# Analysis of Candidate Genes for the Improvement of Litter Size in Pigs

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# Analysis of Candidate Genes for the Improvement of Litter Size in Pigs

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**Dedicated to Marco**

**and Mino**

# Abstract

A candidate gene approach was used to examine the effects of six genes on litter size in pigs. The selected candidate genes were cathepsin L (*CTSL*), epidermal growth factor (*EGF*), epidermal growth factor receptor (*EGFR*), inter-α trypsin inhibitor heavy chain 4 (*ITIH4*), leukemia inhibitory factor (*LIF*) and leukemia inhibitory factor receptor (*LIFR*). The aims of this dissertation were to map the genes, to partially or completely sequence them, to identify linked genetic markers by exploiting the sequences determined and to employ these markers in association studies to test for significant additive and dominant gene effects on the number of piglets born alive (NBA).

Using radiation hybrid (RH) mapping and fluorescence in situ hybridization (FISH) the genes were assigned to pig chromosomes as follows: *CTSL* to SSC10q11-q12, *EGF* to SSC8q23-q24, *EGFR* to SSC9q26, *ITIH4* to SSC13q21-q22, *LIF* to SSC14q21-q22 and *LIFR* to SSC16q13-q14.

The complete coding sequences were determined for *LIF* and *CTSL,* and their genomic organization was determined. The porcine *LIF* gene spans about 6.3 kb and consists of five exons including three alternative first exons (1D, 1M, 1T) spliced onto common second and third exons. The porcine *CTSL* gene spans about 5.6 kb and contains eight exons. *ITIH4, EGF, EGFR* and *LIFR* were sequenced partially. The sequences determined were screened for gene markers. In the case of the *LIF* gene a single nucleotide polymorphism (SNP) was found in exon 3. Microsatellite markers were identified for each of the other genes. All of the gene-linked markers were shown to be highly polymorphic. Subsequently, they were used in an association study to detect putative effects on the number of piglets born alive (NBA) employing 273 sows of a German synthetic pig line. For the intragenic *LIF* marker there was a negative dominance effect of $-0.72 \pm 0.37$ (p=0.047) observed for the first parity and $-0.50 \pm 0.29$ (p=0.087) for the second to tenth parities. No further statistical significant associations between any of the other microsatellite markers and NBA were detected in this study.

# Zusammenfassung

Im Rahmen einer Kandidatengenanalyse wurden sechs Gene mit molekulargenetischen Methoden analysiert und auf ihre Assoziation mit der Wurfgröße beim Schwein untersucht. Bei den ausgewählten Genen handelte es sich um Cathepsin L (*CTSL*), Epidermal Growth Factor (*EGF*), Epidermal Growth Factor Receptor (*EGFR*), Inter-α Trypsin Inhibitor Heavy Chain 4 (*ITIH4*), Leukemia Inhibitory Factor (*LIF*), Leukemia Inhibitory Factor Receptor (*LIFR*). Die im Hinblick auf diese Gene verfolgten Ziele der vorliegenden Dissertation waren ihre chromosomale Lokalisierung, die Ermittlung partieller bzw. vollständiger genomischer Sequenzen, welche zur Identifizierung intragenischer oder gekoppelter genetischer Marker herangezogen wurden und der abschließende Einsatz dieser neu entwickelten Marker in Assoziationsstudien zur Wurfgröße, um eventuelle signifikante additive und dominante Geneffekte auf die Anzahl der lebend geborenen Ferkel feststellen zu können.

Die Gene wurden durch Radiation Hybrid (RH) Mapping und Fluorescence-*in-situ*-Hybridization (FISH) auf folgenden Chromosomenabschnitten lokalisiert: *CTSL* auf SSC10q11-q12, *EGF* auf SSC8q23-q24, *EGFR* auf SSC9q26, *ITIH4* auf SSC13q21-q22, *LIF* auf SSC14q21-q22 und *LIFR* auf SSC16q13-q14.

Die kompletten kodierenden Sequenzen und die genomischen Strukturen wurden für *LIF* und *CTSL* ermittelt. Das porcine *LIF*-Gen erstreckt sich über 6.3 kb. Es enthält 5 Exons, wobei die ersten 3 alternative erste Exons sind (1D, 1M, 1T), welche an die gemeinsamen Exons 2 und 3 gespleißt werden können. Das porcine *CTSL*-Gen ist ca. 5,6 kb lang und besitzt 8 Exons. *ITIH4, EGF, EGFR* und *LIFR* wurden partiell sequenziert. Auf der Suche nach genetischen Markern wurde für *CTSL, ITIH4, EGF, EGFR* und *LIFR* jeweils ein Mikrosatellit identifiziert. Im Fall des *LIF*-Gens wurde im 3. Exon ein SNP (Single Nucleotide Polymorphism) Marker entdeckt. Sämtliche Marker erwiesen sich in ersten Tests als hochpolymorph. In einer Assoziationsstudie zur Anzahl lebend geborener Ferkel wurde ein negativer Dominanzeffekt des *LIF*-Markers von -0,72 ± 0,370 (p=0,047) für den ersten Wurf und -0,50 ± 0,29 (p=0,087) für den 2. bis 10. Wurf beobachtet. Für die restlichen Marker wurden keine statistisch signifikanten Effekte auf die Anzahl lebend geborener Ferkel festgestellt.

# Contents

# Outline of the thesis

Improvement of litter size is one of the major aims in pig breeding, and therefore much effort is made to improve this trait. The advent of molecular genetics has offered new opportunities in this field. Genetic information is used to develop genetic markers allowing selection with regard to economically important traits. Employed in combination with phenotypic information, traditionally used for animal selection, genetic information represents an effective tool for the improvement of litter size.

This thesis contributes to this research area by focusing on six candidate genes for litter size which are involved in early conceptus development and implantation in pigs. These genes are *LIF* (leukemia inhibitory factor; Yelich et al., 1997), *LIFR* (leukemia inhibitory factor receptor; Modric et al., 2000), *CTSL* (cathepsin L; Geisert et al., 1997), *ITIH4* (inter-α-trypsin inhibitor heavy chain 4; Geisert et al., 1998), *EGF* (epidermal growth factor; Kim et al., 2001) and *EGFR* (epidermal growth factor receptor; Wollenhaupt et al., 1999). Their involvement in the regulation of litter size was examined in this study. A more detailed presentation of the objectives of this thesis, a description of the experimental strategy and a brief survey of the contents of the chapters are given below.

## *Aims of the thesis:*

1) The isolation of genomic DNA clones containing the six selected candidate genes.

2) The determination of the complete or partial genomic sequences of the chosen candidate genes.

3) The identification of DNA polymorphisms such as microsatellites and single nucleotide polymorphisms (SNPs) within the sequences determined.

4) The utilization of identified polymorphic microsatellites and SNPs as genetic markers for association studies to show significant additive and dominant gene effects on the number of piglets born alive.

5) The chromosomal assignment of the six genes by fluorescence in situ hybridization (FISH) and radiation hybrid (RH) mapping.

## *Description of the experiments conducted and their interrelationships*

Genomic clones of five of the six chosen candidate genes (*CTSL, LIF, LIFR, EGF, ITIH4*) were isolated via PCR-based screening of a porcine PAC library (Al-Bayati et al., 1999). For *ITIH4, LIFR* and *CTSL* the PCR oligonucleotide primer pairs required for this purpose were derived from their cDNA sequences available in the EMBL nucleotide database under the

accessions S82800 (*ITIH4*), U91518 *(LIFR)* and D37917 (*CTSL*). Comparison with the corresponding human genomic sequences was undertaken to guarantee that the primers did not span two exons. Had this been the case they would not have been suitable for the amplification of specific PCR products on genomic DNA. For *LIF* and *EGF* known porcine primer pairs (Rettenberger et al., 1996; Mendez et al., 1999) were utilized for the isolation of PAC clones.

A genomic clone containing the *EGFR* gene was isolated by screening of a porcine BAC library (Fahrenkrug et al., 2001) with a $^{32}$P-labeled cDNA probe. The cDNA clone required for this purpose was obtained from the Resource Center/Primary Database (http://www.rzpd.de/).

Subsequent to cultivation of the clones on LB agar with the appropriate selective antibiotic, the PAC and BAC DNA were isolated and cleaved with different restriction enzymes. One enzyme at a time was used per digest. The restriction fragments were separated on 0.8 % agarose gels and transferred to nylon membranes via Southern-blotting. The appropriate PCR products served as hybridization probes for the identification of fragments which contained parts of the searched candidate genes. These PCR products were generated using the primers which were also used for the PCR-based screening of the PAC library mentioned above. A new primer pair for the *EGFR* BAC clone, isolated by radioactive hybridization, was derived from the porcine cDNA sequence (EMBL accession AY117054).

The identified fragments were cloned into the polylinker of the vector pGEM-4Z. These constructs were transformed into *E. coli* and amplified together with the bacteria. Afterwards the recombinant plasmid DNA was isolated and sequenced with a LICOR 4200 automated sequencer. A collection of plasmid subclones were sequenced for the determination of the complete genomic sequence of a gene. Remaining gaps were closed by a primer walking strategy until both strands were completely sequenced.

Microsatellites were detected either by scanning the candidate genes' complete genomic sequences determined or - if complete sequencing was not the aim for a certain gene or if no intragenic microsatellite could be identified - by generating and sequencing a collection of plasmid subclones of the respective PAC or BAC clone. These determined partial DNA sequences of a clone were scanned for intergenic microsatellites in close linkage with the respective gene.

The identification of SNPs in the exons of candidate genes was achieved by means of a mutation analysis. This approach is based on sequence comparison of orthologous exons of different animals. The sequence comparison of this work included seven animals of different

pig breeds (Angeln Saddleback, Wild boar, Pietrain, Duroc, German Landrace, German Large White, a synthetic line from a German commercial company, and a second synthetic line with 50% Meishan).

Detected DNA polymorphisms (microsatellites and SNPs) were examined for their suitability as DNA markers by determining their number of alleles, their degree of heterozygosity and their PIC (Polymorphism Information Content) in a small sample of sows of a German synthetic pig line. Suitable markers were employed in association studies with 272 sows of a German synthetic pig line to test for significant additive and dominant gene effects on the number of piglets born alive.

The chromosomal localizations of the candidate genes were physically determined by fluorescence in situ hybridization (FISH) employing the isolated genomic clones as hybridization probes. These results were confirmed by Radiation Hybrid (RH) mapping using intronic PCR primers derived from the candidate gene sequences determined.

## Survey of the contents

The intention of Chapter I is to provide an overview of methods and approaches employed in the improvement of litter size in pigs.

Chapters II and III deal with the *LIF* gene and the *CTSL* gene, respectively. These chapters describe the clone isolation, complete sequencing and mapping (aims 1, 2, 5) for the respective genes. Furthermore, the development of an intragenic SNP marker (aim 3) is reported in Chapter II.

Clone isolation for and chromosomal localization of the genes encoding for EGF and ITIH4 are addressed in Chapters IV and V, respectively.

Chapter VI is concerned with clone isolation, microsatellite marker development and mapping for *LIFR* and *EGFR*.

The development of microsatellite markers for CTSL, *EGF* and *ITIH4* (aim 3) is described in Chapter VII. Additionally this chapter deals with the investigation of associations (aim 4) between the five developed microsatellite markers and litter size. The analogous association study for the SNP marker identified in the *LIF* gene is reported in Chapter VIII.

Chapter IX provides a general conclusion refering to the chapters I-VIII.

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# **Chapter I**

## **Approaches to the improvement of litter size in pigs**

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*Abstract*

One of the major determinants for litter size in pigs is prenatal mortality. It occurs most frequently during the first few weeks of gestation and can be attributed to abnormalities in developmental processes during embryogenesis including trophoblastic elongation and blastocyst implantation. Improvement of litter size has been attempted by means of phenotypic selection. However, another promising approach in pursuit of this aim has been the use of genotypic information for selection. Reproductive traits in general are well-suited for application of marker-assisted selection (MAS). This method combines the use of genotypic information of single genes and breeding values predicted from phenotypic information, resulting in an improvement of both, accuracy and intensity of selection. The possibility of exerting selection criteria at the molecular level shortens the generation interval because the selection decision can take place early in the life of an animal. Moreover, in consideration of the sex-limited nature of reproductive traits, genotypic information allows for selection in the gender in which the trait cannot be directly observed. Accordingly, there has been considerable interest in mapping and identifying genes involved in the regulation of reproductive traits. This review has attempted to provide a comprehensive, but not exhaustive, account of the efforts being made and approaches being used in this field. One approach has been to choose candidate genes *a priori* because of the physiological importance of the proteins they encode and to examine the association between a genetic polymorphism identified in the candidate gene locus and reproductive trait phenotype. In another approach pre-existing or designed families have been used in linkage analysis to map the location of quantitative trait loci (QTL) for the reproductive trait of interest. However, a better understanding of porcine reproduction requires that these functional genomics approaches be merged and integrated with detailed analysis of the proteome to establish linkages between predisposition and physiology.

*General background*

For the pig producer, the clear requirement is to produce quality lean pork at minimum cost and in a manner that is acceptable to the public (Webb, 1998). Reproductive traits, especially litter size and pre-weaning viability, are important components for reducing the costs of producing pork (Tess et al., 1983a, b; de Vries, 1989; Rothschild and Bidanel, 1998). Therefore, much effort is made to improve these traits. Increasing the number of pigs weaned per sow will increase economic returns for pig producers with minimal additional inputs (Rothschild, 1996). The focus of this paper will be mainly on approaches to the improvement

of litter size, but other traits that affect reproductive efficiency in sows such as pre-weaning viability and piglet birth weight will also be addressed briefly because these traits are correlated with litter size. These interrelationships have to be considered when aiming at the improvement of litter size.

*Meishan versus European breeds - Determinants of litter size*

In pig breeding the term litter size is used for the total number of piglets born (TNB) and the number of piglets born alive (NBA). TNB is the sum of NBA and the number of stillborn piglets (NSB). Litter size is determined by the interaction of numerous physiological components. Though the number of ovulated eggs (= ovulation rate, OR) determines the maximum number of possible offspring, litter size does not increase with ovulation rate. In fact the rate of prenatal survival decreases with increasing ovulation rate (Haley and Lee, 1993). Besides ovulation and fertilization rates, the rate of prenatal loss strongly influences litter size (Ashworth, 1998). Several studies indicate that prenatal losses – which are classified as embryonic and fetal losses according to the developmental stage of the conceptuses – occur in every stage of pregnancy and thus have an essential impact on litter size (Pope, 1994). Losses up to the $30^{th}$ day of pregnancy are referred to as embryonic losses. They range between 20 and 30%. Losses during the fetal development can reach 10-20%. It is clear that several external and internal factors are involved in embryonic and fetal losses (Pope, 1994; Ashworth, 1998). For an improvement of litter size in pigs by minimizing these losses, many conditions have to be optimized such as nutrition, husbandry and management of the sows, but in addition to these external factors, there are in particular the genetic factors which have a large influence on this trait (Pope, 1994). The Chinese Meishan pig is well known for its high prolificacy (Haley and Lee, 1993). In comparison with western pig breeds, the average litter size in the Meishan is 3.6 NBA (number of piglets born alive) higher (Bidanel, 1997). According to Haley et al. (1995) the superiority of the Meishan sows for the trait litter size is solely determined by the maternal genotype. Consequently the genotype of the piglets has no influence (Haley and Lee, 1993). Haley et al. (1995) found ovulation rates in Meishan sows higher by five egg cells than in Large White sows. However, other studies showed that ovulation- and conception rates in Meishan and Yorkshire sows were nearly identical (Bolet et al., 1986; Ford, 1997). Therefore, a lower rate of embryonic mortality seems to be the main reason for the increased prolificacy of the Meishan pig (Ashworth, 1998).

The first, critical phase of porcine gestation up to day 30 is characterized by the expression of genes which affect alterations in conceptus, uterine and placental development in the way of

reciprocal signaling between the blastocyst and the uterus (Geisert and Yelich, 1997). Some of the most critical events for early pig embryonic survival occur from day 9 to day 13 of gestation, including equidistant spacing of embryos throughout the uterus (Dziuk, 1968, 1985), rapid trophoblastic elongation (Anderson, 1978), the establishment of conceptus-uterine attachment (Dantzer, 1985) and the inhibition of immune rejection by the maternal system (Geisert and Yelich 1997). Most conceptus mortality in pig breeds occurs between days 12 and 18 of pregnancy (Pope, 1994) which is mainly assumed to be due to variation in genes affecting the aforementioned critical events.

From the $31^{th}$ day of gestation, the onset of the second critical phase, fetal survival rate determines litter size. In this period the uterine capacity required by the growing fetuses may become limited and not all fetuses may survive (Ford, 1997). At a given litter size, the fetuses in sows with larger uterine capacities will have an advantage in terms of placental development (mass, vascularity and surface area) compared with fetuses in sows with more limiting uterine capacities (van der Lende et al., 2001). Reciprocal embryo transfer studies with Meishan and Yorkshire sows indicate that the maternal genotype determines the size of growing fetuses (Biensen et al., 1998). The placental size and thus the available space in the uterus is also maternally controlled up to day 90 of pregnancy (Ford, 1997; Wilson et al., 1998). From around the day 91 on, fetal breed-specific mechanisms begin to determine placental size (Biensen et al., 1999). Faster growing Yorkshire fetuses need an increasingly expanding placental surface area to ensure their nutrition. At the same time of gestation more but smaller Meishan fetuses have equal nutritional requirements but because of a higher degree of placental vascularization there is no need for an enlargement of the placental surface area (Wilson et al., 1998). Therefore, the resulting higher placental efficiency of Meishan sows which, is measured as the ratio of fetal weight and placental weight, represents a selection advantage for the number of piglets born alive (Wilson et al., 1999).


*Litter size and correlated reproductive traits*

The existence of balanced mutual interrelations between the reproductive traits litter size, birth weight, and pre-weaning survival (piglet survival until weaning) limit their concomitant improvement. These interrelations have to be considered when aiming at the improvement of one or more of these traits.

The genetic correlation of litter size with pre-weaning survival is negative in most pig breeds (Rothschild and Bidanel, 1998; Knol, 2001). However, Lee and Haley (1995) showed that piglets from Meishan litters survive almost as well as those from Large White litters, despite a

four-piglet higher litter size and a 450-g lower average birth weight in the Meishan pigs. This demonstrates that there is some room for concomitant improvement of litter size and survival, but at the expense of lower birth weight. A lower birth weight results in delayed growth performance before and after weaning (Quiniou et al., 2002). Thus, Quiniou et al. (2002) argue for the selection of heavier piglets. The importance of high birth weight for survival has been determined in several studies (e.g. Fireman and Siewerdt, 1997; Daza et al., 1999) this has led to the approach of increasing survival through a genetic increase in birth weight. However, contradictory results were found by Siewerdt and Cardellino (1996) and Grandinson et al. (2000), who reported a negative genetic correlation between birth weight and survival. These results were confirmed by Knol (2001) who evaluated different selection strategies for improved piglet survival and concluded that selection for increased individual birth weight will not significantly increase piglet survival. Direct selection for piglet survival is possible but will affect body composition rather than birth weight. Effects on birth weight will probably be negative rather than positive (Knol, 2001). These studies cast doubt on the strategy of replacing selection for increased survival by selection for increased birth weight. Knol et al. (2002) speculated that it is not the average birth weight, but within-litter variation in birth weight that causes the problems with small piglets. Undersized piglets have a higher probability of dying as a result of trauma, chilling or starvation than do their larger more competetive littermates (van der Lende et al., 2001). The existence of a negative correlation between birth weight and survival is corroborated by the findings of Leenhouwers et al. (2002). These authors found indications that selection for piglet survival will result in a decrease in mean birth weight, mean placental weight and placental variation and in an increase in carcass fat percentage and piglet maturity of piglets at birth. This increased maturity is thought to improve the piglets' ability to cope with hazards during birth and within the first days of life, thus leading to a higher pre-weaning survival rate. The connection between birth weight, maturity and carcass fat percentage found by Leenhouwers et al. (2002) is in agreement with Herpin et al. (1993), who concluded that selection for lean tissue growth leads to heavier but less mature piglets at birth. Selection for litter size and survival may ultimately lead to piglets that closely resemble those from genetically obese lines, such as the Meishan (Knol et al., 2002; Leenhouwers et al., 2002). Compared to Western pig breeds the Meishan is not only superior in litter size, but also competetive in pre-weaning survival as reported by Lee and Haley (1995). This can be explained at least in part by the Meishan's high percentage of body fat. Mersmann et al. (1984) suggest that an increase in body reserves

will help to increase survival, through improved thermoregulation and availability of directly usable energy.

The conflicting results concerning selection for birth weight and the aforementioned room for concomitant improvement of litter size and survival qualify the two last-named traits as selection criteria for the improvement of weaned pigs. In this context, however, it is also necessary to keep the average birth weight within a breed specific optimal range (neither to light nor to heavy) and to develop approaches to decrease within-litter variation.

Recent results, approaches and prospects in the improvement of litter size in pigs are addressed in the following chapters.

*Traditional selection versus molecular genetics – Tools for the improvement of litter size*

Traditionally, livestock improvement programs have utilized animal selection on the basis of observable phenotype which represents the collective effect of all genes and the environment. In France, litter size was improved by the hyperprolific approach (Legault and Gruand, 1976). The success of this approach depends on traditional methods such as strict selection of beneficial phenotypes and artificial insemination. Basically, this approach generates great superiority for litter size by returning the genes from a small proportion of prolific sows in multiplier herds to the nucleus herd (Webb, 1998). By the development of hyperprolific lines from the maternal breeds Large White and Landrace francais, the number of piglets born alive per litter and the number of piglets weaned per sow and year were increased from 10.3 and 16.4, respectively, in the year 1970 to 11.3 and 23.8, respectively, in the year 1997 (Steinheuer et al., 2003a). While litter size was substantially improved and growth rate remained unaffected, the drawbacks were that these dam lines had higher backfat and poorer feed conversion than contemporary lines.

The steady progress in information technology over the last couple of decades makes possible the separation of genetic and environmental effects and the estimation of breeding values by calculation on multiplier animals rather than by relying on phenotypic data. Selection index theory is based on the combination of several traits or sources of information, such that the accuracy of the index as a predictor of the selection goal is maximized. In using selection index and best linear unbiased prediction (BLUP) procedures for genetic evaluation of litter size in their lines Lofgren et al. (1994) and Short et al. (1994) have improved litter size. Thus litter size can be improved by the use of BLUP applications (e.g., STAGES, Schinckel et al, 1986; PEST, Groeneveld et al. 1990; PIGBLUP, Long et al., 1990) in a well-designed selection program.

Yet apart from the successes mentioned there has been no substantial breeding progress in litter size reported in recent years using traditional quantitative genetic methods, despite growing efforts. Litter size of German Landrace sows for example averaged 10.5 newborn piglets in 1935 and peaked between 1960 and 1970 with 10.9 piglets. Since then litter sizes declined to 10.3 piglets in 1999 (Steinheuer et al., 2003a). These difficulties in the improvement of litter size are ascribable to its low heritability which is estimated on average at 0.09 for the number of piglets born alive (Bösch et al., 1999; Hanenberg et al., 2001; Lamberson, 1990; Rothschild and Bidanel, 1998). Furthermore, the trait is sex-limited and is not measurable until sexual maturity, at one year at the earliest.

These biological constraints can potentially be ameliorated by the application of molecular genetics methods, particularly the inclusion of genetic markers in selection strategies. The essence of using genetic markers in breeding programs is that they mark chromosomal regions (and sometimes individual genes), and so make it possible to follow the inheritance of these regions from parents to offspring. Thus, if we know which chromosomal segments contain alleles of value, markers may be used to help identify animals that have inherited these alleles and hence the best of genetic variation, whether or not we have phenotypic records or progeny information on the animals (Visscher et al., 1998).

Advances in molecular technologies such as marker assisted selection (MAS, Soller, 1994) provide the possibility of selecting for litter size directly after birth based on genetic marker information. There are two advantages of such information in comparison to phenotypic information. The first is that their early availability contributes to a shortening of the generation interval. The second advantage is the possibility of enhancing the accuracy of selection and thus the selection response of a trait by direct selection of gene variants in both sexes, thus positively affecting its expression. Litter size is well suited for the application of genetic marker information in animal selection. Considering the sex-limited nature of the trait, the identification of genes which contribute to variation in litter size would lead to tools for selection in the gender in which the trait cannot be observed directly.

Genetic markers suited for MAS can lie within a gene (intragenic marker) or in its neighborhood (intergenic marker). When preferable intragenic markers are not available, flanking - or linked - markers within a distance of 5 cM from the gene can be utilized (Moreau et al., 1998). The disadvantages of flanking markers are the possible loss of a gene with a desired effect on a phenotypic trait due to recombination and the existence of different linkage phases between the alleles of the marker and the gene with the causative mutation. For these reasons the existence of linkage disequilibrium between a marker allele and a trait

locus within a family or population is a precondition for the utilization of linked markers. Linkage disequilibrium is defined as the condition in which the frequency of a particular haplotype for two loci is significantly different from that expected under random mating. The expected frequency is the product of observed allelic frequencies at each locus (Dekkers and Hospital, 2002). For this approach in the population-wide improvement of traits using genetic marker information evidence of linkage disequilibrium can only be detected if the founder animal of a family is heterozygous for the linked marker which is a limiting factor. A close linkage between genetic marker and trait locus is preferred to avoid recombination as far as possible and to favor a population-wide linkage disequilibrium. This makes intragenic markers better suited for application in MAS in comparison to trait locus-linked markers. For the latter, it is too risky to carry out selection solely on the basis of marker effects, without confirming the estimated effects by phenotypic evaluation. This is true in particular if marker effects on a trait were initially detected in a different population or genetic background (Dekkers and Hospital, 2002).

The most widely used markers for the genotyping of animals and subsequent linkage studies are single nucleotide polymorphisms (SNPs) and microsatellites. SNPs are naturally occurring variants in the DNA sequence that differ in a single basepair. The identification of a SNP within a gene includes the possibility that it is a causative mutation with a functional difference in the respective gene affecting the investigated trait. However, causal mutations for traits are hard to find, and difficult to prove, and few examples are available (Andersson, 2001). SNPs are diallelic and consequently yield three genotypes AA, AB and BB. Microsatellites are highly polymorphic but never have an effect on gene function. They are always non-functional and can be found in introns or in close proximity of trait loci. Microsatellites consist of 2 to 10 basepair repeats which are variable in the number of repeats and thus vary in length. The number of length polymorphisms of a microsatellite is equivalent to the number of its alleles. Most microsatellites are multiallelic.

Experimental techniques for the identification of trait-associated intragenic or closely linked gene markers can be derived from two approaches which are addressed in more detail in the next two chapters. One approach is the investigation of candidate genes; the other method is the implementation of linkage studies for the identification of quantitative trait loci (QTL).

