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Identification of Quantitative Trait Loci responsible for embryonic lethality in mice assessed by ultrasonography

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ABSTRACT Recurrent Spontaneous Abortion (RSA) is a frequent pathology affecting 1 to 5% of couples. In ~50 % of cases, the aetiology is unknown suggesting a subtle interaction between genetic and environmental factors. Previous attempts to describe genetic factors using the candidate gene approach have been relatively unsuccessful due to the physiological, cellular and genetic complexity of mammalian reproduction. Indeed, fertility can be considered as a quantitative feature resulting from the interaction of genetic, epigenetic and environmental factors. Herein, we identified Quantitative Trait Loci (QTL) associated with diverse embryonic lethality phenotypes and the subsequent embryonic resorption in 39 inter-specific recombinant congenic mice strains, using *in vivo* ultrasound bio-microscopy. The short chromosomal intervals related to the phenotypes will facilitate the study of a restricted number of candidate genes which are potentially dysregulated in patients affected by RSA.

KEY WORDS: *resorption, ultrasound biomicroscopy, quantitative trait loci, recurrent spontaneous abortion*

Introduction

Reproduction in placental mammals involves the harmonious action of several subtly regulated physiological, molecular and cellular processes, implicating complex successive developmental stages from egg fertilization to foetus delivery. One of the first "challenges" encountered by the zygote occurs during the preimplantation period, when the maternal/zygotic transition switches expression from maternal oocyte-stored mRNA to expression from the embryo genome and activation of genes unexpressed in the oocyte (Schultz, 2002). This transition is observed at the two-cell stage and between the four/eight cell stages in rodents and human, respectively (Artley *et al.*, 1992). After this critical stage of embryonic genome activation, necessary for the blastocyst formation and its initial growth, the implantation involves the close mingling of two genetically different organisms. In order to prevent the rejection of the embryonic hemi-allograft an "immunological conciliation" has therefore to be installed. In rodents the hormonal effect of estrogens and progesterone is crucial for the preparation of the receptive uterus, during the implantation window (Huet-Hudson *et al.*, 1989). The oestrogen metabolites are necessary to activate the blastocyst (Paria *et al.*, 1998) and a complex molecular dialogue is established between the blastocyst and the maternal uterine luminal epithelium. This complex interaction includes various factors, such as steroid hormones, adhesion molecules, extracellular matrix proteases, vasoactive and growth factors, cytokines and developmental genes (Dey *et al.*, 2004, Lim *et al.*, 2002, Wang and Dey, 2006). In eutherian mammals dysregulation in preimplatation, implantation or early postimplantation pathways have been associated with embryonic lethality and infertility, especially in mouse models where specific genes have been invalidated (Dey *et al.*, 2004, Sharma *et al.*, 2006, Wang and Dey, 2006). Nevertheless, the embryonic death can also occur at any postimplantation developmental stage, during organogenesis

Abbreviations used in this paper: EED, early embryonic death; ELR, embryonic lethal rate; IRCS, interspecific recombinant congenic strains; MMU, Mus Musculus chromosome; QTL, quantitative trait loci; RSA, recurrent spontaneous abortion.

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and foetal life, as suggested by the invalidation of numerous genes having a strong impact on fertility (http://www.informatics.jax.org http).

Molecular understanding of embryonic implantation and development is of particular interest for the study of human infertility. For instance, miscarriage constitutes a frequent pregnancy complication representing at least 15% of medically followed pregnancies (Rai and Regan, 2006, Wilcox *et al.*, 1988). Sporadic abortion affects 25-50% of couples, while recurrent spontaneous abortion (RSA), defined as at least two or 3 consecutive pregnancy losses, affects 1 to 5% of couples (Rai and Regan, 2006). The diverse aetiologies associated with RSA, include chromosomal alterations (Stephenson *et al.*, 2002), maternal and foetal structural abnormalities (Philipp *et al.*, 2003, Salim *et al.*, 2003), thrombophilic disorders (Rey *et al.*, 2003) and autoimmune disorders such as the antiphospholipid syndrome (Levine *et al.*, 2002). Nevertheless, in ~50 % of the cases the aetiology remains unknown (Li *et al.*, 2002, Plouffe *et al.*, 1992; Tulppala *et al.*, 1993), indicating that the underlying aetiological grounds constitute a complex lattice of genetic and environmental causes. Previous attempts to describe genetic factors using the candidate gene approach have been relatively unsuccessful (Kaare *et al.*, 2006, Kaare *et al.*, 2007). These disappointing results can be explained, as briefly exposed above, by the physiological, cellular and genetic complexity of every step in mammalian reproduction. Indeed, fertility can be considered as a quantitative feature resulting from the interaction of genetic, epigenetic and environmental factors. In humans, the study of the molecular actors involved in the loss of pregnancy is particularly challenging due to obvious ethical constraints, which strengthens the importance of mice models.

