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Increased litter size and super-ovulation rate in congenic C57BL mice carrying a polymorphic fragment of NFR/N origin at the *Fecq4* locus of chromosome 9

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Summary

By analysing N2 mice from a cross between the inbred C57BL strain B10.Q and the NMRI-related NFR/N strain, we recently identified a quantitative trait locus (QTL) influencing litter size. This locus is now denoted *Fecq4*, and it is present on the murine chromosome 9. In the present paper, we describe how the *Fecq4* fragment originating form the NFR/N mouse strain will affect B10.Q mice by means of breeding capacity, super-ovulation rate and embryonic development *in vitro*. Our results show that both the breeding capacity (number of pups produced/breeding cage during a 5 months period) and the mean litter size are significantly increased in *B10.Q.NFR/N-Fecq4* congenic mice. Furthermore. *B10.Q.NFR/N-Fecq4* congenic mice (both homozygous and heterozygous) did respond much better to super-ovulation than wild-type mice, resulting in a dramatically increased yield of fertilized 1-cell embryos. In addition, embryos containing the *Fecq4* fragment were easy to cultivate *in vitro*, resulting in a higher yield of embryos reaching the blastocyst stage. We propose that *B10.Q.NFR/N-Fecq4* congenic mice may be used to improve breeding or super-ovulation rate in different types of genetically modified mice (on C57BL background) that exhibit severe breeding problems. The *Fecq4* fragment has been described in detail, and the possible role of polymorphic candidate genes near the linkage peak (58 Mb) has been discussed. Genes of the cytochrome P450 family (1, 11 and 19), such as *Cyp19a1*, are assumed to be particularly interesting, since they are known to exhibit female-associated reproductive phenotypes, affecting the ovulation rate, if mutated.

1. Introduction

A common problem in biomedical research is low reproductive performance of genetically modified model mice, which in turn has a negative impact on the production of new knowledge in medicine. However, some strains of mice have, after many years of selection, become more resistant to different types of environmental stress and produce larger litters in most types of animal facilities. Such strains are often denoted 'high breeders', and they carry genes ensuring large litter size, high stress resistance during pregnancy and good nursing properties during the lactation period. The NFR/N strain (an inbred NMRI-derived strain) is an example of an inbred strain with high breeding performance, while common C57BL strains normally produce lower number of liters, and are often denoted 'moderate breeders'. Females of the NFR/N strain are known to produce several large litters during a long period of time. Furthermore, they are known to be excellent mothers exhibiting very good nursing properties. Therefore, it is likely that NFR/N mice carry genetic polymorphisms (several modified genes) that are of importance for successful reproduction in mice.

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By studying the second backcross (N2) of the inbred mouse strains NFR/N and C57B1/10.Q (denoted B10.Q), we previously succeeded to identify a number of new quantitative trait loci (QTLs) involved in the maternal control of pregnancy success (Liljander *et al*., 2006). The loci identified are now denoted *Pregq1, Pregq2, Pregq3* and *Pregq4* affecting pregnancy rate; *Fecq3* and *Fecq4* controlling fecundity (litter size); and *Neogq1*, which is a maternal locus that is affecting the growth of the pups during the early lactation period.

The present paper is focusing exclusively on the *Fecq4* locus at chromosome 9 that seems to contain polymorphic genes that positively can affect the fecundity (litter size) in female mice. We find locus particularly interesting since, in addition, other groups have found QTLs of potential interest for both litter size (Kirkpatrick *et al*., 1998) developments of primary oocytes (Everett *et al*., 2004) and super ovulation rate (Spearow & Barkley, 1999; Spearow *et al*., 1999*a*, *b*) at this region of chromosome 9. The QTL observed by Kirkpatrick *et al*. (1998) was of suggestive significant, while the QTLs for primary oocyte development and super-ovulation rate both were significant. Mice different from C57B1 and NMRI had used in these studies. This convinced us that the *Fecq4* locus contains one or more polymorphic genes that are of general importance for the control of reproductive performance in female mice.

In the present paper, we provide data from *B10.Q.NFR/N-Fecq4* congenic mice (*Fecq4* locus of NFR/N origin in B10.Q mice, from now only denoted *Fecq4* congenics) showing that both litter size, super-ovulation rate and embryo development *in vitro* is significantly elevated in *Fecq4* congenic compared with wild-type B10.Q female mice. We also discuss the possible role of candidate genes within this region of chromosome 9.

