1411    | olfactory receptor 1411 |
| 94438244         | 94439213        | Olfr1410    | olfactory receptor 1410 |
| 94450313         | 94451315        | Olfr12      | olfactory receptor 12 |
| 94467550         | 94472391        | Myeov2      | myeloma overexpressed 2 |
| 94474623         | 94479247        | Otos        | otospiralin |
| 94662091         | 94690602        | Gpc1        | glypican 1 |
| Start of the gene | End of the gene | Gene symbol | Gene name |
|------------------|----------------|-------------|-----------|
| 94700534         | 94733312       | Ankmy1      | ankyrin repeat and MYND domain containing 1 |
| 9473794          | 94739026       | Dusp28      | dual specificity phosphatase 28 |
| 94741810         | 94750986       | Rnpepl1     | arginyl aminopeptidase (aminopeptidase B) like 1 |
| 94764813         | 94778355       | Capn10      | calpain 10 |
| 94809526         | 94815938       | Gpr35       | G protein-coupled receptor 35 |
| 94836739         | 94842672       | Aqp12       | aquaporin 12 |
| 94848683         | 94932228       | Kif1a       | kinesin family member 1A |
| 94965650         | 94988099       | Agst        | alanine-glyoxylate aminotransferase |
| 94981761         | 94991354       | Z3I007803Rik| |
| 94999130         | 95001480       | E030010N08Rik| |
| 95066246         | 95131471       | Sved1       | sashi, nidogen and EGF-like domains 1 |
| 95129616         | 95136276       | Mterf2      | MTERF domain containing 2 |
| 95139582         | 95167980       | Pask        | PAS domain containing serine/threonine kinase |
| 95174050         | 95198024       | Pnp1r7      | protein phosphatase 1, regulatory (inhibitor) subunit 7 |
| 95204301         | 95233908       | Tmem16g     | Ano7: anoctamin 7 |
| 95236345         | 95309214       | Hdlbp       | high density lipoprotein (HDL) binding protein |
| 95252098         | 95252952       | LOC6271682  | |
| 95309449         | 95340136       | sept-02     | septin 2 |
| 95342530         | 9542188        | Farp2       | FERM, RhoGEF and pleckstrin domain protein 2 |
| 95452309         | 95466056       | Stk25       | serine/threonine kinase 25 (yeast) |
| 95516099         | 95526168       | Bok         | BCL2-related ovarian killer protein |
| 95535796         | 95585244       | Thap4       | THAP domain containing 4 |
| 95585438         | 95619935       | Atg4b       | autophagy-related 4B (yeast) |
| 95630090         | 95632281       | Dtymk       | deoxythymidylate kinase |
| 95634370         | 95652507       | Ing5        | inhibitor of growth family, member 5 |
| 95655698         | 95682580       | D2hgdh      | D-2-hydroxyglutarate dehydrogenase |
| 95691749         | 95709600       | Gal3ts2     | galactose-3-O-sulfotransferase 2 |
| 95792483         | 95816522       | LOC666009   | |
| 95820914         | 95841947       | LOC619597   | |
| 95850898         | 95858740       | Sialdase 4  | |
| 95868710         | 95882959       | Pddc1       | programmed cell death 1 |
| 97144149         | 97165471       | Fam174a : family with sequence similarity 174, member A |
| 97418087         | 97498000       | St8sia4     | ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4 |
| 98475880         | 98476840       | Gm1833      | predicted gene 1833 |
| 98648136         | 98702541       | Slco4c1     | solute carrier organic anion transporter family, member 4C1 |
| 98702644         | 98708307       | G6306007    | galactose-3-O-sulfotransferase 2 |
| 98736581         | 98827966       | Slco8b1     | solute carrier organic anion transporter family, member 8B1 |
| 98889854         | 98958709       | Slco6c1     | solute carrier organic anion transporter family, member 6B1 |
| 98993265         | 99132680       | LOC634331   | |
| 99176834         | 99309408       | LOC634346   | |
| 99472308         | 99492382       | D1Ertd622e  | DNA segment, Chr 1, ERATO Doi 622, expressed |
| 99536564         | 99591955       | Pip5k2      | diphosphoinositol pentakisphosphate kinase 2 |
| 99600605         | 99623375       | Gm13: gypsy retrotransposon integrase 1 |
| 99651499         | 99925919       | Pam         | peptidylglycine alpha-amidating monooxygenase |
| 100251529        | 10035978        | Slco6d1     | solute carrier organic anion transporter family, member 6D1 |

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growth factor) signaling (p value: 0.0078), chemokine interactions (p value: 0.011) and mTOR (mammalian Target Of Rapamycin) signaling pathway (p value: 0.014).

