Aus den Arbeiten des Forschungsinstitutes für die Biologie landwirtschaftlicher Nutztiere
in Dummerstorf

Identification and analysis of functional candidate genes for the inverted teat defect in pigs

Dissertation

zur

Erlangung des akademischen Grades

doctor rerum naturalium (Dr. rer. nat.)

der Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Rostock

vorgelegt von

Sylvio Tetzlaff, geb. am 19.12.1977 in Demmin

aus Rostock

urn:nbn:de:gbv:28-diss2009-0154-1

Tag der Verteidigung: 20.04.2009

Gutachter: Prof. Dr. Reinhard Schröder

Institut für Biowissenschaften, Abteilung Genetik, Universität Rostock

Prof. Dr. Klaus Wimmers

Forschungsinstitut für die Biologie landwirtschaftlicher Nutztiere,

Forschungsbereich Molekularbiologie, Dummerstorf

Prof. Dr. Karl Schellander

Institut für Tierwissenschaften,

Rheinische Friedrich-Wilhelms-Universität Bonn

dedicated to my family

Contents

Abbreviations I

1 General introduction 1

2 SNP analysis, genotyping and mapping of the porcine *PTHR1* gene to chromosome 13 (Brief report) 6

3 QTL for the heritable inverted teat defect in pigs 10

4 Association of parathyroid hormone-like hormone (PTHLH) and its receptor (PTHR1) with the number of functional and inverted teats in pigs 35

5 Evidence for association of lymphoid enhancer-binding factor-1 (*LEF1*) with the number of functional and inverted teats in pigs 47

6 Differential expression of growth factors and their receptors points out their involvement in the inverted teat defect in pigs 67

7 General discussion 84

8 Summary 94

9 Zusammenfassung 96

10 References 98

11 Appendix 110

Abbreviations

°C	Degree Celsius
µ	Micro- (10^{6})
%	Percent
A	Adenine, ampere
bp	Base pairs
BLAST	Basic local alignment search tool
BRCA2	Breast cancer susceptibility gene
BTA	*Bos taurus* chromosome
C	Cytosine
c	Centi- (10^{2})
CALML3	Calmodulin-like 3 protein
cDNA	complementary deoxyribonucleic acid
cM	centiMorgan
COM	Commercial pig population
cR	centiRay
Da	Dalton
DE	German Large White
DL	German Landrace
dNTP	Deoxyribonucleoside triphosphate
DNA	Deoxyribonucleic acid
DUMI	Experimental Duroc and Berlin Miniature pig population
ESR	Estrogen receptor
et al.	`et alteri´

F	Phenylalanine
FABP3	Fatty acid-binding protein 3
FBAT	Family based association test
FGF	Fibroblast growth factor
FGFR2	Fibroblast growth factor receptor 2
Fig.	Figure
FSH / B	Follicle-stimulating hormone / beta
FSHR	Follicle-stimulating hormone receptor
G	Guanine
g	Grams
GAPDH	Glyceraldehydes-3-phosphate dehydrogenase
GDF8	Growth differentiation factor 8 / myostatin
GHR	Growth hormone receptor
GTF3C2	General transcription factor IIIC, polypeptide 2
HGF	Hepatocyte growth factor
HGFR	Hepatocyte growth factor receptor
HMG1	High mobility group protein-1
HSA	*Homo sapiens* chromosome
IGF1 / 2	Insulin-like growth factor 1 / 2
IMpRH	INRA-Minnesota porcine Radiation Hybrid
IT	Inverted teat
k	Kilo- (10^3)
L	Leucine
l	Litre
LEF1	Lymphoid enhancer-binding factor-1

LEPR	Leptin receptor
LGR7	Leucine-rich G-protein-coupled receptor 7
LOD	Logarithm of odds
M	Molar
m	Milli- (10^{3})
MAS	Marker-assisted selection
min	Minute
mRNA	Messenger ribonucleic acid
n	Nano- (10^{9}), number
NIT	None affected with inverted teat
no.	Number
NPL	Non parametric LOD score
nt	Nucleotide
OMIA	Online mendelian inheritance in animals
p	Pico- (10^{12})
PCR	Polymerase chain reaction
PDGFA / B	Platelet-derived growth factor alpha / beta
PDGFRA	Platelet-derived growth factor receptor alpha
Pi	Pietrain
PRL	Prolactin
PTH	Parathyroid hormone
PTHLH	Parathyroid hormone-like hormone
PTHR1	PTH/PTHLH receptor 1
qRT-PCR	Quantitative real-time PCR
QTL	Quantitative trait loci

RFLP	Restriction fragment length polymorphism
RLN	Relaxin
RNA	Ribonucleic acid
RPL32	Ribsomal protein L32
RYR1	Ryanodine receptor 1
S	Serine
s / sec	Seconds
SSC	*Sus scrofa* chromosome
SNP	Single nucleotide polymorphism
T	Thymine, temperature
Tab.	Table
TCF7L2	Transcription factor 7-like 2
TGFA / B	Transforming growth factor alpha / beta
TSH / B	Thyroid stimulating hormone / beta
TT	Total number of teats
U	Units
UTR	Untranslated region
V	Volts
VEGF	Vascular endothelial growth factor
W	Watts

1 General introduction

Hereditary defects play a tremendous role in continuance and development of animals. The '*Online Mendelian Inheritance in Animals'* (OMIA) database stays abreast of these changes and illustrates more than 2500 inherited disorders and traits in 135 animal species. For the pig about 200 genetically influenced diseases and defects are described. Several of these inherited disorders affect a functional mammary gland. These hereditary defects constitute markedly important criteria on economics in pig production. A functional mammary gland in porcine animals for breeding plays a crucial role. Hence, many dam line breeding programs in pig production focus on udder quality. In particular, the teat number and functional mammary gland capability are important selection criteria for increasing the survival rate of piglets. The most frequent and economically relevant inherited disorder of the mammary gland in pigs is the inverted teat, resulting in non-functional teats that can normally not be suckled by the offspring. The inverted teat is characterized by failure of teats to protrude from the udder surface. The teat channel is held inward, forming a cavity so that milk flow is prevented (Fig. 1). The defect occurs in commercial pig breeds with frequencies between 7.6 to 30 % (Brevern *et al.* 1994; Jonas *et al.* 2008; Mayer & Pirchner 1995; Niggemayer 1993), with 1.2 to 4.6 affected teats (Hittel 1984; Steffens *et al.* 1996). Thereby a minimum average number of twelve functional teats can often not be achieved in affected sows. The selection of gilts because of affected mammary complexes reduces the capability of selection on production and reproduction traits. Several authors considered a complex inheritance of the liability to develop the defect with the presence of a major gene and several minor genes causing this defect during embryonic mammary gland development (Günther *et al.* 1985; Wiesner & Willer 1978).

However, the mode of inheritance and the number of genes involved are still unknown. Heritability was estimated between 0.2 and 0.5 (Brevern *et al.* 1994; Mayer 1994).

Embryonic mammary gland development is dependent on reciprocal inductive interactions between the mesenchyme and overlying ectoderm (Boras-Granic *et al.* 2006; Foley *et al.* 2001; Pispa & Thesleff 2003; Robinson *et al.* 1999; Sakakura *et al.* 1987). Signals originating in the mesenchyme have been proposed to specify ectodermal cells to form epithelial mammary placodes (Sakakura 1987). Reciprocal signaling from the placodes to the underlying mesenchyme then directs condensation of fibroblasts that form the mammary mesenchyme.

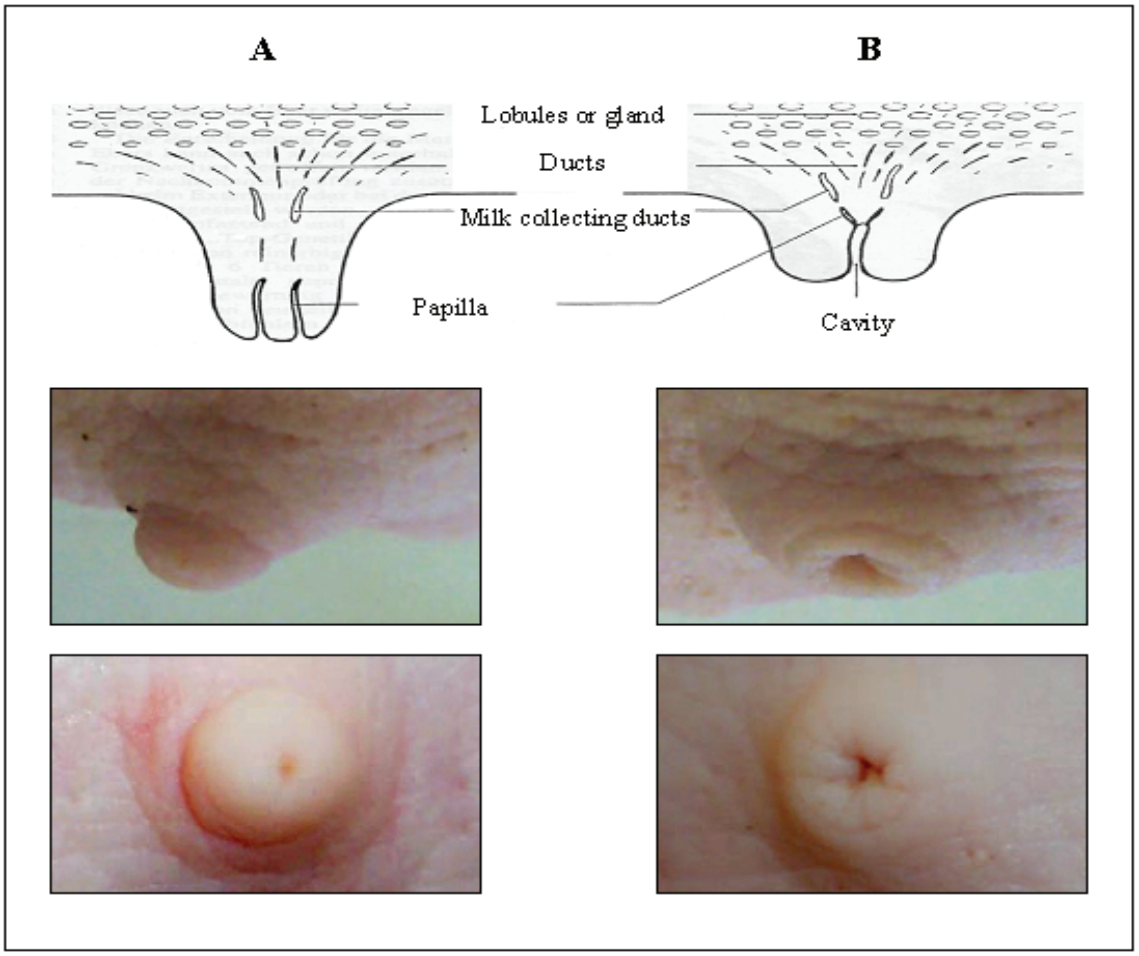


Figure 1 Ultra structure and scheme of a normal developed (A) and an inverted teat (B) (Steffens 1993).

Maintenance of these reciprocal signals stimulates further proliferation and differentiation of the placodes resulting in the development of the ductal mammary gland (Boras-Granic *et al.* 2006; Eblaghie *et al.* 2004). The identification and characterization of candidate genes involved in regulatory pathways that may influence the occurrence of the inverted teat defect will help to better understand the assertive arrangements in functional mammary gland and teat developmental capabilities. These capabilities have to be seen as a result of a complex interaction between multiple signaling factors, pathways and different cell types during several major phases of development (Hovey *et al.* 2001; Imagawa *et al.* 1990; Sakakura 1987).

Thus the candidate gene approach is one major opportunity to detect genes, which affect the development of a functional mammary gland. That approach is based on knowledge of physiology, biochemistry or pathology, which clearly indicates the mechanism of this criterion (Rothschild 2003). As an example, the estrogen receptor (ESR) was presumed to be involved in the genetic variation of reproductive traits. A polymorphism was detected in the *ESR* gene and associated with litter size in pigs (Rothschild *et al.* 1996; Short *et al.* 1997). Rothschild (2003) elucidates that the commercial pig industry is actively using this gene marker information in combination with traditional performance information to improve pig production by marker-assisted selection (MAS). For the inverted teat phenotype the number of currently investigated functional candidate genes is limited. Pathophysiological investigations on this defect would facilitate the identification of functional candidate genes for the inverted teat phenotype. Other strategies to list candidate genes are linkage studies in particular families of affected animals and expression studies of the respective relevant tissue.

The identification of genomic regions for the improvement of different traits is the primary objective of the quantitative trait loci (QTL) approach. For traits like growth,

carcass and meat quality QTL on several porcine chromosomes have been detected (Edwards *et al.* 2008; Murani *et al.* 2006). For linkage analyses usually molecular markers are applied on account of their polymorphic character. Several microsatellites with defined distances on the chromosomes will be genotyped in an experimental F_2 population of a cross between strongly divergent lines, since a high genetic distance could be expected. Afterwards the significance of linkage will be verified with logarithm of the odds (LOD) calculations. Due to the population dependent results of these studies independent validations in commercial populations should be performed (Gerbens *et al.* 2000). Despite the increasing economic importance and the number of ongoing QTL experiments, the information concerning udder quality traits is limited (Oltmanns 2003; Ün 2002). However, QTL for teat number in the pig have been detected on nearly every chromosome (Cassady *et al.* 2001; Hirooka *et al.* 2001; King *et al.* 2003; Rodriguez *et al.* 2005; Wada *et al.* 2000). The QTL approach reveals genomic regions with hundreds of positional candidate genes for a specific trait like inverted teat, so that this approach should be applied in combination with different approaches described in this study.

The third application performed in this thesis is the expression analysis approach. The expression analysis describes the association between the *genome* and the *transcriptome*, more precisely the transcription from DNA to RNA (Velculescu *et al.* 1997). Thus simply the coding region of the *genome* is considered. The basis for studies are differentially expressed genes, which encode the triplets for the different amino acids for the next level, the *proteome* (Wasinger *et al.* 1995; Wilkins 1994). The abundance of RNA influences the further translational potential of a gene in different cell types and tissues and can therefore be determined by quantitative real-time PCR (qRT-PCR). The microarray analysis includes the simultaneously measurement of

transcript abundance of various genes and describes their differential expression in investigated cells or tissues, so that this method may direct in a better understanding of biological processes that could affect a functional development (Moody *et al.* 2003; Pas *et al.* 2005). This approach is especially of interest for traits like the inverted teat, because only a few possible concerned candidate genes are known so far (Chomdej 2005; Jonas *et al.* 2008). Several studies have demonstrated that these approach may be an appropriate instrument for the investigation and understanding of biological processes in a functional mammary gland (Clarkson *et al.* 2004; Master *et al.* 2002).

Combinations of such approaches could reduce the number of discovered genes of more than thousand positional loci on a few often largely unknown candidate genes (Wayne & McIntyre 2002), which are involved as functional positional candidate genes in the mammary gland development.

The objectives of this study were the identification and analysis of functional and positional candidate genes involved in the occurrence of a functional mammary gland. Ultimately, genetic markers should be detected that can be applied in a MAS for a more efficient selection on reproduction traits adapted by different breeding programs for pig populations.

2 SNP analysis, genotyping and mapping of the porcine *PTHR1* gene to chromosome 13 (Brief report)

Tetzlaff S, Ponsuksili S, Murani E, Schellander K, Wimmers K.

Published in Archives of Animal Breeding 2007; 3: 320-321

Background

The parathyroid hormone/parathyroid hormone-like hormone type I receptor (PTHR1) belongs to the family of G protein-coupled receptors for peptide hormones, including parathyroid hormone (PTH) and parathyroid hormone-like hormone (PTHLH), which participate in epithelial-mesenchymal interactions during the formation and differentiation of epithelial organs (Chomdej *et al.* 2004; Foley *et al.* 2001). The function of PTHR1 and its ligands suggest its candidacy for traits related to the development of bones and joints but also of mammary gland. The porcine gene was screened for SNPs and assigned to SSC13.

Procedures

Primer sequences

PTHR-F: 5'-GCTATGGTCCGATGGTGTCT-3'

PTHR-R: 5'-ACTGTCTCCCACTCCTCCTG-3'

SNP analysis, genotyping and mapping

The *PTHR1* gene sequence (GenBank accession no. NM_214382) was used to derive homologous primers every 400-500 bp of the entire mRNA to screen for polymorphisms.

The PCR fragments were amplified and sequenced from 5 pig breeds (Duroc, Hampshire, German Landrace, Pietrain and Berlin-Miniature pig) using standard lab protocols. PCR programs were: initial denaturation for 5 min 95 °C, 40 cycles each 30 s

at 95 °C, 30 s at 64 °C and 1 min at 72 °C and 5 min final extension at 72 °C. Genotyping was performed using the primers PTHR-F and PTHR-R for amplification and *Mbo*II (Fermentas, Leon-Rot, Germany) for restriction digestion. Physical mapping was achieved by screening of the IMpRH panel using the same primers for PCR and by analysis of the results using twopoint and multipoint analysis option of the IMpRH mapping tool (http://www.toulouse.inra.fr). For linkage mapping 19 informative families (n 313) of the DUMI F_2 resource population were genotyped (Hardge *et al.* 1999). Multipoint linkage map was established using the BUILD option of the CRIMAP 2.4 package.

Results

A C/T non-synonymous SNP (L556F) was detected at nucleotide position 1819 in the *PTHR1* gene. Within the DUMI F_1 generation (n 14) we observed 11 heterozygous animals and 3 homozygous for allele C; in the DUMI F_2 generation 116 animals were homozygous for allele C, 149 animals were heterozygous and 48 animals were homozygous for allele T. The *PTHR1* gene was assigned to SSC13 in close proximity to marker SW1400 by twopoint analysis (20 cR; LOD 13.05) using IMpRH panel (Vector: 1101110000 0000000001 0000000000 0000001000 0001000000 1000010001 0100000001 0000000100 0000000000 0101000011 1011000000 01010000). This assignment was further confirmed by genetic mapping of *PTHR1* using multipoint and twopoint analysis that revealed linkage to S0219 (proximal) and SW344 (distal) with distances of 38.4 cM (recombination fraction 0.31, LOD 1.83) and 15.5 cM (recombination fraction 0.15, LOD 12.69), respectively, on the sex averaged map.

Our assignment of *PTHR1* to the q arm of SSC13 is in agreement with the published physical and genetic maps.

Acknowledgements

The authors would like to thank the Federal Ministry of Education and Research (BMBF) and the Förderverein Biotechnologieforschung e.V. (FBF) for financial support.

3 QTL for the heritable inverted teat defect in pigs

Jonas E, Schreinemachers H-J, Kleinwächter T, Ün C, Oltmanns I, Tetzlaff S, Jennen D, Tesfaye D, Ponsuksili S, Murani E, Juengst H, Tholen E, Schellander K, Wimmers K.

Published in Mammalian Genome 2008; 19(2): 127-138

Abstract

The mothering ability of a sow largely depends on the shape and function of the mammary gland. The aim of this study was to identify QTL for the heritable inverted teat defect, a condition characterized by disturbed development of functional teats. A QTL analysis was conducted in a porcine experimental population based on Duroc and Berlin Miniature pig (DUMI). The significant QTL were confirmed by linkage analysis in commercial pigs according to the affected sib pair design and refined by family-based association test (FBAT). Nonparametric linkage analysis revealed five significant and seven suggestive QTL for the inverted teat defect in the porcine experimental population. In commercial dam lines five significant NPL-values were detected. QTL regions in overlapping marker intervals or close proximity in both populations were found on SSC3, SSC4, SSC6, and SSC11. SSC6 revealed QTL in both populations at different positions indicating the segregation of at least two QTL. The results confirm the previously proposed polygenic inheritance of the inverted teat defect and, for the first time, point to genomic regions harboring relevant genes. The investigation revealed variation of the importance of QTL in the various populations due to either differences in allele frequencies and statistical power or differences in the genetic background that modulates the impact of the liability loci on the expression of the disease. The QTL study enabled us to name a number of plausible positional candidate genes. The correspondence of QTL regions for the inverted teat defect and previously mapped QTL for teat number are in line with the etiological relationship of these traits.

Introduction

Inherited disorders significantly affect animal health and welfare issues and economic efficiency of livestock breeding. Though the phenotype itself often suggests monogenic inheritance with the occurrence of either affected or non-affected individuals many inherited diseases are liability traits with polygenic background. In pig breeding the number of piglets reared per sow per year is an important characteristic of efficiency. Next to the number of piglets born alive the mothering ability of the sow, which is related to the number of functional teats, plays an important role in this. The inverted teat defect is the most common and most important disorder of the mammary complex in pig (Brevern *et al.* 1994; Mayer 1994). This disorder is a condition characterized by the failure of teats to protrude from the udder surface. The teat canal is held inward, forming a small crater so that normal milk flow is prevented limiting the rearing capacity and increasing the risk of mastitis. The inverted teat defect has polygenic background and heritability estimates range between 0.2 and 0.5 (Brevern *et al.* 1994; Mayer 1994). It occurs in commercial pig breeds with frequencies between 8 to 30% (Mayer 1994; Steffens *et al.* 1996). In comparison to other hereditary disorders it is difficult to diagnose during routine selection where young sows are adspected while standing or moving within an arena (Steffens 1993). Since the mode of inheritance is not fully understood and the number of genes involved is unknown, it is important to get more knowledge of the genetic cause of the defect. Moreover identification of markers for this liability trait offers perspectives to develop efficient DNA-based selection tools for the improvement of the quality of teats. Therefore the main objective of this study was to identify chromosomal regions and candidate genes for the inverted

teat defect by QTL scan in an experimental population and to validate these results in a commercial population.

Material and Methods

Animals

Analyses were performed on two different populations, an experimental population and animals from commercial dam lines. The German Landrace (DL) and Large White (DE) breeds were chosen for the confirmation studies that are the most commonly used dam lines in Germany. Accordingly, there is selection against the inverted teat defect in these lines.

The experimental population was based on a reciprocal cross between Duroc and Berlin Miniature pig (DUMI population). For the F1 one boar from Duroc breed was mated to four Miniature Pig sows and one Miniature Pig boar was mated to five Duroc sows to produce 43 F1 dams and five F1 boars. The F1 boars and dams were mated to finally produce 905 F2-animals. At 200 days of age two investigators of a team of four trained and skilled persons observed the phenotypes of the teat traits involved in this study. Animals were placed on their backs and teats were evaluated by adspection and palpation. Numbers of functional and inverted teats were recorded. This is similar to time-point of selection of young sows in breeding companies. At this time in 42.2 % of the F2 animals the inverted teat defect was detected.

For the commercial population 2160 animals (castrated male pigs) of the breeds German Landrace (DL) and German Large White (Dl) and their crosses were used. The animals were kept at performance test stations (*LPA Haus Düsse* and *LPA Frankenforst*) under standardized conditions according to the guidelines of the German performance test (ZDS 2003) and monitored at the slaughter line at an age of 180 days of life, on average. Two persons of the team of inspectors examined each animal by adspection and palpation of teats at a separate slaughter line. Out of these 244 animals

were identified with at least one inverted teat. Samples of some of these animals and their affected or non-affected full sib were used for QTL analysis. The samples from the parents of these animals were collected in commercial farms and artificial insemination station, respectively. Only animals where samples of parents and at least one full sib were available were used in this study for the verification of QTL detected in DUMI population. All over 119 families could be analyzed from these samples. Additionally one hundred animals generating eleven families derived from *LPA Grub* from the *Bayerische Landesanstalt für Landwirtschaft* in Bavaria.

Genotyping and mapping

DNA for genotyping was isolated from sperm, muscle or tail using the phenol-chloroform extraction. Each of the 12.5 µl of PCR reaction contained 50 ng genomic DNA, 0.2 µM of each primer, 50 µM of each dNTP, 0.5 U of Taq polymerase and 1.5 mM $MgCl_2$ in 1xPCR buffer. PCR was performed in the thermal cycler PTC 100 (MJ Research USA) at 94°C for 3 min, followed by 30 to 40 cycles at 94°C for 1 min, 55°C to 65°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The annealing temperature was used depending on the optimal temperature for primer. Seventy-two type II markers and twenty-nine type I markers covering the porcine autosomes with mean interval of 23.1 cM were selected from published linkage maps (USDA-MARC and PiGMaP) and used in DUMI population. In the commercial population almost the same set of polymorphic markers was used.

Electrophoresis was performed with LiCor Sequencer System (LI-COR model 4200 automated DNA sequencer) using 12% SequaGel in 1xTBE buffer at a power of 1500V, 50mA, 50W and 50°C temperature. Evaluation of genotypes was done using OneDScan, version 4.10 (Li-COR Biotechnology). Linkage analysis for build up the

maps was performed using CRI MAP, version 2.4 (Green *et al.* 1990). The genetic map obtained essentially has been described previously (Wimmers *et al.* 2006). The QTL analysis for the quantitative trait inverted teat was performed with the software package *Genehunter, Version 2.0 (Whitehead Institute, Cambridge, Massachusetts, USA)* (Kruglyak *et al.* 1996). Genehunter calculated a normalized score Z (v) with a mean of zero and a variance of one under the null hypothesis. The null hypothesis is that there is no linkage between the disease locus and the marker allele (Kruglyak *et al.* 1996).

$$Z(\bar{x}) = \frac{[S(\bar{x}) - \mu]}{s}$$

S(ξ) S_{pairs}

ξ mean of S_{pairs} p ξ S_{pairs}

P a priori probability

s^2 p ξ $(S_{pairs}$ μ$)^2$

Afterward the null hypothesis is calculated under the normalised test statistic NPL (*non parametric linkage*)

NPL ξ Z(v) x P

P *a posteriori* probability

The NPL-analysis calculated the part of the shared allele at each loci on the whole chromosome. These alleles are mentioned as Z0, Z1 and Z2 where is

$$z0 = \frac{a0}{\lambda s}, \quad z1 = a1, \quad z2 = a2\frac{(2\lambda - 1)}{\lambda s}, \text{ with}$$

λs relative risk for the sibling,

a0 ¼,

a1 ½ and

a2 ¼

Chromosome-wide significance was calculated by *Genehunter* and transformed to experiment-wide significance levels using a permutation test (Churchill & Doerge 1994). The experiment-wide significance level was calculated by

$$Pg = \frac{1-(1-Pc)}{r}$$

r length of the specific chromosome/ total length of all chromosomes

Pc chromosome-wide significance level

Further significant and suggestive QTL were defined (Lander & Kruglyak 1995), whereas in a F2 Population the NPL-score of 4.3 is necessary to reach a significance level of 1% using a genome-wide investigation, the significance level of 5% corresponds to a NPL-score of approximately 3.0 (Lander & Kruglyak 1995). The association analysis was performed using the FBAT (family-based association test), a modified test based on TDT (transmission disequilibrium test). In this study the FBAT program (Version 1.4) was used to perform the qualitative and quantitative family-based analyses of both populations (http://www.biostat.harvard.edu/~fbat/fbat.htm). For all the quantitative analyses were done under the condition of an additive model (Horvath *et al.* 2003). FBAT perform the testing by two-step procedure. First the test statistic is defined showing the association between the trait locus and the marker locus. In the second step the distribution of the data of genotypes are tested under the hypothesis. The genotypes of the offspring are treated as random (Rabinowitz & Laird 2000).

The general FBAT statistic U is based on a linear combination of the genotypes of offspring and the traits:

U S E[S] mit S $\Sigma_{ij}\ T_{ij}X_{ij}$

X_{ij} is a function of the genotype of the j-th offspring in family i at the locus tested (depending on the used genetic model).

T_{ij} is a function of the trait (dependent on possible unknown parameters)

T_{ij} Y_{ij}-μ_{ij}

Y_{ij} is the observed trait of the j-th offspring in the family i

μ_{ij} is a random variable

The general FBAT statistic is calculated under the hypothesis „no linkage and no association between marker and gene“. This depends on T_{ij} and on the genotypes of parents. Under this hypothesis, U is, as thought before, as E (U) 0.

With the distribution of the genotypes of offspring (X_{ij} is treated as random and as T_{ij} fix),V Var(U) Var(S) can also be calculated under the hypothesis and can be used for standardization of U.

If X_{ij} is the scalar sum of the genotypes of an individual, then the total statistic of the samples is

Z U/√V

approximately N(0,1). If X_{ij} is a vector, then is

χ^2 U`V-U

an approximately χ^2-distribution with the degrees of freedom similar to V (Horvath *et al.* 2003). In this study the FBAT was performed under the hypothesis „no association and no linkage“.

Results

In the F2 of the experimental population mean number of teat was 12.9. The inverted teat defect occurred at a frequency of 42 % (380 out of 905 animals), with on average 5.2 inverted teats per affected animal at equal frequencies in both sexes. For the commercial breeds out of 2160 animals monitored in the slaughterhouse 244 animals (11.3 %) were found showing one and more inverted teats (mean 2.3). In the commercial dam lines mean numbers of teats were 14.6. Incidence of inverted teats was slightly higher in DL than in DE and F1 animals (12.5 %, 10.7 %, and 10.5 %). In the experimental population on all autosomes except SSC17 QTL were detected reaching 5 % chromosome-wide significance. Within these fourteen QTL reached the experiment-wide significance level. Of these eight were found with highly significant experiment-wide NPL-value (Table 1). In the experimental population twelve suggestive QTL (NPL > 3.0) for the inverted teat defect were detected (Lander & Kruglyak 1995). Of these five significant QTL (NPL > 4.3) were detected. As in the commercial population the affected sib pair design was used, the p-values of the estimated NPL-values detected by the Genehunter program were used for definition of the significance levels. Five QTL positions were found with only one NPL-value exceeding the 5% experiment-wide significance threshold (Table 2). QTL regions in overlapping marker intervals or close proximity in both populations were found on SSC3, SSC4, SSC6, and SSC11 (Fig. 1). The association analysis using the FBAT program revealed more alleles of Duroc origin having positive influence on the development of normal teats (negative Z-score). The following alleles from Duroc origin could be shown being highly associated with normal teat development: on SSC1 allele 165 of marker SW1301 (Z-score -3.8), on SSC4 allele 234 of marker S0097 (Z-score -7.3), on SSC6 allele 154 of marker S0220

(Z-score -4.9) and on SSC14 allele 174 (Z-score -5.1) of marker S0007. On SSC1 the allele 161 of marker SW1301 and on SSC3 allele 286 of marker S0164, originated from Miniature pig, were shown being highly associated with the inverted teat defect (Z-scores 7.2 and 2.5, respectively). The Miniature pig originated alleles 207 of marker SW2443 (SSC2), 302 of marker S0164 (SSC3), and 212 of S0097 (SSC4) showed positive impact on teat development (Z-scores -2.6 and -2.3 and 2.0, respectively). Data of association compared to linkage analysis of both populations are shown in Table 3. The distal region of SSC1 contained a QTL for the inverted teat defect with NPL-value reaching the 1 % chromosome-wide significance in DUMI population (Table 1). There was no QTL found on SSC1 in the commercial dam lines. However, family based association test revealed significant association and linkage of marker SW1301 in both the experimental and the commercial population (Table 3). On SSC2 a significant QTL was detected in the telomeric region showing 5 % experiment-wide significant NPL-value in animals of the experimental population (Table 1). This could be confirmed by association analysis of marker SW2443 (Table 3). The QTL could not be detected when analyzing all animals of commercial population. Linkage analysis of only families of crossbreeds (DE×DL, DL×DE) revealed a QTL with NPL-value significant at 5 % chromosome-wide level at marker SW1564, central on SSC2. The distal region on SSC3 showed a highly significant QTL for the inverted teat defect in the DUMI population (Table 1). The locus was confirmed at 5 % chromosome-wide significance in the commercial animals as well as by association analysis (Tables 2 and 3). Two alleles (218, 286) were identified, inherited from Duroc and Miniature pig, respectively, showing an increase of liability for the inverted teat defect (Table 3). Linkage and association analysis on SSC4 revealed significant QTL with a peak at marker S0097 in animals of experimental population (Table 1). A significant NPL-value

could be detected in animals of commercial families in the region between markers S0214 and S0097, i.e. in close proximity to the QTL found in the DUMI (Table 2). Family based association tests revealed significant association and linkage of alleles of marker S0097 in both populations. On chromosome 5 in the region of the microsatellite marker SW1482 a highly significant QTL was detected (Table 1). The analysis of the crossbreed animals in commercial group revealed a QTL with NPL-value significant at 5 % chromosome-wide significance level more distal on SSC5 (Table 2). The highest NPL-value of 9.25 was detected on SSC6 with peak at marker S0220 (Fig. 1). In the dam lines the highest NPL-value was detect at marker S0059, distal of S0220. This QTL was the only locus with experiment-wide significance in the commercial animals. There was a good agreement of the NPL-plots found in the analysis of all commercial animals and of analysis separated by breeds. An NPL-value of 1.16 implementing chromosome-wide 5 % significance level was detected in the commercial animals with peak at marker S0035 telomeric of SSC6. At this position a QTL reaching 5 % experiment-wide significance level was also obtained in the DUMI population. Alleles of marker S0220 were significant associated to the defect in both populations, whereas alleles of S0059 and S0035 showed only association in the commercial and experimental population, respectively. Results of linkage and association analysis in both populations indicate that there are at least two QTL segregating on SSC6 affecting the inverted teat defect. The distal region of SSC7 showed significant NPL-values for the heritable inverted teat defect, as revealed by full sib analysis, between markers S0115 and S0101 at the experiment-wide 5 % significance level only in the experimental population. SSC8 carried a QTL at the proximal end in DUMI population. The region around marker S0086, which is more distal, showed a significant NPL-value in the analysis of crossbreed animals of commercial population (Table 2). On SSC11 a

QTL reaching the 1 % experiment-wide significance in DUMI population was found surrounding marker S0386 (Table 1). In the commercial animals an NPL-value reaching the 1 % chromosome-wide significance level was detected in close proximity at SW703. The peak was also detected in the families including only animals of the German Landrace breed (Table 2). Both regions could be confirmed by association analysis revealing alleles of marker S0386 being associated with the defect status in experimental population and alleles of marker SW703 being associated with the defect in both populations (Table 3). A significant QTL could be detected on SSC12 between the markers SW605 and L147 in animals of the DUMI population. The NPL-score of 6.7 was the second highest in this analysis (Table 1). There was no evidence found for a QTL neither an associated allele in the commercial animals. The chromosome 14 contained a significant QTL next to the marker S0007, but no significant QTL was detected in the commercial animals (Table 1). Significant association of the marker S0007 was found in the both population (Table 3). On chromosomes 16 and 18 suggestive QTL were detected in DUMI population, additionally also significant associated alleles of markers S0061 and SWR414 were found. In the commercial population significant association of alleles of markers SWR414 on SSC18, was found, however non on SSC16 (Table 3). Even though the NPL-values calculated on SSC9, 10 and 13 being significant and highly significant at the chromosome-wide level they could not be defined as QTL according the definition of Lander and Kruglyak (1995). Significant associated alleles were only detected on the respective markers on SSC9 and 10. On SSC17 no significant QTL was detected.

Table 1 Evidence for QTL significant at 5 % chromosome-wide[1] and experiment-wide[2] level for the inverted teat defect in the DUMI population

SSC	NPL-value	p-value[1]	p-value[2]	closest marker to QTL	marker position[3]	flanking marker	
						upper	lower
1	4.43	≤ 0.01**	0.09	SW1301	141	S0155	-
2	4.56	≤ 0.01**	0.04*	SW2443	0	-	L144
3	3.9	≤ 0.01**	≤ 0.01**	S0164	61	SW72	NTAN1
4	5.2	≤ 0.01**	≤ 0.01**	S0097	120	S0214	-
5	3.83	≤ 0.01**	≤ 0.01**	SW1482	40	-	SW1134
6	2.81	≤ 0.01**	0.02*	S0035	7	-	S0087
6	9.25	≤ 0.01**	≤ 0.01**	S0220	78	S0300	S0059
7	2.63	≤ 0.01**	0.04*	S0101	135	S0115	-
8	3.41	≤ 0.01**	≤ 0.01**	SW2410	1	-	KIT1
9	2.35	≤ 0.01**	0.12	SW911	37	SW21	SW54
10	2.88	≤ 0.01**	0.02*	SW2067	128	ITIH2	-
11	3.35	≤ 0.01**	≤ 0.01**	S0386	60	S0071	SW703
12	6.7	≤ 0.01**	≤ 0.01**	S0143	6	-	SW874
13	1.92	0.02*	0.35	SW398	79	SW344	IL12A
14	3.78	≤ 0.01**	≤ 0.01**	S0007	60	MBL2	VIN
15	2.44	≤ 0.01**	0.08	SW936	89	SW1111	SW1119
16	3.14	≤ 0.01**	0.01*	S0061	93	IL12B	-
17	0.64	0.23	0.99	SW2431	94	SW840	-
18	3.23	≤ 0.01**	0.02*	SWR414	58	SW787	-

*: significant at 5 % level, **: significant at 1 % level, [1]chromosome-wide, [2]experiment-wide, [3]position of marker closest to QTL on public linkage map (USDA-MARCv2).

Table 2 Evidence for QTL significant at 5 % chromosome-wide[1] and experiment-wide[2] level for the inverted teat defect in the commercial dam lines

SSC	NPL-value	closest marker to QTL	marker position[3]	flanking marker		p-value all[#]		DL[#]		DL×DE / DE×DL[#]	
				upper	lower	chr[1]	exp[2]	chr[1]	exp[2]	chr[1]	exp[2]
1	0.69	SW1301	141	S0155	-	0.11	0.48	0,21	0,75	0,18	0,69
2	0.61	SW1564	55	SW240	SW834	0.15	0.96	0,4	0,99	0,05*	0,62
3	1.16	S00164	61	SW72	SW2570	0.02*	0.36	0,56	0,99	0,05*	0,63
4	1.06	S0214	120	S0214	SW857	0.02*	0.33	0,07	0,69	0,09	0,78
5	1.45	SW967	146	IGF1	-	0.07	0.75	0.28	0.99	0.01*	0.19
6	1.16	S0035	7	-	S0087	0.02*	0.16	0,18	0,87	0,07	0,52
6	1.64	S0059	93	S0220	S0003	≤ 0,01**	0.02*	0,03*	0,27	0,05*	0,41
7	0.77	S0101	135	S0115	-	0.1	0.75	0.17	0.92	0.13	0.84
8	0.9	S0086	62	SW2611	S0144	0.06	0.8	0.06	0.79	0.02*	0.79
9	0.36	SW54	67	SW911	S0109	0.33	0.99	0.19	0.97	-	-
10	0.64	SW830	0	-	S0070	0.14	0.92	0.07	0.73	0.29	0.99
11	1.95	SW703	76	S0009	-	≤ 0,01**	0.24	0.04*	0.83	0.05	0.90
12	0.74	SW874	65	GH	SW605	0.21	0.99	-	-	0.17	0.99
13	0.73	SW344	35	S0219	PIT1	0.2	0.97	0.42	0.99	0.04*	0.44
14	0.51	SW857	7	-	S0007	0.21	0.99	0,12	0,99	0,43	0,99
15	0.77	SW936	89	SW1111	SW1119	0.08	0.99	0.15	0.99	0.24	0.99
16	0.5	S0111	0	-	S0026	0.20	0.99	0.31	0.99	0.28	0.99
17	0.7	SW840	49	SW335	GHRH	0.12	0.85	0.19	0.96	0.13	0.89
18	0.17	SW1023	32	-	LEP	0.37	0.99	0.29	0.99	0.42	0.99

[#]all= merged analysis of German Landrace and F1 (DL*DE/DE*DL), DL=German Landrace, DE=Large White, *: significant at 5 % level, **: significant at 1 % level, [1]chromosome-wide, [2]experiment-wide, [3]position of marker closest to QTL on public linkage map (USDA-MARCv2).

Table 3 Overview of results including markers at positions of QTL detected either in the DUMI population or the commercial dam lines or both

SSC	Marker	QTL (Genehunter) NPL value		Association study (FBAT)					
				significant allele†		Z value+		p value	
		DUMI	comm	DUMI	comm	DUMI	comm	DUMI	comm
1	SW1301	4.43*	0.69	161[1]	.	7.197	.	≤ 0.001	.
				163[3]	.	5.604	.	≤ 0.001	.
				165[2]	.	3.757	.	≤ 0.001	.
				171[2]	171	1.897	2.425	0.058	0.015
2	SW2443	4.56*	0.43	203[3]	.	2.455	.	0.014	.
				207[1]	207	2.568	1.866	0.010	0.062
3	S0164	3.9*	1.16#	214[3]		2.496		0.013	
				218[2]		2.958		0.003	
				266[3]	ns	2.395	ns	0.017	ns
				286[1]		2.475		0.013	
				302[1]		2.297		0.022	
4	S0097	5.2*	0.67	205[3]	148	7.19	3.888	≤ 0.001	≤ 0.001
				212[1]	170	1.972	3.458	0.049	≤ 0.001
				234[2]	172	7.296	2.513	≤ 0.001	0.012
	S0058		1.06#		ns		ns		ns
5	SW1482	3.83*		108	ns	2.465	ns	0.014	ns
				110		2.21		0.027	
6	S0035	2.82~	1.16#	179	ns	3.007	ns	0.003	ns
	S0059	0.83	1.64#		144		1.896		0.058
				ns	146	ns	2.419	ns	0.016
					154		2.121		0.034
	S0220	9.25*	0.1	146[3]	.	4.248	.	≤ 0.001	.
					148	.	2.530	.	0.011
				154[2]	.	4.9	.	≤ 0.001	.
8	SW2410	3.41*		ns	ns	ns	ns	ns	ns
11	S0386	3.35*		154	ns	2.037	ns	0.042	ns
				158		3.889		≤ 0.001	
	SW703	2.58	1.95#	127	.	3.741	.	≤ 0.001	.
				133	.	1.941	.	0.052	.
				.	135	.	2.236	.	0.025
				.	137	.	2.076	.	0.038
12	S0143	6.7*		155	ns	7.673	ns	≤ 0.001	ns
				157		7.013		≤ 0.001	
14	S0007	3.78*	0.24	158[3]	.	5.755	.	≤ 0.001	.
				174[2]	.	5.149	.	≤ 0.001	.
				.	177	.	2.041	.	0.041
				.	187	.	2.064	.	0.039
16	S0061	3.14*		166	ns	2.405	ns	0.016	ns
				170		1.899		0.058	
18	SWR414	3.23*	0.13	123	.	2.181	.	0.029	.
				147	.	1.882	.	0.06	.
				.	153	.	2.566	.	0.01

ns: not significant, * QTL according to the definition of Lander and Kruglyak (1995), i.e. NPL >3; ~ QTL reaching 5 % genome-wide significance; # QTL according to the chromosome-wide p-values of $p \leq 0.05$ in all animals of commercial population, + positive Z-scores indicate that alleles can be found more often in affected animals, † names of alleles derived from their length in bp, [1]origin breed Miniature pig, [2]origin breed Duroc, [3]allele inherited from both breeds.