2. Material and methods

(i) *Animals*

NFR/N mice were originally obtained from the National Institute of Health (Maryland, USA) and the B10.Q mice were bought form The Jackson Laboratory (Bar Harbor, Maine, USA). (B10.Q $\propto$ NFR/N) $\propto$ B10.Q N8 mice were bred in IVC-cages in the BMC barrier animal house and at the Biomedical Center and the Pathology animal house at Lund University, Sweden. All mice were fed *ad libitum* with standard rodent pellets (LAB FOR R36, irradiated breeding food for rats and mice, Lactamin AB, Sweden) and water in a climate-controlled environment with a photoperiod of LD 12 : 12. The mice used in the present study had clean health monitoring protocols according to the Federation of European Laboratory Animal Sciences Association (FELASA) recommendations. Ethical permissions: M125-04 (embryo transfer) and M236-06 (reproduction and arthritis).

(ii) *Description of breeding cages*

A total number of 17 *Fecq4* females and 20 control B10.Q females were used in the study of litter size. The set-up of the breeding cages were as follows: two females were kept together with one B10.Q male for five month. During this time the number of litters and pups were counted. All the females were 10 weeks old when the study started.

(iii) *Description of the congenic strain and the* Fecq4 *locus*

The B10.Q.Fecq4 congenic mice were produced by traditional back-crossing through the replacement of a selected region from the NFR/N on chromosome 9. Briefly, mice from an NFR/N $\propto$ B10.Q N2 backcross, heterozygous for selected markers on chromosome 9, were backcrossed to B10.Q for eight generations (N8). At this stage the mice are considered to be 99·6% B10.Q homozygous on all the other chromosomes. Mice heterozygous for markers between D9Mit27 (50·4 Mb) and D9mit124 (75·9 Mb) were then intercrossed two times to produce the congenic line *Fecq4*. All the mice in the study are homozygous and the fragment size is equal in all animals (Fig. 1). To exclude the possibility that there may have been any NFR/N alleles selectively retained during backcrossing at other known QTLs for reproductive loci, we genotyped the following loci: *Fecq1* and *Fecq2* (Kirkpatrick *et al*., 1998), *Fecq3*, *Pregq1*, *Cia40*/*Pregq2*, *Pregq3* and *Pregq4* (Liljander *et al*., 2006*a*, *b*; 2008). The genotype analysis showed that no remaining NFR/N fragments were present at these loci.

(iv) *Super-ovulation and embryo preparation*

The standard protocols for super-ovulation and embryo preparation from oviducts (Nagy *et al*., 2003) were followed. Briefly, age matched, 4 weeks old female mice were first injected i.p. with 5 i.u. of the follicle stimulating hormone PMSG (G-4877, SIGMA) at 2 p.m. in the afternoon. At noon, 46 h later, the females received an i.p. injection of 5 i.u of the ovulation-inducing hormone hCG (C-1063, SIGMA) and were placed together with single-caged adult B10.Q males (1 female/male). Mating normally takes place in the middle of the night, and on the following morning (E.D. day 0·5) the females were inspected for vaginal plugs. The females were sacrificed and oviducts removed. Oviducts from each mouse were put in tubes with medium M2 (M7167, SIGMA). The 1-cell embryos were then prepared under a dissection