Discussion

In the reproductive processes, as in others, hundreds of genes interact into subtle regulatory networks, and this complexity does not permit to easily identify the molecular factors of dysfunctions leading to infertility cases. Moreover, when our interest is turned towards the human clinic, the study of factors involved in reproductive defects is particularly challenging due to obvious ethical constraints, which rends obligatory the use of animal models. However although hundreds of mutant mouse models with infertility/hypofertility phenotypes have been generated [2], the genetic causes of infertilities are far from being elucidated in their whole [3]. This is the case for the RSA pathology which affects a non negligible percentage of the population (1 to 5%) and for which the genetic origin(s) is still little documented. In the aim to identify new genes responsible for embryonic lethality, we used a mouse model of interspecific recombinant congenic strains (IRCS). Although during the gestation/pregnancy development, mice and humans do not establish exactly the same system of placentation, similarities strong enough between these two species exist, making the mouse model useful to identify genes involved in humans. Indeed, in several mouse models involving the complement system [18,19], it has been clearly shown that there is a continuum between embryo resorption and placental diseases, since the same mice have these resorption and preeclampsia-like symptoms [20]. It is also known that C3 defects are clearly linked with VEGF defects, thus inducing defective placentation, leading in the most extreme cases to embryonic death. Therefore, mouse models of embryo resorption via known deregulations of complement system are proved to be suitable models of the human continuum placental vascular disease-spontaneous resorption. A well studied mouse model of immunologically mediated peri-implantation pregnancy loss that shares features with human recurrent miscarriage is derived from DBA/2-mated CBA/J mice (CBA/J × DBA/2) [21,22,23]. Indeed embryos derived from mating CBA/J females with DBA/2 males showed an increased

| Genomic region                  | Expressed Transcripts (>100 AUF) | Deregulated transcripts (2-fold threshold) |
|---------------------------------|---------------------------------|-------------------------------------------|
| Total genome                    | 18,085                          | 3,436 (19%)                               |
| Spretus fragment in MMU1        | 148                             | 53 (36%)                                  |
| Led2minA (84.5–90.5 Mb)         | 55                              | 24 (44%)                                  |
| Led2minB (95.1 Mb to <100.3 Mb) | 25                              | 8 (32%)                                   |

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frequency of resorption (29.4 ± 6.5%), more than three times greater than that seen within these and other strains or strain combinations (CBA/J × CBA/J: 8.9 ± 5.1%; CBA/J × BALB/c: 8.2 ± 5.6%; DBA/2 × DBA/2: 8.5 ± 6.6%; n = 6–32 mice/group; CBA/J × DBA/2 vs. others, p < 0.01) [18]. Spontaneous resorption in the CBA/J × DBA/2 model is attributed to NK cells, macrophages, and Th1-type cytokines, and represent a rejection of the semiallogeneic fetoplacental [24]. Murine resorptions are characterized by focal necrosis at the junction of the fetal trophoblast with decidua, an infiltrate of polymorphonuclear leukocytes (with some lymphocytic cells) at sites of necrosis and along the walls of large vessels in decidua, and by thrombosis and hemorrhage [25, 26, 27, 28]. Infiltration begins on day 6.5 of gestation, 2 days after implantation has occurred, and abortions begin after day 8.5 of pregnancy [24, 28].