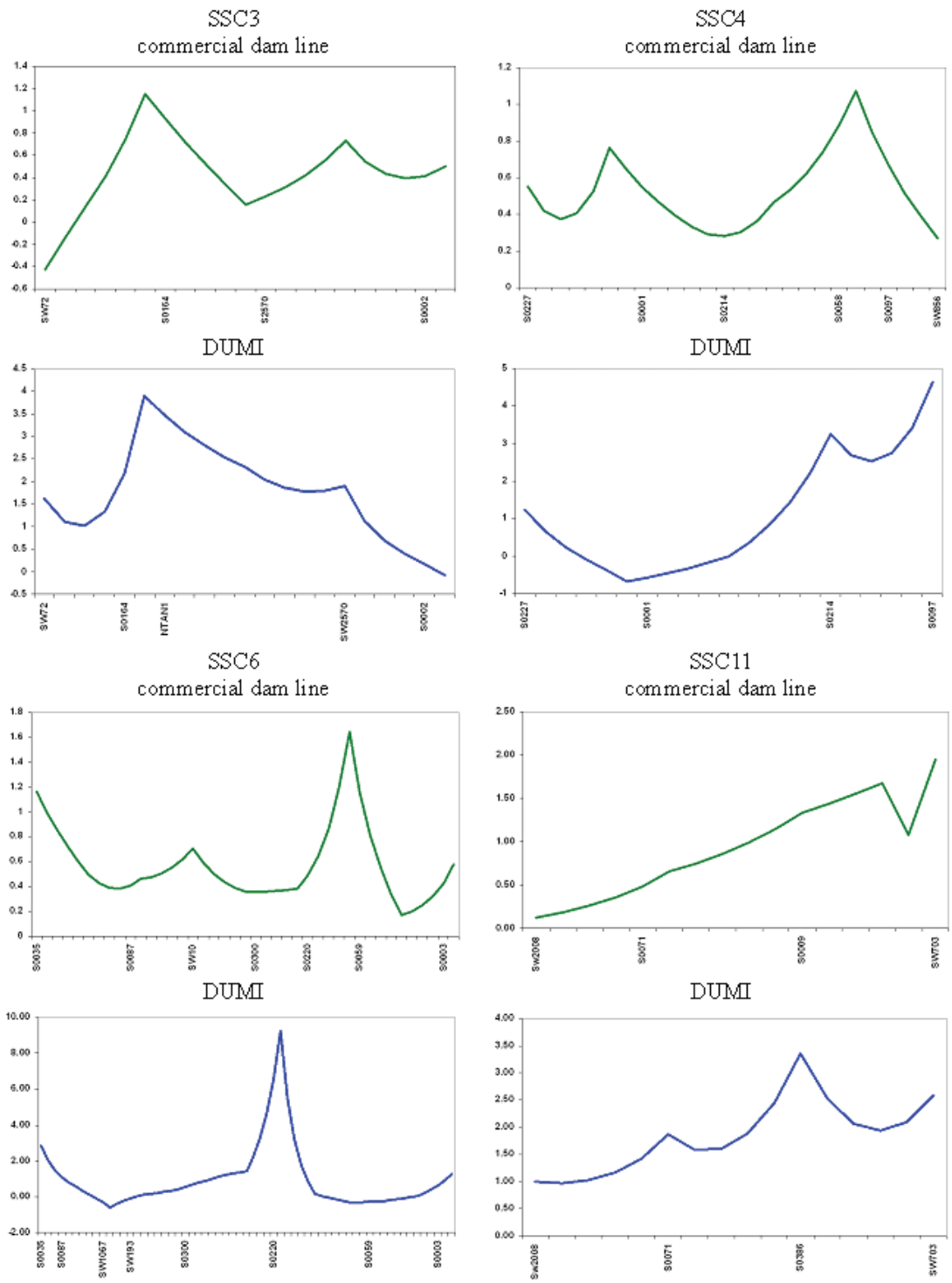


Figure 1 Profiles of multipoint NPL scores for SSC 3, 4, 6, and 11, respectively, obtained from QTL analysis in the experimental population `DUMI´ and commercial dam lines. Positions of markers genotyped are given at the x-axis; numbers at the y-axis indicate NPL values.

Discussion

A linkage study was performed for the heritable defect inverted teat in the pig in an experimental population showing a high incidence of the defect. In order to evaluate the relevance of the results in commercial pig breeds and to confirm the results the linkage study was also done in an independent set of animals of the breed German Landrace and its cross with Large White, that represent the most important dam lines in pig production in Germany. The study showed that the inverted teat defect is governed by genetic variation at several loci distributed throughout the genome. This is in agreement with more recent models of inheritance of the inverted teat defect that propose a polygenic background while early works suggested autosomal recessive inheritance with reduced penetrance (Mayer & Pirchner 1995; Nordby 1934). The emergence of inverted teats depends on insufficient mesenchymal proliferation at the teat ground during teat development (Günther 1984). As far as proliferation processes are concerned the defect of inverted teats is related to the trait of number of teats. Due to this etiological link of the traits QTL for inverted teats and number of teats can be expected to be partly identical. The number of teats may also depend on local signaling between adjacent embryonic tissues that determine the somite patterning and cell fate decisions leading to the establishment of the mammary bud but being less important for the liability to express the inverted teat phenotype. There were a few reports on QTL for the number of teats in the pig that are summarized in a public accessible database, PigQTLDB (Hu *et al.* 2005; Hu & Reecy 2007). Most studies used experimental population of crosses with the breed Meishan (Hirooka *et al.* 2001; Rodriguez *et al.* 2005; Rohrer *et al.* 1999; Yue *et al.* 2003). Meishan is known for its high number of teats of about 17.0 on average (Haley *et al.* 1995) compared to Large White breed with

about 14 teats (Clayton *et al.* 1981; Haley *et al.* 1995). On SSC1 QTL for number of teats were detected between marker SJ029 and SWR485 as well as between marker SW803 and SW373 in resource populations from different groups (Beeckmann *et al.* 2003b; Cassady *et al.* 2001; Geldermann *et al.* 2003; Rohrer *et al.* 1999; Wada *et al.* 2000). In the latter region with upper marker S0155 and peak at marker SW1301 a QTL for the inverted teat defect was detected in the experimental population in this study. Comparing the results from different QTL studies, we can conclude that there might be a number of genes influencing the teat development on chromosome 1 in pig. The relaxin gene (RLN) maps on SSC1 at position 1q28-q29 showing association with the inverted teat defect (Wimmers *et al.* 2002). RLN knockout mice were shown to exhibit impaired mammary development resulting in a phenotype similar to inverted teats that cannot be suckled by the offspring (Zhao *et al.* 1999). Another gene involved in teat development is the estrogen receptor (ESR) gene, in the region 1p24-p25 where QTL for number of teats was found (Wada *et al.* 2000). The ESR gene is already used as marker for the litter size in different porcine populations (Short *et al.* 1997). Even though no QTL could be detected in commercial families, SSC1 contains candidate genes for the development of functional teats. By the results of association analysis using the marker SW1301 on SSC1 more evidence was given corroborating this assumption. QTL for number of teats were located in the proximal region of SSC2 (Hirooka *et al.* 2001; Lee *et al.* 2003a) close to the QTL for inverted teats reported here. Positional candidate genes are insulin-like growth factor 2 (IGF2) and follicle-stimulating hormone beta (FSHB). Insulin-like growth factor-II (IGF2) is a growth factor and mediates prolactin induced in mammary gland development showing dose-dependent mitogenic properties on primary human breast epithelial cells and stroma cells (Hovey *et al.* 2003; Strange *et al.* 2002; Strange *et al.* 2004). The follicle-

stimulating hormone (FSH) is essential for normal reproductive function in males and females; it is more important for female than for male fertility (Tapanainen *et al.* 1997). It was also found in another study that the FSH gene plays a role for the morphological development of the mammary gland within the lactogenesis (McNeilly 1994). For SSC3 one QTL for number of teats is published at 84 cM with a F-statistics of 15.78 (Rohrer 2000). In the resource population a QTL in the same region next to marker S0164 was detected. The transforming growth factor alpha gene (TGFA) and the follicle-stimulating hormone receptor gene (FSHR) map to 3q22-q23 and 3q21, respectively (Remy *et al.* 1995). FSHR mediates effects of FSH on mammary development. Growth promoting TGFA and growth inhibiting TGFB are co-expressed in the bovine mammary gland. Higher mRNA contents of both factors during mammogenesis and involution may indicate autocrine or paracrine functions for these growth factors during proliferation and reorganisation of the mammary tissue (Plath *et al.* 1997). On SSC4 a QTL for the inverted teat defect was found in the commercial population where the thyroid stimulating hormone beta (TSHB) gene at 4q21 is a functional and positional candidate gene. The metabolic hormone TSH is necessary for alveolar morphogenesis in proliferation and lactogenesis of the mammary gland. Thyroid hormone is, as well as prolactin, an important regulator for the functional development of the mammary gland. Lower level of TSH in the serum leads to a loss of the ductal growth and a lower or lacking proliferation of alveolar tissue (Bhattacharjee & Vonderhaar 1984; Vonderhaar & Greco 1979). In studies of Lee and colleagues, a QTL for number of teats was detected between markers SWR453 and SW2425 on SSC5 (Lee *et al.* 2003b). In our study a high NPL-value for the heritable inverted teat defect was detected in the same region. The insulin-like growth factor-1 gene (IGF1) is suggested to be a candidate gene for the teat development in pig, but a microsatellite within IGF1 showed no association

to the inverted teat defect in this study. Another candidate gene, the parathyroid-hormone-like hormone gene (PTHLH); is found to be expressed on SSC5 in the stroma cell of the mammary gland. An overexpression of PTHLH in transgene mice lead to a mismatch of the gland and ductal growth in different stages (Dunbar *et al.* 2001; Wysolmerski *et al.* 1994; Wysolmerski *et al.* 1995). It was found that a C/T non-synonymous single nucleotide polymorphism (S19L) at nucleotide position 375 of the porcine PTHLH cDNA was associated with the inverted teat defect (Chomdej *et al.* 2004; Chomdej 2005). In this study two significant QTL in different regions on SSC6 could be detected. The NPL-value had its peak in the DUMI and the commercial population at the marker S0220 and S0059, respectively. Further in both populations significant NPL-value could be detected at marker S0035. A QTL for number of teats was detected in a three-generation resource population between marker DG93 and SW1328 with peak at 171 cM (Cassady *et al.* 2001). The result of linkage analysis in this study was partly verified by the association analysis in both populations. Our linkage analysis in experimental and commercial families highlight the telomeric region of SSC6 close to S0035 and the central region close to S0059 and S0220 as regions containing QTL for the inverted teat defect segregating in both populations. The comparative map of pig and human indicate synteny of QTL at S0035 with the telomeric q-arm of HSA16. S0220 and S0059 direct to HSA1 (Meyers *et al.* 2005). However, whereas the current status of the comparative map clearly shows correspondence of the genomic region of S0059 to HSA1, this still has to be proven for S0220 that may also correspond to HSA19. Establishment of this relationship will finally contribute to clarification whether the NPL-plot peaks at S0220 in the DUMI represents the same QTL as the NPL-plot peak at S0059 obtained in the dam lines. But anyway there were several genes already mapped on pig chromosome 6 being involved

in the development of the mammary gland. The transforming growth factor beta 1 (TGFB) gene at 6q11-q21 (directing to HSA19) and the leptin receptor (LEPR) gene at 6q33-q35 (directing to HSA1) are suggested to be candidate genes for the teat development. Leptin receptors have been also shown in mammary epithelial cells and it has been suggested that leptin is involved in the control of the proliferation of both breast cells (Dundar *et al.* 2005). Further candidate genes could be found being located in the comparative region between marker S0220 and SW59 such as the wingless-type MMTV integration site family, member 4 (WNT4) gene that is part of the early development of the mammary gland. The LIM domain only 4 (LMO4) gene was mapped at HSA1p22.3 a higher expression of this gene could be shown to suppress the differentiation of epithelial cell in mammary gland (Visvader *et al.* 2001). The impact of the protein tyrosine phosphatase, receptor-type, F (PTPTF or LAR) gene was shown in knockout mice, where female mice with gene deficient could not deliver milk. The LAR-mediated signaling may have an impact on development and function of mammary gland (Schaapveld *et al.* 1997). Another gene involved in the mammary gland development mapped on human chromosome 1 is the fatty acid-binding protein 3 (FABP3 or MDGI) also called mammary-derived growth inhibitor. It was the first gene being identified as growth inhibitor in the lactating bovine mammary gland (Bohmer *et al.* 1987). In summary, several positional and functional candidate genes could be identified, further studies need to be done to identify the exactly position of the QTL on SSC6 and compare it to the human genome map to detect the regions with the highest concordance in these two species. Several QTL for the number of teats have been found on SSC7 (Cassady *et al.* 2001; Wada *et al.* 2000). A QTL for the inverted teat defect on 7q25 close to marker S0101 was detected only in the experimental population. The prolactin (PRL) gene on 7p11-p12 is a functional candidate gene for teat characteristics.

PRL is involved in different physiological processes and a key factor for the development of mammary gland and lactation (Bole-Feysot *et al.* 1998). The activity of PRL over its receptor in the lactation is essential for the regulation of the metabolism of the adipose tissue (Ling *et al.* 2003). On SSC8 different QTL for number of teats were detected, suggesting that there may be different genes involved in the mammary gland development (Beeckmann *et al.* 2003a; Cassady *et al.* 2001; Hirooka *et al.* 2001; King *et al.* 2003). On SSC8 a QTL with peak at SW268 was suggested (King *et al.* 2003). The QTL between SW1070 and S0144 includes the marker S0086, where in our study a chromosome-wide significant NPL-value was detected in the commercial crossbreed population (Beeckmann *et al.* 2003a). A QTL for number of nipples in pigs between marker SY23 and SW905 was detected, where also a QTL with peak at marker SW2410 was detected in the DUMI population (Cassady *et al.* 2001). The relaxin receptor leucin-rich G-protein-coupled receptor 7 (LGR7) was mapped on human chromosome 4 in the comparative region of SSC8. LGR7 is likely to be involved in the development of the mammary gland and a candidate for the inverted teat defect, as its ligand already showed association to the defect (Chomdej 2005; Wimmers *et al.* 2002). RH mapping of LGR7 showed that this gene is located next to SW368. QTL for the number of nipples detected on SSC10 include regions between marker SW1708 and SW2067, marker SW1991 and SW1626, between marker SWR1849 and SW2000 and between marker SW1041 and SW951 (Dragos-Wendrich *et al.* 2003; Hirooka *et al.* 2001; Rodriguez *et al.* 2005; Rohrer 2000). It can be summarized from these studies that in the region between marker SWR1849 and SW2067 QTL for number of nipple were found, in DUMI population the peak of experiment-wide significant NPL-value was detected also at marker SW2067 (Table 1). There are no candidate genes proposed in these studies. The orthologous human genomic region on chromosome 10 harbours

calmodulin-like 3 protein (CALML3), a protein specifically expressed in mammary epithelial cells, that is regulated during differentiation and might be involved in Ca-dependent pathways during mammary development (Yaswen *et al.* 1992). The QTL with the highest peak at the marker S0386 on SSC11 could be confirmed in the commercial families. A QTL for the number of teats was found showing a F-value of 3.25 (Cassady *et al.* 2001). The breast cancer susceptibility gene (BRCA2) that is involved in the teat development is detected on SSC11 (Bignell *et al.* 1997; Musilova *et al.* 2000). QTL for the number of teats were detected in several experimental populations on different regions on SSC12 (Hirooka *et al.* 2001; Rodriguez *et al.* 2005; Yue *et al.* 2003). The detected region in the own study promotes the growth hormone to be as a positional candidate gene for the teat defect. Our detected significant and suggestive QTL in the experimental population on SSC14, 16 and 18 could not be confirmed in commercial population. Interestingly alleles of most significant marker S0007 on SSC14 were shown to be significant and highly significant associated to the defect status in both populations. Only one publication suggests a QTL region on SSCX in pig (Cepica *et al.* 2003). The androgen receptor gene (AR) was mapped to the X chromosome. This gene shows association to the number of teats but no association to the inverted teat defect could be detected (Trakooljul *et al.* 2004). In the present study no linkage mapping on SSCX was conducted. The mothering ability of sow plays an important role for the economic efficiency of pig production. Sows cannot grow up more piglets than they have functional teats. The teats characteristics are used as selection criteria in pig breeding, with typically a minimum of 12 to 14 functional teats and no defect teats requested. To detect the genetic cause of heritable defects, different approaches can be used. In our study the application of linkage analysis, which is a priori hypothesis-free, were chosen. The QTL analysis was performed in different

populations, an experimental population with high defect incidence to increase the power of this approach, and commercial populations to confirm results and to evaluate their relevance for commercial breeding. QTL regions in overlapping marker intervals or close proximity in both populations were found on SSC3, SSC4, SSC6, and SSC11. Further association analysis of each marker separately provided further evidence of regions being involved in the phenotype of inverted teat defect. For some regions with significant NPL-values in both populations or significant QTL in DUMI population significant association and linkage of different alleles to the defect could be shown by family-based association test. However, the genetic origin for the inverted teat defect pig is not detected yet. Future studies are necessary to detect genes that are involved in the development of the inverted teat defect. Combining these results with current attempts to identify genes expressed during mammary gland development will facilitate the detection of causal genes.

Acknowledgements

Funds provided by Development association biotechnology research (FBF) and the Federal Ministry of Education and Research (BMBF) supported this work.

4 Association of parathyroid hormone-like hormone (PTHLH) and its receptor (PTHR1) with the number of functional and inverted teats in pigs

Tetzlaff S, Chomdej S, Jonas E, Ponsuksili S, Murani E, Phatsara C, Schellander K, Wimmers K.

Published in Journal of Animal Breeding and Genetics 2009; 126: 237-241

Summary

Parathyroid hormone-like hormone gene (*PTHLH*) and its receptor, parathyroid hormone/ parathyroid hormone-like hormone receptor 1 (*PTHR1*), play a role in epithelial mesenchymal interactions during growth and differentiation of different tissues and anatomic structures, including teats. Therefore, *PTHLH* and *PTHR1* were evaluated as functional candidate genes for their effects on number and shape of teats in pigs. In particular, focus was on the occurrence and number of inverted teats, the most frequent and economically relevant teat developmental defect in pigs. For this purpose association and linkage of the *PTHLH* gene and the *PTHR1* gene with inverted teat defect and the total number of teats and inverted teats were studied in an experimental Duroc and Berlin Miniature pig (DUMI) population. Polymorphism C1819T of *PTHR1* was significantly associated with inverted teat phenotype (p 0.014), total number of teats (p 0.047) and was close to significance with number of inverted teats (p 0.078). Polymorphism C375T of *PTHLH* was close to significance with the inverted teat phenotype (p 0.122) and showed no significant association with total number of teats (p 0.621) and number of inverted teats (p 0.256) in the DUMI population. Association analyses were also performed for combined effects of *PTHLH* and *PTHR1* in order to address potential interaction, however, revealed no indication of effects of interaction. The function, the position, and the association shown here promote *PTHR1* as a candidate gene for number of teats and in particular for affection by and number of inverted teats.

Keywords
Family based association, inherited disorder, liability trait, mammary gland, mothering ability

Introduction

Many dam line breeding programs in pig populations focus on udder quality. In particular, the teat number and functional mammary gland capability are important selection criteria for increasing the survival rate of piglets. The most frequent and economically relevant inherited disorder of the mammary complex in pigs is the inverted teat, resulting in non-functional teats that cannot be suckled by the offspring. The defect occurs in commercial pig breeds with frequencies between 7.6 to 30 % (Brevern *et al.* 1994; Jonas *et al.* 2008; Mayer & Pirchner 1995; Niggemayer 1993). Wiesner and Willer (1978) considered a complex inheritance of the liability to develop the defect with the presence of a major gene and several minor genes causing this defect. However, the mode of inheritance and the number of genes involved are still unknown. Heritability was estimated between 0.2 and 0.5 (Mayer 1994). In a Duroc and Berlin Miniature pig (DUMI) population 53.6 % suffered from mammary gland abnormalities, 42.2 % had inverted teats and 17.9 % showed extra teats (Hardge *et al.* 1999).

Parathyroid hormone-like hormone gene (*PTHLH*) and the parathyroid hormone/ parathyroid hormone-like hormone receptor 1 (*PTHR1*) were shown to regulate epithelial mesenchymal interactions during the formation of mammary gland (Foley *et al.* 2001). Therefore *PTHLH* and *PTHR1* are functional candidate genes for traits related to mammary gland and teat development.

The aim of this study was to investigate the association and linkage of *PTHLH* and *PTHR1* gene polymorphisms with the inverted teat defect (Chomdej *et al.* 2004; Tetzlaff *et al.* 2007).

Materials and methods

Animals and Phenotypes

Animals (n 313) of an experimental population based on a reciprocal cross of Duroc and Berlin Miniature pig (DUMI population) were used. At 200 days of age two investigators of a team of four trained and skilled persons observed the phenotypes of the teat traits involved in this study. Animals were placed on their backs and teats were evaluated by inspection and palpation. Numbers of functional and inverted teats were recorded.

Genotypes

The *PTHLH* polymorphism analysis was performed by an allelic discrimination assay (Assays-by-DesignSM Service; ABI PRISM® 7000, PE Applied Biosystems) using primers PTHLP-FW: 5'-GAGCGTCGCGGTGTTC-3' and PTHLP-RW: 5'-AGCGCCCGCAGGAG-3' for PCR amplification and allele-specific probes PTHLP-V2: 5'-VIC-CTGAGCTATTCGGTGCC-TAMRA-3' and PTHLP-M2: 5'-FAM-CTGAGCTATTTGGTGCC-TAMRA-3' for discrimination of either C or T at nt 375 of PTHLH (GenBank accession no. AY193782) (Chomdej *et al.* 2004). *PTHR1* polymorphism analysis was conducted by restriction fragment length polymorphism assay. To amplify the region containing a *PTHR1* single nucleotide polymorphism (SNP) primers PTHR-F: 5'-GCTATGGTCCGATGGTGTCT-3' and PTHR-R: 5'-ACTGTCTCCCACTCCT CCTG-3' were used and PCR fragments were subsequently incubated with the restriction enzyme *Mbo*II (Fermentas) suitable to discriminate the C and the T allele at nt 1819 (GenBank accession no. NM_214382) (Tetzlaff *et al.* 2007). Amplification reactions were conducted in a final volume of 15 µl, containing 0.5 unit

of Taq DNA polymerase, 0.2 mM of each dNTP, 0.2 µM of each primer, 1 x buffer by manufacturer's instruction (GeneCraft) and 50 ng DNA.

Expression

In order to survey expression of porcine *PTHLH* and *PTHR1* genes in different tissues and organs quantitative reverse transcriptase PCR (qRT-PCR) was performed using the LightCycler® 480 system (Roche). Total RNA was isolated from epithelial teat tissue, connective teat tissue, liver, kidney, adrenal gland, spleen, tonsil, lymph node, muscle, hypothalamus and pituitary from adult pigs of German Landrace using Tri-Reagent (Sigma) and NucleoSpin® RNA II kit (Macherey-Nagel) including DNase treatment following manufactor's instructions. RNA samples were visualized on 1.5 % formaldehyde containing agarose gel to check the integrity and the concentration was measured by spectrometry with a NanoDrop® ND-1000 spectrophotometer (PEQLAB). First-strand cDNA was synthesized from 1µg of total RNA using random primers and oligo (dT)13 N in the presence of Superscript™ III reverse transcriptase (Invitrogen). The primers for *PTHLH*, *PTHR1* and ribosomal protein L32 (*RPL32*) as an internal reference were described in Table 1. Reactions were performed in a final volume of 12 µl using 6.0 µl of LightCycler® 480 SYBR Green I Master (Roche), 600 nM of each primer and 100 ng cDNA. Amplification conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, annealing temperature (Table 1) for 10 sec and 72 °C for 15 sec. At the completion of the amplification protocol, all samples were subjected to a melting curve to verify the absence of any non-specific products.

Table 1 Gene-specific primers (5´-3´) used for porcine *PTHLH* and *PTHR1* gene amplification in qRT-PCR, *RPL32* was treated as internal reference

Primer set	Sequence	Annealing (°C)	Product size (bp)
PTHLH	GCAAGGAGCAGGAAAAGAAG AGAGCAATGGGGAGACAGTT	64	261
PTHR	TGACCTTCTTCCTTTACTTCCTG GCTCTCACACTGACCCACAC	60	178
RPL32	AGCCCAAGATCGTCAAAAAG TGTTGCTCCCATAACCAATG	60	165

Statistics

In order to evaluate the genes for association and linkage with total number of teats, number of inverted teats and the affection status the Family-Based Association Test was used in the experimental DUMI population (FBAT, http://www.biostat.harvard.edu/~fbat/default.html). The quantitative association analyses were done in bi-allelic tests under the condition of an additive genetic model (Horvath *et al.* 2001). A statistical evaluation of the interaction between *PTHLH* and *PTHR1* gene polymorphisms and their influence on inverted teat trait was executed with *LOGISTIC* procedure of the SAS software package 9.1 (SAS Institute, Cary, NC, USA) using father, mother and gender as additional explanatory variables of interest in the model.

Results

Genotype analysis

Two non-synonymous C>T SNPs were detected at nucleotide position 375 (S19L) of the porcine *PTHLH* cDNA and at nucleotide position 1819 (L556F) of the porcine *PTHR1* cDNA (Chomdej *et al.* 2004; Tetzlaff *et al.* 2007). Mendelian inheritance of these polymorphic sites was monitored in individuals of the experimental DUMI population. The frequencies of genotype combinations are presented in Table 2.

Table 2 *PTHR1/PTHLH* genotype combinations at C1819T/C375T and their frequencies in F2 DUMI resource population (n 200)

Affection	Genotype combinations n (%)									Significance
PTHR1	CC	CC	CC	CT	CT	CT	TT	TT	TT	
PTHLH	CC	CT	TT	CC	CT	TT	CC	CT	TT	
IT	5 (2.5)	19 (9.5)	3 (1.5)	7 (3.5)	18 (9.0)	12 (6.0)	2 (1.0)	6 (3.0)	3 (1.5)	n. s.
NIT	11 (5.5)	20 (10.0)	10 (5.0)	20 (10.0)	32 (16.0)	10 (5.0)	7 (3.5)	10 (5.0)	5 (2.5)	n. s.

IT, affected at least 1 inverted teat observed; NIT, none affected - no inverted teat observed; n (%) number and percentage of observed genotype combinations, combined genotypes of alleles at C1819T and C375T.

Abundance of genotype combination was not significantly different between IT and NIT.

Expression of porcine *PTHLH* and *PTHR1* genes

The qRT-PCR of 11 tissues including epithelial and connective teat tissue from adult pigs indicated differential expression of porcine *PTHLH* and *PTHR1* genes as shown in Figure 1. The *PTHLH* gene was expressed in multiple tissues thereby clearly detectable in epithelial and connective teat tissue. A lower expression was detected in kidney and adrenal gland. The expression pattern of *PTHR1* gene revealed that liver and kidney were the major sites of expression. Low abundance was observed in muscle and pituitary.

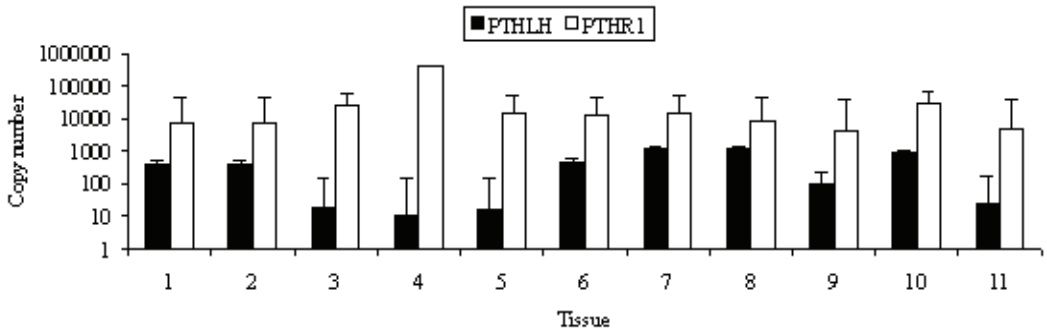


Figure 1 Tissue-specific expression pattern of *PTHLH* and *PTHR1* genes assayed by qRT-PCR. Copy number of *RPL32* gene was measured to normalize for equal RNA amounts.1, epithelial teat tissue; 2, connective teat tissue; 3, liver; 4, kidney; 5, adrenal gland; 6, spleen; 7, tonsil; 8, lymph node; 9, muscle; 10, hypothalamus; 11, pituitary.

Association with inverted teat trait

Association analyses were first performed separately for each locus. The results of SNPs C375T of *PTHLH* and C1819T of *PTHR1* with inverted teat as the affection trait are summarized in Table 3. SNP C1819T of *PTHR1* gene was significantly associated with total teat number (p 0.047), the affection trait (p 0.013) and close to

significance with total inverted teat number (p 0.078). SNP C375T of *PTHLH* showed no significant association with total teat number (p 0.621) and total inverted teat number (p 0.256) but was close to significance with inverted teat as the affection trait (p 0.122).

Table 3 Association of *PTHLH* and *PTHR1* with the affection by inverted teats (Aff), total number of inverted teats (TIT) and total number of teats (TT) in the DUMI resource population as revealed by FBAT analysis

	allele	Freq	Aff	TT	TIT
Marker			*p*	*p*	*p*
PTHLH *(C375T*)	C	0.5	0.122	0.621	0.256
PTHR1 (C1819T)	C	0.6	0.013	0.047	0.078

Discussion

This investigation is the first to attend to association and linkage of *PTHLH* and its receptor *PTHR1* with the inverted teat defect in pigs. The emergence of inverted teats depends on insufficient mesenchymal proliferation at the teat ground during teat development. As far as proliferation processes are concerned the defect of inverted teats is related to the trait of number of teats. The number of teats may also depend on local signaling between adjacent embryonic tissues (Jonas *et al.* 2008). Foley et al. (2001) elucidated the influence of *PTHLH* and *PTHR1* on the regulative interaction of epithelial mesenchymal proliferation during the formation of the mammary gland. Prior experiments with knockout mice indicated involvement of *PTHLH* in the ontogenesis of a functional mammary gland, because these mice developed the inverted teat phenotype (Dunbar *et al.* 2001; Wysolmerski *et al.* 1998). Furthermore the expression results of both genes in relevant tissues for the inverted teat defect approved the results reported in other species (Kobayashi *et al.* 2005; Kong *et al.* 1994). Thus *PTHLH* and *PTHR1* genes were contemplated as functional positional candidate genes for the inverted teat phenotype. Our results for the SNP C1819T in *PTHR1* have revealed a significant association and linkage to teat number as well as occurrence of inverted teats. However, association with teat number as well as with inverted teat defect and the SNP C375T in *PTHLH* could not be verified. Furthermore the amino acid exchanges of both SNPs were analyzed by SIFT (sorting intolerant from tolerant; http://blocks.fhcrc.org/sift/SIFT.html). SIFT is based on the premise that important amino acids will be conserved among sequences in a protein family, so changes at amino acid conserved in the family should affect protein function (Ng & Henikoff 2002). Thus, phylogenetic relationships do not promote functional impact of the amino

acid exchanges. However, the amino acid exchanges are between polar (serine) and neutral (leucine) and neutral (leucine) and aromatic (phenylalanine) amino acids, respectively, therefore impact on structural and biochemical properties of the proteins cannot be ruled out. For *PTHLH* the polymorphism is located in its signal peptide that directs post-translational transport. The SNP at nt 1819 of *PTHR1* is located in its intracellular tail, which is involved in signal transduction. SNPs in coding regions of the genes are not likely to affect their expression. Any functional effects have to be confirmed. When analyzing genes of hormones and their receptors effects of interaction can be expected, however no interaction between *PTHLH* and *PTHR1* genotypes were observed. That might be due to the limited number of animals. Quantitative trait loci (QTL) for teat traits on Chromosome 5 (SSC5) and 13 (SSC13) have been identified by several authors (Jonas *et al.* 2008; Lee *et al.* 2003b; Yue *et al.* 2003). Although *PTHLH* and *PTHR1* were displayed in close proximity to these QTL (Chomdej *et al.* 2004; Tetzlaff *et al.* 2007), only the SNP in *PTHR1* gene likely is a marker in close linkage disequilibrium to a causative polymorphism affecting teat number and liability for the inverted teat defect; that gene even represent good candidate for the causal polymorphism. The SNPs of both genes were found to segregate among pigs of commercial breeds, German Landrace, Large White, Pietrain, Hampshire and Duroc, with allele ‘C’ at both SNPs being the prominent one (Chomdej *et al.* 2004; Tetzlaff *et al.* 2007). Effects of both genes on teat phenotypes but also on other traits will be evaluated in animals of commercial dam lines in order to further qualify the genes as functional positional candidate genes in a gene assisted selection. Further analyses of trait association in other populations as well as functional assays will provide more insight into the causal nature of the polymorphisms.

Acknowledgements

The authors would like to thank the Federal Ministry of Education and Research (BMBF) and the Förderverein Biotechnologieforschung der Deutschen Schweineproduktion e.V. (FBF) for financial support (FUGATO - HeDiPig, FKZ 0313392A). The experiments described in this study were performed in accordance with all appropriate regulations.

5 Evidence for association of lymphoid enhancer-binding factor-1 (*LEF1*) with the number of functional and inverted teats in pigs

Tetzlaff S, Jonas E, Phatsara C, Murani E, Ponsuksili S, Schellander K, Wimmers K.

Published in Cytogenetic and Genome Research 2009; 124: 139-146

Abstract

The lymphoid enhancer-binding factor-1 (*LEF1*) belongs to a family of regulatory proteins that share homology with the high mobility group protein-1 (*HMG1*). The *LEF1* gene is a mediator in the canonical Wnt-signaling pathway required for morphogenesis of early mammary gland during embryogenesis. Here we describe the molecular characterization of porcine *LEF1* gene and its association with number of teats and inverted teats in experimental and commercial populations. The 2357 bp cDNA sequence contains an 1197 bp open reading frame encoding a protein of 398 amino acids. The porcine *LEF1* protein shares high identity with other mammalian *LEF1*. The *LEF1* gene contains 12 exons and maps to pig chromosome 8 (SSC8). We identified two single nucleotide polymorphisms (SNPs), a T1351C transition and an A1666C transversion, in the 3′ end of *LEF1*. SNP A1666C was highly significant associated with presence of infected teats ($p \leq 0.01$), total number of teats ($p \leq 0.01$) and total number of inverted teats ($p \leq 0.01$); SNP T1351C was close to significance with total number of inverted teats ($p \leq 0.1$) in the experimental DUMI population. SNP T1351C was significantly associated with total number of inverted teats (p 0.04) and close to significance with affected teats (p 0.06) in commercial populations. Haplotype analysis confirmed the tendency towards association with affected teats (p 0.06) in the experimental DUMI population. The function, the position, and the association shown here promote *LEF1* as a candidate gene for number of teats and in particular for presence and number of inverted teats.

Introduction

In dam line breeding programs in pig populations udder quality is a selection criterion addressed in order to maintain and improve mothering ability. In particular, the teat number and functional mammary gland capability are important selection criteria for increasing the survival rate of piglets. The most frequent and economically relevant inherited disorder of the mammary gland in pigs is the inverted teat, resulting in non-functional teats that cannot be suckled by the offspring. The defect occurs in commercial pig breeds with frequencies between 7.6 to 30 % (Brevern *et al.* 1994; Jonas *et al.* 2008; Mayer & Pirchner 1995; Niggemayer 1993). Several authors considered a complex inheritance of the liability to develop the defect with the presence of a major gene and several minor genes causing this defect in the embryonic mammary gland development (Günther *et al.* 1985; Wiesner & Willer 1978). However, the mode of inheritance and the number of genes involved are still unknown. Heritability was estimated between 0.2 and 0.5 (Mayer 1994). In an experimental Duroc and Berlin Miniature pig (DUMI) population 53.6 % suffered from mammary gland abnormalities, 42.2 % had inverted teats and 17.9 % showed misplaced teats (Hardge *et al.* 1999). Embryonic mammary gland development is dependent on reciprocal inductive interactions between the mesenchyme and overlying ectoderm (Boras-Granic *et al.* 2006; Foley *et al.* 2001; Pispa & Thesleff 2003; Robinson *et al.* 1999; Sakakura *et al.* 1987). Signals originating in the mesenchyme have been proposed to specify ectodermal cells to form epithelial mammary placodes (Sakakura 1987). Reciprocal signaling from the placodes to the underlying mesenchyme then directs condensation of fibroblasts that form the mammary mesenchyme. Maintenance of these reciprocal

signals stimulates further proliferation and differentiation of the placodes resulting in the development of the ductal mammary gland (Boras-Granic *et al.* 2006).

The lymphoid enhancer-binding factor-1 is one of the earliest known regulators involved in formation of the mammary gland (Boras-Granic *et al.* 2006; Mailleux *et al.* 2002; Vangenderen *et al.* 1994). *LEF1* is an architectural protein chaperoning a number of distinct factors controlling transcription of target genes. In the context of the canonical Wnt-signaling cascade *LEF1*-ß-catenin-containing complexes, resulting in activated transcription of Wnt-target genes, replace complexes of *LEF1* and the transcriptional repressors (Behrens *et al.* 1996; Boras-Granic *et al.* 2006).

The canonical Wnt-signaling pathway is important for placodes induction and differentiation of mammary epithelium (Hennighausen & Robinson 2001). A role of Wnt-induced signaling in mammary bud development is supported by studies of transgenic mouse embryos (Chu *et al.* 2004; Veltmaat *et al.* 2004). During the early stages of mammary development, *LEF1* is expressed in epithelial cells of the mammary bud and is subsequently induced in the mesenchyme surrounding each bud during embryogenesis (Foley *et al.* 2001). Induction of *LEF1* expression in the mammary mesenchyme is dependent on paracrine signaling from the mammary epithelium by the parathyroid hormone-like hormone and the parathyroid hormone/ parathyroid hormone-like hormone receptor 1 (Foley *et al.* 2001). Defective mammopoeisis arises in *LEF1*-deficient animals; embryos initially exhibit a reduced number of mammary buds and aborted development of those that do form (Boras-Granic *et al.* 2006; Vangenderen *et al.* 1994). Therefore *LEF1* is a functional candidate gene for traits related to mammary gland and teat development.

The aim of this study was to characterize the porcine *LEF1* gene and perform the association and linkage analysis of identified polymorphisms with the inverted teat defect.

Materials and methods

The nucleotide sequence reported in this article has been submitted to GenBank (accession number EU719607).

cDNA characterization

A BLAST search using the human *LEF1* mRNA sequence (GenBank accession number NM_016269) identified overlapping porcine expressed sequence tags (CX063890, BP162088, AJ958238 and AJ957434). These ESTs were concatenated and gene specific primers were designed (Table 1). Amplification reactions were conducted in a final volume of 15 µl, containing 0.5 unit of Taq DNA polymerase (GeneCraft), 0.2 mM of each dNTP, 0.2 µM of each primer, 1 x reaction buffer (GeneCraft), and 100 ng of liver cDNA. The cycling protocol was 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 sec, annealing temperature (Table 1) for 30 sec and 72 °C for 1 min, and a final extension step at 72 °C for 5 min. The PCR products were checked on a 1.5 % agarose gel, purified with the NucleoSpin Extract II kit (Macherey-Nagel) and bi-directional sequenced using the BigDye Terminator Cycle Sequencing kit (version 1.1) and an ABI Prism™ 310 Genetic Analyser (Applied Biosystems). The PCR primers described in Table 1 were also used for SNP screening in 5 pig breeds (Duroc, Hampshire, German Landrace, Pietrain and Berlin-Miniature pig).

Annotation of the *LEF1* exons on the porcine cDNA sequence was done using the human and murine *LEF1* mRNA sequence (NM_016269, NM_010703) and the Evidence Viewer tool (http://www.ncbi.nlm.nih.gov/sutils/evv.cgi/) for cDNA cDNA sequence comparisons. Translation of the cDNA sequence and analysis of the protein sequence were done with the Open Reading Frame Finder tool

(http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein sequence alignments were performed with the MultAlign analysis tool (http://bioinfo.genopole- toulouse. prd.fr/multalin/multalin.html).

Table 1 Primers used for amplification of porcine *LEF1* cDNA in this study

Exon	Primer	Sequence (5′-3′)	Annealing (°C)	Product size (bp)
1 – 2	LEF 1	CCTTCCCTTCCAATTCTCCT ACCTCGTGTCCGTTGCTC	60	322
1 – 4	LEF 2	GATTACAGAGTGGTCGGGATG GTGATGAGGGGAGTGAGAGG	60	491
2 – 7	LEF 3	AACACCCTGATGACGGAAAG ATGAGGGATGCCAGTTGTGT	60	515
5 - 10	LEF 4	TGGACAGATTACCCCACCTC TTTCTCTCTCTTCCTCTTCTTTTTC	60	536
9 – 12	LEF 5	TTAGCACGGAAGGAAAGACA GCAGAGACAGGGGAGGAAAG	60	476
12	LEF 6	GCAGCTTTCCTCCCCTGT CAATCGTTAGATGACAGTTTGG	60	799

Chromosome localization of the LEF1 gene

The physical mapping of *LEF1* was performed using the INRA-University of Minnesota porcine radiation hybrid panel (IMpRH). The chromosomal assignment was calculated using *twopoint* and *multipoint* analysis option of the IMpRH mapping tool (http://www.toulouse.inra.fr). PCR used for mapping was conducted in a total volume of 20 µl containing 0.5 unit of Taq DNA polymerase (GeneCraft), 0.2 mM of each dNTP, 0.2 µM of each primer (5′-TGGAAAACGGAGCTCATT-3′ and 5′-GCAGAGACAGGGGAGGAAAG-3′), 1 x reaction buffer (GeneCraft), and 50 ng panel DNA. The PCR protocol consisted of 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 sec, 60 °C for 30 sec and 72 °C for 1 min, and a final extension step at 72

°C for 5 min. The PCR products were visualized on a 2 % agarose gel. The linkage map including the *LEF1* gene was calculated with the CRIMAP 2.4 software package using the *build* and *twopoint* analysis option (Washington University School of Medicine, St. Louis, MO, USA) and 19 families (n 200) of the experimental DUMI resource population that were segregating at the SNP A1666C.

Expression

In order to survey differential expression of porcine *LEF1* gene quantitative reverse transcription PCR (qRT-PCR) was performed using the LightCycler 480 system (Roche). Total RNA was isolated from epithelial and connective teat tissue from affected and none affected animals (n 30) of German Landrace in full sib pair design and additionally from epithelial teat tissue, connective teat tissue, liver, kidney, adrenal gland, spleen, tonsil, lymph node, muscle, hypothalamus and pituitary from adult pigs of German Landrace using Tri-Reagent (Sigma) and NucleoSpin RNA II kit (Macherey-Nagel) including DNase treatment following manufacturer's instructions. RNA samples were visualized on 1.5 % formaldehyde containing agarose gel to check the integrity and the concentration was measured by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB). First-strand cDNA was synthesized from 1µg of total RNA using random primers and oligo (dT)13 N in the presence of Superscript III reverse transcriptase (Invitrogen). The ribosomal protein L32 (*RPL32*) was treated as an internal reference. Reactions were performed in a final volume of 12 µl using 6.0 µl of LightCycler 480 SYBR Green I Master (Roche), 600 nM of each primer (LEF_up: 5´-AAA GAC AAT CAC TGC CAA ACC-3´, LEF_dw: 5´-GCA GAC AGA AAC GGA GAG GA-3´, RPL_up: 5´- AGC CCA AGA TCG TCA AAA AG-3´ and RPL_dw: 5´-TGT TGC TCC CAT AAC CAA TG-3´) and 100 ng cDNA. Amplification conditions

were 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, 60 °C for 10 sec and 72 °C for 15 sec. At the completion of the amplification protocol, all samples were subjected to a melting curve to verify the absence of any non-specific products.

Animals and Phenotypes

Animals derived from the `DUMI´ three generation F2 resource population by reciprocal crossing of Berlin Miniature Pig and Duroc (Hardge *et al.* 1999). At 200 days of age animals (n 375) of the DUMI resource population were placed on their backs and teats were evaluated by inspection and palpation by two investigators. Numbers of functional and inverted teats were recorded. To validate the results obtained in the experimental population, animals of commercial herds (n 458 animals, comprising of 130 full sib families with at least one affected offspring (n 147)) were used. Samples of German Landrace and German Large White pigs as well as their crossbreeds were collected from a performance test station and the teat traits were recorded in the slaughterhouse.

Genotyping

The *LEF1* polymorphism analysis of T1351C was performed by a Pyrosequencing assay (Pyrosequencing™ Assay Design Software; PSQ™HS 96A, Biotage AB) using primers 5´-Biotin-GAC CCC GGG ACC TCT TCT-3´ and 5´-GCA GTG ACC TCA GGG TGA AG-3´ for PCR amplification and 5´-CCA TGG AGA TGG GCC-3´ as sequencing primer. *LEF1* polymorphism A1666C was detected by restriction fragment length polymorphism assay. To amplify the region containing *LEF1* SNP A1666C primers 5´-GCA GCT TTC CTC CCC TGT-3´ and 5´-GCA GAC AGA AAC GGA GAG GA-3´ were used and PCR fragments were subsequently incubated with the

restriction enzyme *Eco*24I (Fermentas) suitable to discriminate the A and the C allele at nucleotide position 1666 and visualized in a 2 % agarose gel. Naming of both SNPs refers to the position in the cDNA sequence of *LEF1* (GenBank accession number EU719607).