Genotyping of an identified marker is followed by statistical verification of possible significant trait variants between bearers of different marker alleles (Milan, 2000). Suitable for selection are most notably population-wide verifiable, marker-associated additive gene effects. However, due to epistatic and pleiotropic effects a trait-affecting gene (trait locus) can

have a minor effect in one population but a major effect in another (Linville et al., 2001). A verified additive effect can help to facilitate animal selection and mating decisions to enhance the favorable genotype in the population. Previous simulation studies showed that the highest breeding progress is achieved by using genotypic and phenotypic information contemporaneously. An overly strong emphasis on genotypic information diminishes breeding progress in traits not included in those data. On the other hand selection based solely on phenotypic information is less efficient for traits that can be recorded early in life by genotyping (Dekkers and Hospital, 2002). Breeding strategies using genomic and phenotypic data are reviewed in detail by Visscher et al. (1998) and Dekkers and Hospital (2002). Two of these strategies are recurrent selection and introgression programs. Recurrent selection is the main vehicle for genetic improvement in livestock and aims at the improvement of a breed or line as a source of superior germplasm for commercial production through within-breed or within-line selection (Dekkers and Hospital, 2002). The aim of an introgression program is to introduce particular alleles for trait loci from one breed or line (the donor) into another (the recipient), with the aid of genetic markers, by repeated backcrosses to the superior line. At some point crosses within the backcross line would be used to fix the introgressed allele, and then selection would continue within the line. If the two lines are of similar genetic merit, the best alleles from both lines might be selected directly from an $F_2$ intercross with the aid of genetic markers. Between these two extremes, there is a continuum of possibilities with varying numbers of rounds of backcrossing prior to intercrossing the animals and selecting within the intercross (Visscher et al., 1998).

*Candidate genes for litter size*

One way of gathering genomic information is the candidate gene approach which was proposed as procedure to identify genes with significant phenotypic performance effects and possible use in genetic improvement programmes. A gene will be suggested as a potential candidate gene for litter size because of the important physiological role it plays in reproduction (physiological candidate genes) (Rothschild, 1998). Moreover, candidate genes can be chosen by regarding genes in regions associated with possible QTL (positional candidate genes), by utilizing information about orthologous genes in syntenic chromosomal regions of other species (positional comparative candidate genes) (Haley, 1999), and by considering differentially expressed genes in the tissue under investigation (Wilson et al., 2000; Liang and Pardee, 1992). Clearly, several of these criteria should apply in the choice of a candidate gene. Polymorphisms in selected genes (e.g. microsatellites or SNPs) are usually

identified on the basis of DNA sequence analysis. The detection of a significant phenotypic effect on litter size for an identified candidate gene polymorphism in association studies serves as evidence for concluding that the gene is a major one for litter size (or a marker for a closely-linked major gene).

Especially well suited for such studies are reciprocal $F_2$ and $R_1$ generations bred from extreme populations for the trait in question, such as Wild Boar x Landrace or Meishan x Landrace (Geldermann et al. 1999), for example. Approaches to develop experimental populations for the use in candidate gene analyses are described by Linville et al. (2001) and van Rens et al. (2002). These populations are also well-suited for QTL studies, a topic addressed in the next chapter. The population of pigs used in the experiment of Linville et al. (2001) originated from Index (I) and Control (C) lines described by Johnson et al. (1999). These had a common base of Landrace/Large White composite population. Pigs were randomly assigned, within litter, to either line I or line C at Generation 0 and then selected for increased values of an index of ovulation rate and embryonic survival (line I) or randomly (line C). At Generation 8 of index selection, the lines IOL and COL were formed from line I and line C, respectively. Eight generations of two-stage selection in lines IOL and COL were practiced. Stage-one selection included all gilts from 50% of litters with the greatest number of fully formed pigs at birth. Stage-two selection included the 50% of these gilts with the greatest ovulation rate. At generation 0 of two stage selection, line I, and thus line IOL, differed from line C by 4.22 ova and 1.94 pigs. After eight generations of two stage selection, lines IOL and C differed in mean estimated breeding value by 6.1 ova and 4.7 fully formed pigs, whereas lines COL and C differed by 2.24 ova and 2.9 fully formed pigs.

The aim of a study conducted by van Rens et al. (2002) was to investigate the effect of estrogen receptor (ESR) genotype on litter size and placental traits in pigs (results presented later). To design a population optimally suited for this purpose van Rens et al. (2002) used two half sibling Large White boars (ESR genotype AA) and 8 Meishan (2 BB and 6 AB) sows as parents. From the $F_1$ offspring, 6 AB boars and 21 AB gilts were selected to produce the $F_2$ population. Females of the second to fifth litter of the $F_1$ crossbred sows were used as the experimental animals in this research.

Most candidate gene analyses for reproduction in pigs have focussed on litter size or its component traits, especially NBA. Associations between several candidate gene-linked polymorphisms and NBA have been reported by several authors for different pig breeds and lines (Table 1).

Table 1: Survey of candidate gene effects on the trait number of piglets born alive (NBA)

| Reference | DNA marker | No. of sows No. of litters | Breed / line | Additive (a) and dominance (d) effects |
|---|---|---|---|---|
| Rothschild et al. (1996) | ESR | 276 litters | PIC lines with Meishan contribution | a = 0.8 (p < 0.01) d = 0.6 (p < 0.01) |
| Short et al. (1997a) | ESR | 4262 sows 9015 litters | Large White synthetic line | a = 0.31 (p < 0.01) d = 0.14 (p < 0.05) |
| Chen et al. (2000) | ESR | 262 sows | 5 populations of chinese and western breeds | a = 0.315 to 1.79 (p < 0.001) depending on the breed |
| Linville et al. (2001) | ESR | 523 sows | 3 PIC lines | a = 0.474 (n.s.) d = 1.58 (n.s.) |
| Van Rens et al. (2002) | ESR | 275 sows | Large White x Meishan F2 crossbreed | AB gilts had 1.22 NBA per litter more than AA gilts (p < 0.05) |
| Vincent et al. (1998) | PRLR | 1077 sows 2714 litters | 5 PIC lines | a = -0.33 to 0.47 (p < 0.05) d = -0.33 to 0.63 (p < 0.01) depending on the line |
| Southwood et al. (1999) | PRLR | 2615 litters | 5 PIC lines | a = 0.1 to 0.9 |
| Drögemüller et al. (2001) | PRLR | 2159 sows 8336 litters | German Landrace, Duroc and synthetic lines | a= 0.71 (p < 0.05) for Duroc |
| Linville et al. (2001) | PRLR | 524 sows | 3 PIC lines | a = -0.007 (n.s.) d = -0.466 (n.s.) |
| Ollivier et al. (1997) | RBP4 | 129 sows | Large White hyperprolifique line | a = 0.08 (n.s.) |
| Rothschild et al. (2000) | RBP4 | 1300 sows 2555 litters | Large White, Landrace and synthetic lines | a = 0.15 (n.s.) d = -0.01 (n.s.) |
| Linville et al. (2001) | RBP4 | 190 sows | 3 PIC lines | a = 0.526 (n.s.) d = 0.313 (n.s.) |
| Steinheuer et al. (2003b) | RBP4 | 51 boars | German Landrace | a = -0.472 (p < 0.001) d = 0.604 (p < 0.001) |
| Short et al. (1997b) | OPN | n/a | n/a | one allele showed an association with NBA (p < 0.05) |
| Hamann et al. (2000) | OPN | 2144 sows 8300 litters | German Landrace, Duroc and synthetic lines | some genotypes showed a significant association with NBA |
| Li et al. (1998) | FSHB | n/a | n/a | a = 1.06 (first parity) a = 1.01 (second parity) |
| Linville et al. (2001) | FSHB | 520 | 3 PIC lines | a = 0.12 (n.s.) d = 0.759 (n.s.) |
| Linville et al. (2001) | EGF | 189 | 3 PIC lines | could not be estimated with contrasts because only two genotypes occurred |
| Linville et al. (2001) | PTGS2 | 523 | 3 PIC lines | a = 0.403 (n.s.) d = 0.076 (n.s.) |

a = additive effect; d = dominance effect; n/a = not available; n.s. = not significant

PIC (Pig Improvement Company, Franklin, USA)

ESR (estrogen receptor), PRLR (prolactin receptor), RBP4 (retinol-binding protein 4), OPN (osteopontin), FSHB (follicle-stimulating hormone beta), EGF (epidermal growth factor), PTGS2 (prostaglandin-endoperoxide synthase 2)

The first successful verification of an association between a candidate gene and litter size was described by Rothschild et al. (1996) for a *PvuII*-restriction fragment length polymorphism (RFLP) of the estrogen receptor (*ESR*) gene on chromosome 1 in both a Meishan by Large White and a European breed synthetic population. Conceptus-derived estrogen plays a major role in the establishment of gestation by signaling to the uterus and maintenance of pregnancy by extending the life-span of corpora lutea. The results of Rothschild et al. (1996) were confirmed by Short et al. (1997a) in four synthetic lines of European breeds. Short et al. (1997a) ascribed their smaller effects in comparison to the results of Rothschild et al. (1996) to their considerably larger sample size which is a main parameter in determining the relative efficiency of MAS (Gimelfarb and Lande, 1994; Zhang and Smith, 1993). In contrast to the effects of the ESR *PvuII*-polymorphism reported by Rothschild et al. (1996), Short et al. (1997a), Chen et al. (2000), Van Rens et al. (2002), and Gibson et al. (2002) were not able to detect any significant association between the ESR polymorphism and litter size in a Meishan x Large White $F_2$ population. In agreement with this result Drögemüller et al. (1999), Linville et al. (2001) and Isler et al. (2002) found no confirmation of the effect of the ESR polymorphism on litter size, previously reported by Rothschild et al. (1996) and Short et al. (1997a).

Another candidate gene for litter size, the prolactin receptor *(PRLR)* gene on porcine chromosome 16, plays a role in the maintenance of gravidity. An interaction of estrogen and prolactin is responsible for the redirection of luteolytic prostaglandin F ($PGF_{2\alpha}$) secretion from an endocrine pathway, toward the endometrial stroma and vasculature, to an exocrine one, toward the uterine lumen (Gross et al., 1990). Consequently, $PGF_{2\alpha}$ is sequestered in the uterine lumen and does not become available, via the utero-ovarian vasculature, to exert its luteolytic effect. A diallelic polymorphism in the *PRLR* gene has been reported to be associated with differences in litter size (Vincent et al., 1998). This result is in agreement with other reports (Southwood et al., 1999; Drögemüller et al., 2001), whereas Linville et al. (2001) found no association.

The retinol-binding protein 4 (*RBP4*) gene on chromosome 14 has been suggested as a candidate gene for litter size based on its role in providing the conceptus with appropriate amounts of retinoic acid in the early critical phase of pregnancy around day 12 and in buffering retinoic acid oversupply (Harney et al. 1993; Rothschild et al., 2000). Retinoic acid is implicated in the regulation of gene transcription (Yelich et al., 1997a). At the time of implantation and trophoblastic elongation RBP4 is sequestered in the uterine lumen (Harney et al., 1993). Examinations of the *RBP4* gene as candidate gene affecting litter size showed

non significant effects of a diallelic RFLP marker within an intron of the gene (Ollivier et al,. 1997; Rothschild et al., 2000; Linville et al. 2001). Steinheuer et al. (2003b), however, observed a significant effect on NBA in German Landrace. No association of the *RBP4* polymorphism with litter size was found by Drögemüller et al. (2001).

The osteopontin (*OPN*) gene has been implicated in transport and buffering of $Ca^{2+}$ from the maternal circulation to the conceptus; this is supported by evidence of expression of the gene in cells of mouse placenta and decidua (Waterhouse et al., 1992). The existence of binding sites for estrogen and glucocorticoids within the promoter of the *OPN* gene in mice (Craig and Denhardt, 1991) argues for a regulation of its transcription by steroid hormones known to be involved in reproduction. For the aforementioned reasons and due to the fact that there is a corresponding location with a QTL for litter size on chromosome 8 (Short et al., 1997b), *OPN* was considered a candidate gene for litter size. A highly polymorphic microsatellite marker linked with *OPN* was examined for its association with litter size. Significant effects of some of its 13 alleles were detected in studies of Short et al. (1997b) and Hamann et al. (2000).

Follicle-stimulating hormone beta *(FSHB)* was chosen as a candidate gene because it functions in the maturation of small and medium follicles into large follicles that ovulate (Wang and Greenwald, 1993; Mannaertz et al., 1994). In a candidate gene analysis, Li et al. (1998) found additive effects on litter size associated with a marker within *FSHB*. However, these effects were not confirmed by a study of Linville et al. (2001).

In a candidate gene analysis for loci affecting litter size, Linville et al. (2001) also examined in addition to the mutations of the genes mentioned (*ESR, PRLR, FSHB* and *RBP4*), polymorphisms of the genes epidermal growth factor *(EGF)* and prostaglandin-endoperoxidase synthase 2 *(PTGS2)*, and found that no estimates of allele substitution effect were significant for any of these genes.

The variability of results between studies and populations employing the same polymorphism show the difficulties in confirming previously published candidate gene effects in different populations. However, even a lack of association between a gene-associated polymorphism and a phenotype does not necessarily mean that the gene product is not important in regulating the trait. Rather it shows the necessity of investigating different pig breeds and larger sample sizes to evaluate the usefulness of markers for MAS-based improvement of litter size. The inconsistent results could be due to different sample sizes employed in the studies (Table 1) and / or to different breed- or population-specific allele distributions. Another reason for the conflicting results might be the occurrence of different population-specific linkage phases between candidate gene marker and causative mutation caused by

recombination. Moreover, as a result of epistatic and pleiotropic effects, a trait locus might have a minor effect in one population but a major effect in another.

In the near future further analyses of new candidate genes are to be expected. Jiang et al. (2002) reported the development of new SNP markers for genes that were found to be involved in reproduction such as amphiregulin *(AREG)*, fibrinogen gamma chain *(FGG)* and estrogen sulfotransferase *(STE)*. These markers can now be used in association studies to estimate their effects on reproductive traits.

The possibilities for the identification of new candidate genes are continuously being improved in several ways. First, knowledge about the physiological role of genes in reproduction is growing steadily. Second, QTL studies give indications of the chromosomal localization of putative new candidate genes, a point which is addressed in the following chapter. The third improvement is due to investigations concerning differential gene expression in tissues relevant for reproduction ( e.g. Vallée et al., 2002). All these efforts contribute to the establishment of a catalogue of genes involved in regulating litter size. In combining all the information available, these methods provide accumulating evidence that will facilitate the choice of new candidate genes.

*Quantitative trait loci for litter size and its component traits*

As with with other complex traits in animals, litter size is a quantitative or polygenic trait in which the influences of many genes combine to contribute to the phenotype. Unlike qualitative (i.e., Mendelian) traits which are generally mediated by a single gene, quantitative traits vary continuously across a population and derive from a constellation of both genetic and environmental influences. According to Geldermann et al. (1985) a quantitative trait locus (QTL) is a single gene locus, or a marked DNA region that contains the gene, with a measurable effect on the genetic variance of a trait. Such a QTL or so-called major gene should determine the phenotypic variance of a trait in a predominant manner and should therefore cause more than 10% of the phenotypic variance of the targeted trait. The mapping of QTL for reproductive traits is achieved by analysis of pre-existing or designed families with recorded performances in linkage studies with anonymous markers (e.g. microsatellites or SNPs) covering the whole genome, usually one marker every 20 cM. For detection of a QTL, a marker has to be identified in close proximity to the unknown trait-affecting gene. The closer the linkage, the higher the likelihood that they are inherited together. If such a marker is also highly polymorphic then the transmission of QTL alleles can be derived from the inheritance of the marker alleles on the progeny. When linkage between marker and QTL is

close then the likelihood is high that a certain marker allele cosegregates with a certain QTL allele in all offspring. This is a precondition for the estimation of a QTL effect on the targeted phenotypic trait by means of marker alleles or marker genotypes. For this reason the estimated effect depends on the likelihood of how often a certain QTL allele is inherited with a certain marker allele. This is determined by the rate of recombination and by the linkage phase between QTL and marker. Furthermore, the estimated QTL effect is dependent on additive and dominant effects of the unknown trait-affecting gene and the possible existence of epistasis. Statements about linkage between marker locus and QTL become increasingly less reliable if recombination frequencies exceed 10 to 20% (Knippers, 1997). A recombination occurring between marker locus and QTL changes the linkage phase between marker alleles and alleles of the trait-influencing QTL. This means that verification of a marker allele alone is not sufficient to make a statement about a QTL allele. Accordingly, the linkage phase between marker locus and QTL has to be determined in every population and family investigated.

To maximize the probability of detecting QTL, breeds are used whose performance differs markedly, under the assumption that some key genes affecting the trait have different alleles in the two breeds. Approaches to develop experimental populations well-suited for QTL studies and candidate gene analyses (Linville et al., 2001; van Rens et al., 2002) are addressed in the previous chapter. Specifics of the statistical analyses vary in different published studies, but authors have commonly reported results relative to a genome-wise error rate which accounts for multiple hypothesis testing implicit in a genome-wide QTL search (Kirkpatrick, 2002). Lander and Kruglyak (1995) proposed the terms suggestive linkage and significant linkage to characterize results expected by chance alone once per genome-wide search or 0.05 times per genome-wide search, respectively. These terms are used here in an approximate sense to refer to results from several genome-wide QTL searches that have been reported. An equation used to convert point-wise (nominal) probabilities for QTL to genome-wide level of significance was presented by Lander and Kruglyak (1995):

$$\text{genome-wide significance} = (C + 2 \cdot G \cdot \rho \cdot f \cdot df_n) \cdot (1 - \text{prob}(f, df_n, df_d))$$

where $C = 19$ (representing the 18 autosomes and the X chromosome), $G = 25$ (the length of the swine genome in morgans), $\rho$ is the autocorrelation function ($\rho = 1$ for a backcross and 1.5 for an $F_2$ population), and f is the $F$- ratio, with $df_n$ numerator degrees of freedom and $df_d$ denominator degrees of freedom. This is the expected number of false positives per genome scan.

A survey on localized QTL for litter size or its component traits in pigs is given in Table 2 and leads to interesting comparisons between results of these studies among each other but also with the candidate gene studies described in the previous chapter.

Table 2: Chromosomal localizations of QTL for litter size and its component traits.

| chromosome | QTL suggestive linkage | significant linkage | Position (cM) | reference |
|---|---|---|---|---|
| 3 | OR | | 36 | Rohrer et al., 1999 |
| 4 | NSB | | 1 | Wilkie et al., 1999 |
| 7 | TNB[1] | | 10 | de Koning et al., 2001 |
| 8 | | OR | 5 | Rohrer et al., 1999 |
| | OR[a] | | 110 | Rathje et al., 1997 |
| | OR | | 101 | Wilkie et al., 1999 |
| | | OR | 99 | Braunschweig et al., 2001 |
| | UC | | 71 | Rohrer et al., 1999 |
| 9 | OR[a] | | 67 | Rohrer et al., 1999 |
| 10 | OR | | 89 | Rohrer et al., 1999 |
| 12 | TNB[2] | | 71 | de Koning et al., 2001 |
| 13 | NSB | | 101 | Cassady et al., 2001 |
| 14 | TNB[2] | | 62 | de Koning et al., 2001 |
| 15 | OR[a] | | 79 | Rohrer et al., 1999 |
| 17 | TNB[2] | | 43 | de Koning et al., 2001 |

OR: ovulation rate (measured as the number of corpora lutea present on each ovary after slaughter or measured by laporatomy), UC: uterine capacity, NSB: number of stillborn piglets, TNB: total number of born piglets

[1]first parity     [2]second parity

[a] suggestive linkage exceeded, statistical evidence would be expected to occur between 1 and 1.76 times at random in a genome scan (further explanation in the text)

Some correspondence between QTL mapping results is expected since several studies have employed Meishan x Large White crosses, or crosses of selected and unselected Large White-derived lines. In fact several studies found evidence for ovulation rate QTL on chromosome 8. However these QTL were mapped at opposite ends of the chromosome by Rohrer et al. (1999) and Rathje et al. (1997). In subsequent studies the location determined by Rathje et al. (1997) was confirmed by studies of Wilkie et al. (1999) and Braunschweig et al. (2001) but a report of Cassady et al. (2001) casts doubt on the report by Rathje et al. (1997) of a chromosome-8 QTL. The lines used in both studies were basically the same with the difference that the experiments of Cassady et al. (2001) included more animals. However, this subsequent analysis failed to provide additional support for the preliminary observations of Rathje et al. (1997). Furthermore, linkage for the QTL mapped by Rathje et al. (1997) and Wilkie et al. (1999) is just on the nominal and suggestive level, respectively, whereas Rohrer et al. (1999) and Braunschweig et al. (2001) found significant linkage on a genome-wide level but with conflicting results. So maybe there is more than one QTL for ovulation rate on chromosome 8. For a clarification further confirmation studies are necessary to address this subject.

Overall, the QTL studies for litter size or its component traits reported to date, show relatively inconsistent results concerning QTL locations. This is maybe a consequence of the highly polygenic control of this trait, by loci with small effects that interact with each other and with the environment (Pomp et al., 2001). Realistically, differences are to be expected in light of the different lines used to create the populations under study and the genetic heterogeneity between and within lines. A third reason for these differences are the varying frequencies of the QTL in the populations and lines used for the association or linkage analyses. Furthermore, the sample sizes employed limit the power of the used methods to detect QTL of modest effect (Kirkpatrick, 2002).

*Merging of QTL- and candidate gene approach – Evidence for a 'polygenic paradox'*
After identification of a QTL, the ultimate goal is to identify the responsible gene itself and the causative mutation. The first steps toward this challenging aim are the fine mapping of the QTL and merging of the mapped QTL with putative physiological candidate genes in this chromosomal region. The possibility that a gene is really involved in a trait of interest is greatly enhanced by coincidence between the chromosomal localizations of a QTL and a newly mapped candidate gene when there is a congruency between the affected QTL-linked reproductive trait and the physiological role the candidate gene takes in reproduction. The

strategy of first mapping the trait locus and then looking for genes with a putative effect on this trait within that particular chromosomal region, has been termed 'positional candidate cloning'. This approach has already proven to be very useful (see Copeland et al., 1993) and will be one of the major future strategies for identifying trait genes in humans (Collins, 1995) and in farm animals.

However, in addition to the inconsistent results between different QTL studies concerning litter size and its component traits, there has also been relatively little correspondence between these QTL and many of the most prominent candidate genes, selected on the basis of physiological evidence (see Table 1) which is illustrated by the Tables 2 and 3, respectively. Putative correspondence exists between the QTL for TNB on chromosome 14 and the gene RBP4 even though there are inconsistent mapping results for this gene. Other potential correspondences might exist between the genes *AREG, EGF FGG* and *OPN* and the QTL for ovulation rate and uterine capacity on chromosome 8. However, there are no further putative correspondences. To date no QTL for litter size or one of its component traits have been found on chromosome 1 in the region corresponding to the *ESR* locus, and investigation specifically of the *ESR* marker failed to show evidence of association. At first sight this is remarkable because the *ESR* marker has been found to be a promising gene marker for litter size as indicated in the previous chapter (Table 1) but the inability to detect a QTL in this case might be explained by the fact that the *ESR* effect mainly occurs in Meishan. The smaller effects of the ESR polymorphism in Western pig breeds might be also associated with the lower frequencies of the favorable allele *B*. Steinheuer (2001) found frequencies of 0.05 and 0.02 in German Landrace (n = 28) and Pietrain (n = 256), respectively, for the favorable *B* allele.

Likewise no QTL for litter size have been identified on the chromosomes 16 and 2, where two other well studied candidate genes have been mapped, namely the *PRLR* gene on chromosome 16 and the *FSHB* gene on chromosome 2. Kirkpatrick (2002) stated that this lack of correspondence could be for various reasons: some candidate genes may have modest effects which are undetectable with the sample sizes employed in the genome-wide QTL search; there may be a lack of segregation of the candidate gene alleles in some populations; the candidate gene marker may simply be a linked marker with heterogeneity of linkage phase eliminating association; or the originally observed candidate gene effects may in some cases have been due to chance (statistical thresholds employed in candidate gene studies have often been less stringent relative to genome-wide QTL searches). A further reason may be that the candidate gene polymorphisms and the flanking markers used in QTL studies are not at the

Table 3: Chromosomal localizations of candidate genes for litter size and its component traits

| gene | chromosome | position | | reference |
| | | cytogenetic map | linkage map (cM) | |
|---|---|---|---|---|
| ESR | 1 | p2.5-p2.4 | | Ellegren et al., 1994b |
| | | | 0.00 | Archibald et al., 1995 <br> Ellegren et al., 1994a |
| | | | 19.00 | Rohrer et al., 1996 |
| | | | 22.30 | Ellegren et al., 1994b <br> Marklund et al., 1996 |
| FSHB | 2 | p1.6-p1.2 | | Mellink et al., 1995 |
| | | | 28.00 | Ellegren et al., 1994a |
| | | | 35.40 | Marklund et al., 1996 |
| | | | 37.00 | Archibald et al., 1995 |
| | | | 42.00 | Zhang et al., 1995 |
| | | | 55.50 | Rohrer et al., 1994 |
| AREG | 8 | | 65.00 | Rohrer et al., 1994 |
| EGF | 8 | q2.3-q2.4 | | Spötter et al., 2001b |
| | | | 84.00 | Rohrer et al., 1996 |
| FGG | 8 | q1.1-q1.2 | | Lahbib-Mansais et al., 2000 |
| | | | 20.00 | Archibald et al., 1995 |
| OPN | 8 | | | Zhang et al., 1992 |
| PTGS2[1] | 9 | | | Gladney et al., 1999 |
| | | q2.4-q2.5 (S0114) | | Lopez-Corrales et al., 1999 |
| | | | 101.00 (S0114) | Archibald et al., 1995 |
| | | | 117.00 (S0114) | Groenen et al., 1996 |
| | | | 118.90 (S0114) | Rohrer et al., 1996 |
| RBP4[1] | 14 | | | Messer et al., 1996 |
| | | | 60.00 (S0007) | Rohrer et al., 1996 |
| | | | 75.00 (S0007) | Archibald et al., 1995 |
| | | | 82.50 (S0007) | Marklund et al., 1996 |
| | | | 107.70 (S0007) | Kapke et al., 1996 |
| PRLR[1] | 16 | | | Vincent et al., 1997 |
| | | | 0.00 (S0006) | Archibald et al., 1995 <br> Marklund et al., 1996 |
| | | | 22.10 (S0006) | Rohrer et al., 1996 |
| STE | - | - | - | - |

[1] PTGS2, RBP4 and PRLR were assigned to the chromosomes 9, 14 and 16, respectively. The given positions on cytogenetic and linkage maps for these genes refer to the closely linked (LOD scores > 10) microsatellite markers S0114, S0007 and S0006, respectively.

same time heterozygous in the sires of the founder generation and as a consequence thereof, cosegregation of the linked marker alleles and the alleles of the polymorphisms of the candidate genes does not occur. There were no studies in which linkage phases between candidate gene polymorphisms and linked marker alleles were proven. Therefore, direct comparisons between QTL for litter traits and candidate genes are not possible.