In the present work we used a mouse model including interspecific recombinant congenic strains (IRCS). The originality of this whole model is based on the presence of a small homozygous fragments of Mus spretus genome fixed on a Mus musculus B6 genetic background [16]. Thus, a strain differs of each other and from the B6 parental strain by the spretus segments. Mus musculus and Mus spretus diverged ~2 million years ago meaning that the association of their two genomes has the potential to lead to genetic incompatibilities [14]. Using this model, in past studies, we were able to localize various QTL modulating male and female reproductive processes. We identified a QTL responsible for ~25% of the embryonic resorptions present in the IRCS-66H-MMU1 strain containing a solely chromosomal fragment of spretus origin located on MMU1. This QTL was called Led2 and has been mapped to an interval of 32 Mb which contains 215 genes [15]. The aim of the present study was to redefine this region and to identify candidate genes potentially involved in embryonic lethality.

To accomplish the fine mapping of this QTL, we generated recombinant substrains from 66H-MMU1 by backcrosses, each of them presenting a unique sub-fragment of the Led2 QTL. Each recombinant strain segments were crossed with B6 males, resulting in a fetus/placenta complex with heterozygous B6/seg genes (at the Led2 locus) and uterine homozygous spretus genes (at the Led2 locus). During each gestation, the substrains were phenotyped in vivo by ultrasonography. This non-invasive technology, based upon a high frequency ultrasound device [29] allows in vivo real time high resolution observations of embryonic development [15, 30] and resorption (~70 µm and ~40 µm lateral and axial resolution, respectively) and permits to carry out longitudinal analysis of gestation. We observed an increase of the embryonic death rate in R3 and R5 substrains (Group 2). The analysis of the genotype/phenotype segregation allowed us to determine two reduced QTL regions (Led2minA and Led2minB) of approximately 6 Mb each, present together in spretus version only in R3 and R5 strains. In the other recombinant substrains which have not the phenotype, the one or the other of the region is present but not the two regions together. So we defined the first reduced spretus region called Led2minA which encompasses D1Mit50 to D1Mit305 region (>84.5 Mb to 90.5 Mb) and the second called Led2minB located at the rs5692309 marker (>92.5 Mb to <100.3 Mb). Our statistical analysis succeeded in proving the presence of Led2minA QTL responsible for a main effect on embryonic death but it failed for Led2minB. However, notable differences between the embryonic death rates of certain strains (R6 compared to R3 or R5) led to suppose that this latter region could also have a small effect in the phenotype. Taken together, these data did not support the presence of an epistatic interaction between Led2minA and Led2minB.

Reverse crosses using IRCS Group 2 males and B6 females revealed that the genes expressed at heterozygous state in the placental tissues are not deleterious for the gestation. Therefore, we deduced that the high rate of embryonic death occurring during the gestation resulted from dysfunction of genes expressed in the uterine tissue. This is in accordance with the normal embryonic development observed in group 2 IRCS females. Then, we carried out a microarray analysis searching to identify uterine deregulated genes in IRCS animals from Group 2. Although we observed deregulated genes located in all chromosomes (19%) we noticed that those situated on the spretus fragment were preferentially modified (~40%). This concentration of deregulated genes located on the spretus fragment has already been reported in a previous study of our group performed on testis transcriptome [31]. It has been showed that at genomic scale differential SNPs between Mus musculus and Mus spretus are frequent since they appear, in average, every 100 bp. When located on the promoter regions of spretus origin, these nucleotide substitutions could modify the transactivation/transrepression properties of transcription factors of C57BL/6J nature, thus modifying the spretus gene expressions. Additionally, dysfunctions leading to embryonic death could result from non-synonymous coding polymorphisms, accumulated during evolution in the spretus genome. These phenomena should be originated from evolution of separated genomic regions that produces transcription factors/DNA (“transcriptomic shock”) and/or protein-protein (“proteomic shock”) incompatibilities [32].

