The estrogen receptor locus is associated with a major gene influencing litter size in pigs

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ABSTRACT Identification of individual major genes affecting quantitative traits in livestock species has been limited to date. By using a candidate gene approach and a divergent breed cross involving the Chinese Meishan pig, we have shown that a specific allele of the estrogen receptor (*ER*) locus is associated with increased litter size. Female pigs from synthetic lines with a 50% Meishan background that were homozygous for this beneficial allele produced 2.3 more pigs in first parities and 1.5 more pigs averaged over all parities than females from the same synthetic lines and homozygous for the undesirable allele. This beneficial *ER* allele was also found in pigs with Large White breed ancestry. Analysis of females with Large White breed background showed an advantage for females homozygous for the beneficial allele as compared to females homozygous for the other allele of more than 1 total pig born. Analyses of growth performance test records detected no significant unfavorable associations of the beneficial allele with growth and developmental traits. Mapping of the *ER* gene demonstrated that the closest known genes or markers were 3 centimorgans from *ER*. To our knowledge, one of these, superoxide dismutase gene (*SOD2*), was mapped for the first time in the pig. Analysis of *ER* and these linked markers indicated that *ER* is the best predictor of litter size differences. Introgression of the beneficial allele into commercial pig breeding lines, in which the allele was not present, and marker-assisted selection for the beneficial allele in lines with Meishan and Large White background have begun.

Genetic linkage and cytological maps in humans and livestock offer the promise of discovering individual loci that control important biological functions and diseases. Individual genes that cause disease or influence physiological functions in humans have been reported (1) but the list in livestock is limited (2). Moreover, the genetic dissection of complex quantitative traits, such as those involving production, health, and reproduction traits, represents a formidable challenge to researchers (3). However, the ability to make specific crosses and the increasing availability of genetic markers and candidate genes have made it possible to identify quantitative trait loci (QTL) that have a large influence on specific traits of economic interest in plants (4, 5) and animals (6, 7).

Reproductive traits are of primary interest in livestock because they play a major role in efficiency of production. Selection for increased number of offspring has been employed in model species like mice (8) and in pigs (9–11) and sheep (12) with only limited success because of the low heritability and the sex-limited nature of reproductive traits. Understanding the genetic control of complex traits such as reproduction requires a fundamental understanding of the genes and biochemical processes involved. One approach is to

use large numbers of available genetic markers and linkage maps to identify regions of the genome influencing quantitative traits. Once chromosomal regions containing important QTL have been identified, the search then focuses on these regions to isolate the individual genes involved in the process of interest. This approach has been extremely useful in identifying disease loci in humans (13, 14). The causative mutation for an important pig disease, porcine stress syndrome, was identified in this manner (15). In sheep, an autosomal mutation called the Booroola gene, designated fecundity Booroola or *FecB* (16), and an X chromosome-linked gene, designated *FecX* (17), that increase ovulation rate and litter sizes have been identified by using anonymous markers. An alternate strategy to using genome-wide scans is to employ the candidate gene approach, which makes use of the catalog of human genes identified by medical and biochemical research to identify genes in livestock that might be expected to play a role in the biological process of interest.

Steroid hormones and their receptors play an important role in reproductive processes (18). Cells in target tissues have receptor proteins that specifically bind the hormone during the initial stage in its action. Estrogen is intimately involved with pregnancy and its function is mediated through the estrogen receptor (*ER*). Mutations in this protein have been implicated in spontaneous abortion and in human breast cancer (19). It has been recently shown that transgenic mice containing a nonfunctional *ER* gene have considerable phenotypic changes in the reproductive system (20). Therefore, *ER* was chosen as a candidate gene to study litter size in pigs.

Use of animals from diverse genetic backgrounds can increase the ability to detect major QTL. The Chinese Meishan pig has approximately four more piglets per litter but is slower growing and fatter than American or European commercial breeds (21, 22). The importation of Chinese pigs into the United States and several countries in Europe in the 1970s and 1980s was motivated in large part by the goal to discover the genes that control greater litter size.

To determine a genetic cause of the increased litter size observed in Meishan pigs, a study was initiated to determine the role of the *ER* gene on litter size in pigs. Additionally, since no consensus linkage map existed in pigs, markers from several linkage maps in the region near *ER* were genotyped to determine whether any of the other loci near *ER* were more highly associated with differences in litter size.

MATERIALS AND METHODS

Animals. All pigs used in these experiments were bred and raised at genetic nucleus herds owned and operated by either

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Abbreviations: lod, Logarithm of odds; cM, centimorgan(s); *ER*, estrogen receptor; QTL, quantitative trait loci; TNB, total number born; NBA, number born live; ADG, average daily gain; BF, backfat thickness; FN, number of functional nipples.