In this study, we aimed at identifying Quantitative Trait Loci (QTL) associated with embryonic lethality and the subsequent embryonic resorption. For this purpose, we used an original mice model of 53 Interspecific Recombinant Congenic Strains (IRCS), each of them containing around of 2% of *Mus spretus* genome stably fixed at a homozygous state in a C57BL/6J genetic background (Burgio *et al.*, 2007, L'Hote *et al.*, 2007, Montagutelli and Abitbol, 2004). This model permits the unambiguous mapping of relevant genes into small chromosomal intervals since the *spretus* fragments have recently been precisely mapped using ~800 informative markers. Postimplantation embryo development, from E8.5 to E14.5 of IRCS females crossed with C57BL/6J males was assessed *in vivo*, using high resolution ultrasound biomicroscopy. We identified 3 QTL, located on MMU1, MMU13 and MMU19 responsible of embryonic lethality. The study of the mouse gene composition in the spretus fragments suggested several genes putatively involved in the phenotypes. The human orthologues constitute interesting targets that could be molecularly evaluated as candidates for Recurrent Spontaneous Abortion.

TABLE 1

C57BL/6J (CONTROL GROUP), IRCS AND SUBSTRAINS AFFECTED BY EMBRYONIC LETHALITY

Strain	TNA	ETN
C57/BL6 (parent)	22	195
IRCS (whole set)	207	1603
66H	9	76
66H-MMU1	8	69
66H-MMU13	20	177
66H-MMU18	7	60
66H-MMU13+18	9	66
103C	6	51
135B	6	48
135E	5	43

TNA: total number of animals examined (mothers). ETN: total number of embryos followed.

Results and Discussion

Embryonic death phenotypes are reliably revealed by ultrasound biomicroscopy

In order to identify QTL related with embryonic death we examined 207 pregnant mice from 39 out of 53 available IRCS and from a control group consisting of 22-C57BL/6J animals (Table 1). The gestation was obtained by crossing each IRCS female with a C57BL/6J male, in order to maintain a constant paternal genetic background. Genetically the maternal uterine tissue is of IRCS type, while the placenta is heterozygous (B6/SEG) at the position of the *spretus* segment. This design allows the detection of specifically maternal phenotypic traits inducing embryonic lethality. The defects can theoretically be due to dysregulations of uterine and/or placental genes. Females from the control group were crossed with C57BL/6J males. Embryonic development between E8.5 and E14.5 was followed *in vivo* on a total of 1603 heterozygous (C57BL/6J / *spretus*) embryos using high frequency ultrasound biomicroscopy (Table 1). This systematic ultrasonographic observation is based upon a high frequency ultrasound device, which represents an advantageous imaging modality for *in vivo* and non-invasive analysis of mouse embryo development (Foster *et al.*, 2000), including functional parameters as arterial and venous flow measurements inside the umbilical cord by pulse Doppler. In accordance with ethical rules on animal experimentation, this approach considerably reduces the number of the sacrificed animals and makes it possible to carry out longitudinal studies.

Each IRCS and C57BL/6J female was also subjected to a pre-gestation ultrasonographic examination (US1) in order to evaluate putative anatomical malformations of the reproductive (vagina, uterine horns and ovaries) and urinary tracts (bladder and kidney) and to investigate signs related with local or systemic pathologies. These observations allowed us to exclude 3 individuals presenting a severe loose of kidney tissue and its replacement by a non-echogenic content, suggesting a hydronephrotic process (data not shown). Furthermore, in one individual, we could not observe uterine horn structures owing possibly to a low diameter of these structures (<0.5 mm, below the detection capacities of the equipment). This animal was mated several times but never became pregnant. Dissection confirmed the presence of hypoplasic uterine horns characterised by a thin and brittle tissue.