Statistics

In order to evaluate the genes for association and linkage with total number of teats, total number of inverted teats, and the affection status, i.e. the presence or absence of inverted teats the Family-Based Association Test was used in the experimental and the commercial population. Analysis was done for both SNP separately and for the corresponding haplotypes in the experimental population applying FBAT and HBAT, accordingly (FBAT, http://www.biostat.harvard.edu/~fbat/default.html). The quantitative association analyses were done in bi-allelic tests under the condition of an additive genetic model (Horvath *et al.* 2001). Frequencies of genotypes and haplotypes were calculated using Proc 'freq' and 'haplotype' of the SAS software package 9.1 (SAS Institute, Cary, NC, USA). In the commercial population only SNP *T1351C* was segregating.

Results

cDNA and protein characterization

The *LEF1* cDNA is composed of 2357 bp, contains an 1197-bp open reading frame encoding a protein of 398 amino acids with a molecular mass of 44.2 kDa and an isoelectric point of 6.9; flanked by a 62-bp-long 5´UTR and 1098 bp of 3´UTR. Using the human reference cDNA for cDNA cDNA sequence comparisons we deduced that the porcine *LEF1* gene consists of 12 exons in agreement with human and murine *LEF1* exon structure that conform perfectly to the GT/AG rule. The deduced amino acid porcine *LEF1* sequence displays perfect alignment with the human; mouse and rat *LEF1* with 98, 98 and 97 % sequence identity, respectively (Fig. 1).

Chromosomal mapping

The *LEF1* gene was assigned to SSC8 by multipoint analysis using IMpRH panel (Vector: 1101100011 1100000000 0110000001 0110000010 0000000100 1000011001 0000001000 0011000001 0101000000 0000010100 1100001111 11001100). The results of twopoint analysis suggest the gene in close proximity to marker *SW1337* (26cR; LOD 14.47) presented in Figure 2. This assignment was further confirmed by linkage mapping of the loci *SW2410* (0 cM), *S0086* (48.3 cM), *LEF1* (87.9 cM), *S0144* (114.8 cM) and *SW61* (135.3 cM) on the sex-averaged map of SSC8 (Fig. 2).

```
mouse           MPQLSG  GGGGGDPELCATDEMIPFKDEGDPQKEKIFAEISHPEEEGDLADIKSSLVNE 58
rat             MPQLSG  GGGGGDPELCATDEMIPFKDEGDPQKEKIFAEISHPEEEGDLADIKSSLVNE 58
pig             MPQLSGA GGGGGDPELCATDEMIPFKDEGDPQKEKIFAEISHPEEEGDLADIKSSLVNE 59
human           MPQLSGGGGGGGGDPELCATDEMIPFKDEGDPQKEKIFAEISHPEEEGDLADIKSSLVNE 60
                ******  ***************************************************

mouse           SEIIPASNGHEVVRQAPSSQEPYHDKAREHPDEGKHPDGGLYNKGPSYSSYSGYIMMPNM 118
rat             SEIIPASNGHEVVGQTQSSQEPYHDKAREHPDDGKHPDGGLYNKGPSYSSYSGYIMMPNM 118
pig             SEIIPASNGHEVARQAQSSQESYHDKAREHPDDGKHPDGGLYNKGPSYSSYSGYIMMPNM 119
human           SEIIPASNGHEVARQAQTSQEPYHDKAREHPDDGKHPDGGLYNKGPSYSSYSGYIMMPNM 120
                ************. *: :***.**********:**************************

mouse           NSDPYMSNGSLSPPIPRTSNKVPVVQPSHAVHPLTPLITYSDEHFSPGSHPSHIPSDVNS 178
rat             NSDPYMSNGSLSPPIPRTSNKVPVVQPSHAVHPLTPLITYSDEHFSPGSHPSHIPSEVNP 178
pig             NNDPYMSNGSLSPPIPRTSNKVPVVQPSHAVHPLTPLITYSDEHFSPGSHPSHIPSDVNS 179
human           NNDPYMSNGSLSPPIPRTSNKVPVVQPSHAVHPLTPLITYSDEHFSPGSHPSHIPSDVNS 180
                *.*******************************************************:**.

mouse           KQGMSRHPPAPEIPTFYPLSPGGVGQITPPIGWQGQPVYPITGGFRQPYPSSLSGDTSMS 238
rat             KQGMSRHPPAPEMPTFYPLSPGGVGQITPPLGWQGQPVYPITGGFRQAYPSSLSGDTSMS 238
pig             KQGMSRHPPAPEIPTFYPLSPGGVGQITPPLGWQGQPVYPITGGFRQPYPSSLSVDTSMS 239
human           KQGMSRHPPAPDIPTFYPLSPGGVGQITPPLGWQGQPVYPITGGFRQPYPSSLSVDTSMS 240
                ***********::*****************:****************.****** *****

mouse           RFSHHMIPGPPGPHTTGIPHPAIVTPQVKQEHPHTDSDLMHVKPQHEQRKEQEPKRPHIK 298
rat             RFSHHMIPGPPGPHTTGIPHPAIVTPQVKQEHPHTDSDLMHVKPEHEQRKEQEPKRPHIK 298
pig             RFSHHMIPGPPGPHTTGIPHPAIVTPQVKQEHPHTDSDLMHVKPQHEQRKEQEPKRPHIK 299
human           RFSHHMIPGPPGPHTTGIPHPAIVTPQVKQEHPHTDSDLMHVKPQHEQRKEQEPKRPHIK 300
                ******************************************:***************

mouse           KPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHM 358
rat             KPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHM 358
pig             KPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHM 359
human           KPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHM 360
                ************************************************************

mouse           QLYPGWSARDNYGKKKKRKREKLQESTSGTGPRMTAAYI 397
rat             QLYPGWSARDNYGKKKKRKREKLQESTSGTGPRMTAAYI 397
pig             QLYPGWSARDNYGKKKKRKREKLQESTSGTGPRMTAAYI 398
human           QLYPGWSARDNYGKKKKRKREKLQESASGTGPRMTAAYI 399
                **************************:************
```

Figure 1 Alignment of different mammalian LEF1 proteins. The deduced amino acid sequence of the porcine LEF1 is shown aligned to that from human (*Homo sapiens*, GenBank accession number NP_057353), mouse (*Mus musculus*, NP_034833) and rat (*Rattus norvegicus*, NP_569113). The numbers represent the position of the amino acids in the respective protein sequences. Asterisks beneath the alignment indicate identical amino acid residues in all sequences, while colons and dots represent very similar and similar amino acids, respectively.

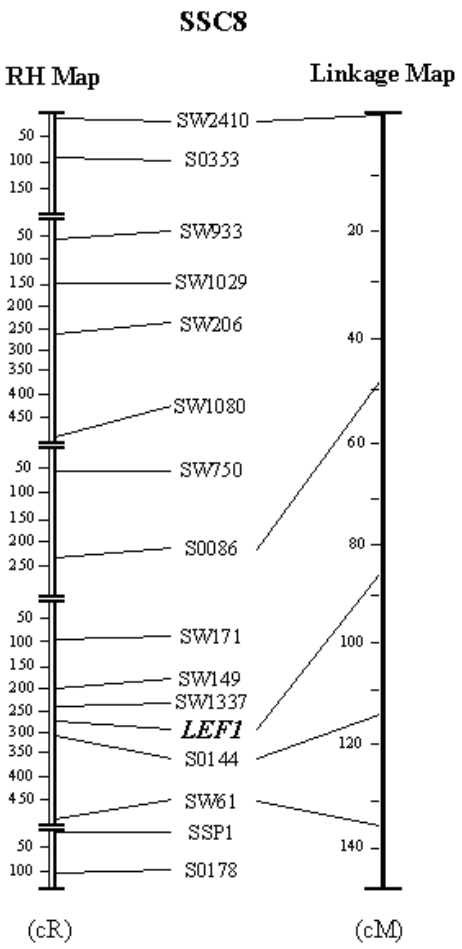


Figure 2 Comparative mapping of *Sus scrofa* chromosome 8. The RH map (in cR) provided by INRA Toulouse is compared to the linkage map (in Kosambi cM) obtained from the F2 DUMI resource population. Position of the *LEF1* gene is displayed in bold and italic letters.

Expression of porcine LEF1 gene

The qRT-PCR of 12 tissues including epithelial and connective teat tissue from adult pigs indicated differential expression of porcine *LEF1* gene as shown in Figure 3. The *LEF1* gene was expressed in multiple tissues thereby clearly detectable in epithelial and connective teat tissue. The expression pattern of *LEF1* gene revealed that lymph node and lymphocyte were the major sites of expression. Analysis towards phenotype dependent expression of *LEF1* yield to none significant differences in expression in epithelial and connective teat tissue of affected and none affected sibs (Fig 4).

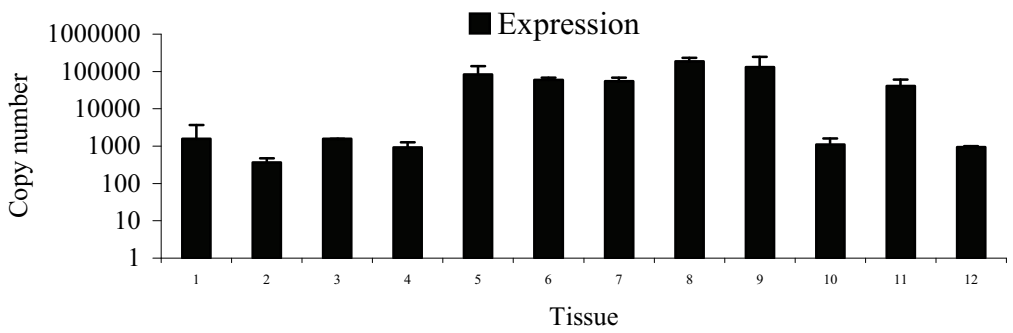


Figure 3 Tissue specific expression pattern of *LEF1* gene assayed by qRT-PCR. Copy number of *RPL32* gene was measured to normalize for equal RNA amounts.1, epithelial teat tissue; 2, connective teat tissue; 3, liver; 4, kidney; 5, adrenal gland; 6, spleen; 7, tonsil; 8, lymph node; 9, lymphocyte, 10, muscle; 11, hypothalamus; 12, pituitary.

Table 2 *LEF1* haplotypes at T1351C/A1666C and their frequencies in F2 DUMI resource population (n 200)

Population	Affection Status	Haplotypes (n (%))			Significance
		C-C	C-A	T-A	
DUMI	IT	15 (12.0)	41 (32.8)	69 (54.8)	n. s.
DUMI	NIT	15 (19.3)	26 (34.7)	34 (45.4)	n. s.

IT, affected with inverted teat; NIT, none affected with inverted teat; n., number of observations; haplotypes, haplotypes of alleles at T1351T and C1666A. Abundance of haplotypes for both groups was not significantly different.

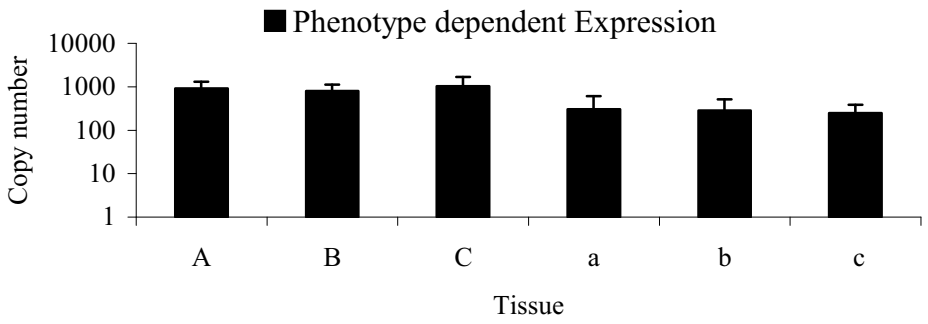


Figure 4 Phenotype dependent expression pattern of *LEF1* gene assayed by qRT-PCR. Copy number of *RPL32* gene was measured to normalize for equal RNA amounts. A, epithelial teat tissue from unaffected animals; B, epithelial teat tissue from normal teat of affected animals; C, epithelial teat tissue from inverted teats; a, connective teat tissue from unaffected animals; b, connective teat tissue from normal teat of affected animals; c, connective teat tissue from inverted teats.

Genotype analysis

Two SNPs were detected at nucleotide position 1351 (T > C) and at nucleotide position 1666 (A > C) in the 3′ end of the porcine *LEF1* cDNA. Mendelian inheritance of these polymorphic sites was monitored in individuals of the experimental DUMI population. The frequencies of haplotypes are presented in Table 2.

Association with teat traits

Association analyses were first performed separately for each locus. SNP A1666C was highly significant associated with the presence of inverted teats ($p \leq 0.01$), total number of teats ($p \leq 0.01$) and total number of inverted teats ($p \leq 0.01$); SNP T1351C was close to significance for the trait total number of inverted teats ($p \leq 0.1$) in the experimental DUMI population (Table 3). Analysis of the three haplotypes segregating in the experimental DUMI population confirmed the association with the presence of inverted teats at p 0.06 (Table 3). In the commercial population SNP T1351C was significantly associated with total number of inverted teats (p 0.04) and close to significance with the affected teats (p 0.06). SNP A1666C did not segregate in the commercial population (Table 3).

Table 3 Association of *LEF1* gene with inverted teats (Aff), total number of inverted teats (TIT) and total number of teats (TT) in the experimental (DUMI) and commercial (COM) population as revealed by FBAT analysis

Characteristic	Population	Allele	Freq	Aff	TT	TIT
Marker				*p*	*p*	*p*
T1351C	DUMI	T	0.5	0.45	0.75	≤0.1
A1666C	DUMI	C	0.4	≤0.01	≤0.01	≤0.01
T1351C	COM	T	0.96	0.06	0.31	0.04
A1666C	COM	A	1.0	n. a.	n. a.	n. a.

Discussion

This investigation is the first to characterize the porcine *LEF1* gene and to demonstrate association and linkage with the inverted teat defect in the pig. The emergence of inverted teats depends on insufficient mesenchymal proliferation at the teat ground during teat development. As far as proliferation processes are concerned the defect of inverted teats is related to the trait of number of teats. The number of teats may also depend on local signaling between adjacent embryonic tissues (Jonas *et al.* 2008). Boras-Granic et al. (2006) elucidated the influence of lymphoid enhancer-binding factor-1 on the regulative interaction of epithelial and mesenchymal proliferation during the formation of the mammary gland. Knockout experiments indicated involvement of *LEF1* in the ontogenesis of a functional mammary gland, because *LEF1*-deficient mice developed a defective mammary gland with a reduced number of mammary buds and aborted development of those that do form (Boras-Granic *et al.* 2006; Vangenderen *et al.* 1994). The expression of *LEF1* gene in epithelial and mesenchymal compartments of the teats, i.e. tissues relevant for the inverted teat defect, indicates a functional role of *LEF1* in teat development also in pigs and by this approves the results reported in other species (Boras-Granic *et al.* 2006; Vangenderen *et al.* 1994). Also, very high sequence similarities of the deduced porcine *LEF1* amino acids sequence with other mammalian species supports the concept of equivalent biological roles for different regulatory mechanisms of proliferation during ontogenesis in the various species (Eastman & Grosschedl 1999; Kratochwil *et al.* 1996). Quantitative trait loci (QTL) for teat traits on chromosome 8 have been identified by several authors (Beeckmann *et al.* 2003a; Cassady *et al.* 2001; Sato *et al.* 2006). *LEF1* was displayed in close proximity to these QTL and thus is a positional candidate gene. Our results for the SNP A1666C in the

experimental DUMI population revealed a highly significant association and linkage to teat number as well as occurrence of inverted teats. Analysis of the SNP T1351C and the haplotypes support this finding. Moreover, association of *LEF1* with number of inverted teats as well as with presence or absence of inverted teats could be verified in commercial dam lines for the segregating SNP T1351C at p 0.04 and 0.06, respectively. The polymorphisms detected are likely markers in close linkage to a causative polymorphism affecting teat number and liability for the inverted teat defect. However, the allele frequencies are unfavourable with regard to the power to unravel association with the SNP A1666C not segregating and the SNP T1351C showing low frequency in the animals of commercial breeds investigated. In the experimental DUMI population alleles at both SNPs exist at similar frequencies, but highly significant association was shown only for SNP A1666C that obviously exhibits consistent linkage phase to a causative polymorphic site across all families. These different results enhance the conclusion that different population and family-specific effects are due to genetic drift and/or recombination between the SNPs of the *LEF1* gene and the causative polymorphism (Du *et al.* 2007; Sved 1971). Moreover, for complex traits, like liable traits, a single gene often plays a very small role in a complex interaction of many genes in a pathway that act differently and promote various regulative measures in different populations or breeds. Thus various modes of compensation may be active in different individuals. Comparative sequencing revealed no polymorphisms in the coding regions of the gene, thus polymorphisms affecting the expression of the genes are likely to be causal for any effects on teat development. SNPs in untranslated regions of genes were shown to affect expression for example by creating a potential target site for microRNAs (Clop *et al.* 2006); however *in silico* analysis with the TargetScan tool (predicts biological targets of microRNAs by searching for the presence of conserved

8mer and 7mer sites that match the recognition region of each microRNA; http://www.targetscan.org) of the polymorphic sites described here did not indicate conservation and particular functional roles of the polymorphic sites. While using samples of peripubertal animals we could not show trait dependent expression, however this does not rule out polymorphisms effecting the expression of *LEF1* at other ontogenetic stages that final lead to different teat phenotypes. In conclusion, whereas *LEF1* was promoted as functional positional candidate gene for the inverted teat defect the identification of causal polymorphism remains open. Effects of *LEF1* gene on teat phenotypes but also on other traits will be evaluated in an increased number of animals of commercial dam lines in order to further qualify the gene as functional positional candidate gene in gene assisted selection. Further analyses of trait association in other populations as well as functional assays will provide more insight into the causal nature of the polymorphisms.

Acknowledgements

The authors would like to thank the Federal Ministry of Education and Research (BMBF) and the Förderverein Biotechnologieforschung e.V. (FBF) for financial support (FUGATO). The experiments described in this study were performed in accordance with all appropriate regulations.

6 Differential expression of growth factors and their receptors points out their involvement in the inverted teat defect in pigs

Tetzlaff S, Murani E, Schellander K, Ponsuksili S, Wimmers K.

Published online in Journal of Animal Science 2009, July 31

Abstract

In this study eight genes of growth factors and their receptors were investigated that are known to play a significant role in signaling pathways involved in the ontogenetic but also tumorigenic development of breast and mammary gland. Differential expression of *FGFR2, GHR, HGF, HGFR, PDGFA, PDGFRA, PDGFB, VEGF* was analyzed in mesenchymal and epithelial teat tissue of animals affected and non-affected by inverted teat defect. Comparisons were made at the level of affection between samples derived from non-affected animals and affected animals, including specimens of normal and inverted teats. In addition, comparisons were made at the level of the teat phenotype with normal teats of non-affected animals vs. either the normal or the inverted teat of affected animals. All genes tested, expect *HGFR*, showed significant differential expression at $P < 0.05$ in either the mesenchymal and/or the epithelial teat tissue. In general, we observed more pronounced differences when comparing samples obtained from inverted tissues vs. samples from normal once. Therefore the results of our study suggest that the gene expression of the growth factors and their receptors associates directly with the teat phenotype and rather than on the affection status of the investigated animals suggesting that local processes and a tissue-specific compensation by means of differential expression of growth factors and their receptors are responsible for the development of impaired teat phenotypes.

Keywords: expression profile, inherited disorder, inverted teat, pig, qRT-PCR, signaling pathways

Introduction

In pigs a functional mammary gland is an important selection criterion for increasing the survival rate of piglets. The most relevant inherited disorder of the mammary complex is the inverted teat, resulting in non-functional teats. The defect occurs in commercial breeds with frequencies between 7.6 to 30 % (Jonas *et al.* 2008). The emergence of inverted teats depends on insufficient mesenchymal proliferation at the developing teat ground (Jonas *et al.* 2008). Several authors elucidate the influence of hormones and other genetic factors on the regulative interaction of epithelial mesenchymal proliferation during the formation of the mammary gland (Foley *et al.* 2001; Sakakura 1987).

Feldman et al. (1993) have argued that mammary development depends on interaction of mammogenic and lactogenic factors. In particular, growth hormone (GH) and its receptor (GHR) mediate differentiation and development of the immature mammary gland. Further, the hepatocyte growth factor (HGF) could be shown as a factor with pleiotropic activities throughout mammary development including stimulation of cell proliferation in epithelial cells and their interaction with mesenchymal cells during embryogenesis (Lock *et al.* 2002; Rosen *et al.* 1994). Platelet derived growth factors (PDGF) and their receptors (PDGFR), vascular endothelial growth factor (VEGF), belonging to the PDGF/VEGF signaling superfamily, and fibroblast growth factors (FGF) and their receptors (FGFR) are important regulators for tissue-tissue interactions to control proliferation of epithelial and mesenchymal cells during development of epidermal appendages (Hoch & Soriano 2003; Hovey *et al.* 2001; Sakakura 1987).

Transcript abundance of eight growth factors and receptors known to be relevant for assertive arrangements in a functional mammary gland was comparatively analyzed in

epithelial and mesenchymal tissue of normal and inverted teats to provide experimental evidence for their involvement in the development of the disorder.

Materials and methods

Tissue collection and RNA preparation

To collect samples of epithelial and mesenchymal teat tissue animals of commercial herds of crossbreeds of German Landrace (GL), German Large White (LW) and Pietrain (Pi x (GL x LW)) pigs (n 28 animals, comprising of 14 full sib families with one affected and one non-affected litter mates) were used. Epithelial and mesenchymal tissue was gathered from the teat of the non-affected sibling and from a normal teat and an inverted teat of the affected sibling. A total of 84 tissue samples were collected in an experimental slaughterhouse where two persons of the team of inspectors examined also the teat phenotypes by adspection and palpation of teats at a separate slaughter. Tissue samples were directly frozen in liquid nitrogen and stored at 80 °C.

Total RNA from each tissue was isolated using Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany) and NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) including DNase treatment. RNA samples were visualized on 1.5 % formaldehyde containing agarose gel to check the integrity and the concentration was measured by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). In addition absence of DNA contamination was checked using the RNA as a template in a PCR amplifying a fragment of the glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) gene. All RNAs were stored at 80 °C until downstream analysis was performed.

Quantitative real-time PCR (qRT-PCR)

In order to survey differential expression of the epithelial and mesenchymal tissue samples qRT-PCR was performed using the LightCycler® 480 system (Roche, Mannheim, Germany). First-strand cDNA was synthesized from 1µg of total RNA using random primers and oligo d(T) 13VN in the presence of Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). The ribosomal protein L32 (*RPL32*) and the general transcription factor IIIC, polypeptide 2, (*GTF3C2*) were treated as internal references. Reactions were performed in a final volume of 12 µl using 6.0 µl of LightCycler 480 SYBR Green I Master (Roche), 600 nM of each primer (Table 1) and 100 ng cDNA. Amplification conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, annealing (Table 1) for 10 sec and 72 °C for 15 sec. At the completion of the amplification protocol, all samples were subjected to melting curve analyses and gel electrophoresis to verify the absence of any non-specific product. PCR products from each of the respective transcripts were used to generate external standard curves for the calculation of copy numbers. Normalization for each sample used the geometric means of the relative concentration of each internal reference gene (*RPL32* and *GTF3C2*). For statistical analysis of qRT-PCR data tissue-specific normalized means of transcript abundance levels were compared with Student's *t*-test. Differences between tissue samples were considered significant at $P < 0.05$ (Figures 1 and 2).

Table 1 Primers used for qRT-PCR

Gene		Sequence 5´-3´	Accession no.	T (°C)	Size (bp)
FGFR2	f	GGATTACAACTCGCCTCTCCT	**AB271924**	62	196
	r	GCTTCCTTGGGCTTCTCTTT			
GHR	f	GAAGACATTTACATCACCACAGAAA	**NM214254**	64	197
	r	ACATAGCCACACGATGAGAGAAAC			
GTF3C2[1]	f	TTGAAAGCCAAGCCCACTTC	**CK466410**	64	230
	r	GGGGAGGCAGGTCTAAAGGT			
HGF	f	ATGGTACTTGGTGTCATTGTTCCT	**CU656003**	62	183
	r	TTGATGTAAAGAGAGTTGTGTTAATGG			
HGFR	f	CCCACCCTCATTACATCACC	**EW531089**	62	245
	r	TTCTTCCTCATCCATCATTTACAAC			
PDGFA	f	GCACAGTCAGATCCACAGCA	**BP439777**	62	163
	r	GATGCTTCTCTTCCTCCGAAC			
PDGFRA	f	ACAAGCTGTATCACTGCCTTTGTTT	**EW193418**	62	179
	r	CTTCCTGTCGCGTTAGTTCTC			
PDGFB	f	CGTCTGTCTCGATGCCTGATT	**EW082988**	62	169
	r	GTCAGTAGAGGAAGAGAGCGATG			
RPL32[1]	f	AGCCCAAGATCGTCAAAAG	**AY550039**	62	165
	r	TGTTGCTCCCATAACCAATG			
VEGF	f	GACGAAGGTCTGGAGTGTGTG	**AF318502**	62	157
	r	TTTCTTGCCTCGCTCTATCTT			

[1]Reference genes.

Results

Our investigation was performed on eight genes using mesenchymal and epithelial tissue samples from discordant animals of commercial populations. All genes tested, expect *HGFR*, showed significant differential expression at $P < 0.05$ in terms of the inverted teat defect (Figures 1 and 2). In this study a total of 26 significant differences in expression could be found in mesenchymal and epithelial tissue. In particular, *FGFR2*, *HGF*, *PDGFA*, *PDGFRA* and *VEGF* were significant differential expressed in mesenchymal tissue of total normal teat (B) and inverted teat (E); furthermore, *FGFR2*, *HGF*, *PDGFA* and *PDGFRA* exhibited significant differential expression in mesenchymal teat tissue of non-affected sibs (C) in comparison with inverted teat tissue (E) of the affected sibs; *HGF* and *PDGFRA* showed significant differential expression in normal (D) and inverted (E) mesenchymal teat tissue of the affected animals. A significant differential expression profile of mesenchymal tissue from affected animals (A) compared with non-affected animals (C) could be demonstrated for *HGF* and *PDGFA* (Figure 1). Six of our investigated genes showed significant differences of expression in epithelial teat tissue; *HGF*, *GHR*, *PDGFA*, *PDGFRA* and *VEGF* were significant differential expressed in epithelial tissue of total normal teat (B) and inverted teat (E); furthermore, *HGF*, *PDGFA*, *PDGFRA, PDGFB* and *VEGF* exhibited significant differential expression in epithelial teat tissue of non-affected sibs (C) in comparison with inverted teat tissue of the affected sibs (E); *HGF* and *PDGFRA* showed significant differential expression in comparison of normal (D) and inverted epithelial teat tissue (E) from the affected animals. A significant differential expression between epithelial teat tissue of affected animals (A) and non-affected animals (C) could be demonstrated in the *PDGFA* gene (Figure 2).

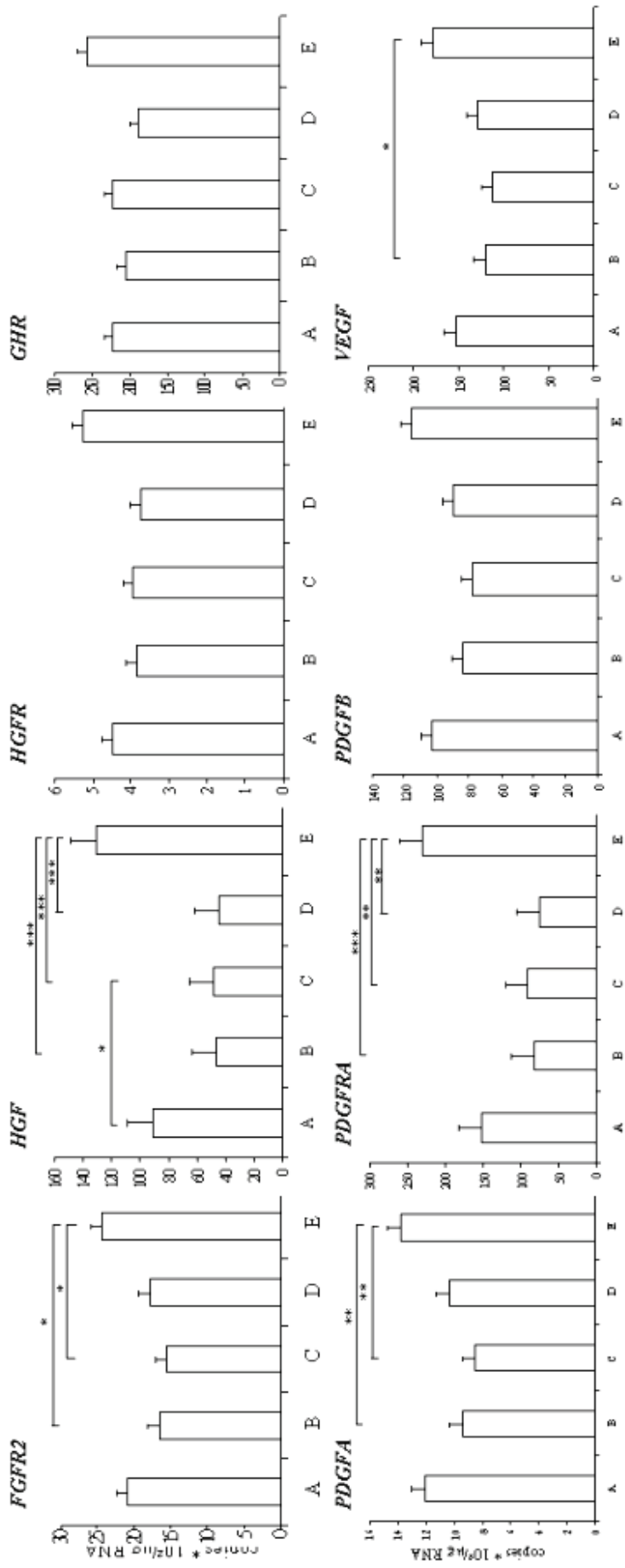


Figure 1 Gene expression profiles of mesenchymal teat tissue: Transcript abundance was determined by qRT-PCR from mesenchymal teat tissue samples of non-affected animals (n = 14), from normal teat tissue of affected animals (n = 14) and inverted teat tissue (n = 14). Mean abundance and standard errors are shown. A, expression value of tissue from affected animals; B, total normal teat tissue; C, teat tissue from non-affected animals; D, normal teat tissue from affected animals; E, inverted teat tissue; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

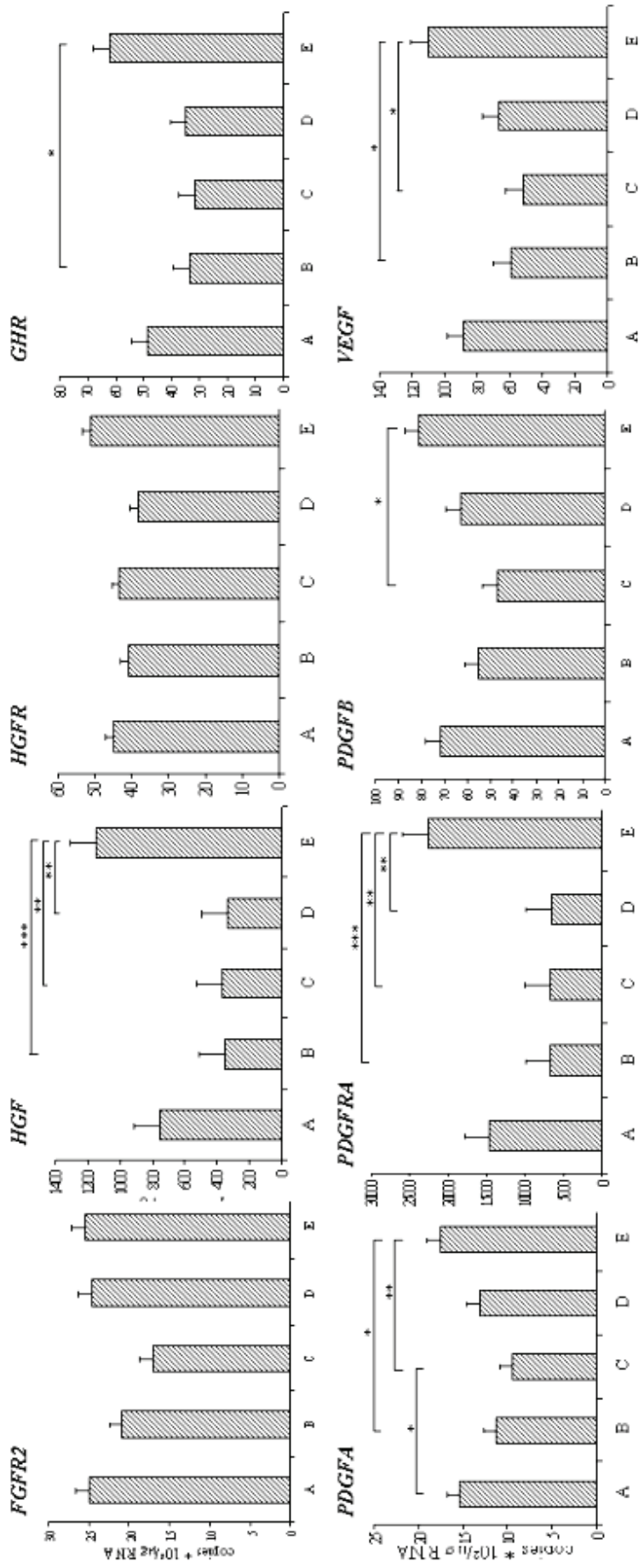


Figure 2 Gene expression profiles of epithelial teat tissue: Transcript abundance was determined by qRT-PCR from epithelial teat tissue samples of non-affected animals (n = 14), from normal teat tissue of affected animals (n = 14) and inverted teat tissue (n = 14). Mean abundance and standard errors are shown. A, expression value of tissue from affected animals; B, total normal teat tissue; C, teat tissue from non-affected animals; D, normal teat tissue from affected animals; E, inverted teat tissue; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

Discussion

Teat developmental and functional mammary gland capabilities have to be seen as a result of a complex interaction between multiple factors, signaling pathways and different cell types during several major phases of development (Hovey *et al.* 2001; Imagawa *et al.* 1990; Sakakura 1987). In particular, a number of paracrine growth factors have been proposed to be involved in teat and mammary gland development. Microarray analyses revealed that signaling pathways of several growth factors are involved in normal and inverted teat development with several genes along the signaling pathways showing trait dependent regulation (Figure 3; manuscript in preparation). The hierarchical superior components of these pathways, the growth factors and their receptors are not fully covered be the microarray. Here we aimed to quantify the transcripts of eight growth factors and growth factor receptors depending on the affection status of the organism and the teat phenotype using the more to exact and sensitive real-time PCR technique.

Seven out of eight genes addressed here showed differential expression in either the mesenchymal and/or the epithelial teat tissue. In general, we observed more pronounced differences when comparing samples obtained from inverted tissues vs. samples from normal once. Therefore, the results of our study suggest that the gene expression of the growth factors and their receptors associates directly to the teat phenotype and rather than to the affection status of the investigated animals. This indicates that local processes and tissue-specific compensation by means of differential expression of growth factors and their receptors in the explicit teat are the main drivers of inverted teat development (Figures 1 and 2).

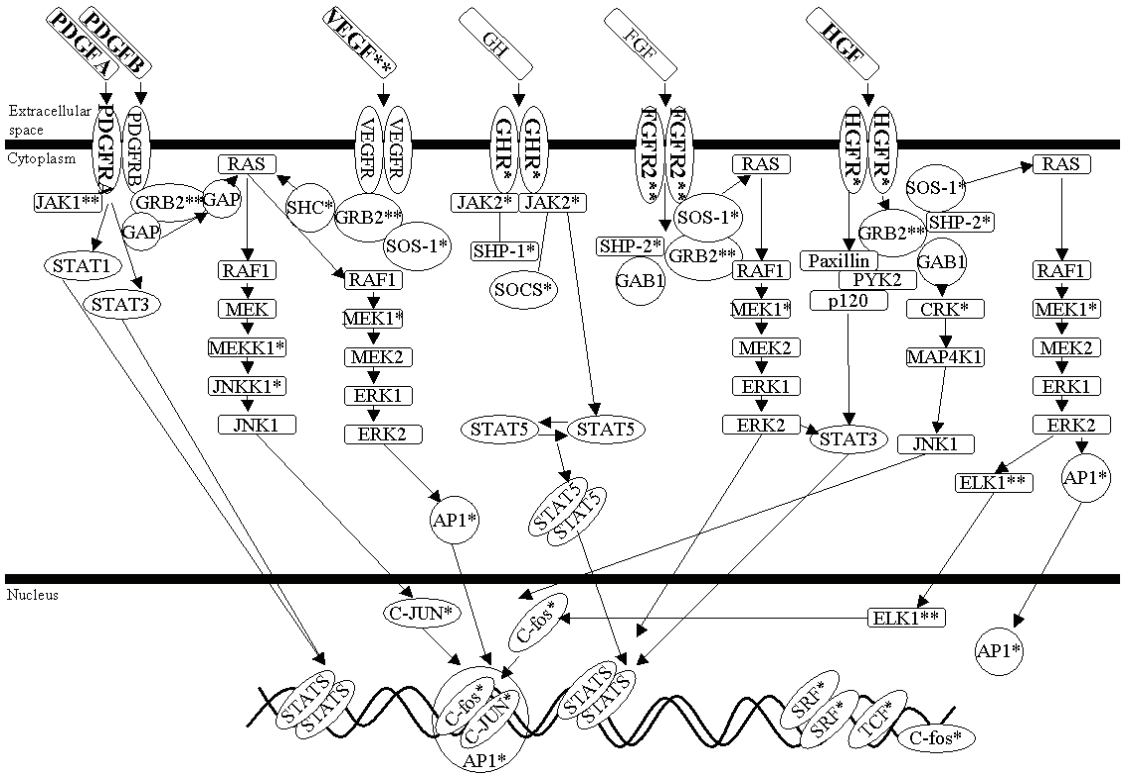


Figure 3 Simplified canonical pathways of growth factor signaling involved in the development of a functional mammary gland: Genes in bold letters are described in this investigation. * indicates fold change ≥ 1.2; ** indicates regulation significant at $p \leq 0.05$ in previous own microarray experiments. AP1, activator protein 1; C-fos, C-fos protein; C-JUN, C-JUN transcription factor; CRK, V-CRK sarcoma virus CT10 oncogene homolog; ELK1, member of ETS oncogene family; ERK1-2, extracellular signal-regulated kinase 1-2; GAB1, GRB2-associated binding protein 1; GAP, glyceraldehyde-3-phosphate dehydrogenase; GRB2, growth factor receptor bound protein 2; JAK1-2, Janus tyrosine kinase 1-2; JNK1, Jun N-terminal kinase 1; JNKK1, Jun N-terminal kinase kinase 1; MAP4K1, mitogen-activated protein kinase kinase kinase kinase 1; MEK1-2, mitogen-activated protein kinase kinase 1-2; MEKK1, mitogen-activated protein kinase kinase kinase 1; p120, catenin, delta 1; PYK2, pyruvate kinase 2; RAF1, V-RAF-1 murine leukemia viral oncogene homolog 1;RAS, rat sarcoma viral oncogene homolog; SHC, SHC (Src homology 2 domain containing) transforming protein 1;

SHP1-2, SHP1-2 containing protein tyrosine phosphatase; SOCS, suppressor of cytokine signaling; SOS-1, son of sevenless homolog 1; SRF, serum response factor; STAT1-5, signal transducer and activator of transcription 1-5; TCF, transcription factor.

FGFR2 belongs to the family of four *fibroblast growth factor receptors* in mammals. This gene is known to be expressed during early mammary gland development and plays roles in regulation of cell proliferation and differentiation in mammary epithelial and mesenchymal cells (Eblaghie *et al.* 2004; Kim *et al.* 2007). Several genes encoding FGF ligands like FGF7 and FGF10 are known to bind to *FGFR2* as the receptor, which has been reported to be already expressed in mammary ectoderm (Mailleux *et al.* 2002). Mouse embryos lacking *FGF10* gene function fail to develop mammary placodes, suggesting that *FGF10* signaling through its receptor *FGFR2* is required to initiate development of mammary glands (Mailleux *et al.* 2002). The FGFR2 protein consists of an extracellular region, composed of three immunglobulin-like domains, a single hydrophobic membrane-spanning segment and a cytoplasmatic tyrosine kinase domain. The extracellular part of the protein interacts with FGF10, starting a cascade of downstream signals, ultimately influencing the development of mammary glands. The significant differences in expression between normal vs. inverted mesenchymal teat tissue suggest its importance for a functional mammary gland not only through development in embryogenesis. Human *FGFR2* maps to a region that is syntenic to the distal arm of SSC14 (Table 2), where a quantitative trait loci (QTL) for inverted teats was found in an experimental cross population (Jonas *et al.* 2008).

Table 2 List of candidate genes, their functions and chromosomal assignments

Gene	Function	Porcine chromosomal assignment	Human chromosomal assignment
FGFR2 *(fibroblast growth factor receptor 2)*	early embryonic development	SSC14q	HSA10q26
GHR (growth hormone receptor)	Differentiation and development	SSC16q1.3-1.4[1]	HSA5p13
HGF (*hepatocyte growth factor)*	Cell proliferation, cell-cell interaction	SSC9q21[2]	HSA7q21.1
HGFR (hepatocyte growth factor receptor)	Cell proliferation, cell-cell interaction	SSC9q15-21	HSA7q31
PDGFA (platelet-derived growth factor alpha)	Regulation of cell proliferation	SSC3p	HSA7p22.3
PDGFRA *(platelet-derived growth factor receptor alpha)*	Regulation of cell proliferation	SSC8p12[3]	HSA4q11
PDGFB (platelet-derived growth factor beta)	Regulation of cell proliferation	SSC5p	HSA22q13.1
VEGF *(vascular endothelial growth factor)*	Cell proliferation and growth	SSC7q11-13	HSA6p21.3

[1] Chowdhary et al. (1994).

[2] Pinton et al. (2000).

[3] Johansson et al. (1992).

The *GHR* gene encodes a protein that is a transmembrane receptor for growth hormone and has been found in mammary glands of pigs (Jammes *et al.* 1991). Binding of growth hormone to the receptor leads to receptor dimerization and a further activation of an intra- and intercellular transduction pathway for growth and development of the mammary gland (Feldman *et al.* 1993). Feldman et al. (1993) elucidate its central and primary role in mammary gland development. Growth hormone together with its receptor can act directly on mammary tissue stimulating cell growth and further the end bud formation (Silberstein & Daniel 1987). The position of *GHR* on SSC16 announced by Chowdhary et al. (1994) fell in the confidence interval of the QTL for inverted teats in commercial breeds reported by Jonas et al. (2008).

Hepatocyte growth factor is a mesenchymally derived factor with pleiotropic activities mediated through its HGF receptor. HGF stimulates cell proliferation in a wide range of cellular targets, including epithelial cells as well as epithelial-mesenchymal interactions during embryogenesis (Lock *et al.* 2002; Rosen *et al.* 1994). However, it is unclear how the *HGF* receptor conducts the signaling pathways leading to its pleiotropic activities. Maroun et al. (2000) have demonstrated that for promotion of epithelial morphogenesis recruitment of the Gab family of docking proteins (Gab1 and Gab2) are necessary but not sufficient. These proteins are phosphorylated through tyrosine kinase downstream from the *HGF* receptor, activated by *HGF.* The differential expression of *HGF* in mesenchymal and epithelial teat tissue (Figures 1 and 2) confirms its role in biological pathways involved in the occurrence of a functional mammary gland. *HGFR* gene expression was not found to be different in the investigated teat tissues. This might indicate that HGFR with its ligands has eminent relevance for distinct pathways for motility, growth, and/or morphogenesis in various tissues. Local effects at the teat and mammary gland are regulated on the level of the growth factor, whereas general effects are determined at the receptor level or more distally. Moreover, the chromosomal assignment of *HGF* (Pinton *et al.* 2000) and *HGFR* to the proximal region of SSC9 (Table 2) revealed no allocation to a QTL for teat developmental traits.