However, Pomp et al. (2001) gave a further explanation for the low correspondence of QTL and genes chosen as candidate genes based on the physiological role they play in a trait (physiological genes). Pomp et al. proposed a 'polygenic paradox' whereby QTL and physiological genes appear to represent two distinct subsets of genes. This means that evidence for an important role of a protein in regulation of a phenotype does not necessarily implicate the underlying gene as a QTL. Pomp et al. (2001) hypothesized that QTL primarily represent regulatory elements or initiation factors in a cascade of events, culminating in expression of physiological genes. Fuller understanding of this 'polygenic paradox' will require broad evaluation at the DNA, mRNA, protein and detailed phenotypic levels using a wide variety of techniques including DNA sequencing, evaluation of gene expression, and even mutational and transgenic analysis all of which were united under the term 'functional genomics' by Pomp et al. (2001). The aim is to apply these techniques in genome-wide or system-wide experimentation, expanding the scope of biological investigation from studying single genes or proteins to studying all genes or proteins and their multiple interactions at once in a systematic manner. The merging of accumulating information of such studies will establish linkages between QTL and physiological genes, enhancing our understanding of how complex traits in domestic animals are controlled and regulated, and facilitating improvement of economically important traits. In particular, 'functional genomics' will be a very powerful tool for studying the quantitative polygenic control of reproduction, as well as for understanding the underlying biology and physiology (Pomp et al., 2001). Gene expression profiles between divergent breeds will allow for the dissection of selection response (or genetic variation) into two major categories: (I) loci that have been selected for (by definition, the QTL); and (II) genes expression of which (quantity or quality of mRNA) has changed as a result of direct or indirect interaction with QTL (Figure 1) (Pomp et al., 2001). Key components underlying the biology of reproduction are most likely to be identified within the second category. This assumption supports the traditional hypothesis that quantitative traits are under polygenic control and that each of these genes has only a small or moderate effect. In other words there are many QTL operating in tandem and with potentially complex interactions to control reproduction at the genetic level. Although the heritable

genetic variation must, by definition, reside within the QTL, it is likely that these genes are regulatory and initiate critical changes in the transcription or translation of other genes, within

QTL1 ──────▶ RNA1 ──────▶ Protein1

QTL2 ──────▶ RNA2 ──────▶ Protein2

Gene3 ──────▶ RNA3 ──────▶ Protein3 ──────▶ LITTER SIZE

Gene3 ──────▶ RNA3 ──────▶ Protein3

QTL5 ──────▶ RNA5 ──────▶ Protein5

QTL5 ──────▶ RNA5 ──────▶ Protein5
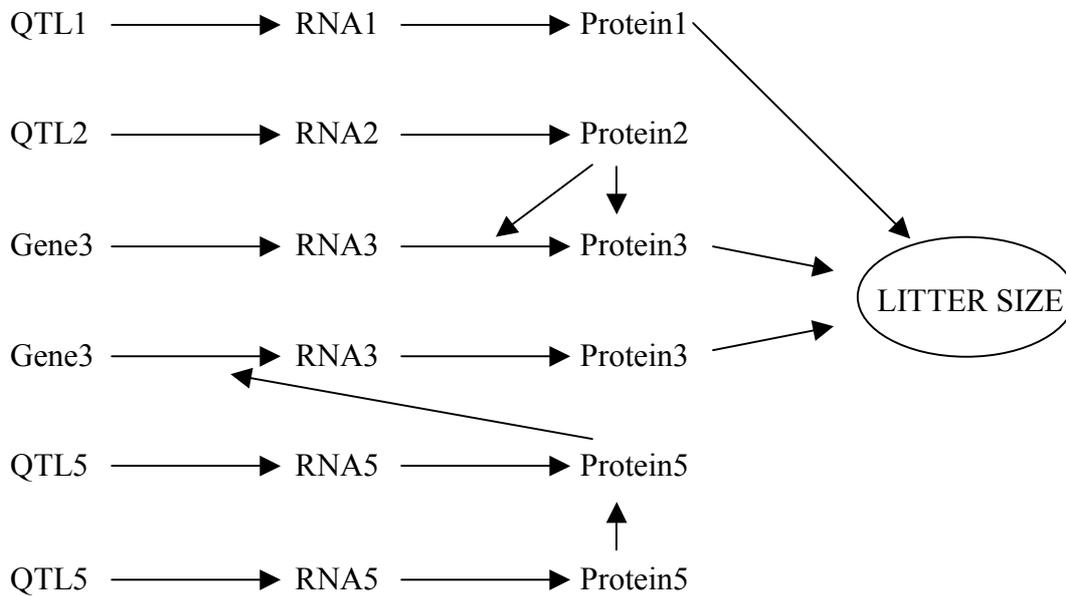
Figure 1: Schematic diagram representing a simplified hypothesis of the genetic architecture of a complex polygenic trait such as litter size or ovulation rate in pigs as hypothesized by Pomp et al. (2001). In this hypothesis, some QTL may directly influence a reproductive phenotype (QTL1), but more often others (QTL2, 5, 6) will exert effects by interacting with and regulating expression of 'physiological genes'.

which heritable sequence variation does not occur (Figure 1). Pomp et al. (2001) illustrate their hypothesis with a putative control mechanism for the *FSHB* gene which is clearly a critical rate-limiting protein in the determination of ovulation rate in pigs, and has been implicated as an important correlated response to selection for increased ovulation rate and litter size. However, there is no evidence that the *FSHB* locus is a QTL for reproduction. Thus, QTL for reproduction must stimulate changes in *FSHB* mRNA profiles through direct interaction, or through a cascade of regulatory events which may also be manifested at the mRNA or protein levels. Although several chromosomal regions have been found to harbor genes with effects on *FSH* concentrations, the *FSHB* locus itself has not been implicated as a QTL.

*From genomics to proteomics – Narrowing the gap between sequence and function*

Apart from the identification and mapping of candidate genes for litter size there has also been considerable interest in the elucidation of their physiological roles and modes of action, and several reports shed light on these subjects (Geisert and Yelich, 1997; La Bonnardière, 1993; Ying et al., 2000; Roberts et al., 1993; Green et al., 1998; Vallet et al., 2002; Simmen and Simmen, 1990). But the mammalian genome consists of approximately 30,000 genes and in many cases their respective proteins have more than one function depending on different possible interactions among each other. So this is at present an almost infinite research area. An important step in examining functions of genes is to determine their spatial and temporal expression patterns which implies the construction of - preferably comprehensive - cDNA libraries either of different tissues, or of the same tissue at various points in time, under different treatments, or from different genotypes, depending on the goal of the particular study. Such studies may also provide further evidence for candidate genes chosen based on their physiological role or their position in regions associated with possible QTL. This subject is addressed later in this chapter. In the first place, methods for the investigation of differential gene expression and some of their reported applications concerning reproduction in pigs are reviewed briefly.

A useful strategy in the construction of a cDNA library is to sequence only 600 bases at both ends of the cDNA which is enough to allow the identification of the transcripts (Hatey and Milan, 2002). These sequences are called expressed sequence tags (ESTs) and their generation was demonstrated by Adams et al. (1991). However, methods used for the evaluation of differentially expressed genes in the early stages have been laborious and not suitable for large-scale analysis. They allowed only the simultaneous examination of limited sets of genes and comprised methods such as Northern blotting and reverse transcriptase (RT-) PCR. Yelich et al. (1997b) used RT-PCR to investigate the ontology of elongation and gene expression in the early developing conceptus. Gene expression was detected for leukemia inhibitory factor (*LIF*) and integrin beta 1 (*ITGB1*) but not for estrogen, progesterone, oxytocin, and prostaglandin $F_{2\alpha}$ receptors. Better approaches have been developed to enable investigations of changes in gene expression in whole tissue, such as differential screening of cDNA libraries (e.g. Tosser-Klopp et al., 1997) or Differential Display (DD-) PCR (Liang and Pardee, 1992), the latter of which was used to isolate differentially expressed transcripts in the peri-implantation (days 11-12) endometrium of unilaterally pregnant pigs (Green et al., 1996). Chang et al. (2000) used DD-PCR to isolate transcripts from oviductal epithelia of gilts carrying embryos at various stages of early development. A technique even more efficient in

detecting differentially expressed genes compared to DD-PCR is subtractive suppressive hybridization (SSH; Diatchenko et al, 1996). This method includes two steps, the first of which is a subtraction that sorts out sequences specific for one sample from those common to both samples that are being compared, followed by the second step, a suppression, i.e. a reduction in the amount of the most abundant sequences in order to also obtain the rare ones. In an effort to identify genes associated with the Meishans high survival rate, Vallée et al. (2002) used the SSH technique to compare Meishan x Landrace and Landrace breeds at day 15 of gestation; they constructed two different cDNA libraries to identify differentially expressed genes in endometrial and embryonic tissues taken at the implantation period. They detected a total of 137 genes for the endometrial library and 166 genes for the embryo library which were differentially expressed between Meishan x Landrace and Landrace sows. Ross et al. (2003) used SSH to characterize differential gene expression during rapid trophoblastic elongation in the pig. Of the 384 transcripts screened, sequences were obtained for 142 that were confirmed to be differentially expressed. A comparison of the results of these studies and candidate genes for litter size in pigs (Tables 1 and 3) concerning possible correspondences is addressed later on in this chapter.

The advent of cDNA microarray technology permits the analysis of the expression of thousands of genes simultaneously, making it perhaps the most valuable tool for studying biological events in domestic animals. These devices are based on the hybridization of known sequences with the mRNA population to be studied in such conditions that the hybridization level is proportional to the amount of the corresponding sequence in the mRNA population analyzed (Hatey and Milan, 2002). For this purpose, known sequences, either cDNAs or oligonucleotides representing expressed genes, are spotted at high density onto a solid support (nylon, plastic, glass) and hybridized under stringent conditions with fluorescent targets produced from mRNA of two distinct biological samples (for example two tissues at various points in time, or under different treatments, or from different genotypes). The intensity of each fluorescent dye at each spot is detected with a microarray laser scanner. The data is usually represented as the ratio of the expression detected between the two RNA samples used (Pomp et al., 2001). As a first approach human arrays can be used in heterologous conditions. However, for a more accurate estimation of gene expression, it is important to develop species specific arrays. Caetano et al. (2002) utilized a specific cDNA microarray with 4608 probes from a normalized ovarian follicle cDNA library to identify differentially expressed genes associated with ovulation rate in ovarian/follicular tissue of two different lines. The tissues were collected during the follicular phase of the estrous cycle. A total of 92 clones showed

significant expression differences. Such experiments produce large sets of data which require specialized computer software for their management and analysis (Ermolaeva et al., 1998; Basset et al., 1999). This makes bioinformatics one of the most important and challenging aspects of using microarrays in the dissection of complex traits, such as reproduction at the transcription level.

The ability to examine the transcriptome on a system-wide basis permits not only a thorough comparison between treatments, developmental stages or genotypes, but also provides the opportunity to identify biological connections between genes and to uncover and link biochemical pathways that play critical roles in regulating important reproductive phenotypes (Pomp et al., 2001). However, the methods described here measure amounts of mRNA which are themselves phenotypes, under the potentially strong influence of environmental factors and interactions with other genetic components. Importantly, these amounts of mRNA may not be directly correlated with concentrations or activity of their respective translated proteins, or with the economically relevant end-point phenotypes such as litter size. Although 'functional genomics' may yield a large amount of information, significant efforts will be required to confirm and corroborate the influences of changes in gene expression within the broader, complex genetic and physiological models that are currently used. This bridge between genetics and physiology will be critical for implementing a fully integrated research program combining quantitative genetics, genomics, proteomics, metabolics and phenomics. It is likely that such an approach will be required to fully clarify the complex and polygenic nature of reproductive traits in pigs, and lead to discoveries that will have a strong impact on improvement of reproduction in the pork industry (Pomp et al., 2001).

Despite the mentioned flaws, the question arises whether expression studies could be useful in the confirmation of candidate genes, previously implicated in the regulation of litter size based on positional or physiological evidence. Some of the investigated candidate genes for litter size (Tables 1 and 3) were reported to be differentially expressed in the conceptus and / or tissues with reproductive function, including *EGF* (Kim et al., 2001), *OPN* (Garlow et al., 2002), *PTGS2* (Wilson et al. 2002), *RBP4* (Harney et al., 1993; Yelich et al., 1997a), and *STE* (Kim et al., 2002). These findings support their implication in litter size. However, these investigations of differential expression of single genes by Northern and slot blot hybridization were not confirmed by one or more of the mentioned expression studies using more progressive methods facilitative of large-scale analysis (Green et al., 1996; Chang et al., 2000; Vallée et al., 2002; Caetano et al., 2002; Ross et al., 2003) with one putative exception for RBP4. Vallée et al. (2002) identified a transcript with 98% homology to porcine retinol-

binding protein but left open with which of the 4 RBPs. Methods like DD-PCR, SSH and cDNA microarray allow the identification of numerous but not all differentially expressed genes in a tissue and there are several possible reasons, the first of which is the incompleteness of the utilized cDNA libraries. Further reasons may be a differential expression of a gene to weak to detect, the occurrence of congruent alleles in cDNA libraries of different investigated animals or breeds, or the differential expression of a gene at another point in time than investigated. However, the identification of numerous previously unknown transcripts and a low level of consistency between expression studies is not so surprising. It rather demonstrates the highly polygenic control of litter size and its component traits.

*The mouse model – a source of candidate genes for fertility traits in pigs?*
There is a steadily growing body of knowledge about the physiological role of genes in reproduction, especially in mice. With the advent of transgenic and gene-targeting methodologies to determine the biological roles of genes during development, the mouse has become an increasingly important model. Traditional gene-targeting approaches lead to an annulment of gene expression in early gestation and affect the development of the fetus and / or placenta (Han and Carter, 2001). Albeit many genes have been shown to be essential for prenatal survival in this manner, several genes or groups of genes are more or less resistant to gene-targeting approaches. Recent studies using gene knockout as a tool have indicated that in many cases the lack of expression of a single growth factor gene has relatively little effect on growth and survival, in a few cases causing only some minor abnormalities (Wei et al., 2001). This has led to the theory that many functions of growth factor families are subject to redundancy, and that just because a gene is highly conserved and redundantly expressed this does not mean it is essential (Shastry, 1994). This functional compensation for the absence or defect in a gene or pathway is not restricted to the growth factor families. For example, the process of uterine implantation appears to be compatible with a number of different null mutations in genes coding for cell adhesion molecules and their ligands (Poirier and Kimber, 1997), for cytokines (Lim et al., 2002) and for proteases (Sol-Church et al., 1999), all of which are also involved in early development. However, gene-targeting is a powerful tool which facilitates the investigation of gene functions in mice (e.g. Carlone and Skalnik, 2001; Allan et al., 2001) compared to other species. The resulting wealth of knowledge of mice has brought great advantages to this research field. Most of these reports concentrate on embryonic survival in the critical early period of gestation. Some deal with the preimplantation development of the embryo and its regulation by growth factors (e.g. Kaye

and Harvey, 1995; Stewart and Cullinan, 1997), but the majority of these reports are concerned with the periimplantation period and genes involved in its regulation. There are very comprehensive reports on this subject which propose a mouse model for the periimplantation period (e.g. Paria et al., 2001, 2002; Cross, 2001; Lim et al., 2002; Rinkenberger et al., 1997). Uterine and embryonic factors in implantation include steroid hormone signaling, adhesion molecules, histamine and prostaglandin signaling, growth factor signaling, cytokine signaling pathways, transcription factors and cannabinoid signaling pathways. Several of these genes are summarized in Table 4, and their putative interconnection in pathways is illustrated in a simplified manner in Figure 2. The question arises whether this wealth of knowledge about gene function is also applicable to other species such as the pig, based on the assumption of homologous physiological roles for orthologous genes in two different species. If so, this knowledge could be used for example as a source for the choice of new candidate genes for litter size in pigs. Opinions differ somewhat on the extrapolation of data obtained from a mouse model to other species. In any case, a comparison of pigs and mice is difficult since many parameters related to early pregnancy differ, one major point of which is the different type of placentation. Although the early stages of embryonic development, including establishment of cell lineages that make up the placenta, proceed similarly among all vertebrates, the form taken by the placenta is extremely variable (Amoroso, 1981). In primates and rodents, trophoblasts are invasive, breaching uterine vessels. As a result, maternal blood is in direct contact with trophoblasts (hemochorial placenta). In the pig, however, no invasion of the trophoblast into the maternal uterine tissue occurs. Trophoblasts fail to make direct contact with the maternal blood supply; rather they are apposed to uterine epithelium throughout the course of pregnancy (epitheliochorial placenta; Cross et al., 1994). Because of these differences Chen et al. (1999) and La Bonnardiere et al. (1993) deemed it unwise to extrapolate data from a mouse model to other species. Since the process of implantation is complex and varies across species, no unified theme has yet been formulated (Paria et al., 2001). However, there are also basic similarities among various species (see for example the *Hox* genes, McGinnis and Krumlauf, 1992), especially during the preimplantation period before close physical association with the maternal physiology is established via the uterine endometrium (Kaye and Harvey, 1995). Moreover, the mouse model has already proved useful for an better understanding of mammalian implantation (Paria et al., 2002). Most reports about the periimplantation period and genes involved in its regulation try to draw parallels to other species (Paria et al., 2001,

Table 4: Uterine and embryonic factors in blastocyst implantation in mice.

| | |
|---|---|
| **uterine factors in implantation** | |
| steroid hormone signaling | |
|     estrogen ($E_2$), estrogen receptor (ESR), progesterone ($P_4$), progesterone receptor (PGR) | |
| adhesion molecules | |
|     selectins, galectins, heparan sulfate proteoglycans (HSPGs), Mucin 1 (Muc1), integrins, cadherins, trophinin complex | |
| histamine and prostaglandin signaling | |
|     histamine, histidine decarboxylase (HDC), prostaglandins (e.g. $PGI_2$, $PGE_2$), prostaglandin receptors, peroxisome proliferator-activated receptors (e.g. PPARD), cyclooxygenases (COX1, COX2), retinoid X receptors (RXRs) | |
| growth factor signaling | |
|     transforming growth factor betas (TGFBs), fibroblast growth factors (FGFs), insuline like growth factors (IGFs), platelet-derived growth factors (PDGFs), vascular endothelial growth factors (VEGFs), epidermal growth factor (EGF), transforming growth factor alpha (TGFA), heparin-binding EGF (HB-EGF), amphiregulin, betacellulin, epiregulin, neuroregulins, receptors for EGF-like growth factors ErbB1 (EGFR), ErbB2, ErbB3, ErbB4) | |
| cytokine signaling pathway | |
|     tumor necrosis factor alpha (TNFA), interleukins (IL1, IL6, IL11), granulocyte/macrophage-colony stimulating factor (GM-CSF) leukemia inhibitory factor (LIF) | |
| transcription factors | |
|     Homeobox Gene (HOXA-10, HOXA-11), Homeobox (H6 family) 3 Gen (HMX3) | |
| cannabinoid signaling pathway | |
|     anandamide, 2-arachidonoylglycerol (2-AG) | |
| **embryonic factors in implantation** | |
| steroid hormone signaling | |
|     estrogen receptor (ESR) | |
| adhesion molecules | |
|     integrins, E-cadherin, zonula occludens-1 (ZO-1), occludin | |
| growth factor signaling | |
|     transforming growth factor alpha (TGFA), amphiregulin, cripto, ErbB1/EGFR, ErbB2, ErbB4 | |
| cytokine signaling pathway | |
|     LIF receptor (LIFR), gp130 | |
| cannabinoid signaling | |
|     cannabinoid receptors (CNR1, CNR2) | |

2002; Lim et al., 2002; Cross, 2001; Rinkenberger et al., 1997; Cross et al., 1994) such as human, rabbit, hamster, guinea pig and farm animals including pigs, sheep and cattle. For the improvement of litter size in pigs the mouse model provides starting points for research and in the choice of new candidate genes, where it should be used as one criterion, preferably in combination with the additional selection criteria mentioned above.

BLASTOCYST

activation

ErbBs ↑

CNR1 ↓

**IMPLANTATION**

catecholstrogen

PPARD/
RXR

?

OVARY

PGI$_2$

PGE$_2$

estrogen ⟶ estrogen ⟶ LIF ⟶ HB-EGF ⟶ COX2 ⟵ HOX10
HOX11

receptive state

progesterone ⟶ progesterone

prereceptive state

UTERUS
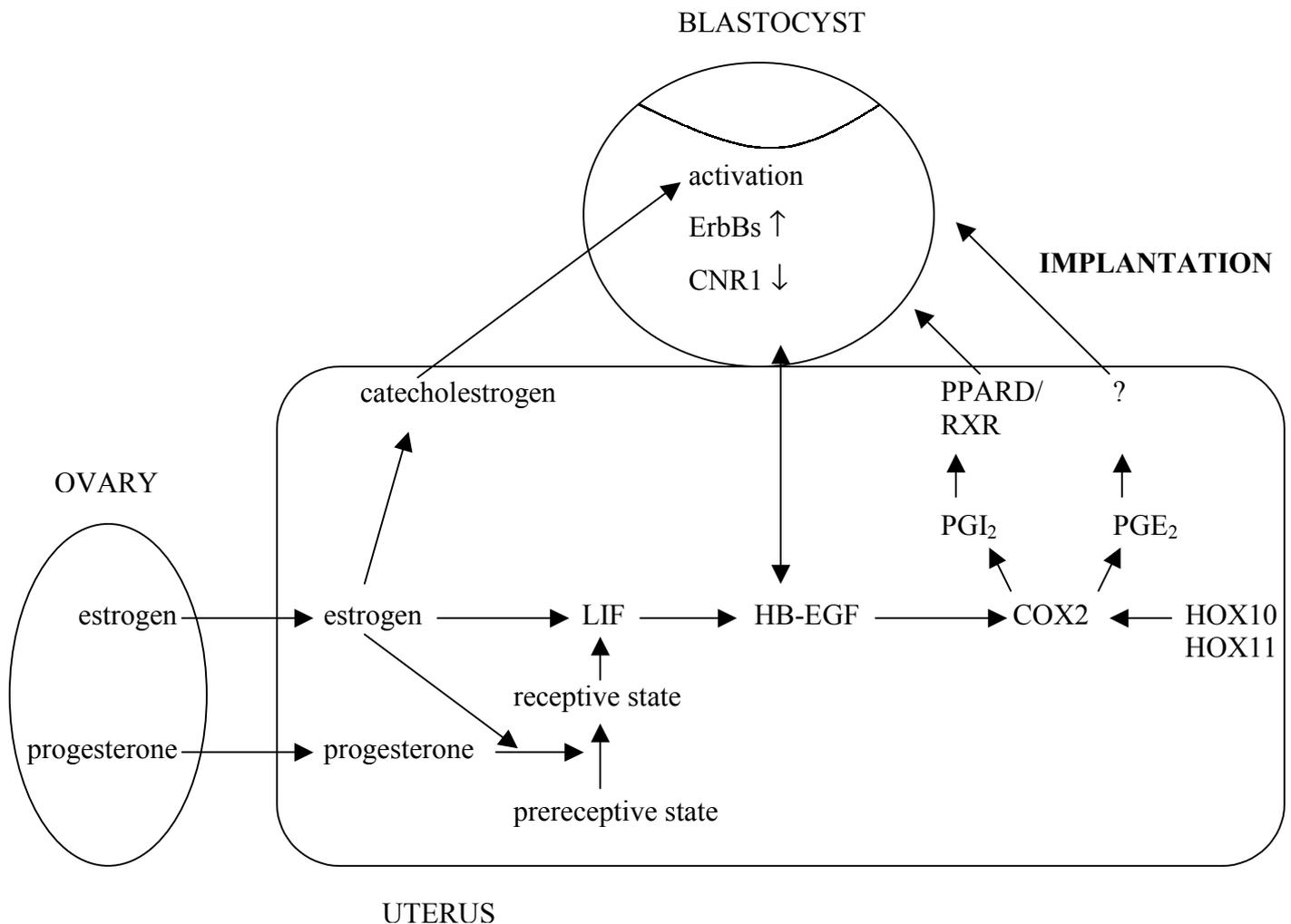
Figure 2: Simplified illustration of a putative molecular cascade of events in blastocyst implantation in the mouse. For full names of the genes, see Table 4.

*Candidate gene analysis for litter size in pigs – a case study*

A candidate gene approach was used to examine the effects of six genes on litter size in pigs. The new candidate genes selected were cathepsin L (*CTSL*), epidermal growth factor (*EGF*), epidermal growth factor receptor (*EGFR*), inter-α trypsin inhibitor heavy chain 4 (*ITIH4*), leukemia inhibitory factor (*LIF*), leukemia inhibitory factor receptor (*LIFR*). In the following, the reasons for choosing these genes for examination are reviewed briefly.

Cathepsins are lysosomal cysteine proteases that have been implicated as modulators of invasive implantation in cats (Li et al., 1992) and rats (Elangovan and Moulton, 1980). CTSL activity in the pig uterus is induced by progesterone and increases at the time of trophoblast

elongation with peak activity on day 15 of pregnancy (Geisert et al., 1997). Although the pig forms a diffuse epitheliochorial type of placental attachment, the high affinity of CTSL for collagen (Kirschke et al., 1982) and elastin (Mason et al., 1982) suggests that it may play a role in placental attachment on days 13-18 of gestation through limited proteolysis of the uterine epithelial glycocalyx (Geisert and Yelich, 1997). Uterine growth and expansion during early pregnancy involves elastase activity and collagen remodelling (Renegar, 1982) in which CTSL could play a role in both uterine and placental development.

ITIH4 is a glycoprotein that belongs to the inter-α-trypsin inhibitor family of serine protease inhibitors which act as acute phase reactants after trauma (Buchmann et al., 1990). Endometrial gene expression of *ITIH4* in pig was detected during estrus cycle (days 0–18) and early pregnancy (days 10–18). Gene expression of *ITIH4* is enhanced during the midluteal phase (days 12 and 15) of the estrus cycle and the period of trophoblast attachment (days 12–18). It was not detected in day-10 or day-12 pig conceptus tissues (Geisert et al., 1998). Synthesis of the glycoprotein by the uterine epithelium is stimulated by progesterone (Geisert et al., 1995). Regulation of cleavage for release of the polypeptide during pregnancy and early conceptus development suggests that it may play a role in conceptus-uterine interactions for the establishment of pregnancy in pigs, probably as an acute phase protein for protection of the uterus from the inflammatory response induced by conceptus attachment to the uterine epithelium (Geisert et al., 1998; Gonzales-Ramon et al., 1995). In addition to this possible role, the multipolypeptide chain of porcine ITIH4 could also serve to stabilize the epithelial glycocalyx (Chen et al., 1994) and inhibit conceptus endometrial invasion. Alteration in ITIH4 may not be the only factor involved with trophoblast attachment; however, cleavage of ITIH4 could induce local alterations in receptivity to the conceptus that permits the conceptus to contact integrins for firm attachment to the uterine epithelium (Bowen et al., 1997).