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PIC USA or PIC UK. Purebred PIC USA Meishan pigs were initially genotyped and their litter records were collected and analyzed. However, due to the limited size of the pure Meishan line, animals from synthetic lines involving the Meishan breed were used. Females from a 50% United States/50% Meishan synthetic line and females from a 50% European/50% Meishan synthetic line were used for the first analysis. Since it was important to reduce the effect of background genes and to clearly examine the effect of the *ER* locus, data involving only families were collected. These data consisted of litter records from groups of sisters in which at least two *ER* genotypes existed. Initially, collection of such data from existing records was limited. However, planned matings, involving sires and dams that had been previously genotyped, were made to speed the process of collection of data from families of sisters with different *ER* genotypes. The parental genotypes involved in these matings were AA $\times$ AB, AB $\times$ AB, and AB $\times$ BB (alleles at pig *ER* described below). In analysis 1, the total number of females from the two lines was 161 from 50 families (Table 1). For analysis 2, other synthetic commercial lines were screened for the previously identified *ER* polymorphism. Animals of PIC lines with Large White ancestry had this *ER* polymorphism and were then used in the second phase of this study. The total number of animals and records for analysis 2 is given in Table 1.

Several performance traits were recorded. Litter traits (records) included total number born (TNB), which was calculated as live births plus stillborn animals (excluding mummified fetuses), and number born alive (NBA). Growth performance traits were measured on nearly all females from analyses 1 and 2. These performance records included average daily gain (ADG) over a test period, ultrasonically measured backfat thickness (BF), and number of functional nipples (FN).

Molecular Biology. Blood or other tissue samples were collected by PIC personnel under approved animal care procedures and shipped either to Iowa State University or a PIC laboratory for analysis. Initial analyses consisted of isolation of genomic DNA, digestion with *Pvu* II endonuclease, electrophoresis, blotting, and hybridization with a 1.3-kb cDNA insert of the human *ER* gene probe (23), as described by Rothschild *et al.* (24). This cDNA, which covers the entire coding region, has been shown to be well conserved across species (25, 26). Fragment sizes were estimated by using molecular size standards. Results of an example Southern blot analysis are shown in Fig. 1. Based on previous data, the allele with a 4.3-kb fragment was designated the A allele and the allele with a 3.7-kb fragment was called the B allele (27).

To genotype a larger number of animals a second method of detection of the *ER* polymorphism was developed. A polymerase chain reaction (PCR) test was developed by obtaining sequence data from the genomic regions surrounding the

FIG. 1. Restriction fragment length polymorphism analysis of 10 unrelated pigs. The 4.3-kb (A allele) and 3.7-kb (B allele) fragments are shown with homozygous and heterozygous animals.

polymorphic *Pvu* II site. This test** is now in use to determine all *ER* genotypes.

Statistical Analyses. Data from analyses 1 and 2 were analyzed separately since they represented synthetic lines with very different genetic backgrounds (Meishan vs. Large White). An animal model with full relationships (28) was employed for each analysis. The assumed statistical models used to analyze the litter size traits TNB and NBA included the fixed effects of generation, contemporary group farrowed, and the *ER* genotype effect. Random effects included the animal and random residual effects. Heritabilities were estimated from the data for both TNB and NBA and were 0.09 for analysis 1 and were 0.12 and 0.17 for TNB and NBA, respectively for analysis 2. For the performance traits, the assumed model in analysis 1 included the fixed effects of contemporary group tested, generation, and *ER* genotype. Heritabilities were assumed to be 0.25 (ADG), 0.30 (BF), and 0.09 (FN). For analysis 2, the model was similar but a fixed effect for line was included and an interaction of line by *ER* was included since there are some genetic differences between the lines. For analysis 2, the assumed heritabilities were 0.46 (ADG), 0.48 (BF), and 0.17 (FN). Additive effects and their significance were estimated by using linear regression techniques, regressing the litter trait on number of copies of the B allele of *ER*. The dominance deviation was calculated as the deviation of the heterozygotes from the mean of the homozygotes, using the least squares means. Significance was tested as the lack of fit to the additive model.

Markers and Genotyping. No consensus linkage map around *ER* existed at the initiation of this project. Markers expected to be near *ER* from the two previously published linkage maps were chosen (29, 30). Porcine microsatellite markers in this study included *Sw552*, *Swr485*, *Sw64*, *Sw137*, and *S0008*, and methods used in their analyses were as described (30, 31). Additionally, superoxide dismutase 2 (*SOD2*) was chosen based on syntenic information from the human map and animals were genotyped for *SOD2* as described (32). Eighteen families of female offspring only (83 total) were genotyped for the above genes/markers.