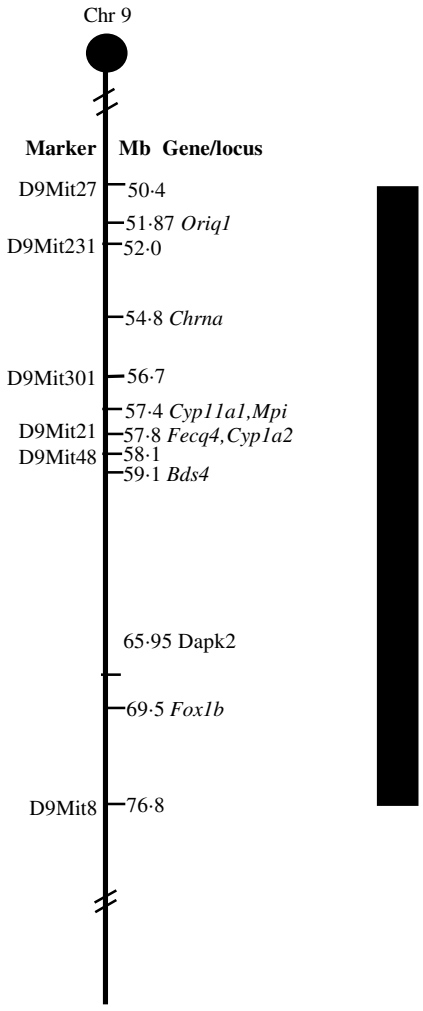

Fig. 1. The dark area indicates the NFR/N congenic fragment, *Fecq4* in the congenic mice *B10.Q.NFR/N-Fecq4*. The markers are placed according to Mouse Ensemble built 36 (http://www.ensembl.org/Mus_musculus/index.html).

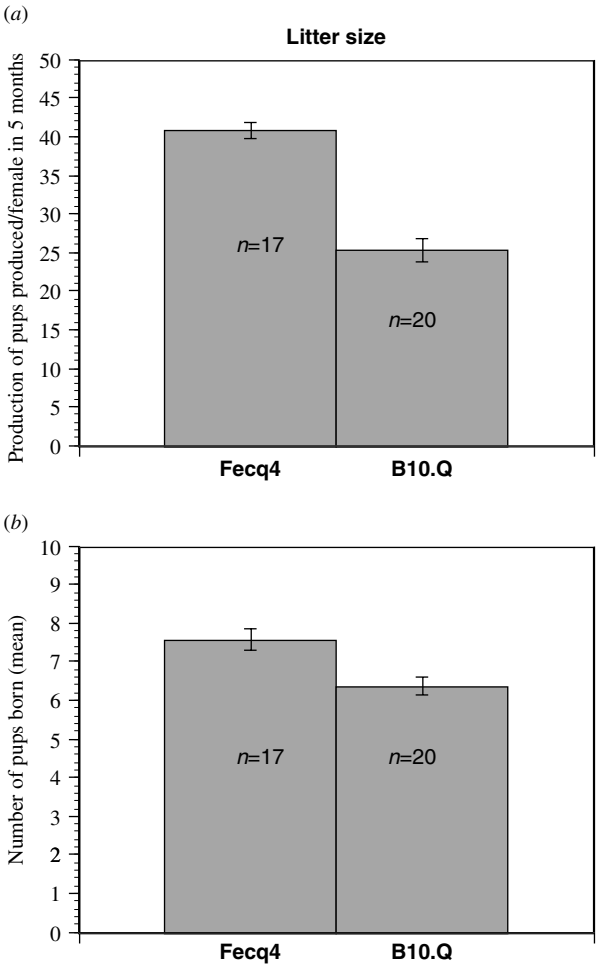

Fig. 2. (*a*) The total production of pups per female in congenic B10.Q.NFR/N-*Fecq4* (*Fecq4*) and B10.Q control mice over a five months period (mean value S.E.). $P = 0{\cdot}035$.
(*b*) The mean value (S.E.) for the litter size from each female over a five-month of period. Although *B10.Q.NFR/N-Fecq4* mice exhibited a higher litter size, the difference was not quite significant ($P = 0{\cdot}094$).

microscope, in drops of M2 medium supplemented with hyaluronidase (makes the embryos separate), and washed several times in medium M2 and before subjected to *in vitro* cultivation.

(v) In vitro *cultivation of pre-implantation embryos*

The *in vitro* cultivation of the pre-implantation embryos was, in principle, performed according to the standard protocol (Nagy *et al*., 2003). Briefly, the one-cell embryos were transferred to small Petri dishes containing 30 μl drops of medium M16 (M7292, SIGMA) covered with mineral oil. Each drop contained the total yield of 1-cell embryos from one individual mouse. The embryos were incubated at 37 °C in humidified air containing 5% CO_2. The number of viable embryos of expected developmental stage (2-cell embryos day 1·5, morula day 2·5 and blastocysts day 3·5 and 4·5) was counted once every day.