Leukemia inhibitory factor (LIF) is a member of the IL-6 family of pleiotropic cytokines and was initially identified by its capacity to induce macrophage differentiation of the myeloid leukaemic cell line M1 (Tomida et al., 1984; Hilton et al., 1988a, 1988b). The effects of LIF in many physiological systems include proliferation, differentiation, and cell survival (for reviews see Hilton, 1992; Metcalf, 1992). These biological effects of LIF are mediated by binding to a specific LIF receptor subunit (LIFR) (Gearing et al., 1991) that is, being a member of the cytokine-binding family of receptor subunits. Formation of a high-affinity signaling complex requires the association of the LIF-LIFR complex with another transmembrane signal-transducing molecule gp130 (Gearing et al., 1992a, 1992b) which itself

exhibits features of the cytokine family of receptors (Hibi et al., 1990). The essential role of endometrial synthesized LIF in blastocyst growth and implantation in mice (Stewart et al., 1992; Stewart, 1994; Savatier et al., 1996) implies that the LIF/LIFR system may also serve a vital function in conceptus development and implantation in pigs (Geisert and Yelich, 1997). This implication is supported by the detection of LIF gene expression in porcine endometrium at the time of blastocyst attachment (Anegon et al., 1994; Modric et al., 2000), and the presence of LIFR mRNA in porcine periimplantation conceptuses (Yelich et al., 1997b; Modric et al., 2000).

The cellular effects of epidermal growth factor (EGF) and EGF-like proteins, including transforming growth factor α (TGFα), heparin-binding EGF, and amphiregulin are mediated through binding to the membrane-bound EGF receptor (EGFR) (Prigent and Lemoine, 1992). All of these ligands are expressed by the pig endometrium during early pregnancy (Brigstock et al., 1990, 1996a, 1996b; Kennedy et al., 1994; Kim et al., 1995). A quantitative trait locus (QTL) for uterine capacity was identified on the long arm of chromosome 8 near 71 cM (Rohrer et al., 1999). This region is near the known location of the EGF gene (Mendez et al., 1999; Spötter et al., 2001b). Thus, the chromosomal location of EGF, its specific biochemical actions including cell proliferation (Haining et al., 1991) and initiation of DNA synthesis (Tomooka et al., 1986), its ability to improve the embryonic development *in vitro* (Wood and Kaye, 1989; Paria and Dey, 1990), and its increased luminal content on day 12 of pregnancy followed by a decline to day 16 (Diehl et al., 1994) indicate that the EGF/EGFR system may play a significant role in embryonic and maternal interactions (Wollenhaupt et al., 1999). This is further supported by the finding that endometrial and conceptus tissues express *EGFR* (Zhang et al., 1992a, 1992b; Kennedy et al., 1994).

After the isolation of genomic PAC and BAC clones for the six selected candidate genes they were localized by radiation hybrid (RH) mapping and fluorescence in situ hybridization (FISH; Spötter et al., 2001a, 2001b, 2001c, 2003; Kuiper et al., 2001). These mapping results are displayed in Table 5 and help to refine the existing porcine gene maps.

The complete coding sequences were determined for *LIF* (Spötter et al., 2001c) and *CTSL* (Spötter et al., 2001a) and their genomic organization is illustrated in Figure 3. The porcine *LIF* gene spans about 6.3 kb and consists of five exons including three alternative first exons (1D, 1M, 1T) spliced onto common second and third exons. The most frequent transcript, the LIF-D mRNA, measures 3.9 kb, and the resulting primary peptide consists of 202 amino acids. The porcine *CTSL* gene spans about 5.6 kb and contains eight exons. The mRNA

Table 5: Mapping results of six candidate genes for litter size in pigs.

| Gene | Localization (SSC) | Reference |
|------|--------------------|-----------|
| *LIF* | 14q21-q22 | Spötter et al., 2001c |
| *LIFR* | 16q13-q14 | Spötter et al., 2003 |
| *EGF* | 8q23-q24 | Spötter et al., 2001b |
| *EGFR* | 9q26 | Spötter et al., 2003 |
| *ITIH4* | 13q21-q22 | Kuiper et al., 2001 |
| *CTSL* | 10q11-q12 | Spötter et al., 2001a |
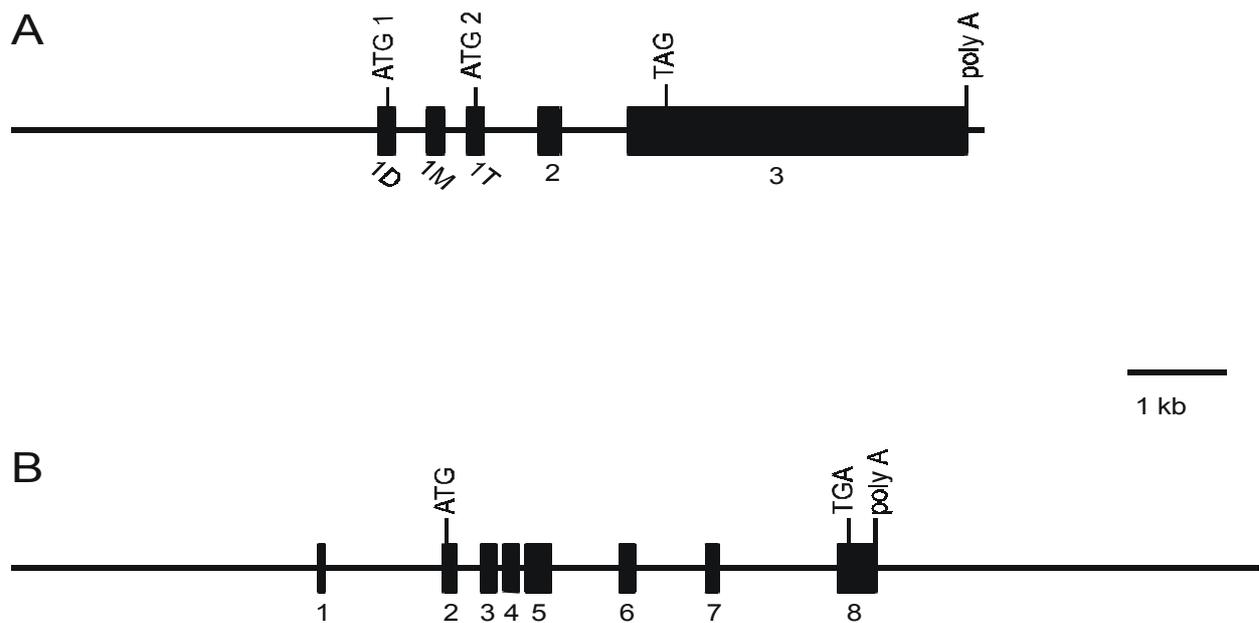


Figure 3: Chromosomal organization of the porcine genes encoding for LIF (A) and CTSL (B)

measures 1.4 kb and the primary peptide is composed of 334 amino acids. These sequences are publicly available in the EMBL nucleotide database under the accessions AJ315771 (CTSL) and AJ296176 (*LIF*). *ITIH4, EGF, EGFR* and *LIFR* were sequenced partially. The sequence information determined here was used to identify gene markers for all six genes. In case of the *LIF* gene a SNP (C/T transition) was found in exon 3 which can easily be genotyped by *Dra*III RFLP (Spötter et al., 2001c). For each of the other genes microsatellite markers were developed (Spötter et al., 2003). The six newly developed gene-linked markers were shown to be highly polymorphic and heterozygous in 273 sows of a German synthetic pig line (see Table 6). They have been physically anchored by FISH and RH mapping and should prove useful for future QTL fine mapping studies.

A negative dominance effect for the LIF marker was observed in an association study employing the six newly developed markers and 273 sows of a German synthetic pig line. This effect was $-0.72 \pm 0.370$ (p=0.047) for the first parity and $-0.50 \pm 0.29$ (p=0.087) for the second to tenth parities. No further statistical significant associations between any of the other microsatellite markers and NBA were observed in this study. It is important to stress that the

Table 6: Characterization of six newly developed gene markers.

| Gene | Marker type | Number of alleles | Allele size (min-max) | Expected heterozygosity | PIC[1] |
|------|-------------|-------------------|-----------------------|-------------------------|--------|
| EGF | microsatellite | 7 | 141-155 | 0.78 | 0.75 |
| ITIH4 | microsatellite | 4 | 148-162 | 0.47 | 0.44 |
| CTSL | microsatellite | 9 | 180-208 | 0.78 | 0.75 |
| LIFR | microsatellite | 7 | 133-149 | 0.80 | 0.77 |
| EGFR | microsatellite | 9 | 110-136 | 0.80 | 0.77 |
| LIF | SNP | 2 | 144, 266 | 0.40 | 0.40 |

[1]Polymorphism Information Content

lack of association between a gene-associated polymorphism and a phenotype does not mean that the gene product is not important in regulating the trait. Rather it is necessary to evaluate the developed markers in larger populations and in different pig breeds because associations

between the marker and trait may vary between populations, lines, or families. This was shown in several studies with DNA markers, such as *ESR* and *PRLR*.

The selection of new candidate genes for litter size is facilitated by expression studies, QTL studies and knowledge about the role of homologous genes in other species, such as the mouse. An enlargement of the marker set characterized above and the chromosomal localization of more genes implicated in reproduction and their sequencing is desirable in the context of basic research and especially in regard to the elucidation of gene function. All this contributes to solving the puzzle of the regulation of reproduction and will possibly help improve litter size in pigs.

*Conclusions*

Continued development of molecular tools and databases insure that many steps of the process of developing markers for selection will continue to occur at an increasingly rapid pace. However, the bottleneck in many cases will be at the initial step of obtaining the requisite phenotypic information. While technological developments steadily reduce genotyping and marker development costs, costs of collecting relevant phenotypic information or developing resource populations are unlikely to decline. This represents one of the most significant limitations to the application of genetic markers in selection for reproductive traits (Kirkpatrick, 2002).

The key risk of genetic improvement with steadily increasing accuracy of selection is the continous accumulation of homozygosity for genetic variation. This conflict between using markers for selecting the best animals (which are likely to be related) and selecting the least related to minimise inbreeding needs to be addressed (Visscher et al., 1998). However, many recent studies on the improvement of long-term response to selection using index and BLUP selection have shown that there is room for substantially decreasing levels of inbreeding without sacrificing response to selection (Brisbane and Gibson, 1995; Wray and Goddard, 1994; Villanueva et al., 1994). Methods already being developed to balance inbreeding and selection optimally may need to be extended to generate new heterozygosity through composite lines (Webb, 1998). Furthermore, theoretical work is needed to accommodate multilocus Mendelian inheritance and phenomena such as epistasis, genetic background effects and interactions between the environment and genetics. Unless genetic markers capture most of the genetic variation for the trait, which is far from the case at present, selection must be based on a combination of marker and conventional phenotypic data. Although several useful genes (primarily gene-linked markers) have been identified in pigs,

their application has been limited and their success inconsistent because the genes were not identified in breeding populations, or because they interact with other genes or the environment (Dekkers and Hospital, 2002). Most applications of molecular genetics to breeding programmes have attempted to incorporate molecular data into existing breeding programs. The effective use of molecular data might, however, require a substantial redesign of such programs so as to take better advantage of the unique features of molecular data (Dekkers and Hospital, 2002). Benefits from MAS are highest when selection takes place in stages or in groups of animals where previously no selection was possible (Visscher et al., 1998). This is the case for traits that are age-linked (measurable only in animals of a certain age), sex-limited (measurable only in one sex), and / or of low heritability, such as litter size, but also for traits such as disease resistance that are difficult to improve through conventional means because of the difficulty and expense of recording phenotype. However, until the complete analysis of complex traits has been achieved, observable genotype will remain an irreplaceable tool in genetic improvement programs, since it takes into account the collective effect of all genes (Dekkers and Hospital, 2002).

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# Chapter II

**Molecular characterization and chromosomal assignment of the porcine gene for leukemia inhibitory factor LIF**

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# Molecular characterization and chromosomal assignment[1] of the porcine gene for leukemia inhibitory factor LIF

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[1] This is a more precise localization of a gene previously mapped on SSC14 (Rettenberger et al., 1996; IMpRH map)

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ABSTRACT

Leukemia inhibitory factor (LIF) is a pleiotrophic cytokine involved in early conceptus development in pig. We isolated a PAC clone containing the porcine LIF gene and determined the complete DNA sequence of the gene, which spans about 6.3 kb and consists of 5 exons including three alternative first exons (1D, 1M, 1T) spliced onto common second and third exons. The LIF-D transcript encodes a protein of 202 amino acids sharing 87%, 84%, and 78% identity with respectively human, ovine, and murine leukemia inhibitory factors. The LIF-M and LIF-T transcripts both encode a truncated protein of 158 amino acids. Two SNP markers within untranslated regions of the LIF cDNA were identified. One SNP is located in the 5'-UTR of the alternative exon 1T while the other SNP is located in the 3'-UTR of exon 3. Based on fluorescence in situ hybridization and radiation hybrid mapping, the porcine LIF gene was assigned to chromosome 14q2.1→q2.2.

The cytokine LIF was initially identified by its capacity to induce macrophage differentiation of the myeloid leukaemic cell line M1 (Tomida et al., 1984; Hilton et al., 1988 a, b). The pleiotrophic effects of LIF in many physiological systems include proliferation, differentiation and cell survival (for reviews see Hilton, 1992; Metcalf, 1992), all of which are associated with blastocyst development and implantation. Little is known about the regulation of these events, except that a complex interaction between peptide and steroid hormones synchronizes the preparation of the uterus for implantation with the development of the embryo (Stewart et al., 1992). Uterine expression of LIF and that of its receptors has been demonstrated in a number of mammalian species indicating that LIF may have widespread importance in the establishment of pregnancy, although the variations in the reaction of the uterus in preparation for and during implantation are considerable among species (Vogiagis and Salamonsen, 1999, Stewart et al., 1992, Yelich et al., 1997).

The human and murine LIF genes have been cloned and completely sequenced (Stahl et al., 1990). Partial sequence informations about other mammalian LIF genes have also been reported (Willson et al., 1992). Investigations about the gene structure of mammalian LIF genes revealed a complex organization with three alternative first exons, which can be spliced onto common second and third exons, yielding three independently regulated transcripts (Haines et al., 1999).

In this report we describe the cloning and sequencing of the complete porcine LIF gene followed by the search for new genetic markers (SNPs) within the gene. The fluorescence in situ hybridization method (FISH) was used to refine the map location of the porcine LIF gene which has previously been assigned to SSC 14 by somatic cell hybrid analysis (Rettenberger et al., 1996).

MATERIALS AND METHODS

*Cloning and sequencing of the porcine LIF gene*

For the isolation of porcine PAC clones containing the LIF gene a porcine PAC library (Al-Bayati et al., 1999) was initially screened with PCR primers LIFV (5'-GGG CAG TTC TTA GCT GTC TCC TCT C-3') and LIFR (5'-TTT CAA AGT CTA CCT AAG GGG CAG C-3') (Rettenberger et al., 1996). One LIF PAC clone designated IVMP 714A1245 was isolated. DNA was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany). PAC DNA was restricted with different enzymes, separated on 0.8% agarose gels, and transferred to nylon membranes. Hybridizations with the above mentioned PCR product using the ECL enhanced chemiluminescence system (AmershamPharmacia, Freiburg, Germany) identified genomic fragments that contained parts of the porcine LIF gene. Selected fragments were cloned into the polylinker of pGEM-4Z (Promega, Mannheim, Germany). Recombinant plasmid DNA was sequenced with the ThermoSequenase kit (AmershamPharmacia, Freiburg, Germany) and a LICOR 4200 automated sequencer. After sequencing a collection of plasmid subclones, remaining gaps were closed by a primer walking strategy until both strands were completely sequenced. Sequence data were analyzed with Sequencher 4.0.5 (GeneCodes, Ann Arbor, MI).

*Mutation analysis*

To identify variations within the porcine LIF sequence, exons with their flanking regions were PCR amplified with DNA isolated from seven different pig breeds (Angeln Saddleback, Wild boar, Pietrain, Duroc, German Landrace, German Large White, a synthetic line from a German commercial company, and a second synthetic line with 50% Meishan) and a wild boar. The following PCR primers were used: LIF_1D1 (5'-CTT GCC TAG TTT CAA GCC ACC T-3') and LIF_1D2 (5'-AAG GCA GAG CGG GAA AAG TAG T-3') for the PCR product containing exon 1D, LIF_1MT1 (5'-TTC TTT CTG TCT TTC CGC TTT C-3') and

LIF_1MT2 (5'-ATC CCT CAA AAC TTC CTG TCC C-3') for the PCR product containing exons 1M and 1T, LIF_21 (5'-CCC CTT GCT ACC AGA GGT AGA G-3') and LIF_22 (5'-CTC TGC CAA TAT GTA ACA GGG C-3') for the PCR product containing exon 2, and LIF_31 (5'-ACT TCT GGT TCT CAG GAC GGT C-3') and LIF_32 (5'-CAC TTG GGT CTG GTG ATG TTC T-3') for the PCR product containing exon 3. The PCR products were sequenced using internal IRD700 labelled primers.

*Fluorescence in situ hybridization*

The PAC clone containing the porcine LIF gene was labeled with digoxigenin by nick translation using a Nick-Translation-Mix (Boehringer Mannheim Corp., Mannheim, Germany). FISH on GTG-banded pig chromosomes was performed using 750 ng of digoxigenin labeled PAC DNA. 1 μg sheared total porcine DNA and 10 μg salmon sperm DNA were used as competitors in this experiment. After hybridization over night, signal detection was performed using a Digoxigenin-FITC Detection Kit (Quantum Appligene, Heidelberg, Germany). The chromosomes were counterstained with DAPI and slides were mounted in propidium iodide/antifade. Metaphases that were previously photographed were reexamined after hybridization with a Zeiss Axioplan 2 microscope equipped for fluorescence.

*Probe name:* IVMP 714A1245

*Probe type:* PAC clone from porcine PAC library (Al Bayati et al., 1999)

*Insert size:* 75 kb

*Vector:* pCYPAC2

*Proof of authenticity:* DNA sequencing

*Gene reference:* Stahl et al. (1990)

RESULTS AND DISCUSSION

A porcine PAC clone containing a 75-kb insert was isolated from a genomic library using a PCR based screening procedure and primers for the porcine LIF gene. The DNA sequence of a 10281 bp *Sac*I fragment harboring the complete LIF gene was determined and deposited in the EMBL nucleotide database under the accession AJ296176.

The genomic structure of the porcine LIF gene was determined by comparison to the human LIF gene. Similar to the human gene the porcine LIF gene consists of three alternative first exons, which can be spliced onto common second and third exons. Thus there are three different transcripts with different 5'-ends (Fig. 1). The gene spans approximately 6.3 kb of genomic sequence. All exon/intron boundaries conform to the GT/AG rule (Table 1).
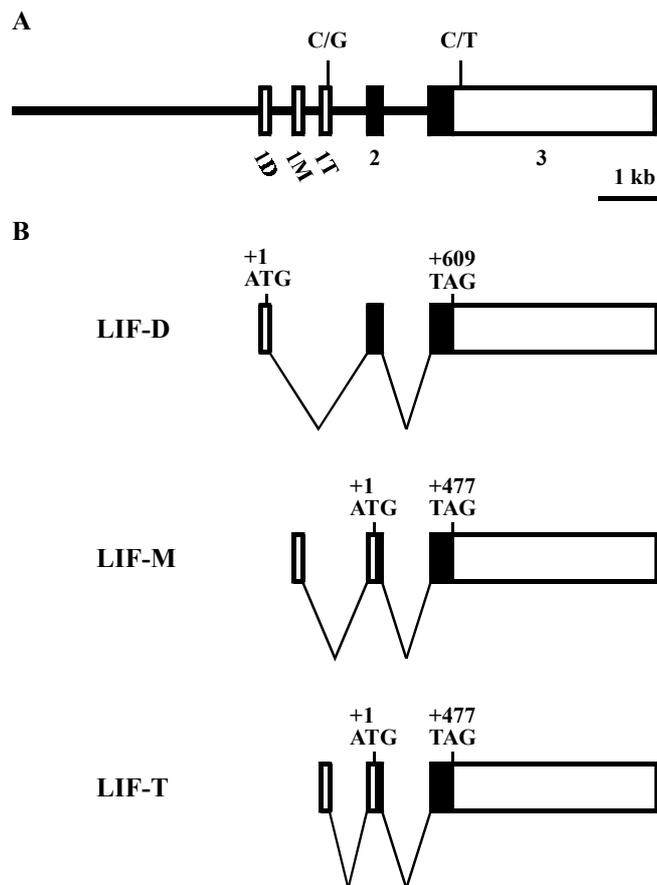


**Fig. 1.** (A) Genomic structure of the porcine LIF gene. Translated exons are shown as solid boxes. Untranslated exons, or untranslated regions of exons are shown as hatched boxes. The positions of the two identified SNPs are marked above the boxes with the alternative bases. (B) Three different splice forms of the porcine LIF mRNA are generated by alternative usage of three different first exons. The resulting two open reading frames are indicated by numbering.

The 3.9 kb LIF-D mRNA harbors an open reading frame of 606 bp coding for a protein of 202 amino acids. The porcine protein shares 87%, 84%, and 78% identity with respectively human, ovine, and murine leukemia inhibitory factors. The first exons of the LIF-M and

LIF-T transcripts contain no in-frame AUG, causing translation to initiate at an AUG encoded by exon 2, which is common to both transcripts (Fig. 1B). The translation product of LIF-M and LIF-T consists of 158 amino acids and lacks the secretory signal sequence of the LIF-D protein.

**Table 1:** Exon-intron junctions of the porcine LIF gene

|       | Exon Size |                                   |       | Intron Size |         |
|-------|-----------|-----------------------------------|-------|-------------|---------|
| Exon  | (bp)      | 5' Intron/Exon…Exon/Intron 3'     | Intron| (bp)        | Phase   |
| 1D    | 83        | *ATGAAC*…CGGCAG**gt**aaat          | 1D    | 1657        | 1       |
| 1M    | 24        | *CTGGAA…TGCTAG***gt**gagc          | 1M    | 1175        | 5'-UTR  |
| 1T    | 123       | *CCACCT…CACTTG***gt**acaa          | 1T    | 697         | 5'-UTR  |
| 2     | 179       | gcctgtttgc**ag**CAGTTG…CTCTAC**gt**aagt | 2 | 718         | 0       |
| 3     | >3640     | tctgcccctc**ag**TACACA…*ATTAATAAAGAACCTGA* |   |             |         |

Exon sequences are shown in uppercase letters, and intron sequences in lowercase letters. Untranslated regions are shown in italics. The conserved GT/AG exon/intron junctions are shown in boldface type. For the three alternative first exons the transcription start sites are shown instead of intron/exon junctions; for the last exon the polyadenylation signal is shown in bold italics instead of an exon/intron junction.

The transcription start site of the LIF-D transcript could be assigned based on the homology between the human and porcine LIF genes. Similar to the human gene, a TATA box (TATATAA) is located 31 bp upstream of this transcription start site in the porcine gene. Downstream of exon 1D, homology with the human gene supports the existence of two more alternative transcription start sites delineating exons 1M and 1T. Again similar to the human gene, no TATA boxes could be found upstream of these transcription start sites. The 5'-UTRs of the LIF-D, LIF-M, and LIF-T transcripts consist of 64, 137, and 236 nucleotides respectively, while the 3'-UTR of all LIF transcripts contains approximately 3200 nucleotides.

A search for sequence variations within the LIF gene in different pigs revealed no polymorphism that would affect the amino acid sequence of porcine LIF. However, two SNPs in untranslated regions could be identified. A transversion polymorphism (C/G) was found at

position 4950 of EMBL Acc. AJ296176 within the alternative first exon 1T. A transition polymorphism (C/T) was found at position 6988, 24 bp downstream of the stop codon in exon 3. The SNP in exon 3 can easily be genotyped by *Dra*III RFLP. In 17 unrelated Pietrain boars the allele distribution was 53:47 for the C/G polymorphism at position 4950 and for the C/T polymorphism at position 6988. Allele frequencies of the SNP in exon 3 in several different pig breeds are given in table 2. These two SNPs represent physically anchored genetic markers that might be useful in future QTL studies.

**Table 2:** Allele distributions for the C/T polymorphism in exon 3

| Breed | Sample Size | Allele Distribution (%) | |
|---|---|---|---|
| | (No. of genotyped boars) | C | T |
| Pietrain | 17 | 53 | 47 |
| Duroc | 27 | 33 | 67 |
| German Landrace | 70 | 56 | 44 |
| German Large White | 18 | 25 | 75 |
| German synthetic line | 41 | 33 | 67 |

In order to refine the established chromosomal localization of the porcine LIF gene on chromosome 14 (http://fabctr.umn.edu/RHMaps/chromosome/chromosome14.html) the isolated PAC clone was used as probe in a FISH experiment on porcine metaphase chromosomes. The porcine LIF gene could be assigned to SSC14q2.1→q2.2 (Fig. 2). This chromosomal assignment was confirmed by independent analysis of the IMpRH panel using newly designed PCR primers from the LIF gene. The orthologous human, murine, and bovine LIF genes map to HSA22q12, MMU11A1-A2, and BTA17 respectively. This is in agreement with established comparative maps of human, mouse, cattle and pig. (Bucan et al., 1993; Rettenberger et al., 1996; Band et al., 2000).
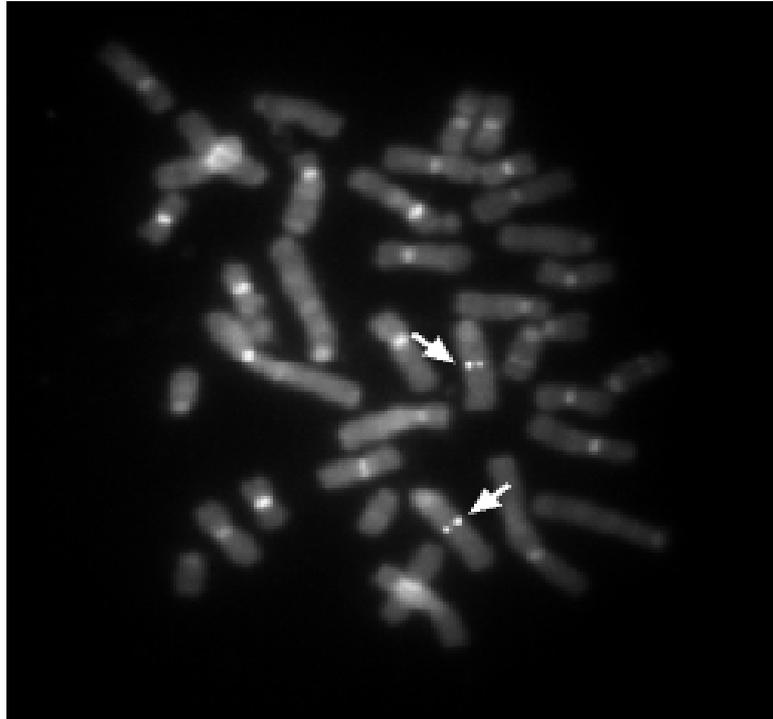
**Fig. 2.** Chromosomal assignment of the porcine LIF gene by FISH analysis. The digoxigenin labeled PAC IVMP 714A1245 containing the porcine LIF gene was hybridized to GTG-banded metaphase chromosomes of a normal pig. Double signals indicated by arrows are visible on both chromosomes 14q2.1→q2.2. The chromosomes were counterstained with propidium iodide and subsequently identified by DAPI staining.