Platelet derived growth factors and their receptors are important regulators for tissue-tissue interactions to control proliferation of epithelial and mesenchymal cells during embryonic development of epidermal appendages (Hoch & Soriano 2003; Karlsson *et al.* 1999; Sakakura 1987; Xu *et al.* 2005). PDGFs have characteristic domains, which include eight conserved cysteines that are involved in inter- and intramolecular bonds. The signaling network consists of four homo- or heterodimers of two subunit proteins, A-, B-, C- and D-chain. Their receptors are two tyrosine kinases (PDGFRA and

PDGFRB) that are able to form homo- or heterodimers after the binding of PDGF ligand. Different studies suggest that PDGFB has a limited function in regulating organogenesis, whereas PDGFA and its PDGFRA receptor are more broadly required during embryogenesis (Hoch & Soriano 2003; Xu *et al.* 2005). Functionally, PDGFA and PDGFRA signaling plays an important role in cell proliferation, cell migration and in regulating epithelial-mesenchymal interactions during developmental processes (Xu *et al.* 2005). In addition, Table 2 presents the chromosomal assignment of *PDGFA* to the distal region of SSC3 and *PDGFRA* to SSC8p12, as described in Johansson et al. (1992). This assignment for *PDGFA* revealed no allocation to a QTL for teat developmental traits. However, *PDGFRA* fell in the confidence interval of QTL for inverted teats and number of teats (Beeckmann *et al.* 2003a; Jonas *et al.* 2008). Furthermore, the chromosomal assignment by means of published human-porcine comparative maps allocates *PDGFB* to the distal region of SSC5 (Table 2). That region exhibits several QTL for mammary gland capabilities, including a QTL for inverted teats and a QTL for teat number (Jonas *et al.* 2008; Rodriguez *et al.* 2005).

Vascular endothelial growth factor belongs to the PDGF/VEGF signaling superfamily. VEGF is involved in functional development of the mammary gland via the establishment and maintenance of a vascular supply to support the mammary fat pad and associated epithelial proliferation (Hoch & Soriano 2003; Hovey *et al.* 2001). VEGF as a glycosylated homodimer stimulates vascular permeability and promotes cell proliferation through liberation of several proteases (Hovey *et al.* 2001). *VEGF* assigned to the proximal region of SSC7 (Table 2), where no QTL for inverted teats is located (Jonas *et al.* 2008). QTL for teat number were found close to *VEGF* on SSC7 (Sato *et al.* 2006; Wada *et al.* 2000).

Expression analyses of the porcine transcriptome in terms of teat developmental and functional mammary gland capabilities revealed a number of functional candidate genes that show specific differential expression profiles of epithelial and mesenchymal tissue of normal and inverted teats, which is in line with their known physiological function. Furthermore, their assignment to QTL regions for teat developmental and mammary gland capabilities corroborate its candidacy for the occurrence of the inverted teat defect.

Acknowledgements

The authors would like to thank the Federal Ministry of Education and Research (BMBF) and the Förderverein Biotechnologieforschung der Deutschen Schweineproduktion e.V. (FBF) for financial support (FUGATO - HeDiPig, FKZ 0313392A). The experiments described in this study were performed in accordance with all appropriate regulations.

7 General discussion

The subjects of animal genomics and genetics are rapidly developing through the last decades. Recent technological advances are improving, especially the accessibility of the genome in identifying genes, fast and efficient screening of genetic variation present in the genome, gene expression profiling, and statistical methods to identify and use genomic regions of particular interest for breeding purposes. The investigations described in this thesis contribute to these subjects but furthermore to a better understanding of the assertive arrangements in functional mammary gland and teat developmental capabilities. In this chapter results that have been obtained in this investigation will be exploited and discussed in retrospect with the original objectives and future prospects will be given. At first the aspect and the enormous impact of the inverted teat defect will be discussed followed by a discussion of the main findings. Finally, the main conclusions and implications are exposed.

The inverted teat defect

The reasoning of the investigation described in this thesis is that the inverted teat defect is one of the most frequent and economically relevant hereditary defects in pig populations (Blendl *et al.* 1980). This defect that reduces the number of functional teats and associated results in reduction of raising performance in breeding herds (Wiesner & Willer 1978). In German pig populations up to 30 % of animals suffer from inverted teats (Brevern *et al.* 1994; Mayer & Pirchner 1995; Niggemayer 1993). It is a fact that the inverted teat defect has a genetic background, but the number of genes involved and their hereditary is still unknown. Heritability estimates range from 0.2 to 0.5 (Brevern *et al.* 1994; Hittel 1984; Mayer 1994). The inverted teat is characterized by failure of teats

to protrude from the udder surface. This incidence of inverted teats depends on insufficient mesenchymal proliferation at the teat ground during embryogenesis and postnatal development (Günther 1984). As far as proliferation processes are concerned the defect of inverted teats is related to the trait of number of teats, which may also depend on local signaling between adjacent embryonic tissues that determine the somite patterning and cell fate decisions leading to the establishment of the mammary bud (Jonas *et al.* 2008). The regulation of mammary development is affected by complex interactions of different hormones, their receptors and other genetic factors which collaborate in multiple signaling pathways and different cell types during several major phases of development (Hovey *et al.* 2001; Imagawa *et al.* 1990; Sakakura 1987). Furthermore, the influence of hormones and different genetic factors on a functional mammary gland have been investigated in several mammalian species (Boras-Granic *et al.* 2006; Eblaghie *et al.* 2004; Foley *et al.* 2001; Sakakura 1987). Thus knowledge of physiology and genetic background of a functional mammary gland are the major origins for identification and analysis of functional candidate genes, implemented in this thesis.

Functional candidate gene analysis

In several previous studies a number of different candidate genes have been successfully investigated in identifying linkage association or causative relations. Thus, a detected polymorphism in the *ESR* gene is associated with litter size in pigs (Rothschild *et al.* 1996; Short *et al.* 1997). Furthermore, the myostatin gene (*GDF8*) could be ascertained as responsible for a marked increase in skeletal muscle mass in mammalian species (Grobet *et al.* 1997; Kambadur *et al.* 1997; McPherron *et al.* 1997; Welle *et al.* 2007). This phenotype evolves from five mutations that destruct the

myostatin protein in cattle (Grobet *et al.* 1998). The farthest studied and discussed gene in the pig is the ryanodine receptor 1 gene (*RYR1*). Fuji et al. (1991) discovered a point mutation that affects the stress susceptibility and accompanying a decreased meat quality in pigs.

In this thesis the parathyroid hormone/parathyroid hormone-like hormone type I receptor was investigated as the receptor of parathyroid hormone and parathyroid hormone-like hormone, which participate in the formation and differentiation of the mammary gland (Chomdej *et al.* 2004; Foley *et al.* 2001). Several investigations with knockout mice indicated involvement of *PTHLH* and its receptor in the ontogenesis of a functional mammary gland, because these mice developed the inverted teat phenotype (Dunbar *et al.* 2001; Wysolmerski *et al.* 1998). The polymorphism in the *PTHR1* gene has revealed a significant association and linkage to occurrence of inverted teats as well as teat number. The point mutation of *PTHR1* is located in its intracellular tail, which is involved in signal transduction. When analyzing genes of hormones and their receptors effects of interaction can be expected, however no interactions between *PTHLH* and *PTHR1* genotypes were observed in the investigated experimental DUMI population.

Furthermore lymphoid enhancer-binding factor-1 revealed a regulative influence on the formation of the mammary gland (Boras-Granic *et al.* 2006). Experiments in other species indicated involvement of *LEF1* in the ontogenesis of a functional mammary gland (Boras-Granic *et al.* 2006; Vangenderen *et al.* 1994). One of the detected two polymorphisms in the experimental DUMI population revealed a highly significant association and linkage to teat number as well as occurrence of inverted teats. Analyses of the haplotypes support this finding. Moreover, association of *LEF1* with inverted teats could be verified in commercial dam lines. The polymorphisms detected are likely markers in close linkage to a causative polymorphism affecting teat number and liability

for the inverted teat defect. However, the reverse association exhibits consistent linkage phase to a causative polymorphic site across all families. These different findings enhance the conclusion that different population and family-specific effects are due to genetic drift and/or recombination between the polymorphisms of the *LEF1* gene and the causative polymorphism (Du *et al.* 2007; Sved 1971).

QTL analysis

Quantitative trait loci analyses are usually combined with candidate genes and require validation in several populations. Different studies of QTL for reproduction, milk yield or meat quality traits in other species account for that approach (Jennen *et al.* 2005; Jiang *et al.* 2005). Thus, detected QTL in commercial broiler lines could be confirmed and showed consequently the potential for applying these results in a marker-assisted selection (de Koning *et al.* 2003). Studies in dairy cattle offered QTL for milk yield and composition on bovine chromosome 26 (BTA26) and the transcription factor 7-like 2 gene (*TCF7L2*) as a marker for that QTL could be demonstrated as a significantly associated positional candidate gene for protein yield in milk. Furthermore, these results could be assigned and confirmed in several bovine populations (Jiang *et al.* 2005).

In the pig the strategy of developing experimental populations from divergent breeds is applied to increase heterozygosity (Geldermann *et al.* 1996; Hardge *et al.* 1999). Thereby linkage disequlibria are generated between the molecular markers and the QTL. That describes the assumption to identify QTL (Lynch & Walsh 1997). Several QTL for different traits on nearly every chromosome in pig populations could be detected (Hu *et al.* 2005). Thus QTL fot the inverted teat defect in an experimental DUMI population were detected on several chromosomes (Oltmanns 2003; Ün 2002). Because of the absence of confirmed QTL analyses for the inverted teat phenotype in

other experimental and commercial populations these data were used and reanalyzed to perform the analyses parallel to the QTL analyses in a commercial population (Jonas *et al.* 2008). The strongly decreased number of QTL in the commercial population is likely caused by the eminent selection pressure that generates homozygosity on several with the QTL linked loci. Although, these results for the inverted teat QTL in the commercial population elucidate that QTL for inverted teat could be confirmed in different pig populations. Due to the experimental design and different heterozygosity of molecular markers loci may not be detected or different loci for quantiative traits may be discovered (Silva *et al.* 2008; Wattrang *et al.* 2005). Consequentially, such verified QTL may be exploited in MAS programs in commercial populations and their molecular basis may be revealed by positional cloning (Evans *et al.* 2003).

The functional candidate gene *PTHR1* was displayed in close proximity to the QTL for inverted teat on SSC13, so that the polymorphism in the *PTHR1* gene likely is a marker in close linkage disequilibrium to a causative polymorphism affecting teat number and liability for the inverted teat phenotype. Thus *PTHR1* was contemplated as a functional positional candidate gene for the inverted teat phenotype. Furthermore, QTL for teat traits on chromosome 8 have been identified by several authors (Beeckmann *et al.* 2003a; Cassady *et al.* 2001; Sato *et al.* 2006). *LEF1* was displayed in close proximity to these QTL and thus is not only a functional, but also a positional candidate gene.

Expression analysis

Gene expression describes the transcription of the information covered in the DNA (Velculescu *et al.* 1997). The basis for studies are different expressed genes, which influence in their transcript abundance the further translational potential of a gene in different cell types and tissues and can therefore be determined by several techniques.

In this thesis the microarray technology combined with the quantitative real-time PCR were used. Microarrays simultaneously measure the transcript abundance from thousands of genes and made it possible to study complex regulatory processes within the target cell (Master *et al.* 2002). Own microarray results in mesenchymal and epithelial teat tissue of animals affected and non-affected by the inverted teat defect showed more than 300 differentially regulated genes in epithelial and more than 200 differentially regulated genes in mesenchymal teat tissue. To interpret such large amounts of generated data, algorithms have been used to organize genes into groups of related expression patterns (Eisen *et al.* 1999; Tamayo *et al.* 1999). The expression patterns of our microarrays indicated differential expression of genes of biological processes like `development´, `cell communication´, `signal transduction and intracellular signaling cascades´ for comparisons at the level of affection between samples derived from non-affected animals and affected animals. For comparisons between specimens of normal vs. inverted teats, the microarray results suggested biological processes involved in `cell growth´, `cell junction´, `growth regulation and development´. These clustered expression analyses are used to identify groups of genes that function in common pathways and identify significant molecular differences between the investigated cells or tissues (Master *et al.* 2002). Mammary development is characterized by consecutive stages of cell growth, differentiation, high metabolic activity and apoptosis. This comprises tremendous variances in epithelial and mesenchymal tissue architecture, morphogenesis, vascularization and organized remodeling (Clarkson *et al.* 2004). Visvader and Lindemann (2003) elucidate in the mouse model that these events are stringently controlled at the transcriptional level by circulating hormones and locally derived factors. Many growth factors have been shown to affect this developmental progress (Carvalho *et al.* 2005; Eroglu *et al.* 2006; Lim *et*

al. 2005). The in this thesis investigated expression patterns of porcine mammary tissues confirmed the involvement of regulative signaling pathways in the occurrence of a functional mammary gland, as ascertained for the mouse model. Thus, signaling pathways, like the FGF, GH, HGF, PDGF and VEGF signaling pathway, could be shown to activate through their growth factors related cascades and mechanisms on intracellular level or more distally (Eblaghie *et al.* 2004; Feldman *et al.* 1993; Hoch & Soriano 2003; Hovey *et al.* 2001; Lock *et al.* 2002). Based on the microarray analyses growth factors and their receptors were selected for further quantitative real-time analyses. In addition, to minimize the enormous costs of the microarray analyses the studies were combined with qRT-PCR. The benefit of this technique is that more samples could be detected, quantified and the differential expression of single genes could be analyzed precisely. This approach results in significant differentially expressed growth factors and receptors. Gene expression depends directly on the teat phenotype. This indicates that locally derived factors and receptors are mainly responsible for inverted teat development (Visvader & Lindeman 2003). Primarily the *HGF* and *PDGFRA* genes with their highly significant differential expression in the investigated mesenchymal and epithelial tissues are very interesting candidate genes for additional studies. Furthermore, several of that growth factors and receptors fell in the confidence interval of QTL for inverted teat and teat number in the experimental and the commercial population. Thus, these genes are not only functional candidate genes but also positional candidate genes.

Conclusions and future prospects

In this thesis the *PTHR1* gene and its role in the occurrence of the inverted teat phenotype has been described. Therefore associations of the detected polymorphism in

an experimental pig population could be demonstrated. Effects of the interacting *PTHLH* gene and its receptor *PTHR1* could not be verified. Prospective the association on teat phenotypes but also on other traits should be evaluated in animals of commercial dam lines in order to further qualify the gene as a functional positional candidate gene in a marker-assisted selection.

Furthermore, the *LEF1* gene was discussed as a functional and positional candidate gene for the inverted teat phenotype. Polymorphisms, identified in animals of an experimental and a commercial pig population, left no conclusions from the causal polymorphism that remains open. Effects of the *LEF1* gene on teat phenotypes but also on other traits should be evaluated in an increased number of animals from the commercial population. In addition, trait associations in further pig populations should be verified in order to supplementary succeed the gene as functional and positional candidate gene in a marker-assisted selection.

Further analyses of trait associations of the investigated genes in other populations as well as functional assays will provide more insight into the causal nature of the polymorphisms in the development of a functional mammary gland in the pig but these findings could be also interesting for other species.

Prior polymorphic genes or genomic regions may be applied in breeding programs supported by marker-assisted selection following criteria should be validated. First trait associations in the regarding pig population need to be verified. Furthermore, the linkage phase between the unfavourable allele and the mutation responsible for the genetic variation of the inverted teat phenotype should be ascertained.

Moreover, the QTL analyses with the additional fine mapping of microsatellites on chromosomes 3, 4, 6 and 11 showed consensuses in genomic regions of the experimental and the commercial population. Thus, these loci may be seen as

interesting regions for further analyses of positional candidate genes for the inverted teat defect. Effects of that QTL should be validated as well in further populations, before implementing in a marker-assisted selection.

The microarray technology was used to identify groups of genes that function in common pathways involved in the occurrence of the inverted teat phenotype and identify significant expression differences between the teat tissues. In conclusion, the in this thesis described signaling pathways showed significant differences in the investigated teat tissues. Consequentially, the growth factors and receptors activating further cascades were investigated with the quantitative real-time PCR method. These results revealed functional candidate genes for further investigations. Thus, SNP screenings and trait associations could be performed in experimental and commercial pig populations.

The constantly increasing number of functional and positional candidate genes for the inverted teat phenotype elucidates that the cause of this inherited disorder is an argument for the complexity of that defect and that further investigations for detection of the basically causative gene are necessary and promising. In the future identifications and investigations of candidate genes should be advanced. Furthermore, major developmental phases, prenatal but also postnatal, of the mammary gland should be considered in further investigation, because several studies clarify the crucial influence of major phases in mammary gland development and the occurrence of the inverted teat phenotype. The ongoing of the porcine genome sequencing project established the chance to implement the information of more than 1500 positional candidate genes integrated in the detected and confirmed QTL regions for inverted teats on chromosomes 3, 4, 6 and 11 via the microarray technology on a specific *custom-made* array for that defect. In addition, to the porcine whole genome microarray from

Affymetrix company this enables the simultaneously analysis of more than thousand positional candidate genes to discover further regulative mechanisms and involved candidate genes that are specific for the occurrence of inverted teat.

Finally, the development of the SNP chip technology will facilitate the detection of genetic variations on a genome wide level to discover genes or more precisely their variations responsible for specific traits. Though, this technique should be further advanced to exhibit a powerful tool in the implementation for researchers in genetically influenced diseases and inherited disorders.

8 Summary

Genetic disorders in mammals play a tremendous role in continuance and development of animals. Different investigations document a multitude of genetically influenced diseases, which could not be fully elucidated until now. In the pig the inverted teat is one of the economically relevant inherited defects. This thesis covers the identification and analysis of functional candidate genes involved in the occurrence of the inverted teat defect.

A detected SNP in the functional candidate gene *PTHR1*, which is involved with its ligand *PTHLH* in mesenchymal/epithelial interactions during mammary gland development, could be shown as significantly associated with the inverted teat phenotype as well as teat number in an experimental pig population. The physical mapping on SSC13 revealed *PTHR1* in close proximity to a QTL for inverted teats.

The *LEF1* gene is involved in the ontogenesis of a functional mammary gland, as indicated in other species. Due to molecular characterization of *LEF1* two SNP could be detected. One SNP in the experimental DUMI population revealed a highly significant association and linkage to teat number as well as occurrence of inverted teats. Moreover, association of *LEF1* with inverted teats could be verified in a commercial population. The detected SNP are likely markers in close linkage to a causative polymorphism affecting teat number and liability for the inverted teat defect. The QTL analyses for inverted teat phenotype in an experimental and a commercial pig population revealed significant QTL on twelve chromosomes for the experimental and on five chromosomes for the commercial population. QTL with overlapping intervals in both populations could be detected on SSC3, SSC4, SSC6 and SSC11. These QTL results confirm the formerly elucidated polygenic inheritance of inverted teats and

enable the information of novel positional candidate genes. Adjacent microarray analyses of epithelial and mesenchymal teat tissue of commercial animals in an affected sib pair design offered differentially regulated genes involved in biological processes like growth, development and cell communication. Constitutive expression analyses of eight genes: *FGFR2*, *GHR*, *HGF*, *HGFR*, *PDGFA*, *PDGFRA*, *PDGFB* and *VEGF* revealed significant differential expressions. No differential expression was detected for *HGFR* in the compared animals with normal against inverted teats. These expression results elucidate local effect on the teat and mammary gland determined by growth factors and their receptors.

In summary, the in this thesis identified and analyzed genes may be established as functional and in part positional candidate genes for the inverted teat defect.

9 Zusammenfassung

Genetische Defekte in Säugetieren nehmen eine bedeutende Rolle in ihrem Fortbestand und ihrer Entwicklung ein. Verschiedene Untersuchungen belegen eine Vielzahl von genetisch verursachten Krankheitsbildern, die zum Teil bis heute nicht vollständig wissenschaftlich aufgeklärt werden konnten. Beim Schwein stellt der Gendefekt `Stülpzitze´ einen der am ökonomisch relevantesten dar. Diese Arbeit beinhaltet die Identifizierung und Analyse von funktionellen Kandidatengenen für die Ausprägung der Stülpzitze beim Schwein.

Ein detektierter SNP im funktionellen Kandidatengen *PTHR1,* welches mit seinem Liganden *PTHLH* an mesenchymalen/epithelialen Interaktionen während der Entwicklung der Milchdrüse beteiligt ist, zeigte signifikante Assoziationen mit dem Phänotyp Stülpzitze als auch mit der Zitzenzahl in einer experimentellen Schweinepopulation. Die physische Kartierung auf SSC13 ergab überdies eine Lage in die Nähe zu einem QTL für die Stülpzitze. Des Weiteren ist das *LEF1* Gen in die Ontogenese einer funktionellen Milchdrüse involviert, was bereits an anderen Spezies nachgewiesen wurde. Beruhend auf der molekularen Charakterisierung von *LEF1* wurden zwei SNP entdeckt. Ein SNP war in der experimentellen Population mit der Zitzenzahl als auch der Stülpzitze hoch signifikant assoziiert und gekoppelt. Darüber hinaus ließ sich die Assoziation mit der Stülpzitze in einer kommerziellen Population verifizieren. Die entdeckten SNP sind höchst wahrscheinlich gekoppelte Marker eines kausalen Polymorphismus, der die Zitzenzahl und die Ausprägung der Stülpzitze beeinflusst. QTL Analysen für das Merkmal Stülpzitze sowohl in einer experimentellen als auch kommerziellen Population zeigten QTL Regionen auf zwölf Chromosomen der experimentellen und auf fünf Chromosomen der kommerziellen Population. Auf den

Chromosomen 3, 4, 6 und 11 wurden QTL mit überlappenden Intervallen in beiden Populationen ermittelt. Diese Ergebnisse bestätigen die Hypothese einer polygenen Vererbung der Stülpzitze und ermöglichen die Information über und weitere Nutzung neuer positioneller Kandidatengene. Anschließende Mikroarray Untersuchungen epithelialen und mesenchymalen Zitzengewebes kommerzieller Tiere im *affected sib pair* Design ergaben differentiell regulierte Gene, die in biologische Prozesse wie Wachstum, Entwicklung und Zellkommunikation involviert sind. Konstitutive Expressionsanalysen von acht aus den Mikroarrayergebnissen hervorgehenden funktionellen Kandidatengenen: *FGFR2*, *GHR*, *HGF*, *HGFR*, *PDGFA*, *PDGFRA*, *PDGFB* und *VEGF* zeigten signifikant differentielle Expressionen. Lediglich *HGFR* ließ keinerlei Expressionsunterschiede in den miteinander verglichenen Zitzengeweben von betroffenen und nicht betroffenen Geschwistertieren erkennen. Die Expressionsergebnisse der untersuchten Wachstumsfaktoren und ihrer Rezeptoren deuten auf einen Effekt lokalen Ursprungs im betroffenen Zitzengewebe hin.

Zusammenfassend lässt sich herausstellen, dass die in dieser Arbeit untersuchten Gene für den Erbdefekt der Stülpzitze als funktionelle und zum Teil auch positionelle Kandidatengene zu sehen sind.

10 References

Beeckmann, P., Moser, G., Bartenschlager, H., Reiner, G., Geldermann, H. (2003a) Linkage and QTL mapping for Sus scrofa chromosome 8. *Journal of Animal Breeding and Genetics*, **120**, 66-73.

Beeckmann, P., Schroffel, J., Moser, G., Bartenschlager, H., Reiner, G., Geldermann, H. (2003b) Linkage and QTL mapping for Sus scrofa chromosome 1. *Journal of Animal Breeding and Genetics*, **120**, 1-10.

Behrens, J., vonKries, J.P., Kuhl, M., Bruhn, L., Wedlich, D., Grosschedl, R., Birchmeier, W. (1996) Functional interaction of beta-catenin with the transcription factor LEF-1. *Nature.*, **382**, 638-642.

Bhattacharjee, M., Vonderhaar, B.K. (1984) Thyroid-Hormones Enhance the Synthesis and Secretion of Alpha-Lactalbumin by Mouse Mammary Tissue Invitro. *Endocrinology*, **115**, 1070-1077.

Bignell, G., Micklem, G., Stratton, M.R., Ashworth, A., Wooster, R. (1997) The BRC repeats are conserved in mammalian BRCA2 proteins. *Human Molecular Genetics*, **6**, 53-58.

Blendl, H.M., Hußlein, E., Bleicher, H. (1980) Zitzenbeurteilung beim Schwein - ein wesentliches Selektionskriterium. *Bayerisches landwirtschaftliches Jahrbuch*, **58**, 250-255.

Bohmer, F.D., Kraft, R., Otto, A., Wernstedt, C., Hellman, U., Kurtz, A., Muller, T., Rohde, K., Etzold, G., Lehmann, W., Langen, P., Heldin, C.H., Grosse, R. (1987) Identification of A Polypeptide Growth Inhibitor from Bovine Mammary-Gland - Sequence Homology to Fatty Acid-Binding and Retinoid-Binding Proteins. *Journal of Biological Chemistry*, **262**, 15137-15143.

Bole-Feysot, C., Goffin, V., Edery, M., Binart, N., Kelly, P.A. (1998) Prolactin (PRL) and its receptor: Actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocrine Reviews*, **19**, 225-268.

Boras-Granic, K., Chang, H., Grosschedl, R., Hamel, P.A. (2006) Lef1 is required for the transition of Wnt signaling from mesenchymal to epithelial cells in the mouse embryonic mammary gland. *Developmental Biology*, **295**, 219-231.

Brevern, N.V., Schimpf, B., Wörner, R., Swalve, H. (1994) Parameterschätzung für Zitzenmerkmale bei Hybridsauen. *Züchtungskunde*, **66**, 339-348.

Carvalho, I., Milanezi, F., Martins, A., Reis, R.M., Schmitt, F. (2005) Overexpression of platelet-derived growth factor receptor a in breast cancer is associated with tumour progression. *Breast Cancer Research*, **7**, R788-R795.

Cassady, J.P., Johnson, R.K., Pomp, D., Rohrer, G.A., Van Vleck, L.D., Spiegel, E.K., Gilson, K.M. (2001) Identification of quantitative trait loci affecting reproduction in pigs. *Journal of Animal Science*, **79**, 623-633.

Cepica, S., Reiner, G., Bartenschlager, H., Moser, G., Geldermann, H. (2003) Linkage and QTL mapping for Sus scrofa chromosome X. *Journal of Animal Breeding and Genetics*, **120**, 144-151.

Chomdej, S. (2005) Molecular genetic analysis of positional candidate genes for mammary gland characteristics in pigs. Dissertation, Rheinische Friedrich-Wilhelms-Universität Bonn, Germany.

Chomdej, S., Ponsuksili, S., Schellander, K., Wimmers, K. (2004) Sequencing, SNP identification and mapping of the porcine PTHLH gene to chromosome 5. *Animal Genetics*, **35**, 151-152.

Chowdhary, B.P., Ellegren, H., Johansson, M., Andersson, L., Gustavsson, I. (1994) In-Situ Hybridization Mapping of the Growth-Hormone Receptor (Ghr) Gene Assigns A Linkage Group (C9, Psa, Ghr, and S0105) to Chromosome-16 in Pigs. *Mammalian Genome*, **5**, 160-162.

Chu, E.Y., Hens, J., Andl, T., Kairo, A., Yamaguchi, T.P., Brisken, C., Glick, A., Wysolmerski, J.J., Millar, S.E. (2004) Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. *Development.*, **131**, 4819-4829.

Churchill, G.A., Doerge, R.W. (1994) Empirical Threshold Values for Quantitative Trait Mapping. *Genetics*, **138**, 963-971.

Clarkson, R.W.E., Wayland, M.T., Lee, J., Freeman, T., Watson, C.J. (2004) Gene expression profiling of mammary gland development reveals putative roles for death receptors and immune mediators in post-lactational regression. *Breast Cancer Research*, **6**, R92-R109.

Clayton, G.A., Powell, J.C., Hiley, P.G. (1981) Inheritance of Teat Number and Teat Inversion in Pigs. *Animal Production*, **33**, 299-304.

Clop, A., Marcq, F., Takeda, H., Pirottin, D., Tordoir, X., Bibe, B., Bouix, J., Caiment, F., Elsen, J.M., Eychenne, F., Larzul, C., Laville, E., Meish, F., Milenkovic, D., Tobin, J., Charlier, C., Georges, M. (2006) A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nature Genetics*, **38**, 813-818.

de Koning, D.J., Windsor, D., Hocking, P.M., Burt, D.W., Law, A., Haley, C.S., Morris, A., Vincent, J., Griffin, H. (2003) Quantitative trait locus detection in commercial broiler lines using candidate regions. *Journal of Animal Science*, **81**, 1158-1165.

Dragos-Wendrich, M., Moser, G., Bartenschlager, H., Reiner, G., Geldermann, H. (2003) Linkage and QTL mapping for Sus scrofa chromosome 10. *Journal of Animal Breeding and Genetics*, **120**, 82-88.

Du, F.X., Clutter, A.C., Lohuis, M.M. (2007) Characterizing linkage disequilibrium in pig populations. *International Journal of Biological Sciences*, **3**, 166-178.

Dunbar, M.E., Dann, P., Brown, C.W., Van, H.J., Dreyer, B., Philbrick, W.P., Wysolmerski, J.J. (2001) Temporally regulated overexpression of parathyroid hormone-

related protein in the mammary gland reveals distinct fetal and pubertal phenotypes. *J Endocrinol*, **171**, 403-416.

Dundar, B., Dundar, N., Erci, T., Bober, E., Buyukgebiz, A. (2005) Leptin levels in boys with pubertal gynecomastia. *Journal of Pediatric Endocrinology & Metabolism*, **18**, 929-934.

Eastman, Q., Grosschedl, R. (1999) Regulation of LEF-1/TCF transcription factors by Wnt and other signals. *Current Opinion in Cell Biology*, **11**, 233-240.

Eblaghie, M.C., Song, S.J., Kim, J.Y., Akita, K., Tickle, C., Jung, H.S. (2004) Interactions between FGF and Wnt signals and Tbx3 gene expression in mammary gland initiation in mouse embryos. *Journal of Anatomy*, **205**, 1-13.

Edwards, D.B., Ernst, C.W., Raney, N.E., Doumit, M.E., Hoge, M.D., Bates, R.O. (2008) Quantitative trait locus mapping in an F2 Duroc x Pietrain resource population: II. Carcass and meat quality traits. *J Anim Sci*, **86**, 254-266.

Eisen, M.B., Spellman, P.T., Brown, P.O., Botstein, D. (1999) Cluster analysis and display of genome-wide expression patterns (vol 95, pg 14863, 1998). *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 10943.

Eroglu, A., Gulec, S., Kurtman, C., Cam, R., Akar, N. (2006) Vascular endothelial growth factor 936 C/T polymorphism in cancer patients. *Annals of Oncology*, **17**, 1467-1468.

Evans, G.J., Giuffra, E., Sanchez, A., Kerje, S., Davalos, G., Vidal, O., Illan, S., Noguera, J.L., Varona, L., Velander, I., Southwood, O.I., de Koning, D.J., Haley, C.S., Plastow, G.S., Andersson, L. (2003) Identification of quantitative trait loci for production traits in commercial pig populations. *Genetics*, **164**, 621-627.

Feldman, M., Ruan, W.F., Cunningham, B.C., Wells, J.A., Kleinberg, D.L. (1993) Evidence That the Growth-Hormone Receptor Mediates Differentiation and Development of the Mammary-Gland. *Endocrinology*, **133**, 1602-1608.

Foley, J., Dann, P., Hong, J., Cosgrove, J., Dreyer, B., Rimm, D., Dunbar, M.E., Philbrick, W., Wysolmerski, J.J. (2001) Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. *Development.*, **128**, 513-525.

Fuji, J., Otsu, K., Zorzato, F., De Leon, S., Khanna, V.K., Weiler, J.E., O'Brien, P.J., MacIennan, D.H. (1991) Identification of a mutation in porcine ryanodine receptor associated with malignant hyperthermia. *Science*, **253**, 448-451.

Geldermann, H., Muller, E., Beeckmann, P., Knorr, C., Yue, G., Moser, G. (1996) Mapping of quantitative-trait loci by means of marker genes in F-2 generations of wild boar, Pietrain and Meishan pigs. *Journal of Animal Breeding and Genetics-Zeitschrift fur Tierzuchtung und Zuchtungsbiologie*, **113**, 381-387.

Geldermann, H., Muller, E., Moser, G., Reiner, G., Bartenschlager, H., Cepica, S., Stratil, A., Kuryl, J., Moran, C., Davoli, R., Brunsch, C. (2003) Genome-wide linkage

and QTL mapping in porcine F2 families generated from Pietrain, Meishan and Wild Boar crosses. *Journal of Animal Breeding and Genetics*, **120**, 363-393.

Gerbens, F., de Koning, D.J., Harders, F.L., Meuwissen, T.H.E., Janss, L.L.G., Groenen, M.A.M., Veerkamp, J.H., van Arendonk, J.A.M., Te Pas, M.F.W. (2000) The effect of adipocyte and heart fatty acid-binding protein genes on intramuscular fat and backfat content in Meishan crossbred pigs. *Journal of Animal Science*, **78**, 552-559.

Green P., Falls K., Crooks S. (1990) Documentation for CRI-MAP, version 2.4. Washington University School of Medicine, St. Louis, MO, USA.

Grobet, L., Martin, L.J.R., Poncelet, D., Pirottin, D., Brouwers, B., Riquet, J., Schoeberlein, A., Dunner, S., Menissier, F., Massabanda, J., Fries, R., Hanset, R., Georges, M. (1997) A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nature Genetics*, **17**, 71-74.

Grobet, L., Poncelet, D., Royo, L.J., Brouwers, B., Pirottin, D., Michaux, C., Menissier, F., Zanotti, M., Dunner, S., Georges, M. (1998) Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. *Mammalian Genome*, **9**, 210-213.

Günther, C., Dämmrich, K., Plonait, H. (1985) Morphologie und Pathogenese der sogenannten Stülpzitzen des Schweins. *Züchtungskunde*, **57**, 256-266.

Günther, C. (1984) Morphologie der sogenannten "Stülpzitze" beim Schwein im Vergleich zum histologischen Bild einer normalen Zitze. Dissertation, Freie Universität Berlin, Germany.

Haley, C.S., Lee, G.J., Ritchie, M. (1995) Comparative Reproductive-Performance in Meishan and Large White-Pigs and Their Crosses. *Animal Science*, **60**, 259-267.

Hardge, T., Koepke, K., Reissmann, M., Wimmers, K. (1999) Maternal influences on litter size and growth in reciprocal crossed Miniature Pigs and Durocs. *Archiv für Tierzucht-Archives of Animal Breeding*, **42**, 83-92.

Hennighausen, L., Robinson, G.W. (2001) Signaling pathways in mammary gland development. *Developmental Cell*, **1**, 467-475.

Hirooka, H., de Koning, D.J., Harlizius, B., van Arendonk, J.A.M., Rattink, A.P., Groenen, M.A.M., Brascamp, E.W., Bovenhuis, H. (2001) A whole-genome scan for quantitative trait loci affecting teat number in pigs. *Journal of Animal Science*, **79**, 2320-2326.

Hittel, J. (1984) Genetische und umweltbedingte Einflüsse auf die Ausprägung von Zitzenanomalien beim Schwein. Dissertation, Georg-August-Universität Göttingen, Germany.

Hoch, R.V., Soriano, P. (2003) Roles of PDGF in animal development. *Development.*, **130**, 4769-4784.

Horvath, S., Xu, X., Laird, N.M. (2001) The family based association test method: strategies for studying general genotype-phenotype associations. *European Journal of Human Genetics*, **9**, 301-306.

Horvath, S., Xu, X., Lake, S.L., Weiss, S.T., Silverman, E.K., Laird, N.M. (2003) Family-based tests for associating haplotypes with general phenotype data: application to asthma genetics. *American Journal of Human Genetics*, **73**, 610.

Hovey, R.C., Goldhar, A.S., Baffi, J., Vonderhaar, B.K. (2001) Transcriptional regulation of vascular endothelial growth factor expression in epithelial and stromal cells during mouse mammary gland development. *Molecular Endocrinology*, **15**, 819-831.

Hovey, R.C., Harris, J., Hadsell, D.L., Lee, A.V., Ormandy, C.J., Vonderhaar, B.K. (2003) Local insulin-like growth factor-II mediates prolactin-induced mammary gland development. *Molecular Endocrinology*, **17**, 460-471.

Hu, Z.L., Dracheva, S., Jang, W.H., Maglott, D., Bastiaansen, J., Rothschild, M.F., Reecy, J.M. (2005) A QTL resource and comparison tool for pigs: PigQTLDB. *Mammalian Genome*, **16**, 792-800.

Hu, Z.L., Reecy, J.M. (2007) Animal QTLdb: beyond a repository. *Mammalian Genome*, **18**, 1-4.

Imagawa, W., Bandyopadhyay, G.K., Nandi, S. (1990) Regulation of Mammary Epithelial-Cell Growth in Mice and Rats. *Endocrine Reviews*, **11**, 494-523.

Jammes, H., Gaye, P., Belair, L., Djiane, J. (1991) Identification and Characterization of Growth-Hormone Receptor Messenger-Rna in the Mammary-Gland. *Molecular and Cellular Endocrinology*, **75**, 27-35.

Jennen, D.G.J., Vereijken, A.L.J., Bovenhuis, H., Crooijmans, R.M.P.A., van der Poel, J.J., Groenen, M.A.M. (2005) Confirmation of quantitative trait loci affecting fatness in chickens. *Genetics Selection Evolution*, **37**, 215-228.

Jiang, Z., De, S., Garcia, M.D., Griffin, K.B., Wu, X.L., Xiao, Q., Michal, J.J., Sharma, B.S., Jansen, G.B. (2005) An independent confirmation of a quantitative trait locus for milk yield and composition traits on bovine chromosome 26. *Journal of Animal Breeding and Genetics*, **122**, 281-284.

Johansson, M., Ellegren, H., Marklund, L., Gustavsson, U., Ringmarcederberg, E., Andersson, K., Edforslilja, I., Andersson, L. (1992) The Gene for Dominant White Color in the Pig Is Closely Linked to Alb and Pdgfra on Chromosome-8. *Genomics*, **14**, 965-969.

Jonas, E., Schreinemachers, H.J., Kleinwachter, T., Un, C., Oltmanns, I., Tetzlaff, S., Jennen, D., Tesfaye, D., Ponsuksili, S., Murani, E., Juengst, H., Tholen, E., Schellander, K., Wimmers, K. (2008) QTL for the heritable inverted teat defect in pigs. *Mammalian Genome*, **19**, 127-138.

Kambadur, R., Sharma, M., Smith, T.P.L., Bass, J.J. (1997) Mutations in myostatin (GDF8) in double-muscled Belgian blue and Piedmontese cattle. *Genome Research*, **7**, 910-916.

Karlsson, L., Bondjers, C., Betsholtz, C. (1999) Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle. *Development.*, **126**, 2611-2621.

Kim, Y., Bingham, N., Sekido, R., Parker, K.L., Lovell-Badge, R., Capel, B. (2007) Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 16558-16563.

King, A.H., Jiang, Z.H., Gibson, J.P., Haley, C.S., Archibald, A.L. (2003) Mapping quantitative trait loci affecting female reproductive traits on porcine chromosome 8. *Biology of Reproduction*, **68**, 2172-2179.

Kobayashi, T., Kronenberg, H.M., Foley, J. (2005) Reduced expression of the PTH/PTHrP receptor during development of the mammary gland influences the function of the nipple during lactation. *Developmental Dynamics*, **233**, 794-803.

Kong, X.F., Schipani, E., Lanske, B., Joun, H., Karperien, M., Defize, L.H.K., Juppner, H., Potts, J.T., Segre, G.V., Kronenberg, H.M., Abousamra, A.B. (1994) The Rat, Mouse and Human Genes Encoding the Receptor for Parathyroid-Hormone and Parathyroid Hormone-Related Peptide Are Highly Homologous. *Biochemical and Biophysical Research Communications*, **200**, 1290-1299.

Kratochwil, K., Dull, M., Farinas, I., Galceran, T., Grosschedl, R. (1996) Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. *Genes & Development*, **10**, 1382-1394.

Kruglyak, L., Daly, M.J., ReeveDaly, M.P., Lander, E.S. (1996) Parametric and nonparametric linkage analysis: A unified multipoint approach. *American Journal of Human Genetics*, **58**, 1347-1363.

Lander, E., Kruglyak, L. (1995) Genetic Dissection of Complex Traits - Guidelines for Interpreting and Reporting Linkage Results. *Nature Genetics*, **11**, 241-247.

Lee, S.S., Chen, Y., Moran, C., Cepica, S., Reiner, G., Bartenschlager, H., Moser, G., Geldermann, H. (2003a) Linkage and QTL mapping for Sus scrofa chromosome 2. *Journal of Animal Breeding and Genetics*, **120**, 11-19.

Lee, S.S., Chen, Y., Moran, C., Stratil, A., Reiner, G., Bartenschlager, H., Moser, G., Geldermann, H. (2003b) Linkage and QTL mapping for Sus scrofa chromosome 5. *Journal of Animal Breeding and Genetics*, **120**, 38-44.

Lim, Y.J., Kim, J.W., Song, J.Y., Hong, M.S., Jin, S.Y., Yoon, S.H., Park, H.J., Choe, B.K., Lee, J.J., Yim, S.V., Hong, S.I., Baik, H.H., Ha, E., Park, Y.H. (2005) Epidermal growth factor gene polymorphism is different between schizophrenia and lung cancer patients in Korean population. *Neuroscience Letters*, **374**, 157-160.

Ling, C., Svensson, L., Oden, B., Weijdegard, B., Eden, B., Eden, S., Billig, H. (2003) Identification of functional prolactin (PRL) receptor gene expression: PRL inhibits lipoprotein lipase activity in human white adipose tissue. *Journal of Clinical Endocrinology and Metabolism*, **88**, 1804-1808.

Lock, L.S., Maroun, C.R., Naujokas, M.A., Park, M. (2002) Distinct recruitment and function of Gab1 and Gab2 in Met receptor-mediated epithelial morphogenesis. *Molecular Biology of the Cell*, **13**, 2132-2146.

Lynch, M., Walsh, B. (1997) Genetics and analysis of quantitative traits. Sinauer Associates, Inc., Sunderland, Massachusetts.

Mailleux, A.A., Spencer-Dene, B., Dillon, C., Ndiaye, D., Savona-Baron, C., Itoh, N., Kato, S., Dickson, C., Thiery, J.P., Bellusci, S. (2002) Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo. *Development.*, **129**, 53-60.

Maroun, C.R., Naujokas, M.A., Holgado-Madruga, M., Wong, A.J., Park, M. (2000) The tyrosine phosphatase SHP-2 is required for sustained activation of extracellular signal-regulated kinase and epithelial morphogenesis downstream from the Met receptor tyrosine kinase. *Molecular and Cellular Biology*, **20**, 8513-8525.

Master, S.R., Hartman, J.L., D'Cruz, C.M., Moody, S.E., Keiper, E.A., Ha, S.I., Cox, J.D., Belka, G.K., Chodosh, L.A. (2002) Functional microarray analysis of mammary organogenesis reveals a developmental role in adaptive thermogenesis. *Molecular Endocrinology*, **16**, 1185-1203.

Mayer, J. (1994) Genetische Parameter von Gesäugeanomalien beim Schwein. Dissertation, Technische Universität München - Weihenstephan, Germany.

Mayer, J., Pirchner, F. (1995) Asymmetry and Inverted Nipples in Gilts. *Archiv für Tierzucht-Archives of Animal Breeding*, **38**, 87-91.

McNeilly, S. (1994). Suckling and the control of gonadotropin secretion, The Physiology of Reproduction, edited by E. K. Neill, New York: Raven Press.

McPherron, A.C., Lawler, A.M., Lee, S.J. (1997) Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature.*, **387**, 83-90.

Meyers, S.N., Rogatcheva, M.B., Larkin, D.M., Yerle, M., Milan, D., Hawken, R.J., Schook, L.B., Beever, J.E. (2005) Piggy-BACing the human genome - II. A high-resolution, physically anchored, comparative map of the porcine autosomes. *Genomics*, **86**, 739-752.

Moody, D.E., Rosa, A.J., Reecy, J.M. (2003) Current status of livestock DNA microarrays. *Agricultural Biotechnology Net*, **5**, 1-8.

Murani, E., Muraniova, M., Ponsuksili, S., Schellander, K., Wimmers, K. (2006) Molecular characterization and evidencing of the porcine CRH gene as a functional-positional candidate for growth and body composition. *Biochem Biophys Res Commun*, **342**, 394-405.

Musilova, P., Kubickova, S., Vozdova, M., Rubes, J. (2000) Mapping of the oncogene c-myc (MYC) and the breast cancer susceptibility gene (BRCA2) in the pig by FISH. *Animal Genetics*, **31**, 154.

