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Genetic analysis and expression study of candidate genes affecting leg weakness-related traits in pigs

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“*To my dear parents and family,*

แด่พ่อแม่ที่รักและครอบครัว,

Zu Meinen lieben Eltern und Familie”

-

“*To my respective teachers and lovely friends,*

แด่ครูอาจารย์ที่เคารพ และเพื่อนที่รัก,

Zu Meinen jeweiligen Lehern und liebe Freunde ”

Genetische Analyse und Expressionstudie zu Kandidatengenen, die die Merkmale der Beinschwäche beim Schwein beeinflussen

Beinschwäche Probleme sind von großer Bedeutung in der Schweinezucht Industrie, besonders im Bezug auf den Tierschutz. Diese Studie wurde durchgeführt, um chromosomale Regionen und Kandidatengene, die die Beinschwäche-Merkmale beim Schwein beeinflussen, zu identifizieren. Es wurden insgesamt 310 F_2 Schweine aus einer Duroc×Piétrain Ressourcen-Population mittels 82 genetischer Marker genotypisiert. Sowohl Vorder- und Hinterbeine als auch die Füße der Schweine wurden nach den Deutschen Standardsystem für die Lineare Fundamentbeurteilung bewertet. Der Osteochondrose (OC) Status wurde histologisch am Kopf und Condylus Medialis des linken Femurs sowie Humerus bewertet. Die Merkmale für die Knochenmineraldichte (BMD) wurden an Ulna und Radius mit Hilfe der Dual-Röntgen-Absorptiometrie gemessen. Insgesamt wurden 16 QTLs auf 10 Autosomen für die Beinschwäche-Merkmale identifiziert. Alle QTLs hatten ein chromosomweites Signifikanznivea von 5% bzw. erreichten die QTLs auf SSC2 und 3 für BMD und OC am Kopf des Humerus ein genomweites Signifikanz von ebenfalls 5%. Dieses Ergebnis eignet sich für weitere Untersuchungen. Gene, die bekannt dafür sind, während der endochondralen Ossifikation eine signifikante Rolle zu spielen, wurden als funktionelle beziehungsweise positionelle Kandidatengene für diese Studie ausgewählt. Signifikante Assoziationen wurden zwischen dem SNP von *MMP3* (g.158 C>T) und der OC Gruppe am Femurkopf ($P<0,05$) sowie der BMD Gruppe ($P<0,05$) in der DuPi Population festgestellt. Die Gelenkknorpel von 12 Kastraten (6 Kranke und 6 Gesunde, wobei jeweils ein krankes und ein gesundes Tier ein Vollgeschwister-Paar bildeten) wurden für die Genexpressionsstudie verwendet. Die Gene *TGFβ1* und *RUNX2* wurden im Vergleich zu gesunden Knorpeln verstärkt in den OC ($P<0,05$) exprimiert. Dagegen war die Expression von *MGP* bei den OC geringer. Bei der Expression von *COL2A1*, *MMP9* und *SOX9* konnte kein Unterschied zwischen den beiden Gruppen festgestellt werden. Zudem war die Expression des MGP Proteins beim Western Blot ebenfalls geringer in der OC Gruppe. Für MGP konnte eine CpG-Region im Promotor identifiziert werden. Dabei zeigte sich, dass die DNA-Methylierung von drei CpG Bindungsstellen in der OC Gruppe im Vergleich zu der Gruppe mit gesunden Knorpeln höher war. Bei der Immunfluoreszenzuntersuchung von normalen Gelenkknorpeln, die von Schweinen unterschiedlichen Alters stammten, war das MGP Signal am stärksten bei jungen Schweinen und nahm mit zunehmendem Alter ab. Die MGP Proteinexpression war um die Knorpel Kanäle am höchsten. Diese Ergebnisse zeigen, dass das *MGP* Gen ein potentielles Kandidatengen für die Entwicklung von OC bei Schweinen sein könnte. Zusammenfassend lässt sich sagen, dass die Ergebnisse Informationen zu den QTL Regionen und den Kandidatengenen für Beinschwäche-Merkmale beim Schwein liefern.

Genetic analysis and expression study of candidate genes affecting leg weakness-related traits in pigs

Leg weakness (LW) problems are a great concern in the pig industry especially with regard to animal welfare. The present study was carried out to identify chromosome regions and candidate genes affecting LW-related traits in pigs. A total of 310 F_2 pigs from a Duroc×Pietrain resource population were genotyped using 82 genetic markers. Front and rear legs and feet of pigs were scored according to the German standard scoring system. Osteochondrosis (OC) was histologically scored at the head and condylus medialis of the left femur and humerus. Bone mineral density (BMD) traits were measured in the whole ulna and radius bones using dual energy X-ray absorptiometry. A total of 16 QTL were identified for LW-related traits on ten porcine autosomes. All QTL reached the 5% chromosome-wide significance level. QTL on SSC2 and SSC3 for bone mineral content (BMC) and OC at head of humerus, respectively, reached the 5% genome-wide significance level which is worthwhile for further investigation. Genes known to play significant roles in endochondral ossification were selected as functional/positional candidate genes in this study. Significant association was found between SNP of *MMP3* (g.158 C>T) with OC at head of femur (*P*<0.05) and BMD (*P*<0.05) in the DuPi population. Articular cartilages from 12 castrated males (6 were healthy and 6 were diseased, and each healthy and disease pairs were full-sibling used for genes expression study. *TGFβ1* and *RUNX2* genes were higher expressed in OC (P<0.05) compared to healthy cartilage. On the other hand, *MGP* was lower expressed in OC but the expression of *COL2A1*, *MMP9* and *SOX9* mRNA abundance was not different. Moreover, the expression of MGP protein was lower (*P*<0.05) in the OC group when quantified by Western blot. One CpG region was identified in *MGP* promoter and DNA methylation of three CpG sites were higher in OC compared with normal cartilage. Immunofluorescence of normal articular cartilage collected from pigs at different ages revealed that MGP signals were higher in younger pigs and decreased in the older pigs. The MGP protein was expressed more near to the cartilage canals. These results suggest that the *MGP* gene might be a potential candidate gene for the development of OC in pigs. In summary, the results provided information on QTL regions and candidate genes for leg weakness-related traits in pigs.

Contents Pages

Abstract		III
List of abbreviations		VIII
List of tables		X
List of figures		XI
1	Introduction	1
2	Literature review	3
2.1	Leg weakness in pigs	3
2.2	Feet and leg scores	4
2.3	Osteochondrosis	5
2.4	Bone mineral density and content	9
2.5	Quantitative trait loci analysis for leg weakness-related traits	9
2.6	Candidate genes for leg weakness-related traits	10
2.6.1	Transforming growth factor beta 1 gene	14
2.6.2	Collagen type II alpha1 and collagen type X alpha1 genes	14
2.6.3	Matrix metallopeptidase 3 and 9 genes	16
2.6.4	Runt-related transcription factor 2 and sex determining region Y-box 9 genes	16
2.6.5	Matrix gla-protein gene	17
3	Materials and Methods	
3.1	Materials	18
3.1.1	Design of the study	18
3.1.2	Experimental animals	19
3.1.2.1	F_2 Duroc × Pietrain (DuPi) resource population	19
3.1.2.1.1	Feet and leg scores	20
3.1.2.1.2	Histology of articular cartilage for OC lesion observation	20
3.1.2.1.3	Bone mineral density traits	22
3.1.1.2	Animal for association analysis	23
3.1.3	Chemicals and equipments	24
3.1.3.1	Chemicals and kits	24
3.1.3.2	Reagents	25
3.1.3.3	Media	28

3.1.3.4	Enzymes	28
3.1.3.5	Equipments	29
3.1.3.6	Software used	29
3.2	Methods	
3.2.1	DNA isolation from cartilage and muscle tissues	30
3.2.2	DNA isolation from agarose gel	30
3.2.3	RNA extraction from articular cartilage	31
3.2.4	cDNA synthesis	32
3.2.5	Amplification of 3´ and 5´ ends of *MGP* by RACE PCR	32
3.2.6	PCR conditions	33
3.2.7	Protein extraction from articular cartilage	33
3.2.8	Ligation and transformation	33
3.2.9	Sequencing	34
3.2.10	Genomic bisulfite PCR and sequencing	35
3.2.11	Screening for polymorphic sites	35
3.2.12	Genotyping	35
3.2.13	Marker data for QTL analysis	38
3.2.14	Western blot	38
3.2.15	Immunofluorescence	39
3.2.16	Promoter prediction, CpG island identification of porcine *MGP* gene	40
3.3	Statistical analysis	40
3.3.1	QTL mapping	40
3.3.2	Association analysis	42
4	Results	
4.1	Phenotype characteristics of the DuPi resource population	43
4.1.1	Leg and feet scores	43
4.1.2	Osteochondrosis scores	44
4.1.3	DXA traits	44
4.2	Phenotype characteristic of the animals used for association analysis	48
4.3	QTL for leg weakness-related traits	50
4.3.1	QTL for leg and feet scores	50
4.3.2	QTL for osteochondrosis scores	50
4.3.3	QTL for bone density traits	50

4.4	Gene characterization and detection of polymorphisms	52
4.4.1	Collagen type II alpha I	52
4.4.2	Collagen type X alpha I	53
4.4.3	Matrix gla protein	54
4.4.4	Matrix metallopeptidase 3	55
4.4.5	Transforming growth factor beta 1	55
4.5	Association of candidate genes with leg weakness-related traits	56
4.6	Expression of candidate genes in OC cartilage	60
4.7	Molecular characterization of the porcine *MGP* gene	62
4.7.1	Characterization of the porcine *MGP* gene	62
4.7.2	MGP expression in healthy and osteochondrosis cartilage	63
4.7.3	MGP localization in articular cartilage	65
4.7.4	DNA methylation pattern of the *MGP* promoter	65
5	Discussion	
5.1	Characterization of traits related to leg weakness in pigs	68
5.2	QTL for leg weakness related traits	69
5.3	Association of candidate genes with leg weakness-related traits	72
5.4	Gene expression in porcine articular cartilage with and without OC lesion	74
5.5	Molecular characterization of the porcine *MGP* as a candidate gene for OC	76
6	Summary	79
7	Zusammenfassung	81
8	References	83

List of abbreviations

A	: Adenine
BMA	: Bone mineral area
BMC	: Bone mineral content
BMD	: Bone mineral density
bp	: Base pairs
BSA	: Bovine serum albumin
C	: Cytosine
COL2A1	: Collagen type 2 alpha 1
COL10A1	: Collagen type 10 alpha 1
cM	: Centimorgan
ddH_2O	: Distilled & deionized water
ddNTP	: Dideoxyribonucleoside triphosphate
DMSO	: Dimethyl sulfoxide
DNA	: Deoxynucleic acid
dNTP	: deoxyribonucleoside triphosphate
	(usually one of dATP, dTTP, dCTP and dGTP)
DTT	: Dithiothreitol
DXA	: Dual energy x-ray absorptionmetry
E.coli	: *Escherichia coli*
EDTA	: Ethylenediaminetetraacetic acid (powder is a disodium salt)
EtBr	: Ethidium bromide
EtOH	: Ethanol
FBAT	: Family based association tests
G	: Guanine
HWE	: Hardy Weinberg equilibrium
IGF2	: Insulin-like growth factor 2
IPTG	: Isopropylthio-ß-D-galactoside
ISH	: In situ hybridization
kb	: Kilobases
$MgCl_2$	: Magnesium chloride
MGP	: Matrix gla protein