Mapping data

*Location:* 14q2.1→q2.2

*Number of cells examined:* 21

*Number of cells with specific signals:* 0 (2), 1 (0), 2 (7), 3 (1), 4 (11) chromatids per cell

*Most precise assignment:* 14q2.1→q2.2

*Location of background signals (site with >2 signals):* none observed

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# **Chapter III**

**Characterization and comparative mapping of the porcine CTSL gene**

**indicates a novel synteny between HSA9q21→q22 and SSC10q11→q12**

A. Spötter, C. Drögemüller, H. Kuiper, B. Brenig, T. Leeb, and O. Distl

# Characterization and comparative mapping of the porcine CTSL gene indicates a novel synteny between HSA9q21→q22 and SSC10q11→q12

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ABSTRACT

Cathepsin L (CTSL) is a lysosomal cysteine protease with potent elastase and collagenase activities. Its high activity in the uterine lumen during the period of placental attachment has led to speculation that CTSL may play an important role during embryonic implantation in the pig. Cathepsins have also been implicated in blastocyst implantation in other species like cat, rat and man. We isolated a PAC clone containing the porcine CTSL gene and determined the complete DNA sequence of the gene, which spans about 5.6 kb and consists of eight exons. The CTSL transcript encodes a primary peptide of 334 amino acids sharing 73%–78% identity with other mammalian cathepsin L precursor proteins. Based on fluorescence in situ hybridization and radiation hybrid mapping, the porcine CTSL gene was assigned to chromosome 10q11→q12.

The proteolytic activity of cathepsin L as well as its high affinity for collagen (Kirschke et al., 1982) and elastin (Mason et al., 1982) has led to the hypothesis that cathepsin L may be involved with the early stages of implantation in the endothelial-chorial placenta found in the cat (Verhage et al., 1989; Li et al., 1991). Although no direct evidence is available on the structural changes associated with implantation in the cat, two other species (rat and man) show remarkable changes in uterine elastin and collagen during pregnancy (Li et al., 1991). In the rat, uterine elastin content increases 300-fold over that of nonpregnant controls (Starcher et al., 1985). Aflin et al. (1988) have proposed that invasion of the human trophoblast into the decidua is associated with the breakdown of type IV collagen containing microfibrils. Thus it is tempting to speculate that cathepsin L may be important in the implantation process. Geisert et al. (1997) found that cathepsin L is also present in the pig, a species with noninvasive epitheliochorial placentation. The role of endometrial cathepsin L in the pig is obviously not for invasion through uterine surface epithelium. Rather it may possibly effect the alterations in uterine and placental development that are necessary for placental attachment and growth (Geisert et al., 1997). These events involve an increase in elastase activity and collagen remodelling in which cathepsin L could play a role. Uterine expression of cathepsin L has been demonstrated in a number of mammalian species indicating that this enzyme may have widespread importance in the early stages of blastocyst implantation and thus influencing embryo survival.

The complete coding sequences of the human and murine CTSL genes have been determined (Joseph et al., 1988). The cathepsin L genes of both species contain eight exons and seven introns. The human gene located on HSA9q21→q22 (Fan et al., 1989) spans approximately 5.1 kb (Chauhan et al., 1993), whereas the murine gene on MMU13 (Pilz et al., 1995) spans approximately 7.4 kb (Chauhan et al., 1993).

In this report we describe the cloning and sequencing of the complete porcine cathepsin L gene. The location of the porcine CTSL gene on SSC 10 was determined by radiation hybrid mapping and fluorescence in situ hybridization (FISH).

MATERIALS AND METHODS

*Cloning and sequencing of the porcine CTSL gene*

For the isolation of porcine PAC clones containing the CTSL gene a porcine PAC library (Al-Bayati et al., 1999) was initially screened with PCR primers CTSL_5 (5'-CCT CAA GGC AAT CAG GGC TGC A-3') and CTSL_6 (5'-CAC AGT TGC GAC TGC CTT-3') which were derived from the porcine cDNA (Acc. D37917) sequence. One CTSL PAC clone designated IVMP 714I16_0039 was isolated. DNA was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany). PAC DNA was restricted with different enzymes, separated on 0.8% agarose gels, and transferred to nylon membranes. Hybridizations with the above mentioned PCR product using the ECL enhanced chemiluminescence system (AmershamPharmacia, Freiburg, Germany) identified genomic fragments that contained parts of the porcine CTSL gene. Selected fragments were cloned into the polylinker of pGEM-4Z (Promega, Mannheim, Germany). Recombinant plasmid DNA was sequenced with the ThermoSequenase kit (AmershamPharmacia, Freiburg, Germany) and a LICOR 4200 automated sequencer. After sequencing a collection of plasmid subclones, remaining gaps were closed by a primer walking strategy until both strands were completely sequenced. Sequence data were analyzed with Sequencher 4.0.5 (GeneCodes, Ann Arbor MI). Further analyses were performed with the online tools of the European Bioinformatics Institute (http://www.ebi.ac.uk/) and the RepeatMasker searching tool for repetitive elements (Smit AFA and Green P; RepeatMasker at http://ftp.genome.washington.edu/RM/RepeatMasker .html).

*RH-mapping*

The INRA-University of Minnesota porcine Radiation Hybrid panel (IMpRH; Yerle et al., 1998) was obtained from INRA, Laboratoire de Genetique Cellulaire, BP 27, Castanet-Tolosan, France. The 118 DNAs of the RH panel were subjected to PCR amplification. A 512-bp fragment of a part of the CTSL intron 5 was amplified using specific primers CTSL_RH1 (5'-AAA TGG AGC TCC TTC TCT TG-3') and CTSL_RH2 (5'-AGA TCC CTT AGG TCT ACT TGG-3'). Temperature and time profile were 35 cycles of 94°C for 30 s, 56°C for 1 min, and 72°C for 30 s. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Positive signals were scored and the results were statistically analyzed using the IMpRH mapping tool (http://imprh.toulouse.inra.fr/).

*Fluorescence in situ hybridization*

The PAC clone containing the porcine CTSL gene was labeled with digoxigenin by nick translation using a Nick-Translation-Mix (Boehringer Mannheim Corp., Mannheim, Germany). FISH on GTG-banded pig chromosomes was performed using 750 ng of digoxigenin labeled PAC DNA. 1 μg sheared total porcine DNA and 10 μg salmon sperm DNA were used as competitors in this experiment. After hybridization over night, signal detection was performed using a Digoxigenin-FITC Detection Kit (Quantum Appligene, Heidelberg, Germany). The chromosomes were counterstained with DAPI and slides were mounted in propidium iodide/antifade. Metaphases that were previously photographed were reexamined after hybridization with a Zeiss Axioplan 2 microscope equipped for fluorescence.

*Probe name:* IVMP 714I16_0039

*Probe type:* PAC clone from porcine PAC library (Al Bayati et al., 1999)

*Insert size:* 75 kb

*Vector:* pCYPAC2

*Proof of authenticity:* DNA sequencing

*Gene reference:* Chauhan et al. (1993)

RESULTS AND DISCUSSION

A porcine PAC clone containing a 75-kb insert was isolated from a genomic library using a PCR based screening procedure and primers for the porcine CTSL gene. The DNA sequence of two adjacent *Sac*I fragments (5518 and 7203 bp) harboring the complete CTSL gene was determined and deposited in the EMBL nucleotide database under the accession AJ315771. The genomic structure of the porcine CTSL gene was determined by comparison to the porcine CTSL cDNA and to the human CTSL gene. Similar to the human orthologue the porcine CTSL gene consists of eight exons and seven introns. The gene spans approximately 5.6 kb of genomic sequence. All exon/intron boundaries conform to the GT/AG rule (Table 1). Among the introns within the coding region, introns 2–5 interrupt the open reading frame

**Table 1:** Exon-intron junctions of the porcine CTSL gene

| Exon | Exon Size (bp) | 5' Intron/Exon…Exon/Intron 3' | Intron | Intron Size (bp) | Phase |
|------|------|------|------|------|------|
| 1 | >66 | …..…*CCGCAG***gt**cagc | 1 | 1168 | 5'-UTR |
| 2 | 137 | ttccttccct**ag***GTTTTT*…GGCATG**gt**tggt | 2 | 281 | 0 |
| 3 | 123 | cattgcctct**ag**AATGAA…GACATG**gt**gagt | 3 | 103 | 0 |
| 4 | 147 | ttgtccttaa**ag**ACCAAT…AATCAG**gt**atga | 4 | 98 | 0 |
| 5 | 225 | ttttatttt**c**agGGTCAG…GGAAGG**gt**aaat | 5 | 698 | 0 |
| 6 | 166 | tgcctttaaa**ag**GAAACA…AGTCAG**gt**aggt | 6 | 720 | 1 |
| 7 | 118 | tcactctccc**ag**GCATTT…GAACAG**gt**atga | 7 | 1218 | 2 |
| 8 | >382 | ttcttctttc**ag**TTGGGG…*TAAAATAAATTTGAATT* | | | |

Exon sequences are shown in uppercase letters, and intron sequences in lowercase letters. Untranslated regions are shown in italics. The conserved AG/GT dinucleotides at exon/intron junctions are shown in boldface type. For the last exon the polyadenylation signal is shown in bold italics instead of an exon/intron junction.
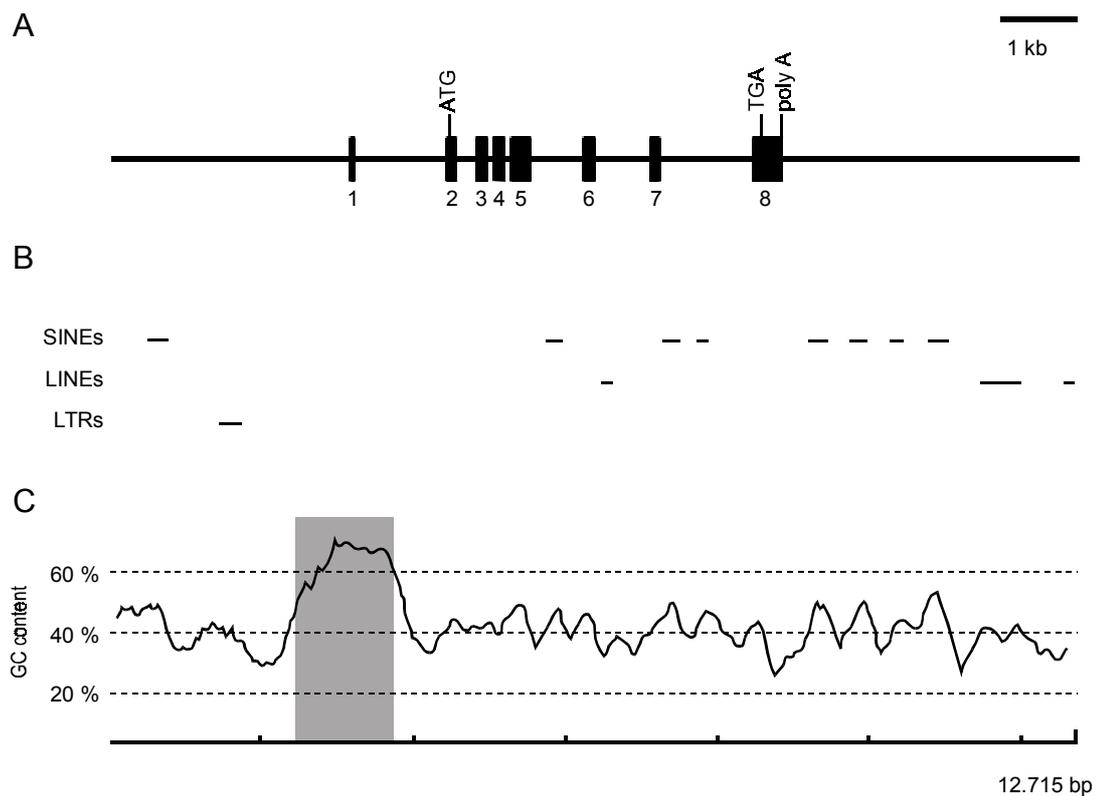
**Fig. 1**. (**A**) Genomic structure of the porcine CTSL gene. (**B**) Approximate sizes and positions of the identified repetitive elements. (**C**) GC-content of the porcine CTSL gene. The GC-content was calculated using a 300-bp window. The shaded box highlights a CpG island around the first exon of the porcine CTSL gene.

between codons (type 0 intron), intron 6 interrupts the codon after the first nucleotide (type 1 intron), and intron 7 interrupts the codon after the second nucleotide (type 2 intron). This is in agreement with the human and murine cathepsin L genes.

A search for known porcine repetitive elements within the determined DNA sequence revealed the existence of eight SINE elements, three LINE elements and one LTR element (Fig. 1B). Repetitive sequences make up 24.4% of the 12715 bp that were determined. The overall GC-content of the reported sequence was 43.2%. A CpG island spanning 1260 bp (bases 2482–3741) was detected around the first exon (Fig. 1C), indicating the presence of a functional promoter region.

The 1.4-kb CTSL mRNA harbors an open reading frame of 1002 bp coding for a primary peptide of 334 amino acids. The porcine peptide shares 78% and 73% identity with human and murine cathepsin L precursor proteins, respectively (Fig. 2). The first exon of the CTSL

gene contains no in-frame ATG, causing translation to initiate at an ATG encoded by exon 2 (Fig. 1A).

```
porcine    MKPSLFLTALCLGIASAAPKLDQNLDADWYKWKATHGRLYGMNEEGWRRAVWEKNMKMIE 60
human      -N-T-I-A-F-------TLTF-HS-E-Q-T----M------------------------- 60
murine     -NLL-L-AV----TALAT--F--TFS-E-HQ--S—R-----T---E----I-----R--Q 60
           *:   *:*:.:*** * *: .:*:.:.*:* :**: * **** *** ****:*****:**:

porcine    LHNQEYSQGKHGFSMAMNAFGDMTNEEFRQVMNGFQNQKHKKGKVFHESLVLEVPKSVDW 120
human      ------RE---S-T----------S------------R-PR-----Q-P-FY-A-R---- 120
murine     ---G---N-Q-G---E---------------V--YRH------RL-Q-P-M-KI------ 120
           *** ** :*:*.*:* ********.******:**:::* :**::*:*.*. : *:****

porcine    REKGYVTAVKNQGQCGSCWAFSATGALEGQMFRKTGKLVSLSEQNLVDCSRPQGNQGCNG 180
human      -------P-------------------------R-I-----------G----E---- 180
murine     ----C—P----------------S-C------L-----I-----------HA-------- 180
           **** **.***************:*.****** ***:*:***********  .***:****

porcine    GLMDNAFQYVKDNGGLDTEESYPYLGRETNSCTYKPECSAANDTGFVDIPQREKALMKAV 240
human      ----Y-----Q------S------EAT-E.--K-N-KY-V----------KQ-------- 239
murine     ----F----I-E-----S------EAKDG.--K-RA-FAV----------Q-------- 239
           **** ****:::*****:****** . :   **.*..: :.***********::********

porcine    ATVGPISVAIDAGHSSFQFYKSGIYYDPDCSSKDLDHGVLVVGYGFEGTDSNSSKFWIVK 300
human      --------------E--L---E---FE-----E-M------------S-E-DNN-Y-L-- 299
murine     ---------M--S-P-L---S-----E-N----NL------L----Y------KN-Y-L-- 299
           *********:**.* *: **..***:*:***::*****:****:*.*:*:..*:*:**

porcine    NSWGPEWGWNGYVKMAKDQNNHCGISTAASYPTV. 334
human      ----E---MG--------RR-----AS-------. 333
murine     ----S---ME--I-I---RD----LA------V-N 334
           **** ***  **:*:***: ****::.*****.*
```

**Fig. 2.** Alignment of the primary CTSL amino acid sequences of pig, man and mouse. Identical amino acids are marked by asterisks, while conserved amino acid exchanges are marked by colons (strong conservation) or dots (weak conservation).

In order to determine the chromosome location of the porcine CTSL gene the isolated PAC clone was used as probe in a FISH experiment on porcine metaphase chromosomes. The porcine CTSL gene could be assigned to SSC10q11→q12 (Fig. 3). This chromosome assignment was confirmed by the results of the IMpRH panel. The CTSL gene was placed by multipoint analyses between the microsatellite marker SW173 (Rohrer et al., 1996) and the EST SSC10G07 (Jørgensen et al., 1997) with lod scores of 2.78 and 3.22, respectively. This region has been mapped to porcine chromosome 10q11→q12. The human CTSL gene has been mapped to HSA9q21→q22 (Fan et al., 1989; Chauhan et al., 1993). According to the comparative map of pig and human (Goureau et al., 1996; http://www.toulouse.inra.fr /lgc/pig/compare/compare.htm) the SSC10q11→q12 region shares conserved synteny with HSA9p12→p13 which is confirmed by the location of the genes AP4A (Jørgensen et al.,

1997) and ACO1 (synonymous with IREB1; Winterø et al., 1998). Our mapping results show that the established synteny between proximal HSA9p and proximal SSC10q is more complex than previously thought and that the comparative maps of pig and man in this region have to be refined. In particular it seems that the conservation of synteny extends beyond the centromere of HSA9 so that conserved sequences from HSA9q can also be found on SSC10q11→q12. Therefore, it would be helpful to have additional information on the mapping positions in pig of other orthologous genes situated between HSA9p13 and HSA9q21→q22 to delineate the extended borders of the previously established conserved segment.
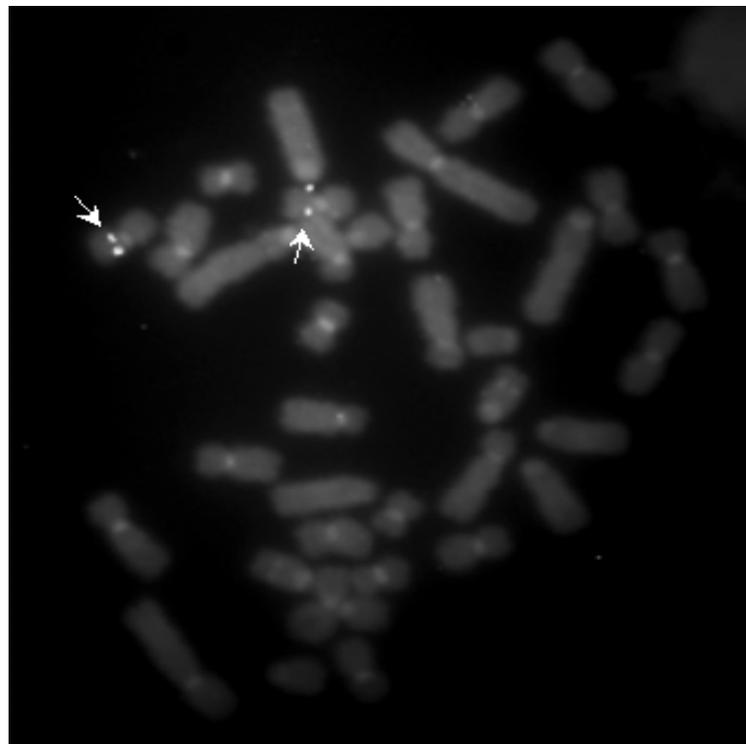


**Fig. 3.** Chromosome assignment of the porcine CTSL gene by FISH analysis. The digoxigenin labeled PAC IVMP 714I16_0039 containing the porcine CTSL gene was hybridized to GTG-banded metaphase chromosomes from a normal pig. Double signals indicated by arrows are visible on both chromosomes 10q11→q12. The chromosomes were counterstained with propidium iodide and DAPI.

*Mapping data*

*Location:* 10q11→q12

*Number of cells examined:* 35

*Number of cells with specific signals:* 0 (11), 1 (2), 2 (11), 3 (3), 4 (8) chromatids per cell

*Most precise assignment:* 10q11→q12

*Location of background signals (site with >2 signals):* none observed

## ACKNOWLEDGEMENTS

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# Chapter IV

# Assignment of the porcine epidermal growth factor (*EGF*) gene to SSC8q2.3-q2.4 by fluorescence in situ hybridization and radiation hybrid mapping

A. Spötter, H. Kuiper, C. Drögemüller, B. Brenig, T. Leeb, and O. Distl

# Assignment[1] of the porcine epidermal growth factor (*EGF*) gene to SSC8q2.3-q2.4 by fluorescence in situ hybridization and radiation hybrid mapping

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[1]This is a more precise localization of *EGF* previously mapped to SSC8q2.3→q2.7 by Mendez et al. (1999)

*Source/description*

Epidermal growth factor (EGF) is a polypeptide that has many different biological properties. *In vitro*, it is a potent mitogen for many cultured cells and *in vivo*, it stimulates the proliferation and differentiation of skin tissue and corneal, lung and tracheal epithelia[1]. EGF is also involved in early conceptus development in pig[2]. It is produced by the conceptus and in the uterus of the sow. In the fetus EGF stimulates growth and proliferation of skin epithelia[3]. In the uterus the mitogen may induce endometrial growth and differentiation to create an environment favourable for the developing conceptus. For the isolation of the porcine PAC clone containing the *EGF* gene a porcine PAC library[4] was initially screened with PCR primers EGF_1 (5'-GAA ACA ATT CCC GTG TTC TCT-3') and EGF_2 (5'-TCA CTT CCA CAC CTG TAA CAT CT-3')[5]. PCR amplification (25 µl final volume) was performed using 25 ng of genomic porcine DNA, 1x PCR buffer (QIAGEN Hilden, Germany), 100 µM each dNTP, 10 pmol each primer, and 2.5 U *Taq* polymerase (QIAGEN Hilden, Germany). The thermocycler profile was 94°C for 4 min; 38 cycles of 94°C for 30 s, 60°C for 60 s, and 72°C for 30 s; followed by a final cooling step at 4°C for 10 min. One *EGF* PAC clone designated TAIGP714I1851 was isolated. DNA from the clone was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany).

*Chromosome preparation*

Porcine metaphase spreads for FISH on GTG-banded chromosomes were prepared using phytohemagglutinin stimulated blood lymphozytes from a normal pig. Cells were harvested and slides prepared using standard cytogenetic techniques. Prior to fluorescence in situ hybridization the chromosomes were GTG-banded and well-banded spread metaphase chromosomes were photographed using a highly sensitive CCD camera and IPLab 2.2.3 (Scanalytics, Inc.).

*Fluorescence in situ hybridization*

A 70 kb PAC clone containing the porcine EGF gene (TAIGP714I1851) was labeled with digoxigenin by nick translation using a Nick-Translation-Mix (Boehringer Mannheim Corp., Mannheim, Germany). FISH on GTG-banded pig chromosomes was performed using 750 ng of digoxigenin labeled PAC DNA. 1 µg sheared total porcine DNA and 10 µg salmon sperm DNA were used as competitors in this experiment. After hybridization overnight, signal detection was performed using a Digoxigenin-FITC Detection Kit (Quantum Appligene, Heidelberg, Germany). The chromosomes were counterstained with DAPI and slides were

mounted in propidium iodide/antifade. Metaphases that were previously photographed were re-examined after hybridization with a Zeiss Axioplan 2 microscope equipped for fluorescence.

*Radiation hybrid (RH) mapping*

The INRA-University of Minnesota porcine Radiation Hybrid panel[6] (ImpRH) was obtained from INRA, Laboratoire de Genetique Cellulaire, BP 27, 31326 Castanet-Tolosan, France. The 118 DNAs of the RH panel were subjected to PCR amplification. A 550-bp fragment of the EGF gene was amplified using the above mentioned primers EGF_1 and EGF_2[5]. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Positive signals were scored and the results were statistically analyzed using the IMpRH mapping tool (http://imprh.toulouse.inra.fr/)

*Chromosomal location*

The chromosomal location of the porcine *EGF* gene on SSC8q2.3-q2.4 was determined by FISH of the PAC clone TAIGP714I1851 to metaphase chromosomes (Fig. 1). This localization was confirmed by analyzing a porcine radiation hybrid panel. The retention frequency of the PCR primers EGF_1 and EGF_2 was 16 %. Two-point analysis revealed close linkage of *EGF* to microsatellite markers *SW149*[7], *SW1337* and *SW2160*[8] at distances of 4.2, 3.9 and 5.9 cR, respectively. The corresponding LOD scores were 9.52, 9.9 and 6.47, respectively. This result is in concordance with the FISH assignment of the porcine *EGF* gene to SSC8q2.3-q2.4.