Overall, we performed a total of 1581 ultrasonographic examinations from pregnant (US2 and US3) and non-pregnant (US1) animals, including the test group. In accordance with similar studies (Ji and Phoon, 2005), these analyses enabled us to establish a perfect correlation between the total embryo number observed by ultrasonography and their presence in the uterine horns, verified after the animal dissection. The sensibility of the technique permitted us to identify and count unam-

biguously the implanted embryos from E7.5. However, we did not examine pregnant mice before E8.5 since before this developmental stage it was difficult to distinguish living embryos and resorbed structures (Figure 1A-C). The living status of the embryo was determined by the visualisation of heartbeats and by assessment of blood flows in the umbilical cord, using pulsed Doppler mode.

Phenotypically, the dead embryos were classified into two categories (see Figure 1): early embryonic death (EED, embryo totally resorbed at US2) and late embryonic death (LED, death observed during US2 or US3, when visible organs were progressively resorbed). Nevertheless, this apparent dichotomised classification could also be considered as embryonic lethality as a whole (EL) (see below).

In most cases, in embryos of the EED phenotype, the amniotic and exocoelomic cavities were never observed (Figure 1G). At US2, by contrast, contiguous embryos displayed a normal development. Furthermore, in EED embryonic resorptions, the cardiac heartbeat, normally detected by ultrasonography from E8.5-E9.5, was absent, indicating that the EED occur mainly between embryo implantation (E4.5) and the onset of the heartbeats (E8.5). Embryonic resorption (ER) displayed a characteristic high central echogenic zone corresponding to the embryo implantation site (Figure 1G).

Embryos presenting the LED phenotype displayed a heartbeat arrest and an absence of umbilical cord flow (as assessed by Doppler) visible at US3. Alternatively, they also corresponded to US2 observations of dead embryos with a clear organogenesis (a well-formed, non beating heart). In these embryos, intra-embryonic structures displayed initially a normal anatomical aspect including thoracic and abdominal viscera development similar to the surrounding embryos that would accomplish a normal development to birth. Embryos affected by LED showed a characteristic 5-step involution pathway leading, *in fine,* to ER (Figure 1A-G). After the first step of the LED (the heartbeat arrest), the embryonic structures became disorganised and disappeared progressively. During this step an intensive proteolytic activity probably took place since the intra-embryonic structures were eventually totally resorbed after 24-48 hours, depending on their relative toughness. Thus, the embryo head was the last structure to be completely resorbed leading to the apparition of an anechogenic cavity full of liquid (steps 2-3, Figures 1D-E). The two remaining steps of the LED pathway include the liquid replacement by a dense tissue (Step 4, Figure 1F) and finally the complete ER (step 5, Figure 1G). These findings represent the first *in vivo* real time description of the involution of mice defective embryos.

Four interspecific recombinant congenic strains (IRCS) are affected by embryonic lethality

We analyzed by ultrasound biomicroscopy a total of respectively 1603 and 195 embryos from IRCS and C57BL/6J (control group) pregnant mice. There was a perfect correlation between the total number of implanted embryo observed at US2 and US3, showing that structures are highly reliably detected, and demonstrating the examination accuracy. C57BL/6J mice displayed an average of 8.9 (± 1.2) implanted embryos per individual, including EED and LED structures (Table 2). This result was not significantly different from the average calculated using the total number of heterozygous embryos implanted in the IRCS (8.2 ± 1.9). Among the 39 IRCS studied, we identified 4 strains (66H, 103C, 135B and 135E) displaying a highly significant increase in embryonic lethality ratio (ELR-defined as the proportion of dead embryos at any stage of development relative to the totality of the implanted embryos), compared with the control group (results from the statistical analysis are summarized in Table 2 and Figure 2). Indeed, while the ELR in C57BL/6J was estimated at 4.6 %, the 66H, 103C, 135B and 135E strains showed an increase to respectively, 14.5% (p = 0.025), 19.6% (p= 0.0012), 25% (p= 0.0002) and 18.6% (p= 0.0048). The embryonic lethality observed in 66H and 103C strains was mainly due to the EED phenotype, since respectively 10.5% and 11.8% (p=0.034) of the implanted embryos displayed at US2 the characteristic aspect of the ER. Conversely, 16.7% ($p = 6.25 \times 10^{-7}$) of the implanted embryos in the 135B strain and 16.3% ($p = 2.77 \times 10^{-10}$) in the 135E strain could rather be classified as LED, since the highest proportion of their ELR, could be observed at US3. Interestingly, in 66H and 103C the remaining fraction of the ELR