(vi) *Micro-satellite genotyping*

Toe biopsies were collected from all females and DNA was isolated according to a previously described protocol (Laird *et al*., 1991). The congenic fragment was genotyped with ten fluorescence-labelled micro-satellite markers (INTERACTIVA, Ulm, Germany) in order to verify the size of the congenic fragment. PCR amplification was performed according to a previously described protocol (Laird *et al*., 1991). The following program was used to amplify the DNA: denaturation at 95 °C for 3 min, annealing at 56 °C for 45 s, polymerization at 72 °C for 1 min, 30 cycles of 95 °C for 30 s, 56 °C for 45 s, 72 °C for 1 min and a final extension step of 7 min at 72 °C. The PCR products were analysed on a MegaBACE™ 1000 (Amersham Pharmacia Biotech) according to the manufacture's protocol. Data were analysed with

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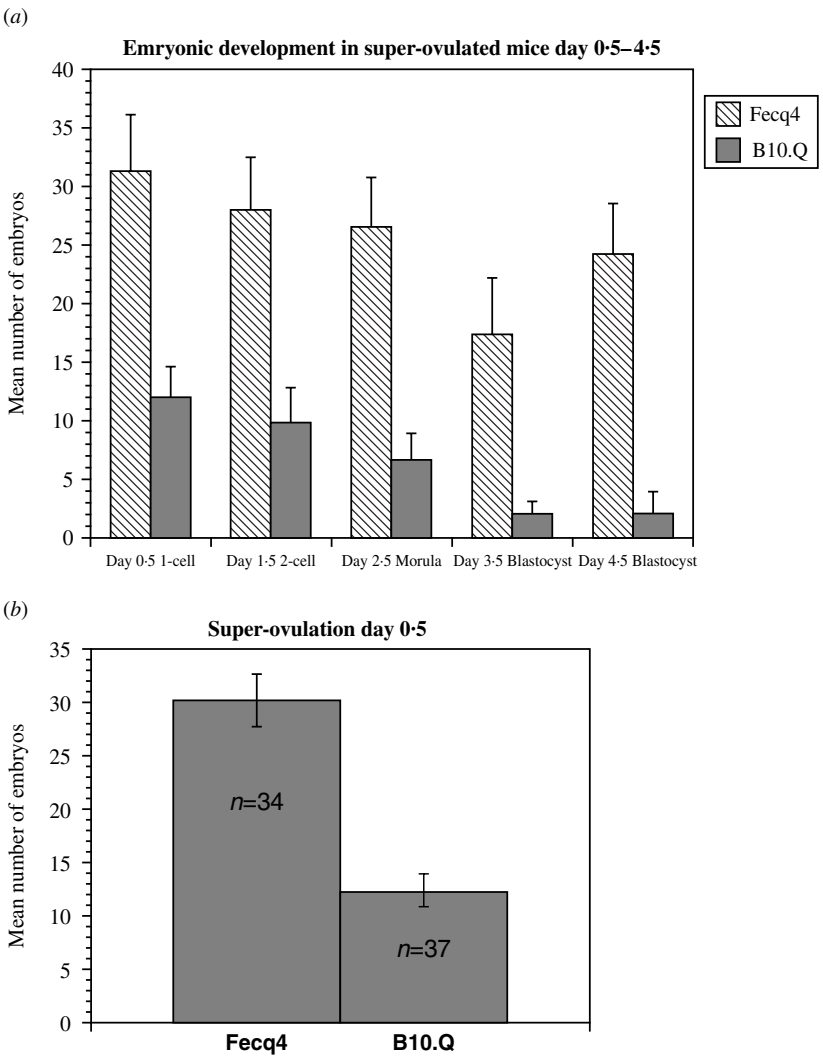

Fig. 3. (*a*) Difference in the number of embryos obtained after super-ovulation in *B10.Q.NFR/N-Fecq4* (*Fecq4*) and B10.Q control females. Mean value (S.E.) from ten ($n=10$) *Fecq4* and ten ($n=10$) B10.Q females at 4 weeks of age. (*b*) Difference in the yield of embryos after super-ovulation in *B10.Q.NFR/N-Fecq4 congenic mice* (*Fecq4*) and B10.Q controls. Mean value (S.E.) from $n=34$ *Fecq4* congenic females and $n=37$ B10.Q females ($P=0{\cdot}001$).