*Comment:* In this report we mapped a porcine PAC clone containing the *EGF* gene to SSC8q2.3-q2.4 which is consistent with comparative mapping information in human[9] (http://www.toulouse.inra.fr/lgc/pig/compare/compare.htm). The human *EGF* gene was assigned to HSA4q25. This chromosome shares extensive conserved regions with SSC8.
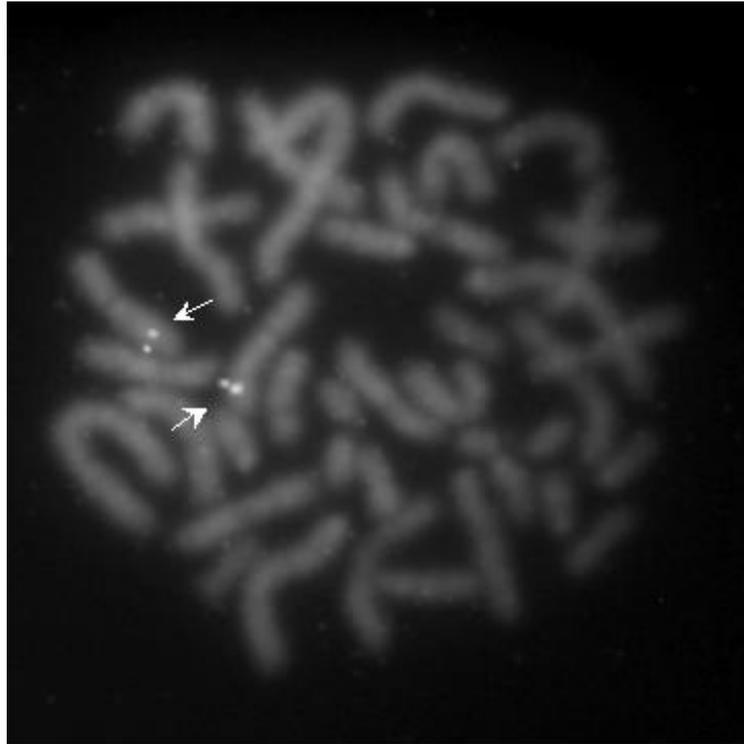
**Figure 1** Chromosomal assignment of the porcine *EGF* gene by FISH analysis. The digoxigenin labeled PAC clone TAIGP714I1851Q5 containing the porcine EGF gene was hybridized to GTG-banded metaphase chromosomes of a normal pig. Double signals indicated by arrows are visible on both chromosomes 8q2.3-q2.4. The chromosomes were counterstained with propidium iodide and subsequently identified by DAPI staining.

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# Chapter V

**Assignment[1] of the porcine inter-α trypsin inhibitor heavy chain 4 (*ITIH4*) gene to SSC13q2.1→q2.2 by fluorescence in situ hybridization and radiation hybrid mapping**

H. Kuiper, A. Spötter, C. Drögemüller, B. Brenig, T. Leeb, and O. Distl

# Assignment[1] of the porcine inter-α trypsin inhibitor heavy chain 4 (*ITIH4*) gene to SSC13q2.1→q2.2 by fluorescence in situ hybridization and radiation hybrid mapping

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[1] This is the first physical assignment of a gene previously mapped to SSC13 per genetic linkage mapping by Baskin et al. (1998)

Running title: Porcine *ITIH4* gene

[#] These authors contributed equally to the work.

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RATIONALE AND SIGNIFICANCE

ITIH4 is a glycoprotein that belongs to the inter-α-trypsin inhibitor family of serine protease inhibitors which act as acute phase reactants after trauma (Buchmann et al., 1990). Endometrial gene expression of ITIH4 in pig was detected during oestrus cycle (days 0–18) and early pregnancy (days 10–18). Gene expression of ITIH4 is enhanced during the midluteal phase (days 12 and 15) of the oestrus cycle and the period of trophoblast attachment (days 12–18). It was not detected in day 10 or day 12 pig conceptus tissues (Geisert et al., 1998). Synthesis of the glycoprotein by the uterine epithelium is stimulated by progesterone (Geisert et al., 1995). The role of ITIH4 in uterine function and in the conceptus has not been established. Regulation of cleavage for release of the polypeptide during pregnancy and early conceptus development suggests that it may play a role in conceptus-uterine interactions for the establishment of pregnancy in pigs, probably as an acute phase protein for protection of the uterus from the inflammatory response induced by conceptus attachment to the uterine epithelium (Geisert et al., 1998; Gonzales-Ramon et al., 1995). In this study, we assigned the ITIH4 gene to SSC13q2.1→q2.2.

MATERIALS AND METHODS

*Isolation and characterization of the ITIH4 clone*

For the isolation of the porcine PAC clone containing the ITIH4 gene a porcine PAC library (Al-Bayati et al., 1999) was initially screened with PCR primers ITIH4_1 (5'-GTT CGG GAA GCC ATA GAC G-3') and ITIH4_2 (5'-CGG AAG TTG TCC TGC GTG AC-3') which were derived from the porcine cDNA sequence (Acc. S82800). One ITIH4 PAC clone designated IVMP 714I12_0006 was isolated.

*Chromosome preparation*

Porcine metaphase spreads were prepared using phytohemagglutinin stimulated blood lymphocytes from a normal pig. Cells were harvested and slides prepared using standard cytogenetic techniques. Prior to fluorescence in situ hybridization the chromosomes were GTG-banded and well-banded metaphase chromosome spreads were photographed using a highly sensitive CCD camera and IPLab 2.2.3 (Scanalytics, Inc.).

*Fluorescence in situ hybridization*

A 180-kb PAC clone containing the porcine ITIH4 gene was labeled with digoxigenin by nick translation using a Nick-Translation-Mix (Boehringer Mannheim Corp., Mannheim, Germany). FISH on GTG-banded pig chromosomes was performed using 750 ng of digoxigenin labeled PAC DNA. 1 µg sheared total porcine DNA and 10 µg salmon sperm DNA were used as competitors in this experiment. After hybridization overnight, signal detection was performed using a Digoxigenin-FITC Detection Kit (Quantum Appligene, Heidelberg, Germany). The chromosomes were counterstained with DAPI and slides were mounted in propidium iodide, antifade. Metaphases that were previously photographed were reexamined after hybridization with a Zeiss Axioplan 2 microscope equipped for fluorescence.

*Probe name:* IVMP 714I12_0006

*Probe type:* PAC clone from porcine PAC library (Al Bayati et al., 1999)

*Insert size:* 180 kb

*Vector:* pCYPAC2

*Proof of authenticity:* DNA sequencing

*Gene reference:* Hashimoto et al. (1996)

*Radiation hybrid (RH) mapping*

DNA from the clone designated IVMP 714I12_0006 was isolated using the Qiagen plasmid maxi kit (Qiagen, Hilden, Germany). The INRA-University of Minnesota porcine Radiation Hybrid panel (ImpRH; Yerle et al., 1998) was obtained from INRA, Laboratoire de Genetique Cellulaire, BP 27, 31326 Castanet-Tolosan, France. The 118 DNAs of the RH panel were subjected to PCR amplification. A 350-bp fragment of the ITIH4 gene was amplified using the above-mentioned primers ITIH4_1 and ITIH4_2. Temperature and time profile were 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 30 s. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Positive signals were scored and the results were statistically analyzed using the IMpRH mapping tool (http://imprh.toulouse.inra.fr/).

RESULTS AND DISCUSSION

The chromosome location of the porcine ITIH4 gene was determined by FISH using the PAC clone IVMP 714I12_0006 to probe metaphase chromosomes (Fig. 1).

*Mapping data:*

*Location:* SSC13q2.1→q2.2

*Number of cells examined:* 45

*Number of cells with specific signals:* 0 (2), 1 (7), 2 (10), 3 (12), 4 (14) chromatids per cell

*Most precise assignment:* SSC13q2.1→q2.2

*Location of background signals (site with >2 signals):* none observed
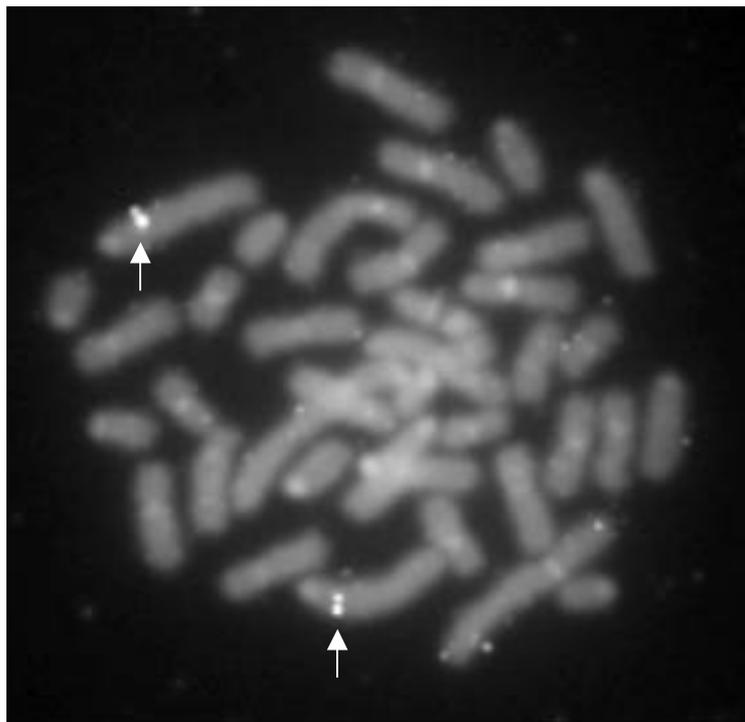


**Fig. 1.** Chromosomal assignment of the porcine *ITIH4* gene by FISH analysis. The digoxigenin labeled PAC IVMP 714I12_0006 containing the porcine *ITIH4* gene was hybridized to GTG-banded metaphase chromosomes of a normal pig. Double signals indicated by arrows are visible on both chromosomes 13q2.1→q2.2. The chromosomes were counterstained with propidium iodide and subsequently identified by DAPI staining.

In order to confirm the location of the porcine ITIH4 gene a porcine radiation hybrid panel was analyzed. The PCR primers derived from the PAC clone containing the gene showed a retention frequency of 22%. Two-point analysis revealed close linkage of ITIH4 to EST SSC24F05 (Jørgensen et al., 1997) at a distance of 5.1 cR (LOD score 7.35) and to microsatellite marker SW864 (Davies et al., 1995) at a distance of 7.3 cR (LOD score 5.02). On the porcine linkage map SW864 is closely linked to the microsatellites SW2458 and SW1400 (Rohrer et al., 1996) which have been physically assigned to SSC13q2.1→q2.2 (Alexander et al., 1996)

In this report we mapped a porcine PAC clone containing the ITIH4 gene to SSC13q2.1→q2.2. The human ITIH4 gene was assigned to HSA3p21.2→p14.1. This is consistent with the established comparative map of pig and human (Goureau et al., 1996; http://www.toulouse.inra.fr/lgc/pig/compare/compare.htm) indicating significant homology between SSC13 and HSA3. On both the human and the porcine chromosome ITIH4 was located close to CAMP (cathelicidin antimicrobial peptide, also known as FALL39) and DAG1 (dystroglycan1) and proximal from TF (transferrin), CP (ceruloplasmin) and SIAT1 (sialyltransferase) which points to a largely conserved gene order in this genome region. Yet it must be stressed that until more orthologous genes have been mapped precisely there is still the possibility of small gene order rearrangements within this large homologous region.


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# Chapter VI

**Mapping and microsatellite marker development for the porcine leukemia inhibitory factor receptor (*LIFR*) and epidermal growth factor receptor (*EGFR*) genes**

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# Mapping[*] and microsatellite marker development for the porcine leukemia inhibitory factor receptor (*LIFR*) and epidermal growth factor receptor (*EGFR*) genes

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[*] To our knowledge this is the first time these genes have been mapped.

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ABSTRACT

Leukemia inhibitory factor receptor (LIFR), epidermal growth factor receptor (EGFR), and their respective ligands have been implicated in regulating growth and development of the early pig conceptus. We isolated a PAC clone containing the porcine gene for LIFR and a BAC clone with the porcine EGFR gene, respectively. On each of these clones one microsatellite marker was identified by sequencing a collection of subclones. These gene-associated markers were evaluated by genotyping of 202 unrelated boars of four different breeds. Based on fluorescence in situ hybridization and radiation hybrid mapping, the porcine LIFR gene was assigned to SSC16q13→q14. The EGFR gene mapped to SSC9q26.

Leukemia inhibitory factor (LIF) is a member of the IL-6 family of pleiotropic cytokines and was initially identified by its capacity to induce macrophage differentiation of the myeloid leukaemic cell line M1 (Tomida et al., 1984; Hilton et al., 1988a, 1988b). The effects of LIF in many physiological systems include proliferation, differentiation, and cell survival (for reviews see Hilton, 1992; Metcalf, 1992). These biological effects of LIF are mediated by binding to a specific LIF receptor subunit (LIFR) (Gearing et al., 1991) that is, being a member of the cytokine-binding family of receptor subunits. Formation of a high-affinity signaling complex requires the association of the LIF-LIFR complex with another transmembrane signal transducing molecule gp130 (Gearing et al., 1992a, 1992b), which itself exhibits features of the cytokine family of receptors (Hibi et al., 1990). The essential role of endometrial synthesized LIF in blastocyst growth and implantation in mice (Stewart et al., 1992; Stewart, 1994; Savatier et al., 1996) implies that the LIF/LIFR system may also serve a vital function in conceptus development and implantation in pigs (Geisert and Yelich, 1997). This implication is supported by the detection of LIF gene expression in porcine endometrium at the time of blastocyst attachment (Anegon et al., 1994; Modric et al., 2000), and the presence of LIFR mRNA in porcine peri-implantation conceptuses (Yelich et al., 1997; Modric et al., 2000).

The cellular effects of epidermal growth factor (EGF) and EGF-like proteins, including transforming growth factor α (TGFα), heparin-binding EGF, and amphiregulin are mediated through binding to the membrane bound EGF receptor (EGFR) (Prigent and Lemoine, 1992). All of these ligands are expressed by the pig endometrium during early pregnancy (Brigstock et al., 1990, 1996a, 1996b; Kennedy et al., 1994; Kim et al., 1995). A quantitative trait locus

(QTL) for uterine capacity was identified on the long arm of chromosome 8 near 71 cM (Rohrer et al., 1999). This region is near the known location of the EGF gene (Mendez et al., 1999; Spötter et al., 2001). Thus, the chromosomal location of EGF, its specific biochemical actions including cell proliferation (Haining et al., 1991) and initiation of DNA synthesis (Tomooka et al., 1986), its ability to improve the embryonic development *in vitro* (Wood and Kaye, 1989; Paria and Dey, 1990), and its increased luminal content on day 12 of pregnancy followed by a decline to day 16 (Diehl et al., 1994) indicate that the EGF/EGFR system may play a significant role in embryonic and maternal interactions (Wollenhaupt et al., 1999). This is further supported by the finding that endometrial and conceptus tissues express EGFR (Zhang et al., 1992a, 1992b; Kennedy et al., 1994).

In this study we describe the isolation of one PAC and one BAC clone containing the porcine genes for LIFR and EGFR, respectively. On each of these clones one microsatellite marker was identified by sequencing a collection of subclones. These new and highly polymorphic DNA markers will be used for upcoming association studies to test for significant additive and dominant gene effects on the embryonic survival and the number of piglets born alive

We used the radiation hybrid mapping (RH) method and the fluorescence in situ hybridization (FISH) method to assign the LIFR gene to SSC16q13→q14 and the EGFR gene to SSC9q26.

MATERIALS AND METHODS

*Isolation and subcloning of genomic clones for sequence analysis based identification of microsatellites*

For the isolation of porcine PAC clones containing the LIFR gene the porcine PAC library TAIGP714 (Al-Bayati et al., 1999) was initially screened with PCR primers LIFRpacA (5'-CAG AGA AGA GCA TGT TTG TC-3') and LIFRpacB (5'-GTC GAT GTA AAT GAC CTG TG-3'), which were derived from the porcine cDNA (Acc. U91518) sequence. One LIFR PAC clone designated TAIGP714F2198 was isolated. For the isolation of BAC clones containing the porcine EGFR gene, high-density clone filters of the porcine genomic BAC library RPCI-44, constructed in pTARBAC2 (Fahrenkrug et al., 2001), were screened with $^{32}$P labeled cDNA probes according to the RPCI protocols (http://www.chori.org/bacpac/). The cDNA clones required for this purpose were obtained from the Resource Center/Primary Database (http://www.rzpd.de/). One EGFR BAC clone designated RPCI44-91P11 was isolated. PAC and BAC DNAs were isolated using the Qiagen plasmid maxi kit (Qiagen,

Hilden, Germany). This DNA was restricted with different enzymes and separated on 0.8% agarose gels. The resulting fragments were cloned into the polylinker of pGEM-4Z (Promega, Mannheim, Germany). Recombinant plasmid DNA was sequenced with the ThermoSequenase kit (AmershamPharmacia, Freiburg, Germany) and a LICOR 4200 automated sequencer. Sequence data were analyzed with Sequencher 4.0.5 (GeneCodes, Ann Arbor, MI). A search for genetic markers in the determined DNA sequences of the two isolated clones resulted in the development of one microsatellite marker for each gene.

*Determination of microsatellite marker characteristics*

Genotyping for EGFR and LIFR was performed using the PCR primer pairs EGFR_MSc (5'-CTT GTT GTA AAG GGT GCC TG-3')/EGFR_MSd (5'-GGC GAA TGT TTT GTT CTC CT-3') and LIFR_MSa (5'-GAA ATC ATG AGG AGG GTA C-3')/LIFR_MSb (5'-GTT TTG ATA TAG GAG TGT GTG-3') respectively. The PCR amplification (15 µl final volume) for both primer pairs was performed using 20 ng of genomic porcine DNA, 1× PCR buffer (GL BioTech, Bremen, Germany), 0.5 µl enhancer (GL BioTech) 100 µM each dNTP, 4 pmol each primer, and 1 U *Taq* polymerase (GL BioTech). Conditions were 94°C 4 min, followed by 35 cycles of: 94°C, 30 s; 58°C, 60 s; 72°C, 30 s for both primer pairs. Marker characteristics (Tables 1 and 2) were determined by genotyping boars belonging to four different pig breeds (Duroc, German Landrace, German Large White, and a synthetic line from a German commercial pig breeding company).

*RH-Mapping*

The INRA-University of Minnesota porcine Radiation Hybrid panel (IMpRH; Yerle et al., 1998) was obtained from INRA, Laboratoire de Genetique Cellulaire, BP 27, 31326 Castanet-Tolosan, France. The 118 DNAs of the RH panel were subjected to PCR amplification. A 448-bp fragment of the porcine LIFR gene was amplified using the above-mentioned primers LIFRpacA and LIFRpacB. A 180-bp fragment of the porcine EGFR gene was amplified using the primers EGFRpacA (5'-CAA GGT ACA AGT AAC AAG C-3') and EGFRpacB (5'-ATG TAG GTG ATC TCC AAG-3'). Temperature and time profile were 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 56°C (LIFR) and 52°C (EGFR) respectively for 1 min, and 72°C for 30 s. The PCR products were analyzed by agarose gel electrophoresis and ethidium bromide staining. Positive signals were scored and the results were statistically analyzed using the IMpRH mapping tool (http://imprh.toulouse.inra.fr/).

*Fluorescence in situ hybridization*

The genomic clones containing the porcine genes for LIFR and EGFR, respectively, were labeled with digoxigenin by nick translation using a Nick-Translation-Mix (Boehringer Mannheim Corp., Mannheim, Germany). FISH on GTG-banded pig chromosomes was performed using 750 ng (50 ng/μl) of digoxigenin labeled PAC or BAC DNA respectively. 1 μg sheared total porcine DNA and 10 μg salmon sperm DNA were used as competitors in this experiment. After hybridization overnight, signal detection was performed using a Digoxigenin-FITC Detection Kit (Quantum Appligene, Heidelberg, Germany). The chromosomes were counterstained with DAPI and slides were mounted in propidium iodide/antifade. Metaphases that were previously photographed were reexamined after hybridization with a Zeiss Axioplan 2 microscope equipped for fluorescence.

RESULTS AND DISCUSSION

The porcine PAC clone TAIGP714F2198 containing the LIFR gene was isolated from a genomic library using a PCR based screening procedure and primers for the porcine LIFR gene. The porcine BAC clone RPCI44-91P11 containing the EGFR gene was isolated by hybridization of high-density clone filters of a genomic library with ^{32}P labeled cDNA probes of the porcine EGFR gene.

A search for microsatellites within the genomic clones resulted in the development of two microsatellite markers, one on each clone. Both microsatellites are $(CA)_n$ dinucleotide repeats. Marker characteristics were determined by genotyping boars of four different pig breeds (Tables 1 and 2) using the above mentioned PCR primer pairs EGFR_MSc/EGFR_MSd and LIFR_Msa/LIFR_MSb respectively.

Concerning the LIFR linked microsatellite the total number of different alleles observed over all four breeds was nine and their sizes ranged between 133 and 149 bp. The highest number of alleles (nine) was found in German Landrace (GL) and the lowest (five) in German Large White (GW). One reason for this finding might be the different sample sizes of genotyped boars, which were 89 in GL and 19 in GW. The rare alleles might also occur in a larger sample size of GW. Another reason for the different number of alleles between the breeds might be breed specific alleles. To a greater degree the latter seems also to be the reason for the allele distribution of the EGFR linked microsatellite in the different breeds. Here the highest number of alleles (nine) was found in the German synthetic line (CC) with 48

**Table 1:** Characterization of the newly developed *LIFR*-linked microsatellite marker

| breed | sample size | no. of alleles | allele size min-max (bp) | expected heterozygosity | PIC[1] |
|---|---|---|---|---|---|
| Duroc | 43 | 7 | 133-149 | 0.72 | 0.67 |
| German Landrace | 89 | 9 | 133-149 | 0.79 | 0.76 |
| German Large White | 19 | 5 | 133-149 | 0.58 | 0.55 |
| German synthetic line | 47 | 7 | 133-149 | 0.81 | 0.78 |

[1]Polymorphism Information Content

**Table 2:** Characterization of the newly developed *EGFR*-linked microsatellite marker

| breed | sample size | no. of alleles | allele size min-max (bp) | expected heterozygosity | PIC[1] |
|---|---|---|---|---|---|
| Duroc | 45 | 6 | 110-136 | 0.65 | 0.59 |
| German Landrace | 88 | 6 | 114-136 | 0.71 | 0.68 |
| German Large White | 20 | 5 | 122-136 | 0.59 | 0.51 |
| German synthetic line | 48 | 9 | 110-136 | 0.82 | 0.79 |

[1]Polymorphism Information Content

genotyped boars and the lowest (five) again in GW. Six alleles were identified in the GL boars. So here the number of alleles is not positively correlated with the number of genotyped animals as is the case dealing with the LIFR linked microsatellite (Table 1). A total of ten different alleles was observed over all four breeds concerning the EGFR associated marker and their sizes ranged between 110 and 136 bp. For both markers the values for expected heterozygosity and PIC (Polymorphism Information Content) are distributed similarly between the different breeds. The highest values were found in the CC boars followed by GL

and Duroc. The lowest values for expected heterozygosity and PIC occurred in GW. The higher degree of heterozygosity of the CC animals compared to GW and Duroc is not surprising, taking into account that this line has a multibreed origin. In conclusion, the two newly developed microsatellite markers were shown to be highly polymorphic and

**A)**



**B)**



**C)**
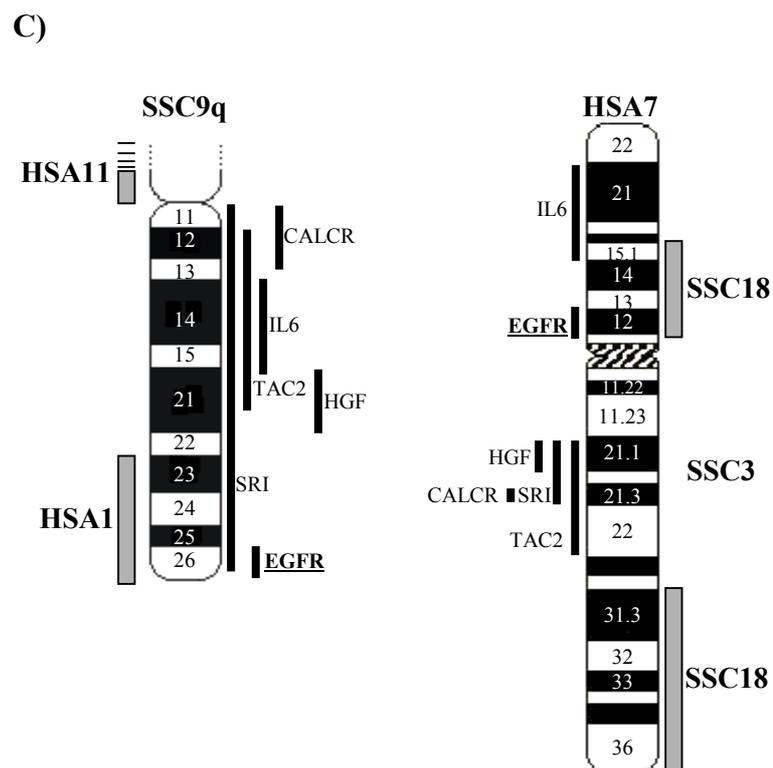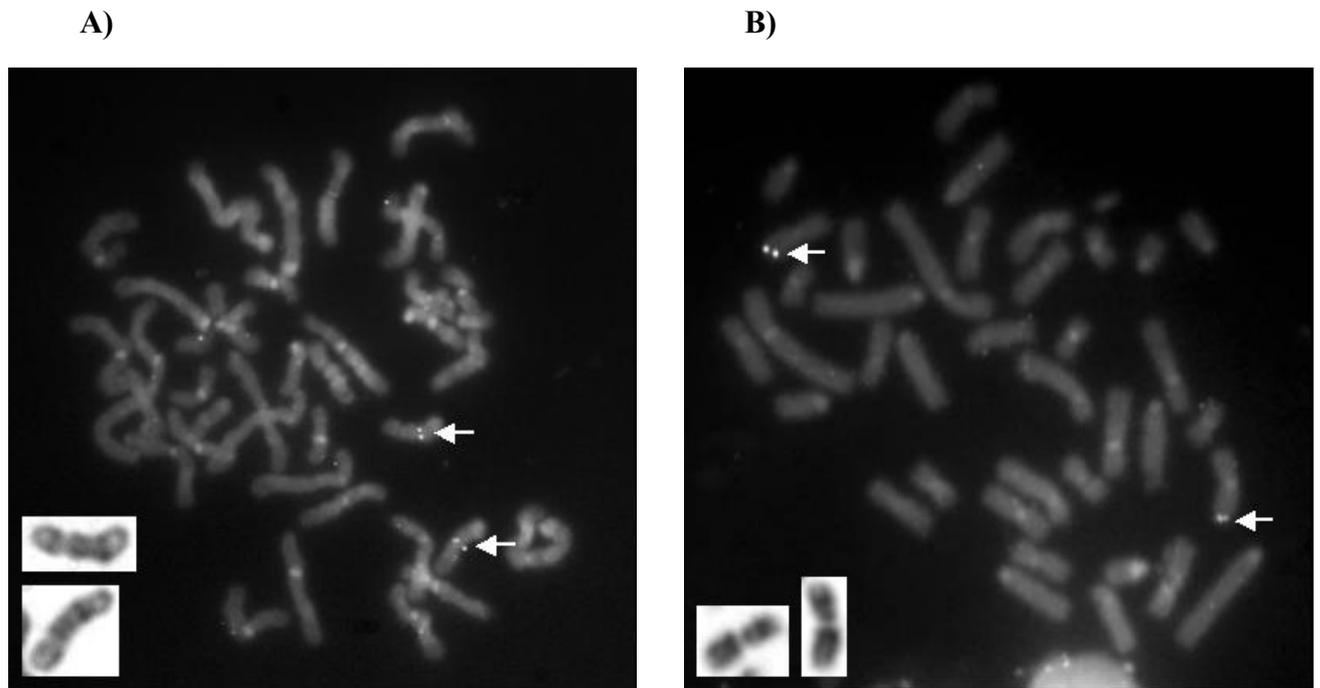
**Fig. 1.** Chromosomal assignment of the porcine genes for LIFR (**A**) and EGFR (**B**) by FISH analysis. The digoxigenin labeled genomic clones containing the porcine genes for LIFR and EGFR, respectively, were hybridized to GTG-banded metaphase chromosomes of a normal pig. The chromosomes were counterstained with propidium iodide and subsequently identified by DAPI staining. Double signals indicated by arrows are visible on both chromosomes 16q13→q14 (**A**) and 9q26 (**B**). The respective GTG-banded metaphase chromosomes are displayed in the left bottom corners of (**A**) and (**B**).

