Deciphering the genetics of pig complex traits through QTL mapping and positional candidate cloning

Dissertation

to obtain the Ph. D. degree

in the Faculty of Agricultural Sciences,

Georg-August-University Göttingen, Germany

presented by

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Göttingen, December, 2006

D7

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Date of dissertation: 1th February, 2007

Dedicated To My Beloved Family...

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List of Publications

The present dissertation is based on the following publications:

1. Knorr C, Beck J, Beuermann C, Chen K, Ding N, Gatphayak K, Huang LS, Laenoi W and Brenig B. (2006) Chromosomal assignment of porcine oncogenic and apoptopic genes CACNA2D2, TUSC4, ATP2A1, COL1A1, TAC1, BAK1 and CASP9. *Anim Genet*, 5, 523-525.
2. Ding NS, Guo YM, Knorr C, Ma JW, Lan LT, Ai HS, Haley CS, Brenig B and Huang LS (2006) Genome-wide QTL mapping for teat number in an Erhualian × White Duroc pig resource population. *BMC Genetics* (submitted).
3. Ding NS, Knorr C, Baumgartner BG，Beck J, Huang LS and Brenig B (2007) Molecular characterization and evaluation of the porcine *SOX9* gene as a positional candidate for inguinal/scrotal hernia. *In preparation*.

Abstract

Most traits in livestock are controlled by multiple genes and are therefore called genetically complex traits. Emerging genetic resources and technologies, genomic strategies, enable the systematic identification of quantitative trait loci (QTLs), quantitative trait genes (QTGs) and quantitative trait nucleotides (QTNs) underlying these complex traits.

In this thesis, we firstly report the molecular characterization and evaluation of the porcine *SOX9* gene as a positional candidate for inguinal/scrotal hernia. The *SOX9* gene was assigned to SSC12p13-p14 by *FISH* and RH-panel analysis. The genomic DNA sequence of 11,330 bp containing the whole *SOX9* gene was sequenced. Four polymorphisms (SNP-5'UTR-G-420A, SNP-5'UTR-G195T, an 18 bp deletion at 223 bp of the 5'UTR and SNP-intron2-G2462T) were detected and genotyped. Association analyses between the *SOX9* gene and pig hernia inguinalis/scrotalis were performed with HaploView software. The results showed that the 18-bp deletion had a significant effect on hernia inguinalis/scrotalis in pig ($p<0.05$). Subsequently, a protein factor binding specifically to the 18-bp-deletion allele was detected using electrophoretic mobility shift assay (EMSA). Further transfection assays showed that the deletion leads to a dramatic increase in the transcription activation compared to the insertion. We propose that this deletion creates a potential binding site of a transcription factor to enhance SOX9 expression level which might contribute to pig inguinalis/scrotalis through its functions on the development of the male gonad, collagen metabolism and apoptosis.

In the present thesis, we also performed a genome-wide scan to identify quantitative trait loci (QTLs) affecting the traits associated with teat number. A

total of 151 microsatellites evenly distributed over 18 porcine autosomes and the X-chromosome were genotyped for 560 F_2 pigs in a White Duroc×Erhualian intercross. A linkage map with a total length of 2471.4 cM and the average marker interval of 19.3 cM was produced. Linear regression method was used to map QTL via QTL Express. Four genome-wide significant QTLs for total teat number (TTN) were detected on SSC4 ($p<0.05$), SSC7 ($p<0.01$), SS12 ($p<0.01$) and SSC13 ($p<0.05$). Four chromosomal regions prominently affecting the teat number of right side (RTN) were identified on SSC3 ($p<0.01$), SS7 ($p<0.001$), SSC12 ($p<0.01$) and SSC15 ($p<0.01$), while only two significant QTLs related to left teat number (LTN) were found on SSC7 ($p<0.01$) and SSC12 ($p<0.05$). The estimated additive effects indicated that Erhualian alleles at the most significant QTLs had positive additive effects compared with the Duroc alleles, except for one QTL on SSC7 at which White Duroc alleles showed increasing teat numbers. Imprinting effects on TTN were detected on SSC7 and SSC12. The study appears to be the first presentation of QTLs for left and right side teat number in pigs.

Abbreviations

ABCG2	ATP binding cassette sub family G member 2
AI	Artificial insemination
AMH	Anti- Müllerian hormone
ASP	Affected sib pairs
BLUP	Best Linear Unbiased Prediction
bp	Base pair
CALCA	CALC(Calcitonin)-A/α-CGRP
CEA	Carcinoembryonic antigen
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1
CREB	cAMP-response element-binding protein
Col2α1	Collagen α1 (II)
Col11α2	Collagen α2 (XI)
Col27α1	Collagen α1 (XXVII)
CSL	Cranial suspensory ligament
CGRP	Calcitonin gene-related peptide
DNA	Deoxyribonucleic acid
DGAT1	Diacylglycerol-acyltransferase 1
dpc	Days post coitum
EBV	Estimated breeding value
ELN	Elastin
EMSA	Electrophoretic mobility shift assay
FA	Fluctuating aysmmetry
FISH	Fluorescence in situ hybridization
GDF8	Growth differentiation factor 8 (myostatin)
GFN	Genitofemoral nerve
GREAT/LGR8	G protein-coupled receptor affecting testicular descent/Relaxin receptor 8
GUSB	β-glucuronidase
IBD	Identical-by-descent
IGF2	Insulin-like growth factor 2

INSL3	Insulin-like peptide 3
IP3	Inositol 1,4,5-trisphosphate
LD	Linkage disequilibrium
LTN	Left teat number
MAS	Marker-assisted selection
MIS	Müllerian inhibiting substance
MMPs	Matrix metalloproteinases
mRNA	Messenger Ribonucleic Acid
PCD	Programmed cell death
PV	Processus vaginalis
QTG	Quantitative trait gene
QTL	Quantitative trait locus
QTN	Quantitative trait nucleotide
RLF	Relax-like factor
RH	Radiation hybrid
RTN	Right teat number
SF1	Steroidogenic factor 1
SNP	Single nucleotide polymorphism
SOX9	SRY-Related High-Mobility Group (HMG) Box 9
SRY	Sex-Determining Region on the Y Chromosome
SSC	*Sus scrofa* Chromosome
TDT	Transmission disequilibrium test
TTN	Total teat nuber
UTR	Untranslated region

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Introduction

The pig is among of the world's most important livestock because it not only provides the major red meat consumed (43%) worldwide (Rothschild *et al*., 1998), but also serves as an important model for human health and represents a significant future source of organs for transplantation (Rothschild, 2003). It is well-known that most of the biological or economically important traits and the majority of common genetic diseases in pigs have a complex multifactorial inheritance. Yet, very little is known about the detailed molecular architecture of these traits, such as how many genes are involved, what is the distribution of gene effects, how important are gene-by-gene and gene-by-environment interaction, how important are epigenetic mechanisms, etc. So, the genetic dissection of complex traits is one of the most difficult and important challenges. With the advancements in pig genetics and genomics, this arduous mission becomes feasible (Womack, 2005).

1 Strategies for dissecting complex traits

To identify the QTGs (Quantitative Trait Genes) and QTNs (Quantitative Trait Nucleotides) underlying economically important traits and complex disorders such as hernia inguinalis/scrotalis in pig, the QTLs are localized by within family linkage analyses as the first step, then fine-mapped by exploiting population-wide linkage disequilibrium and ultimately characterized by genomic sequencing and functional validation assay.