**This PCR test for *ER* genotype is performed by PIC under a license with Roche Molecular Systems, Inc.

Table 1. Summary of data structure from females of the PIC lines

Parameter	Value
Analysis 1—Meishan synthetic lines	
No. of sires	27
No. of dams	50
No. of females	161
No. of first parity records	161
No. of all parity records	276
Analysis 2—Large White synthetic lines	
No. of sires	181
No. of dams	565
No. of females	1079
No. of first parity records	1079
No. of all parity records	1912

Table 2. Effect of the ER genotypes on reproductive traits in Meishan synthetic line females

	First parity			All parities		
	N	TNB	NBA	N	TNB	NBA
ER genotype						
AA	62	10.1 ^a	9.1 ^a	98	11.0 ^a	10.2 ^a
AB	73	11.4 ^b	10.5 ^b	125	12.4 ^b	11.5 ^b
BB	26	12.4 ^b	11.4 ^b	53	12.5 ^b	11.7 ^b
Effect						
Additive		1.2	1.2		0.9	0.8
Dominance		0.2	0.2		0.7	0.6

N, total number of records. Additive effect was estimated as the linear regression of litter size on ER (for all values, $P < 0.01$). Dominance effect was estimated from the least squares means.

^{a,b}Means in the same column with different superscripts significantly differ at $P < 0.01$.

Linkage Analyses. To perform our analyses, data from 18 full-sib families were used. Only female data were included, so the map represents a female linkage map only. Animals were genotyped twice for accuracy and data entry was also double-checked for accuracy. Linkage analyses were performed by using MAPMAKER (33). Pairwise analysis of linkage was performed with the two point option of MAPMAKER. Logarithm of odds (lod) scores greater than 3.0 were assumed significant. The order of the markers and genes was obtained by comparing $\log_{10}$ likelihoods of the permutations of all markers and genes included using the MAPMAKER compare option. The best order was selected by using the highest $\log_{10}$ likelihood from among all permutations. Distances in Kosambi centimorgans (cM) between the genes and markers were estimated for the multipoint map using the map option in MAPMAKER.

RESULTS

The ER Gene Is Associated with Increased Litter Size.

Initial analyses involving 21 purebred PIC USA Meishan females suggested that the B allele was associated with an increase in TNB and NBA of approximately 3 pigs per litter (data not shown). However, the limited data made this result statistically nonsignificant. Analysis 1 involved the 50 families of sisters from the two synthetic lines with 50% Meishan genes and results are in Table 2. For first parity litters, the difference between BB and AA homozygotes was 2.3 pigs per litter ($P < 0.01$) for both TNB and NBA. The additive substitution effect for both TNB and NBA was 1.2 pigs per litter per B allele ($P < 0.01$) and no dominance was detected. For all parities, the effect of the B allele was slightly less than that seen in first parities. The BB homozygote females produced 1.5 more pigs

Table 3. Effect of the ER genotypes on reproductive traits in Large White lines

	First parity			All parities		
	N	TNB	NBA	N	TNB	NBA
ER genotype						
AA	444	9.5 ^a	8.7 ^a	759	9.8 ^c	9.0 ^c
AB	391	9.9 ^{a,b}	9.2 ^{a,b}	677	10.4 ^d	9.5 ^d
BB	244	10.7 ^b	9.9 ^b	476	10.7 ^d	9.9 ^d
Effect						
Additive		0.6	0.6		0.5	0.5
Dominance		-0.2	-0.1		0.2	0.0

Additive effect was estimated as the linear regression of litter size on ER (for all values, $P < 0.01$). Dominance effect was estimated from the least squares means.

^{a,b}Means in the same column with different superscripts significantly differ at $P < 0.05$.

^{c,d}Means in the same column with different superscripts significantly differ at $P < 0.01$.

Table 4. Summary of effects of ER genotypes on performance measures

ER genotype	Meishan synthetic females				Large White synthetic females			
	N	ADG, g/day	BF, mm	FN	N	ADG, g/day	BF, mm	FN
AA	50	793	18.4	13.7	444	853	11.6	13.5
AB	61	803	19.1	14.1	391	853	11.7	13.4
BB	17	808	19.8	14.3	244	870	12.0	13.2

Means do not differ significantly ($P > 0.10$).

per litter ($P < 0.01$) for both TNB and NBA than did AA females. The additive allelic substitution effect was estimated to be 0.9 pig per litter per B allele for TNB ($P < 0.01$) and 0.8 pig per litter per B allele for NBA ($P < 0.01$). No dominance effects were observed for parity 1 data but effects appear dominant over all parities, although the data were insufficient to reject the hypothesis of an additive model.