(**C**) Syntenic relationship between SSC9q and HSA7. Mapped genes are displayed as black bars. Syntenic relationship to other chromosomes is indicated by grey bars with exception of SSC3 because in this case chromosomal correspondences have not been confirmed by chromosomal painting. This indication system is in accordance with the conventions of the comparative map of pig and human (http://www.toulouse. inra.fr/lgc/pig/compare/compare .htm).

heterozygous in a sample of four different pig breeds. They have been physically anchored by FISH and radiation hybrid mapping and should prove useful for future QTL fine mapping studies. The two gene associated microsatellites will also be used for upcoming association studies to test for significant additive and dominant gene effects on the embryonic survival and number of piglets born alive.

The chromosome locations of the porcine genes for LIFR and EGFR were determined by FISH of the PAC clone TAIGP714F2198 and the BAC clone RPCI44-91P11, respectively, to metaphase chromosomes (Fig. 1).

*Mapping data: LIFR*

*Location:* 16q13→q14

*Number of cells examined:* 30

*Number of cells with specific signals:* 0 (3), 1 (4), 2 (8), 3 (4), 4 (11) chromatids per cell

*Most precise assignment:* 16q13→q14

*Location of background signals (site with >2 signals):* none observed

*Mapping data: EGFR*

*Location:* 9q26

*Number of cells examined:* 30

*Number of cells with specific signals:* 0 (1), 1 (0), 2 (4), 3 (9), 4 (16) chromatids per cell

*Most precise assignment:* 9q26

*Location of background signals (site with >2 signals):* none observed


In order to confirm these localizations, a porcine radiation hybrid panel was analyzed. The PCR primers derived from the PAC clone containing the LIFR gene, showed a retention frequency of 36%. Two-point analysis revealed close linkage of LIFR to the microsatellite markers SW1645 (Rohrer et al., 1996) at a distance of 0.9 cR (LOD score 23.68) and SW403 (Rohrer et al., 1994) at a distance of 2.2 cR (LOD score 15.95). On the porcine linkage map the next physically assigned marker to SW1645 and SW403 is, with a distance of 5.6 cM, the microsatellite S0298 at SSC16q14 (Hoyheim et al., 1996). Therefore these findings are in agreement with the FISH result.

The PCR primer pair derived from the BAC clone RPCI44-91P11 containing the EGFR gene, showed a retention frequency of 30%. Two-point analysis revealed close linkage of the EGFR gene to the microsatellites SW749 (Rohrer et al., 1996) at a distance of 6.7 cR (LOD score 5.47) and SW1651 (Rohrer et al., 1996) at a distance of 8.2 cR (LOD score 4.00). SW1651 has been physically assigned to SSC9q26 (Lopez-Corrales et al., 1999), which is in perfect accordance with the FISH result.

In this report we mapped a porcine PAC clone containing the LIFR gene to 16q13→q14. The human LIFR gene has been assigned to HSA5p13→p12. This is consistent with the established comparative map of pig and human (Goureau et al., 1996; http://www.toulouse. inra.fr/lgc/pig/compare/compare.htm) indicating significant conserved synteny between SSC16 and HSA5. On both the human and the porcine chromosome LIFR was localized in the same chromosomal region as GHR (growth hormone receptor), NPR3 (natriuretic peptide receptor C/guanylate cyclase C), C9 (complement component 9) and PRLR (prolactin receptor), which points to a largely conserved gene order in these genome regions.

We assigned a porcine BAC clone containing the EGFR gene to SSC9q26. In the current comparative map of pig and human, SSC9 shares conserved synteny with HSA1, HSA7 and HSA11. The human EGFR has been mapped to HSA7p12. Figure 1C illustrates the

relationship between SSC9q and HSA7. The current status of the comparative map is that the homology HSA7p12 region shares homology with SSC3 and SSC18 but not with SSC9. Hence our mapping result is not in agreement with the established homology between HSA7 and SSC9. However, it must be stressed that until more orthologous genes have been mapped precisely within these large homologous regions there is still the possibility of gene order rearrangements and refinement of evolutionary chromosomal breakpoints. Thus, our mapping results show the need of refinement of the established homology between SSC3, SSC9, SSC18 and HSA7 which seems to be more complex than previously thought.

## ACKNOWLEDGEMENTS

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# **Chapter VII**

# **Development of new genetic markers and their association with litter size in pigs**

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# Development of new genetic markers and their association with litter size in pigs

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SUMMARY

Five new microsatellite markers at candidate gene loci for litter size have been identified based on partial sequence analysis of porcine PAC and BAC clones containing the respective candidate genes encoding for epidermal growth factor (EGF), inter-$\alpha$ trypsin inhibitor heavy chain 4 (ITIH4), cathepsin L (CTSL), leukemia inhibitory factor receptor (LIFR), and epidermal growth factor receptor (EGFR). These gene associated markers were characterized and evaluated for their association with the number of piglets born alive (NBA) in a German synthetic pig line. Genotyping was performed on 273 sows. Information on 955 litter records from these 273 sows was used in the analyses with respect to litter size. In the genotyped sample the developed microsatellite markers were shown to be highly polymorphic. However, estimation of allele substitution effects revealed no significant associations between any of the examined markers and the studied phenotypes regarding litter size.

Key Words: DNA Markers, Litter Size, Pigs

INTRODUCTION

The use of marker assisted selection (MAS) in combination with traditional selection methods can generate a substantial increase in genetic response. This selection strategy is particularly suited for traits that are age-limited (measurable only in animals of a certain age), sex-limited (measurable only in one sex), and/or of low heritability, such as litter size (Soller, 1994) where moderate increases can equal large gains in profit. In comparison to phenotypic information the use of genetic markers has two advantages. The first is that their early availability contributes to a shortening of the generation interval and the second is the possibility to enhance the accuracy of selection and thus the selection response of a trait by direct selection of gene variants in both sexes affecting its expression positively.

The candidate gene approach was proposed as procedure to identify genes with significant phenotypic performance effects and possible use in genetic improvement programs. Candidate genes are considered for investigation based on physiological, immunological or endocrine evidence. Polymorphisms within such a gene or a closely linked genetic marker are used for genotyping performance-tested resource populations to estimate their effects. The decision to incorporate genetic markers into MAS schemes requires reliable information about additive and dominance effects of each marker in the population of interest (Soller, 1994). There is evidence that the genes for EGF, ITIH4, CTSL, LIFR, and EGFR are involved in early conceptus development and/or conceptus-uterine interactions for the establishment of

pregnancy in pigs and thus affecting litter size. In the following the reasons for their choice as candidate genes in the current study are briefly discussed.

*EGF*, encoding for a polypeptide with many different biological properties, and *EGFR*, encoding for its membrane bound receptor, were investigated as potential candidate genes for litter size for several reasons. A quantitative trait locus (QTL) for uterine capacity was identified on the long arm of the porcine chromosome 8 near 71 cM (Rohrer et al., 1999). This region is near the known location of the *EGF* gene on SSC8q2.3-q2.4 (Spötter et al., 2001a). Accordingly, indications for a significant role of the EGF/EGFR system in embryonic and maternal interactions (and thus litter size) are the chromosomal location of EGF, and furthermore, its specific biochemical actions including cell proliferation (Haining et al., 1991) and initiation of DNA synthesis (Tomooka et al., 1986), its ability to improve the embryonic development *in vitro* (Wood and Kaye, 1989; Paria and Dey, 1990), and its increased luminal content on day 12 of pregnancy followed by a decline to day 16 (Diehl et al., 1994), which is the time around embryo implantation. These indications are further supported by the finding that endometrial and conceptus tissues express *EGFR* (Kennedy et al., 1994).

The plasma glycoprotein ITIH4 is associated with the time of conceptus attachment to the uterine surface and conceptus survival in pigs (Geisert et al., 1995, 1998). Geisert et al. (1997) proposed a model in which ITIH4 is no longer described as a part of the plasma but present on the uterine epithelial surface. Alteration in ITIH4 may not be the only factor involved with trophoblast attachment; however, cleavage of ITIH4 could induce local alterations in receptivity to the conceptus that permits the conceptus to contact integrins for firm attachment to the uterine epithelium (Bowen et al., 1997). Thus *ITIH4* is a potential candidate gene for litter size. In addition to the possible role in trophoblast attachment, the multipolypeptide chain of the pig ITIH4 could also serve to stabilize the epithelial glycocalyx of the uterus and protect it from free radical damage (Geisert and Yelich, 1997). *ITIH4* was assigned to SSC13q2.1-q2.2 (Kuiper et al., 2001).

Cathepsin L (CTSL) is a lysosomal cysteine protease with potent elastase (Mason et al., 1982) and collagenase (Kirschke et al., 1982) activities. Its high activity in the uterine lumen during the period of placental attachment has led to speculation that CTSL may play an important role during embryonic implantation in the pig (Geisert et al., 1997). Cathepsins have also been implicated in blastocyst implantation in other species like rat (Starcher and Percival, 1985) and man (Aflin et al., 1988). In the pig, endometrial cathepsin L may possibly effect the alterations in uterine and placental development that are necessary for placental attachment and growth of the embryo (Geisert and Yelich, 1997). These events involve an increase in

elastase activity and collagen remodelling in which cathepsin L could play a role and thus influence the number of piglets born alive. *CTSL* was mapped to SSC10q1.1-q1.2 (Spötter et al., 2001b).

The gene for LIFR, a receptor subunit that binds to LIF, a pleiotropic cytokine, was chosen as a candidate gene for litter size due to the essential role of the LIF/LIFR system in blastocyst growth and implantation in mice (Stewart et al., 1992; Stewart, 1994; Savatier et al., 1996). This implies that the LIF/LIFR system may also serve a vital function in conceptus development and implantation - and thus litter size - in pigs (Geisert and Yelich, 1997). This implication is supported by the detection of *LIF* gene expression in porcine endometrium at the time of blastocyst attachment (Anegon et al., 1994; Modric et al., 2000), and the presence of *LIFR* mRNA in porcine peri-implantation conceptuses (Yelich et al., 1997; Modric et al., 2000). The *LIFR* gene was assigned to SSC16q1.3-q1.4 (Spötter et al., 2003).

The objectives of the current study were to develop new highly polymorphic DNA markers for *EGF, ITIH4, CTSL, LIFR*, and *EGFR,* to characterize these markers, and to examine their effects on litter size in a sample of 273 sows of a German synthetic pig line.

## MATERIALS AND METHODS

*Animals and Methods*

All animals were reared on a single farm and were subjected to the same fertility management, e.g., estrous control, insemination regime. The employed population consisted of 273 sows belonging to a German synthetic line (CC) of Duroc and Large White origin. The number of piglets born alive (NBA) was recorded in 955 litters of sows farrowing up to 10 times. In table 1, an overview is given on the number of animals genotyped, the available phenotypic records and the means for litter size.

Table 1. Available phenotypic records, NBA means and ranges in a synthetic German pig line

| parity | sows | records | NBA | | |
| --- | --- | --- | --- | --- | --- |
| | | | means | SD | min-max |
| 1st | 273 | 273 | 9.80 | ±2.22 | 3-15 |
| 2nd-10th | 219 | 682 | 10.73 | ±2.56 | 3-20 |

The Dneasy 96 Tissue Kit (QIAGEN, Hilden, Germany) was used to extract DNA from frozen ear tissue.

The isolation and physical mapping of porcine PAC clones containing the *ITIH4*, *EGF*, *CTSL,* and *LIFR* genes respectively, and a porcine BAC clone containing the *EGFR* gene, was described by Kuiper et al. (2001) and Spötter et al. (2001a, 2001b, 2003). The isolated PAC and BAC DNAs were restricted with different enzymes and separated on 0.8% agarose gels. The resulting fragments were cloned into the polylinker of pGEM-4Z (Promega, Mannheim, Germany). Recombinant plasmid DNA was sequenced with the ThermoSequenase kit (AmershamPharmacia, Freiburg, Germany) and a LI-COR 4200 automated sequencer. Sequence data were analyzed using Sequencher 4.0.5 software (GeneCodes, Ann Arbor, MI). A search for genetic markers in the determined DNA sequences of the respective clones TAIGP714I1851Q5 (*EGF*), TAIGP714I126 (*ITIH4*), TAIGP714I1639 (*CTSL*), and TAIGP714F2198 (*LIFR*) all of which were derived from a porcine PAC library (Al-Bayati et al., 1999), and the porcine BAC clone RPCI44-91P11 (*EGFR*) isolated from a porcine BAC library (Fahrenkrug et al., 2001) resulted in the identification of one microsatellite marker for each gene. Flanking PCR primers were derived from the DNA fragments containing the microsatellites. For EMBL nucleotide database accessions of these DNA fragments, primer sequences, their respective annealing temperatures (AT) and repeat type of each microsatellite see table 2. Using this newly developed genetic markers the mentioned sows from a synthetic line were genotyped at the *EGF-, ITIH4-, CTSL-, LIFR-,* and *EGFR* locus. The PCR amplification (15 µl final volume) was performed using 20 ng of genomic porcine DNA, 1x PCR buffer (Promega, Mannheim, Germany), 100 µM each dNTP, 5-15 pmol each primer, and 1 U *Taq* polymerase (Promega, Mannheim, Germany). Temperature and time profile were 35 cycles of 94°C for 30 s, AT for 1 min, and 72°C for 30 s. The forward primers were 5' IRD700 labelled to enable fluorescent PCR fragment detection on a LI-COR 4200 automated sequencer. Raw data were genotyped using Gene Profiler 3.55 software (Scanalytics, Inc., Fairfax, USA).

*Statistical Analysis*

An animal model with the additive genetic relationship matrix, including pedigree information on 488 animals up to 15 generations of the CC line, was employed for the association analyses between genotypes of marker loci and phenotypic traits. The approach was to analyze the effects of individual alleles and their allelic substitution effects by linear regression. Using this approach, the number of copies of each microsatellite allele (0, 1, or 2)

Table 2. Microsatellite primer sequences, annealing temperatures (AT), repeat types and EMBL accessions

| primer name | sequence (5'-3') | AT (°C) | Repeat type | EMBL accessions |
|---|---|---|---|---|
| EGF_MS_f | ATGGGCTGTGTATGTATG | 52 | $(CA)_n$ | AJ547861 |
| EGF_MS_r | GGGATGAGTGAAAGTGTG | | | |
| ITIH4_MSf | CTCACACACCCATAATCCC | 58 | $(CA)_n$ | AJ547862 |
| ITIH4_MSr | GCCAGCCTCTTCCTTCTC | | | |
| CTSL_MS_f | GAGCTCTTGGCCTAGAAAAGG | 60 | $(CA)_n$ | AJ547863 |
| CTSL_MS_r | TGCTAGGACAGTGGTTTCTTG | | | |
| LIFR_MSf | GAAATCATGAGGAGGGTAC | 58 | $(CA)_n$ | AJ547859 |
| LIFR_MSr | GTTTTGATATAGGAGTGTGTG | | | |
| EGFR_MSf | CTTGTTGTAAAGGGTGCCTG | 58 | $(CA)_n$ | AJ547860 |
| EGFR_MSr | GGCGAATGTTTTGTTCTCCT | | | |

were fit as linear covariates for each animal. Data were analyzed separately for the three most frequent alleles of each marker. A separate analysis was performed for the records of the first parities and for the records of $2^{nd}$ to $10^{th}$ parities of the sows. The litter size trait, number of piglets born alive (NBA), was analyzed using PEST (Groeneveld, 1990) employing following linear animal models:

First parity records: $NBA_{ijkm} = \mu + YS_i + b*n\_alleles_j + a_m + e_{ijk}$

Records from all parities: $NBA_{ijklmn} = \mu + YS_i + b*n\_alleles_j + PN_k + pe_l + a_m + e_{ijklmn}$

Year-season-classes (YS) for farrowing were treated as fixed effect. Random effects included the additive genetic (a, m=1-488) effect of the sow and a residual effect. The number of alleles (b*n_alleles) for each of the analyzed gene associated microsatellite markers was included as a linear covariate. For the analyses of available records from parities 2-10 of a sow, the model was extended to include parity number (PN) as a fixed effect and the random permanent environmental effect of the sows (pe, l=1-273).

RESULTS AND DISCUSSION

In the current study we report the identification of five new microsatellite markers associated with candidate genes for litter size in pigs. To our knowledge this is the first study that investigates an association between the trait litter size and the genes *ITIH4, CTSL, LIFR*, and *EGFR*. However, that does not apply for *EGF*. Linville et al. (2001) detected no effect of an *EGF* linked diallelic marker, first described by Mendez et al. (1999), on ovulation rate and number of fully formed, live (NBA), stillborn, and mummified piglets.

A characterization of the five new microsatellite markers genotyped in this study is displayed in table 3. The number of alleles per marker ranges from 4 (*ITIH4*) to 9 (*EGFR*) and the values for expected heterozygosity are within a scope of 0.46 (*ITIH4*) and 0.80 (*EGFR*). The PIC is strongly correlated with the degree of heterozygosity and ranges from 0.43 (*ITIH4*) to 0.77 (*EGFR*). The microsatellite markers were shown to be highly polymorphic and heterozygous in the tested population. They have been physically anchored by FISH and radiation hybrid mapping (Kuiper et al., 2001; Spötter et al., 2001a, 2001b, 2003) and should prove useful for future QTL fine mapping studies.

Table 3. Allele frequencies, heterozygosity and PIC of the new microsatellite markers

| Allele number | EGF | | ITIH4 | | CTSL | | LIFR | | EGFR | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Allele size (bp) | Allele frequency (%) | Allele size (bp) | Allele frequency (%) | Allele size (bp) | Allele frequency (%) | Allele size (bp) | Allele frequency (%) | Allele size (bp) | Allele frequency (%) |
| 1 | 141 | 4.8 | 148 | 71.0 | 184 | 15.1 | 133 | 21.6 | 110 | 2.2 |
| 2 | 143 | 19.4 | 154 | 14.7 | 188 | 8.3 | 139 | 8.3 | 112 | 2.6 |
| 3 | 149 | 26.4 | 156 | 5.7 | 194 | 0.6 | 141 | 0.9 | 114 | 23.8 |
| 4 | 151 | 4.9 | 162 | 8.6 | 196 | 10.7 | 143 | 23.8 | 116 | 1.3 |
| 5 | 153 | 15.2 | | | 202 | 22.1 | 145 | 27.9 | 122 | 26.9 |
| 6 | 155 | 29.3 | | | 204 | 7.8 | 147 | 4.4 | 124 | 6.2 |
| 7 | | | | | 208 | 35.4 | 149 | 13.1 | 126 | 8.4 |
| 8 | | | | | | | | | 128 | 24.2 |
| 9 | | | | | | | | | 136 | 4.4 |
| Expected heterozygosity | 0.78 | | 0.46 | | 0.78 | | 0.79 | | 0.80 | |
| PIC[1] | 0.75 | | 0.43 | | 0.75 | | 0.76 | | 0.77 | |

[1]Polymorphism Information Content

The examination of possible marker effects on litter size in this study was carried out by genotyping a sample of 273 sows with 955 litter records from the CC line and estimating allelic substitution effects. Allele frequencies ranged from less than 1% to more than 70% (see table 3). For each marker the three most frequent alleles were analyzed for association with NBA. For the *ITIH4* linked marker just the two most frequent alleles were analyzed because of the low frequency of the other alleles. Substitution effects from the linear regression model were small and did not exceed the range of –0.29 to 0.43 (table 4). However, no significant impact on NBA of any of the markers was observed.

It is important to stress that the lack of association between a gene associated polymorphism and a phenotype does not mean implicitly that the gene product is not important in regulating the trait. Associations between a marker and a trait may vary between populations, breeds, or lines. This was shown in several studies with DNA markers of candidate genes for litter size in pigs.

The effect of the *B* allele of a diallelic marker at the estrogen receptor *(ESR)* locus differed from 0.6 to 2 piglets more per litter (Short et al., 1997). Another study showed no significant effect of the *ESR* genotype on litter size in 59 sows from a hyperprolific Large White line and a control Large White line (Legault et al., 1996). This result was confirmed by Gibson et al. (2002), who found no association of the ESR PvuII mutation with sow productivity in a Meishan x Large White F2 population.

The *B* allele of a diallelic marker at the prolactin receptor *(PRLR)* locus indicated an additive effect on NBA across all parities in a Duroc population (Drögemüller et al., 2001). Vincent et al. (1998) have shown the *A* allele of this marker to be significantly associated with increased litter size in three of five commercial lines involving Meishan, Large White, Landrace and Duroc.

Different linkage phases between the investigated markers and a causal mutation due to recombination may explain the observed differences between the lines. Also, still unknown QTLs with effects on litter size could be linked to these gene associated markers.

However, the above-mentioned studies refer to diallelic DNA markers which are more powerful in statistical applications than multiallelic microsatellites because effects associated with microsatellite alleles with a lower frequency are difficult to test. Consequently, these difficulties are increasing with decreasing sample size. This was the reason for analyzing only the most frequent alleles in the current study.

The only reported implementation of a multiallelic microsatellite marker in an association study concerning litter size in pigs refers to the Osteopontin *(OPN)* gene. A highly

Table 4. Estimated allele substitution effects for NBA in first and later parities in a synthetic German pig line

| locus | parity | allele length (bp) | allele substitution effect | SE | p |
|---|---|---|---|---|---|
| *EGF*_MS | 1. | 143 | 0.21 | 0.28 | 0.46 |
| | | 149 | -0.28 | 0.25 | 0.28 |
| | | 155 | 0.16 | 0.24 | 0.50 |
| | 2.-10. | 143 | 0.20 | 0.19 | 0.30 |
| | | 149 | 0.07 | 0.19 | 0.73 |
| | | 155 | -0.14 | 0.18 | 0.43 |
| *ITIH4*_MS | 1. | 148 | 0.003 | 0.25 | 0.99 |
| | | 154 | 0.43 | 0.33 | 0.19 |
| | 2.-10. | 148 | 0.05 | 0.18 | 0.78 |
| | | 154 | -0.07 | 0.25 | 0.78 |
| *CTSL*_MS | 1. | 184 | 0.32 | 0.32 | 0.31 |
| | | 202 | -0.29 | 0.28 | 0.30 |
| | | 208 | -0.09 | 0.25 | 0.70 |
| | 2.-10. | 184 | 0.26 | 0.21 | 0.23 |
| | | 202 | -0.14 | 0.20 | 0.48 |
| | | 208 | -0.01 | 0.18 | 0.94 |
| *LIFR*_MS | 1. | 133 | 0.14 | 0.23 | 0.56 |
| | | 143 | -0.28 | 0.23 | 0.22 |
| | | 145 | 0.21 | 0.21 | 0.31 |
| | 2.-10. | 133 | -0.02 | 0.16 | 0.91 |
| | | 143 | 0.01 | 0.16 | 0.97 |
| | | 145 | -0.03 | 0.15 | 0.85 |
| *EGFR*_MS | 1. | 114 | -0.12 | 0.26 | 0.64 |
| | | 122 | -0.11 | 0.27 | 0.70 |
| | | 128 | -0.01 | 0.27 | 0.99 |
| | 2.-10. | 114 | 0.01 | 0.18 | 0.96 |
| | | 122 | 0.23 | 0.18 | 0.20 |
| | | 128 | -0.15 | 0.20 | 0.45 |

polymorphic microsatellite marker linked with that gene showed significant effects of some of its 13 alleles on litter size in a commercial Large-White lines (Southwood et al. 1998) and in a study of Hamann et al. (2000) employing 1578 German Landrace sows, 212 Duroc sows, as well as 268 sows of a synthetic line. Steinheuer et al. (2002) reported a negative allelic

substitution effect of an osteopontin marker allele on NBA for German Landrace boars (-0.177±0.111; p<0.01).

All of the studies discussed above demonstrate the difficulties in confirming previously published candidate gene effects in different genetic groups and show the necessity for further investigation of different pig breeds and larger sample sizes to evaluate the usefulness of the five newly developed microsatellite markers for MAS based improvement of litter size.

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# **Chapter VIII**

**Evidence of a new *LIF* associated genetic Marker for Litter Size in a**

**synthetic Pig Line**

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# Evidence of a new *LIF* associated genetic Marker for Litter Size in a synthetic Pig Line[1]

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*Abstract*

The leukemia inhibitory factor (*LIF*) gene is a candidate gene for litter size in pigs. A diallelic RFLP marker based on a SNP, detected in the third exon of the porcine *LIF* was evaluated for its association with the number of piglets born alive in a German synthetic pig line. Information on 955 litter records from 272 genotyped sows was used in the analyses with respect to litter size. Additionally, the growth and carcass traits average daily weight gain and back fat thickness were tested for associations with the *LIF* marker in this population. At the *LIF* locus the allele frequencies were 0.27 for the *A* allele and 0.73 for the *B* allele. There was no indication of an additive effect on the number of piglets born alive but a small negative dominance effect was observed, which amounted to $-0.72 \pm 0.37$ (p=0.047) for the 1$^{st}$ parity and $-0.50 \pm 0.29$ (p=0.087) for the 2$^{nd}$ to 10$^{th}$ parity. No associations were detected between the marker alleles and the growth and carcass traits.

Key Words: DNA Markers, Litter Size, Pigs, *LIF*

INTRODUCTION

Marker assisted selection (**MAS**) in conjunction with traditional selection methods is most effective for traits that are either expressed later in lifetime, that are sex dependent, or of low heritability, such as litter size where moderate increases can equal large gains in profit (Soller, 1994). Genetic markers allow identification of both males and females carrying beneficial alleles early in life, thereby improving accuracy, reducing the generation interval, and accelerating the genetic improvement of the trait.