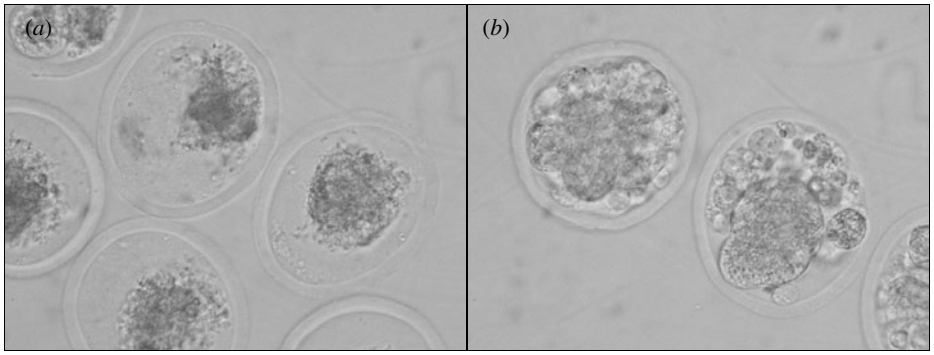

Fig. 4. Common shape of unfertilized B10.Q embryos. The pictures show the most common shapes of unfertilized B10.Q wild-type and *B10.Q.NFR/N-Fecq4* congenic mouse embryos after 24 h of *in vitro* cultivation. Characteristically, the embryos die quickly by lysis (*a*) or undergo uncontrolled cell division leading to a 'morula-like' shape (*b*). These two 'death pathways' of unfertilized embryos were equally common in the strains studied. A small proportion ($<5\%$) of the unfertilized B10.Q wild-type and *B10.Q.NFR/N-Fecq4* congenic embryos did undergo parthenogenetic cell division, which is almost impossible to distinguish from ordinary cell division.

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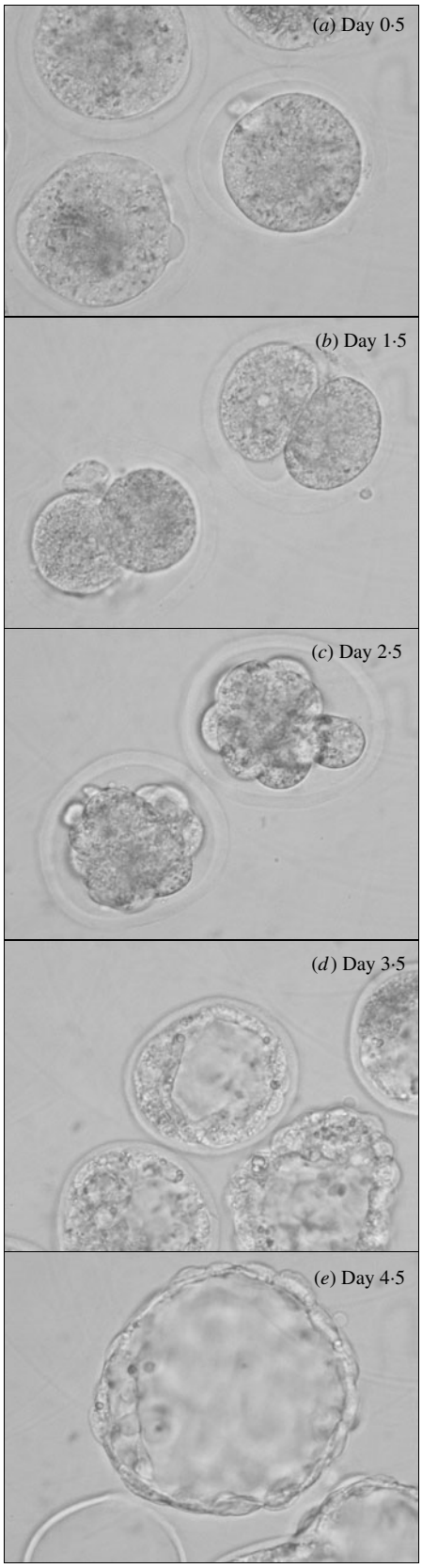

Fig. 5. Development of embryos day 0·5–4·5.

Genetic Profiler 1·5 through comparison from parental mouse strains.

(vii) *Statistical analysis*

Statistical analyses were performed by the two-tailed Mann–Whitney *U*-test.