MMP3	: Metalloproteinase 3
MMP9	: Metalloproteinase 9
mRNA	: Messenger RNA
MW	: Molecular weight
NaCl	: Sodium chloride
OC	: Osteochondrosis
PAGE	: Polyacrylamide gel electrophoresis
PCR	: Polymerase chain reaction
QTL	: Quantitative trait loci
RACE	: Rapid amplication of cDNA end
RFLP	: Restriction fragment length polymorphism
RNA	: Ribonucleic acid
rpm	: Revolutions per minute
RUNX2	: Runt-related transcription factor 2
SDS	: Sodium dodecyl sulfate
SNP	: Single nucleotide polymorphism
T	: Thymine
TAE	: Tris-acetate buffer
TBE	: Tris- borate buffer
TE	: Tris- EDTA buffer
TGFβ1	: Transforming growth factor beta 1
UTR	: Untranslated region
X-gal	: 5-Bromo-4-chloro-3-indolyl-beta-D-galactoside

List of tables

Table 1: QTL for leg weakness-related traits in different pig breeds 11

Table 2: Descriptive statistics of performance and carcass traits of the DuPi resource population 19

Table 3: Scoring criteria for legs and feet scores 21

Table 4: Number and percentage of osteochondrosis scores at different joints 23

Table 5: List of primers name, sequences and their applications in this study 36

Table 6: List of primers and enzymes used for PCR-RFLP analysis 37

Table 7: Numbers and percentage of animals at different leg and feet and osteochondrosis scores 45

Table 8: Statistics and phenotype correlation between leg and feet and OC scores 46

Table 9: Descriptive statistics of BMD, BMC and BMA of the DuPi resource population 46

Table 10: Effect of production and carcass traits on leg and feet scores, OC score and DXA traits 46

Table 11: Rotated factor pattern and final communality estimates from principal component analysis of leg weakness-related traits 48

Table 12: Number of the animals at different OC scores and different joints positions 49

Table 13: Location and estimated effected of the identified QTL for leg weakness-related traits 51

Table 14: List of detected SNP in porcine *COL2A1* gene 53

Table 15: Allele and genotype frequencies and Hardy Weinberg equilibrium of three SNPs in the *MMP3*, *COL10A1* and *TGFβ1* genes 58

Table 16: Association of *MMP3* g.158 C>T with bone density traits in DuPi resource population 58

Table 17: Association of the analyzed SNPs of the *MMP3, COL10A1* and *TGFβ1* Genes with leg weakness related traits 59

Table 18: The association analyses between SNP of *TGFβ1* and principal components 60

Table 19: Description of exon and intron boundary of porcine *MGP* gene 62

List of figures

Figure 1:	Example of feet evaluation in pigs	4
Figure 2:	Example of front and rear leg evaluation in pigs	5
Figure 3:	Osteochondrosis lesion in articular cartilage of pigs	6
Figure 4:	Causal diagram showing likely etiologic factors of osteochondrosis and how they may influence the development of the disease	7
Figure 5:	Histological image of porcine articular cartilage with salfanin-O and Masson-goldner staining	12
Figure 6:	Cellular events and molecular markers of chondrogenesis, chondrocyte differentiation, and articular cartilage development and maintenance	13
Figure 7:	Different types of collagen molecule in articular cartilage	15
Figure 8:	Schematic display of the research design used in this study	18
Figure 9:	Sample of histological template for the evaluation of OC score	22
Figure 10:	Sample of PCR-RFLP for genotyping *COL2A1* and *MMP3* genes	37
Figure 11:	Evidence of QTL for leg weakness related traits on SSC2 and 3	52
Figure 12:	Porcine *COL2A1* gene structure and position of the identified SNP	53
Figure 13:	Porcine *COL10A1* gene structure and position of the identified SNP	54
Figure 14:	Porcine *MGP* gene structure and position of the identified SNP	54
Figure 15:	Porcine *MMP3* gene structure and position of the identified SNP	55
Figure 16:	Porcine *TGFβ1* gene structure and position of the identified SNP	56
Figure 17:	Relative expression of *MMP3, TGFβ1, COL10A1* and *MGP* genes in healthy and osteochondrosis cartilage	61
Figure 18:	Relative expression of *COL2A1*, *MMP9*, *SOX9* and *RUNX2* in healthy and osteochondrosis cartilage	61

Figure 19:	A comparison of the peptide sequence of the porcine MGP protein with the peptide of the human bovine, rat and mouse	63
Figure 20:	Expression analysis of *MGP* gene and protein in articular cartilage	64
Figure 21:	Immunolocalization of MGP protein in articular cartilage of 1-day, 2-month and 6-month olds pigs	66
Figure 22:	Porcine *MGP* gene structure and DNA methylation percentage of *MGP* promoter	67

1 Introduction

Interest in animal welfare continues to increase throughout the world. Health problems of the animal affect their well-being and cause considerable economic losses for farmers. Leg weakness (LW) is one of the health problems commonly observed in pig farms. LW can occur at any age of pigs, but severe signs usually occur in sows and boars. Pigs that face LW problem will be culled earlier in the selection process from the breeding herd. Therefore, pig breeders are including factors that affect leg soundness into their breeding programs. A number of traits can be used to evaluate or predict LW in pigs. Leg and feet scores refer to the structure and the overall soundness of both front and back of the legs and feet. Furthermore, accumulating data suggest that the primary cause of LW in pig is osteochondrosis (OC). OC is a common and clinically important joint disorder not only in pigs but also in other domestic animals such as horse, dog and chicken. The pathogenesis of OC is still not fully understood and believed to be multi-factorial, including environmental, nutritional, metabolic and genetics factors. However, genetics seem to be the main predisposition factor towards OC. Selecting pig considering OC therefore might be helpful to decrease the incidence of leg weakness in pigs. Moreover, other determinants or predictors that can be used to estimate the fracture risk of the bone and the straightness or the weakness of the legs are bone mineral density traits. Low bone density and content can lead to loss of soundness of the limb bone.

The effects of different factors, such as nutrition and management, were elucidated and described in addition; genetic is reported to be one of the important factors for LW. However, the genetic mechanisms underlying all these leg weakness-related traits are complex and not easy to understand. Therefore, studies on the molecular genetic mechanisms of traits that cause of leg weakness are important and challenging. So far, limited research has been applied to molecular genetic approaches for identifying the genes responsible for leg weakness-related traits in pigs. The nature of the genes affecting leg weakness-related traits is still largely unknown. Quantitative trait loci (QTL) studies for leg weakness-related traits using cross breds between exotic and commercial pig breeds are reported. Many candidate genes involved in the pathways that regulate chondrocyte behavior during endochondral ossification were identified.

However, only a limited number of reports is available confirming the function and the association of these candidate genes with leg weakness in pigs. Despite a number of factors is known to influence the development of leg weakness or OC specially body and leg structure of the animal, growth rate, mechanical stress and body weight. Using an intercross between the fast growing breeds such as Duroc and Pietrain could considered as a good model for QTL study concerning traits related with leg weakness. To gain more knowledge on genes underlying leg weakness-related traits and to identify the chromosome region linked with these traits, the present study therefore aimed to:

1. Localize quantitative trait loci (QTL) influencing leg weakness related traits in F_2 Duroc × Pietrain resource population.
2. Identify single nucleotide polymorphisms of positional/functional candidate genes and evaluate their association with leg weakness-related traits.
3. Investigate the expression of candidate genes to detect the potential differences expression between healthy and OC cartilage.
4. Perform the molecular characterization of matrix gla protein gene (*MGP*) as a functional candidate gene for OC in pigs.

2 Literature review

2.1 Leg weakness in pigs

The leg weakness problem has become an important issue in all commercial animals including chicken, cattle, goat as well as in pigs. Leg weakness reduces animal well-being and causes economic losses in all production stages. An evidence to support this is that between 20 to 50% of boars completing performance test have to be rejected from the breeding herd due to leg weakness problems (Lee et al. 2003, Webb et al. 1983). Due to the fact that affected animals might not be able to access food and water lead to a decrease in productivity and performance. Many factors such as physiological, biochemical, nutritional, environmental effects and genetics may be involved in the development of leg weakness. Research has highlighted the association between the increased incidance of leg weakness and the large improvements in production traits, such as high growth rates and carcass composition (Serenius et al. 2004, Stern et al. 1995). In addition, genetic correlations between leg weakness related traits and longivity of breeding sows are reported (Jorgensen 2000, Rothschild et al. 2007).

To date, the genetics underlying or causing leg weakness are still not clear. The heritability estimates for leg weakness from sires of Duroc, Landrace, and Yorkshire were 0.23, 0.30 and 0.39, respectively (Huang et al. 1995). In Landrace and Large White sows, the heritability for overall leg score is estimated to be between 0.27 and 0.38, respectively (de Sevilla et al. 2009). Duroc pigs were reported to have more problems of structure in the front legs than in the rear legs (Goedegebuure et al. 1988). Selection of Duroc pigs with different degrees of leg weakness was found to be associated with changes in front-leg bone and other factors such as muscle lengths and weights (Draper et al. 1992). Despite the potential influence of genetic factor in leg weakness, little is known of the genetics of those traits that are directly or indirectly associated with leg weakness. Therefore, studies on the genetic basis of these traits will be advantageous and may help to select pigs with reduced leg weakness problems.

2.2 Feet and leg scores

Besides body condition score, two of the most important traits used for evaluating feet and leg health status of the animals are the feet and leg scores. The scoring is usually done by visual evaluation of boars or sows that will be used for breeding proposes. Examples of feet and leg evaluation from the National Swine Improvement Federation (NSIF) are shown in Figure 1 and 2. Desirable and undesirable feet are indicated in Figure 1. The system for evaluating front and rear leg structure is indicated in Figure 2. The main propose of evaluating feet and legs is to decrease the contributing factor that could lead to defects in locomoting ability. If the animals show bad feet and leg scores, this may result in a high number of piglets having leg weakness problems. Therefore, leg and feet scores have been considered as common traits in conventional breeding approaches. The effect of leg conformation on survivability and performance traits has been subjected to intensive research in sows, as it also plays a role in sow longevity (de Sevilla et al. 2008, de Sevilla et al. 2009, Serenius et al. 2004). Nevertheless, studies related to the genetics of overall or specific feet and leg conformation defects in growing and finishing pig are very limited.