Analyses of pure breeds (27) and PIC synthetic lines suggested that the *Pvu* II ER polymorphism existed only in commercial lines with the Large White background. To examine further the associated effect of the ER gene on litter size, analysis of litter records from synthetic PIC lines with Large White ancestry was conducted. Results from analysis 2 are presented in Table 3. First parity differences between BB and AA females were 1.2 pigs per litter for both TNB and NBA ($P < 0.05$). The additive substitution effect was 0.6 pig per litter per B allele ($P < 0.01$). For all parities, differences between the BB and AA females were 0.9 pig per litter for both TNB and NBA ($P < 0.01$). Additive allelic substitution effects were estimated to be 0.5 pig per litter per B allele ($P < 0.01$).

Analyses of performance traits and FN are in Table 4. BB females grew slightly faster, were fatter, and had more FN than AA females but none of the relationships were significant between ER and ADG, BF, or FN. The differences in FN appeared to exist for the 50% Meishan synthetic lines but results were not confirmed for lines of Large White background.

A Female Consensus Linkage Map Near ER. Results from the pairwise linkage analysis using MAPMAKER are presented in Table 5. All markers and genes had some significant linkages except *S0008*. The multipoint linkage map based on the highest $\log_{10}$ likelihood value developed from these results (Kosambi

Table 5. Summary of two-point lod scores for genes and markers near ER on pig chromosome 1

Locus 1	Locus 2	θ	lod score
<i>Swr485</i>	<i>Sw552</i>	0.06	6.25
<i>ER</i>	<i>Sw552</i>	0.12	12.71
<i>ER</i>	<i>Swr485</i>	0.02	13.87
<i>Sw137</i>	<i>Sw552</i>	0.20	1.46
<i>Sw137</i>	<i>Swr485</i>	0.08	7.89
<i>Sw137</i>	<i>ER</i>	0.03	7.20
<i>Sw64</i>	<i>Sw552</i>	0.17	6.57
<i>Sw64</i>	<i>Swr485</i>	0.10	16.06
<i>Sw64</i>	<i>ER</i>	0.06	9.23
<i>Sw64</i>	<i>Sw137</i>	0.06	6.97
<i>S0008</i>	<i>Sw64</i>	0.25	2.10
<i>S0008</i>	<i>Sw137</i>	0.18	2.19
<i>S0008</i>	<i>ER</i>	0.16	2.38
<i>S0008</i>	<i>Swr485</i>	0.27	2.07
<i>SOD2</i>	<i>Swr485</i>	0.09	1.56
<i>SOD2</i>	<i>Sw552</i>	0.00	2.41
<i>SOD2</i>	<i>ER</i>	0.00	1.20
<i>SOD2</i>	<i>Sw64</i>	0.00	3.01

θ is a recombination distance estimate; lod scores exceeding 3.0 are significant ($P < 0.01$).

centimorgans in brackets) is: *Sw552*–[8.3 cM]–*Swr485*–[3.2 cM]–*ER*–[2.9 cM]–*SOD2*–[2.9 cM]–*Sw137*–[2.6 cM]–*Sw64*–[25.4 cM]–*S0008*. The distance between *Sw64* and *S0008* is considered to be poorly estimated due to the lack of significant lod scores. To our knowledge, this is the first reported mapping of *SOD2* in the pig and results that link *SOD2* to *ER* are similar to that linkage found in humans. The location of *SOD2* is tentative because the limited number of informative meioses prevented determining its exact location.

DISCUSSION

Genetic improvement of litter size has been limited in the past because of the low heritability and sex-limited expression of reproductive rate and the fact that identification of major genes or QTL affecting reproductive traits in livestock has been minimal. While markers for major genes or QTL for litter size in the sheep have been recently detected (16, 17), there has been little evidence to date for major genes affecting litter size in pigs (34). Identification of a specific gene or an anonymous genetic marker for litter size traits in pigs could have a major impact on the improvement of reproductive performance by increasing accuracy of selection. The research reported here was directed at determining whether the *ER* gene, a steroid binding hormone receptor gene, was associated with increased litter size. Our results demonstrate that genetic differences at the *ER* locus exist and that one *ER* allele, discovered initially in the Meishan, is significantly associated with higher litter size. The gene effect appears to be essentially additive in nature in first parity litters, although data over all parities are insufficient to rule out nonadditive effects. The effect of the *ER* allele B in first parity litters in the Meishan synthetic lines is an increase in litter size of more than 20% and represents a real economic advantage since an extra pig per litter is worth from \$15–\$30 in the United States depending on market conditions.