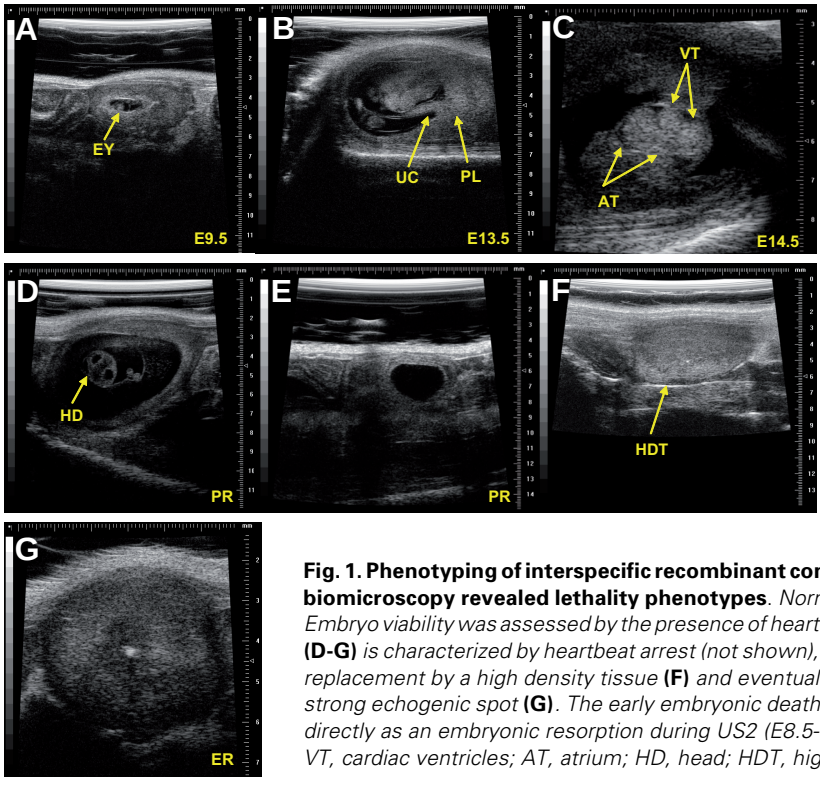

Fig. 1. Phenotyping of interspecific recombinant congenic strain (IRCS) embryo development using ultrasound biomicroscopy revealed lethality phenotypes. *Normal embryo development at E9.5* **(A)**, *E13.5* **(B)** *and E14.5* **(C)**. *Embryo viability was assessed by the presence of heartbeats and a positive umbilical cord Doppler. The LED phenotype* **(D-G)** *is characterized by heartbeat arrest (not shown), progressive resorption (PR) of the embryonic structures* **(D,E)**, *replacement by a high density tissue* **(F)** *and eventually, by the general feature of final embryonic resorption (ER), a strong echogenic spot* **(G)**. *The early embryonic death (EED) phenotype does not involve these phases as it appears directly as an embryonic resorption during US2 (E8.5-E12.5, Fig. 1G). EY, embryo; PL, placenta; UC, umbilical cord; VT, cardiac ventricles; AT, atrium; HD, head; HDT, high density tissue.*