1.1 QTL Mapping

The use of segregation analysis in informative families or experimental crosses to map QTLs has been well established (Lynch *et al*., 1998). The basic resources

critical to QTL mapping are appropriate pedigreed populations with phenotype records and genomic DNA samples (Mehar *et al*., 2004). Designs for detecting QTL in livestock vary from experimental backcross and F_2 populations to half-sib designs that use existing family structures within a commercial breeding population. In comparison to plant species and laboratory animals, genome mapping in livestock faces the following challenges: 1) inbred lines are not commonly available, 2) maintenance of experimental populations can be prohibitively expensive, 3) reproductive capacity and generation interval are often limiting in the choice of experimental design (de Koning *et al.*, 2003). These factors have to be taken into account in both the design and analysis of QTL experiments. Using a sparse marker map (10-20 cM marker interval), several designs have been used to detect quantitative trait loci (QTLs) across the genome in pig.

Experimental crosses: Experimental crosses have been often implemented in pigs because generation intervals are relatively short and the number of offspring is moderate to high. Such crosses have been commonly established between outbred lines with remarkable differences in performances to produce F_2 or backcross populations (Knott *et al*., 1998; Rohrer, 2000). In comparison to either single backcross, the F_2 design is more powerful for detecting QTL with additive and dominance effects.

The QTL analysis model used in outbred crosses is largely the same as that used in the analysis of inbred crosses: marker alleles in the second generation are traced back to their line origin and contrasts for putative QTLs are estimated as differences between lines. Because crosses are between outbred lines, the analysis needs to accommodate the fact that founder lines may share alleles at the marker level. Because lines may not be fixed for alternate QTL alleles, these analyses estimate contrasts between average effects of QTL alleles derived from the

parental breeds, which only represent estimates of the actual effects if alternative QTL alleles are fixed in the founder lines (Perez-Enciso *et al*., 2000). So the major disadvantage of outbred-line crosses is that the degree of homozygosity at marker loci is lower than that in inbred-line crosses which are hardly available in pigs, and then may decrease the power of QTL mapping. This model is straightforward to implement using least squares methodology and has been extended to accommodate more complex genetic models like imprinting, sex-linked and/or sex-specific QTL, and epistatic QTL interactions (Haley *et al.*, 1994; Perez-Enciso *et al.*, 2002; Carlborg *et al*., 2002). Most of these models have been implemented in a user-friendly free analysis package accessible via the web at http://qtl.cap.ed.ac.uk/.

Commercial breeding family: To map QTLs for complex diseases in pigs, experimental crosses are extremely difficult to develop because of low incidence, fertility malady，time consuming and high expenditure, e.g. anal atresia and pig hernia, although one experimental population has been successfully developed to map bovine umbilical hernia (Ron *et al*., 2004). So, a more common approach is to exploit the existing pedigrees with field data recording, especially those in artificial insemination (AI) stations and commercial populations. In these populations, genotypes and phenotypes are collected in a large number of half-sib offspring and their progenies or affected individuals and their unaffected relatives. Nonparametric approach based on identical-by-descent (IBD) allele sharing among affected individuals, and transmission/disequilibrium tests based on comparison of the number of times a marker allele is transmitted versus not-transmitted from a marker heterozygote parent to affected offspring, are often used to identify chromosomal regions associated with traits in this kind of populations. The half-sib design and affected sib pairs design have been successfully implemented in genome-wide QTL analysis of pig diseases

(Grindflek *et al*., 2006; Knorr *et al*., 2006).

In total, the power of such analyses to detect and map QTLs depends on how large a fraction of the phenotypic variation is explained by a given locus and the type and size of the segregating population.

1.2 From QTL to QTN

The principal challenge for deciphering complex traits lies not in detecting QTLs, but in unraveling the genes responsible for these traits. To identify genes and mutations that underlie QTLs, the chromosomal region of a QTL is obtained from initial QTL mapping, its confidential interval is normally larger than 20 cM and contains several hundred genes, and the region needs to be refined using high-resolution mapping approaches. To achieve this goal, additional markers are often to be developed and utilized for second-round scanning. A targeted, inexpensive and fast method to develop microsatellites from large-insert libraries had been introduced (Chen *et al*., 2005). Single nucleotide polymorphisms (SNPs) have also been developed quickly in farm animals. Furthermore, large-size family with some strategies is a powerful approach for fine mapping of QTLs. Multiple continuous generation in conventional backcrosses and F_2 intercrosses families and advanced intercross lines (AIL) which can generate more recombination events have been proved to be an effective potential way to narrow the QTL region significantly (Darvasi, 1998; Wang *et al*., 2003). More powerful statistical methods, including linkage disequilibrium (LD), identical-by-descent (IBD) and transmission disequilibrium test (TDT) have been proved to enable fine mapping of QTL regions in livestock (Riquet *et al.*, 1999; Farnir *et al.*, 2002).

The positional candidate approach is subsequently implemented to scrutinize for candidate genes according to their physiological function in the refined QTL region. The ultimate goal is to identify quantitative trait nucleotides (QTNs) in the

candidate genes and validate the biological effects of QTNs using functional genomics tool (Andersson *et al*., 2004). The whole flowchart is shown in Figure 1. To date, the successful identification of the causative mutations that underlie complex traits in domestic animals is summarized in Table 1.

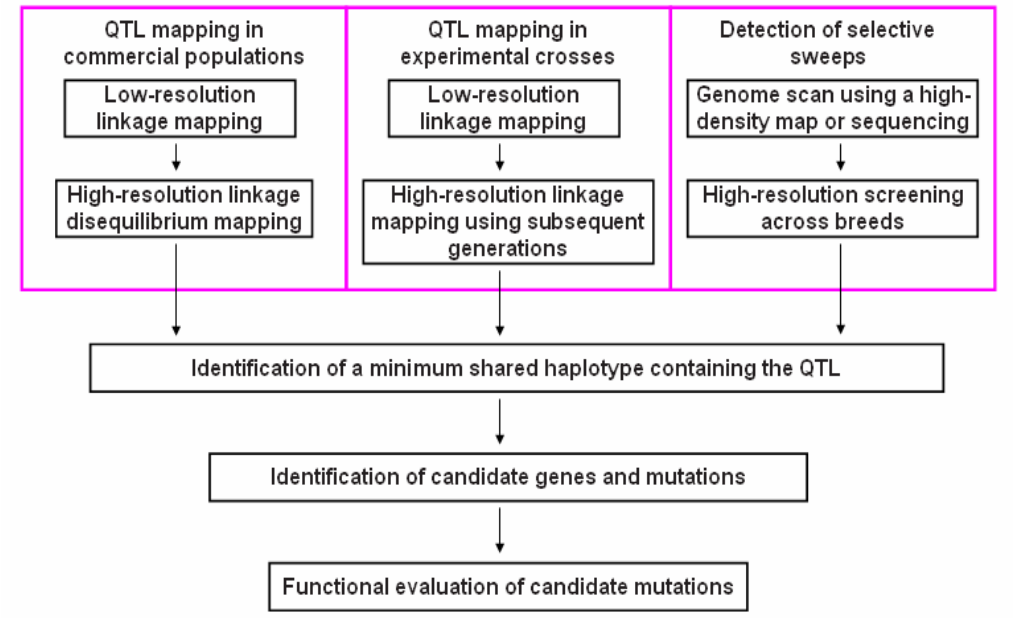

Figure 1. Approaches to mapping and positional cloning of QTLs in domestic animals.

(Cited from Andersson *et al*., 2004)

Table 1. QTNs for complex traits have been identified in livestock.