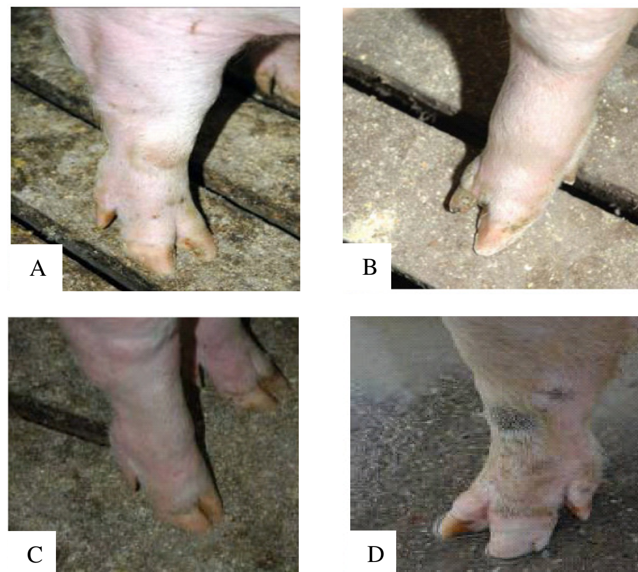

Figure 1: Example of feet evaluation in pigs (A) ideal toe size and spacing (B) uneven toe size and spacing (C) small foot size (D) cracked hooves. (Adapted from the national pork boad)

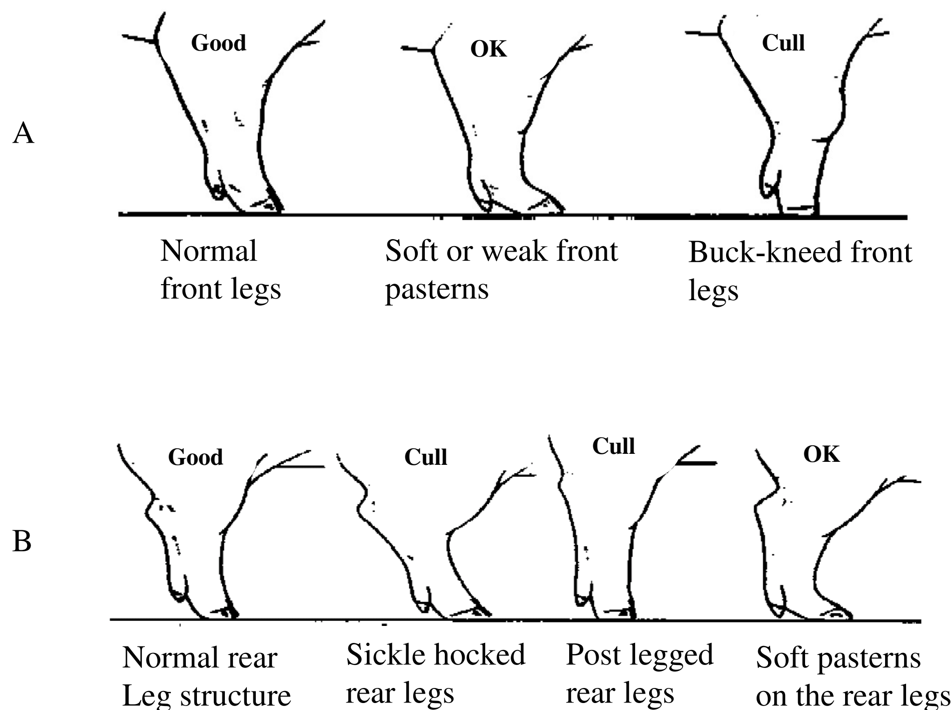

Figure 2: Example of front and rear leg evaluation in pigs (A) front leg side view (B) rear leg side view (Adapted from the national pork board)

2.3 Osteochondrosis (OC)

OC is referred as a cause of leg weakness in pigs (Jorgensen 2000, Stern et al. 1995, Ytrehus et al. 2007). It is a common joint disorder found in pig herds. A sample of OC lesions is shown in Figure 3. OC leads to economic losses due to culling of pigs at many stages of production (Guo et al. 2009, Jørgensen 2000, Kadarmideen et al. 2004). The disease occurs with high frequency in growing pigs of all commercial breeds (Jorgensen and Nielsen 2005, Lee et al. 2003). The heritability estimates for OC were 0.08 to 0.39 (Jorgensen 2000), 0.1 to 0.5 (Lundeheim 1987, Stern et al. 1995) and 0.06 to 0.42 (Kadarmideen et al. 2004) in different breeds. Moreover, OC is also reported to have negative effects on important performance traits such as sow longevity, growth and feed conversion (de Sevilla et al. 2008, de Sevilla et al. 2009, Kadarmideen et al. 2004, Stern et al. 1995).

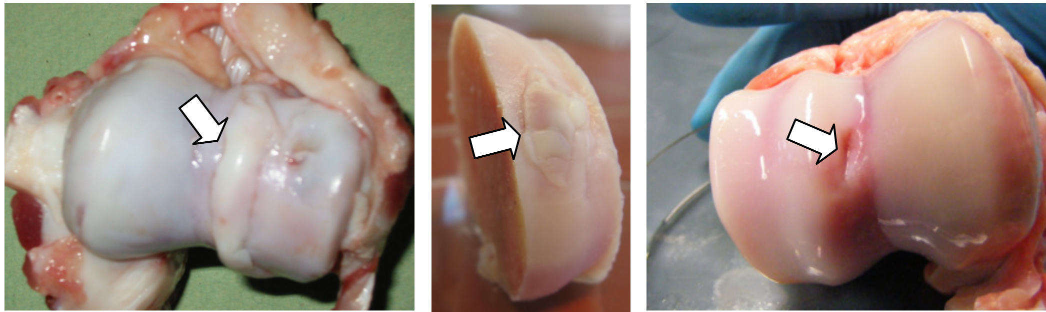

Figure 3: Osteochondrosis lesions (arrows) in articular cartilage of pigs

The term OC has been previously well described and includes three stages: the modifiers latent (lesion confined to epiphyseal cartilage), OC manifesta (lesion accompanied by delay in endochondral ossification), and OC dissecans (cleft formation through articular cartilage) (Ytrehus et al. 2007). The etiology and pathogenesis of OC is not yet clarified. It is characterized by disturbed bone formation, cartilage retention or necrosis of the cartilage canal of articular cartilage (Jorgensen 2000, Kadarmideen et al. 2004). Abnormal vascularisation or the focal failure of blood supply to the growth cartilage, leading to a focal disturbance of endochondral ossification, was proposed as one of the main causes (Ytrehus et al. 2007). The presence of necrotic areas with degenerating vessels in OC cartilage canals also suggests that any defect in cartilage canal at focal sites might lead to the disruption of blood supply to the growing cartilage, that contribute to OC development (Carlson et al. 1991). The pathogenesis model (Figure 4) of OC has been previously descript (Ytrehus et al. 2007). This model includes the fragility of cartilage and/or bone, primary dyschondroplasia, necrosis of sub-chondral bone and ischemic necrosis of growth cartilage. The authors suggested that the primary event in OC is the cessation of cartilage canal blood supply that occurs as a result of damage to temporal and newly formed vessels. In addition, the early lesions (subclinical) of OC are characterized by areas of chondronecrosis that are also closely associated with necrotic, nonperfused vessels in cartilage canals. Nutrition of the fetal cartilaginous precursors of the skeleton takes place through cartilage canals passing through the cartilage matrix. During the process of endochondral ossification, the cartilage canal layer at the articular side becomes thinner to convert into epiphyseal

bone and into the final articular cartilage layers in the mature animal (van Weeren 2006).

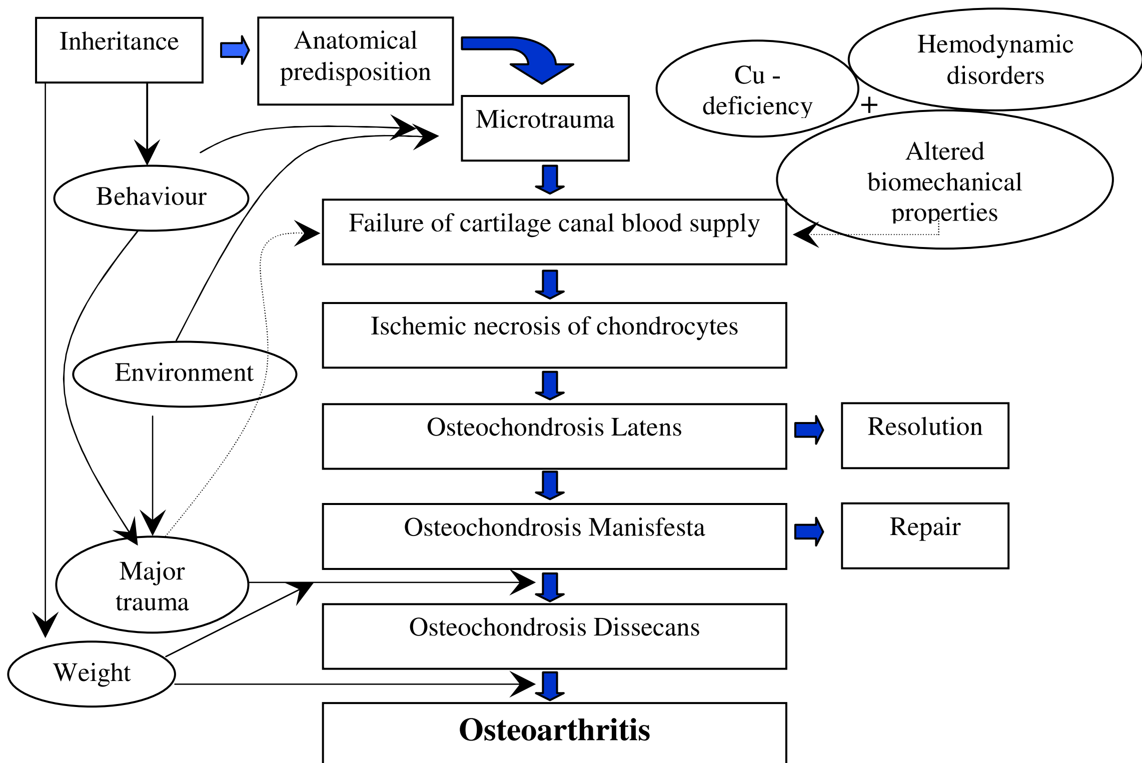

Figure 4: Causal diagram showing likely etiologic factors of osteochondrosis and how they may influence the development of the disease. The factors playing the most important role in primary osteochondrosis are emphasized with bold text boxes and solid arrow lines, whereas factors that may play a role in secondary osteochondrosis are depicted with dotted arrow lines (Adapted from Ytrehus et al. 2007)

As OC progresses, structures in the articular cartilage joint are degenerated. Therefore, many researchers have focused their studies on molecular mechanisms that are altered between normal and degenerated lesion region of articular cartilage. Although articular cartilage does not contain blood vessels, prior to the formation of a secondary ossification centre, temporary vessel-containing structures, ‘cartilage canals’, are found within the growing cartilage, which are involved in the nourishment of the cartilage for an appropriate ossification process (Blumer et al. 2004). The typical lesions of articular

osteochondrosis in pigs were found in the medial aspect and the sagittal ridge of the distal condyle of the humerus and the medial condyle of the femur (Ytrehus et al. 2007). Jorgensen (2000) stated that OC lesions in the elbow joint have greater influence on animal welfare than lesions in the knee joint, and lesions in the elbow joint show the highest correlations with clinical leg weakness (Jorgensen 2000). The OC scores in the knee joint were found to be higher than in the other joints (Stern et al. 1995). The primary changes of this condition were described as early as 2 weeks after birth (Hill et al. 1990) but are commonly observed by the time when pigs reached an age of 2 months (Ytrehus et al. 2007). However, most of the studies on osteochondrosis in pigs were performed as evaluation of lesions in slaughtered pigs (100-120 kg live weight), thus, they described the frequency of manifest lesions (Ytrehus et al. 2004a).

Prevalence of osteochondrosis in purebred Duroc sires and Landrace x Yorkshire sows have been reported for Denmark (Jorgensen 2005). It was found that the pathological abnormalities on the joint surface of the medial humeral condylus were 38.0% of 9,360 pigs and 11.7% suffered from osteochondritis dissecans. This result was similar to the earlier study in Danish Landrace boars (Jorgensen et al. 1995) and Norwegian Landrace x Yorkshire crossbred pigs (Ytrehus et al. 2004). In addition, many researches have found a different frequency between sexes for osteochondrosis, in which castrated male shows higher OC frequency than female pigs (Kadarmideen et al. 2004, Ytrehus et al. 2004). However, sex differences for OC are not clarified, since some authors reported no difference between castrated male and female pigs (Jorgensen et al. 1995). Additional factors that contribute to OC may include external factors such as nutrition and management and internal factors such as anatomic characteristics, biomechanical influences, trauma, failures of vascularization as well as genetics (Figure 4). As mentioned, the exact etiology of OC is not known or not fully understood. However, the literature to date provides relatively strong evidence for the roles of genetics and anatomic factors. There is compelling evidence that inheritance is important in the etiology of OC (Ytrehus et al. 2007), not only in pigs but in other species such as horse and dog (Janutta et al. 2006, LaFond et al. 2002, van Grevenhof et al. 2009, Wittwer et al. 2007) supporting a heritable component of the disease.