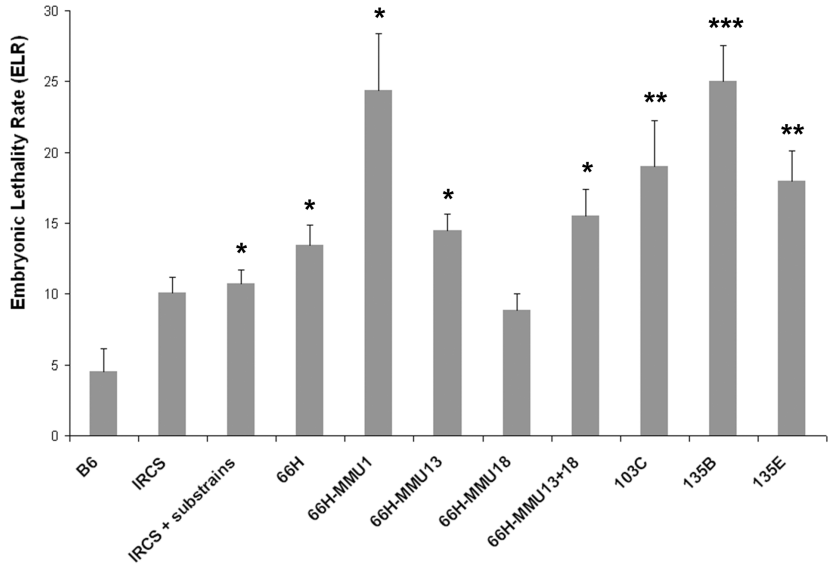

Fig. 2. Results of the interspecific recombinant congenic strain (IRCS) phenotyping in terms of Embryonic Lethality Rate (ELR) defined as the proportion of death embryos at any stage of development to the totality of implanted embryos. *Results are presented as the average rate of dead embryos. Error bars correspond to SEM, calculated from the variation between individual females.*

(represented by LED) also shown significant rates compared with the control group (66H-LED: 4%, p=0.018), 103C-LED: 7.8%, p=6.48 x 10^{-5}). These findings are to be compared with the complete absence of the LED phenotype in the control group.

Genetically, the 66H strain encompasses three spretus genomic fragments located on chromosomes MMU1, MMU13 and MMU18. To precisely dissect the genotype/phenotype interaction, we created and phenotyped three substrains derived from 66H each containing a unique *spretus* fragment: 66H-MMU1, 66H-MMU13 and 66H-MMU18. Each fragment encompasses a unique chromosomal region present in the parental 66H strain. We observed an increase of the ELR in both 66H-MMU1 (24, 6 %, p= 0,013) and 66H-MMU13 (14,7%, p=0,01) whereas in 66H-MMU18 the lethality phenotype was absent.

Interestingly, the LED rate in 66H (containing the three MMU1, MMU13 and MMU18 segments) was estimated at 4% (not significantly different from C57BL/6J). In contrast, LED was high in 66H-MMU1 and 66H-MMU13 (17.4%, p=0.006 and 10.2%, p=0.0001, respectively) suggesting an epistatic (protective?) effect of the 66H-MMU18 *spretus* fragment as shown in Table 2. In fact in the 66H strain, the presence of the three fragments was mainly associated with EED. This could indicate that the MMU18 segment encompasses genetic factors that promote embryonic resorption between E4.5 and E8.5 in the presence of factors expressed from 66H-MMU1 and 66H-MMU13 fragments. Other sub-strains containing different segment combinations were created from the F2 population. The 66H-MMU13+18 substrain showed an increase in ELR (16.7%, p=0.011) mainly represented by the LED phenotype. In this case the effect of MMU18 genetic composition seems not to enable to compensate the LED phenotype produced by the MMU13 *spretus* fragment. The 66H-MMU1+MMU13 and 66H-MMU1+18 were not phenotyped as these strains are highly hypofertile in terms of mating difficulties (defined as the time necessary to became pregnant, Table 2).

Mapping of quantitative trait loci (QTL) involved in embryonic resorption

In our model, the significant phenotypic differences observed between the IRCS and the C57/ BL6 control group could be mapped to chromosomal fragments from *Mus spretus* located in the C57BL/6J genetic background. Therefore, these regions should contain genes responsible for the observed phenotype. Three QTL of embryonic lethality were finally unambiguously mapped on single chromosome segments from strains containing a unique *spretus* fragment (66H-MMU13, 66H-MMU1 and 135E).

The 66H-MMU13 *spretus* fragment (~3.4 Mb) is comprised between the rs120693734 and D13Mit47 polymorphic genetic markers. This relatively short chromosomal region (QTL-*Led1*) is responsible for the LED phenotype. This region contains 31 genes (Table 3). Among these, *Btf3, PolK* and *Foxd1* are