Species	Trait	Gene	QTN	Reference
pig	muscle development	*IGF2*	intron3-G3072A	Van Laere *et al*., 2003
cattle	milk yield and composition	*DGAT1*	K232A	Grisart *et al*., 2002
	milk yield and composition	*ABCG2*	Y581S	Cohen-Zinder *et al*., 2005
sheep	muscular hypertrophy	*GDF8*	3'UTR-G6723A	Clop *et al*., 2006

2 Pig teat number

2.1 Inheritance of teat number

With the improvement of pig reproductive performance, the teat number, especially the number of functional nipples becomes a more important trait regarding the mothering ability of sows; because a sow seldom weans more piglets than the number of the functional nipples it has (Hirooka *et al.*, 2001). The pig industry has traditionally applied selection pressure to teat number (Pumfrey *et al.*, 1980). The heritability of teat number varies in a wide range from 0.07 to 0.79, but most of estimates are in a low to medium interval from 0.20 to 0.50 (Borchers *et al.*, 2002).

The relationship between teat number and maternal performance is still controversial. Jungst *et al.* (1983) indicated that litter weights at 21 and 42 day were not affected by the number of teat. Pumfrey *et al.* (1980) showed that correlations between teat number and litter size, weight at birth, weight at weaning and ovulation rate were negative. But a recent investigation of the effects of teat number on litter size in Duroc, Landrace and Yorkshire gilts showed that 14 or more teats compared to 11-13 teats increased litter size at birth and 21 day weaning (Kim *et al.*, 2005).

In addition, teat number in pigs is a discontinuous and often canalized trait presenting bilateral symmetry with only minor difference between the two sides (Toro *et al.*, 1986), which makes it a good candidate to evaluate fluctuating asymmetry (FA) and developmental instability (Fernández *et al.*, 2004). So the factors that affect the number of teats in pigs are of interest for both biological and practical reasons (Drickamer *et al.*, 1999). In a Yorkshire population, no relationship between body length and number of nipples was detected (Johnson *et*

al., 2003). A major gene responsible for the number of false teats (FTN) is strongly suggested to be present in a Chinese European pig line using likelihood and Bayesian analyses (Sanchez *et al.*, 2003). Considering the limited information about the inheritance of teat number in comparison to other productive traits in pigs, a better understanding of the genetic control of this trait is still a real challenge.

2.2 QTLs for teat number

As a subtrait of fecundity in pigs, teat number is easily recorded in many mapping populations. A number of QTLs have been identified through whole-genome scans (Table 2) in recent years. It is worth to note that QTLs on SSC8, 10 and 12 overlap very well in several different experiments, in which Meishan pigs were used as the founder animals. These observations favor the hypothesis that one or more major genes for teat number are located respectively in these regions. Like most of economically important traits, the question now remains as the fine mapping of these QTLs and afterwards, the identification of the QTGs and QTNs. We will still need to know whether there are other QTLs for this trait which we have yet not identified.

3 Pig inguinal/scrotal hernia

Hernia is one of the most common congenital and developmental undesirable defects in pigs, commonly referred to as rupture in the industry, representing protrusion of part of an organ or tissue through the structures normally containing it (OMIA-Online Mendelian Inheritance in Animals, http://www.angis.org.au/bin/Databases/BIRX/birx_doc?omia+1082). Hernias can be classified as diaphragmatic, inguinal/scrotal, and umbilical/abdominal, depending on the location. The latter two are the more common ones.

Table 2. QTLs for teat number in pigs.

Population	SSC: Position (cM)	P-value[1]	a^2	d^3	References
White composite× Meishan (reciprocal back-cross)	10: 80	p<0.01	-	-	Rohrer, 2000
Goettingen Miniature ♂ × Meishan ♀	1: SJ029-SW485	p<0.01	0.7**	-	Wada *et al.*, 2000
	7: SW859-SW147	p<0.001	0.47**	0.79**	
High-indexing line × control line (Both lines derived from Large White × Landrace)	8: 19	p<0.05	-0.29*	-	Cassady *et al.*, 2001
	11: 46	p<0.05	-	0.67*	
	15: 109	p<0.05	-	-	Holl *et al.*, 2004
Meishan ♂ × Dutch Landrace/Large White ♀	2: 2	p<0.001	-0.15	-	Hirooka *et al.*, 2001
	10: 107	p<0.001	0.35	-	
	12: 80	p<0.001	0.20	-	
Meishan x White (reciprocal crosses)	8: 49	p<0.05[3]	-	0.58**	King *et al.*, 2003
Meishan ♂ × Pietrain ♀	1: 169; 2: 73; 10: 142; X: 11	p<0.05	-	-	Geldermann *et al.*, 2003
Wild ♂ ×Pietrain ♀	1: 171; 5: 28	p<0.05	-	-	Geldermann *et al.*, 2003
Wild ♂ × Meishan ♀	1: 68; 8: 63; 10: 93; 12: 41	p<0.05	-	-	Geldermann *et al.*, 2003
Iberian ♂ × Meishan ♀	5: 29	p<0.05	0.62	-	Rodriguez *et al*., 2005
	10: 72	p<0.001	0.71	-	
	12: 67	p<0.05	0.45	-	
Meishan♀× Duroc ♂	3: 117; 7: 97.1; 8: 62.9	p<0.01	-	-	Sato *et al*., 2006
	8: 29.7; 12: 41.4	p<0.05	-	-	

[1]Genomewide significance level, [2]additive effects, [3]dominance effects, *p<0.05, **p<0.01, ***p<0.001, [3]Chromosome-wide significance.

The economic loss caused by this defect in the pig industry is considerable vast each year in the world. Afflicted individuals cannot feed effectively and their growth is affected. This leads to higher feed costs, slower throughput, and lack of product uniformity, cheaper price for meat-selling and the consequent loss in income. Quite often such afflicted individuals also require extra health considerations – sick pens, drugs, labor, etc. – and hence lead to higher rearing costs. In a nucleus breeding population, such individuals cannot be considered for

use as breeding stock and effectively end up as culls. Moreover, affected animal's welfare can't be guaranteed. There is also the risk that herniated pigs could die as a result of strangulation of the intestine in the umbilical or scrotal ring. Some groups in the world have devoted to decipher the genetic structure of this disorder. Identification of causative genes or causal mutations controlling pig herniation will allow for the reduction of occurring of hernia through marker-assisted selection (MAS).

At the same time, pig hernia is also a good model for research on human hernia (Chang *et al*., 1994). The progress will boost the investigating of molecular mechanism of human hernia formation.

3.1 Epidemiology and inheritance of pig inguinal/scrotal hernia

Incidence: The frequencies of pig inguinal/scrotal hernia differ among different environments and breeds. Due to its nature, pig inguinal/scrotal hernia is a sex-limited congenital malformation. It occurs with prevalence between 0.5-1.5% in different breeds. Researchers observed a significant difference among the progeny of Duroc, Landrace, and Yorkshire in developing scrotal hernia with incidences of 0.6%, 1% and 1.5%, respectively (Vogt *et al*., 1990). It was also proposed that the incidence of inguinal/scrotal was relatively lower than 1% in Norwegian pigs (Lingaas *et al.*, 1991). Thaller also showed a prevalence of 0.57% of scrotal hernia in Landrace and Pietrain populations (Thaller *et al.*, 1996). Another study reported that the incidence rates of scrotal hernia were 1.36% and 1.31% in the Dutch herd-book Landrace and Large White Breeds, respectively. And the corresponding incidence rates for Hypor's European Landrace (D-line) and Large White (C-line) lines were 0.54% and 0.22%, respectively (Charagu, 2006). So, an unusually high incidence, high than 2% herniated pigs of all piglets born, in a certain period of time should be concerned and further investigated, and all herniated animals and their littermates should be culled from nucleus breeding

stock (Patrick *et al.*, 2006).