Ng, P.C., Henikoff, S. (2002) Accounting for human polymorphisms predicted to affect protein function. *Genome Research*, **12**, 436-446.

Niggemayer, A. (1993) Besamungsstation kontrolliert Zitzenvererbung. *Schweinezucht und Schweinemast*, **3**, 11.

Nordby, J.E. (1934) Congenital defects in the mammae of swine. *Journal of Heredity*, **25**, 499-502.

Oltmanns, I. (2003) QTL-Analyse an den Chromosomen 2, 3, 5, 7, 9, 11, 13, 15 und 17 in einer porcinen Ressourcepopulation. Dissertation, Rheinische Friedrich-Wilhelms-Universität Bonn, Germany.

OMIA (2008) Online Mendelian Inheritance in Animals, OMIA. Reprogen, Faculty of Veterinary Science, University of Sydney and Australian National Genomic Information Service (ANGIS), University of Sydney,World Wide Web URL: http://omia.angis.org.au/.

Pas, M.F.W.T., De Wit, A.A.W., Priem, J., Cagnazzo, M., Davoli, R., Russo, V., Pool, M.H. (2005) Transcriptome expression profiles in prenatal pigs in relation to myogenesis. *Journal of Muscle Research and Cell Motility*, **26**, 157-165.

Pinton, P., Schibler, L., Cribiu, E., Gellin, J., Yerle, M. (2000) Localization of 113 anchor loci in pigs: improvement of the comparative map for humans, pigs, and goats. *Mammalian Genome*, **11**, 306-315.

Pispa, J., Thesleff, I. (2003) Mechanisms of ectodermal organogenesis. *Developmental Biology*, **262**, 195-205.

Plath, A., Einspanier, R., Peters, F., Sinowatz, F., Schams, D. (1997) Expression of transforming growth factors alpha and beta-1 messenger RNA in the bovine mammary gland during different stages of development and lactation. *Journal of Endocrinology*, **155**, 501-511.

Rabinowitz, D., Laird, N. (2000) A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. *Human Heredity*, **50**, 211-223.

Remy, J.J., Lahbibmansais, Y., Yerle, M., Bozon, V., Couture, L., Pajot, E., Grebert, D., Salesse, R. (1995) The Porcine Follitropin Receptor - Cdna Cloning, Functional Expression and Chromosomal Localization of the Gene. *Gene*, **163**, 257-261.

Robinson, G.W., Karpf, A.B.C., Kratochwil, K. (1999) Regulation of mammary gland development by tissue interaction. *Journal of Mammary Gland Biology and Neoplasia*, **4**, 9-19.

Rodriguez, C., Tomas, A., Alves, E., Ramirez, O., Arque, M., Munoz, G., Barragan, C., Varona, L., Silio, L., Amills, M., Noguera, J.L. (2005) QTL mapping for teat number in an Iberian-by-Meishan pig intercross. *Animal Genetics*, **36**, 490-496.

Rohrer, G.A. (2000) Identification of quantitative trait loci affecting birth characters and accumulation of backfat and weight in a Meishan-White Composite resource population. *Journal of Animal Science*, **78**, 2547-2553.

Rohrer, G.A., Ford, J.J., Wise, T.H., Vallet, J.L., Christenson, R.K. (1999) Identification of quantitative trait loci affecting female reproductive trails in a multigeneration Meishan-White composite swine population. *Journal of Animal Science*, **77**, 1385-1391.

Rosen, E.M., Nigam, S.K., Goldberg, I.D. (1994) Scatter Factor and the C-Met Receptor - A Paradigm for Mesenchymal Epithelial Interaction. *Journal of Cell Biology*, **127**, 1783-1787.

Rothschild, M., Jacobson, C., Vaske, D., Tuggle, C., Wang, L.Z., Short, T., Eckardt, G., Sasaki, S., Vincent, A., McLaren, D., Southwood, O., vanderSteen, H., Mileham, A., Plastow, G. (1996) The estrogen receptor locus is associated with a major gene influencing litter size in pigs. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 201-205.

Rothschild, M.F. (2003) Advances in pig genomics and functional gene discovery. *Comparative and Functional Genomics*, **4**, 266-270.

Sakakura, T. (1987). Mammary embryogenesis, The Mammary Gland: Development, Regulation and Function, edited by M. C. Neville & C. W. Daniel, pp. 37-66. New York: Plenum Press.

Sakakura, T., Kusano, I., Kusakabe, M., Inaguma, Y., Nishizuka, Y. (1987) Biology of Mammary Fat Pad in Fetal Mouse - Capacity to Support Development of Various Fetal Epithelia Invivo. *Development.*, **100**, 421-430.

Sato, S., Atsuji, K., Saito, N., Okitsu, M., Sato, S., Komatsuda, A., Mitsuhashi, T., Nirasawa, K., Hayashi, T., Sugimoto, Y., Kobayashi, E. (2006) Identification of quantitative trait loci affecting corpora lutea and number of teats in a Meishan x Duroc F-2 resource population. *Journal of Animal Science*, **84**, 2895-2901.

Schaapveld, R.Q.J., Schepens, H.T.G., Robinson, G.W., Attema, J., Oerlemans, F.T.J.J., Fransen, J.A.M., Streuli, M., Wieringa, B., Hennighausen, L., Hendriks, W.J.A.J. (1997) Impaired mammary gland development and function in mice lacking LAR receptor-like tyrosine phosphatase activity. *Developmental Biology*, **188**, 134-146.

Short, T.H., Rothschild, M.F., Southwood, O.I., McLaren, D.G., deVries, A., vanderSteen, H., Eckardt, G.R., Tuggle, C.K., Helm, J., Vaske, D.A., Mileham, A.J., Plastow, G.S. (1997) Effect of the estrogen receptor locus on reproduction and production traits in four commercial pig lines. *Journal of Animal Science*, **75**, 3138-3142.

Silberstein, G.B., Daniel, C.W. (1987) Investigation of Mouse Mammary Ductal Growth-Regulation Using Slow-Release Plastic Implants. *Journal of Dairy Science*, **70**, 1981-1990.

Silva, K.D., Paixao, D.M., Silva, P.V., Solero, B.P., Pereira, M.S., Lopes, P.S., Guimaraes, S.E.F. (2008) Mapping of quantitative trait loci and confirmation of the FAT1 region on chromosome 4 in an F2 population of pigs. *Genetics and Molecular Biology*, **31**, 475-480.

Steffens, S. (1993) Stülpzitzen - ein Problem in der Schweinezucht. *Schweinezucht und Schweinemast*, **3**, 8-11.

Steffens, S., Schoon, H.A., Bollwahn, W. (1996) Zitzenkörperhypoplasien und -aplasien (Stülpzitzen) bei weiblichen und männlichen Schweinen. Große Beilage. *Tierärztliche Praxis*, **24**, 31-35.

Strange, K.S., Wilkinson, D., Edin, G., Emerman, J.T. (2004) Mitogenic properties of insulin-like growth factors I and II, insulin-like growth factor binding protein-3 and epidermal growth factor on human breast stromal cells in primary culture. *Breast Cancer Research and Treatment*, **84**, 77-84.

Strange, K.S., Wilkinson, D., Emerman, J.T. (2002) Mitogenic properties of insulin-like growth factors I and II, insulin-like growth factor binding protein-3 and epidermal growth factor on human breast epithelial cells in primary culture. *Breast Cancer Research and Treatment*, **75**, 203-212.

Sved, J.A. (1971) Linkage disequilibrium and homozygosity of chromosome segments in finite populations. *Theor Popul Biol*, **2**, 125-141.

Tamayo, P., Slonim, D., Mesirov, J., Zhu, Q., Kitareewan, S., Dmitrovsky, E., Lander, E.S., Golub, T.R. (1999) Interpreting patterns of gene expression with self-organizing maps: Methods and application to hematopoietic differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 2907-2912.

Tapanainen, J.S., Aittomaki, K., Huhtaniemi, I.T. (1997) New insights into the role of follicle-stimulating hormone in reproduction. *Annals of Medicine*, **29**, 265-266.

Tetzlaff, S., Ponsuksili, S., Murani, E., Wimmers, K. (2007) SNP analysis, genotyping and mapping of the porcine *PTHR1* gene to chromosome 13. *Archiv für Tierzucht-Archives of Animal Breeding*, **50**, 320-321.

Trakooljul, N., Ponsuksili, S., Schellander, K., Wimmers, K. (2004) Polymorphisms of the porcine androgen receptor gene affecting its amino acid sequence and expression level. *Biochimica et Biophysica Acta-Gene Structure and Expression*, **1678**, 94-101.

Ün, C. (2002) QTL Untersuchungen auf Gesäugequalität mittels Mikrosatellitenmarker auf Chromosomen 1, 4, 6, 8, 10, 12, 14, 16 und 18 in einer porcinen Ressourcepopulation. Dissertation, Rheinische Friedrich-Wilhelms-Universität Bonn, Germany.

Vangenderen, C., Okamura, R.M., Farinas, I., Quo, R.G., Parslow, T.G., Bruhn, L., Grosschedl, R. (1994) Development of Several Organs That Require Inductive

Epithelial-Mesenchymal Interactions Is Impaired in Lef-1-Deficient Mice. *Genes & Development*, **8**, 2691-2703.

Velculescu, V.E., Zhang, L., Zhou, W., Vogelstein, J., Basrai, M.A., Bassett, D.E., Hieter, P., Vogelstein, B., Kinzler, K.W. (1997) Characterization of the yeast transcriptome. *Cell*, **88**, 243-251.

Veltmaat, J.M., Van Veelen, W., Thiery, J.P., Bellusci, S. (2004) Identification of the mammary line in mouse by Wnt10b expression. *Developmental Dynamics*, **229**, 349-356.

Visvader, J.E., Lindeman, G.J. (2003) Transcriptional regulators in mammary gland development and cancer. *International Journal of Biochemistry & Cell Biology*, **35**, 1034-1051.

Visvader, J.E., Venter, D., Hahm, K., Santamaria, M., Sum, E.Y.M., O'Reilly, L., White, D., Williams, R., Armes, J., Lindeman, G.J. (2001) The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. *Proceedings of the National Academy of Sciences of the United States of America*, **98**, 14452-14457.

Vonderhaar, B.K., Greco, A.E. (1979) Lobulo-Alveolar Development of Mouse Mammary-Glands Is Regulated by Thyroid-Hormones. *Endocrinology*, **104**, 409-418.

Wada, Y., Akita, T., Awata, T., Furukawa, T., Sugai, N., Inage, Y., Ishii, K., Ito, Y., Kobayashi, E., Kusumoto, H., Matsumoto, T., Mikawa, S., Miyake, M., Murase, A., Shimanuki, S., Sugiyama, T., Uchida, Y., Yanai, S., Yasue, H. (2000) Quantitative trait loci (QTL) analysis in a Meishan x Gottingen cross population. *Animal Genetics*, **31**, 376-384.

Wasinger, V.C., Cordwell, S.J., Cerpapoljak, A., Yan, J.X., Gooley, A.A., Wilkins, M.R., Duncan, M.W., Harris, R., Williams, K.L., Humpherysmith, I. (1995) Progress with Gene-Product Mapping of the Mollicutes - Mycoplasma-Genitalium. *Electrophoresis*, **16**, 1090-1094.

Wattrang, E., Almqvist, M., Johansson, A., Fossum, C., Wallgren, P., Pielberg, G., Andersson, L., Edfors-Lilja, I. (2005) Confirmation of QTL on porcine chromosomes 1 and 8 influencing leukocyte numbers, haematological parameters and leukocyte function. *Animal Genetics*, **36**, 337-345.

Wayne, M.L., McIntyre, L.M. (2002) Combining mapping and arraying: An approach to candidate gene identification. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 14903-14906.

Welle, S., Bhatt, K., Pinkert, C.A., Tawil, R., Thornton, C.A. (2007) Muscle growth after postdevelopmental myostatin gene knockout. *American Journal of Physiology-Endocrinology and Metabolism*, **292**, E985-E991.

Wiesner, E., Willer, S. (1978) Problems of Occurrence of Inverted Nipples in Swine. *Monatshefte fur Veterinarmedizin*, **33**, 189-190.

Wilkins, M. (1994) 2D Electrophoresis: from protein maps to genomes, Symposium, Sienna, Italy.

Wimmers, K., Chomdej, S., Schellander, K., Ponsuksili, S. (2002) Linkage mapping of SNPs in the porcine relaxin gene. *Animal Genetics*, **33**, 323-324.

Wimmers, K., Fiedler, I., Hardge, T., Murani, E., Schellander, K., Ponsuksili, S. (2006) QTL for microstructural and biophysical muscle properties and body composition in pigs. *Bmc Genetics*, **7**.

Wysolmerski, J.J., Broadus, A.E., Zhou, J., Fuchs, E., Milstone, L.M., Philbrick, W.M. (1994) Overexpression of Parathyroid Hormone-Related Protein in the Skin of Transgenic Mice Interferes with Hair Follicle Development. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 1133-1137.

Wysolmerski, J.J., Mccaugherncarucci, J.F., Daifotis, A.G., Broadus, A.E., Philbrick, W.M. (1995) Overexpression of Parathyroid Hormone-Related Protein Or Parathyroid-Hormone in Transgenic Mice Impairs Branching Morphogenesis During Mammary-Gland Development. *Development.*, **121**, 3539-3547.

Wysolmerski, J.J., Philbrick, W.M., Dunbar, M.E., Lanske, B., Kronenberg, H., Broadus, A.E. (1998) Rescue of the parathyroid hormone-related protein knockout mouse demonstrates that parathyroid hormone-related protein is essential for mammary gland development. *Development.*, **125**, 1285-1294.

Xu, X., Bringas, P., Soriano, P., Chai, Y. (2005) PDGFR-alpha signaling is critical for tooth cusp and palate morphogenesis. *Developmental Dynamics*, **232**, 75-84.

Yaswen, P., Smoll, A., Hosoda, J., Parry, G., Stampfer, M.R. (1992) Protein Product of A Human Intronless Calmodulin-Like Gene Shows Tissue-Specific Expression and Reduced Abundance in Transformed-Cells. *Cell Growth & Differentiation*, **3**, 335-345.

Yue, G., Russo, V., Davoli, R., Sternstein, I., Brunsch, C., Schroffelova, D., Stratil, A., Moser, G., Bartenschlager, H., Reiner, G., Geldermann, H. (2003) Linkage and QTL mapping for Sus scrofa chromosome 13. *Journal of Animal Breeding and Genetics*, **120**, 103-110.

Zhao, L., Roche, P.J., Gunnersen, J.M., Hammond, V.E., Tregear, G.W., Wintour, E.M., Beck, F. (1999) Mice without a functional relaxin gene are unable to deliver milk to their pups. *Endocrinology*, **140**, 445-453.

11 Appendix

Arch. Tierz., Dummerstorf **50** (2007) 3, 320-321

[1]Research Institute for the Biology of Farm Animals, Wilhelm Stahl Allee 2, 18196 Dummerstorf, Germany
[2]Institute of Animal Breeding Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany

SYLVIO TETZLAFF[1], SIRILUCK PONSUKSILI[1], EDUARD MURANI[1],
KARL SCHELLANDER[2] and KLAUS WIMMERS[1]

SNP analysis, genotyping and mapping of the porcine *PTHR1* gene to chromosome 13 (Brief report)

(SNP-Analyse, Genotypisierung und Kartierung des porcinen *PTHR1* Gens auf Chromosom 13)

Background: The parathyroid hormone/parathyroid hormone like hormone type I receptor (PTHR1) belongs to the family of G protein-coupled receptors for peptide hormones, including parathyroid hormone (PTH) and parathyroid hormone like hormone (PTHLH), which participate in epithelial-mesenchymal interactions during the formation and differentiation of epithelial organs (FOLEY et al., 2001; CHOMDEJ et al., 2004). The function of PTHR1 and its ligands suggest its candidacy for traits related to the development of bones and joints but also of mammary gland. The porcine gene was screened for SNPs and assigned to SSC13.

Procedures:
Primer sequences:
PTHR-F: 5'-GCTATGGTCCGATGGTGTCT-3'
PTHR-R: 5'-ACTGTCTCCCACTCCTCCTG-3'
SNP analysis, genotyping and mapping:
The *PTHR1* gene sequence (GenBank accession no. NM_214382) was used to derive homologous primers every 400-500 bp of the entire mRNA to screen for polymorphisms.
The PCR fragments were amplified and sequenced from 5 pig breeds (Duroc, Hampshire, German Landrace, Pietrain and Berlin-Miniature pig) using standard lab protocols. PCR programs were: initial denaturation for 5 min 95 °C, 40 cycles each 30 s at 95 °C, 30 s at 64 °C and 1 min at 72 °C and 5 min final extension at 72 °C. Genotyping was performed using the primers PTHR-F and PTHR-R for amplification and *Mbo*II (Fermentas, Leon-Rot, Germany) for restriction digestion. Physical mapping was achieved by screening of the IMpRH panel using the same primers for PCR and by analysis of the results using twopoint and multipoint analysis option of the IMpRH mapping tool (http://www.toulouse.inra.fr). For linkage mapping 19 informative families (n=313) of the DUMI F_2 resource population were genotyped (HARDGE et al., 1999). Multipoint linkage map was established using the BUILD option of the CRIMAP 2.4 package.

Results: A C/T non-synonymous SNP (L556F) was detected at nucleotide position 1819 in the *PTHR1* gene. Within the DUMI F_1 generation (n=14) we observed 11 heterozygous animals and 3 homozygous for allele C; in the DUMI F_2 generation 116 animals were homozygous for allele C, 149 animals were heterozygous and 48 animals were homozygous for allele T. The *PTHR1* gene was assigned to SSC13 in close proximity to marker SW1400 by twopoint analysis (20cR; LOD 13.05) using IMpRH panel (Vector: 1101110000 0000000001 0000000000 0000001000 0001000000

1000010001 0100000001 0000000100 0000000000 0101000011 1011000000 01010000). This assignment was further confirmed by genetic mapping of *PTHR1* using multipoint and twopoint analysis that revealed linkage to S0219 (proximal) and SW344 (distal) with distances of 38.4 cM (recombination fraction=0.31, LOD=1.83) and 15.5 cM (recombination fraction=0.15, LOD=12.69), respectively, on the sex averaged map. Our assignment of *PTHR1* to the q arm of SSC13 is in agreement with the published physical and genetic maps.

Acknowledgements: The authors would like to thank the Federal Ministry of Education and Research (BMBF) and the Förderverein Biotechnologieforschung e.V. (FBF) for financial support.

References

CHOMDEJ, S.; PONSUKSILI, S.; SCHELLANDER, K.; WIMMERS, K.:
Sequencing, SNP identification and mapping of the porcine PTHLH gene to chromosome 5. Animal Genetics **35** (2004), 151-152

FOLEY, J.; DANN, P.; HONG, J.; COSGROVE, J.; DREYER, B.; RIMM, D.; DUNBAR, M.E.; PHILBRICK, W.; WYSOLMERSKI, J.J.:
Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. Development **128** (2001), 513-525

HARDGE, T.; KOEPKE, K.; REISSMANN, M.; WIMMERS, K.:
Maternal influences on litter size and growth in reciprocal crossed Miniature Pigs and Durocs. Arch. Tierz., Dummerstorf **42** (1999), 83-92

Correspondence to: Sylvio Tetzlaff, tetzlaff@fbn-dummerstorf.de

Mamm Genome (2008) 19:127 138
DOI 10.1007/s00335 007 9086 5

QTL for the heritable inverted teat defect in pigs

Elisabeth Jonas · Heinz-Josef Schreinemachers · Tina Kleinwächter · Cemal Ün · Ina Oltmanns · Sylvio Tetzlaff · Danyel Jennen · Dawid Tesfaye · Siriluck Ponsuksili · Eduard Murani · Heinz Juengst · Ernst Tholen · Karl Schellander · Klaus Wimmers

Received: 25 June 2007 / Accepted: 28 November 2007 / Published online: 25 January 2008

Abstract The mothering ability of a sow largely depends on the shape and function of the mammary gland. The aim of this study was to identify QTL for the heritable inverted teat defect, a condition characterized by disturbed development of functional teats. A QTL analysis was conducted in a porcine experimental population based on Duroc and Berlin Miniature pigs (DUMI). The significant QTL were confirmed by linkage analysis in commercial pigs according to the affected sib pair design and refined by family-based association test (FBAT). Nonparametric linkage (NPL) analysis revealed five significant and seven suggestive QTL for the inverted teat defect in the porcine experimental population. In commercial dam lines five significant NPL values were detected. QTL regions in overlapping marker intervals or close proximity in both populations were found on SSC3, SSC4, SSC6, and SSC11. SSC6 revealed QTL in both populations at different positions, indicating the segregation of at least two QTL. The results confirm the previously proposed polygenic inheritance of the inverted teat defect and, for the first time, point to genomic regions harboring relevant genes. The investigation revealed variation of the importance of QTL in the various populations due to either differences in allele frequencies and statistical power or differences in the genetic background that modulates the impact of the liability loci on the expression of the disease. The QTL study enabled us to name a number of plausible positional candidate genes. The correspondence of QTL regions for the inverted teat defect and previously mapped QTL for teat number are in line with the etiologic relationship of these traits.

E. Jonas · H. J. Schreinemachers · T. Kleinwächter · C. Ün · I. Oltmanns · D. Jennen · D. Tesfaye · H. Juengst · E. Tholen · K. Schellander
Institute of Animal Breeding Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany

S. Tetzlaff · S. Ponsuksili · E. Murani · K. Wimmers (✉)
Research Institute for the Biology of Farm Animals (FBN), Wilhelm Stahl Allee 2, 18196 Dummerstorf, Germany
e mail: wimmers@fbn dummerstorf.de

Introduction

Inherited disorders significantly affect animal health and welfare issues and economic efficiency of livestock breeding. Although the phenotype itself often suggests monogenic inheritance with the occurrence of either affected or nonaffected individuals, many inherited diseases are liability traits with polygenic background. In pig breeding the number of piglets reared per sow per year is an important characteristic of efficiency. Next to the number of piglets born alive, the mothering ability of the sow, which is related to the number of functional teats, plays an important role in this. The inverted teat defect is the most common and most important disorder of the mammary complex in pig (Brevern et al. 1994; Mayer 1995). This disorder is a condition characterized by the failure of teats to protrude from the udder surface. The teat canal is held inward, forming a small crater so that normal milk flow is prevented, thus limiting the rearing capacity and increasing the risk of mastitis. The inverted teat defect has a polygenic background and heritability estimates range between 0.2 and 0.5 (Brevern et al. 1994; Mayer 1995). It occurs in commercial pig breeds with frequencies between 8% and 30% (Große Beilage et al. 1996; Mayer 1995). In comparison to other hereditary disorders, it is difficult to diagnose during routine selection where young sows are inspected while standing or moving within an

arena (Steffens 1993). Since the mode of inheritance is not fully understood and the number of genes involved is unknown, it is important to get more knowledge of the genetic cause of the defect. Moreover, identification of markers for this liability trait offers perspectives to develop efficient DNA-based selection tools for the improvement of the quality of teats. Therefore, the main objective of this study was to identify chromosomal regions and candidate genes for the inverted teat defect by a QTL scan in an experimental population and to validate these results in a commercial population.

Material and methods

Animals

Analyses were performed on two different populations, an experimental population and animals from commercial dam lines. The German Landrace (DL) and Large White (DE) breeds were chosen for the confirmation studies that are the most commonly used dam lines in Germany. Accordingly, there is selection against the inverted teat defect in these lines.

The experimental population was based on a reciprocal cross between Duroc and Berlin Miniature pigs (DUMI population). For F_1 one boar from the Duroc breed was mated to four Miniature pig sows and one Miniature pig boar was mated to five Duroc sows to produce 43 F_1 dams and five F_1 boars. The F_1 boars and dams were mated to finally produce 905 F_2 animals. At 200 days of age the phenotypes of the teat traits were observed by two investigators of a team of four trained and skilled persons involved in this study. Animals were placed on their backs and teats were evaluated by inspection and palpation. Numbers of functional and inverted teats were recorded. This is similar to the time-point of selection of young sows in breeding companies. At this time the inverted teat defect was detected in 42.2% of the F_2 animals.

For the commercial population, 2160 animals (castrated male pigs) of the German Landrace (DL) and German Large White (DE) breeds and their crosses were used. The animals were kept at performance test stations (*LPA Haus Düsse* and *LPA Frankenforst*) under standardized conditions according to the guidelines of the German performance test (ZDS 2003) and monitored at the slaughter line when 180 days old on average. Each animal was examined by two inspectors by inspection and palpation of teats at a separate slaughter line. Of these, 244 animals were identified with at least one inverted teat. Samples of some of these animals and their affected or nonaffected full-sibs were used for QTL analysis. The samples from the parents of these animals were collected in commercial farms and the artificial insemination station, respectively. Only animals for which samples of parents and at least one full-sib were available were used in this study for the verification of QTL detected in DUMI population. Overall, 119 families could be analyzed from these samples. In addition, 100 animals generating 11 families derived from *LPA Grub* from the *Bayerische Landesanstalt für Landwirtschaft* in Bavaria.

Genotyping and mapping

DNA for genotyping was isolated from sperm, muscle, or tail using phenol-chloroform extraction. Each of the 12.5 µl of PCR reaction contained 50 ng genomic DNA, 0.2 µM of each primer, 50 µM of each dNTP, 0.5 U of *Taq* polymerase, and 1.5 mM $MgCl_2$ in 1× PCR buffer. PCR was performed in the thermal cycler PTC 100 (MJ Research, Waltham, MA) at 94°C for 3 min, followed by 30 40 cycles at 94°C for 1 min, 55 65°C for 1 min, 72°C for 1 min, and final extension at 72°C for 10 min. The annealing temperature was used depending on the optimal temperature for primer. Seventy-two type II markers and 29 type I markers covering the porcine autosomes with a mean interval of 23.1 cM were selected from published linkage maps (USDA-MARC and PiGMaP) and used in the DUMI population. In the commercial population, almost the same set of polymorphic markers was used.

Electrophoresis was performed with the LI-COR model 4200 automated DNA sequencer (LI-COR Biosciences, Lincoln, NE) using 12% SequaGel in 1 × TBE buffer at a power of 1500 V, 50 mA, and 50 W at 50°C. Evaluation of genotypes was done using OneDScan v4.10 (LI-COR Biosciences). Linkage analysis for building of the maps was performed using CRIMAP v2.4 (Green 1992). The genetic map obtained has been described previously (Wimmers et al. 2006). The QTL analysis for the inverted teat quantitative trait was performed with the software package Genehunter v2.0 (Whitehead Institute, Cambridge, MA) (Kruglyak et al. 1996). Genehunter calculated a normalized score $Z(v)$ with a mean of zero and a variance of one under the null hypothesis. The null hypothesis is that there is no linkage between the disease locus and the marker allele (Kruglyak et al. 1996).

$$Z(\overline{x}) = \frac{[S(\overline{x}) - \mu]}{s}$$

$$S(\xi) = S_{\text{pairs}}$$

$$\xi = \text{mean of } S_{\text{pairs}} = p\xi S_{\text{pairs}}$$

$$p = a\ priori \text{ probability}$$

$$s^2 = p\xi(S_{\text{pairs}} \quad \mu)^2$$

Afterward the null hypothesis is calculated under the normalised test statistic NPL (nonparametric linkage)

$$\mathrm{NPL} = \xi Z(v) \times P$$

P = a posteriori probability

The NPL analysis calculated the part of the shared allele at each loci on the whole chromosome. These alleles are mentioned as $Z0$, $Z1$, and $Z2$ where

$$z_0 = \frac{a_0}{\lambda_s},\ z_1 = a_1,\ z_2 = a_2 \frac{(2\lambda - 1)}{\lambda_s}$$

where λ_s is the relative risk for the sibling, $a_0 = ¼$, $a_1 = ½$, and $a_2 = ¼$. Chromosome-wide significance was calculated using Genehunter and transformed to experiment-wide significance levels using a permutation test (Churchill and Doerge 1994). The experiment-wide significance level was calculated by

$$P_{\mathrm{g}} = \frac{1 - (1 - P_{\mathrm{c}})}{r}$$

where r is the length of the specific chromosome divided by the total length of all chromosomes and P_{c} is the chromosome-wide significance level.

Further significant and suggestive QTL were defined (Lander and Kruglyak 1995), whereas in a F_2 population the NPL score of 4.3 is necessary to reach a significance level of 1% using a genome-wide investigation, the significance level of 5% corresponds to a NPL score of approximately 3.0 (Lander and Kruglyak 1995).

The association analysis was performed using the FBAT (family-based association test), a modified test based on TDT (transmission disequilibrium test). In this study the FBAT program (version 1.4) was used to perform the qualitative and quantitative family-based analyses of both populations (http://www.biostat.harvard.edu/~fbat/fbat.htm). For all, the quantitative analyses were done under the condition of an additive model (Horvath et al. 2003). FBAT performed the testing by a two-step procedure. First, the test statistic was defined showing the association between the trait locus and the marker locus. Second, the distribution of the genotype data is tested under the hypothesis. The genotypes of the offspring are treated as random (Rabinowitz and Laird 2000). The general FBAT statistic U is based on a linear combination of the genotypes of offspring and the traits:

$$U = S - E[S]$$

$$S = \sum_{ij} T_{ij} X_{ij}$$

where X_{ij} is a function of the genotype of the jth offspring in family i at the locus tested (depending on the used genetic model) and T_{ij} is a function of the trait (dependent on possible unknown parameters).

$$T_{ij} = Y_{ij} - \mu_{ij}$$

where Y_{ij} is the observed trait of the jth offspring in the family i and μ_{ij} is a random variable. The general FBAT statistic is calculated under the hypothesis that there is "no linkage and no association between marker and gene." This depends on T_{ij} and on the genotypes of the parents. Under this hypothesis, U is such that $E(U) = 0$. With the distribution of the genotypes of offspring (X_{ij} is treated as random and as T_{ij} fixed), $V = \mathrm{Var}(U) = \mathrm{Var}(S)$ can also be calculated under the hypothesis and can be used for the standardization of U. If X_{ij} is the scalar sum of the genotypes of an individual, then the total statistic of the samples is

$$Z = U/\sqrt{V}$$

approximately $N(0,1)$. If X_{ij} is a vector, then

$$\chi^2 = U'V - U$$

an approximately χ^2 distribution with the degrees of freedom similar to V (Horvath et al. 2003). In this study the FBAT was performed under the hypothesis: "no association and no linkage."

Results

In the F_2 of the experimental population, the mean number of teats was 12.9. The occurrence of the inverted teat defect was 42% (380 of 905 animals), with on average 5.2 inverted teats per affected animal at equal frequencies in both sexes. For the commercial breeds, of the 2160 animals monitored in the slaughterhouse, 244 animals (11.3%) were found to have one and more inverted teats (mean = 2.3). In the commercial dam lines, the mean number of teats was 14.6. The incidence of inverted teats was slightly higher in DL than in DE and F_1 animals (12.5%, 10.7%, and 10.5%, respectively).

In the experimental population, QTL were detected on all autosomes except SSC17, reaching 5% chromosome-wide significance. Fourteen QTL reached the experiment-wide significance level. Of these 14, eight were found with highly significant experiment-wide NPL values (Table 1). In the experimental population, 12 suggestive QTL (NPL > 3.0) for the inverted teat defect were detected (Lander and Kruglyak 1995). Of these, five significant QTL (NPL > 4.3) were detected. As in the commercial population, the affected sib-pair design was used and the p values of the estimated NPL values detected by the Genehunter program were used for definition of the significance levels. Five QTL positions were found, with only one NPL value exceeding the 5% experiment-wide

Table 1 Evidence for QTL significant at 5% chromosome wide[a] and experiment wide[b] levels for the inverted teat defect in the DUMI population

SSC	NPL value	*p* value[a]	*p* value[b]	Closest marker to QTL	Marker position[c]	Flanking marker	
						Upper	Lower
1	4.43	≤0.01**	0.09	SW1301	141	S0155	
2	4.56	≤0.01**	0.04*	SW2443	0		L144
3	3.9	≤0.01**	≤0.01**	S0164	61	SW72	NTAN1
4	5.2	≤0.01**	≤0.01**	S0097	120	S0214	
5	3.83	≤0.01**	≤0.01**	SW1482	40		SW1134
6	2.81	≤0.01**	0.02*	S0035	7		S0087
6	9.25	≤0.01**	≤0.01**	S0220	78	S0300	S0059
7	2.63	≤0.01**	0.04*	S0101	135	S0115	
8	3.41	≤0.01**	≤0.01**	SW2410	1		KIT1
9	2.35	≤0.01**	0.12	SW911	37	SW21	SW54
10	2.88	≤0.01**	0.02*	SW2067	128	ITIH2	
11	3.35	≤0.01**	≤0.01**	S0386	60	S0071	SW703
12	6.7	≤0.01**	≤0.01**	S0143	6		SW874
13	1.92	0.02*	0.35	SW398	79	SW344	IL12A
14	3.78	≤0.01**	≤0.01**	S0007	60	MBL2	VIN
15	2.44	≤0.01**	0.08	SW936	89	SW1111	SW1119
16	3.14	≤0.01**	0.01*	S0061	93	IL12B	
17	0.64	0.23	0.99	SW2431	94	SW840	
18	3.23	≤0.01**	0.02*	SWR414	58	SW787	

* Significant at 5% level; ** significant at 1% level

[a] Chromosome wide, [b] experiment wide, [c] position of marker closest to QTL on public linkage map (USDA MARCv2)

significance threshold (Table 2). QTL regions in overlapping marker intervals or close proximity in both populations were found on SSC3, SSC4, SSC6, and SSC11 (Fig. 1).

The association analysis using the FBAT program revealed more alleles of Duroc origin with a positive influence on the development of normal teats (negative *Z* score). The following alleles of Duroc origin could be shown to be highly associated with normal teat development: on SSC1, allele 165 of marker SW1301 (*Z* score = −3.8); on SSC4, allele 234 of marker S0097 (*Z* score = −7.3); on SSC6, allele 154 of marker S0220 (*Z* score = −4.9); and on SSC14, allele 174 of marker S0007 (*Z* score = −5.1). The allele 161 of marker SW1301 on SSC1 and the allele 286 of marker S0164 on SSC3 originated from the Miniature pig and were shown to be highly associated with the inverted teat defect (*Z* scores = 7.2 and 2.5, respectively). The Miniature pig-originated alleles 207 of marker SW2443 (SSC2), 302 of marker S0164 (SSC3), and 212 of S0097 (SSC4) showed positive impact on teat development (*Z* scores = −2.6, −2.3, and −2.0, respectively). Data of association compared to linkage analysis of both populations are shown in Table 3.

The distal region of SSC1 contained a QTL for the inverted teat defect with NPL value reaching the 1% chromosome-wide significance in the DUMI population (Table 1). No QTL were found on SSC1 in the commercial dam lines. However, the family-based association test revealed significant association and linkage of marker SW1301 in both the experimental and the commercial population (Table 3).

A significant QTL was detected on SSC2 in the telomeric region with a 5% experiment-wide significant NPL value in animals of the experimental population (Table 1). This could be confirmed by association analysis of the marker SW2443 (Table 3). The QTL could not be detected when analyzing all animals of the commercial population. Linkage analysis of only families of crossbreeds (DE × DL, DL × DE) revealed a QTL with a NPL value significant at the 5% chromosome-wide level at marker SW1564, central on SSC2.

The distal region on SSC3 showed a highly significant QTL for the inverted teat defect in the DUMI population (Table 1). The locus was confirmed at 5% chromosome-wide significance in the commercial animals as well as by association analysis (Tables 2 and 3). Two alleles (218, 286) were identified, inherited from Duroc and Miniature

Table 2 Evidence for QTL significant at 5% chromosome wide[a] and experiment wide[b] level for the inverted teat defect in the commercial dam lines

SSC	NPL value	Closest marker to QTL	Marker position[c]	Flanking marker		*p* value					
				Upper	Lower	All[d]		DL[d]		DL × DE / DE × DL[d]	
						chr[a]	exp[b]	chr[a]	exp[b]	chr[a]	exp[b]
1	0.69	SW1301	141	S0155		0.11	0.48	0.21	0.75	0.18	0.69
2	0.61	SW1564	55	SW240	SW834	0.15	0.96	0.4	0.99	0.05*	0.62
3	1.16	S00164	61	SW72	SW2570	0.02*	0.36	0.56	0.99	0.05*	0.63
4	1.06	S0214	120	S0214	SW857	0.02*	0.33	0,.7	0.69	0.09	0.78
5	1.45	SW967	146	IGF1		0.07	0.75	0.28	0.99	0.01*	0.19
6	1.16	S0035	7		S0087	0.02*	0.16	0.18	0.87	0.07	0.52
6	1.64	S0059	93	S0220	S0003	≤0.01**	0.02*	0.03*	0.27	0.05*	0.41
7	0.77	S0101	135	S0115		0.1	0.75	0.17	0.92	0.13	0.84
8	0.9	S0086	62	SW2611	S0144	0.06	0.8	0.06	0.79	0.02*	0.79
9	0.36	SW54	67	SW911	S0109	0.33	0.99	0.19	0.97		
10	0.64	SW830	0		S0070	0.14	0.92	0.07	0.73	0.29	0.99
11	1.95	SW703	76	S0009		≤0.01**	0.24	0.04*	0.83	0.05	0.90
12	0.74	SW874	65	GH	SW605	0.21	0.99			0.17	0.99
13	0.73	SW344	35	S0219	PIT1	0.2	0.97	0.42	0.99	0.04*	0.44
14	0.51	SW857	7		S0007	0.21	0.99	0.12	0.99	0.43	0.99
15	0.77	SW936	89	SW1111	SW1119	0.08	0.99	0.15	0.99	0.24	0.99
16	0.5	S0111	0		S0026	0.20	0.99	0.31	0.99	0.28	0.99
17	0.7	SW840	49	SW335	GHRH	0.12	0.85	0.19	0.96	0.13	0.89
18	0.17	SW1023	32		LEP	0.37	0.99	0.29	0.99	0.42	0.99

* Significant at 5% level; ** significant at 1% level

[a] Chromosome wide, [b] experiment wide, [c] position of marker closest to QTL on public linkage map (USDA MARCv2)

[d] All merged analysis of German Landrace and F_1 (DL*DE/DE*DL); DL German Landrace; DE Large White

pig, respectively, showing an increase of liability for the inverted teat defect (Table 3).

Linkage and association analyses of SSC4 revealed significant QTL with a peak at marker S0097 in animals of the experimental population (Table 1). A significant NPL value could be detected in animals of commercial families in the region between markers S0214 and S0097, i.e., in close proximity to the QTL found in the DUMI (Table 2). Family-based association tests revealed significant association and linkage of alleles of marker S0097 in both populations.

On SSC5 a highly significant QTL was detected in the region of the microsatellite marker SW1482 (Table 1). The analysis of the crossbreed animals in the commercial group revealed a QTL on SSC5 with a NPL value significant at a 5% chromosome-wide significance level more distal (Table 2).

The highest NPL value of 9.25 was detected on SSC6 with a peak at marker S0220 (Fig. 1). In the dam lines the highest NPL value was detected at marker S0059, distal of S0220. This QTL was the only locus with experiment-wide significance in the commercial animals. There was good agreement between the NPL plots found in the analysis of all commercial animals and analysis separated by breeds. A NPL value of 1.16 implementing a chromosome-wide 5% significance level was detected in the commercial animals with a peak at marker S0035 telomeric of SSC6. At this position a QTL reaching a 5% experiment-wide significance level was also obtained in the DUMI population. Alleles of marker S0220 were significantly associated with the defect in both populations, whereas alleles of markers S0059 and S0035 showed association only in the commercial and experimental populations, respectively. Results of linkage and association analyses in both populations indicate that there are at least two QTL segregating on SSC6 that affect the inverted teat defect.

The distal region of SSC7 showed significant NPL values for the heritable inverted teat defect, as revealed by full-sib analysis, between markers S0115 and S0101 at the experiment-wide 5% significance level only in the experimental population.

SSC8 carried a QTL at the proximal end in the DUMI population. The region around marker S0086, which is more distal, showed a significant NPL value in the analysis of crossbreed animals of the commercial population (Table 2).

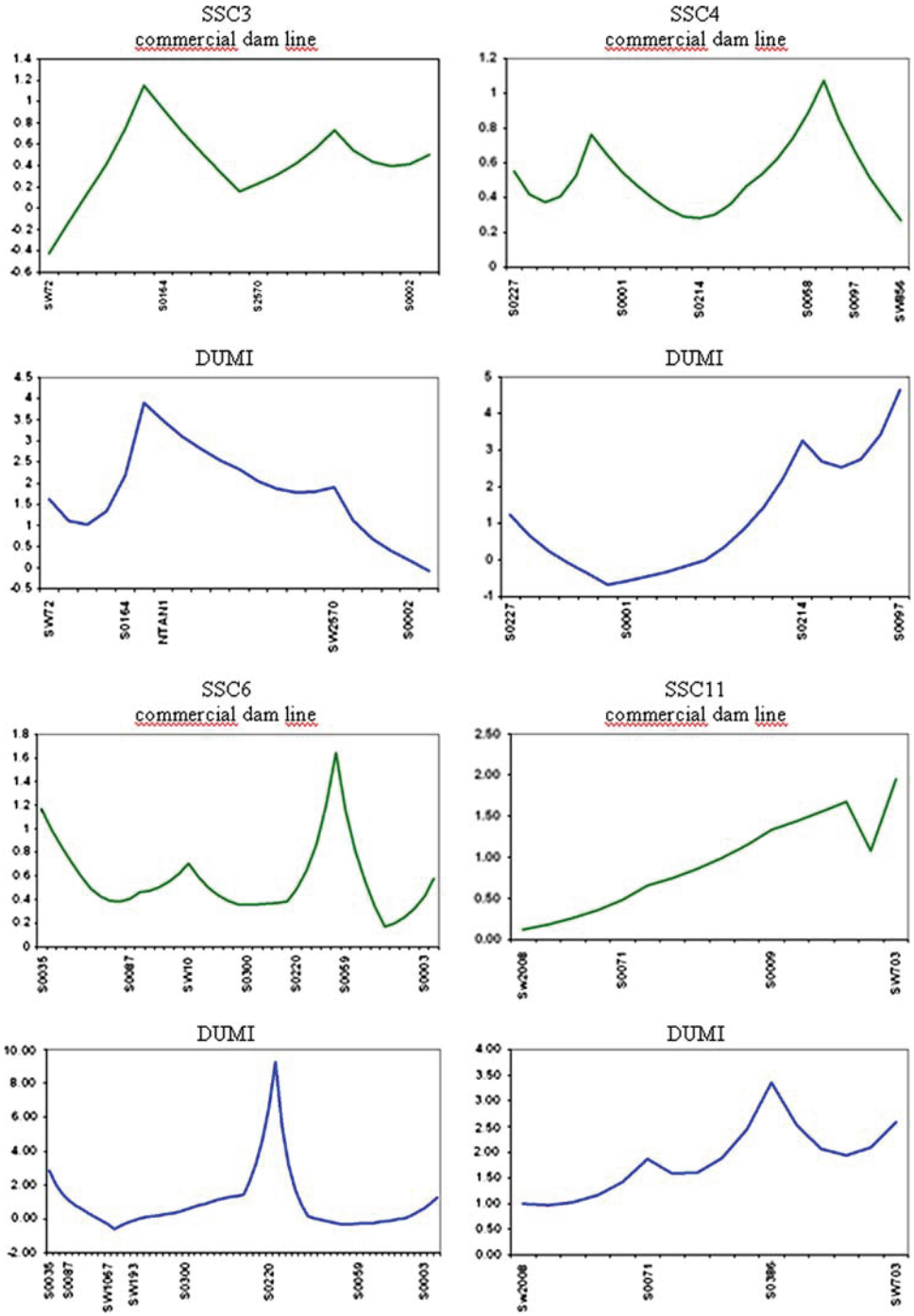


Fig. 1 Profiles of multipoint NPL scores for SSC3, 4, 6, and 11, respectively, obtained from QTL analysis in the experimental population DUMI and commercial dam lines. Positions of markers genotyped are given on the *x* axis; numbers on the *y* axis indicate NPL values

On SSC11 a QTL reaching the 1% experiment-wide significance level in the DUMI population was found surrounding marker S0386 (Table 1). In the commercial animals, a NPL value reaching the 1% chromosome-wide significance level was detected in close proximity at SW703. The peak was also detected in the families that included only animals of the German Landrace breed (Table 2). Both regions could be confirmed by association analysis that revealed alleles of marker S0386 being associated with the defect status in the experimental population and alleles of marker SW703 being associated with the defect in both populations (Table 3).