2.4 Bone mineral density and content

Bone mineral status can be used as parameter to assess bone growth, and the risk of bone fracture and structural soundness in pigs (Mao et al. 2008, Mitchell et al. 2001). The measurement of bone density, reflecting the strength of bones, is represented by the calcium content. Bone mineral density (BMD) and bone mineral content (BMC) is widely measured by using dual-energy absorptiometry (DXA). This method is suitable for examining bone and body composition in longitudinal studies (Grier et al. 1996). The accuracy and precision of DXA use in piglets was evaluated, providing more information about the use of DXA in subjects with low body mass (Koo et al. 1995). In addition, DXA was not only used to measure skeletal mineral status but also to study pork carcass composition (Mitchell et al. 1998, Mitchell et al. 2000). Despite the fact that several publications in human have demonstrated the association of some candidate genes with BMD, there are very few studies on candidate gene or association studies dealing with BMD in pigs. Only one publication has been reported on QTL affecting BMD of the distal femur in White Duroc × Erhualian intercross (Table 1) (Mao et al. 2008). Therefore, genes that contribute to variation in susceptibility to low BMD in pig remain to be identified.

2.5 Quantitative trait loci (QTL) analysis for leg weakness related traits

With the advanced developments in molecular genetic analysis during the past few decades, QTL experiments in pig have rapidly increased. A well known QTL database called PigQTLdb has been developed and has become a valuable tool for pig research (Rothschild et al. 2007). Up to date over 5,986 QTL for 581 different traits are reported (30 September, 2010). It has helped researchers to share the published data, in particular for traits that have low heritability or traits for which measurement of phenotype is difficult (Dekkers 2004). Among them, QTL for leg score (Guo et al. 2009b), feet score (Lee et al. 2003), OC score (Andersson-Eklund et al. 2000, Christensen et al. 2009) and femur BMD (Mao et al. 2008) were identified in different breeding lines such as White Duroc x Erhualian, Wild boar x Large White, Large White x Meishan and Danish Duroc x Danish Large White intercrosses (Table 1). A number of factors are known to

influence the development of leg weakness or OC, especially body weight, growth rate, body and leg structure and mechanical stress of the animal (Jorgensen and Nielsen 2005). It has been reported that the degree of leg weakness and OC of one sex in a breed cannot be translated to the other sex within that breed, nor to the same sex of another breed (Van der Wal et al. 1978). This means that the frequency of leg weakness and OC varies depending on breed or genetic background of the pigs. Performing QTL studies using crossbreds of commercial breeds therefore might provide more insight in to the genetics of leg weakness in pigs. Furthermore, identification of QTL for leg weakness related traits will help to identify the candidate genes that are possibly associated with the development of these traits.

2.6 Candidate genes for leg weakness related traits

Most of the traits related with health and welfare of the animals are complex, difficult to define and are often characterised by low heritability. Thus, applying conventional genetics in combination with molecular genetic approaches is of worldwide interest. It is well known that there are two approaches for genetic dissections of complex and quantitative traits: genome-wide scanning and candidate gene approaches (Zhu and Zhao 2007). Candidate genes are generally known as the genes that play an important role in directly or indirectly affecting the trait of interest. The candidate gene approach has been applied for identifying many genetic diseases both in man and animals (Kadarmideen 2008, Kubota et al. 1997, Alexander et al. 2009, Fan et al. 2009). Therefore, identification of candidate genes involved in leg weakness related traits remains challenging. Focusing on candidate genes that play roles in the biology of bone and cartilage development could be of interest to better understand the biological background of traits related to leg weakness. However, as mentioned previously, body weight, growth rate or body and leg structure of the animal are also considered to be main contributing factors.

Table 1: QTL for leg weakness-related traits in different pig breeds

Related traits previously reported	Pig chromosome (SSC)	Breed	Reference
Osteochondrosis (femur) Femur measurements (weight, length, wild)	5, 3, 15 17	European wild boars X Swedish Large White	Andersson-Eklund et al. 2000
Osteochondrosis Front feet scores Front leg score Back legs scores Back feet score	7 1, 13, 14 13, 14 1, 14 15	Large White x Meishan pigs	Lee et al. 2003
Scapula length Ulna length Humerus length Femur length Tibia length BMD of the distal femur	1, 2, 3, 4, 5, 7, 13, 14, 15, X 1, 4, 5, 7, 13, 14, X 1, 2, 4, 7, 14, 15, X 1, 3, 4, 7, 10, 15, X 1, 7, 13, 15, X 4, 11,	White Duroc × Erhualian intercross	Mao et al. 2008
Front leg score at 213 d Rear leg score at 213 d Gait score of front legs at 153 d Gait score of rear legs at 153 d Gait score of front legs at 223 d Gait score of rear legs at 223 d Length of biceps brachii muscle Weight of biceps brachii muscle	1, 2, 3, 4, 7, 13 2, 4, 5, 7, 13, 16, X 1, 7, 8 1, 7, 9 4, 7, 8, 10 4, 7 1, 2, 3, 4, 8, 18, 1, 2, 4, 7, 8, 10, 15, X	White Duroc x Erhualian intercross	Guo et al. 2009
Osteochondrosis	1, 2, 4, 5, 6, 7, 10, 13, 15	Duroc X Landrace and Large white crossbred	Christensen et al. 2009

Therefore, genes involved in animal growth including bone and cartilage as well as molecular networks underlying articular cartilage development should also be considered. Articular cartilage, also called hyaline cartilage, is an important tissue for the passive movement skeletal system. Morphologically, articular cartilage (Figure 5) is divided into four layers, having structural and biochemical differences: superficial, middle, deep, and calcified. Each zone contains a distinct subpopulation of chondrocytes that differs in morphology, distribution within the matrix as well as metabolic activity (Simon et al. 2004). During postnatal development, articular cartilage undergoes architectural reorganization associated with growth and changes in biomechanical demands on the tissue

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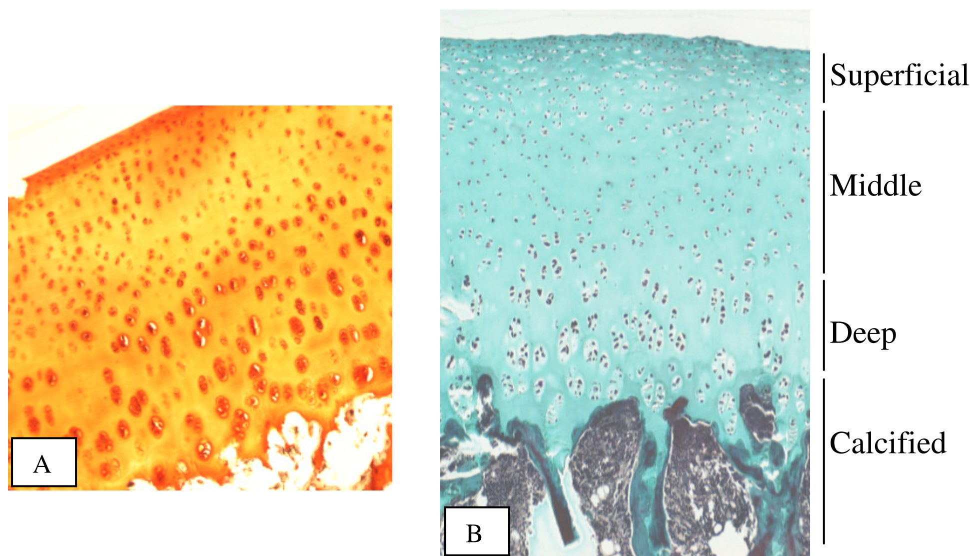

Figure 5: Histological image of porcine articular cartilage with salfanin-O (A) and Masson-Goldner (B) staining (40x)

Chondrogenesis and endochondral ossification are the cartilage differentiation processes that lead to skeletal formation and growth in the developing vertebrate as well as skeletal repair in the adult (Zuscik et al. 2008). Chondrogenesis (Figure 6) is a process for creation of chondrocytes both during embryogenesis as well as in adult life (Zuscik et al. 2008). Chondrocyte cells together with the collagen, proteoglycans and non-collagenous protein are organize into a unique and highly specialized tissue. Chondrocytes synthesize all the matrix components and regulate extracellular matrix (ECM) metabolism (Bhosale and Richardson 2008).

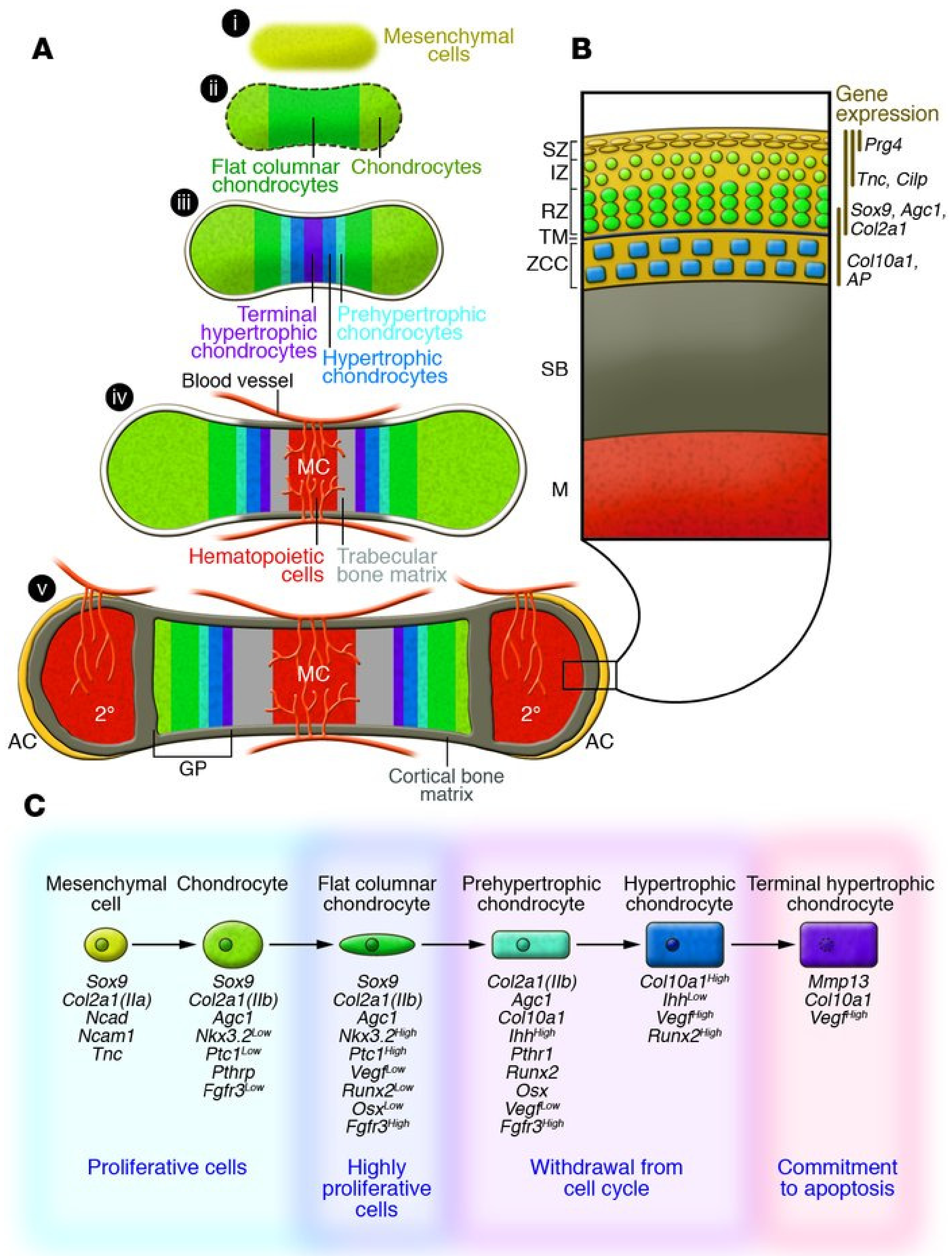

Figure 6: Cellular events and molecular markers of chondrogenesis, chondrocyte differentiation, and articular cartilage development and maintenance (Michael et al. 2008)

Endochondral ossification is the process by which the embryonic cartilaginous model of most bones contributes to longitudinal growth and is gradually replaced by bone. During endochondral ossification, chondrocytes proliferate, undergo hypertrophy and die; the cartilage extracellular matrix they construct is then invaded by blood vessels, osteoclasts, bone marrow cells and osteoblasts, the last of which depositing bone on remnants of cartilage matrix (Mackie et al. 2008). Therefore, candidate genes encoding for growth factors, transcription factors and structure molecules involved in endochondral ossification are considered in this study.