Heritability: Heritability of the susceptibility to scrotal hernia development reported in the literature is relatively moderate. Vogt *et al*. (1990) showed the heritability for this abnormality was 0.29±0.17, 0.34±0.23, and 0.34±0.19 in Duroc, Landrace, and Yorkshire-sired pig groups, respectively based on 5,711 Duroc-sired, 2,227 Landrace-sired and 2,494 Yorkshire-sired male pigs born over a 9-year period. Percentage of affected pigs among male full siblings of affected males was about 3.0 times greater than the overall percentage affected in their respective breed groups. But an even earlier estimation showed a higher heritability of scrotal hernia in two herds up to 0.65 and 0.86, respectively (Mikami *et al.*, 1979). Recognition of genetic modeling of inguinal/scrotal hernias went through a series of changes from a single gene with incomplete dominance (Berge *et al.*, 1941) to double recessive genes (Hutson *et al.*, 1978), and from polygenic inheritance (Magee, 1951; Knap, 1986; Wrathall, 1988) to major gene effects with a polygenic background variation with relatively low heritability between 0.029 and 0.187 (Thaller *et al.*, 1996).

3.2 Etiology of inguinal/scrotal hernia

As a complex genetic defect, it has been a long way to understand the nature of inguinal/scrotal herniation. Even then there are debates regarding the etiopathogenesis of this disease, three aspects have been highlighted to explore the probable causes of hernia inguinalis/scrotalis in past decades: patent processus vaginalis, altered collagen metabolism and testicular descent.

3.2.1 Obliteration of processus vaginalis

In human, indirect hernias are developed from incomplete obliteration of the processus vaginalis (PV), the embryological protrusion of peritoneum that precedes the descent of the testis. The testes originate along the urogenital tract in

the retroperitoneum and migrate during the second trimester of pregnancy to the deep inguinal ring, where they arrive after 6 months of gestation. During the last trimester, they proceed through the abdominal wall via the inguinal canal and descend into the scrotum. Normally the processus vaginalis postnatally obliterates (Figure 2). Failure of this process results in a patent processus vaginalis (PPV) and possibly in an indirect inguinal hernia. Failure of closure of the processus vaginalis accounts for nearly all the inguinoscrotal abnormalities seen in infancy and childhood (Bonnard *et al*., 2003). Van Wessem *et al.* (2003) showed that the etiology of indirect inguinal hernia is congenital.

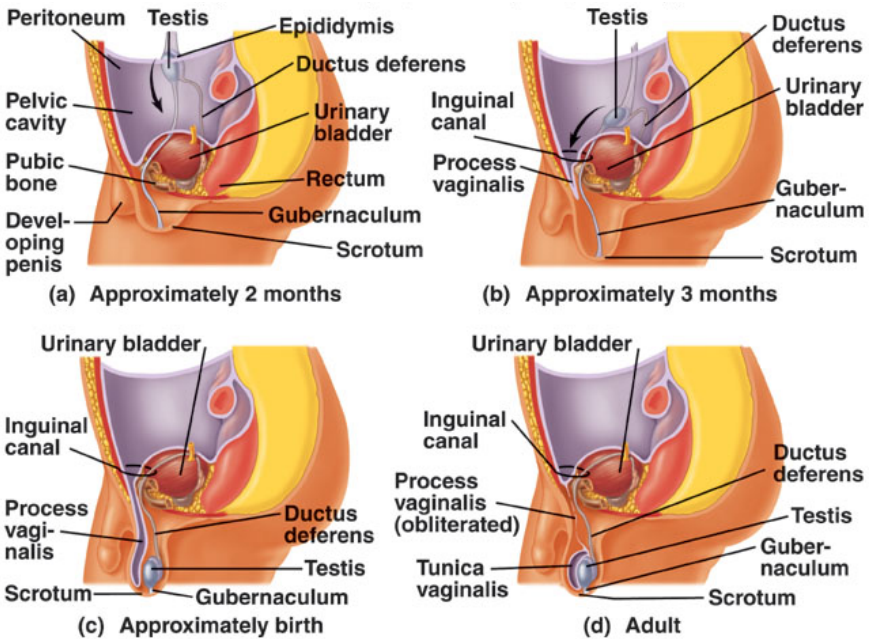

Figure 2. Scheme of the development and obliteration of process vaginalis in the descent of testes in human. (Robert *et al.*, 2004)

The testis is descended through the processus vaginalis via the propulsive force generated by the muscles derived from the gubernaculum (Tanyel *et al.*, 2000). Since the obliterated processus vaginalis is devoid of smooth muscle, and since sacs associated with inguinal hernia present more smooth muscle, the obliteration of the processus vaginalis after descent appears to mandate the disappearance of

smooth muscle (Tanyel *et al.*, 1999), i.e. disappearance of smooth muscle is necessary for the closure of the processus vaginalis. So after propelling the testis, the smooth muscle should undergo programmed cell death (PCD) for obliteration of the processus vaginalis, its failed apoptosis may persist processus vaginalis (Tanyel, 2000). Dedifferentiation into myofibroblast appears to be an important step that increases susceptibility of smooth muscle for programmed cell death (Tanyel *et al.*, 2002). There is no evidence of depletion of stores in cremaster muscles or overload of calcium in sacs associated with inguinal hernia (Tanyel *et al.*, 2003), while inhibition of Ca^{2+} load inhibits PCD.

The probable pathway of obliteration of processus vaginalis was proposed by Tanyel *et al*. (2004) and Tanyel (2004a) (Figure 3). The initial step appears to be the activation of phospholipase C via G-protein-linked signal transduction. Depletion of Ca^{2+} stores with an increase in cytosolic Ca^{2+} is succeeded by mitochondrial Ca^{2+} overload. Increases in Bax and Fas and regulated targeting of Bax to mitochondria initiate the cascade of PCD, which involves a family of proteases called caspases which are activated through two main pathways: extrinsic pathway and intrinsic pathway (Reed, 2000). The disappearance of the smooth muscle is followed by the disappearance of the mesothelium, thus the obliteration of the PV. In this pathway, activation of phospholipase C in smooth muscle is accomplished by parasympathetic influences. The parasympathetic system depends less on androgens. The increase in parasympathetic tonus is accomplished through a decrease in sympathetic tonus via up-regulation of afferent neurotransmitters under control of androgen receptors.

The dominance of parasympathetic tonus through a decrease in sympathetic tonus via an androgen receptor-dependent increase in afferent neurotransmitters at a critical time appears to be mandatory for the obliteration of PV through the PCD

of smooth muscle. Absence or inadequacy, inappropriate duration, and inappropriate timing of parasympathetic dominance affect both calcium signaling and the inducers of apoptosis and results in an inguinal hernia, hydrocele, or abnormal testicular localization.