Some reduction in the effect of the B allele is seen when all parities are averaged for the Meishan synthetics. In fact, the effect of the *ER* allele B in second and later parity records (0.5 pig for TNB) is much more similar to the 0.6–0.5 pig effect of the B allele seen in the Large White synthetic lines. The litter size difference between first and later parities may reflect that genetic differences for litter size in older and more physiologically similar females are reduced or masked by environmental effects such as nutritional effects and stress related to larger first litters. Also, one unanswered question is the actual role that *ER* may play in controlling litter size. It is our hypothesis that it affects embryo survival but further research to test this hypothesis is needed.

Further investigation of several breeds (27) and synthetic lines has revealed that this polymorphism existed in animals of the Large White (called Yorkshire in the United States) breed. Analysis of PIC USA lines with Large White breed backgrounds again demonstrated that the beneficial *ER* allele had a significant effect in first parity litters and also a significant effect for the average of all parity litter records. The effect of the beneficial allele for the average of all parity litters was slightly less than that seen in first parity litters in these lines. The estimated effect of the *ER* beneficial allele is less in these Large White-derived lines when compared to the Meishan synthetic lines, suggesting that background genetics may play a role in *ER* gene expression or that *ER* is not the major gene but a linked gene. Of further interest is why this beneficial allele has been detected only in the Large White or Yorkshire breeds. One could speculate that previous selection for litter size may have increased the frequency of a rare beneficial allele already in the breed. However, its origin may derive from early importation of Chinese pigs into England prior to the 1800s and their interbreeding with pigs that eventually became the Large White breed (35).

Of further interest would be to see whether the B allelic frequency is increasing in either group of synthetic lines. In the Meishan synthetic lines, the B allele came only from the Meishan breed and no direct selection for litter size has occurred in these lines to date. In the Large White synthetics, the frequency of the B allele has been seen to increase during the period of selection for larger litter size.

Since no consensus linkage map for chromosome 1 near *ER* existed at the time of this research, we deemed it important to learn which markers may be close to *ER* and to determine whether any were better predictors of litter size than *ER*. The map we have produced for this area of chromosome 1 integrates markers and genes of recently published maps (29, 30, 36) and adds the closely linked *SOD2* gene. While the map order presented represents the best order determined by the data, it does not solidly place *SOD2* at that exact location and further work to confirm the order of *SOD2* relative to the other markers would be helpful. The mapping of *SOD2* to this region confirms the significant conservation of synteny seen in humans, with *ER* and *SOD2* being closely linked, and also confirms the syntenic group relationship between the long arm of human chromosome 6 and the short arm of pig chromosome 1. Interestingly in mice, *ER* and *SOD2* are located on different chromosomes confirming that the pig is more closely related to humans than to the mouse.

Further statistical analysis with a limited number of Meishan synthetic animals genotyped for the markers linked to *ER* demonstrated that *ER* was the best predictor of litter size differences. These results do not by themselves confirm that *ER* is the major gene itself but suggest that it is at least a very closely linked marker for litter size. To date in the Meishan synthetics, three or four generations of *inter se* matings have taken place and there is no evidence for a reduction in the effect associated with *ER* as generations increased.

From a commercial standpoint, it is important that beneficial alleles for a given trait have no antagonistic relationship with other performance traits. The purpose of importing the Meishan breed and using it in synthetic line development was to improve prolificacy. However, the Meishan's slow growth and excessive fat make it difficult to develop both a prolific and lean synthetic line involving the Meishan breed. The beneficial *ER* allele had no significantly negative pleiotropic effects on growth rate or on backfat. While there was an indication that the beneficial *ER* allele may act to increase FN in the Meishan synthetic lines, no such differences were seen in analyses involving the Large White synthetic lines. If real, such an effect might be the result of an interaction of *ER* with growth factors (20) in the Meishan synthetic females. Continued evaluation of the effects of this gene on all traits is necessary before it is fixed in order to avoid possible associated negative effects.

Discovery of the significant effect of the *ER* locus on litter size has made it possible to use marker-assisted selection to improve prolificacy, growth, and backfat simultaneously in PIC synthetic populations. Additionally, the favorable allele is being incorporated into conventional dam lines that do not have the allele by using marker-assisted introgression. These results demonstrate the usefulness of the candidate gene approach to discover QTL with significant effects on economic traits in pigs. Additional genetic improvement using marker-assisted selection and genes with significant effects like those associated with the *ER* locus should have a major impact on the efficiency of production of high quality pork for the consumer.

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