2.6.1 Transforming growth factor beta 1

Transforming growth factor beta 1 (*TGFβ1*), is a multifunctional peptide dimer from a family of important regulators of chondrocytes and other cells that controls growth, differentiation and other functions (Arevalo-Silva et al. 2001). It has been reported that in normal pig epiphyses, *TGFβ1* was present in the chondrocytes of the epiphyseal hyaline cartilage. This growth factor was found to be deficient in chondrocytes at sites of osteochondrosis (Thorp et al. 1995). However, in horse with OC lesions, the expression of *TGFβ1* was higher, but not significantly in affected tissues (Semevolos et al. 2001). This growth factor is therefore thought to be involved in the cascade of events associated with chondrocyte function during endochondral ossification. Moreover, active *TGFβ1* has been reported to play a key role in osteophyte formation (Scharstuhl et al. 2002). Taken together, these data suggest that *TGFβ1* may play a role in the development of OC and that this gene is a candidate for further functional and association studies in relation to the incidence of leg weakness or OC in pigs.

2.6.2 Collagen type II alpha 1 and collagen type X alpha 1 (*COL2A1* and *COL10A1*)

Articular cartilage consist of an extensively cross-linked collagen network. Different types of collagen molecules are expressed in articular cartilage. In young cartilage, the main type is a copolymer of collagens type II, IX and XI (Figure 7a) (Eyre et al. 2006). Once the chondrocyte cells have initiated hypertrophy, the collagen type X is

synthesized and is the only known hypertrophic chondrocyte-specific molecular marker (Zheng et al. 2003). Possible involvements of type II and X collagen in the development of OC are reported. Increases in type II collagen cleavage by collagenases in OCD lesions were observed in osteoarthritis (Laverty et al. 2002). Moreover, studies in pig have shown that collagen type II was reduced and collagen type X increased in OA lesion and near to the lesion samples (Jefferies et al. 2002). Therefore, *COL2A1* and *COL10A1* can be expected to be good candidate genes for OC.

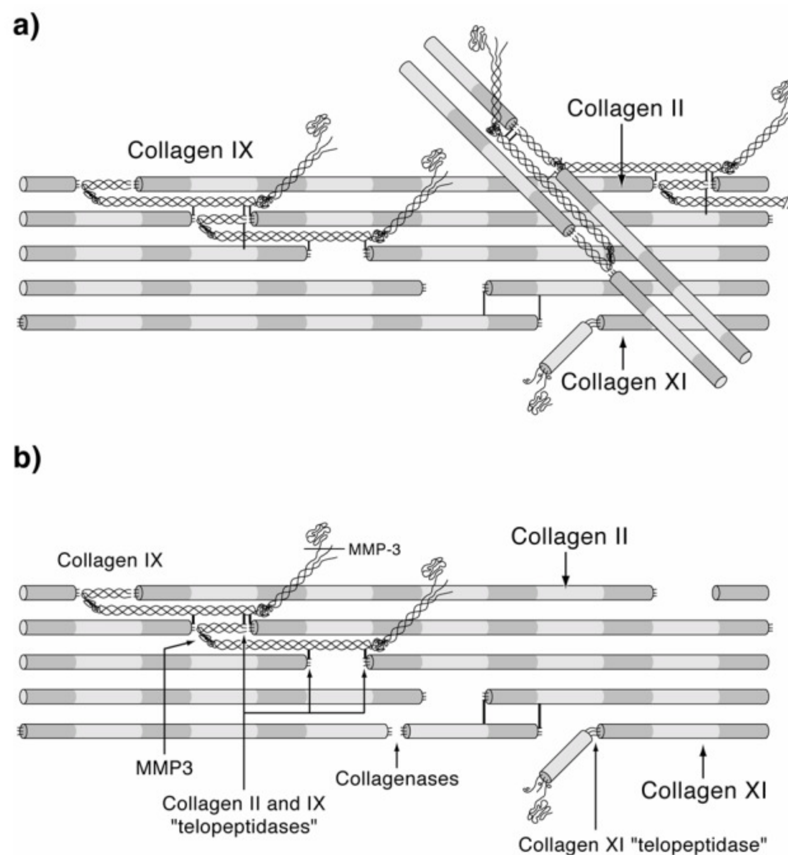

Figure 7: Different types of collagen molecules in articular cartilage. (a) Interaction model between surface collagen IX molecules and the collagen II polymer that can accommodate all the known IX-to-IX and IX-to-II cross-links and potential interfibrillar cross-links. (b) Known and speculated sites of peptide bond cleavage in the heteropolymer required for degradation and/or lateral growth of fibrils (Eyre et al. 2006)

2.6.3 Metalloproteinase 3 and 9 (*MMP3* and *MMP9*)

Proteins of the matrix metalloproteinase family are involved in the breakdown of the extracellular matrix in normal physiological processes and disease condition (Kevorkian et al. 2004). MMPs are involved in cartilage collagen breakdown (Figure 7b). The progression of OC to OA with the degradation of cartilage matrix components is generally agreed to be due to an over synthesis and activation of extracellular proteinases. *MMP3* was significantly decreased in OA but *MMP9* was significantly increased in OA cartilage (Kevorkian et al. 2004). Several studies in humans have measured MMPs in synovial fluid or articular cartilage and reported the differential expression of those enzymes in different stages of cartilage disease (Clements et al. 2009, Davidson et al. 2006, Kevorkian et al. 2004, Yeh et al. 2009). However, the molecular mechanisms underlying cartilage destruction are still poorly understood.

2.6.4 Transcription factors sex determining region Y-box9 (*SOX9*) and runt-related transcription factor 2 (*RUNX2*)

Two master transcription factors, *SOX9* and *RUNX2*, are involved in skeletal development. *SOX9* is expressed in all chondrocytes and is essential for the expression of the proteoglycan aggrecan, which is secreted by chondrocytes to form the characteristic glycosaminoglycan-rich extracellular matrix of cartilage (Tew et al. 2008). *RUNX2* plays important roles in multiple steps of skeletal development. *RUNX2* determines the lineage of osteoblasts from multipotent mesenchymal cells, enhances osteoblast differentiation at an early stage and inhibits osteoblast differentiation at a late stage (Furuichi and Komori 2004, Komori 2002). Both transcription factors were found to be involved in the development of OA. *RUNX2* down regulation resulted in reduced *MMP-13* expression in osteoarthritis chondrocytes. Inhibition of *SOX9* increased *RUNX2* and *MMP-13* mRNA expression in normal chondrocytes (Orfanidou et al. 2009). As previously mentioned, the OA could be the progress from the OC defect. Relative expression of both transcription factors might be important in the regulation of other genes which play roles in the cellular changes occurring in OC development.

2.6.5 Matrix Gla-protein (*MGP*)

MGP is highly accumulated in bone and cartilage and is commonly found at sites of calcification in blood vessels (Luo et al. 1997). *MGP* has been identified as a potential calcification inhibitor of extracellular matrix in cartilage and vascular tissue (Eferl et al. 2004). It contains five vitamin-K-dependent-γ carboxyglutamic acid residues which have high affinity to calcium and phosphate ions and can bind to hydroxyapatite crystals of mineralized tissue (Coen et al. 2009). *MGP*-deficient mice have extensive and inappropriate calcifications in cartilage and arteries leading to osteopenia (a condition where BMD is lower than normal), fractures and blood vessel ruptures (Luo et al. 1997, Schurgers et al. 2005) which might be important causes of OC. Abnormalities in the human *MGP* gene have been linked with Keutel syndrome, a condition characterized by abnormal calcium deposition in cartilage (Munroe et al. 1999). Genetic variation at the human *MGP* locus was reported to be associated with bone mineral density (BMD) in elderly women (Tsukamoto et al. 2000). Moreover, epigenetic change or methylation of CpG sites leading to the abnormal expression of specific genes is reported in osteoarthritis (Roach and Aigner 2007) in human. Therefore, we hypothesized that *MGP* might be a promising candidate gene for the development of OC in pigs. However, to the best of our knowledge, there is no detailed structural and functional characterization of the *MGP* gene and protein in normal and OC cartilage of pigs.

3 Materials and methods

3.1 Materials

3.1.1 Design of the study

A schematic representation of the overall study design of this study is shown in Figure 8.