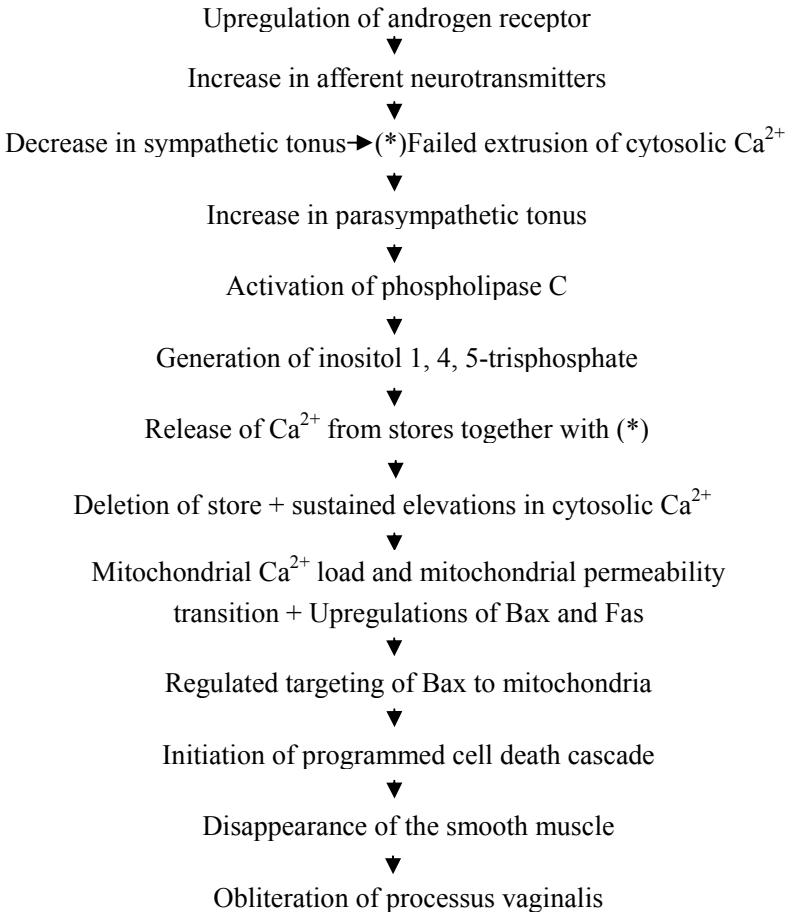

Figure 3. The possible pathway of obliteration of processus vaginalis. (Tanyel *et al.*, 2004)

3.2.2 Testicular descent

In the early fetus, the undetermined ambisexual gonad lies in peri-renal position. Through a cascade pathway regulated by a series of genes, including the testis-determining gene (*SRY*), the steroidogenic factor 1 (*SF1*) and the

SRY-related HMG box gene (*SOX9*), the testis was determined. Then the primitive sex cords in the medullary region of the primitive gonad differentiate into Sertoli cells. The Sertoli cells secrete the glycoprotein hormone Müllerian inhibiting substance (MIS) which causes regression of the Müllerian duct in the male fetus. Leydig cells are differentiated from the mesenchymal cells of the genital ridge and secrete testosterone which influences the differentiation of the Wolffian duct into the epididymis, vas deferens and seminal vesicle (Clarnette *et al*., 1997). These anatomical changes are presented in the testicular descent (Figure 4).

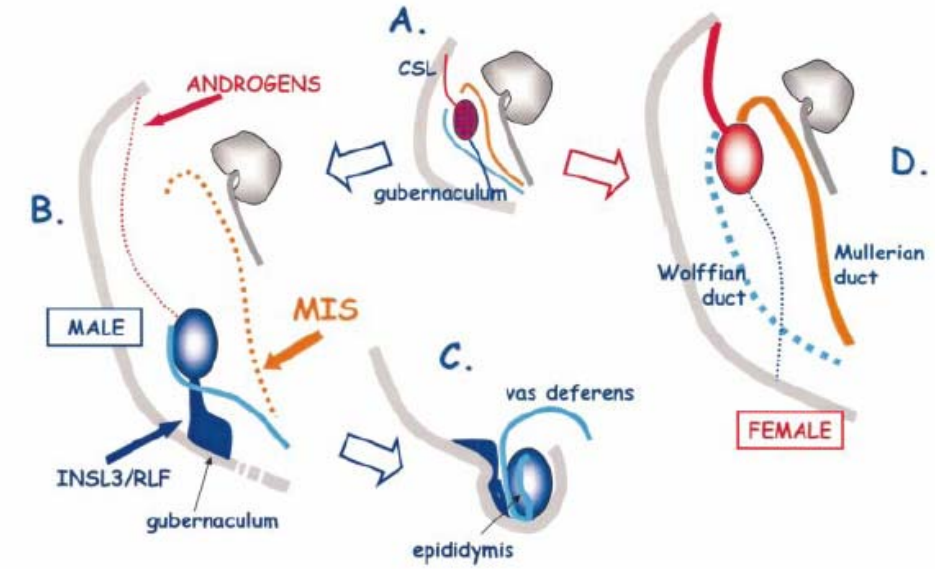

Figure 4. Schematic illustration of the events involved in the descent of the differentiating testis from a peri-renal to an inguinal position.
CSL: the cranial suspensory ligament; INSL3/RLF: insulin-like factor-3/relaxin-like factor; dot line: disappearance of anatomical structure. (Ivell *et al.*, 2003)

The processus vaginalis is closely associated with the mechanism of testicular descent. Testicular descent is a complex developmental process. During the last twenty years, the understanding of this process has been enriched. The process of testicular descent is now proposed to be occurred in two sequential steps with different anatomical mechanisms and different hormonal controls. Two

comprehensive reviews have led to a profound insight into this two-stage hypothesis (Hutson *et al*., 2005) and its molecular basis (Ivell *et al*., 2003). The first phase was suggested to be controlled by Müllerian inhibiting substance (MIS) (also known as anti-Müllerian hormone, AMH), while the second phase was proposed to be dependent on androgens (Table 3).

Table 3. Anatomical phases of testicular descent and their hormonal control.

(Hutson *et al*., 2004)

	Transabdominal Phase	Inguinoscrotal Phase
Anatomy	1. Regression of cranial suspensory ligament 2. Enlargement of genitoinguinal ligament (gubernaculum) ♦ Anchors testis near groin as embryo grows	1. Gubernaculum migrates from external 2. Processus vaginalis grows inside gubernaculum 3. Testis descends inside processus vaginalis 4. Processus vaginalis obliterates after descent
Hormonal Control	1. Testosterone triggers suspensory ligament involution 2. Insl3 (+MIS) stimulates swelling	1. Testosterone controls migration indirectly via GFN and release of CGRP (sensory nerve endings) ♦ Stimulates growth of gubernacular tip by trophism and chemotaxis

Transabdominal phase: The gubernaculum (genitoinguinal ligament) plays a pivotal role in the first phase of testicular descent. It enlarges by mitosis and deposition of hyaluronic acid (Heyns *et al*., 1990) and holds the testis near the inguinal canal (Van der Schoot *et al*., 1992), in which muscles form around the mesenchymal end of the gubernaculum. It is assumed that myogenesis occurs within gubernaculum to provide physical force for the descent of the testis (Tanyel, 2004b). Until now, at least three kinds of hormones are suggested to control the first step. Androgen causes cranial suspensory ligament (CSL) regression and insulin-like peptide 3 (INSL3) (also known as relaxin-like factor, RLF) causes the gubernacular swelling reaction (Nef *et al*., 1999; Zimmermann *et al*., 1999; Kubota *et al*., 2001; Smith *et al*., 2001; Tomiyama *et al*., 2003), which is

augmented by Müllerian inhibiting substance/anti-Müllerian hormone (MIS/AMH) (Hutson *et al*., 1987) and androgen (Kubota *et al*., 2002).

Inguinoscrotal phase: the gubernaculum is proposed to migrate from the external inguinal ring to the scrotum. Simultaneously the gubernaculum becomes hollowed out by a diverticulum of the peritoneum, tunica vaginalis, to allow the intra-abdominal fetal testis to reach the scrotum. Following complete descent, the peritoneum proximal to the testis obliterates to prevent development of an inguinal hernia. This inguinoscrotal phase is controlled by release of calcitonin gene-related peptide (CGRP) from genitofemoral nerve (GFN) under stimulation of androgen (Hutson e*t al*., 1997). CGRP has been shown to cause obliteration of the patent peritoneal sac in inguinal hernia in babies; however, the signaling pathway is still unknown.