3. Results

(i) *Breeding capacity of* Fecq4 *congenic mice*

Fecq4 congenic mice produced significantly more pups per female than homozygous B10.Q littermates when counting the production of pups over a five months period of time (Fig. 2*a*). Furthermore, the *Fecq4* congenic mice also produced significantly more litters ($P = 0{\cdot}011$) over the same period of time. Although *Fecq4* congenic mice characteristically showed a larger litter size (7·6 pups/*Fecq4* congenic female compared with 6·3 pups/B10.Q female), this difference was not significant ($P = 0{\cdot}094$, Fig. 2*b*).

(ii) *Oocyte yield in response to super-ovulation and embryo development* in vitro

As outlined in Fig. 3*a* and *b* the congenic *Fecq4* mice responded more efficiently to a standard super ovulation protocol compared with the B10.Q wt mice. The congenic mice used in the present study were all homozygous for the congenic NFR/N fragment. The congenic and the wild-type mice were all of the same age (4 weeks), and both congenic and wt controls were allowed to mate with adult, fertile B10.Q male mice. The *in vitro* development of fertilized embryos was more efficient in the embryos that were heterozygous for the *Fecq4* fragment. In fact the median egg yield in response to hormone treatment (day 0·5) was doubled, but the development to the blastocyst stage (day 3·5–4·5) was almost 4–5-fold increased. The lower yield of blastocysts on day 3·5 compared with day 4·5 was due to the fact that some embryos still were at the morula stage on day 3·5. Unfertilized negative control embryos (embryos prepared from super ovulated females that were not allowed to go with males) characteristically died by two main pathways: lysis or uncontrolled cell division (Fig. 4).

Figure 5 shows *in vitro* development of pre-implantation embryos day 0·5–4·5. The characteristic difference between day 3·5 and day 4·5 blastocysts is obvious. On day 3·5 many morulae have not yet developed the blastocoels, and those blastocysts that are visible are at an early development stage. On day 4·5 almost all embryos has reached the blastocyst stage, and many of them have also hatched (been released from egg shell – the zona pellucida) and expanded.

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Table 1. *Candidate genes*

Gene	Position (Mb)*	Description	Reproductive or inflammatory phenotypes of mutation
Cyp19a1	53·9	Cytochrome P450, family 19, subfamily a, polypeptide	Defects affecting fertility such as impairments of ovulation, spermiogenesis and mounting behaviour.
Chrna3	54·0	Cholinergic receptor, nicotinic, alpha polypeptide 3	High postnatal and post weaning mortality.
Mpi	57·3	Mannose phosphate isomerase	Embryonic lethality during organogenesis.
Cyp11a1	57·8	Cytochrome P450, family 11, subfamily, a, polypeptide	Steroid deficiency, males are feminized with female external genitalia and underdeveloped gonads.
Bbs4	59·1	Bardet–Biedl syndrome 4 homolog (human)	Embryonic lethality and infertility.
Dapk2	66·0	Discs, large (Drosophila) homolog-associated protein 2	–
Foxb1	69·6	Forkhead box B1	High embryonic lethality and postnatal mortality.

Summary of possible candidate genes on chromosome 9 for *B10.Q.NFR/N-Fecq4* congenic mouse. (http://www.informatics.jax.org/).

4. Discussion

A limited number of linkage analyses based on QTL mapping have been performed for identification of gene regions controlling litter size in female mice (Kirkpatrick *et al*., 1998; Peripato *et al*., 2002, 2004). As previously mentioned, Kirkpatrick *et al*. (1998) reported a locus of suggestive significance for the trait litter size close to the *Fecq4* locus, while Peripato *et al*. (2002, 2004) did not detect any significant loci for this trait on chromosome 9. However, the fact that Everett *et al*. (2004), Spearow & Barkley (1999) and Spearow *et al*. (1999*a*, *b*) did detect loci, significantly linked to ovulation traits, in the vicinity of the *Fecq4* locus convinced us that this region of chromosome 9 indeed contain polymorphic genes important for optimizing breeding success in female mice. It is possible that polymorphisms or mutations in the same gene/s affect both spontaneous ovulation and artificial hormone-induced ovulation (super ovulation), as well as litter size. For these reasons, we found it important to produce a congenic *Fecq4* mouse line that could be investigated for the traits mentioned above, and that could be used for the final identification of the critical gene/s. In addition, the *Fecq4* congenic strain could be a useful tool to optimize breeding in model mice of the C57BL strain that exhibit female-associated breeding problems. Congenic *Fecq4* mice could also be valuable in transgenic technology, since the C57BL strain is preferable for the production of 'classical transgenic' (by pro-nucleus injection) mice, and the response to super ovulation will determine the yield of injectable embryos.