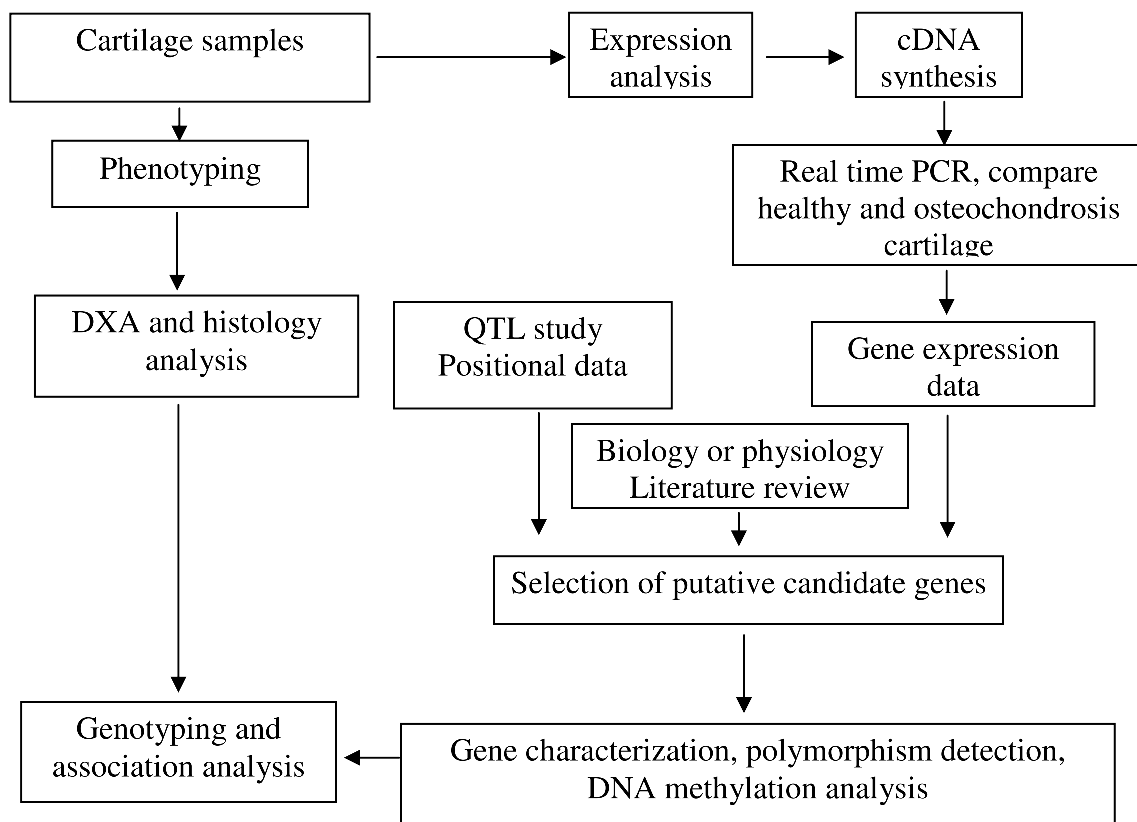

Figure 8: Schematic display of the research design used in this study

3.1.2 Experimental animals

3.1.2.1 F_2 Duroc × Pietrain (DuPi) resource population

Samples used in this study consisted of 310 F_2 DuPi resource population animals including 149 castrated males and 161 females. A more detailed description of this population is shown in Table 2. All pigs were kept at the experimental research farm 'Frankenforst' of the University of Bonn and exposed to uniform environmental conditions. Piglets were weaned at 28 days of age and placed in collective pens in the post-weaning unit until 10 weeks of age. Male piglets were castrated. All animals were individually weighed at birth, at weaning, at the beginning and at the end of the testing, respectively. Pigs were slaughtered in a slaughterhouse of the research farm Schwarzenau in Bavaria. Immediately after slaughter, the left elbow and knee joints were separated and the left femur and humerus bones were dissected from the carcass. The distal and proximal ends of the femur (head of femur and condylus medialis femuris, respectively) and the humerus (head of humerus and condylus medialis humeri, respectively) bones were sawn into longitudinal slabs encompassing both the articular cartilage and epiphyseal plate or growth plate. In total, 1,240 cartilage samples were collected and stored at -20 ºC for histological evaluation and at -80 ºC for gene and protein expression studies.

Table 2: Descriptive statistics of performance and carcass traits of the DuPi resource population

Item	Means	SD	Min	Max
Birth weight (kg)	1.49	0.26	0.80	2.40
ADG (g)	809.75	86.92	581.00	1042.00
End weight (kg)	109.75	8.76	80.00	133.00
Slaughter weight (kg)	87.57	7.16	63.50	105.00
Carcass length	97.89	2.90	87.00	105.00
Age (day)	185.00	15.56	151.00	230.00

3.1.2.1.1 Feet and leg scores

The animals were evaluated for leg scores in a range from one to five, in which three is the optimum level (Table 3). The data were then transformed into a linear scale, by using the absolute value of the original scores after subtracting three. Each ‘leg score’ is an assessment of the strength of legs, the straightness and the stability of the joints. The feet were scored between one and three, where one indicated poor and three indicated good. The ‘feet score’ is an assessment of the angle and strength of attachment of the feet to the legs and the soundness of the toes and weight distribution on the toes.

3.1.2.1.2 Histology of articular cartilage for osteochondrosis lesion observation

As OC lesions are often bilaterally symmetrical (Ytrehus et al. 2007), it was decided to examine only the left legs of the carcass. The OC was scored on the basis of microscopic or histological examination (Figure 9). The recorded OC lesions were score from 1 to 4, with 4 = ‘normal’ and 3 to 1 = ‘mildly to severely affected’. Osteochondral lesions were observed in the following areas: head of the humerus (HH), condylus medialis humeri (CMH), head of the femur (HF) and condylus medialis femoris (CMF). The histological assessment was on the basis of the thickness of cartilage, degradation of cartilage, and vessels and cartilage canals structure. In brief, the specimens were fixed in Bouin's fixative for 48 h, decalcified in RDO (Apex Engineering Products Co., Plainfield, IL, USA) and dehydrated through a series of ascending alcohol concentrations (70, 80, 90, and 100%, respectively). Subsequently, the sections were incubated in Rotihistol® (Roth) and embedded in paraffin. Section of 7-10 µm thickness were cut and stained following the method of Masson-Goldner. The stained sections were examined microscopically at 40x magnification.

Table 3: Scoring criteria for legs and feet scores

Traits	Attribute	Scores				
		5	4	3	2	1
Legs	Strength of legs	Poor	Moderate	Very good	Moderate	Poor
	Straightness	Flexing	Slightly bent	Straight	Buckled	Very buckled
				Scores		
				3	2	1
Feet	Toes-soundness			Good	Moderate	Poor
	Toes-weight distribution			Even	Moderate	Uneven
	Angle of foot attachment			Good	Moderate	Low
	Damage			None	Slight or temporary	Permanent

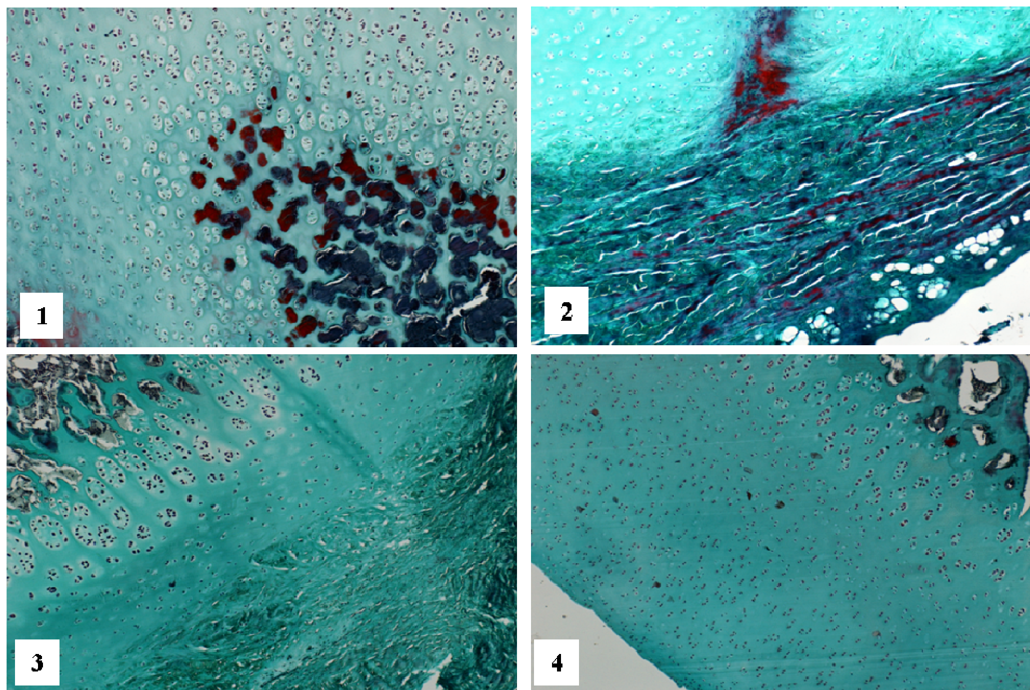

Figure 9: Sample of histological templates for the evaluation of OC score. OC lesions are classified into 4 scores; (1) massive alterations of the cartilage including necrotic or ossified areas and also when cartilage becomes bony, (2) severe changes in the surface and deeper area of the articular cartilage like surface erosion, fibrillations, hyperplasia and chondrocyte necrosis, (3) cartilage surface consists of few changes in surface and fibrillation, (4) cartilage surface is smooth, the matrix and chondrocytes are well organized or only marginally rough surface or weakly eosinophilic matrix or fibrillation are observed.

3.1.2.1.3 Bone mineral density traits

The whole ulna and radius bones from the left carcass were stripped of all surrounding tissues. The samples were sent to the testing and educational farm Oberschleißheim of the Ludwig-Maximilians-University of Munich to be examined using X-ray. BMD, BMC and BMA were observed by a dual-energy X-ray absorptiometry (DXA).

3.1.2.2 Animal for association analysis

Data from 299 pigs with OC were supplied from the SUISAG Company. The pigs were the station-tested animals and were crossbreeds by using Large White × Landrace as dam lines and Large White × Duroc as sire lines. Further description of the animals and the scoring criteria are given in the report of Kadarmideen et al. (2004). The data include OC scores at seven different joint areas: head of humerus (HH), condylus medialis humeri (CMH), condylus lateral humeri (CLH), radius/ulna proximal (RAD), distal epiphysis ulna (DEU), head of femur (HF) and condylus medialis femoris (CMF). A summary of the OC phenotype distribution of the animals is shown in Table 4.

Table 4: Number and percentage of osteochondrosis scores at different joints of the commercial population

Trait (n=299)			Number of joints (%)			
OC score	6	5	4	3	2	1
HH	0	0	1 (0.33)	2 (0.67)	0	296 (99)
CMH	0	0	0	28 (9.63)	31 (10.37)	240 (80.27)
CLH	0	0	1 (0.33)	3 (1)	4 (1.34)	291 (97.32)
RAD	0	0	0	0	25 (8.36)	274 (91.64)
DEU	2 (0.67)	70 (23.41)	47 (15.72)	15 (5.02)	134 (44.82)	31 (10.37)
HF	0	0	0	0	1 (0.33)	298 (99.67)
CMF	0	0	2 (0.67)	21 (7.02)	87 (29.10)	189 (63.21)

3.1.3 Chemicals and equipments

3.1.3.1 Chemicals and kits

Amersham Biosciences (Freiburg): GFXTM PCR DNA and gel band purification kit

Applied Biosystems (Foster City): SYBR® Green universal PCR master mix

BD Biosciences Clontech (USA): SMARTer™ RACE cDNA amplification kit

Beckman Coulter (Krefeld): CEQ™ 8000 genetic analysis system, dye terminator cycle wequencing (DTCS), glycogen

Biomol (Hamburg): phenol, phenol/chlorophormllsoamyl alcohol (25:24:1).

Invitrogen Life Technologies (Karlsruhe): DTT, random primers

MBI Fermentas (St. Leon-Rot): glycogen

Promega (Mannheim): BSA, pGEM®-T vector, RQ1 RNase-free Dnase, RNasin ribonuclease inhibitor, 2X rapid ligation buffer, T4 DNA ligase, *E.coli* strain JM109

Qiagen (Hilden): RNeasy® mini kit, QIAquick PCR purification kit, mini elute™ reaction cleanupkit

Roth (Karlsruhe): acetic acid, agar-agar, ampicillin, bromophenol blue, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid (EDTA), ethanol, ethidium bromide, hydrochloric acid, isopropyl-D-thiogalactoside (IPTG), peptone, potassium dihydrogen phosphate, 2-propanol, sodium acetate, sodium carbonate, sodium chioride, sodium hydroxide, trichloromethane/chiorophorm, tris, x-gal(5-bromo-4-chloro-3-indolylbeta-D-galactopyranoside), yeast extract

Sigma-Aldrich Chemie GmbH (Munich): agarose, ammonium acetate, calcium chloride, formaldehyde, genElute™ plasmid miniprep kit, glutamine, hepes, isopropanol, magnesium chloride, 2-Mercaptoethanol, penicillin, phenol red solution, 10X PCR reaction buffer, potassium chloride, sodium dodecyl sulfate (SDS),

Stratagene (Amsterdam): 5 c DH *Escherichia coli* competent cells

USB (Ohio): ExoSAP-IT

Zymo Research: DNA methylation bisulfite assay kit

3.1.3.2 Reagents

All solutions were prepared with double deionised water.