Based on advanced of mechanism of testicular descent, the gubernaculum is the key anatomical structure in control of testicular descent; it plays an essential role in the complex mechanism of testicular descent and inguinal hernia closure.

3.2.3 Collagen hypothesis

It is well known that an indirect inguinal hernia usually occurs through an unobliterated processus vaginalis, whereas a direct inguinal hernia appears through a weak area and repeated elevation of intra-abdominal pressure. It is likely that more factors than a patent processus vaginalis along are needed to develop an indirect inguinal hernia. High abdominal pressure and weakening of the abdominal muscles can activate herniation. Interestingly, older human patients with inguinal hernia are usually reported to have a defect in the transversalis fascia (Peacock *et al*., 1974). Moreover, there are often high recurrence rates of inguinal hernias after physical strength has been restored by surgical mesh-free procedures (Lichtenstein *et al*., 1993). Obviously, the defect in the fascia

transversalis plays a pivotal role in the development of inguinal hernias.

The transversalis fascia is a fascial envelop of the abdomen and competency of the deep inguinal ring depends on the integrity of this fascia. And the fascia transversalis is a connective tissue composed of a framework of elastic and collagen fibers, which supports the abdominal tension forces. The distension and biological elasticity of the fascia transversalis significantly increased in hernia patients (Pans *et al.*, 1997). Significantly lower amounts of collagen and higher amounts of elastic fibers in transversalis fascia are observed in patients with direct inguinal hernia than those with indirect inguinal hernia (Pans *et al*., 2001; Rodrigues *et al.*, 2002). A point mutation (28197A>G) in the *elastin* (*ELN*) gene leads to an S422G amino acid substitution in the elastin hydrophobic domain, it shows a statistically significant association with inguinal hernia (Rodrigues *et al*., 2006), possibly due to abnormal elastic fiber and impaired fascia transversalis function. A marked reduction of the collagen I/III ratio respective in the fascia transversalis, hernial sac and skin of individuals with inguinal hernias compared with controls was demonstrated by immunohistochemistry and Western Blot analysis (Klinge *et al*., 1999a, 1999b and 1999c). In a sequent experiment, the ratio of type I to type III procollagen mRNA was also significantly decreased in cultured fibroblasts from the skin of patients with primary inguinal hernia as compared to controls (Rosch *et al*., 2002). The decreased ration was mainly due to the increase of type III collagen.

The main collagen types found in connective tissues are fibrillar types I, II, III and XI, with collagen type I being the most abundant protein in humans. The amount and the ration of synthesized and deposited collagens type I and type III determine the quality of connective tissue. Particularly mature type I collagen, predominantly found in dense bundles in connective tissues like tendons or ligaments, is responsible for the tensile strength of tissue. In contrast, type III collagen, consisting

of thinner fibres, represents immature collagen found in early wound healing and in flexible tissues (Wiedemann *et al*., 1975). Moreover, collagen types I and III play an important role in regulating fibrillogenesis, formation of fibril diameters and bundle architecture. With reduced ratio of type I to type III collagen, the geometrical arrangement and diameter of collagen fibrils changes and cross-linking decreases, that leads to reduced mechanical stability (Fleischmajer *et al*., 1990).

So the altered metabolism of collagen may play a pivotal role in the pathogenesis of inguinal hernia formation. It had been reported that connective tissue disorders, such as osteogenesis imperfecta, Marfan's, Ehlers-Danlos, hip dislocation of childhood may predispose individuals to the development of inguinal hernia probably through the interference of collagen metabolism. Autosomal dominant polycystic kidney, known to have abnormal extracellular matrix production is associated with a 43% incidence of hernias (Morris-Stiff *et al*., 1997). In a study in Sweden by Uden and Lindhagen, children with congenital hip dislocation revealed that boys had a 3 times increase in incidence of herniation, while girls showed a 5 times increase (Uden *et al*., 1988). In a series of hypermobile children, 33% had hernias, compared to 5% in a normal population (Friedman *et al*., 1993).

Bendavid (2004) proposed one single unified theory of hernia formation through reviewing progresses of herniation in multiple disciplines including anatomy, genetics, biochemistry, pathology and molecular biology. The theory is that the pathological changes in collagen set the stage for the development of a hernia. This new concept of hernia biology was also put forward by Jansen *et al.* (2004) (Figure 5).

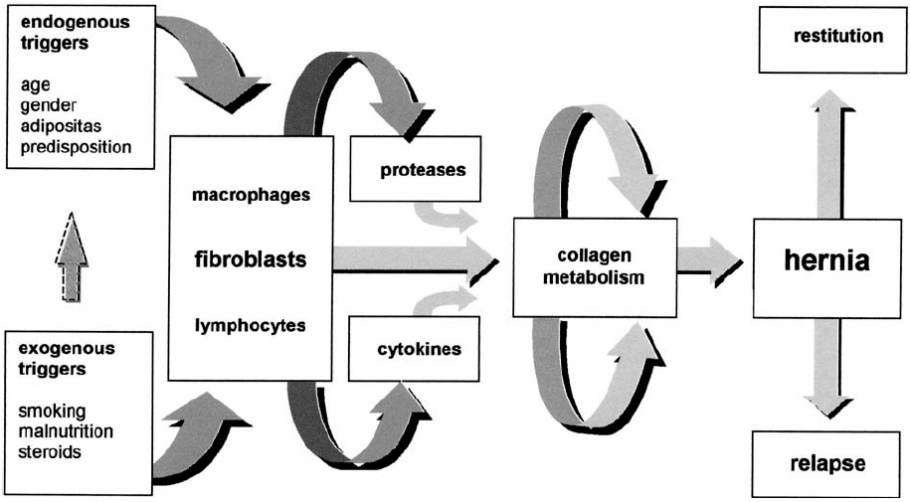

Figure 5. Pathogenesis of hernia formation. (Jansen *et al.*, 2004)

From above, the genes related to collagen metabolism, including collagen genes and genes of matrix metalloproteinases (MMPs) which degrade and remodel collagens can be considered as candidates for inguinal herniation.

3.3 Molecular progress for hernia inguinalis/scrotalis in pigs

In recent years, several groups have devoted to deciphering the genetic architecture of inguinal/scrotal hernia. Grindflek *et al*. (2006) detected 9 significant QTLs ($p<0.01$) on 8 porcine chromosomes in a population of 194 individuals including 103 affected sib pairs (ASP) through a genome-wide linkage analysis. And the effects of 6 genomic regions on 6 chromosomes were genome level significant ($p<0.01$) using transmission/disequilibrium test (TDT). Two significant regions ($p<0.01$) at SW963 on SSC5 and at SW1891 on SSC17 were significant convincing by both ASP test and TDT. Five regions located on SSC1, SSC2, SSC6, SSC15 and SSCX were found to be highly significant ($p<0.005$) by either the ASP test or the TDT. Moreover, six significant haplotypes ($p<0.01$) were constructed, of which one haplotype SW963-SWR1526 was transmitted to hernia pigs with four times higher frequency than to healthy pigs ($p<0.00005$).

Du *et al*. (2004) performed a whole-genome scan using 7 independent scrotal hernia-affected paternal families from 3 commercial pig lines, and identified 3 chromosomes with suggestive statistical evidence for segregation of scrotal hernia genes by an identity-by-descent based nonparametric linkage analysis. QTLs on SSC2 and SSC12 were confirmed by genotyping 27 additional paternal scrotal families for 33 microsatellites markers on the three chromosomes. The QTL regions were refined by identifying 137 polymorphic SNPs (107 on SSC2 and 30 on SSC12) and genotyping approximately 2000 pigs from 147 Pietrain paternal families (with at least 1 affected progeny or at least 80 progeny all absent of scrotal hernia)(Du, personal communication).