TABLE 2

STATISTICAL ANALYSIS FROM 39 OF THE 53 IRCS PHENOTYPED USING *IN VIVO* ULTRASOUND BIOMICROSCOPY

Strain	Average Implanted embryos (± Stdev)	Embryonic resorption	Early Embryonic Death	Late Embryonic Death	Mating difficulty
C57/BL6	8,9 ± 1,2	4,6%	4,6%	0%	▲
IRCS (whole set)	8,2 ± 1,9	9,6% (NS)	4,8%	**4,7%**	▲▲
IRCS (whole set + substrains)	8,3 ± 2	**10,5% (p=0,04)**	4,8%	**5,7%**	▲▲
66H	8,4 ± 3,5	**14,5% (p=0,025)**	**10,5%**	4%	▲▲
66H-MMU1	8,6 ± 3	**24,6 % (p= 0,013)**	7,2%	**17,4%**	▲▲▲▲
66H-MMU13	8,8 ± 2,1	**14,7% (p=0,01)**	4,5%	**10,2%**	▲
66H-MMU18	8,6 ± 1	8,3% (NS)	6,7%	1,6%	▲▲
66H-MMU13+18	7,3 ± 2,5	**16,7% (p= 0,011)**	4,6%	**12,1%**	▲▲
103C	8,5 ± 2,7	**19,6% (p=0,0012)**	**11,8%**	7,8%	▲▲
135B	8 ± 2	**25% (p=0,0002)**	8,3%	**16,7%**	▲▲▲
135E	8,6 ± 1,3	**18,6% (p=0,0048)**	2,3%	**16,3%**	▲▲

The number of triangles represents mating difficulty in terms of period of time to obtain a gestation: ▲: easy. ▲▲: mildly difficult. ▲▲▲ highly difficult. ▲▲▲▲: very highly difficult.

TABLE 3

GENES LOCATED ON MMU13 BETWEEN RS120693734 AND D13MIT47 MARKERS

Start (bp)	End (bp)	Gene Symbol	Mutants with an effect on Embryonic letality	Gene Name
96702488	96719173	F2R	Gene targeting	coagulation factor II (thrombin) receptor
97489065	97516268	1200014M14Rik		RIKEN cDNA 1200014M14 gene
97517456	97536685	9330128J19Rik		RIKEN cDNA 9330128J19 gene
97581386	97643184	Polk		polymerase (DNA directed), kappa
97643434	97738249	Col4a3bp		collagen, type IV, alpha 3 (Goodpasture antigen) binding protein
97750033	97771547	Hmgcr	Gene targeting	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
98046888	98048213	Gm73		gene model 73, (NCBI)
98122569	98135028	1700029F12Rik		RIKEN cDNA 1700029F12 gene
98172342	98230214	B230112C05Rik		RIKEN cDNA B230112C05 gene
98198909	98200046	EG620648		predicted gene, EG620648
98231080	98238565	5730427N09Rik		RIKEN cDNA 5730427N09 gene
98238636	98277162	Gfm2		G elongation factor, mitochondrial 2
98277106	98298969	Hexb		hexosaminidase B
98341869	98353733	Enc1		ectodermal-neural cortex 1
98660157	98684151	EG328314		predicted gene, EG328314
98844851	98847536	5330416C01Rik		RIKEN cDNA 5330416C01 gene
98860287	98860745	ENSMUSG00000069117		predicted gene, ENSMUSG00000069117
98999618	99306324	Rgnef		Rho-guanine nucleotide exchange factor
99347002	99363149	Utp15		UTP15, U3 small nucleolar ribonucleoprotein,
99363348	99374073	Ankra2		ankyrin repeat, family A (RFXANK-like), 2
99380049	99393927	EG667718		predicted gene, EG667718
99402661	99407249	ENSMUSG00000071224		predicted gene, ENSMUSG00000071224
99410052	99417124	Btf3	Gene trap approach	Basic transcription factor 3
99416579	99417461	ENSMUSG00000048603		predicted gene, ENSMUSG00000048603
99454399	99456860	Foxd1	Gene targeting	forkhead box D1
99502252	99502685	ENSMUSG00000059468		predicted gene, ENSMUSG00000059468
99589690	99589980	ENSMUSG00000071222		predicted gene, ENSMUSG00000071222
99657899	99658521	ENSMUSG00000068602		predicted gene, ENSMUSG00000068602
99713670	99714225	EG218501		predicted gene, EG218501
99735135	99737495	Tmem174		transmembrane protein 174
99786395	99794988	Tmem171		transmembrane protein 171
99823567	99915303	Fcho2		FCH domain only 2
100524598	100616697	Mtap1b	Gene targeting	microtubule-associated protein 1B

In the 66H-MMU13 substrain this region originates from *Mus spretus*.

transcriptions factors. It may be possible that their sequence or expression levels impair the normal transactivation of target genes involved in implantation and/or early development.