10X TBE

Tris borate, pH 8.0	90	mM
EDTA, pH 8.0	20	mM
Water added to	1000	mL

10X TE

1 M Tris pH 8.0	50	mL
0.5M EDTA pH 8	0.5	mL
ddH_2O	445	mL

Autoclave to sterilize (121 ^oC for 30 min)

10X PBS

NaCl	87.76	g
$Na_2HPO_4.2H_2O$	15.00	g
NaH_2PO_4	2.04	g
Water added to	1000	mL

1X PBS-Tween (PBST)

1X PBS	999.50	mL
Tween®20	0.5	mL

4X Sample loading buffer

Tris (1 M, pH 6.8)	13.00	mL
SDS	6.00	g
2-Mercaptoethanol	10.00	mL
Glycerine	20.00	mL
Bromophenol blue	10.00	mg
Water added to	50.00	mL

50X TAE

Tris base	242	g
Glacial acetic acid	57.1	mL
0.5 M EDTA pH 8.0	100	mL
Water added to	1000	mL

Agarose gel loading buffer:

Bromophenol blue	0.25	%
Xylene cyanol	0.25	%
EDTA	120	mM

Ampicillin (10 mg/ml):

Ampicillin powder	2	g
Sterile, distilled water,	40	mL
Filtrate with 0.45 µl filter		

Blue dextran loading buffer:

Blue dextran	50	mg
EDTA (0.5M)	50	µl
Water	950	µl
Formamide	5	mL

DEPC-treated water:

DEPC 1 mL/L water

Incubation at 37 °C for overnight and heat inactivated by autoclaving

Digestion buffer

9%NaCl	6.50	mL
Tris (1M, pH 7.5)	5.0	mL
EDTA (0.5 M, pH 8.0)	0.20	mL
SDS (10%)	1.00	mL
Mercaptoethanol	2.00	mL

Proteinase K : 10 mg/mL

X-Gal (50 mg/ml) :

X-Gal	50 mg
N, N-dimethylformamide	1 mL

Stored at -20 °C

3.1.3.3 Media

LB-agar plate

Sodium chloride	8.0 g
Peptone	8.0 g
Yeast extracts	4.0 g
Agar-Agar	12.0 g
Sodium hydroxide (40.0 mg/ml)	480 µl
Water added to	800 mL
Autoclave (121 ^{o}C for 30 min)	

LB-broth

Sodium chloride	8.0 g
Peptone	8.0 g
Yeast extracts	4.0 g
Sodium hydroxide (40.0 mg/ml)	480 µl
Water added to	800 mL
Autoclave (121 ^{o}C for 30 min)	

3.1.3.4 Enzymes

SuperScript™ II Rnase H^{-}Reverse Transcriptase (Gibco BRL®, Karlsruhe), (Invitrogen Life Technologies, Karlsruhe)

Pfu DNA polymerase (Promega)

Taq DNA polymerase (Sigma)

3.1.3.5 Equipments

Apotome microscope
Carl Zeiss

Centrifugation

HERMLE Z 323K, Wehingen

Electrophoresis/DNA Analyzer: Automated sequencer

LI-COR, DNA Analyzer, GENE READER 4200 (MWG-BIOTECH)

Electrophoresis system

For agarose gel: BioRad, Göttingen

Vertical apparatus: Consort, Turnhout

Power supply: PAC 3000 BioRad

Thermocycler:

PTC-100 Programmable Thermal Controller (MJ Research, Inc. USA)

3.1.3.6 Software used

CRIMAP Version 2.4 (Green et al. 1990)

Image Analysis (version 4.10) LI-COR Biotechnology, USA

SAS version 9.02 SAS Institute Inc., Cary, NC

BLAST program: http://www.ncbi.nlm.nih.gov/blast/

DNA to protein translate tool: http://us.expasy.org/tools/dna.html

MapChart2.2: http://www.biometris.nl/uk/Software/MapChart/

Multiple Sequence Alignment:

http://saturn.med.nyu.edu/searching/promultali.html

QTL-express http://qtl.cap.ed.ac.uk/

Restriction enzyme analysis : http://tools.neb.com/NEBcutter/index.php3

Primer design: http://www-genome.wi.mit.edu/cgibin/primer/primer3_www.cgi

BiQ Analyzer: http://biq-analyzer.bioinf.mpi-sb.mpg.de/

CpGProd software: http://pbil.univ-lyon1.fr/software/cpgprod_query.html

Image-J software: http://rsbweb.nih.gov/ij/

Multiple Sequence Alignment: http://pbil.ibcp.fr/htm/index.php

QUMA: http://quma.cdb.riken.jp/

TSSP: http://www.softberry.ru

3.2 Methods

3.2.1 DNA isolation from cartilage and muscle tissues

For genotyping, genomic DNA was isolated from tail samples using the standard phenol chloroform method. Samples of about 1 g were cut into small pieces and were digested by proteinase K (10 mg/ml). The digestion buffer included 700 µl of digestion buffer, 70 µl of 10% SDS. Samples were incubated overnight at 37 °C. Seven hundred µl of phenol-chloroform were added and then the tube was shaken until an emulsion was formed and centrifuged at 10,000 rpm for 10 min. The upper part was transferred into a 2 ml tube, 700 µl of chloroform were added and shook gently. Samples were centrifuged at 10,000 rpm for 10 min. The upper part was carefully collected into 1.7 ml tube, 700 µl of isopropanol and 70 µl of 3M sodium acetate were added into the tube and the samples were shaken gently until precipitation of DNA and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was discarded, then 200 µl of 70% ethanol were added, the pellet liberated from the tube surface then centrifuged at 10,000 rpm for 5 min. The aqueous phase was discarded and the pellet was left to dry. Five hundred microlitres of 1X TE were added and the DNA samples left overnight at room temperature. The DNA concentration were measured and diluted to a concentration of 50 ng/µl. For DNA methylation analysis, genomic DNA was isolated from articular cartilage using Tri reagent according to the manufacturers' protocol.

3.2.2 DNA isolation from agarose gel

The band of interest was excised with a sterile razor blade, placed in a microcentrifuge tube, frozen at –20 °C for at least 1 hour. The band was chopped and 500 µl TE buffer were added. The gel was homogenized by repeated pipeting using a syringe attached

with a needle. Five hundred microlitres of phenol-chloroform (1:1) was added to the DNA solution and vigorously vortexed for a few seconds to form an emulsion. After centrifugation at 12,000 g for 15 min the upper aqueous layer was carefully removed to a new tube. An equal volume of chloroform was added to the aqueous phase, vortexed and centrifuged for 15 min at 12,000 g to allow phase separation. The aqueous layer was transferred to a new tube. One-tenth volume of 3M sodium acetate (pH 5) and two volumes of cold 100% ethanol were added to the DNA sample in a microcentrifuge tube and mixed by inversion. This precipitation was performed by incubation at –20 ^{o}C overnight or at –80 ^{o}C for at least 30 min. To recover the precipitated DNA, the mix was centrifuged at 12,000 *g* in a microcentrifuge for 20 min at 4 ^{o}C. After the supernatant was discarded, the DNA pellet was washed with a cold 75% ethanol (corresponding to about two volume of the original sample) being first incubated at room temperature for 5-10 min and centrifuged again for 5 min. The supernatant was drained being inverted on a paper towel until the DNA pellet was air dried. Finally, the DNA pellet was dissolved with 5 µl of distilled H_2O.

3.2.3 RNA extraction from articular cartilage

Articular cartilage samples were used for RNA isolation. Frozen tissue samples (1 to 10 mg) were powdered by mortar and pestle, 1 ml of TRIZOL® reagent was added and homogenised. The homogenised samples were incubated for 5 min at room temperature. Then 0.2 ml of chloroform were added to homogenised samples, mixed thoroughly by shaking and incubated for 15 min at room temperature. Samples were centrifuged at 12,000 *g* for 15 min at 2 to 8 °C. The RNA remains exclusively in the upper aqueous phase which was transferred to a fresh tube. The RNA in the aqueous phase was precipitated by adding 0.5 ml of ethanol. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 *g* for 10 min at 2 to 8 °C. The supernatant was removed and the RNA pellet was washed once with 1 ml of 75% ethanol. After centrifugation and removal of supernatant, the RNA pellet was air dried and dissolved in 50 µl RNase-free water and stored at –80 °C for further use. The RNA was treated with DNase to remove residual DNA. The DNA digestion was performed by mixing, the following components in a 0.5 ml RNase free tube: 25 µl RNA, 5 µl 10X

buffer, 2 µl RQ1 RNase-Free DNase, 1 µl RNase inhibitor (40 U/µl), and 17 µl RNase-free water and incubated at 37 °C for 1 hour. After incubation, the RNA was purified by RNeasy Mini Kit (Qiagen). To assess the quality of RNA, 2 µl RNA sample was electrophoresed on 1.2% agarose gel.

3.2.4 cDNA synthesis

The total RNA was used to synthesize first strand cDNA. The reaction was performed in a nuclease-free microcentrifuge tube, 1 µl oligo dT(11) (500 µg/ml), 1 to 5 ng total RNA, 1 µl 10 mM dNTP mix and 12 µl sterile water were added in a 0.2 ml PCR tube. The mixture was heated at 70 °C for 10 min and immediately chilled on ice. The contents of the tube were collected in bottom by brief centrifugation and 4 µl 5 X first-strand Buffer (Gibco BRL, Karlsruhe Germany), 2 µl 0.1 M DTT and 1 µl RNase OUT Recominant Ribonuclease Inhibitor (40 U/µl) were added. The reaction was gently mixed and incubated at 40 °C for 2 min. One microlitre (200 units) of Superscript™ II RNase H reverse transcriptase was added and further incubated at 42 °C for 90 min. The reaction was inactivated by heating at 70 °C for 15 min. The cDNA was diluted 1:4 in RNase free water and was used as a template for subsequent PCR amplifications. The cDNA samples were stored at -20 °C for further use.

3.2.5 Amplification of 3' and 5' ends of *MGP* by RACE PCR

Total RNA from cartilage was used for 3' and 5' RACE (Rapid Amplification of cDNA Ends) using the SMARTer™ RACE cDNA Amplification Kit (Clontech). First-strand cDNA synthesis was performed, and then 3' and 5' RACE-PCR were individually performed using the universal primers and the gene specific primers (GSP). The PCR was performed using the following conditions: 35 cycles of 94 °C for 30 s, 60 °C for 30s and 72 °C for 2 min. The PCR products were sequenced after gel purification and cloning.

3.3.6 PCR conditions

PCR amplification was performed in 20 µl reaction volume using 25 ng of genomic DNA, 0.2 µM of each primer, 50 µM of each dNTP, 0.5 U of Taq polymerase in 10X PCR reaction buffer supplied with 1.5 mM $MgCl_2$. Thermocyling was performed using the following program: initial denaturation for 5 min at 95 °C, 35 cycles of each 30 sec at 95 °C, 30 sec at annealing temperature fore each primer, and 30 sec at 72 °C, followed by 10 min final extension at 72 °C.