### Table 4. Microarray validation by RT-QPCR on 7 genes of the QTL region.

| Gene symbol | Microarrays | RT-PCR |
|-------------|-------------|--------|
|             | Expression level in B6 uterus (AUF) | Ratio of expression R3/B6 | Ratio of expression R3/B6 | % PCR efficiency | R-square values |
| Ncl         | 1555        | 0.16   | 0.42249197 | 101% | 0.9986 |
| Cab39       | 1800        | 4.5    | 5.43093265 | 102% | 0.99 |
| Psmd1       | 34109       | 0.90   | 0.69502902 | 104% | 0.9934 |
| Cops7b      | 12526       | 0.62   | 0.66533846 | 105% | 0.9999 |
| Elf4e2      | 652         | 0.5    | 0.50713771 | 105% | 0.9913 |
| Usp40       | 2154        | 0.21   | 0.39715333 | 100% | 0.9992 |
| Trip12      | 12096       | 0.77   | 0.60419569 | 101% | 0.9991 |
| Cyclophilin A (Reference gene) | 38918 | 0.95 | 95% | 0.9994 |

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### Table 5. Genes of *Led2min* region expressed in uterus (>500 AUF) and displaying a deregulation (R3/B6 ratio) and/or non synonymous SNP.

| Gene symbol | Expression level in B6 uterus (AUF) | SNP number | Ratio of expression R3/B6 |
|-------------|-------------------------------------|------------|--------------------------|
| **Led2minA** |                                     |            |                          |
| Trip12      | 12096                               | 4          | 0.77                     |
| Fbxo36      | 992                                 | 6          | 0.12                     |
| AS30040E14Rik | 521                               | 0          | 0.28                     |
| Sp110       | 18202                               | ND         | 0.52                     |
| SP100       | 3798                                | 19         | 0.55                     |
| Cab39       | 1800                                | 0          | 4.5                      |
| Itm2c       | 13239                               | 1          | 0.44                     |
| Spata3      | 996                                 | 7          | 1.33                     |
| 2810455M11Rik | 1922                             | 4          | 0.24                     |
| Psmd1       | 34109                               | 2          | 0.90                     |
| Htr2b       | 1982                                | 4          | 0.13                     |
| Ncl         | 1555                                | 5          | 0.16                     |
| Pdedd       | 9972                                | 0          | 0.15                     |
| Cops7b      | 12526                               | 1          | 0.62                     |
| Elf4e2      | 652                                 | 1          | 0.50                     |
| Ngef        | 1627                                | 4          | 0.54                     |
| Usp40       | 2154                                | 7          | 0.21                     |
| Glrp1       | 517                                 | 9          | 0.11                     |
| Ah14c       | 10043                               | 0          | 0.37                     |
| **Led2minB** |                                     |            |                          |
| Col6a3      | 20317                               | 28         | 2.01                     |
| Mlph        | 3388                                | 10         | 0.48                     |
| Rab17       | 2539                                | 4          | 0.34                     |
| Lrflp1      | 14838                               | 14         | 1.2                      |
| Ramp1       | 13416                               | 7+1 stop lost | 0.78                     |
| Ilkap       | 20118                               | 2+1 splice site | 0.78                     |
| Hes6        | 12151                               | 1          | 0.57                     |
| Per2        | 3786                                | 10         | 0.89                     |
| Traf3lp1    | 814                                 | 9          | 0.66                     |
| Asb1        | 4667                                | 1          | 0.57                     |
| Ndufa10     | 32815                               | 2          | 0.87                     |
| Myeov2      | 14896                               | 5          | 0.73                     |
| Gpc1        | 1912                                | 1          | 0.8                      |
| Rnpepl1     | 960                                 | 1          | 0.69                     |
| Sned1       | 4230                                | 9          | 3.51                     |
| Mterfd2     | 24052                               | 7          | 1.32                     |
| Ppp1r7      | 8390                                | 8          | 0.63                     |
| Hdlbp       | 25050                               | 2          | 1.06                     |
| Farp2       | 634                                 | 20         | 0.74                     |
| Thap4       | 2169                                | 1          | 0.77                     |
| Atg4b       | 678                                 | 3          | 0.72                     |
| Dymk        | 5044                                | 1          | 0.41                     |
| D2hgdh      | 945                                 | 3+1 splice site | 0.44                     |

ND: Not determined.

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Focusing on genes of the Led2minA QTL and applying filters from bioinformatics databases, bibliography and our own results, we propose a selection of 7 genes (Trip12, Cab39, Pmnd1, Ncl, Cops7b, Eif4e2 and Usp40) as putative actors of the embryonic death. These genes play a role in VEGF signaling, mTOR signaling and ubiquitination-degradation pathways. Their effects could be reinforced by a small participation of genes situated on Led2minB region and which could act in the same signaling pathways (Asb1, Traf3ip1, Ramp1 and Cab39).