The significant increase in the LED phenotype frequency observed in the 66H-MMU1 strain permitted us to define a second QTL. The 66H-MMU1 *spretus* fragment is delimited by D1Mit50 and rs120683504 markers corresponding to a large 18.3Mb region not shared with any other IRCS strain. This QTL region, named *Led2,* contains 215 genes (143 described and 72 predicted). The expressional profile analysis of these genes was assessed using the Symatlas gene expression database (http://symatlas.gnf.org) which showed that *Bok*, and *Htr2b* genes are strongly expressed in mouse placental tissue, while *Sned1*, and *Gpc1* are highly expressed in uterus. Interestingly, *Bok* is also highly expressed in the ovary, arguing about a potential ovarian dysfunction in 66H-MMU1 substrain. *Bok* (Bcl-2 Related Ovarian Killer) is considered a pro-apoptotic factor (Hsu and Hsueh, 1998, Hsu *et al.*, 1997, Rodriguez *et al.*, 2006) supposedly implicated in ovarian cell apoptosis and atresia. Indeed, and conversely to 66H, 66HMMU-13 and 66HMMU-18, the 66H-MMU1 substrain displayed a strong reproductive dysfunction in terms of pregnancy success as we obtained only 8 pregnant females during 9 months of mating cycles (Table 2). The MMU1 region matches with a previously described QTL involved in premature ovulation and primary oocyte arrest (*Popoa*), that was related with meiotic abnormalities in the LT/SvKau-derived alleles strains, located up to now to a ~50 Mb region (Everett *et al.*, 2004).

We identified an additional QTL (*Led3*) located on MMU19 in 135E strain. This genomic region, encompassing 8Mb, can be unambiguously associated with the phenotype of the 135E strain which carries a unique *spretus* fragment located between D19Mit49 and D19Mit137 markers.

The 135B strain encompasses two *spretus* fragments located on MMU12 (17.8 Mb, between D12Mit135 and rs120692608 markers) and MMU13 (8.8 Mb between rs120693763 and D13Mit32 markers). Although the 66H-MMU13 and 135B-MMU13 *spretus* fragments are not overlapping they are nearly contiguous (separated by a 40Kb C57BL/6J genomic region). We could hypothesise that in the context of a *spretus* chromosomal continuity on MMU13, the presence of this C57BL/6J "disruptive" region could interfere with a common regulatory region potentially linked with both 66H-MMU13 and 135B LED phenotypes. In this context, the classical genetic approach to elucidate the unambiguous effect of a *spretus* fragment should consist on the separation of the 135B chromosomal fragments.

Finally, we could detect a significant increase of the EED phenotype in the 103C strain. In this case, 4 *spretus* fragments are fixed on the C57BL/6J genetic background: MMU4 (between D4Mit34-rs120686944), MMU7 (between rs120688996-rs120689110), MMU9 (between rs120690534- rs120690604) and MMU19 (between D19Mit32- rs120696784).

Conclusion

The use of IRCS to finely dissect genetic factors causing various phenotypes is appropriate for dissecting reproductive phenotypes, as shown in this study and the previous one using the same material for male phenotypes (L'Hote *et al.*, 2007). This approach implicates extremely challenging successive mice crosses since IRCS are hypofertile. Indeed, during the IRCS strain production, more than 50% did not survive, suggesting that the genetic consequences of consanguinity or in many cases, the mingling of the *Mus musculus* and *Mus spretus* genomes was not viable. The available IRCS mice survived only in the cases of favorable gene combinations. Thus, the totality of the 53 IRCS may harbour specific genome composition enabling them to have survived for at least 10 years. We can hypothesize that the embryonic resorption phenotype would have been originally present in a larger number of strains and that 66H, 103C, 135B and 135E strains have been early preserved by genetic compensatory events.

Up to now, diverse mice crosses have shown high rate of embryonic resorption. For instance, the different mating combinations of CBA/2 X DBA/2 mice display a natural resorption rate of about 20–40% and up to 60%, if the females are injected with polyIC, a synthesized double-strand RNA able to activate natural killer (NK) cells (Shimada *et al.*, 2003). Other studies showed that 15 to 20% of embryos undergo resorption in the spontaneous abortion murine model consisting of females CBA/2x males DBA/J, in which an early infiltration of the decidua with macrophages expressing inducible nitric oxide synthase has been demonstrated (Haddad *et al.*, 1995). Recently, Kaare *et al.* analysed the entire open reading frame of the AMN gene in patients affected with recurrent spontaneous abortion but no causal mutations could be associated (Kaare *et al.*, 2006). This group based the candidate gene approach in the observation that a transgene-induced insertion in the *Amn* gene was prenatally lethal in mouse due to gastrulation defects (Wang *et al.*, 1996) generating the embryonic resorption.