Pig Improve Company (PIC) had conducted a comprehensive gene discovery program to identify markers for scrotal hernia (Plastow *et al*., 2003). Using candidate gene approach (over 40 markers from more than a dozen genes were ultimately investigated) as well as a genome scan approach (using AFLP^{TM} and microsatellite markers), two markers in two different genomic regions were detected to have a strong association with the BLUP EBV system for scrotal hernia in one of the pure lines.

In our group, a genome-wide scan based on affected porcine half-sib families was performed. The pedigree consists of 84 families with a total of 512 animals including 81 boars, 156 sows and 275 affected piglets among them, was genotyped for 139 DNA-markers distributed across the 18 autosomes. Significant QTLs on chromosomes 3, 6, 7, 12 and 15 were mapped by non-parametric linkage analysis (Knorr *et al.*, 2006). Fine mapping is ongoing. A number of candidate genes for hernia inguianlis/scrotalis, including *INSL3*, *GUSB* and *CALCA* were also evaluated (Knorr *et al.*, 2002a, 2002b and 2004; Beck *et al.*, 2006).

4 The *SOX9* gene

The *SOX* (SRY-box) genes are developmental regulators which are characterized by the presence of a 79 amino acid high mobility group (HMG) DNA-binding domain with >50% homology to the sex-determining gene *SRY*. The *SOX* gene family can be further subdivided into twelve subgroups defined by additional homologies outside of the DNA-binding domain (Bowles *et al*., 2000). *SOX9* belongs to subgroup E and is well characterized. *SOX9* gene is involved in a wide range of developmental processes, including chondrogenesis，sex determination (Foster *et al.*, 1994; Wagner *et al*., 1994) and the development of the neural crest (Spokony *et al*., 2002) and the spinal cord glial cells (Stolt *et al*., 2003).

4.1 Biological/physiological function of SOX9

4.1.1 Function in the development of male gonad

As a target gene of SRY, *SOX9* plays key roles in regulating the male developmental pathway (Figure 6). There has been a substantial amount of evidence supporting an important role for *SOX9* in the sex determination, a process defined as the commitment of the indifferent gonad to a testis or an ovary. Huang *et al.* (1999) showed that a duplication of the genomic region containing the *SOX9* gene caused female-to-male sex reversal. Ectopic expression of *SOX9* in undifferentiated gonads induces testis development resulted in all XX transgenic mice (Vidal *et al*., 2001). Moreover, transgenic insertion of a tyrosinase minigene ~1 Mb upstream of the *SOX9* gene in Odsex mice was associated with XX male development lacking *SRY*, presumably due to the inactivation of a repressor element that normally inhibits SOX9 expression in ovaries (Bishop *et al*., 2000). In pigs, *SOX9* was down-regulated in XX gonads and up-regulated in XY from 28 dpc (days post coitum), the up-regulation of the *SOX9* gene was concomitant with appearance of differentiated Sertoli cells

(Parma *et al*., 1999). Pig SOX9 can also retroactivate the pig *SRY* promoter via a SOX9 binding site at position -205 from the ATG translational start site (Daneau *et al.*, 2002). These evidences show that the constitutive expression of *SOX9* in the dimorphic gonad appears to be required and sufficient to promote testis determination (Chaboissier *et al*., 2004; Qin *et al*., 2005).

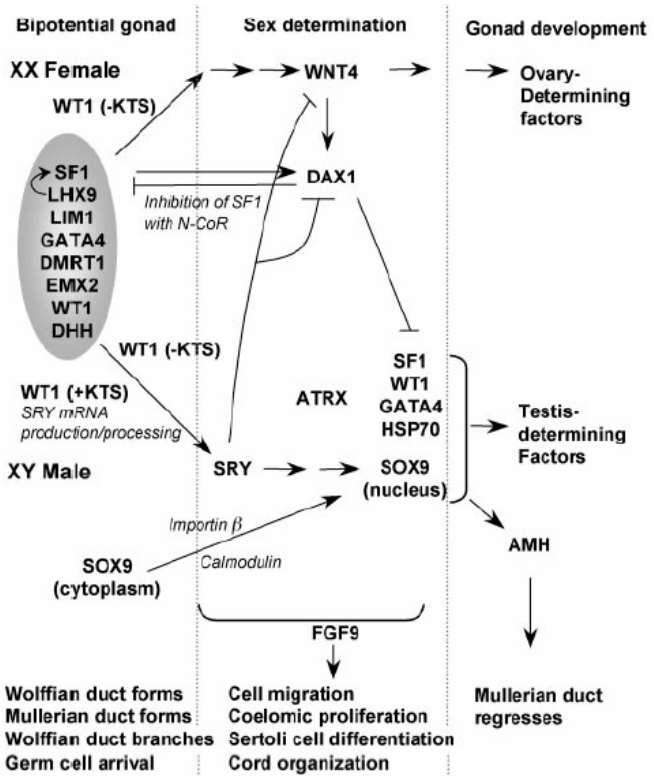

Figure 6. Model for initiation of sex determination (Harley *et al.*, 2005) .

SOX also acts an important role in sex differentiation. SOX9 can bind to the promoter region of the human anti- Müllerian hormone gene (*AMH*) which is responsible for regression of Müllerian ducts during male sex differentiation, and activates transcription of the *AMH* gene together with steroidogenic factor 1 (SF-1) (de Santa Barbara *et al*., 1998). AMH is an essential component of the male sexual differentiation pathway, secreted by Sertoli cells, causing the regression of the Müllerian ducts. *SOX9* is essential for *AMH* transcription. Mice with targeted

mutations in the SOX9 site within the *AMH* promoter result in a complete absence of *AMH* transcript and complete retention of Mullerian duct-derived organs (Arango *et al*., 1999).

In addition, it was speculated that *SOX9* has function on testicular descent via a cascade pathway despite no direct evidences were reported. It has been shown that SOX9 can up-regulate the expression of *SF-1* (Shen *et al*., 2002). SF-1 affects transcription of INSL3 by its binding within *INSL3* promoter (Zimmermann *et al*., 1998; Koskimies *et al*., 2002; Truong *et al*., 2003) and *INSL3* receptor *GREAT/LGR8* (G protein-coupled receptor affecting testicular descent/Relaxin receptor 8) (Adham *et al*., 2004). At last, *INSL3* and its receptor *GREAT/LGR8* act as the critical regulator of the gubernacular differentiation by a speculated involvement of the control of the collagen metabolism and reorganization of extracellular matrix (Adham *et al*., 2004). Furthermore, AMH plays a role in augmenting gubernacular growth (Kubota *et al*., 2002). Therefore, SOX9 is associated with male sex-determination pathway, testicular descent and collagen metabolism.

4.1.2 Function in collagen metabolism

It has been well established that *SOX9* has essential roles in successive steps of the chondrocyte differentiation (Akiyama *et al*., 2002 and 2004) and cartilage formation which is composed of collagen fibrils assembled from mature type II, type IX and type XI collagen molecules (Bell *et al*., 1997; Bi *et al*., 1999; Lefebvre *et al*., 1997). *SOX9* loss-of-function mutations cause the skeletal malformation syndrome campomelic dysplasia, a lethal skeletal malformation syndrome and XY sex reversal (Foster *et al*., 1994; Wagner *et al*., 1994). Haploinsufficiency of SOX9 results in defective cartilage primordia and premature skeletal mineralization (Bi *et al*., 2001). Expressions of the α1 chain of

type II collagen gene (*Col2a1*), collagen α2 (XI) gene (*Col11a2*) and type XXVII collagen gene (*Col27a1*) are cis-regulated by SOX9 through its interaction with the SOX9-binding site on the enhancer region (Bell *et al.*, 1997; Bridgewater *et al*., 1998; Jenkins *et al*., 2005).