Trip12, Pmnd1, Cops7b and Usp40 from Led2minA and Asb1 from Led2minB are involved in protein degradation process through the ubiquitin-proteasome pathway. Trip12 exerts a ligase activity related to ubiquitination [33]. Usp40 functions as a deubiquitinating enzyme in the same degradation pathway [34] and Asb1 is a member of the ankyrin repeat and SOCS box (ASB) family. These family proteins interact with Cul5-Rbx2 to form E3 ubiquitin ligase [35]. Pmnd1 is a component of the 26S proteasome. Cops7b is a subunit of the eight-subunit heteromeric C9p9 signalosome complex. Genetic invalidations of some C9p9 subunits have been associated with developmental defects of post-implantation embryos [36,37,38]. Additionally, Usp40 functions as a deubiquitinating enzyme in the same degradation pathway [34]. Asb1 is a member of the ankyrin repeat and SOCS box (ASB) family. These family proteins interact with Cul5-Rbx2 to form E3 ubiquitin ligase [35].

In the same manner, Led2minA Ncl gene and Led2minB Ramp1 and Cab39 genes are involved in angiogenesis. Ncl encodes nucleolin and treatment of endothelial cells with anti-nucleolin antibody induces apoptosis of these cells [39]. Moreover, nucleolin associates with VEGF-C62 and can be potentially involved in epithelial cell adhesion and proliferation [39,40]. Concerning Ramp1 gene, RAM1 (receptor activity modifying protein) forms a functional receptor for CALCA (Caltocin gene-related peptide) which is a proangiogenic growth factor in the human placental development and plays a critical role in embryonic development and fetal growth [41]. Concerning the Collagen type VI a3 gene, COL IV is a main endometrial extracellular matrix component, and an abnormal increased deposition of collagen might impair uterine function, possibly by interfering with vascularization or retarding remodeling events at implantation [42].

Finally, Cab39 (also called M2E5) and Eif4e2 from Led2minA and Traf3ip1 from Led2minB, participate in the mTOR signaling pathway, a regulatory step of protein synthesis and growth. Cab39 effect has been described upstream of mTOR activation while Eif4e2 is a downstream signaling target involved in translation initiation. Interestingly, Mioo gene disruption in mice leads to early embryonic death [43]. Homozygous Traf3ip1 (Tumor necrosis factor alpha receptor 3 interacting protein 1) mutant mice are not viable. Traf3ip1 mutant mouse line was generated and the enlarged mutant cell size in culture was associated with elevated basal mTOR pathway activity [44]. Otherwise, mTOR pathway is implicated on the VEGF pathway activation. It is worth noting that the VEGF and mTOR pathways have been identified as significantly deregulated in our functional clustering analysis.

Conclusions

We used an in vivo approach of the embryonic development on a mouse IRCS model to refine a chromosome 1 region (Led2) responsible for embryonic death. The present study succeeded in fine-mapping Led2minA QTL which has a main effect on the embryonic death (about 30%) and pointed out a second region Led2minB which could have a minor effect on the same phenotype. Collecting and analyzing experimental, bioinformatics and literature data on the expression and function of genes present in the two regions (Led2minA and Led2minB), we propose 7 genes from Led2minA that could be related with the phenotype. It appears that the vascularization could be the common denominator at these categories of genes involving angiogenesis and the fluidity of the extracellular matrix. The actual identification of the gene(s) involved in this phenotype will necessarily pass through further molecular approaches. An important outcome of this study is the possibility to evaluate novel promising candidates of RSA in humans [13]. This might contribute to elucidate the molecular basis of this multifactorial and complex human disorder and to propose new diagnostic markers.

Supporting Information

Table S1 Sequences of used real time RT-PCR primers.

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Author Contributions

Conceived and designed the experiments: DV CS AZ XM. Performed the experiments: MV GB GR FM VF. Analyzed the data: MV DV CS AZ PL. Contributed reagents/materials/analysis tools: MV GR FM VF. Wrote the paper: DV CS AZ MV PL.

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