The candidate gene approach identifies genes likely to cause variation in a trait based on physiological, immunological or endocrine evidence. Polymorphism within that gene or a closely linked genetic marker is typed in a performance-tested resource population to determine its effect. The decision to incorporate genetic markers into MAS schemes requires reliable information about additive and dominance effects of each marker in the population of interest (Rothschild, 1998). A successful application of the candidate gene approach in pig production was demonstrated by Rothschild et al. (1996). They have reported a specific allele of the estrogen receptor (ESR) locus to be associated with increased litter size in a divergent breed cross involving the Chinese Meishan pig. There is evidence that the *LIF* gene is involved in early conceptus development and conceptus-uterine interactions for the establishment of pregnancy in pigs and thus in the determination of litter size (Yelich et al., 1997; Geisert and Yelich, 1997).

The objective of the current study was to examine the effect of a porcine RFLP marker in the *LIF* gene on litter size in a sample of 272 sows of a German synthetic pig line. In order to identify possible pleiotropic effects of the employed SNP marker (Spötter et al., 2001) on growth and carcass traits, average daily weight gain and backfat thickness were as well analyzed for an association with the RFLP marker in this population.

MATERIALS AND METHODS

*Animals and Methods*

All animals were reared on a single farm and were subjected to the same fertility management, e.g., estrous control, insemination regime. The employed population consisted of 272 sows belonging to a German synthetic line (**CC**) of Duroc and Large White origin. Number of piglets born alive (**NBA**) was recorded in 955 litters of sows farrowing up to 10 times. Back fat thickness (**BF**) was scored by ultrasonic measurement at d 168, and average daily gain (**DG**) was determined by dividing weight at day 168 through age in days. In Table 1, an overview is given on the number of animals genotyped, the available phenotypic records and the means for NBA. Table 2 displays the mean values of BF and DG in the tested population.

Table 1. Available phenotypic records, NBA means and ranges in a synthetic German pig line

| parity | sows | records | NBA means | SD | min-max |
|--------|------|---------|-----------|------|---------|
| 1st | 272 | 272 | 9.80 | 2.22 | 3-15 |
| 2nd-10th | 219 | 682 | 10.73 | 2.56 | 3-20 |

Table 2. Means and ranges for back fat thickness (BF) and average daily gain (DG) in a synthetic German pig line

| Trait | N | Mean | SD | min-max |
|-------|-----|-------------|-------|---------|
| BF | 272 | 15.42 (mm) | 2.20 | 10-24 |
| DG | 272 | 682 (g/day) | 33.65 | 526-747 |

The Dneasy 96 Tissue Kit (QIAGEN, Hilden, Germany) was used to extract DNA from frozen ear tissue.

For genotyping the *LIF Dra*III polymorphism – a C/T transition at position 6988 of the porcine *LIF* gene sequence (EMBL nucleotide database accession AJ296176; Spötter et al., 2001) 24 bp downstream of the stop codon in exon 3 - the forward primer LIF3SNPa: 5´-ATG TGG ATG TGG CCT ACG G-3´ and the reverse primer LIF3SNPb: 5´-GGG AAC AAG GTG GTG ATG G-3´ (Spötter et al., 2001) were used to amplify a 407 bp fragment. The PCR amplification (20 µl final volume) was performed using 20 ng of genomic porcine DNA, 1x PCR buffer (Promega, Mannheim, Germany), 100 µM each dNTP, 4 pmol each primer, and 1 U *Taq* polymerase (Promega). Conditions were 94°C 4 min, followed by 35 cycles of: 94°C, 30 s; 58°C, 60 s; 72°C, 30 s. Three microliters of the PCR product were digested with 3 U *Dra*III (N.E.B., Frankfurt/Main, Germany) and separated on a 1% agarose gel. A 407 bp fragment was observed for the *AA* genotype and a 266 bp and a 144 bp fragment for the *BB* genotype. The sum of the two cleaving products is greater than the original 407 bp fragment because of the 5' overhangs generated by *Dra*III digestion.

*Statistical Analysis*

Allele and genotype frequencies were calculated. Hardy-Weinberg equilibrium in the studied population was tested by comparing expected and observed genotype frequencies using a chi-square test.

An animal model with the additive genetic relationship matrix for the sows, including pedigree information on 488 animals up to 15 generations of the CC line, was employed for the association analysis between genotypes of the RFLP marker and the different phenotypic traits. Additionally, information on the mates of the 272 genotyped sows (88 boars) were considered. An analysis was performed for the records of the first parities and for the records of $2^{nd}$ to $10^{th}$ parities of the sows simultaneously. The litter size trait NBA, was analyzed using PEST (Groeneveld, 1990) using following linear animal model:

First parity records:         $NBA_{ijmn}= \mu + YS_i + GT_j + peb_m + a_n + e_{ijmn}$

Records from all parities:     $NBA_{ijklmno}= \mu + YS_i + GT_j + PN_k + pes_l + peb_m + a_n + e_{ijklmno}$

Year-season-classes (**YS**) for farrowing and marker genotypes (**GT**) were treated as fixed effects. Random effects included the additive genetic (**a**, n=1-488) effect of the sow, a random permanent environmental effect of the boar (**peb**, m=1-88), and a random residual effect (**e**). For the analyses of all available records from different parities of the sows, the model was extended to include parity number (**PN**) as fixed effect and the random permanent

environmental effect of the sow (**pes**, l=1-272). For the analysis of BF and DG the following linear animal model was employed:

$BF_{ijkl} (DG_{ijkl}) = \mu + YB_i + GT_j + G_k + a_l + e_{ijkl}$

Year of birth (**YB**), gender (**G**), and the marker genotypes (GT) were regarded as fixed effects and the additive genetic effect (a) of the sows as random.

Additive genetic effects were estimated by pair-wise comparison of the least square means of the two homozygous genotypes, while the dominance effects were calculated as the deviation of the least square means of the heterozygotes from the average of the two homozygous genotypes. The estimated effects were tested for significance by using the *t*-test.


RESULTS

The C/T transition polymorphism identified in the 3rd exon of the *LIF* gene could easily be genotyped by *DraIII* RFLP (Spötter et al., 2001). PCR on genomic porcine DNA with the above mentioned primers LIF3SNPa and LIF3SNPb generated a 407 bp fragment. After a *Dra*III digest uncleaved fragments were designated allele *A* and cleaved fragments allele *B*. The frequency of the *LIF* allele *A* was 0.27 and that of the allele *B* 0.73. All three genotypes *AA*, *AB*, and *BB* occurred in the genotyped population which was found to be in Hardy-Weinberg equilibrium for the genotyped locus ($\chi^2$=0.303, P=0.860).

Additive and dominance effects of the genotypes are shown in Table 3. A significant negative dominance effect of –0.72 ± 0.37 (P=0.047) was detected in the first parity. For 2nd to 10th parity a negative dominance effect of –0.50 ± 0.29 (P=0.086) was estimated. There was no additive effect of *LIF* on litter size in these data. However, there is a trend for animals carrying the *A* allele to have increased number of piglets born alive across all parities (Table 3). Table 4 displays the results of the statistical evaluation for BF and DG. This analysis was accomplished in order to identify possible pleiotropic effects of the employed marker. No significant effects on BF and DG were estimated in this population, even if the heterozygotes tended to lower BF and DG values than both of the homozygotes.

Table 3. Effect of the *LIF* genotypes on NBA in a synthetic German pig line

| | 1st Parity | | | | 2nd to 10th Parity | | | |
|---|---|---|---|---|---|---|---|---|
| | N | NBA | SE | P | N | NBA | SE | P |
| *LIF* Genotype | | | | | | | | |
| AA | 18 | 10.24 | 0.79 | - | 14 | 11.45 | 0.58 | - |
| AB | 111 | 9.36 | 0.53 | - | 88 | 10.70 | 0.40 | - |
| BB | 144 | 9.88 | 0.49 | - | 117 | 10.82 | 0.40 | - |
| Effect | | | | | | | | |
| Additive | | 0.18 | 0.30 | 0.545 | | 0.34 | 0.25 | 0.169 |
| Dominance | | -0.72 | 0.37 | 0.047 | | -0.50 | 0.29 | 0.086 |

Table 4. Effect of the *LIF* genotypes on BF and DG in a synthetic German pig line

| | BF | | | | DG | | | |
|---|---|---|---|---|---|---|---|---|
| | N | Mean (mm) | SE | P | N | Mean (g/day) | SE | P |
| *LIF* Genotype | | | | | | | | |
| AA | 18 | 16.18 | 0.65 | - | 18 | 630.54 | 11.10 | - |
| AB | 111 | 15.52 | 0.43 | - | 111 | 624.30 | 6.88 | - |
| BB | 144 | 15.82 | 0.42 | - | 144 | 630.41 | 6.65 | - |
| Effect | | | | | | | | |
| Additive | | 0.18 | 0.30 | 0.548 | | 0.06 | 5.08 | 0.990 |
| Dominance | | -0.48 | 0.34 | 0.154 | | -6.18 | 6.01 | 0.304 |

DISCUSSION

Development of porcine genome maps offers the opportunity to identify individual genes controlling reproduction. Applications of MAS will increase as more associations between markers and traits are identified (Rothschild, 1998). This technology seems to be especially promising for fertility traits like litter size, due to the low heritability and the existence of appropriate genetic markers. To our knowledge this is the first study to investigate an association between the trait litter size and the porcine *LIF* gene. *LIF*, a pleiotropic cytokine, was chosen as a candidate gene for litter size due to its essential role in blastocyst growth and

implantation in mice (Stewart et al., 1992; Stewart, 1994; Savatier et al., 1996). The role of this gene in early conceptus development in mice implies that *LIF* may also serve a vital function in conceptus development and implantation - and thus litter size - in pigs (Geisert and Yelich, 1997). This implication is supported by the detection of *LIF* gene expression in porcine endometrium at the time of blastocyst attachment (Anegon et al., 1994; Modric et al., 2000), and the presence of leukemia inhibitory factor receptor *(LIFR)* mRNA in porcine peri-implantation conceptuses (Yelich et al., 1997; Modric et al., 2000). LIFR is a specific LIF receptor subunit (Gearing et al., 1991) and a member of the cytokine-binding family of receptor subunits. Formation of a high-affinity signaling complex requires the association of the LIF-LIFR complex with another transmembrane signal transducing molecule gp130 (Gearing et al., 1992a; 1992b), which itself exhibits features of the cytokine family of receptors (Hibi et al., 1990).

The *LIF* associated negative dominance effect of $-0.72 \pm 0.37$ (P=0.047) for first parity NBA missed the significance level of $P < 0.05$ very closely. This tendency is confirmed by the estimated negative dominance effect for $2^{nd}$ to $10^{th}$ parity NBA. However, these results need to be verified by association studies with larger sample sizes. Verification is also necessary for the nonsignificant trend for animals with the *A* allele to have increased NBA across all parities (Table 3). There is evidence for the existence of both *LIF*-SNP alleles in different populations (Spötter et al., 2001). Associations between the marker and the trait may vary between populations, lines, or families. This was shown in several studies with diallelic DNA markers for reproductive traits. The effect of the *B* allele of a diallelic marker at the estrogen receptor (*ESR)* locus differed from 0.6 to 2 piglets more per litter (Short et al., 1997). Another study showed no significant effect of the *ESR* genotype on litter size in 59 sows from a hyperprolific Large White line and a control Large White line (Legault et al., 1996). Vincent et al. (1998) have shown that the *A* allele of a diallelic marker at the prolactin receptor (*PRLR)* locus is significantly associated with increased litter size in three of five commercial lines involving Meishan, Large White, Landrace and Duroc. Inconsistently, Drögemüller et al. (2001) reported an additive effect of the *B* allele of this marker on NBA, across all parities in a Duroc population. The above mentioned studies demonstrate the difficulties in confirming previously published candidate gene effects in different genetic groups and show the need for studies of marker effects in different lines because allele effects differ between lines or populations. The observed differences between the lines may be explained through variations in the genetic background or different linkage phases between the markers and a causal

mutation due to recombination. Also, still unknown QTL with effect on litter size could be linked to these gene associated markers.

A correlation between the effect of the RFLP marker on NBA and both of the tested growth and carcass traits (DG and BF) was not ascertained in the genotyped population. The estimated effects clearly missed the significance level of $P < 0.05$ (Table 4).

IMPLICATIONS

This study is the first report on an investigation of the effect of a newly developed *LIF* associated RFLP marker on litter size in pigs. The estimated negative dominance effect and the nonsignificant trend for *AA* genotypes to have increased NBA, both across all parities, provide some evidence for a selection for the *A* allele. On the one hand the low frequency of this allele in the genotyped population renders it well suited for animal selection, on the other hand alleles of a low occurrence are all the more difficult to test significantly the smaller the employed sample size, which makes it hard to give reliable recommendations concerning selection. However, in this study the *LIF* marker definitely proved to be a strong candidate for confirmation studies, employing a larger sample size. Moreover, further investigation of different pig breeds is necessary to evaluate the usefulness of this RFLP as a marker for MAS based improvement of litter size in different genetic backgrounds.

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# Chapter IX

# General discussion

# General discussion

A candidate gene analysis for litter size in pigs was carried out for the six genes *LIF* (leukemia inhibitory factor), *LIFR* (leukemia inhibitory factor receptor), *CTSL* (cathepsin L), *ITIH4* (inter-α-trypsin inhibitor heavy chain 4), *EGF* (epidermal growth factor), and *EGFR* (epidermal growth factor receptor). These genes have been mapped physically by fluorescence in situ hybridization (FISH) and genetically by radiation hybrid (RH) mapping. Mapping results were in agreement with the established comparative map of pig and human (Goureau et al., 1996; http://www.toulouse. inra.fr/lgc/pig/compare/compare.htm) except for the *CTSL* gene. The inconsistency detected in this case demonstrates that the comparative maps of pig and man need further refinement. A high-resolution comparative map is a precondition for the fine mapping of QTL by MAS. This in turn is helpful in finding the genes that influence an investigated trait, for example by the positional candidate approach, which is based on the fine mapping of a QTL and a subsequent analysis of this confined region for putative candidate genes. Consequently the genomic region that has to be searched for candidate genes becomes smaller with increasing marker density of the map used.

This work contributes to the development of a high-resolution comparative map of pig and human by the chromosomal assignment of six candidate genes for litter size and to the provision of resources needed for fine mapping of QTL by reporting six newly developed and physically anchored genetic markers. In the cases of *LIFR*, *CTSL*, *ITIH4*, *EGF* and *EGFR* these markers are microsatellites located on the same genomic clone as the respective genes. The size of these PAC and BAC clones ranged between 75 and 200 Kbp Consequently a close linkage of the candidate genes with the respective markers can be expected within a distance of 0.5 cM. For the *LIF* gene, two intragenic SNPs were identified in untranslated regions of the gene. One of them can easily be gentotyped using PCR-RFLP.

The opportunity to exploit very comprehensive mapping information in humans and mice on the basis of comparative gene mapping can also be utilized for the choice of new positional candidate genes for litter size. Although it is a well established fact that intrachromosomal rearrangements may have occurred within regions of conserved synteny between species (e.g. Johansson et al., 1995), colinearity to the human map is expected for large parts of farm animal genomes at least for those species within the mammalian class (Andersson, 1998). The regions of conserved synteny to the human genome have been established by comparative chromosome painting (Zoo-FISH) for several species including the pig (Goureau et al., 1996). Comparative mapping of pig genes assigned solely genetically or by radiation hybrid mapping can be achieved by allocating their map positions (in centi Morgan (cM) and centi Ray (cR),

respectively) to the respective chromosome bands using a porcine comparative cytogenetic, genetic and radiation hybrid map (Milan et al., 2000). The introduced maps were used to scan human chromosome regions, corresponding to porcine QTL for litter size, for genes with putative physiological relevance in this trait (Table 1). Accordingly but the other way round, chromosomal locations of genes already implicated in early embryonic development of mice were examined for correspondence to porcine QTL for litter size (Table 2). A comparative map of pig and mouse was not necessary because in all cases the orthologous human genes have also been mapped. The first approach (see Table 1) yielded 10 putative candidate genes for litter size, 5 of which (DTR, STAT1, MMP1, GRB2, EN1) have never been implicated in the regulation of this trait in pigs and mice before. With the second approach 9 putative candidate genes for litter size were identified. To date, their association with this trait in pigs was not investigated. This search for new candidate genes concerning litter size in pigs provides a starting-point for the development of new gene markers and their investigation for litter size in pigs. An enlargement of the existing set of gene markers for litter size would enhance the chances for an improvement of the trait. A combined approach using polymorphisms of candidate genes and genome-wide equidistantly distributed microsatellites for linkage analysis would greatly enhance the unraveling of new QTL and mutations of genes involved in litter size traits.

In this study the complete coding sequences of the genes for CTSL and LIF were determined and their genomic organization was ascertained. In the case of *CTSL* this provides a precondition and starting point for a mutation analysis to identify putative causative mutations with a potential influence on litter size or its component traits. A mutation analysis of the *LIF* gene was carried out. Screening of the genomic *LIF* sequence for polymorphisms revealed the existence of two SNPs in untranslated regions of the gene. One of these SNPs was genotyped in an association study for litter size employing 273 sows with 955 litters belonging to a German synthetic line. There was no indication of an additive effect on the number of piglets

Table 1: From porcine QTL to murine genes – new putative comparative candidate genes for litter size identified by comparative mapping between pig and human.

| Porcine QTL | | | Putative candidate genes for litter size in the corresponding regions of the human genome (GENE / HSA position / function) |
|---|---|---|---|
| Trait | SSC (cM) | Corresponding cytogenetic position | |
| UC | 8 (71) | q12-q25 | **GNRHR**: gonadotropin-releasing hormone receptor / HSA4q21 / Luteinizing (LH) and follicle-stimulating (FSH) hormones regulate gonadal function and gametogenesis, and are critical for normal |

sexual maturation and reproductive function. LH and FSH are synthesized and secreted from pituitary gonadotropes under the regulation of hypothalamic GNRH (Kaiser, 1998).
**FGF2**: fibroblast growth factor 2 (basic) / HSA4q26-q27 / FGF family members possess broad mitogenic and cell survival activities, and are involved in a variety of biological processes, including embryonic development, cell growth, morphogenesis, tissue repair, tumor growth and invasion (Lim et al., 2002).

| | | | |
|---|---|---|---|
| OR | 9 (67) | p21-p12 | **MMP1**: matrix metalloproteinase 1 (interstitial collagenase) / HSA 11q22 / Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis (Hurst et al., 1999). |
| TNB | 12 (71) | p11-q13 | **HOXB**: homeo box B / HSA 17q21-q22 / homeobox genes encode a highly conserved family of transcriptionfactors that play an important role in morphogenesis in all multicellular organisms. Mammals possess four similar homeobox gene clusters, HOXA, HOXB, HOXC and HOXD, located on different chromosomes, consisting of 9 to 11 genes arranged in tandem. This gene is one of several homeobox HOXB genes located in a cluster on chromosome 17. The exact role of this gene has yet to be determined. For the role of HOX genes in murine implantation refer to Lim et al. (2002)<br>**GRB2**: growth factor receptor-bound protein 2 / HSA 17q24-q25 / signal transduction gene in the human and the mouse. GRB2 could be implicated in reciprocal signaling between the blastocyst and the uterus based on information about the role of GAB1 (GRB2-associated binding protein 1) in these processes (Hemberger and Cross, 2001) |
| TNB | 14 (62) | p25-p29 | **CYP17A1**: cytochrome P450, family 17, subfamily A, polypeptide 1 / HSA 10q24 / This gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. This is a key enzyme in the steroidogenic pathway that produces progestins, mineralocorticoids, glucocorticoids, androgens, and estrogens (Kado et al., 2002). |
| OR | 15 (79) | p23-p24 | **EN1**: engrailed homolog 1 / HSA 2q13-q21 / Homeobox-containing genes are thought to have a role in controlling development. In Drosophila, the 'engrailed' (en) gene plays an important role during development in segmentation, where it is required for the formation of posterior compartments. The human engrailed homologs 1 and 2 encode homeodomain-containing proteins and have been implicated in the control of pattern formation during development of the central nervous system (Loomis et al., 1998).<br>**HOXD**: homeo box D / HSA 2q31 / This gene is one of several homeobox HOXD genes located at 2q31-2q37 chromosome regions. Deletions that removed the entire HOXD gene cluster or 5' end of this cluster have been associated with severe limb and genital abnormalities. This gene is one of several homeobox HOXD genes located in a cluster. The exact role of this gene has yet to be determined. For the role of HOX genes in murine implantation refer to Lim et al. (2002).<br>**STAT1**: signal transducer and activator of transcription / HSA 2q32 / member of the STAT protein family. In response to cytokines and growth factors, STAT family members are phosphorylated by the |

| | | | |
|---|---|---|---|
| | | | receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein can be activated by various ligands including interferon-alpha, interferon-gamma, EGF, PDGF and IL6 (Pan et al., 2003). |
| TNB | 17 (43) | p12-p22 | **DTR**: diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor) / HSA 5q23 / Heparin-binding epidermal growth factor is implicated in blastocyst implantation in mice (Lim et al., 2002). |

born alive (NBA) but a significant negative dominance effect of $-0.72 \pm 0.37$ (p=0.047) was observed for the first parity and of $-0.50 \pm 0.29$ (p=0.087) for the second to tenth parity. However, this effect needs to be confirmed by analysis of a larger sample size. Moreover, further investigation of different pig breeds is necessary to evaluate the usefulness of this SNP marker for MAS based improvement of litter size. Association studies for NBA with the same population as for the *LIF* gene failed to provide evidence for an effect of any of the five microsatellite markers, developed for the other investigated candidate genes. Yet, it cannot be excluded that these markers might show significant effects on litter size traits in more powerful study designs or other pig breeds or lines with a different genetic background. For this reason further evaluation of these markers is appropriate. Such studies should also consider additional litter size traits such as uterine capacity, number of corpora lutea, and total number of born piglets to find possible associations.

When effects of any of the mentioned microsatellite markers on litter size are detected, a mutation analysis of the respective genes should be carried out in a manner analogous to that for the *LIF* gene. The identification of intragenic SNP markers includes the possibility of finding mutations that cause the phenotypic effects. However, evidence has to be provided that a certain mutation is in fact the causative, trait influencing mutation and not just another one in very close linkage to it.

Several possible strategies for the identification of mutations are available. Northern blot analysis of mRNA from affected and non-affected individuals may reveal differences in the level, or in the tissue distribution, of gene expression, implying the presence of a regulatory mutation in the promoter or in another regulatory element. Moreover, a mRNA of aberrant size may be due to a deletion/insertion or a splice defect. Such investigations will be greatly facilitated in the near future by the development of cDNA microarrays. This technology

Table 2: From murine genes to porcine QTL – new putative comparative candidate genes for litter size identified by comparative mapping between pig and human.

| -Gene<br>-Localization (HSA)<br>-Corresponding SSC region | Function of the orthologous murine gene | Putative corresponding porcine QTL (localization) |
|---|---|---|
| -Bystin-like (BYSL)<br>-HSA6p21.1<br>-SSC7p | adhesion molecule, mediates embryo attachment (Aoki et al., 2000; Suzuki et al., 2000) | TNB (SSC7p13-p12, 10 cM) |
| -Kallikrein 9 (KLK9)<br>-HSA19<br>-SSC2, 6, 5,7 | implicated in embryo implantation (Geisert et al., 2001) | TNB (SSC7p13-p12, 10 cM) |
| -Peroxisome Proliferator Activator Receptor Delta (PPARD)<br>-HSA6p21.2<br>-SSC7p | implicated in embryo implantation (Lim et al., 2002) | TNB (SSC7p13-p12, 10 cM) |
| -Neural Cell Adhesion Molecule 1 (NCAM1)<br>-HSA11q23-q24<br>-SSC9p24-p11 | adhesion molecule, mediates embryo attachment (Kallapur and Akeson, 1992) | OR (SSC9p21-p12, 67cM) |
| -Integrin, Beta 1 (ITGB1)<br>-HSA10p11.2<br>-SSC10q12-q17 | adhesion molecule, mediates embryo attachment (Beauvais-Jouneau and Thiery, 1997) | OR (SSC10q13-q17, 10cM) |
| -Erythroblastic Leukemia Viral Oncogene Homolog 2 (ERBB2)<br>-HSA 17q11.2-q12<br>-SSC12 | interaction with EGF-like growth factors shown to be of importance for implantation (Olayioye et al., 2000) | TNB (SSC12p11-q13, 71cM) |
| -Cannabinoid Receptor 2 (CNR2)<br>-HSA1p<br>-SSC4q, SSC6q, SSC14q26-q29 | role in defining the window of uterine receptivity for implantation (Schmid et al., 1997) | TNB (SSC14q25-q29, 62cM) |
| -Erythroblastic Leukemia Viral Oncogene Homolog 4 (ERBB4)<br>-HSA2q34<br>-SSC15p22-p26 | interaction with EGF-like growth factors shown to be of importance for implantation (Olayioye et al., 2000) | OR (SSC15p22-p24, 79cM) |
| -Fibronectin 1 (FN1)<br>-HSA2q34-q36<br>-SSC15p22-p26 | adhesion molecule, mediates embryo attachment (Wartiovaara et al., 1979) | OR (SSC15p22-p24, 79cM) |

permits the analysis of thousands of genes simultaneously. Normal mRNA expression in affected individuals suggests that the mutation is a structural one, and the next step is to screen for mutations using cDNA clones or RT-PCR products based on sequence analysis. If a regulatory mutation is expected because of aberrant mRNA expression or if the coding

sequence does not differ between affected and non-affected individuals it will be necessary to clone the gene and make a sequence comparison using genomic DNA. When a putative mutation has been identified it is necessary to provide evidence that the mutation causes the phenotypic effect. For a monogenic trait with complete penetrance there has to be a correlation between the mutation and the phenotype, depending on whether the trait shows a dominant or recessive inheritance. Moreover, individuals showing the phenotype but lacking the mutation may occur, but they should then exhibit other mutations in the same gene. Further indication for a causal relationship may also be provided if a missense mutation causes a substitution in a highly conserved and functionally important part of the protein or if a nonsense mutation is identified and the phenotype is obviously due to the lack of gene expression. Yet, even if there are striking indications of a causal relationship it is often difficult to exclude the possibility that the observed mutation is only very closely linked to the causative mutation. Conclusive evidence may be achieved by expressing the normal and variant forms of the gene product *in vitro* and comparing their functions. In many cases, however, it will be necessary to study the phenotypic effect *in vivo* by producing transgenic animals carrying the specific mutation (Andersson, 1998).

Such bridges between genetics and physiology will be critical for implementing a fully integrated research programme combining quantitative genetics, genomics, proteomics, metabolics and phenomics to fully dissect the complex and polygenic nature of porcine reproductive traits (Pomp et al., 2001) in different environments and genetic backgrounds.

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