4.1.3 Function in apoptosis

As a transcription factor with a crucial role in normal development, SOX9 induces genes involved in cellular differentiation, resulting in the formation of mature cells susceptible to senescence and apoptosis. Until now, accumulated evidences suggest that SOX9 controls cell apoptosis. Akiyama *et al*. (2002) performed an elaborate experiment and proved SOX9 controls, either directly or indirectly, anti-apoptotic molecules such as *Noggin* and *Chordin* that inhibit signals (e.g. BMPs) responsible for formation of interdigital spaces. Inactivation of *SOX9* in $SOX9^{flox/flox}$ mice, *Prx1-Cre* limb mesenchyme results in markedly increase apoptosis, an increased expression of *Bax*, and an increase in cleaved caspase 3 production.

Drivdahl *et al*. (2004) showed that SOX9 acts as a tumor suppressor in M12 prostate cancer cells by inhibiting proliferation through causing cell cycle arrest in G0/G1and increasing sensitivity to apoptosis. In the colon carcinoma cell line HT29C1.16E, SOX9 down-regulated the carcinoembryonic antigen (CEA), a tumor marker that is up-regulated in many types of human cancers, and up-regulated CEACAM1 which is a human tumor suppressor (Jay *et al.*, 2005; Zalzali *et al.*, 2006). The function of *SOX9* on apoptosis may depend on the context of cells. Further evidences are required.

4.2 Molecular structure and features of the *SOX9* gene

Human *SOX9* had been localized to 17q24.3-q25.1 (Tommerup *et al*., 1993). While the porcine *SOX9* gene was assigned to SSC12p13-11 by porcine somatic

cell hybrid panel analysis (Lahbib-Mansais *et al.*, 1997), this region is homologous to human *SOX9* location. The human *SOX9* gene contains three exons and encodes a protein of 509 amino acids (Wagner *et al*., 1994). An analysis of the structure and function of the mouse *SOX9* promoter identified a proximal promoter region spanning from -193 to -73 bp, which is in part responsible for the sex- and tissue-specific expression of the SOX9 gene (Kanai *et al*., 1999). This proximal promoter region is moderately conservative between mouse and human, and contains several conserved regulatory elements, including CCAAT box, GATA and CREB binding sites (Colter *et al*., 2005). Moreover, the spatiotemporal expression pattern of *SOX9* is regulated by a complex set of widely spaced tissue-specific enhancers, located in the immediate vicinity of the transcription start site , up to 251 kb 5' and up to 95 kb 3' to *SOX9* (Bagheri-Fam *et al*., 2006). Morishita *et al.* (2001) also identified a 30-bp element in the first intron of human SOX9 gene acting as an enhancer.

Three functional domains are recognized for SOX9 so far, a high-mobility group (HMG) DNA-binding domain, a C-terminal transactivation domain and a DNA-dependent dimerization domain (Sudbeck *et al*., 1996; Sock *et al*., 2003). The sequence-specific DNA binding, DNA bending, and transactivation properties of SOX proteins suggest that these proteins act as transcription factors with characteristics of both classical transcription factors and architectural chromatin factors (Bell *et al*., 1997; Lefebvre *et al*., 1997). Moreover, SOX9 may have an additional function during pre-mRNA splicing (Ohe *et al*., 2002). In vitro studies demonstrate that the HMG domains of all SOX proteins tested to date bind with high affinity to a consensus DNA sequence (A/T A/T CAA A/T G) (Denny *et al*., 1992). Several investigators have suggested that SOX proteins bind to this core motif with different affinities, which is determined by sequences adjacent to the core motif (Collignon *et al*., 1996; Kamachi *et al*., 1999; Mertin *et al*., 1999).

Mertin *et al*. (1999) showed the 5'AG and 3'GG flanking nucleotides enhance the affinity of SOX9 HMG domain. It has been known that mutations in the DNA-binding or transcriptional-activation domain of *SOX9* can cause campomelic dysplasia (Foster *et al*., 1994; Wagner *et al*., 1994). The second domain essential for SOX9 function is a proline/glutamine/serine (PQS)-rich C-terminal transcription-activation domain. The potency of this domain is enhanced by a proline/glutamine/alanine (PQA)-rich motif which is unable to activate transcription alone. The SOX9 protein is known to activate transcription of the type II collagen gene and anti-Müllerian hormone gene. The third domain was just recently characterized as a dimerization domain in a conserved region immediately preceding the HMG domain (Sock *et al*., 2003).

5 Aims of the thesis

The objectives of this thesis were:

- To identify QTLs affecting pig teat number in a White Duroc×Erhualian resource population using genome-wide microsatellite scanning.
- To characterize the porcine *SOX9* gene as a positional candidate gene for hernia inguinalis/scrotalis, evaluate the association of its genetic variation with pig inguinal/scrotal hernia, and characterize the probable molecular mechanism underlying the genetic effects.

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Acknowledgements

It is my proud privilege to thank all the people who meticulously supervised me and kindly help me during my PhD study at the Institute of Veterinary Medicine (IVM) in the Faculty of Agricultural Sciences, Georg-August-University of Göttingen.

I would like to deliver my deepest sense of gratitude and indebtedness to **Prof. Dr. Dr. Bertram Brenig**, an excellent supervisor and Head of IVM, for his greatest guidance, constructive suggestion and continuous support that allowed me to have the great opportunity to work in the IVM and conduct my doctorate successfully. I am highly thankful to my co-supervisor **Prof. Dr. Christoph Knorr** for his affectionate counselling, splendid pilot to the fascinating field of molecular genetics and kindest help whenever I needed during all these years. I also want to deliver my sincere thanks to my co-supervisor **Prof. Dr. Lusheng Huang** (Jiangxi Agricultural University (JXAU), China) for his elaborate organization that made me fulfilled part work of the thesis in JXAU, and his valuable direction and encouragement.

I specially want to thank **Prof. Dr. Henner Simianer** and **Prof. Dr. Dr. Matthias Gauly** for kindly being my co-examiner.

I owe sincere appreciations to my good friend **Dr. Bernhard Baumgartner** for his kind help on expression work, thoughtful comments and enthusiasms always available to answer my questions and bring fun to my life in Göttingen. I also want to heartily thank **Miss Julia Beck** for her great help especially at the beginning of my study, for her excellent *FISH* analysis and beneficial guidance on computer technology. I would like to express my thanks to **Viola Raupach** and **Susen Lattermann** for their technical assistance. Many thanks are also due to **Dr. Alexandra Baumgartner, Dr. Ekkehard Schütz** and other graduate students **Christian Beuermann**, **Monique Germerodt**, **Rifat Morina**, **Claudia Floren** and other colleagues **Sara Henneke** and **Stefan Balzer** for their assistance during my research. Moreover，I also want to express my sincere thanks to **Miss Joana Luis Armada Bras** for her kind help on EMSA and expression work.

I am very grateful to my Chinese colleagues at JXAU **Dr. Jun Ren**, **Mrs. Jun Gao** and **Mr. Yuanmei Guo** for their help on the part work of this thesis in China.

Finally, I would like to devote my deep thanks to **my large family** for their eternal support and immense encouragement. I would greatly appreciate my fiancée **Shuting Zhu** for her unfailing soulful love that gave me the constant inspiration to fulfill my PhD study.

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