A significant QTL could be detected on SSC12 between the markers SW605 and L147 in animals of the DUMI population. The NPL score of 6.7 was the second highest in this analysis (Table 1). There was no evidence found for a QTL nor an associated allele in the commercial animals.

SSC14 contained a significant QTL next to the marker S0007, but no significant QTL was detected in the

Table 3 Overview of results, including markers at positions of QTL detected in either the DUMI population or the commercial dam lines or both

SSC	Marker	QTL (Genehunter) NPL value		Association study (FBAT)					
				Significant allele[e]		*Z* value[d]		*P* value	
		DUMI	comm	DUMI	comm	DUMI	comm	DUMI	comm
1	SW1301	4.43[a]	0.69	161[1]	.	7.197	.	≤0.001	.
				163[3]	.	5.604	.	≤0.001	.
				165[2]	.	3.757	.	≤0.001	.
				171[2]	171	1.897	2.425	0.058	0.015
2	SW2443	4.56[a]	0.43	203[3]	.	2.455	.	0.014	.
				207[1]	207	2.568	1.866	0.010	0.062
3	S0164	3.9[a]	1.16[c]	214[3]	ns	2.496	ns	0.013	ns
				218[2]		2.958		0.003	
				266[3]		2.395		0.017	
				286[1]		2.475		0.013	
				302[1]		2.297		0.022	
4	S0097	5.2[a]	0.67	205[3]	148	7.19	3.888	≤0.001	≤0.001
				212[1]	170	1.972	3.458	0.049	≤0.001
				234[2]	172	7.296	2.513	≤0.001	0.012
	S0058		1.06[c]		ns		ns		ns
5	SW1482	3.83[a]		108	ns	2.465	ns	0.014	ns
				110		2.21		0.027	
6	S0035	2.82[b]	1.16[c]	179	ns	3.007	ns	0.003	ns
	S0059	0.83	1.64[c]	ns	144	ns	1.896	ns	0.058
					146		2.419		0.016
					154		2.121		0.034
	S0220	9.25[a]	0.1	146[3]	.	4.248	.	≤0.001	.
					148	.	2.530	.	0.011
				154[2]	.	4.9	.	≤0.001	.
8	SW2410	3.41[a]		ns	ns	ns	ns	ns	ns
11									
	S0386	3.35[a]		154	ns	2.037	ns	0.042	ns
				158		3.889		≤0.001	
	SW703	2.58	1.95[c]	127	.	3.741	.	≤0.001	.
				133	.	1.941	.	0.052	.
				.	135	.	2.236	.	0.025
				.	137	.	2.076	.	0.038
12	S0143	6.7[a]		155	ns	7.673	ns	≤0.001	ns
				157		7.013		≤0.001	
14	S0007	3.78[a]	0.24	158[3]	.	5.755	.	≤0.001	.
				174[2]	.	5.149	.	≤0.001	.
				.	177	.	2.041	.	0.041
				.	187	.	2.064	.	0.039
16	S0061	3.14[a]		166	ns	2.405	ns	0.016	ns
				170		1.899		0.058	
18	SWR414	3.23[a]	0.13	123	.	2.181	.	0.029	.
				147	.	1.882	.	0.06	.
				.	153	.	2.566	.	0.01

ns not significant

[a] QTL according to the definition of Lander and Kruglyak (1995), i.e., NPL > 3, [b] QTL reaching 5% genome wide significance, [c] QTL according to the chromosome wide *p* value of ≤0.05 in all animals of commercial population, [d] Positive *Z* scores indicate that alleles can be found more often in affected animals, [e] Names of alleles derived from their length in bp, [1] Origin breed Miniature pig, [2] Origin breed Duroc, [3] Allele inherited from both breeds

commercial animals (Table 1). Significant association of the marker S0007 was found in both populations (Table 3).

On SSC16 and SSC18 suggestive QTL were detected in DUMI population; in addition, significant associated alleles of markers S0061 and SWR414 were also found. In the commercial population, significant association of alleles of markers SWR414 on SSC18 was found, however, none were found on SSC16 (Table 3).

Even though the NPL values calculated on SSC9, 10 and 13 were significant and highly significant at the chromosome-wide level, they could not be defined as QTL according the definition of Lander and Kruglyak (1995). Significant associated alleles were detected only on the respective markers on SSC9 and 10. On SSC17 no significant QTL was detected.

Discussion

A linkage study was performed for the heritable defect inverted teat in the pig in an experimental population that showed a high incidence of the defect. To evaluate the relevance of the results in commercial pig breeds and to confirm the results, a linkage study was done in an independent set of animals of the German Landrace breed and its cross with Large White; these breeds represent the most important dam lines in pig production in Germany. The study showed that the inverted teat defect is governed by genetic variation at several loci distributed throughout the genome. This is in agreement with more recent models of inheritance of the inverted teat defect that propose a polygenic background, while early works suggested autosomal recessive inheritance with reduced penetrance (Nordby 1934; Mayer and Pirchner 1995).

The emergence of inverted teats depends on insufficient mesenchymal proliferation at the teat ground during teat development (Günther 1984). As far as proliferation processes are concerned, the defect of inverted teats is related to the number-of teats trait. Because this etiologic link of the traits, QTL for inverted teats and number of teats can be expected to be partly identical. The number of teats may also depend on local signaling between adjacent embryonic tissues that determine the somite patterning and cell fate decisions leading to the establishment of the mammary bud but is less important for the liability to express the inverted teat phenotype. There are a few reports on QTL for the number of teats in the pig that are summarized in a publicly accessible database, PigQTLDB (Hu and Reecy 2007; Hu et al. 2005). Most studies used an experimental population of crosses with the breed Meishan (Hirooka et al. 2001; Rodriguez et al. 2005; Rohrer et al. 1999; Yue et al. 2003). Meishan is known for its high number of teats, about 17.0 on average (Haley et al. 1995), compared with that of the Large White breed, with about 14 teats (Clayton et al. 1981; Haley et al. 1995).

On SSC1 QTL for the number of teats were detected between markers SJ029 and SWR485 and between markers SW803 and SW373 in resource populations from different groups (Beeckmann et al. 2003b; Cassady et al. 2001; Geldermann et al. 2003; Rohrer et al. 1999; Wada et al. 2000). In the latter region with upper marker S0155 and peak at marker SW1301, a QTL for the inverted teat defect was detected in the experimental population in this study. Comparing the results from different QTL studies, we can conclude that there might be a number of genes influencing teat development on SSC1. The relaxin gene (*RLN*) maps to SSC1 at position 1q28 q29, showing association with the inverted teat defect (Wimmers et al. 2002). *RLN* knockout mice were shown to exhibit impaired mammary development resulting in a phenotype similar to inverted teats that cannot be suckled by the offspring (Zhao et al. 1999). Another gene involved in teat development is the estrogen receptor (*ESR*) gene, in the region 1p24 p25 where a QTL for the number of teats was found (Wada et al. 2000). The *ESR* gene is already used as a marker for the litter size in different porcine populations (Short et al. 1997). Even though no QTL could be detected in commercial families, SSC1 contains candidate genes for the development of functional teats. By the results of association analysis using the marker SW1301 on SSC1, there is more evidence corroborating this assumption.

QTL for the number of teats were located in the proximal region of SSC2 (Hirooka et al. 2001; Lee et al. 2003a), close to the QTL for inverted teats reported here. Positional candidate genes are insulin-like growth factor 2 (*IGF2*) and follicle-stimulating hormone beta (*FSHB*). Insulin-like growth factor-2 is a growth factor that mediates prolactin induced in mammary gland development that shows dose-dependent mitogenic properties on primary human breast epithelial cells and stroma cells (Hovey et al. 2003; Strange et al. 2002, 2004). The follicle-stimulating hormone (FSH) is essential for normal reproductive function in males and females; it is more important for female than for male fertility (Tapanainen et al. 1997). In another study it was also found that the *FSH* gene plays a role in the morphologic development of the mammary gland within lactogenesis (McNeilly 1994).

For SSC3 one QTL for the number of teats is published at 84 cM with an *F* statistic of 15.78 (Rohrer 2000). In the resource population a QTL in the same region next to marker S0164 was detected. The transforming growth factor alpha (*TGFA*) gene and the follicle-stimulating hormone receptor (*FSHR*) gene map to 3q22 q23 and 3q21, respectively (Remy et al. 1995). FSHR mediates effects of FSH on mammary development. Growth-promoting TGFA and growth-inhibiting TGFB are

coexpressed in the bovine mammary gland. Higher mRNA contents of both factors during mammogenesis and involution may indicate autocrine or paracrine functions for these growth factors during proliferation and reorganization of the mammary tissue (Plath et al. 1997).

On SSC4 a QTL for the inverted teat defect was found in the commercial population where the thyroid-stimulating hormone beta (*TSHB*) gene at 4q21 is a functional and positional candidate gene. The metabolic hormone TSH is necessary for alveolar morphogenesis in proliferation and lactogenesis of the mammary gland. Thyroid hormone, as well as prolactin, is an important regulator of the functional development of the mammary gland. A lower level of TSH in the serum leads to a loss of the ductal growth and a lower or no proliferation of alveolar tissue (Bhattacharjee and Vonderhaar 1984; Vonderhaar and Greco 1979).

In studies by Lee et al. (2003b), a QTL for the number of teats was detected between markers SWR453 and SW2425 on SSC5. In our study a high NPL value for the heritable inverted teat defect was detected in the same region. The insulin-like growth factor-1 (*IGF1*) gene is suggested to be a candidate gene for teat development in pig; however, a microsatellite within *IGF1* showed no association with the inverted teat defect in this study. Another candidate gene, the parathyroid-hormone-like hormone (*PTHLH*) gene on SSC5 is found to be expressed in the stroma cell of the mammary gland. An overexpression of PTHLH in transgene mice leads to a mismatch of the gland and ductal growth in different stages (Dunbar et al. 2001; Wysolmerski et al. 1994, 1995). It was found that a C/T nonsynonymous single nucleotide polymorphism (S19L) at nucleotide position 375 of the porcine PTHLH cDNA was associated with the inverted teat defect (Chomdej 2005; Chomdej et al. 2004).

In this study two significant QTL in different regions on SSC6 could be detected. The NPL value had its peak in the DUMI and the commercial population at markers S0220 and S0059, respectively. Furthermore, in both populations a significant NPL value could be detected at marker S0035. A QTL for the number of teats was detected in a three-generation resource population between markers DG93 and SW1328 with a peak at 171 cM (Cassady et al. 2001). The result of linkage analysis in this study was partly verified by the association analysis in both populations.

Our linkage analysis in experimental and commercial families highlight the telomeric region of SSC6 close to marker S0035 and the central region close to markers S0059 and S0220 as regions containing QTL for the inverted teat defect segregating in both populations. The comparative map of pig and human indicate synteny of QTL at marker S0035 with the telomeric q-arm of HSA16. S0220 and S0059 direct to HSA1 (Meyers et al.2005). However, whereas the current status of the comparative map clearly shows correspondence of the genomic region of S0059 to HSA1, it still has to be proven that S0220 may also correspond to HSA19. Establishment of this relationship will finally contribute to clarification of whether the NPL plot peak at S0220 in the DUMI represents the same QTL as the NPL plot peak at S0059 obtained in the dam lines. There were several genes already mapped on SSC6 that are involved in the development of the mammary gland. The *TGFB* gene at 6q11 q21 (directing to HSA19) and the leptin receptor (*LEPR*) gene at 6q33 q35 (directing to HSA1) are suggested to be candidate genes for teat development. Leptin receptors have been also appeared in mammary epithelial cells and it has been suggested that leptin is involved in the control of the proliferation of both breast cells (Dundar et al. 2005). Other candidate genes, such as the wingless-type MMTV integration site family, member 4 (*WNT4*) gene, which plays a part in the early development of the mammary gland, could be found in the comparative region between markers S0220 and SW59. The LIM domain only 4 (*LMO4*) gene was mapped at HSA1p22.3; a higher expression of this gene could be shown to suppress the differentiation of epithelial cells in mammary gland (Visvader et al. 2001). The impact of the protein tyrosine phosphatase, receptor-type, F (*PTPTF* or *LAR*) gene was shown in knockout mice, in which female mice could not deliver milk. LAR-mediated signaling may have an impact on development and function of the mammary gland (Schaapveld et al. 1997). Another gene involved in mammary gland development that mapped onto HSA1 is the fatty acid-binding protein 3 (*FABP3* or *MDGI*) gene, also called mammary-derived growth inhibitor. It was the first gene identified as a growth inhibitor in the lactating bovine mammary gland (Bohmer et al. 1987). In summary, several positional and functional candidate genes could be identified; further studies need to be done to identify the exact position of the QTL on SSC6 and compare it to the human genome map to detect the regions with the highest concordance in these two species.

Several QTL for the number of teats have been found on SSC7 (Cassady et al. 2001; Wada et al. 2000). A QTL for the inverted teat defect on 7q25 close to marker S0101 was detected only in the experimental population. The prolactin (*PRL*) gene on 7p11 p12 is a functional candidate gene for teat characteristics. PRL is involved in different physiologic processes and is a key factor for the development of mammary gland and lactation (Bole-Feysot et al. 1998). The activity of PRL over its receptor in lactation is essential for the regulation of the metabolism of the adipose tissue (Ling et al. 2003).

Different QTL for the number of teats were detected on SSC8, suggesting that there may be different genes involved in mammary gland development (Beeckmann et al. 2003a; Cassady et al. 2001; Hirooka et al. 2001;

King et al. 2003). On SSC8 a QTL with a peak at SW268 was suggested (King et al. 2003). The QTL between SW1070 and S0144 includes the marker S0086, where in our study a chromosome-wide significant NPL value was detected in the commercial crossbreed population (Beeckmann et al. 2003a). A QTL for the number of nipples in pigs between markers SY23 and SW905 was detected, and a QTL with a peak at marker SW2410 was detected in the DUMI population (Cassady et al. 2001). The relaxin receptor leucin-rich G-protein-coupled receptor 7 (LGR7) was mapped on HSA4 in the comparative region of SSC8. LGR7 is likely to be involved in the development of the mammary gland and is a candidate for the inverted teat defect, because its ligand showed an association with the defect (Chomdej 2005; Wimmers et al. 2002). Radiation hybrid (RH) mapping of LGR7 showed that this gene is located next to SW368.

QTL for the number of nipples detected on SSC10 include regions between markers SW1708 and SW2067, between markers SW1991 and SW1626, between markers SWR1849 and SW2000, and between markers SW1041 and SW951 (Dragos-Wendrich et al. 2003; Hirooka et al. 2001; Rodriguez et al. 2005; Rohrer 2000). It can be summarized from these studies that in the region between markers SWR1849 and SW2067, QTL for the number of nipples were found. In DUMI population the peak of the experiment-wide significant NPL value was detected at marker SW2067 (Table 1). There are no candidate genes proposed in these studies. The orthologous human genomic region on chromosome 10 harbors calmodulin-like 3 protein (CALML3), a protein specifically expressed in mammary epithelial cells that is regulated during differentiation and might be involved in Ca-dependent pathways during mammary development (Yaswen et al. 1992).

The QTL with the highest peak at the marker S0386 on SSC11 could be confirmed in the commercial families. A QTL for the number of teats was found with an *F* value of 3.25 (Cassady et al. 2001). The breast cancer susceptibility (*BRCA2*) gene, which is involved in teat development, was detected on SSC11 (Bignell et al. 1997; Musilova et al. 2000).

QTL for the number of teats were detected in several experimental populations in different regions of SSC12 (Hirooka et al. 2001; Rodriguez et al. 2005; Yue et al. 2003). The detected region in our own study promotes the growth hormone as a positional candidate gene for the teat defect.

Our detected significant and suggestive QTL in the experimental population on SSC14, 16, and 18 could not be confirmed in the commercial population. Interestingly, alleles of most significant markers S0007 on SSC14 were shown to be significantly and highly significantly associated with the defect status in both populations.

Only one publication suggests a QTL region on SSCX in pig (Cepica et al. 2003). The androgen receptor (*AR*) gene was mapped to the X chromosome. This gene shows an association with the number of teats but no association with the inverted teat defect could be detected (Trakooljul 2004). In the present study no linkage mapping on SSCX was conducted.

The mothering ability of a sow plays an important role in the economic efficiency of pig production. Sows cannot raise more piglets than they have functional teats. The teat characteristics are used as selection criteria in pig breeding, with typically a minimum of 12 to 14 functional teats and no defect teats requested.

To detect the genetic cause of heritable defects, different approaches can be used. In our study the application of linkage analysis, which is *a priori* hypothesis-free, was chosen. The QTL analysis was performed in different populations: an experimental population with high defect incidence to increase the power of this approach, and commercial populations to confirm results and to evaluate their relevance for commercial breeding. QTL regions in overlapping marker intervals or close proximity in both populations were found on SSC3, SSC4, SSC6, and SSC11. Further association analysis of each marker separately provided further evidence of regions being involved in the phenotype of the inverted teat defect. For some regions with significant NPL values in both populations or significant QTL in the DUMI population, significant association and linkage of different alleles to the defect could be shown by the family-based association test. However, the genetic origin of the inverted teat defect has not been detected yet. Future studies are necessary to detect genes that are involved in the development of the inverted teat defect. Combining these results with current attempts to identify genes expressed during mammary gland development will facilitate the detection of the causal genes.

Acknowledgments This work was supported by funds provided by the Development Association for Biotechnology Research (FBF) and the Research Federal Ministry of Education and Research (BMBF).

References

Beeckmann P, Moser G, Bartenschlager H, Reiner G, Geldermann H (2003a) Linkage and QTL mapping for *Sus scrofa* chromosome 8. J Anim Breed Genet 120:66 73

Beeckmann P, Schroffel J, Moser G, Bartenschlager H, Reiner G, et al. (2003b) Linkage and QTL mapping for *Sus scrofa* chromosome 1. J Anim Breed Genet 120:1 10

Bhattacharjee M, Vonderhaar BK (1984) Thyroid hormones enhance the synthesis and secretion of alpha lactalbumin by mouse mammary tissue in vitro. Endocrinology 115:1070 1077

Bignell G, Micklem G, Stratton MR, Ashworth A, Wooster R (1997) The BRC repeats are conserved in mammalian BRCA2 proteins. Hum Mol Genet 6:53 58

Bohmer FD, Kraft R, Otto A, Wernstedt C, Hellman U, et al. (1987) Identification of a polypeptide growth inhibitor from bovine mammary gland sequence homology to fatty acid binding and retinoid binding proteins. J Biol Chem 262:15137 15143

Bole Feysot C, Goffin V, Edery M, Binart N, Kelly PA (1998) Prolactin (PRL) and its receptor: Actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. Endocr Rev 19:225 268

Brevern N, Schimpf B, Wörner R, Swalve H (1994) Parameter schätzung für Zitzenmerkmale bei Hybridsauen. Züchtungskunde 66:339 348

Cassady JP, Johnson RK, Pomp D, Rohrer GA, Van Vleck LD, et al. (2001) Identification of quantitative trait loci affecting repro duction in pigs. J Anim Sci 79:623 633

Cepica S, Reiner G, Bartenschlager H, Moser G, Geldermann H (2003) Linkage and QTL mapping for *Sus scrofa* chromosome X. J Anim Breed Genet 120:144 151

Chomdej S (2005) Molecular genetic analysis of positional candidate genes for mammary gland characteristics in pigs. Thesis, Rheinische Friedrich Wilhelms Universität, Bonn

Chomdej S, Ponsuksili S, Schellander K, Wimmers K (2004) Sequencing, SNP identification and mapping of the porcine PTHLH gene to chromosome 5. Anim Genet 35:151 152

Churchill GA, Doerge RW (1994) Empirical threshold values for quantitative trait mapping. Genetics 138:963 971

Clayton GA, Powell JC, Hiley PG (1981) Inheritance of teat number and teat inversion in pigs. Anim Prod 33:299 304

Dragos Wendrich M, Moser G, Bartenschlager H, Reiner G, Gelder mann H (2003) Linkage and QTL mapping for *Sus scrofa* chromosome 10. J Anim Breed Genet 120:82 88

Dunbar ME, Dann P, Brown CW, Van Houton J, Dreyer B, et al. (2001) Temporally regulated overexpression of parathyroid hormone related protein in the mammary gland reveals distinct fetal and pubertal phenotypes. J Endocrinol 171:403 416

Dundar B, Dundar N, Erci T, Bober E, Büyükgebiz A (2005) Leptin levels in boys with pubertal gynecomastia. J Pediatr Endocrinol Metab 18:929 934

Geldermann H, Muller E, Moser G, Reiner G, Bartenschlager H, et al. (2003) Genome wide linkage and QTL mapping in porcine F 2 families generated from Pietrain, Meishan and Wild Boar crosses. J Anim Breed Genet 120:363 393

Green P (1992) Construction and comparison of chromosome 21 radiation hybrid and linkage maps using Cri Map. Cytogenet Cell Genet 59:122 124

Große Beilage E, Steffens S, Schoon HA, Bollwahn W (1996) Zitzenkörperhypoplasien und aplasien (Stülpzitzen) bei weibli chen und männlichen Schweinen. Tierärztl Praxis 24:31 35

Günther C (1984) Morphologie der sogenannten "Stülpzitze" beim Schwein im Vergleich zum histologischen Bild einer normalen Zitze. Thesis, Freie Universität, Berlin

Haley CS, Lee GJ, Richie M (1995) Comparative reproductive performance in Meishan and Large White pigs and their crosses. Anim Sci 60:259 267

Hirooka H, de Koning DJ, Harlizius B, van Arendonk JAM, Rattink AP, et al. (2001) A whole genome scan for quantitative trait loci affecting teat number in pigs. J Anim Sci 79:2320 2326

Horvath S, Xu X, Lake SL, Weiss ST, Silverman EK, et al. (2003) Family based tests for associating haplotypes with general phenotype data: application to asthma genetics. Am J Hum Genet 73:610 610

Hovey RC, Harris J, Hadsell DL, Lee AV, Ormandy CJ, et al. (2003) Local insulin like growth factor II mediates prolactin induced mammary gland development. Mol Endocrinol 17:460 471

Hu Z, Reecy JM (2007) Animal QTLdb: beyond a repository A public platform for QTL comparisons and integration with diverse types of structural genomic information. Mamm Genome 18:1 4

Hu Z, Dracheva S, Jang W, Maglott D, Bastiaansen J, et al. (2005) A QTL resource and comparison tool for pigs: PigQTLDB. Mamm Genome 16:792 800

King AH, Jiang ZH, Gibson JP, Haley CS, Archibald AL (2003) Mapping quantitative trait loci affecting female reproductive traits on porcine chromosome 8. Biol Reprod 68:2172 2179

Kruglyak L, Daly MJ, Reeve Daly MP, Lander ES (1996) Parametric and nonparametric linkage analysis: a unified multipoint approach. Am J Hum Genet 58:1347 1363

Lander E, Kruglyak L (1995) Genetic dissection of complex traits guidelines for interpreting and reporting linkage results. Nat Genet 11:241 247

Lee SS, Chen Y, Moran C, Cepica S, Reiner G, et al. (2003a) Linkage and QTL mapping for *Sus scrofa* chromosome 2. J Anim Breed Genet 120:11 19

Lee SS, Chen Y, Moran C, Stratil A, Reiner G, et al. (2003b) Linkage and QTL mapping for *Sus scrofa* chromosome 5. J Anim Breed Genet 120:38 44

Ling C, Svensson L, Oden B, Weijdegard B, Eden B, et al. (2003) Identification of functional prolactin (PRL) receptor gene expression: PRL inhibits lipoprotein lipase activity in human white adipose tissue. J Clin Endocrinol Metab 88:1804 1808

Mayer JPF (1995) Gesäugeasymmetrie und Stülpzitzenbefall bei Jungsauen. Arch Tierz Dummerstorf 38:97 91

Mayer J, Pirchner F (1995) Asymmetry and inverted nipples in gilts. Arch Tierz Dummerstorf 38:87 91

McNeilly S (1994) Suckling and the control of gonadotropin secretion. In: Knobil E, Neill JD (eds) The Physiology of Reproduction. vol 2, Chap. 60, Raven Press, New York, pp 1179 1212

Meyers SN, Rogatcheva MB, Larkin DM, Yerle M, Milan D, et al. (2005) Piggy BACing the human genome II. A high resolu tion, physically anchored, comparative map of the porcine autosomes. Genomics 86:739 752

Musilova P, Kubickova S, Vozdova M, Rubes J (2000) Mapping of the oncogene c myc (MYC) and the breast cancer suscepti bility gene (BRCA2) in the pig by FISH. Anim Genet 31:154 154

Nordby JE (1934) Congenital defects in the mammae of swine. J Hered 25:499 502

Plath A, Einspanier R, Peters F, Sinowatz F, Schams D (1997) Expression of transforming growth factors alpha and beta 1 messenger RNA in the bovine mammary gland during different stages of development and lactation. J Endocrinol 155:501 511

Rabinowitz D, Laird N (2000) A unified approach to adjusting association tests for population admixture with arbitrary pedigree structure and arbitrary missing marker information. Hum Hered 50:211 223

Remy JJ, Lahbib Mansais Y, Yerle M, Bozon V, Couture L, et al. (1995) The porcine follitropin receptor cDNA cloning, functional expression and chromosomal localization of the gene. Gene 163:257 261

Rodriguez C, Tomas A, Alves E, Ramirez O, Arque M, et al. (2005) QTL mapping for teat number in an Iberian by Meishan pig intercross. Anim Genet 36:490 496

Rohrer GA (2000) Identification of quantitative trait loci affecting birth characters and accumulation of backfat and weight in a Meishan White Composite resource population. J Anim Sci 78:2547 2553

Rohrer GA, Ford JJ, Wise TH, Vallet JL, Christenson RK (1999) Identification of quantitative trait loci affecting female repro ductive traits in a multigeneration Meishan White composite swine population. J Anim Sci 77:1385 1391

Schaapveld RQJ, Schepens HTG, Robinson GW, Attema J, Oerle mans F, et al. (1997) Impaired mammary gland development and

function in mice lacking LAR receptor like tyrosine phosphatase activity. Dev Biol 188:134 146

Short TH, Rothschild MF, Southwood OI, McLaren DG, deVries A, et al. (1997) Effect of the estrogen receptor locus on reproduction and production traits in four commercial pig lines. J Anim Sci 75:3138 3142

Strange KS, Wilkinson D, Emerman JT (2002) Mitogenic properties of insulin like growth factors I and II, insulin like growth factor binding protein 3 and epidermal growth factor on human breast epithelial cells in primary culture. Breast Cancer Res Treat 75:203 212

Strange KS, Wilkinson D, Edin G, Emerman JT (2004) Mitogenic properties of insulin like growth factors I and II, insulin like growth factor binding protein 3 and epidermal growth factor on human breast stromal cells in primary culture. Breast Cancer Res Treat 84:77 84

Steffens S (1993) Stülpzitzen ein Problem in der Schweinezucht. SUS Schweinezucht und Schweinemast 3:8 11

Tapanainen JS, Aittomaki K, Huhtaniemi IT (1997) New insights into the role of follicle stimulating hormone in reproduction. Ann Med 29:265 266

Trakooljul N (2004) Molecular and association analyses of the androgen receptor gene as a candidate for production and reproduction traits in pigs. Thesis, Rheinische Friedrich Wilhelms Universität, Bonn

Visvader JE, Venter D, Hahm K, Santamaria M, Sum EYM, et al. (2001) The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. Proc Natl Acad Sci U S A 98:14452 14457

Vonderhaar BK, Greco AE (1979) Lobulo alveolar development of mouse mammary glands is regulated by thyroid hormones. Endocrinology 104:409 418

Wada Y, Akita T, Awata T, Furukawa T, Sugai N, et al. (2000) Quantitative trait loci (QTL) analysis in a Meishan × Gottingen cross population. Anim Genet 31:376 384

Wimmers K, Chomdej S, Schellander K, Ponsuksili S (2002) Linkage mapping of SNPs in the porcine relaxin gene. Anim Genet 34:323 324

Wimmers K, Fiedler I, Hardge T, Murani E, Schellander K, et al. (2006) QTL for microstructural and biophysical muscle properties and body composition in pigs. BMC Genet 7:15

Wysolmerski JJ, Broadus AE, Zhou J, Fuchs E, Milstone LM, et al. (1994) Overexpression of parathyroid hormone related protein in the skin of transgenic mice interferes with hair follicle development. Proc Acad Natl Sci U S A 91:1133 1137

Wysolmerski JJ, McCaugherncarucci JF, Daifotis AG, Broadus AE, Philbrick WM (1995) Overexpression of parathyroid hormone related protein or parathyroid hormone in transgenic mice impairs branching morphogenesis during mammary gland development. Development 121:3539 3547

Yaswen P, Smoll A, Hosoda J, Parry G, Stampfer MR (1992) Protein product of a human intronless calmodulin like gene shows tissue specific expression and reduced abundance in transformed cells. Cell Growth Differ 3:335 345

Yue GH, Beeckmann P, Moser G, Muller E, Bartenschlager H, et al. (2003) QTL alleles on chromosome 7 from fatty Meishan pigs reduce fat deposition. Sci China C Life Sci 46:10 17

ZDS (Zentral Verband der Deutschen Schweineproduktion e. V) (2003) Richtlinie fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit beim Schwein, 10 December 2003, Bonn, Germany

Zhao L, Roche PJ, Gunnersen JM, Hammond VE, Tregear GW, et al. (1999) Mice without a functional relaxin gene are unable to deliver milk to their pups. Endocrinology 140:445 453

J. Anim. Breed. Genet. ISSN 0931-2668

ORIGINAL ARTICLE

Association of parathyroid hormone-like hormone (PTHLH) and its receptor (PTHR1) with the number of functional and inverted teats in pigs

S. Tetzlaff[1], S. Chomdej[2], E. Jonas[2], S. Ponsuksili[3], E. Murani[1], C. Phatsara[2], K. Schellander[2] & K. Wimmers[1]

1 Research Institute for the Biology of Farm Animals, Research Unit 'Molecular Biology', Dummerstorf, Germany
2 Institute of Animal Breeding Science, Animal Breeding and Husbandry Group, University of Bonn, Germany
3 Research Institute for the Biology of Farm Animals, Research Group 'Functional Genome Analysis', Dummerstorf, Germany

Keywords
Family based association; inherited disorder; liability trait; mammary gland; mothering ability.

Correspondence
Klaus Wimmers, Research Institute for the Biology of Farm Animals, Research Unit 'Molecular Biology', Wilhelm Stahl Allee 2, 18196 Dummerstorf, Germany.
Tel: +49 38208 68700;
Fax: +49 38208 68702;
E mail: wimmers@fbn dummerstorf.de

Received: 28 April 2008;
accepted: 10 September 2008

Summary

Parathyroid hormone-like hormone gene (*PTHLH*) and its receptor, parathyroid hormone/ parathyroid hormone-like hormone receptor 1 (*PTHR1*), play a role in epithelial mesenchymal interactions during growth and differentiation of different tissues and anatomic structures, including teats. Therefore, *PTHLH* and *PTHR1* were evaluated as functional candidate genes for their effects on number and shape of teats in pigs. In particular, focus was on the occurrence and number of inverted teats, the most frequent and economically relevant teat developmental defect in pigs. For this purpose, association and linkage of the *PTHLH* gene and the *PTHR1* gene with inverted teat defect and the total number of teats and inverted teats were studied in an experimental Duroc and Berlin Miniature pig (DUMI) population. Polymorphism C1819T of *PTHR1* was significantly associated with inverted teat phenotype ($p = 0.014$), total number of teats ($p = 0.047$) and was close to significance with the number of inverted teats ($p = 0.078$). Polymorphism C375T of *PTHLH* was close to significance with the inverted teat phenotype ($p = 0.122$) and showed no significant association with the total number of teats ($p = 0.621$) and the number of inverted teats ($p = 0.256$) in the DUMI population. Association analyses were also performed for combined effects of *PTHLH* and *PTHR1* in order to address potential interaction, however, revealed no indication of effects of interaction. The function, position and the association shown here promote *PTHR1* as a candidate gene for number of teats and in particular for affection by and number of inverted teats.

Introduction

Many dam line breeding programmes in pig populations focus on udder quality. In particular, the teat number and functional mammary gland capability are important selection criteria for increasing the survival rate of piglets. The most frequent and economically relevant inherited disorder of the mammary complex in pigs is the inverted teat, resulting in non-functional teats that cannot be suckled by the offspring. The defect occurs in commercial pig breeds with frequencies between 7.6% and 30% (Niggemayer 1993; Brevern *et al.* 1994; Mayer & Pirchner 1995; Jonas *et al.* 2008). Wiesner

 doi 10 1111/j 1439 0388 2008 00781 x

and Willer (1978) considered a complex inheritance of the liability to develop the defect with the presence of a major gene and several minor genes causing this defect. However, the mode of inheritance and the number of genes involved are still unknown. Heritability was estimated to be between 0.2 and 0.5 (Mayer 1994). In a Duroc and Berlin Miniature pig (DUMI) population, 53.6% suffered from mammary gland abnormalities, 42.2% had inverted teats and 17.9% showed extra teats (Hardge *et al.* 1999).

Parathyroid hormone-like hormone gene (*PTHLH*) and the parathyroid hormone/parathyroid hormone-like hormone receptor 1 (*PTHR1*) were shown to regulate epithelial mesenchymal interactions during the formation of the mammary gland (Foley *et al.* 2001). Therefore, *PTHLH* and *PTHR1* are functional candidate genes for traits related to mammary gland and teat development. The aim of this study was to investigate the association and linkage of *PTHLH* and *PTHR1* gene polymorphisms with the inverted teat defect.

Materials and methods

Animals and phenotypes

Animals (n = 313) of an experimental population based on a reciprocal cross of the DUMI population were used. At 200 days of age, two investigators of a team of four trained and skilled persons observed the phenotypes of the teat traits involved in this study. The animals were placed on their backs and teats were evaluated by inspection and palpation. The numbers of functional and inverted teats were recorded. Animals without inverted teats were classified as 'non-affected' animals while those with at least one inverted teat were categorized as 'affected'.

Genotypes

The *PTHLH* polymorphism analysis was performed by an allelic discrimination assay (Assays-by-DesignSM Service; ABI PRISM® 7000; PE Applied Biosystems, Darmstadt, Germany) using primers PTHLP-FW: 5′-GAGCGTCGCGGTGTTC-3′ and PTHLP-RW: 5′-AGCGCCCGCAGGAG-3′ for polymerase chain reaction (PCR) amplification and allele-specific probes PTHLP-V2: 5′-VIC-CTGAGCTATTCGGTGCC-TAMRA-3′ and PTHLP-M2: 5′-FAM-CTGAGCTAT-TTGGTGCC-TAMRA-3′ for discrimination of either C or T at nt 375 of PTHLH (GenBank accession no. AY193782; Chomdej *et al.* 2004). *PTHR1* polymorphism analysis was conducted by restriction fragment length polymorphism assay. To amplify the region containing a *PTHR1* single nucleotide polymorphism (SNP) primers PTHR-F: 5′-GCTATG-GTCCGATGGTGTCT-3′ and PTHR-R: 5′-ACTGTCT-CCCACTCCTCCTG-3′ were used and PCR fragments were subsequently incubated with the restriction enzyme *Mbo*II (Fermentas, St. Leon-Rot, Germany) suitable to discriminate the C and the T alleles at nt 1819 (GenBank accession no. NM 214382; Tetzlaff *et al.* 2007). Amplification reactions were conducted in a final volume of 15 μl, containing 0.5 unit of Taq DNA polymerase, 0.2 mM of each dNTP, 0.2 μM of each primer, 1x buffer according to the manufacturer's instruction (GeneCraft, Cologne, Germany) and 50 ng of DNA.

Expression

In order to survey expression of porcine *PTHLH* and *PTHR1* genes in different tissues and organs, quantitative reverse transcriptase PCR (qRT-PCR) was performed using the LightCycler® 480 system (Roche, Penzberg, Germany). Total RNA was isolated from epithelial teat tissue, connective teat tissue, liver, kidney, adrenal gland, spleen, tonsil, lymph node, muscle, hypothalamus and pituitary from adult pigs of German Landrace using Tri-Reagent (Sigma, Taufkirchen, Germany) and NucleoSpin® RNA II kit (Macherey-Nagel, Düren Germany) including DNase treatment following the manufacturer's instructions. RNA samples were visualized on 1.5% formaldehyde containing agarose gel to check the integrity and the concentration was measured by spectrometry with a NanoDrop® ND-1000 spectrophotometer (PEQLAB). First-strand cDNA was synthesized from 1 μg of total RNA using random primers and oligo (dT)13N in the presence of Superscript™ III reverse transcriptase (Invitrogen, Hamburg, Germany). The primers for *PTHLH*, *PTHR1* and ribosomal protein L32 (*RPL32*) as

Table 1 Gene specific primers (5′ 3′) used for porcine *PTHLH* and *PTHR1* gene amplification in qRT PCR, *RPL32* was treated as internal reference

Primer set	Sequence	Annealing (°C)	Product size (bp)
PTHLH	GCAAGGAGCAGGAAAAGAAG AGAGCAATGGGGAGACAGTT	64	261
PTHR	TGACCTTCTTCCTTTACTTCCTG GCTCTCACACTGACCCACAC	60	178
RPL32	AGCCCAAGATCGTCAAAAAG TGTTGCTCCCATAACCAATG	60	165

an internal reference were described in Table 1. The reactions were performed in a final volume of 12 μl using 6.0 μl of LightCycler® 480 SYBR Green I Master (Roche), 600 nM of each primer and 100 ng of cDNA. Amplification conditions were 95°C for 10 min, 40 cycles of 95°C for 15 s, annealing temperature (Table 1) for 10 s and 72°C for 15 s. At the completion of the amplification protocol, all samples were subjected to a melting curve to verify the absence of any non-specific products.

Statistics

In order to evaluate the genes for association and linkage with total number of teats, number of inverted teats and the affection status of the Family-Based Association Test (FBAT) was used in the experimental DUMI population with the empirical variance option and the null hypothesis stating 'no association, but linkage' (FBAT; http://www.biostat.harvard.edu/~fbat/default. html). The quantitative association analyses were performed in bi-allelic tests under the condition of an additive genetic model (Horvath *et al.* 2001). A statistical evaluation of the interaction between *PTHLH* and *PTHR1* gene polymorphisms and their influence on inverted teat trait was executed with *LOGISTIC* procedure of the SAS software package 9.1 (Cary, NC, USA) using father, mother and gender as additional explanatory variables of interest in the model.

Results

Genotype analysis

Two non-synonymous C>T SNP were detected at nucleotide position 375 (S19L) of the porcine *PTHLH* cDNA and at nucleotide position 1819 (L556F) of the porcine *PTHR1* cDNA (Chomdej *et al.* 2004; Tetzlaff *et al.* 2007). Mendelian inheritance of these polymorphic sites was monitored in individuals of the experimental DUMI population. The frequencies of genotype combinations are presented in Table 2.

Expression of porcine *PTHLH* and *PTHR1* genes

The qRT-PCR of 11 tissues including epithelial and connective teat tissues from adult pigs indicated differential expression of porcine *PTHLH* and *PTHR1* genes as shown in Figure 1. The *PTHLH* gene was expressed in multiple tissues thereby clearly detectable in epithelial and connective teat tissues. A lower expression was detected in the kidney and adrenal gland. The expression pattern of *PTHR1* gene revealed that liver and kidney were the major sites of expression. Low abundance was observed in the muscle and pituitary.

Association with inverted teat trait

Association analyses were first performed separately for each locus. The results of SNP C375T of *PTHLH* and C1819T of *PTHR1* with inverted teat as the affection trait are summarized in Table 3. SNP C1819T of *PTHR1* gene was significantly associated with total teat number ($p = 0.047$), the affection trait ($p = 0.013$) and close to significance with total inverted teat number ($p = 0.078$). SNP C375T of *PTHLH* showed no significant association with total teat number ($p = 0.621$) and total inverted teat number ($p = 0.256$) but was close to significance with inverted teat as the affection trait ($p = 0.122$).

Discussion

This investigation is the first to attend to association and linkage of *PTHLH* and its receptor *PTHR1* with the inverted teat defect in pigs. The emergence of inverted teats depends on insufficient mesenchymal

Table 2 *PTHR1 PTHLH* genotype combinations at C1819T/C35T and their frequencies in F2 DUMI resource population (n 200)

Affection	Genotype combinations n (%)									
PTHR1	CC	CC	CC	CT	CT	CT	TT	TT	TT	
PTHLH	*CC*	CT	TT	CC	CT	TT	CC	CT	TT	Significance
IT	5 (2.5)	19 (9.5)	3 (1.5)	7 (3.5)	18 (9.0)	12 (6.0)	2 (1.0)	6 (3.0)	3 (1.5)	n. s.
NIT	11 (5.5)	20 (10.0)	10 (5.0)	20 (10.0)	32 (16.0)	10 (5.0)	7 (3.5)	10 (5.0)	5 (2.5)	n. s.

IT, affected at least 1 inverted teat observed; NIT, none affected no inverted teat observed; n (%); number and percentage of observed genotype combinations, combined genotypes of alleles at C1819T and C375T. Abundance of genotype combination was not significantly different between IT and NIT.

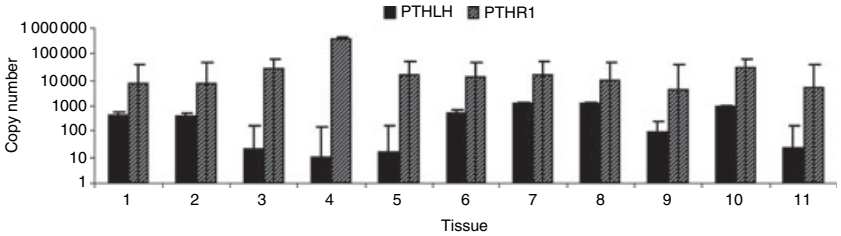


Figure 1 Tissue specific expression pattern of *PTHLH* and *PTHRI* genes assayed by qRT PCR. Copy number of *RPL32* gene was measured to normalize for equal RNA amounts.1, epithelial teat tissue; 2, connective teat tissue; 3, liver; 4, kidney; 5, adrenal gland; 6, spleen; 7, tonsil; 8, lymph node; 9, muscle; 10, hyopthalamus; 11, pituitary.

proliferation at the teat ground during teat development. As far as proliferation processes are concerned, the defect of inverted teats is related to the trait of the number of teats. The number of teats may also depend on local signalling between adjacent embryonic tissues (Jonas *et al.* 2008). Foley *et al.* (2001) elucidated the influence of *PTHLH* and *PTHR1* on the regulative interaction of epithelial mesenchymal proliferation during the formation of the mammary gland. Prior experiments with knockout mice indicated involvement of *PTHLH* in the ontogenesis of a functional mammary gland, because these mice developed the inverted teat phenotype (Wysolmerski *et al.* 1998; Dunbar *et al.* 2001). Furthermore, the expression results of both genes in relevant tissues for the inverted teat defect approved the results reported in other species (Kong *et al.* 1994; Kobayashi *et al.* 2005). Thus, *PTHLH* and *PTHR1* genes were contemplated as functional positional candidate genes for the inverted teat phenotype. Our results for the SNP C1819T in *PTHR1* have revealed a significant association and linkage to teat number as well as occurrence of inverted teats. However, association with teat number as well as inverted teat defect and the SNP C375T in *PTHLH* could not be verified. Furthermore, the amino acid exchanges of both SNP were analysed by SIFT (Sorting Intolerant from Tolerant; http://blocks.fhcrc.org/sift/ SIFT. html). SIFT is based on the premise that important amino acids will be conserved among sequences in a protein family, so changes at amino acid conserved in the family should affect protein function (Ng & Henikoff 2002). Thus, phylogenetic relationships do not promote functional impact of the amino acid exchanges. However, the amino acid exchanges are between polar (serin) and neutral (leucine) and neutral (leucine) and aromatic (phenylalanine) amino acids, respectively, therefore impact on structural and biochemical properties of the proteins cannot be ruled out. For *PTHLH*, the polymorphism is located in its signal peptide that directs post-translational transport. The SNP at nt 1819 of *PTHR1* is located in its intracellular tail, which is involved in signal transduction. SNP in the coding regions of the genes are not likely to affect their expression. Any functional effects have to be confirmed. When analysing genes of hormones and their receptors effects of interaction can be expected, however no interaction between *PTHLH* and *PTHR1* genotypes were observed. That might be because of the limited number of animals. *PTHLH* was mapped slightly distal of quantitative trait loci (QTL) for number of nipples and the inverted teat defect on SSC5 (Lee *et al.* 2003; Chomdej *et al.* 2004; Rodríguez *et al.* 2005; Jonas *et al.* 2008), whereas *PTHR1* was displayed in close proximity to a QTL for the inverted teat defect on SSC13 (Tetzlaff *et al.* 2007; Jonas *et al.* 2008). The SNP in *PTHR1* likely is a marker in close linkage disequilibrium to a causative polymorphism affecting the liability for the inverted teat defect; *PTHR1* even represents a good candidate for the causal polymorphism. The SNP of both genes were found to segregate among pigs of commercial breeds, German Landrace, Large White, Pietrain, Hampshire and Duroc, with allele 'C' at both SNP being the prominent one (Chomdej *et al.* 2004; Tetzlaff *et al.* 2007). Effects of both genes on teat phenotypes but also on other traits will be evaluated in animals of commercial dam lines in order to further

Table 3 Association *of PTHLH* and *PTHR1* with the affection by inverted teats *(AF)*, total number of inverted teats (TIT) and total number of teats (TT) in the DUMI resource population as revealed by FBAT analysis

			Aff	TT	TIT
Marker	allele	Freq	*p*	*p*	*p*
PTHLH(C375T)	C	0.5	0.122	0.621	0.256
PTHR1 (C1819T)	C	0.6	0.013	0.047	0.078

qualify the genes as functional positional candidate genes in a gene-assisted selection. Further analyses of trait association in other populations as well as functional assays will provide more insight into the causal nature of the polymorphisms.