In fact, the *Fecq4* mouse strain has already been used at our laboratory to facilitate the freezing and embryo transfer of two-cell embryos from other congenic strains (strains that were undergoing backcrossing against *B10.Q*, and quickly had to be transferred to another laboratory).

Male mice of two different congenic strains from Dr Holmdahls Laboratory (Medical Inflammation Research, Lund University, Sweden and Karolinska Institute, Department of Medical Biochemistry and Biophysics, Division of Medical Inflammation Research, Sweden), which had to undergo transfer from Lund to Stockholm as frozen embryos, were allowed to mate with super-ovulated, age-matched *Fecq4* and *B10.Q* females. Equal number of *Fecq4* and *B10.Q* females were used for the super-ovulation, but it both cases the final yield of 2-cells were 2–3 times increased if *Fecq4* females had been used in mating. The increased yield of frozen embryos obtained by the mating with the *Fecq4* females turned out to be critical for the final success of the transfer of these congenic lines. An increased embryo yield from the B6 strain is also very valuable in transgenic technologies – especially when doing pronuclear injections.

The B10.Q mouse is a common mouse in all laboratories working with the H2q-dependent collagen II-induced arthritis models and other H2q-dependent models. Since the ordinary C57BL/6 mouse (the B6 mouse) is a more universal model mouse, we have now started to backcross the *Fecq4* congenic strain against B6. We assume that the increase in egg yield by super-ovulation can be of general value in cryopreservation of transgenic or congenic lines on B6 background, or lines that are undergoing backcrossing against B6.

The results of the present study indeed show that the *Fecq4* congenic strain has higher breeding performance than ordinary B10.Q wild-type mice, both with respect to litter size and the production of offspring over time. However, even more pronounced is the elevated response to artificial hormone-induced

ovulation according to the standard super ovulation protocol, and the dramatically increased yield of viable pre-implantation embryos after *in vitro* cultivation. The latter property of our *Fecq4* congenic line could be used to optimize the result of embryo freezing and embryo transfers of straits that exhibit extremely low response in super ovulation, or are difficult to grow *in vitro*. Normally, only one or two further backcrossings are needed to remove the *Fecq4* fragment.

Although the *Fecq4* congenic strain can be useful without further reduction in fragment size, it is of great interest to identify the specific polymorphic or mutated gene(s) that actually are causing the positive effects observed. The *Fecq4* fragment contains over 200 genes and a few of these genes have previously been reported to affect reproductive phenotypes that might influence traits such as super-ovulation and litter size. In Table 1, we have listed some of the most interesting candidate genes that are present in the congenic fragment of chromosome 9. We have paid special attention to genes near the linkage peak (58 Mb) of the previously detected QTL (*Fecq4*), and genes that are known to affect reproductive traits associated with the females.

Although still very speculative, there are a number of genes that we find particularly interesting. Firstly, genes of the cytochrome P450 family (1, 11 and 19) present in this region all exhibit interesting reproductive phenotypes if mutated (Fisher *et al*., 1998). The *Cyp19* gene control the formation of estrogens from C_{19} steroids, and the process is catalysed by aromatase cytochrome P450 (P450arom). In particular, *Cyp19a1* appears to be significant in this context, since null-mutated mice have shown impaired ovulation and impaired reproductive behaviour (mounting). Secondly, a gene of potential interest is the tyrosine kinase gene *Chrna3*, since null mutations will cause embryonic and postnatal death (Xu *et al*., 1999).

Thirdly, the *Mpi* gene (mannose phosphate isomerase), close to the linkage peak of *Fecq4*, is also of potential interest, since null-mutated mice exhibit defects in the development of extra-embryonic tissues and embryonic death (DeRossi *et al*., 2006).

In order to find polymorphisms between NFR/N and B10 mice concerning the genes mentioned above, sequencing analyses are needed. According to known effects of null-mutations, we are paying special attention on the *Cyp19a1* gene, and the possible influence of a mutation/polymorphism between the NFR/N and B10.Q strains.

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