In conclusion, our study describes the first report of an *in vivo* approach of the embryonic development in order to identify genes responsible for complex phenotypic traits in mice. Furthermore, the IRCS model allowed us to propose a restricted number of candidate genes causing embryonic resorption. We believe that these results could be an important starting point in understanding the genetic aetiology of RSA.

Materials and Methods

Animals

The Interspecific Recombinant Congenic Strains used in the present study were produced from an initial cross between *Mus spretus* (SEG/Pas, strain SEG) females and *Mus musculus domesticus* (C57BL/6J, strain B6) males, followed by three backcrosses of the F1 fertile males with B6 females and by at least 20 generations of brother/sister crosses (Burgio *et al.*, 2007, L'Hote *et al.*, 2007). The map of the different strains is given at the Pasteur Institute Website: http://www.pasteur.fr/ip/index.jsp. Thus, each strain contains homozygous genomic fragments (in average 2%) of *Mus spretus* included in a homozygous C57BL/6J genetic background (~98%). In order to create 66H substrains (66H-MMU1, 66H-MMU13 and 66H-MMU18), 66H female mice were crossed with C57Bl/6 males, then a F2 generation was produced. The progeny encompassing a unique *spretus* chromosomal fragment (MMU1, MMU13 or MMU18) was selected using microsatellites, amplified by a further B6 cross and re-established at the homozygous state by a brother/sister cross. Furthermore, during this process mice containing different allelic combinations were also generated (66H-MMU1+13, 66H-MMU1+18 and 66H-MMU13+18). The animals were bred in the Department of Mouse Functional Genetics of the Pasteur Institute (Paris). After weaning, 4 weeks aged mice were housed at the animal facilities of the Cochin Institute (Paris). Animals received food and water *ad libitum* and were exposed to a cycle of 12 h of light and 12 h of dark. Temperature was maintained at 20 degrees. In order to study embryo development and resorption, 8 to 12 week IRCS females were mated with C57BL/6J males, during a period of up to 4.5 days. Indeed, we could confirm that IRCS are intrinsically hypofertile showing a considerable enhancement of pregnancy rate from 10% to 40% when mated during 12 or 108 hours, respectively. All the experimental procedures were conducted in accordance with the policies of the Paris Descartes University, the Cochin Institute and the Guidelines for Biomedical Research Involving Animals.

Ultrasound biomicroscopy and phenotyping

Female mice (n = 229, Table 1) were subjected to at least 3 ultrasonographic examinations, named consecutively ultrasonography 1 (US1) to ultrasonography 3 (US3), using the high frequency ultrasound system Vevo 770 (Visualsonics, Toronto, Canada). Searching to establish potential major anatomical anomalies of the vagina, the uterus and the ovaries, US1 was performed on every female during the morning of the first day of the breeding period initiation. US2 and US3 were performed for all pregnant mice, between E8.5-E12.5 and E10.5-E14.5, respectively. Additional examinations were assessed on the individuals affected by the Late Embryonic Death (LED) phenotype in order to follow the regressive process leading eventually to Embryonic Resorption (ER). In order to prepare the animals for ultrasound examination, anaesthesia was induced in a chamber and maintained with a face mask using 1.5% of isoflurane (Minerve Veterinary Equipment, France). A chemical hair remover was used to eliminate the abdominal hair. The optimized contact between the skin surface and the transducer was obtained using an ultrasonographic contact gel. Body temperature, electrocardiographic and respiratory profiles were monitored using the Vevo 770's integrated heating pad and monitoring device. The ultrasound biomicroscope allows real time *in vivo* observations with a high resolution (~70 μm and ~40 μm lateral and axial resolution, respectively) using high frequency transducers (40-60MHz). Real time visualisation was assessed at 40 frames/minute. Searching the optimal image quality, RMV 704 and 708 transducers were used depending on the depth of each specific embryonic structure and the developmental stage.

Statistical analysis

The statistical value of the observed differences between the IRCS individuals and the control group (C57BL/6J) were evaluated by a student T-test corrected for multiple testing using the Bonferronni correction. Each female was analyzed separately; the variables observed were the number of implanted embryos, the number of dead embryos at US2 and US3, the position of implantation, the sex ratio of surviving pups at three days. Thus, $p<0.05$ was considered significant.

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