Acknowledgements

The authors would like to thank the Federal Ministry of Education and Research (BMBF) and the Förderverein Biotechnologieforschung der Deutschen Schweineproduktion e.V. (FBF) for financial support (FUGATO HeDiPig, FKZ 0313392A). The experiments described in this study were performed in accordance with all appropriate regulations.

References

Brevern N.V., Schimpf B., Wörner R., Swalve H. (1994) Parameterschätzung für Zitzenmerkmale bei Hybridsau en. *Züchtungskunde*, **66**, 339 348.

Chomdej S., Ponsuksili S., Schellander K., Wimmers K. (2004) Sequencing, SNP identification and mapping of the porcine PTHLH gene to chromosome 5. *Anim. Genet.*, **35**, 151 152.

Dunbar M.E., Dann P., Brown C.W., Van H.J., Dreyer B., Philbrick W.P., Wysolmerski J.J. (2001) Temporally reg ulated overexpression of parathyroid hormone related protein in the mammary gland reveals distinct fetal and pubertal phenotypes. *J. Endocrinol.*, **171**, 403 416.

Foley J., Dann P., Hong J., Cosgrove J., Dreyer B., Rimm D., Dunbar M.E., Philbrick W., Wysolmerski J.J. (2001) Parathyroid hormone related protein maintains mammary epithelial fate and triggers nipple skin differ entiation during embryonic breast development. *Devel opment*, **128**, 513 525.

Hardge T., Koepke K., Reissmann M., Wimmers K. (1999) Maternal influences on litter size and growth in reciprocal crossed Miniature Pigs and Durocs. *Arch. Anim. Breed.*, **42**, 83 92.

Horvath S., Xu X., Laird N.M. (2001) The family based association test method: strategies for studying general genotype phenotype associations. *Eur. J. Hum. Genet.*, **9**, 301 306.

Jonas E., Schreinemachers H.J., Kleinwächter T., Un C., Oltmanns I., Tetzlaff S., Jennen D., Tesfaye D., Ponsuksili S., Murani E., Juengst H., Tholen E., Schel lander K., Wimmers K. (2008) QTL for the heritable inverted teat defect in pigs. *Mamm. Genome*, **19**, 127 138.

Kobayashi T., Kronenberg H.M., Foley J. (2005) Reduced expression of the PTH/PTHrP receptor during develop ment of the mammary gland influences the function of the nipple during lactation. *Dev. Dyn.*, **233**, 794 803.

Kong X.F., Schipani E., Lanske B., Joun H., Karperien M., Defize L.H.K., Juppner H., Potts J.T., Segre G.V., Kronenberg H.M., Abousamra A.B. (1994) The rat, mouse and human genes encoding the receptor for parathyroid hormone and parathyroid hormone related peptide are highly homologous. *Biochem. Bio phys. Res. Commun.*, **200**, 1290 1299.

Lee S.S., Chen Y., Moran C., Stratil A., Reiner G., Bart enschlager H., Moser G., Geldermann H. (2003) Link age and QTL mapping for Sus scrofa chromosome 5. *J. Anim. Breed. Genet.*, **120**, 38 44.

Mayer J. (1994) Genetische Parameter von Gesäuge anomalien beim Schwein. Technische Universität Mün chen, Weihenstephan, Deutschland.

Mayer J., Pirchner F. (1995) Asymmetry and inverted nipples in gilts. *Arch. Anim. Breed.*, **38**, 87 91.

Ng P.C., Henikoff S. (2002) Accounting for human poly morphisms predicted to affect protein function. *Genome Res.*, **12**, 436 446.

Niggemayer A. (1993) Besamungsstation kontrolliert Zit zenvererbung. *Schweinezucht und Schweinemast*, **3**, 11.

Rodríguez C., Tomás A., Alves E., Ramirez O., Arqué M., Muñoz G., Barragán C., Varona L., Silió L., Amills M., Noguera J.L. (2005) QTL mapping for teat number in an Iberian by Meishan pig intercross. *Anim Genet.*, **36**, 490 496.

Tetzlaff S., Ponsuksili S., Murani E., Wimmers K. (2007) SNP analysis, genotyping and mapping of the porcine *PTHR1* gene to chromosome 13. *Arch. Anim. Breed.*, **50**, 320 321.

Wiesner E., Willer S. (1978) Problems of occurrence of inverted nipples in swine. *Monatshefte fur Veterinarmedi zin*, **33**, 189 190.

Wysolmerski J.J., Philbrick W.M., Dunbar M.E., Lanske B., Kronenberg H., Broadus A.E. (1998) Rescue of the parathyroid hormone related protein knockout mouse demonstrates that parathyroid hormone related protein is essential for mammary gland development. *Develop ment*, **125**, 1285 1294.

Original Article

Cytogenetic and Genome Research

Cytogenet Genome Res 2009;124:139–146
DOI: 10.1159/000207521

Accepted after revision: November 3, 2008
by K. Reed

Evidence for association of lymphoid enhancer-binding factor-1 *(LEF1)* with the number of functional and inverted teats in pigs

S. Tetzlaff[a] E. Jonas[b] C. Phatsara[b] E. Murani[a] S. Ponsuksili[c] K. Schellander[b] K. Wimmers[a]

[a]Research Institute for the Biology of Farm Animals, Research Unit 'Molecular Biology´, Dummerstorf,
[b]Institute of Animal Breeding Science, Animal Breeding and Husbandry Group, University of Bonn, Bonn,
[c]Research Institute for the Biology of Farm Animals, Research Group 'Functional Genome Analysis´,
Dummerstorf (Germany)

Abstract

The lymphoid enhancer-binding factor-1 (LEF1) belongs to a family of regulatory proteins that share homology with the high mobility group protein-1 (HMG1). The *LEF1* gene is a mediator in the canonical Wnt-signalling pathway required for morphogenesis of early mammary gland during embryogenesis. Here we describe the molecular characterisation of the porcine *LEF1* gene and its association with number of teats and inverted teats in experimental and commercial populations. The 2357-bp cDNA sequence contains an 1197-bp open reading frame encoding a protein of 398 amino acids. The porcine LEF1 protein shares high identity with LEF1 in other mammalian species. The *LEF1* gene contains 12 exons and maps to pig chromosome 8 (SSC8). We identified two single nucleotide polymorphisms (SNPs), a T1351C transition and an A1666C transversion, in the 3′ end of *LEF1*. Associations of the SNP A1666C with presence of inverted teats ($P \leq 0.01$), total number of teats ($P \leq 0.01$) and total number of inverted teats ($P \leq 0.01$) were highly significant; SNP T1351C showed near significance with total number of inverted teats ($P \leq 0.1$) in the experimental DUMI population. SNP T1351C was significantly associated with total number of inverted teats ($P = 0.04$) and close to significance with affected teats ($P = 0.06$) in commercial populations. Haplotype analysis confirmed the tendency towards association with affected teats ($P = 0.06$) in the experimental DUMI population. The function, position, and associations shown here promote *LEF1* as a candidate gene for number of teats and in particular for presence and number of inverted teats.

In dam-line breeding programs for pigs, udder quality is a selection criterion addressed in order to maintain and improve mothering ability. In particular, the teat number and functional mammary gland capability are important selection criteria for increasing the survival rate of piglets. The most frequent and economically relevant inherited disorder of the mammary gland in pigs is the inverted teat, resulting in non-functional teats that cannot be suckled by the offspring. The defect occurs in commercial pig breeds with frequencies between 7.6 and

This work was supported by the Federal Ministry of Education and Research (BMBF) and the Förderverein Biotechnologieforschung e.V. (FBF) (FUGATO).

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1424–8581/09/1242–0139$26.00/0

Accessible online at:
www.karger.com/cgr

Klaus Wimmers
Research Institute for the Biology of Farm Animals, Research Unit
'Molecular Biology', Wilhelm-Stahl-Allee 2, DE–18196 Dummerstorf (Germany)
telephone: +49 38208 68700; fax: +49 38208 68702
e-mail: wimmers@fbn-dummerstorf.de

30% (Niggemayer, 1993; Brevern et al., 1994; Mayer and Pirchner, 1995; Jonas et al., 2008). Several authors considered a complex inheritance of the liability to develop the defect with the presence of a major gene and several minor genes causing this defect in embryonic mammary gland development (Wiesner and Willer, 1978; Günther et al., 1985). However, the mode of inheritance and the number of genes involved are still unknown. Heritability was estimated between 0.2 and 0.5 (Mayer, 1994). In an experimental Duroc × Berlin Miniature pig (DUMI) population 53.6% suffered from mammary gland abnormalities, 42.2% had inverted teats and 17.9% showed misplaced teats (Hardge et al., 1999). Embryonic mammary gland development is dependent on reciprocal inductive interactions between the mesenchyme and overlying ectoderm (Sakakura et al., 1987; Robinson et al., 1999; Foley et al., 2001; Pispa and Thesleff, 2003; Boras-Granic et al., 2006). Signals originating in the mesenchyme have been proposed to specify ectodermal cells to form epithelial mammary placodes (Sakakura, 1987). Reciprocal signalling from the placodes to the underlying mesenchyme then directs condensation of fibroblasts that form the mammary mesenchyme. Maintenance of these reciprocal signals stimulates further proliferation and differentiation of the placodes resulting in the development of the ductal mammary gland (Boras-Granic et al., 2006).

The lymphoid enhancer-binding factor-1 is one of the earliest known regulators involved in formation of the mammary gland (Vangenderen et al., 1994; Mailleux et al., 2002; Boras-Granic et al., 2006). LEF1 is an architectural protein chaperoning a number of distinct factors controlling transcription of target genes. In the context of the canonical Wnt-signalling cascade LEF1-β-catenin-containing complexes, resulting in activated transcription of Wnt-target genes, replace complexes of LEF1 and the transcriptional repressors (Behrens et al., 1996; Boras-Granic et al., 2006).

The canonical Wnt-signalling pathway is important for placode induction and differentiation of mammary epithelium (Hennighausen and Robinson, 2001). A role of Wnt-induced signalling in mammary bud development is supported by studies of transgenic mouse embryos (Chu et al., 2004; Veltmaat et al., 2004). During the early stages of mammary development, LEF1 is expressed in epithelial cells of the mammary bud and is subsequently induced in the mesenchyme surrounding each bud during embryogenesis (Foley et al., 2001). Induction of LEF1 expression in the mammary mesenchyme is dependent on paracrine signalling from the mammary epithelium by the parathyroid hormone-like hormone and the parathyroid hormone/parathyroid hormone-like hormone receptor 1 (Foley et al., 2001). Defective mammopoeisis arises in LEF1-deficient animals; embryos initially exhibit a reduced number of mammary buds and aborted development of those that do form (Vangenderen et al., 1994; Boras-Granic et al., 2006). Therefore *LEF1* is a functional candidate gene for traits related to mammary gland and teat development.

The aim of this study was to characterize the porcine *LEF1* gene and perform the association and linkage analysis of identified polymorphisms with the inverted teat defect.

Materials and methods

The nucleotide sequence reported in this article has been submitted to GenBank (accession number EU719607).

cDNA characterization

A BLAST search using the human *LEF1* mRNA sequence (GenBank accession number NM_016269) identified overlapping porcine expressed sequence tags (CX063890, BP162088, AJ958238 and AJ957434). These ESTs were concatenated and gene-specific primers were designed (Table 1). Amplification reactions were conducted in a final volume of 15 μl, containing 0.5 unit of Taq DNA polymerase (GeneCraft), 0.2 mM of each dNTP, 0.2 μM of each primer, 1× reaction buffer (GeneCraft), and 100 ng of liver cDNA. The cycling protocol was 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, annealing temperature (Table 1) for 30 s and 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR products were checked on a 1.5% agarose gel, purified with the NucleoSpin Extract II kit (Macherey-Nagel) and bi-directionally sequenced using the BigDye Terminator Cycle Sequencing kit (version 1.1) and an ABI Prism™ 310 Genetic Analyzer (Applied Biosystems). The PCR primers described in Table 1 were also used for SNP screening in five pig breeds (Duroc, Hampshire, German Landrace, Pietrain and Berlin Miniature pig).

Annotation of the *LEF1* exons on the porcine cDNA sequence was done using the human and murine *LEF1* mRNA sequence (NM_016269, NM_010703) and the Evidence Viewer tool (http://www.ncbi.nlm.nih.gov/sutils/evv.cgi/) for cDNA – cDNA sequence comparisons. Translation of the cDNA sequence and analysis of the protein sequence were done with the Open Reading Frame Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Protein sequence alignments were performed with the MultAlign analysis tool (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html).

Chromosome localization of the LEF1 gene

The physical mapping of *LEF1* was performed using the INRA-University of Minnesota porcine radiation hybrid panel (IMpRH). The chromosomal assignment was calculated using

Table 1. Primers used for amplification of porcine *LEF1* cDNA in this study

Exon	Primer set	Sequence (5′–3′)	Annealing (°C)	Product size (bp)
1–2	LEF_1	CCTTCCCTTCCAATTCTCCT ACCTCGTGTCCGTTGCTC	60	322
1–4	LEF_2	GATTACAGAGTGGTCGGGATG GTGATGAGGGGAGTGAGAGG	60	491
2–7	LEF_3	AACACCCTGATGACGGAAAG ATGAGGGATGCCAGTTGTGT	60	515
5–10	LEF_4	TGGACAGATTACCCCACCTC TTTCTCTCTCTTCCTCTTCTTTTTC	60	536
9–12	LEF_5	TTAGCACGGAAGGAAAGACA GCAGAGACAGGGGAGGAAAG	60	476
12	LEF_6	GCAGCTTTCCTCCCCTGT CAATCGTTAGATGACAGTTTGG	60	799

the twopoint and multipoint analysis option of the IMpRH mapping tool (http://www.toulouse.inra.fr). PCR used for mapping was conducted in a total volume of 20 μl containing 0.5 unit of Taq DNA polymerase (GeneCraft), 0.2 mM of each dNTP, 0.2 μM of each primer (5′-TGGAAAACGGAGCTCATT-3′ and 5′-GCAGAGACAGGGGAGGAAAG-3′), 1× reaction buffer (Gene-Craft), and 50 ng panel DNA. The PCR protocol consisted of 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 1 min, and a final extension step at 72°C for 5 min. The PCR products were visualized on a 2% agarose gel. The linkage map including the *LEF1* gene was calculated with the CRIMAP 2.4 software package using the build and twopoint analysis option (Washington University School of Medicine, St. Louis, MO, USA) and 19 families (n = 200) of the experimental DUMI resource population that were segregating at the SNP A1666C.

Expression

In order to survey differential expression of porcine *LEF1* gene quantitative reverse transcription PCR (qRT-PCR) was performed using the LightCycler 480 system (Roche). Total RNA was isolated from epithelial and connective teat tissue from affected and unaffected animals (n = 30) of German Landrace in full sib pair design and additionally from epithelial teat tissue, connective teat tissue, liver, kidney, adrenal gland, spleen, tonsil, lymph node, muscle, hypothalamus and pituitary from adult pigs of German Landrace using Tri-Reagent (Sigma) and NucleoSpin RNA II kit (Macherey-Nagel) including DNase treatment following manufacturer's instructions. RNA samples were visualized on 1.5% formaldehyde containing agarose gels to check the integrity and the concentration was measured by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB). First-strand cDNA was synthesized from 1 μg of total RNA using random primers and oligo(dT)13 N in the presence of Superscript III reverse transcriptase (Invitrogen). The ribosomal protein L32 *(RPL32)* was treated as an internal reference. Reactions were performed in a final volume of 12 μl using 6.0 μl of LightCycler 480 SYBR Green I Master (Roche), 600 nM of each primer (LEF_up: 5′-AAAGACAATCACTGCCAAACC-3′, LEF_dw: 5′-GCAGACAGAAACGGAGAGGA-3′, RPL_up: 5′-AGCCCAAGATCGTCAAAAAG-3′ and RPL_dw: 5′-TGTTGCTCCCATAACCAATG-3′) and 100 ng cDNA. Amplification conditions were 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 10 s and 72°C for 15 s. At the completion of the amplification protocol, all samples were subjected to a melting curve to verify the absence of any nonspecific products.

Animals and phenotypes

Animals derived from the 'DUMI' three generation F2 resource population by reciprocal crossing of Berlin Miniature Pig and Duroc (Hardge et al., 1999). At 200 days of age animals (n = 375) of the DUMI resource population were placed on their backs and teats were evaluated by inspection and palpation by two investigators. Numbers of functional and inverted teats were recorded. To validate the results obtained in the experimental population, animals of commercial herds (n = 458 animals, comprising 130 full sib families with at least one affected offspring (n = 147)) were used. Samples of German Landrace and German Large White pigs as well as their crossbreeds were collected from a performance test station and the teat traits were recorded in the slaughterhouse.

Genotyping

The *LEF1* polymorphism analysis of T1351C was performed by a Pyrosequencing assay (Pyrosequencing™ Assay Design Software; PSQ™HS 96A, Biotage AB) using primers 5′-Biotin-GACCCCGGGACCTCTTCT-3′ and 5′-GCAGTGACCTCAGGGTGAAG-3′ for PCR amplification and 5′-CCATGGAGATGGGCC-3′ as sequencing primer. *LEF1* polymorphism A1666C was detected by restriction fragment length polymorphism assay. To amplify the region containing *LEF1* SNP A1666C primers 5′-GCAGCTTTCCTCCCCTGT-3′ and 5′-GCAGACAGAAACGGAGAGGA-3′ were used and PCR fragments were subsequently incubated with the restriction enzyme *Eco*24I (Fermentas) suitable to discriminate the A and the C allele at nucleotide position 1666 and visualized in a 2% agarose gel. Naming of both SNPs refers to the position in the cDNA sequence of *LEF1* (GenBank accession number EU719607).

```
mouse           MPQLSG--GGGGGDPELCATDEMIPFKDEGDPQKEKIFAEISHPEEEGDLADIKSSLVNE 58
rat             MPQLSG--GGGGGDPELCATDEMIPFKDEGDPQKEKIFAEISHPEEEGDLADIKSSLVNE 58
pig             MPQLSGA-GGGGGDPELCATDEMIPFKDEGDPQKEKIFAEISHPEEEGDLADIKSSLVNE 59
human           MPQLSGGGGGGGGDPELCATDEMIPFKDEGDPQKEKIFAEISHPEEEGDLADIKSSLVNE 60
                ******  ***************************************************

mouse           SEIIPASNGHEVVRQAPSSQEPYHDKAREHPDEGKHPDGGLYNKGPSYSSYSGYIMMPNM 118
rat             SEIIPASNGHEVVGQTQSSQEPYHDKAREHPDDGKHPDGGLYNKGPSYSSYSGYIMMPNM 118
pig             SEIIPASNGHEVARQAQSSQESYHDKAREHPDDGKHPDGGLYNKGPSYSSYSGYIMMPNM 119
human           SEIIPASNGHEVARQAQTSQEPYHDKAREHPDDGKHPDGGLYNKGPSYSSYSGYIMMPNM 120
                ************. *: :***.**********:***************************

mouse           NSDPYMSNGSLSPPIPRTSNKVPVVQPSHAVHPLTPLITYSDEHFSPGSHPSHIPSDVNS 178
rat             NSDPYMSNGSLSPPIPRTSNKVPVVQPSHAVHPLTPLITYSDEHFSPGSHPSHIPSEVNP 178
pig             NNDPYMSNGSLSPPIPRTSNKVPVVQPSHAVHPLTPLITYSDEHFSPGSHPSHIPSDVNS 179
human           NNDPYMSNGSLSPPIPRTSNKVPVVQPSHAVHPLTPLITYSDEHFSPGSHPSHIPSDVNS 180
                *.*****************************************************:**.

mouse           KQGMSRHPPAPEIPTFYPLSPGGVGQITPPIGWQGQPVYPITGGFRQPYPSSLSGDTSMS 238
rat             KQGMSRHPPAPEMPTFYPLSPGGVGQITPPLGWQGQPVYPITGGFRQAYPSSLSGDTSMS 238
pig             KQGMSRHPPAPEIPTFYPLSPGGVGQITPPLGWQGQPVYPITGGFRQPYPSSLSVDTSMS 239
human           KQGMSRHPPAPDIPTFYPLSPGGVGQITPPLGWQGQPVYPITGGFRQPYPSSLSVDTSMS 240
                ***********::*****************:****************.****** *****

mouse           RFSHHMIPGPPGPHTTGIPHPAIVTPQVKQEHPHTDSDLMHVKPQHEQRKEQEPKRPHIK 298
rat             RFSHHMIPGPPGPHTTGIPHPAIVTPQVKQEHPHTDSDLMHVKPEHEQRKEQEPKRPHIK 298
pig             RFSHHMIPGPPGPHTTGIPHPAIVTPQVKQEHPHTDSDLMHVKPQHEQRKEQEPKRPHIK 299
human           RFSHHMIPGPPGPHTTGIPHPAIVTPQVKQEHPHTDSDLMHVKPQHEQRKEQEPKRPHIK 300
                ********************************************:***************

mouse           KPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHM 358
rat             KPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHM 358
pig             KPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHM 359
human           KPLNAFMLYMKEMRANVVAECTLKESAAINQILGRRWHALSREEQAKYYELARKERQLHM 360
                ************************************************************

mouse           QLYPGWSARDNYGKKKKRKREKLQESTSGTGPRMTAAYI 397
rat             QLYPGWSARDNYGKKKKRKREKLQESTSGTGPRMTAAYI 397
pig             QLYPGWSARDNYGKKKKRKREKLQESTSGTGPRMTAAYI 398
human           QLYPGWSARDNYGKKKKRKREKLQESASGTGPRMTAAYI 399
                **************************:************
```

Fig. 1. Alignment of different mammalian LEF1 proteins. The deduced amino acid sequence of porcine LEF1 is shown aligned to that of human (*Homo sapiens*, GenBank accession number NP_057353), mouse (*Mus musculus*, NP_034833) and rat (*Rattus norvegicus*, NP_569113). The numbers represent the position of the amino acids in the respective protein sequences. Asterisks beneath the alignment indicate identical amino acid residues in all sequences, while colons and dots represent very similar and similar amino acids, respectively.

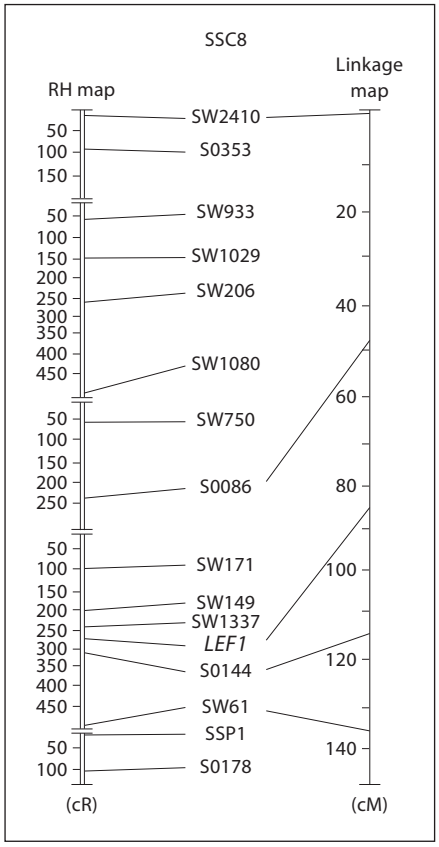
Statistics

In order to evaluate the genes for association and linkage with total number of teats, total number of inverted teats, and the affection status, i.e. the presence or absence of inverted teats the family-based association test was used in the experimental and the commercial population. Analysis was done for both SNP separately and for the corresponding haplotypes in the experimental population applying FBAT and HBAT, accordingly (FBAT, http://www.biostat.harvard.edu/~fbat/default.html). The quantitative association analyses were done in bi-allelic tests under the condition of an additive genetic model (Horvath et al., 2001). Frequencies of genotypes and haplotypes were calculated using Proc 'freq' and 'haplotype' of the SAS software package 9.1 (SAS Institute, Cary, NC, USA). In the commercial population only SNP T1351C was segregating.

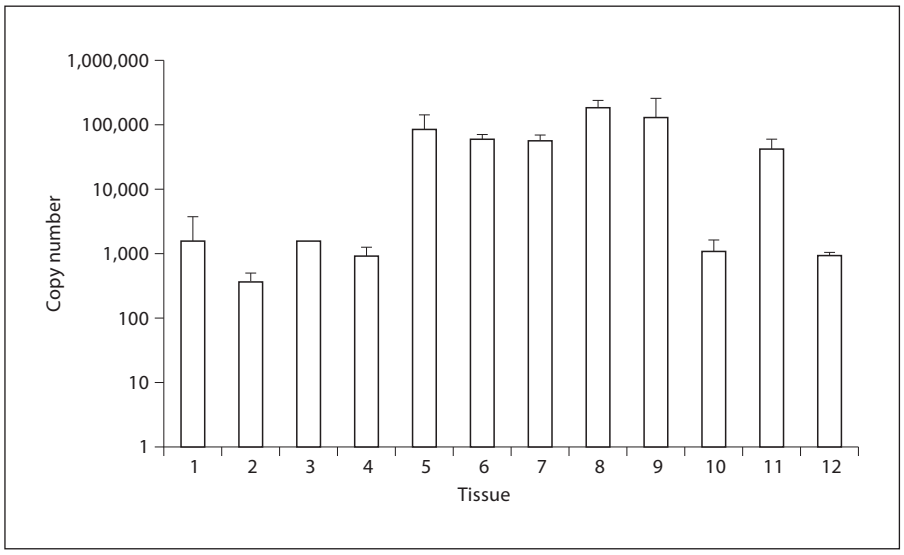
Results

cDNA and protein characterisation

The *LEF1* cDNA is composed of 2357 bp, contains an 1197-bp open reading frame encoding a protein of 398 amino acids with a molecular mass of 44.2 kDa and an



2



3

Fig. 2. Comparative mapping of *Sus scrofa* chromosome 8. The RH map (in cR) provided by INRA Toulouse is compared to the linkage map (in Kosambi cM) obtained from the F2 DUMI resource population. Position of the *LEF1* gene is displayed in italic letters.

Fig. 3. Tissue-specific expression pattern of the *LEF1* gene assayed by qRT-PCR. Copy number of the *RPL32* gene was measured to normalize for equal RNA amounts. 1, Epithelial teat tissue; 2, connective teat tissue; 3, liver; 4, kidney; 5, adrenal gland; 6, spleen; 7, tonsil; 8, lymph node; 9, lymphocyte, 10, muscle; 11, hypothalamus; 12, pituitary.

isoelectric point of 6.9; flanked by a 62-bp-long 5′ UTR and 1098 bp of 3′ UTR. Using the human reference cDNA for cDNA–cDNA sequence comparisons we deduced that the porcine *LEF1* gene consists of 12 exons in agreement with human and murine *LEF1* exon structure that conform perfectly to the GT/AG rule. The deduced amino acid porcine *LEF1* sequence displays perfect alignment with the human, mouse and rat *LEF1* with 98, 98 and 97% sequence identity, respectively (Fig. 1).

Chromosomal mapping

The *LEF1* gene was assigned to SSC8 by multipoint analysis using IMpRH panel (Vector: 1101100011 1100000000 0110000001 0110000010 0000000100 1000011001 0000001000 0011000001 0101000000 0000010100 1100001111 11001100). The results of two-point analysis suggest the gene is in close proximity to marker SW1337 (26cR; LOD 14.47) presented in Fig. 2. This assignment was further confirmed by linkage mapping of the loci SW2410 (0 cM), S0086 (48.3 cM), *LEF1* (87.9 cM), S0144 (114.8 cM) and SW61 (135.3 cM) on the sex-averaged map of SSC8 (Fig. 2).

Expression of porcine LEF1 gene

The qRT-PCR of 12 tissues including epithelial and connective teat tissue from adult pigs indicated differential expression of *LEF1* as shown in Fig. 3. The *LEF1* gene was expressed in multiple tissues and thereby clearly detectable in epithelial and connective teat tissue. The expression pattern of *LEF1* revealed that lymph nodes and lymphocytes were the major sites of expression. Analysis of phenotype dependent expression of *LEF1* yielded no significant differences in expression in epithelial and connective teat tissue of affected and unaffected sibs (Fig. 4).

Genotype analysis

Two SNPs were detected at nucleotide position 1351 (T > C) and at nucleotide position 1666 (A > C) in the 3′ end of the porcine *LEF1* cDNA (Table 2). Mendelian inheritance of these polymorphic sites was monitored in individuals of the experimental DUMI population. The frequencies of haplotypes are presented in Table 3.

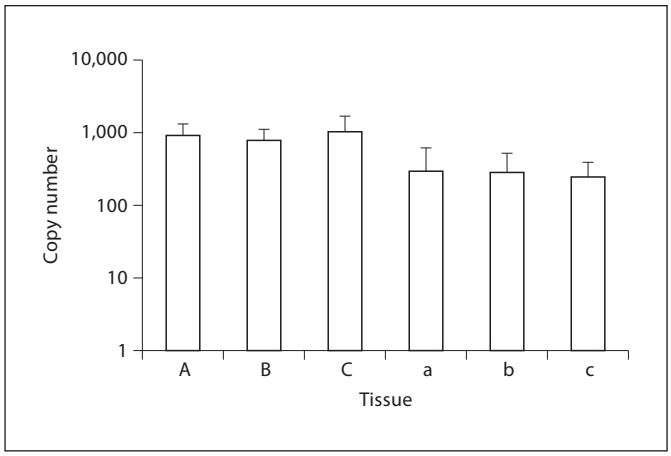


Fig. 4. Phenotype-dependent expression pattern of the *LEF1* gene assayed by qRT-PCR. Copy number of the *RPL32* gene was measured to normalize for equal RNA amounts. A, Epithelial teat tissue from unaffected animals; B, epithelial teat tissue from normal teat of affected animals; C, epithelial teat tissue from inverted teats; a, connective teat tissue from unaffected animals; b, connective teat tissue from normal teat of affected animals; c, connective teat tissue from inverted teats.

Table 2. Association of *LEF1* gene with inverted teats (Aff), total number of inverted teats (TIT) and total number of teats (TT) in the experimental (DUMI) and commercial (COM) population as revealed by FBAT analysis

Characteristic (Marker)	Population	Allele	Freq	Aff *(P)*	TT *(P)*	TIT *(P)*
T1351C	DUMI	T	0.5	0.45	0.75	≤0.1
A1666C	DUMI	C	0.4	≤0.01	≤0.01	≤0.01
T1351C	COM	T	0.96	0.06	0.31	0.04
A1666C	COM	A	1.0	n. a.	n. a.	n. a.

Table 3. *LEF1* haplotypes at T1351C/A1666C and their frequencies in F2 DUMI resource population (n = 200)

Population	Affection status[a]	Haplotypes, n (%)[b]			Significance
		C–C	C–A	T–A	
DUMI	IT	15 (12.0)	41 (32.8)	69 (54.8)	n.s.
DUMI	NIT	15 (19.3)	26 (34.7)	34 (45.4)	n.s.

[a] IT = affected with inverted teat; NIT = unaffected with inverted teat.

[b] n = number of observations; haplotypes, haplotypes of alleles at T1351C and C1666A. Abundance of haplotypes for both groups was not significantly different.

Association with teat traits

Association analyses were first performed separately for each locus. SNP A1666C was highly significantly associated with the presence of inverted teats ($P \leq 0.01$), total number of teats ($P \leq 0.01$) and total number of inverted teats ($P \leq 0.01$); SNP T1351C was close to significance for the trait total number of inverted teats ($P \leq 0.1$) in the experimental DUMI population (Table 2). Analysis of the three haplotypes segregating in the experimental DUMI population confirmed the association with the presence of inverted teats at $P = 0.06$ (Table 3). In the commercial population SNP T1351C was significantly associated with total number of inverted teats ($P = 0.04$) and close to significance with the affected teats ($P = 0.06$). SNP A1666C did not segregate in the commercial population (Table 2).

Discussion

This investigation is the first to characterize the porcine *LEF1* gene and to demonstrate association and linkage with the inverted teat defect in the pig. The emergence of inverted teats depends on insufficient mesenchymal proliferation at the teat ground during teat development. As far as proliferation processes are concerned the defect of inverted teats is related to the trait of number of teats. The number of teats may also depend on local signalling between adjacent embryonic tissues (Jonas et al., 2008). Boras-Granic et al. (2006) elucidated the influence of lymphoid enhancer-binding factor-1 on the regulative interaction of epithelial and mesenchymal proliferation during the formation of the mammary gland. Knockout experiments indicated involvement of *LEF1* in the ontogenesis of a functional mammary gland, because *LEF1*-deficient mice developed a defective mammary gland with a reduced number of mammary buds and aborted development of those that do form (Vangenderen et al., 1994; Boras-Granic et al., 2006). The expression of the *LEF1* gene in epithelial and mesenchymal compartments of the teats, i.e. tissues relevant for the inverted teat defect, indicates a functional role of *LEF1* in teat development also in pigs and by this confirms the results reported in other species (Vangenderen et al., 1994; Boras-Granic et al., 2006). Also, very high sequence similarities of the deduced porcine LEF1 amino acids sequence with other mammalian species supports the concept of equivalent biological roles for different regulatory mechanisms of proliferation during ontogenesis in the various species (Kratochwil et al., 1996; Eastman and

Grosschedl 1999). Quantitative trait loci (QTL) for teat traits on chromosome 8 have been identified by several authors (Cassady et al., 2001; Beeckmann et al., 2003; Sato et al., 2006). *LEF1* was displayed in close proximity to these QTL and thus is a positional candidate gene. Our results for the SNP A1666C in the experimental DUMI population revealed a highly significant association and linkage to teat number as well as occurrence of inverted teats. Analysis of the SNP T1351C and the haplotypes support this finding. Moreover, association of *LEF1* with number of inverted teats as well as with presence or absence of inverted teats could be verified in commercial dam lines for the segregating SNP T1351C at $P = 0.04$ and 0.06, respectively. The polymorphisms detected are likely markers in close linkage to a causative polymorphism affecting teat number and liability for the inverted teat defect. However, the allele frequencies are unfavourable with regard to the power to unravel association with the SNP A1666C not segregating and the SNP T1351C showing low frequency in the animals of commercial breeds investigated. In the experimental DUMI population alleles at both SNPs exist at similar frequencies, but highly significant association was shown only for SNP A1666C that obviously exhibits consistent linkage phase to a causative polymorphic site across all families. These different results enhance the conclusion that different population- and family-specific effects are due to genetic drift and/or recombination between the SNPs of the *LEF1* gene and the causative polymorphism (Sved, 1971; Du et al., 2007). Moreover, for complex traits, like liable traits, a single gene often plays a very small role in a complex interaction of many genes in a pathway that can perform differently and promote various regulative measures in different populations or breeds. Thus various modes of compensation may be active in different individuals. Comparative sequencing revealed no polymorphisms in the coding region of the gene, thus polymorphisms affecting the expression of the gene are likely to be causal for any effects on teat development. SNPs in untranslated regions of genes were shown to affect expression for example by creating a potential target site for microRNAs (Clop et al., 2006); however in silico analysis with the TargetScan tool (predicts biological targets of microRNAs by searching for the presence of conserved 8mer and 7mer sites that match the recognition region of each microRNA; http://www.targetscan.org) of the polymorphic sites described here did not indicate conservation and particular functional roles of the polymorphic sites. While using samples of peripubertal animals we could not show trait dependent expression, however this does not rule out polymorphisms affecting the expression of *LEF1* at other ontogenetic stages that finally lead to different teat phenotypes. In conclusion, whereas *LEF1* was promoted as a functional positional candidate gene for the inverted teat defect the identification of causal polymorphism remains open. Effects of the *LEF1* gene on teat phenotypes but also on other traits will be evaluated in an increased number of animals of commercial dam lines in order to further qualify the gene as functional positional candidate gene in gene assisted selection. Further analyses of trait association in other populations as well as functional assays will provide more insight into the causal nature of the polymorphisms.

Acknowledgements

The experiments described in this study were performed in accordance with all appropriate regulations.

References

Beeckmann P, Moser G, Bartenschlager H, Reiner G, Geldermann H: Linkage and QTL mapping for *Sus scrofa* chromosome 8. J Anim Breed Genet 120:66–73 (2003).

Behrens J, von Kries JP, Kuhl M, Bruhn L, Wedlich D, et al: Functional interaction of beta-catenin with the transcription factor LEF-1. Nature 382:638–642 (1996).

Boras-Granic K, Chang H, Grosschedl R, Hamel PA: Lef1 is required for the transition of Wnt signaling from mesenchymal to epithelial cells in the mouse embryonic mammary gland. Dev Biol 295:219–231 (2006).

Brevern NV, Schimpf B, Wörner R, Swalve H: Parameterschätzung für Zitzenmerkmale bei Hybridsauen. Züchtungskunde 66:339–348 (1994).

Cassady JP, Johnson RK, Pomp D, Rohrer GA, Van Vleck LD, et al: Identification of quantitative trait loci affecting reproduction in pigs. J Anim Sci 79:623–633 (2001).

Chu EY, Hens J, Andl T, Kairo A, Yamaguchi TP, et al: Canonical WNT signaling promotes mammary placode development and is essential for initiation of mammary gland morphogenesis. Development 131:4819–4829 (2004).

Clop A, Marcq F, Takeda H, Pirottin D, Tordoir X, et al: A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. Nat Genet 38:813–818 (2006).

Du FX, Clutter AC, Lohuis MM: Characterizing linkage disequilibrium in pig populations. Int J Biol Sci 3:166–178 (2007).

Eastman Q, Grosschedl R: Regulation of LEF-1/TCF transcription factors by Wnt and other signals. Curr Opin Cell Biol 11:233–240 (1999).

Foley J, Dann P, Hong J, Cosgrove J, Dreyer B, et al: Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. Development 128:513–525 (2001).

Günther C, Dämmrich K, Plonait H: Morphologie und Pathogenese der sogenannten Stülpzitzen des Schweins. Züchtungskunde 57: 256–266 (1985).

Hardge T, Koepke K, Reissmann M, Wimmers K: Maternal influences on litter size and growth in reciprocal crossed Miniature Pigs and Durocs. Arch Anim Breed 42:83–92 (1999).

Hennighausen L, Robinson GW: Signaling pathways in mammary gland development. Dev Cell 1:467–475 (2001).

Horvath S, Xu X, Laird NM: The family based association test method: strategies for studying general genotype-phenotype associations. Eur J Hum Genet 9:301–306 (2001).

Jonas E, Schreinemachers HJ, Kleinwachter T, Un C, Oltmanns I, et al: QTL for the heritable inverted teat defect in pigs. Mamm Genome 19:127–138 (2008).

Kratochwil K, Dull M, Farinas I, Galceran T, Grosschedl R: Lef1 expression is activated by BMP-4 and regulates inductive tissue interactions in tooth and hair development. Gene Dev 10:1382–1394 (1996).

Mailleux AA, Spencer-Dene B, Dillon C, Ndiaye D, Savona-Baron C, et al: Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo. Development 129:53–60 (2002).

Mayer J: Genetische Parameter von Gesäugeanomalien beim Schwein. Technische Universität München – Weihenstephan (1994).

Mayer J, Pirchner F: Asymmetry and inverted nipples in gilts. Arch Anim Breed 38:87–91 (1995).

Niggemayer A: Besamungsstation kontrolliert Zitzenvererbung. Schweinezucht Schweinemast 3:11 (1993).

Pispa J, Thesleff I: Mechanisms of ectodermal organogenesis. Dev Biol 262:195–205 (2003).

Robinson GW, Karpf ABC, Kratochwil K: Regulation of mammary gland development by tissue interaction. J Mammary Gland Biol 4: 9–19 (1999).

Sakakura T: Mammary Embryogenesis, pp 37–66 (Plenum Press, New York 1987).

Sakakura T, Kusano I, Kusakabe M, Inaguma Y, Nishizuka Y: Biology of mammary fat pad in fetal mouse – capacity to support development of various fetal epithelia in vivo. Development 100:421–430 (1987).

Sato S, Atsuji K, Saito N, Okitsu M, Sato S, et al: Identification of quantitative trait loci affecting corpora lutea and number of teats in a Meishan × Duroc F-2 resource population. J Anim Sci 84:2895–2901 (2006).

Sved JA: Linkage disequilibrium and homozygosity of chromosome segments in finite populations. Theor Popul Biol 2:125–141 (1971).

Vangenderen C, Okamura RM, Farinas I, Quo RG, Parslow TG, et al: Development of several organs that require inductive epithelial-mesenchymal interactions is impaired in Lef-1-deficient mice. Gene Dev 8:2691–2703 (1994).

Veltmaat JM, Van Veelen W, Thiery JP, Bellusci S: Identification of the mammary line in mouse by Wnt10b expression. Dev Dyn 229: 349–356 (2004).

Wiesner E, Willer S: Problems of occurrence of inverted nipples in swine. Monatsh Veterinärmed 33:189–190 (1978).

JOURNAL OF ANIMAL SCIENCE
The Premier Journal and Leading Source of New Knowledge and Perspective in Animal Science

Differential expression of growth factors and their receptors points out their involvement in the inverted teat defect in pigs

S. Tetzlaff, E. Murani, K. Schellander, S. Ponsuksili and K. Wimmers

J Anim Sci published online Jul 31, 2009;

Running head: Growth factor expression and inverted teats in pigs

Differential expression of growth factors and their receptors points out their involvement in the inverted teat defect in pigs

S. Tetzlaff,* E. Murani,* K. Schellander,† S. Ponsuksili,‡ and K. Wimmers*[1]

* Research Institute for the Biology of Farm Animals, Research Unit `Molecular Biology´, Wilhelm-Stahl-Allee 2, 18196 Dummerstorf, Germany; † Institute of Animal Breeding Science, Animal Breeding and Husbandry Group, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany; ‡ Research Institute for the Biology of Farm Animals, Research Group `Functional Genome Analysis´, Dummerstorf, Germany

[1]Corresponding author: wimmers@fbn-dummerstorf.de

Published Online First on July 31, 2009 as doi:10.2527/jas.2008-1660

ABSTRACT

In this study 8 genes of growth factors and their receptors were investigated that are known to play a significant role in signaling pathways involved in the ontogenetic, but also tumorigenic, development of breast and mammary glands. Differential expression of *FGFR2, GHR, HGF, HGFR, PDGFA, PDGFRA, PDGFB,* and *VEGF* was analyzed in mesenchymal and epithelial teat tissue of peripubertal pigs affected and non-affected by the inverted teat defect. Comparisons were made at the level of affection between samples derived from non-affected animals and affected animals, including specimens of normal and inverted teats. In addition, comparisons were made at the level of the teat phenotype with normal teats of non-affected animals vs. either the normal or the inverted teat of affected animals. All genes tested, except *HGFR*, showed significant differential expression at $P < 0.05$ in either the mesenchymal and/or the epithelial teat tissue. In general, we observed more pronounced differences when comparing samples obtained from inverted tissues vs. samples from normal ones. Therefore, results of our study suggest that gene expression of the growth factors and their receptors associates directly with the teat phenotype rather than with the affection status of the investigated animals, suggesting that local processes and tissue-specific compensation by means of differential expression of growth factors and their receptors are responsible for the development of impaired teat phenotypes.

Key words: expression profile, inherited disorder, inverted teat, pig, qRT-PCR, signaling pathways

INTRODUCTION

In pigs a functional mammary gland is an important selection criterion for increasing the survival rate of piglets. The most relevant inherited disorder of the mammary complex is the inverted teat, resulting in non-functional teats. The defect occurs in commercial breeds with frequencies between 7.6 and 30% (Jonas et al., 2008). The emergence of inverted teats depends on insufficient mesenchymal proliferation at the developing teat ground (Jonas et al., 2008). Several authors have elucidated the influence of hormones and other genetic factors on the regulative interaction of epithelial mesenchymal proliferation during the formation of the mammary gland (Sakakura, 1987; Foley et al., 2001).

Feldman et al. (1993) have argued that mammary development depends on interaction of mammogenic and lactogenic factors. In particular, growth hormone (GH) and its receptor (GHR) mediate differentiation and development of the immature mammary gland. Further, the hepatocyte growth factor (HGF) could be shown as a factor with pleiotropic activities throughout mammary development, including stimulation of cell proliferation in epithelial cells and their interaction with mesenchymal cells during embryogenesis (Rosen et al.,1994; Lock et al., 2002). Platelet derived growth factors (PDGF) and their receptors (PDGFR), vascular endothelial growth factor (VEGF), belonging to the PDGF/VEGF signaling superfamily, and fibroblast growth factors (FGF) and their receptors (FGFR) are important regulators for tissue-tissue interactions to control proliferation of epithelial and mesenchymal cells during development of epidermal appendages (Sakakura, 1987; Hovey et al., 2001; Hoch and Soriano, 2003; Eblaghie et al., 2004).

Transcript abundance of 8 growth factors and receptors known to be relevant for assertive arrangements in a functional mammary gland were comparatively analyzed in epithelial and

mesenchymal tissue of normal and inverted teats to provide experimental evidence for their involvement in the development of the disorder.

MATERIALS AND METHODS

Tissue Collection and RNA Preparation

To collect samples of epithelial and mesenchymal teat tissue, animals of commercial herds of crossbreeds of German Landrace (GL), German Large White (LW), and Pietrain (Pi x (GL x LW)) pigs (n 28 animals comprising 14 full sib families with 1 affected and 1 non-affected litter mate; castrated males of 169 d of age on average) were used. Epithelial and mesenchymal tissue was gathered from the teat of the non-affected sibling and from a normal teat and an inverted teat of the affected sibling. On average, affected siblings showed 2.9 (1 to 5) inverted teats. A total of 84 tissue samples were collected in an experimental slaughterhouse where 2 persons of the team of inspectors also examined the teat phenotypes by adspection and palpation of teats at a separate slaughter. Tissue samples were directly frozen in liquid nitrogen and stored at 80°C.

Total RNA from each tissue was isolated using the Tri-Reagent (Sigma-Aldrich, Taufkirchen, Germany) and NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) including DNase treatment. RNA samples were visualized on 1.5% formaldehyde containing agarose gels to check the integrity, and the concentration was measured by spectrometry with a NanoDrop ND-1000 spectrophotometer (PEQLAB, Erlangen, Germany). In addition, absence of DNA contamination was checked using the RNA as a template in a PCR amplifying a fragment of the glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*) gene. All RNAs were stored at 80°C until downstream analysis was performed.

Quantitative Real-Time PCR (qRT-PCR)

In order to survey differential expression of the epithelial and mesenchymal tissue samples, qRT-PCR was performed using the LightCycler® 480 system (Roche, Mannheim, Germany). First-strand cDNA was synthesized from 1µg of total RNA using random primers and oligo d(T) 13VN in the presence of Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). The ribosomal protein L32 (*RPL32*) and the general transcription factor IIIC, polypeptide 2, (*GTF3C2*) were treated as internal references. Reactions were performed in a final volume of 12 µL using 6.0 µL of LightCycler 480 SYBR Green I Master (Roche), 600 nM of each primer (Table 1), and 100 ng of cDNA. Amplification conditions were 95°C for 10 min, 40 cycles of 95°C for 15 sec, annealing (Table 1) for 10 sec, and 72°C for 15 sec. At the completion of the amplification protocol, all samples were subjected to melting curve analyses and gel electrophoresis to verify the absence of any non-specific product. PCR products from each of the respective transcripts were used to generate external standard curves for the calculation of copy numbers. Normalization for each sample used the geometric means of the relative concentration of each internal reference gene (*RPL32* and *GTF3C2*). For statistical analysis of qRT-PCR data, tissue-specific normalized means of transcript abundance levels were compared with Student's *t*-test (paired samples). Differences between tissue samples were considered significant at $P < 0.05$ (Figures 1 and 2).

RESULTS

Our investigation was performed on 8 genes using mesenchymal and epithelial tissue samples from discordant animals of commercial populations. All genes tested, expect *HGFR*, showed significant differential expression at $P < 0.05$ in terms of the inverted teat defect (Figures 1 and 2). In this study, a total of 25 significant differences in expression could be found in mesenchymal and epithelial tissue. In particular, *FGFR2*, *HGF*, *PDGFA*, *PDGFRA*, and *VEGF* were significantly differentially expressed in mesenchymal tissue of total normal teat (B) and inverted teat (E); furthermore, *FGFR2*, *HGF*, *PDGFA*, and *PDGFRA* exhibited significant differential expression in mesenchymal teat tissue of non-affected sibs (C) in comparison with inverted teat tissue (E) of the affected sibs; *HGF* and *PDGFRA* showed significant differential expression in normal (D) and inverted (E) mesenchymal teat tissue of the affected animals. A significant differential expression profile of mesenchymal tissue from affected animals (A) compared with non-affected animals (C) could be demonstrated for *HGF* and *PDGFA* (Figure 1). Six of our investigated genes showed significant differences of expression in epithelial teat tissue; *HGF*, *GHR*, *PDGFA*, *PDGFRA*, and *VEGF* were significantly differentially expressed in epithelial tissue of total normal teat (B) and inverted teat (E); furthermore, *HGF*, *PDGFA*, *PDGFRA, PDGFB*, and *VEGF* exhibited significant differential expression in epithelial teat tissue of non-affected sibs (C) in comparison with inverted teat tissue of the affected sibs (E); *HGF* and *PDGFRA* showed significant differential expression in comparison to normal (D) and inverted epithelial teat tissue (E) from the affected animals. Significant differential expression between epithelial teat tissue of affected animals (A) and non-affected animals (C) was demonstrated in the *PDGFA* gene (Figure 2).

DISCUSSION

Teat developmental and functional mammary gland capabilities have to be seen as a result of a complex interaction among multiple factors, signaling pathways, and different cell types during several major phases of development (Sakakura, 1987; Imagawa et al., 1990; Hovey et al., 2001). In particular, a number of paracrine growth factors have been proposed to be involved in teat and mammary gland development. Microarray analyses revealed that signaling pathways of several growth factors are involved in normal and inverted teat development with several genes along the signaling pathways showing trait dependent regulation (Figure 3; manuscript in preparation). The hierarchical superior components of these pathways, the growth factors and their receptors are not fully covered by the microarray. Here we aimed to quantify the transcripts of 8 growth factors and growth factor receptors, depending on the affection status of the organism and the teat phenotype using the more exact and sensitive real-time PCR technique.

Seven of 8 genes addressed here showed differential expression in either the mesenchymal and/or the epithelial teat tissue. In general, we observed more pronounced differences when comparing samples obtained from inverted tissues vs. samples from normal ones. Therefore, results of our study suggest that gene expression of the growth factors and their receptors associates directly with the teat phenotype rather than with the affection status of the investigated animals. This indicates that local processes and tissue-specific compensation by means of differential expression of growth factors and their receptors in the explicit teat are the main drivers of inverted teat development (Figures 1 and 2). Almost consistently, expression levels of the transcripts were greater in epithelial and mesenchymal inverted teat tissue than in normal teat tissue. Greater transcription levels of the growth factors and their receptors may point to greater differentiation and proliferative activity of inverted teats in the crosstalk between

both tissue compartments. This may represent compensatory processes taking place in order to catch up the retarded developmental progress of inverted teats.

FGFR2 belongs to the family of 4 *fibroblast growth factor receptors* in mammals. This gene is known to be expressed during early mammary gland development and plays roles in regulation of cell proliferation and differentiation in mammary epithelial and mesenchymal cells (Eblaghie et al., 2004; Kim et al., 2007). Several genes encoding FGF ligands like FGF7 and FGF10 are known to bind to *FGFR2* as the receptor, which has been reported to be already expressed in mammary ectoderm (Mailleux et al., 2002). Mouse embryos lacking *FGF10* gene function fail to develop mammary placodes, suggesting that *FGF10* signaling through its receptor *FGFR2* is required to initiate development of mammary glands (Mailleux et al., 2002). The FGFR2 protein consists of an extracellular region, composed of 3 immunglobulin-like domains, a single hydrophobic membrane-spanning segment, and a cytoplasmatic tyrosine kinase domain. The extracellular part of the protein interacts with FGF10, starting a cascade of downstream signals, ultimately influencing the development of mammary glands. The significant differences in expression between normal vs. inverted mesenchymal teat tissue suggest its importance for a functional mammary gland not only through development in embryogenesis. Human *FGFR2* maps to a region that is syntenic to the distal arm of SSC14 (Table 2), where a quantitative trait locus (QTL) for inverted teats was found in an experimental cross population (Jonas et al., 2008).

The *GHR* gene encodes a protein that is a transmembrane receptor for growth hormone and has been found in mammary glands of pigs (Jammes et al., 1991). Binding of growth hormone to the receptor leads to receptor dimerization and further activation of an intra- and intercellular transduction pathway for growth and development of the mammary gland (Feldman

et al., 1993). Feldman et al. (1993) elucidate its central and primary role in mammary gland development. Growth hormone together with its receptor can act directly on mammary tissue stimulating cell growth and further the end bud formation (Silberstein and Daniel, 1987). The position of *GHR* on SSC16 announced by Chowdhary et al. (1994) fell in the confidence interval of the QTL for inverted teats in commercial breeds reported by Jonas et al. (2008).

Hepatocyte growth factor is a mesenchymally derived factor with pleiotropic activities mediated through its HGF receptor. HGF stimulates cell proliferation in a wide range of cellular targets, including epithelial cells, as well as epithelial-mesenchymal interactions during embryogenesis (Rosen et al., 1994; Lock et al., 2002). However, it is unclear how the *HGF* receptor conducts the signaling pathways leading to its pleiotropic activities. Maroun et al. (2000) have demonstrated that, for promotion of epithelial morphogenesis, recruitment of the Gab family of docking proteins (Gab1 and Gab2) is necessary, but not sufficient. These proteins are phosphorylated through tyrosine kinase downstream from the *HGF* receptor, activated by *HGF*. The differential expression of *HGF* in mesenchymal and epithelial teat tissue (Figures 1 and 2) confirms its role in biological pathways involved in the occurrence of a functional mammary gland. *HGFR* gene expression was not found to be different in the investigated teat tissues. This might indicate that HGFR with its ligands has eminent relevance for distinct pathways for motility, growth, and/or morphogenesis in various tissues. Local effects at the teat and mammary gland are regulated on the level of the growth factor, whereas general effects are determined at the receptor level or more distally. Moreover, the chromosomal assignment of *HGF* (Pinton et al., 2000) and *HGFR* to the proximal region of SSC9 (Table 2) revealed no allocation to a QTL for teat developmental traits.

Platelet derived growth factors and their receptors are important regulators of tissue-tissue interactions to control proliferation of epithelial and mesenchymal cells during embryonic development of epidermal appendages (Sakakura, 1987; Karlsson et al., 1999; Hoch and Soriano, 2003; Xu et al., 2005). PDGFs have characteristic domains, which include 8 conserved cysteines that are involved in inter- and intramolecular bonds. The signaling network consists of 4 homo- or heterodimers of 2 subunit proteins, A-, B-, C- and D-chain. Their receptors are 2 tyrosine kinases (PDGFRA and PDGFRB) that are able to form homo- or heterodimers after binding of the PDGF ligand. Different studies suggest that PDGFB has limited function in regulating organogenesis, whereas PDGFA and its PDGFRA receptor are more broadly required during embryogenesis (Hoch and Soriano, 2003; Xu et al., 2005). Functionally, PDGFA and PDGFRA signaling plays an important role in cell proliferation, cell migration, and in regulating epithelial-mesenchymal interactions during developmental processes (Xu et al., 2005). In addition, Table 2 presents the chromosomal assignment of *PDGFA* to the distal region of SSC3 and *PDGFRA* to SSC8p12, as described in Johansson et al. (1992). This assignment for *PDGFA* revealed no allocation to a QTL for teat developmental traits. However, *PDGFRA* fell in the confidence interval of QTL for inverted teats and number of teats (Beeckmann et al., 2003; Jonas et al., 2008). Furthermore, chromosomal assignment by means of published human-porcine comparative maps allocates *PDGFB* to the distal region of SSC5 (Table 2). That region exhibits several QTL for mammary gland capabilities, including a QTL for inverted teats and a QTL for teat number (Rodriguez et al., 2005; Jonas et al., 2008).

Vascular endothelial growth factor belongs to the PDGF/VEGF signaling superfamily. VEGF is involved in functional development of the mammary gland via the establishment and maintenance of a vascular supply to support the mammary fat pad and associated epithelial

proliferation (Hovey et al., 2001; Hoch and Soriano, 2003). VEGF as a glycosylated homodimer stimulates vascular permeability and promotes cell proliferation through liberation of several proteases (Hovey et al., 2001). *VEGF* is assigned to the proximal region of SSC7 (Table 2), where no QTL for inverted teats is located (Jonas et al., 2008). QTL for teat number were found close to *VEGF* on SSC7 (Wada et al., 2000; Sato et al., 2006).

Expression analyses of the porcine transcriptome in terms of teat developmental and functional mammary gland capabilities revealed a number of functional candidate genes that show specific differential expression profiles of epithelial and mesenchymal tissue of normal and inverted teats, which is in line with their known physiological function. It is uncertain to what extent differences on the RNA level relate to differences in the level of proteins. However, it is remarkable that our microarray experiments showed trait associated regulation of a significant number of members of the corresponding signalling pathways. This indicates that the transcriptional differences described here are associated with transcriptional differences of downstream members of signalling pathways promoting the biological and physiological significance of our findings. Moreover, assignment of the growth factors and receptors to QTL regions for teat developmental and mammary gland capabilities corroborate their candidacy for the occurrence of the inverted teat defect.

ACKNOWLEDGEMENTS

The authors thank the Federal Ministry of Education and Research (BMBF) and the Förderverein Biotechnologieforschung der Deutschen Schweineproduktion e.V. (FBF) for financial support (FUGATO - HeDiPig, FKZ 0313392A). The experiments described in this study were performed in accordance with all appropriate regulations.

LITERATURE CITED

Beeckmann, P., G. Moser, H. Bartenschlager, G. Reiner, and H. Geldermann. 2003. Linkage and QTL mapping for Sus scrofa chromosome 8. Journal of Animal Breeding and Genetics 120:66-73.

Chowdhary, B. P., H. Ellegren, M. Johansson, L. Andersson, and I. Gustavsson. 1994. In-situ hybridization mapping of the growth-hormone receptor (Ghr) gene assigns a linkage group (C9, Psa, Ghr, and S0105) to chromosome-16 in pigs. Mammalian Genome 5:160-162.

Eblaghie, M. C., S. J. Song, J. Y. Kim, K. Akita, C. Tickle, and H. S. Jung. 2004. Interactions between FGF and Wnt signals and Tbx3 gene expression in mammary gland initiation in mouse embryos. Journal of Anatomy 205:1-13.

Feldman, M., W. F. Ruan, B. C. Cunningham, J. A. Wells, and D. L. Kleinberg. 1993. Evidence that the growth-hormone receptor mediates differentiation and development of the mammary-gland. Endocrinology 133:1602-1608.

Foley, J., P. Dann, J. Hong, J. Cosgrove, B. Dreyer, D. Rimm, M. E. Dunbar, W. Philbrick, and J. J. Wysolmerski. 2001. Parathyroid hormone-related protein maintains mammary epithelial fate and triggers nipple skin differentiation during embryonic breast development. Development 128:513-525.

Hoch, R. V., and P. Soriano. 2003. Roles of PDGF in animal development. Development 130:4769-4784.

Hovey, R. C., A. S. Goldhar, J. Baffi, and B. K. Vonderhaar. 2001. Transcriptional regulation of vascular endothelial growth factor expression in epithelial and stromal cells during mouse mammary gland development. Molecular Endocrinology 15:819-831.

Imagawa, W., G. K. Bandyopadhyay, and S. Nandi. 1990. Regulation of mammary epithelial-cell growth in mice and rats. Endocrine Reviews 11:494-523.

Jammes, H., P. Gaye, L. Belair, and J. Djiane. 1991. Identification and characterization of growth-hormone receptor messenger-Rna in the mammary-gland. Molecular and Cellular Endocrinology 75:27-35.

Johansson, M., H. Ellegren, L. Marklund, U. Gustavsson, E. Ringmarcederberg, K. Andersson, I. Edforslilja, and L. Andersson. 1992. The gene for dominant white color in the pig is closely linked to Alb and Pdgfra on chromosome-8. Genomics 14:965-969.

Jonas, E., H. J. Schreinemachers, T. Kleinwachter, C. Un, I. Oltmanns, S. Tetzlaff, D. Jennen, D. Tesfaye, S. Ponsuksili, E. Murani, H. Juengst, E. Tholen, K. Schellander, and K. Wimmers. 2008. QTL for the heritable inverted teat defect in pigs. Mammalian Genome 19:127-138.

Karlsson, L., C. Bondjers, and C. Betsholtz. 1999. Roles for PDGF-A and sonic hedgehog in development of mesenchymal components of the hair follicle. Development 126:2611-2621.

Kim, Y., N. Bingham, R. Sekido, K. L. Parker, R. Lovell-Badge, and B. Capel. 2007. Fibroblast growth factor receptor 2 regulates proliferation and Sertoli differentiation during male sex determination. Proceedings of the National Academy of Sciences of the United States of America 104:16558-16563.

Lock, L. S., C. R. Maroun, M. A. Naujokas, and M. Park. 2002. Distinct recruitment and function of Gab1 and Gab2 in Met receptor-mediated epithelial morphogenesis. Molecular Biology of the Cell 13:2132-2146.

Mailleux, A. A., B. Spencer-Dene, C. Dillon, D. Ndiaye, C. Savona-Baron, N. Itoh, S. Kato, C. Dickson, J. P. Thiery, and S. Bellusci. 2002. Role of FGF10/FGFR2b signaling during mammary gland development in the mouse embryo. Development 129:53-60.

Maroun, C. R., M. A. Naujokas, M. Holgado-Madruga, A. J. Wong, and M. Park. 2000. The tyrosine phosphatase SHP-2 is required for sustained activation of extracellular signal-regulated kinase and epithelial morphogenesis downstream from the Met receptor tyrosine kinase. Molecular and Cellular Biology 20:8513-8525.

Pinton, P., L. Schibler, E. Cribiu, J. Gellin, and M. Yerle. 2000. Localization of 113 anchor loci in pigs: improvement of the comparative map for humans, pigs, and goats. Mammalian Genome 11:306-315.

Rodriguez, C., A. Tomas, E. Alves, O. Ramirez, M. Arque, G. Munoz, C. Barragan, L. Varona, L. Silio, M. Amills, and J. L. Noguera. 2005. QTL mapping for teat number in an Iberian-by-Meishan pig intercross. Animal Genetics 36:490-496.

Rosen, E. M., S. K. Nigam, and I. D. Goldberg. 1994. Scatter factor and the C-Met receptor - A paradigm for mesenchymal epithelial interaction. Journal of Cell Biology 127:1783-1787.

Sakakura, T. 1987. Mammary embryogenesis. Page 37 in The Mammary Gland: Development, Regulation and Function. M. C. Neville and C. W. Daniel, eds. Plenum Press, New York.

Sato, S., K. Atsuji, N. Saito, M. Okitsu, S. Sato, A. Komatsuda, T. Mitsuhashi, K. Nirasawa, T. Hayashi, Y. Sugimoto, and E. Kobayashi. 2006. Identification of quantitative trait loci affecting corpora lutea and number of teats in a Meishan x Duroc F-2 resource population. J. Anim. Sci. 84:2895-2901.

Silberstein, G. B. and C. W. Daniel. 1987. Investigation of mouse mammary ductal growth-Regulation using slow-release plastic implants. J. Dairy Sci. 70:1981-1990.

Wada, Y., T. Akita, T. Awata, T. Furukawa, N. Sugai, Y. Inage, K. Ishii, Y. Ito, E. Kobayashi, H. Kusumoto, T. Matsumoto, S. Mikawa, M. Miyake, A. Murase, S. Shimanuki, T. Sugiyama, Y. Uchida, S. Yanai, and H. Yasue. 2000. Quantitative trait loci (QTL) analysis in a Meishan x Gottingen cross population. Animal Genetics 31:376-384.

Xu, X., P. Bringas, P. Soriano, and Y. Chai. 2005. PDGFR-alpha signaling is critical for tooth cusp and palate morphogenesis. Developmental Dynamics 232:75-84.

Table 1. Primers used for qRT-PCR

Gene		Sequence 5′-3′	Accession no.	T (°C)	Size (bp)
FGFR2	for	GGATTACAACTCGCCTCTCCT	**AB271924**	62	196
	rev	GCTTCCTTGGGCTTCTCTTT			
GHR	for	GAAGACATTTACATCACCACAGAAA	**NM214254**	64	197
	rev	ACATAGCCACACGATGAGAGAAAC			
GTF3C2[1]	for	TTGAAAGCCAAGCCCACTTC	**CK466410**	64	230
	rev	GGGGAGGCAGGTCTAAAGGT			
HGF	for	ATGGTACTTGGTGTCATTGTTCCT	**CU656003**	62	183
	rev	TTGATGTAAAGAGAGTTGTGTTAATGG			
HGFR	for	CCCACCCTCATTACATCACC	**EW531089**	62	245
	rev	TTCTTCCTCATCCATCATTTACAAC			
PDGFA	for	GCACAGTCAGATCCACAGCA	**BP439777**	62	163
	rev	GATGCTTCTCTTCCTCCGAAC			
PDGFRA	for	ACAAGCTGTATCACTGCCTTTGTTT	**EW193418**	62	179
	rev	CTTCCTGTCGCGTTAGTTCTC			
PDGFB	for	CGTCTGTCTCGATGCCTGATT	**EW082988**	62	169
	rev	GTCAGTAGAGGAAGAGAGCGATG			
RPL32[1]	for	AGCCCAAGATCGTCAAAAG	**AY550039**	62	165
	rev	TGTTGCTCCCATAACCAATG			
VEGF	for	GACGAAGGTCTGGAGTGTGTG	**AF318502**	62	157
	rev	TTTCTTGCCTCGCTCTATCTT			

[1]Reference genes.

Table 2. List of candidate genes, their functions, and chromosomal assignments

Gene	Function	Porcine chromosomal assignment	Human chromosomal assignment
FGFR2 (*fibroblast growth factor receptor 2*)	Early embryonic development	SSC14q	HSA10q26
GHR (growth hormone receptor)	Differentiation and development	SSC16q1.3-1.4[1]	HSA5p13
HGF (hepatocyte growth factor)	Cell proliferation, cell-cell interaction	SSC9q21[2]	HSA7q21.1
HGFR (hepatocyte growth factor receptor)	Cell proliferation, cell-cell interaction	SSC9q15-21	HSA7q31
PDGFA (platelet-derived growth factor alpha)	Regulation of cell proliferation	SSC3p	HSA7p22.3
PDGFRA (platelet-derived growth factor receptor alpha)	Regulation of cell proliferation	SSC8p12[3]	HSA4q11
PDGFB (platelet-derived growth factor beta)	Regulation of cell proliferation	SSC5p	HSA22q13.1
VEGF (vascular endothelial growth factor)	Cell proliferation and growth	SSC7q11-13	HSA6p21.3

[1] Chowdhary et al. (1994).

[2] Pinton et al. (2000).

[3] Johansson et al. (1992).

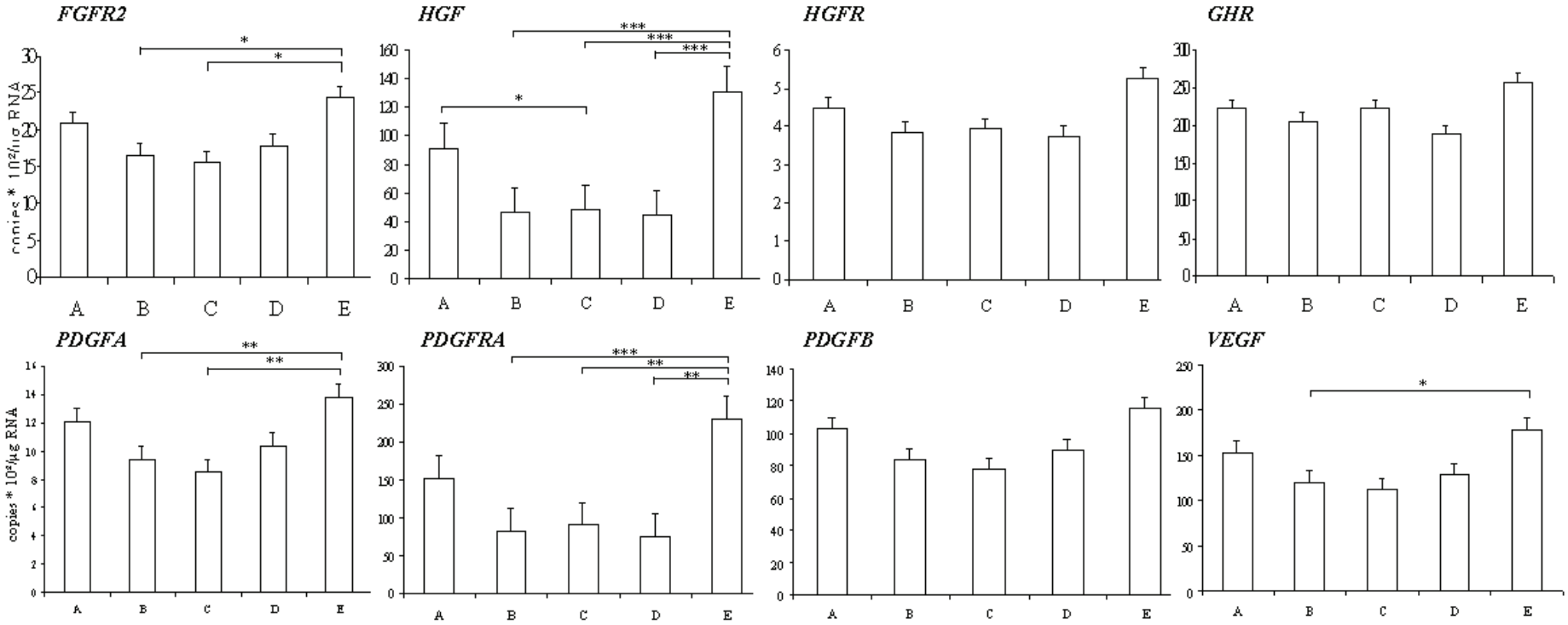


Figure 1. Gene expression profiles of mesenchymal teat tissue. Transcript abundance was determined by qRT-PCR from mesenchymal teat tissue samples of non-affected animals (n = 14); from normal teat tissue of affected animals (n = 14); and inverted teat tissue (n = 14). Mean abundance and standard errors are shown. A, expression value of tissue from affected animals; B, total normal teat tissue; C, teat tissue from non-affected animals; D, normal teat tissue from affected animals; E, inverted teat tissue; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

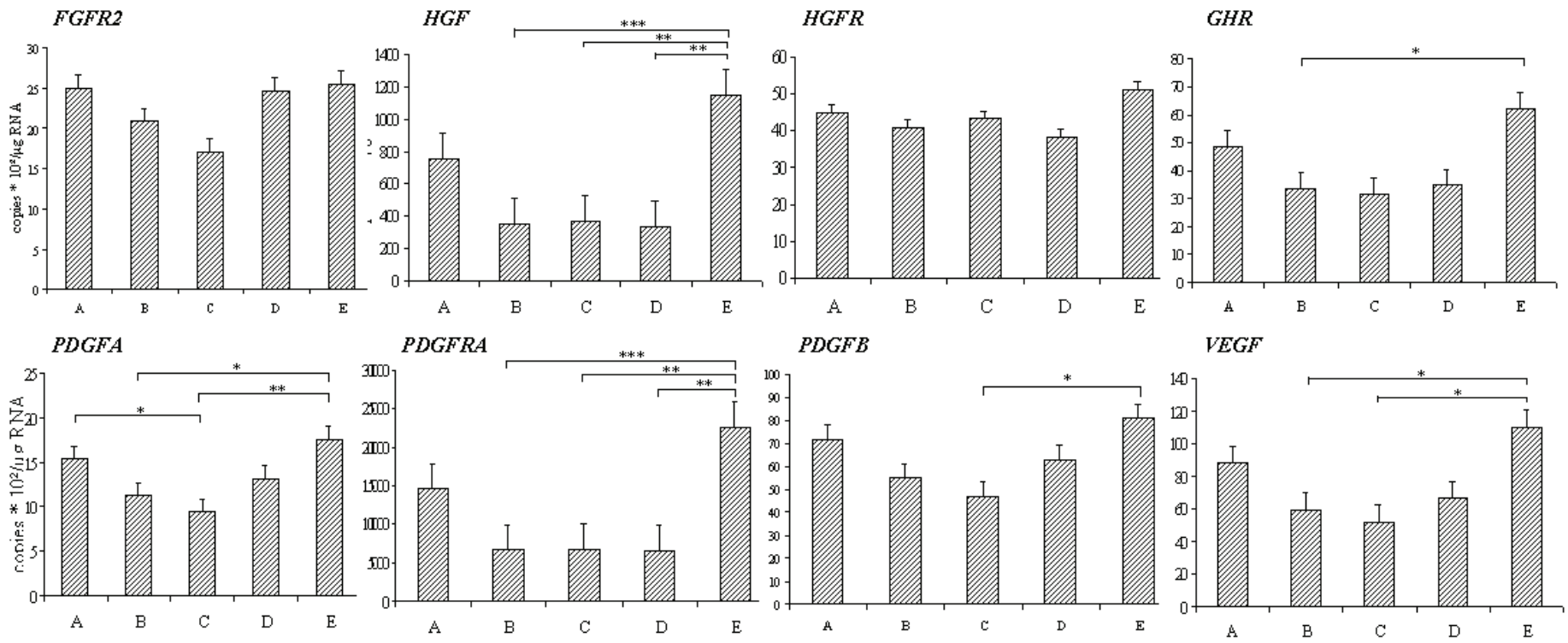


Figure 2. Gene expression profiles of epithelial teat tissue. Transcript abundance was determined by qRT-PCR from epithelial teat tissue samples of non-affected animals (n = 14); from normal teat tissue of affected animals (n = 14); and inverted teat tissue (n = 14). Mean abundance and standard errors are shown. A, expression value of tissue from affected animals; B, total normal teat tissue; C, teat tissue from non-affected animals; D, normal teat tissue from affected animals; E, inverted teat tissue; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

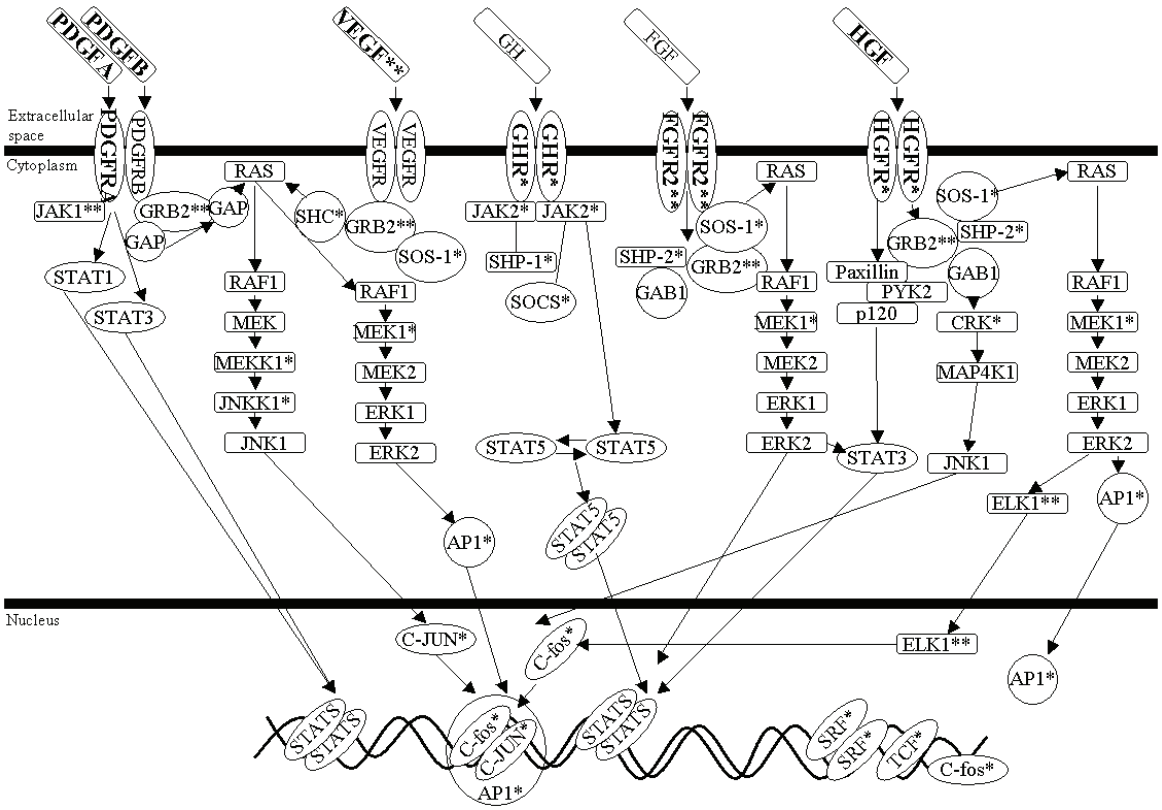


Figure 3. Simplified canonical pathways of growth factor signaling involved in the development of a functional mammary gland: Genes in bold letters are described in this investigation. * indicates fold change > 1.2; ** indicates regulation significant at $P < 0.05$ in previous own microarray experiments. AP1, activator protein 1; C-fos, C-fos protein; C-JUN, C-JUN transcription factor; CRK, V-CRK sarcoma virus CT10 oncogene homolog; ELK1, member of ETS oncogene family; ERK1-2, extracellular signal-regulated kinase 1-2; GAB1, GRB2-associated binding protein 1; GAP, glyceraldehyde-3-phosphate dehydrogenase; GRB2, growth factor receptor bound protein 2; JAK1-2, Janus tyrosine kinase 1-2; JNK1, Jun N-terminal kinase 1; JNKK1, Jun N-terminal kinase kinase 1; MAP4K1, mitogen-activated protein kinase kinase kinase kinase 1; MEK1-2, mitogen-activated protein kinase kinase 1-2; MEKK1, mitogen-activated protein kinase kinase kinase 1; p120, catenin, delta 1; PYK2, pyruvate kinase 2; RAF1, V-RAF-1 murine leukemia viral oncogene homolog 1;RAS, rat sarcoma viral oncogene homolog; SHC, SHC (Src homology 2 domain containing) transforming protein 1; SHP1-2, SHP1-2 containing protein tyrosine phosphatase; SOCS, suppressor of cytokine signaling; SOS-1, son of sevenless homolog 1; SRF, serum response factor; STAT1-5, signal transducer and activator of transcription 1-5; TCF, transcription factor.

Acknowledgements

Without the undeviating help and encouragement of many people this thesis had not been originated, therefore I sincerely would like to thank at this point.

For the abandonment of this interesting topic for my thesis as well as excellent supervising and assistance during my cumulative work in the Research Unit `Molecular Biology´ thanks to Prof. Dr. Klaus Wimmers. The motivating discussions and his constructive criticism significantly contributed to the success of this study.

I am highly thankful to Prof. Dr. Reinhard Schröder, department of Genetics, Institute for Life Sciences, University of Rostock for his willingness and assistance as a co-supervisor.

Drs. Siriluck Ponsuksili and Eduard Murani I am thankful for their thought provoking comments and considerations.

I sincerely thank Mrs. Hanni Tychsen and Mrs. Angela Garve for their competent assistance all the time. Their comprehensive knowledge, their manifold experiences and ideas as well as the humorous laboratory atmosphere always helped me with occurred difficulties.

I am also thankful to people at the Research Unit `Molecular Biology´ and the Research Group `Functional Genome Analysis´, which are not personally mentioned and helped me in one or the other way to accomplish this work.

For her helpful cooperation in the completion of my work and her troubleshooting via E-Mail and telephone I thank Dr. Elisabeth Jonas, Animal Breeding and Husbandry Group, Institute of Animal Breeding Science, University of Bonn.

Special thanks goes to my wife Peggy for her patience, her appreciation and her encouragement in the completion of this work. I am grateful to my daughter Hannah and my son Tobias for their ability to give me the strength to accomplish this work.

Curriculum Vitae

	School
1984-90	Grammar School *Georgi Dimitrow* in Neubrandenburg
1990-96	Cooperative Comprehensive School Datzeberg in Neubrandenburg
14.06.1996	General qualification for university entrance
	Academic studies
01.10.97-31.03.04	Studies of Life Sciences in Rostock University, academics Molecular Biology, Genetics, Animal Physiology and Microbiology
08.04.2004	Granting degree *certified biologist* with grade *good*
	Occupational activities
March ´03-June ´05	Diplomate and free research associate in the Institute of Legal Medicine, Working Unit `Forensic Genetics´, University of Rostock
Since October ´05	Graduation in the Research Institue for the Biology of Farm Animals, Research Unit `Molecular Biology´, Dummerstorf
	Advanced training
December ´03	Innsbruck Medical University Institute of Legal Medicine Innsbruck, Austria

Publications and lectures

von Wurmb-Schwark, N., **Tetzlaff, S.**, Hammer, U., Wegener, R. Der Nachweis von DYS390 und Betaglobin zur Chimärismusanalyse und in Spurenfällen. Vortrag, 10. Frühjahrstagung - Region Nord - der Deutschen Gesellschaft für Rechtsmedizin, 18.-19. Mai 2001, Greifswald, Tagungsband S. 29.

Tetzlaff, S., Weirich, V., Brandstätter, A., Parson, W., Wegener, R. Kam Ötzi aus Mecklenburg? Populationsgenetische Daten zu den hypervariablen Regionen HVI und HVII der mtDNA aus der Region Mecklenburg. Poster, 83. Jahrestagung der Deutschen Gesellschaft für Rechtsmedizin, 22.- 25. September 2004, Göttingen, Tagungsband S. 28.

Weirich, V., **Tetzlaff, S.**, Brandstätter, A., Parson, W., Wegener, R. (2004) Kam Ötzi aus Mecklenburg? Populationsgenetische Daten zu den hypervariablen Regionen HV-I und HV-II der mtDNA aus der Region Mecklenburg. *Rechtsmedizin*, 14(4), 346.

Tetzlaff, S., Brandstätter, A., Wegener, R., Parson, W., Weirich, V. (2007) Mitochondrial DNA population data of HVS-I and HVS-II sequences from a northeast German sample. *Forensic Science International*, 172, 218-224.

Tetzlaff, S. Prächtige Ferkel brauchen wohlgeformte Zitzen: Stülpzitze als Erbkrankheit. Poster, Die lange Nacht der Wissenschaften, 26. April 2007, Rostock. Nachtprogramm S. 13.

Tetzlaff, S., Ponsuksili, S., Murani, E., Schellander, K., Wimmers, K. (2007) SNP analysis, genotyping of the porcine *PTHR1* gene to chromosome 13. *Archives of Animal Breeding*, 3, 320-321.

Tetzlaff, S., Ponsuksili, S., Murani, E., Wimmers, K. Molekulargenetische Charakterisierung des funktionellen Kandidatengens *PTHR1* für den Erbdefekt Stülpzitze beim Schwein. Vortrag, Vortragstagung der Deutschen Gesellschaft für Züchtungskunde und der Gesellschaft für Tierzuchtwissenschaft, 26.-27. September 2007, Hohenheim, Tagungsband S. A02.

Jonas, E., Schreinemachers, H-J., Kleinwächter, T., Ün, C., Oltmanns, I., **Tetzlaff, S.**, Jennen, D., Tesfaye, D., Ponsuksili, S., Murani, E., Juengst, H., Tholen, E., Schellander. K,, Wimmers, K. (2008) QTL for the heritable inverted teat defect in pigs. *Mammalian Genome*, 19(2), 127-138.

Wimmers, K., **Tetzlaff, S.**, Murani, E., Yammuen-Art, S., Phatsara, C., Jonas, E., Schellander, K., Ponsuksili, S. (2008) Identification of positional functional candidate genes for the inverted teat defect in pigs. Vortrag, Pig Genome II-Second European Conference on Pig Genomics, 04.-05. Juni 2008, Ljubljana, Slowenien, Tagungsband S. 18.

Tetzlaff, S., Chomdej, S., Jonas, E., Ponsuksili, S., Murani, E., Phatsara, C., Schellander, K., Wimmers, K. (2009) Association of parathyroid hormone-like hormone (*PTHLH*) and its receptor (*PTHR1*) with the number of functional and inverted teats in pigs. *Journal of Animal Breeding and Genetics*, 126, 237-241.

Tetzlaff, S., Jonas, E., Phatsara, C., Murani, E., Ponsuksili, S., Schellander, K., Wimmers, K. (2009) Evidence for association of lymphoid enhancer-binding factor-1 (*LEF1*) with the number of functional and inverted teats in pigs. *Cytogenetic and Genome Research*, 124, 139-146.

Tetzlaff, S., Murani, E., Schellander, K., Ponsuksili, S., Wimmers, K. (2009) Differential expression of growth factors and their receptors points out their involvement in the inverted teat defect in pigs. *Journal of Animal Science*, published online, 31.07.2009.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig angefertigt und ohne fremde Hilfe verfasst habe, keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe und die den benutzten Werken inhaltlich und wörtlich entnommenen Stellen als solche kenntlich gemacht habe.

Erklärung

zum eigenen Anteil an der Veröffentlichung:

Jonas, E., Schreinemachers, H-J., Kleinwächter, T., Ün, C., Oltmanns, I., Tetzlaff, S., Jennen, D., Tesfaye, D., Ponsuksili, S., Murani, E., Juengst, H., Tholen, E., Schellander. K,, Wimmers, K. (2008) QTL for the heritable inverted teat defect in pigs. *Mammalian Genome*, 19(2), 127-138.

Hiermit erkläre ich, dass ein wesentlicher Bestandteil der genannten Veröffentlichung die Genotypisierung von Mikrosatellitenmarkern darstellt. Im Rahmen der Veröffentlichung wurden von mir 375 Schweine einer experimentellen Population aus Kreuzungstieren der Rassen Duroc und Berliner Miniaturschwein (DUMI) sowie 415 kommerzielle Schweine der Rassen Deutsche Landrasse, Deutsches Edelschwein als auch deren Kreuzungstiere an den Mikrosatelliten SW133, SW782, SW1129, SW122 und SW446 genotypisiert, die zusammen mit den Markern S0300 und S0059 auf dem porcinen Chromosom 6 lokalisiert sind. Im Anschluss wurde eine genetische Kartierung mithilfe der Software CRI MAP, Version 2.4 durchgeführt und eine genetische marker-basierte Karte kalkuliert. Daraufhin erfolgten mittels der Genehunter Software, Version 2.0 Kopplungsanalysen (*quantitative trait loci,* QTL Analysen) mit chromosomen-weitem Signifikanzlevel. Der Permutationstest wurde zur weiteren Ermittlung eines experiment-weiten Signifikanzlevels genutzt. Die Ergebnisse verdeutlichten die Anwesenheit einer signifikanten QTL Region auf dem Chromosom 6 in der untersuchten experimentellen als auch in der kommerziellen Population.