3.2.7 Protein extraction from articular cartilage

The same articular cartilage tissues used for RNA extraction were also used for protein extraction. Proteins were isolated from the phenol-ethanol supernatant obtained after precipitation of DNA with ethanol. Three volumes of acetone were added to the supernatant, mixed by inversion and incubated for 10 min at room temperature. After protein precipitation at 12,000 *g* for 10 min at 4 ºC, protein pellets were washed by 0.5 ml of protein wash (0.3 M guanidine hydrochloride in 95% ethanol and 2.5% glycerol). After dispersing the pellet, an additional 3 time wash with 1 ml of protein wash were added, incubated for 10 min at room temperature and sedimented by centrifugation at 8,000 *g* for 5 min. The final wash was performed in 1 ml of ethanol containing 2.5% glycerol (V:V). Protein pellets were dried for 5-10 min at room temperature and 200 µl of digestion solvent were added to solubilise the pellet and stored at -20 °C for further use.

3.2.8 Ligation and transformation

The purified PCR fragment was ligated into a pGEM-T easy vector (Promega, Mannheim Germany). The reaction was performed in 5 µl total volume, which contained 2.5 µl 2 X ligation buffer, 0.5 µl pGEM®–T Vector, 1.5 µl PCR products and 0.5 µl T 4 DNA ligase. The reaction was gently mixed and incubated for 1 hour at room temperature or at 4 °C overnight. For transformation, competent *E.coli* DH5α were

aliquoted to chilled polypropylene tubes and 3 µl of ligation reaction mix were added by 60 µl of the competent cells. The mixture was incubated on ice for 30 min. The cells were then heat shocked at 42 ^{o}C for 90 sec, incubated on ice for 2 min, and 700 µl LB medium were added at room temperature. The culture was incubated at 37 ^{o}C in a shaking incubator for 90 min. For each ligation reaction, two LB plates supplemented with ampicillin (100 µg/µl) were prepared by adding 0.10 M IPTG and 20 µl of X-Gal and spreaded with a glass pipette being allowed to absorb for 20 min prior to use. The transformed bacterial culture was plated in duplicate and the plates were incubated at 37 ^{o}C overnight. Successful cloning of the insert will produce white colonies as it interrupts the coding of ß-galactosidase. The presence of the DNA insert in white colonies was confirmed by M13 PCR.

3.2.9 Sequencing

PCR products were purified using EXO-SAP IT. Five microlitres of purified sample were used for cycle sequencing, with specific primers and the Quick Start Kit for Dye Terminator Cycle Sequencing or DTCS (BeckmanCoulter, Krefeld, Germany), including DNA polymerase, pyrophosphatase, buffer, dNTPs and dye terminators. Once the sequencing-PCR was performed, 3M NaOAc, 100 mM EDTA and glycogen were added to stop the reaction. To each sample, 60 µl of 98% ethanol (Roth) were added and mixed well by vortexing and then centrifuged for 15 min at 18,000 rpm at 4 °C. All liquid was removed and replaced with 200 µl 70% ethanol without mixing and centrifuged again for 15 min at 18,000 rpm at 4 °C. The ethanol was then removed and the sample was air dried for 10 min. The sample was then resuspended in 40 µl of sample loading solution (SLS) (Beckman Coulter). Cleaned up samples were transferred manually to a CEQ sample plate and overlaid with mineral oil. Samples were sequenced using the CEQ™ 8000 Genetic Analysis System (Beckman Coulter).

3.2.10 Genomic bisulfite PCR and sequencing

Genomic DNA was extracted from 9 OC and 9 healthy articular cartilages, the same samples as used for gene expression analysis. Each DNA (1 µg) was modified with the bisulfite conversion reaction according to the manufacturer protocol (EZ DNA Methylation Kit, Zymo Research). The converted DNA was diluted with 15 µl of ddH_2O. Each converted DNA (2 µl) was used as a template for PCR with primers designed using the MethPrimer program (Li and Dahiya 2002). The PCR products amplified from the bisulfite-treated DNA were further analyzed through cloning and sequencing. Each of the PCR products was purified using QIAquick PCR purification kit (Qiagen), and then individually cloned into the pGEM-T Easy vector (Promega). A minimum of 5 different clones were randomly selected for sequencing with M13 primers. Methylation sites were visualized and quality controlled by using BiQ Analyzer (Bock et al. 2005) and QUMA (Kumaki et al. 2008) software.

3.2.11 Screening for polymorphic sites

In silico analysis by comparing several sequences from the database revealed the possible targets for PCR amplification. The SNP positions were confirmed by comparative sequencing of PCR fragments in a SNP discovery panel of F_2 animals from DuPi. The screening primers used in this study are shown in Table 5.

3.2.12 Genotyping

SNPs were genotyped depending on the suitability of the mutation and the surrounding sequences to design a PCR-RFLP. *COL2A1, COL10A1, MMP3* and *TGFβ1* were genotyped by PCR-RFLP analysis (restriction enzyme and size of the PCR product are shown in Table 6 and Figure 10).

Table 5: List of primer names, sequences and their applications in this study

Primer name		Sequence (5´- 3´)	Purpose
MGPBi	F	TTAATTGGTTAGGATTAAGAGGTTATTTTT	Methylation
MGPBi	R	CCACAACCATATAACAATACCAAATC	Methylation
MGPRTPCR	F	GTGGCAGCCCTGTGCTAT	Real time PCR
MGPRTPCR	R	GGAGGCTTGTTGAGTTCTCG	Real time PCR
MGPG2-F	F	GCAGAGATGGAGAGCGAAAG	PCR for 3' RACE
MGPG2-R	R	GCAAGCTTCCCGGTTTAACT	PCR for 5' RACE
MGPG1-F	F	TCACAAACACTCAATGCCTGA	Genomic sequencing
MGPG1-R	R	TTTCCCAAAGGAGACCCTCT	Genomic sequencing
MGP.W-F1	F	CTGGAGCCAGTGGTTTCTGT	Genomic sequencing
MGP.W-R1	R	TCTGTGTGGTTGATGTGGTG	Genomic sequencing
MGP.W-F2	F	TTGTGTCGGAGAGTATTCATGG	Genomic sequencing
MGP (F2)	F	GCCATGGTTTATGGATACAAC	Genomic sequencing
MGP (R1)	R	AAATAACGATTGTAGGCGGCA	Genomic sequencing
MGPexon1R	R	AGGAGCAGGCTCTTCATGGT	Genomic sequencing
MGPexon2R	R	GCTTTCGCTCTCCATCTCTG	Genomic sequencing
MGPexon3R	R	GGAGGCTTGTTGAGTTCTC	Genomic sequencing
MGPintron1F	F	CCAGGGAGAGGACTGTCTCA	Genomic sequencing
MGPintron3F	F	AGACTCCAGTGGCTGGTTTG	Genomic sequencing
MGPfr185 :	F	GCCACAGCAGAGATGGAGA	Genomic sequencing
MGPrv240 :	R	TTCATAGGATTCCAAGCTCTCA	Genomic sequencing
MGPfr18	F	AGACCCTGAGAGCAACCTCA	Genomic sequencing
MGPrv529	R	CCATGGCACTTTCATTCCTT	Genomic sequencing
MGP152f	F	GTGGCAGCCCTGTGCTAT	Genomic sequencing
MGP152r	R	GGAGGCTTGTTGAGTTCTCG	Genomic sequencing
GAPDH	F	ACCCAGAAGACTGTGGATGG	Real time PCR
GAPDH	R	ACGCCTGCTTCACCACCTTC	Real time PCR
TBP	F	GATGGACGTTCGGTTTAGG	Real time PCR
TBP	R	AGCAGCACAGTACGAGCAA	Real time PCR
TGFβ1	F	CCACTCTCAGCCTCTCTGCT	Real time PCR
TGFβ1	R	TGGGTTCTCGGTATCCTACG	Real time PCR
MMP9	F	GTCCTGGTGCTCCTGGTG	Real time PCR
MMP9	R	AACCCTCATTAGCCCATTCC	Real time PCR
SOX9	F	CTCAAGGGCTACGACTGGAC	Real time PCR
SOX9	R	GAGTTTGCCCAGAGTCTTGC	Real time PCR
COL2A1	F	AAGGTGGGAAACCAGGTGAT	Real time PCR
COL2A1	R	AGGAGCTCCAGCTTCACCAG	Real time PCR
RUNX2	F	CAGACCAGCAGCACTCCATA	Real time PCR
RUNX2	R	ACGCCATCATTCTGGTTAGG	Real time PCR
M13	F	TTGTAAAACGACGGCCAGT	PCR and Seq
M13	R	CAGGAAACAGCTATGACC	PCR and Seq

Table 6: List of primers and enzymes used for PCR-RFLP analysis

Gene	Primer (5′-3′)	Product size	SNP Position	Enzyme
COL2A1	F1:CAAAGATGGCGAGACAGGTG R1: CAGATGGCCCAGGAGCAC	397	Intron 29	*MspI*
COL10A1	F4:TTCAGCCTACCTCCATATGCAT R4:CAACAGCACTACGACCC	400	3’UTR	*TaqI*
MMP3	F1:GACGGGAAAGCTGGATTCTA R1:CCAGGTGCATAGGCATGAG	429	Intron 2	*DdeI*
TGFβ1	F1:CTACTCATCCATCTGAGTG R1:GAAGCAGTAGTTGGTATCCA	932	Intron 6	*BseYI*

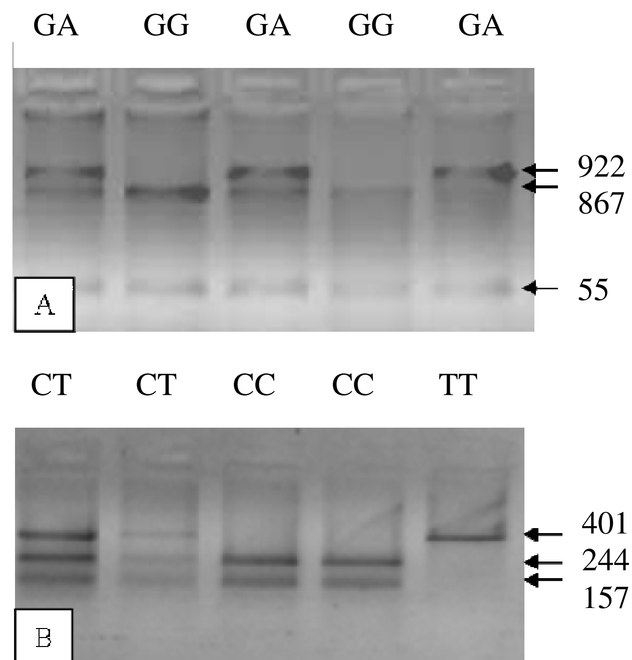

Figure 10: Sample of PCR-RFLP for genotyping (A) *COL2A1*, (B) *MMP3*genes

3.2.13 Marker data for QTL analysis

Marker genotyping data from SQL-based database at the Institute of Animal Science, University of Bonn, Germany were used in this study. A linkage map with the total length of 2588.7 cM and an average marker interval of 31.57 cM was used. P, F1 and F_2 animals of the DuPi population were genotyped with 82 genetic markers to cover all porcine autosomes. The set of markers includes 79 microsatellites and 3 biallelic markers. The order of markers and the genetic distances between them have been described (Uddin et al. 2010). These markers were mainly selected from the USDA/MARC map (http://www.marc.usda.gov). In brief, DNA was isolated from tail muscle samples using the phenol-chloroform clean up and the salt precipitation. DNA was dissolved and kept at 4 °C. Different single and multiplex PCR were applied to amplify the fragments. All PCR reactions were performed using a thermocycler PTC 100 (MJ Research USA). Genotyping, electrophoresis, and allele determination were done using a LI-COR 4200 Automated Sequencer (DNA Analyzer, GENE Reader 4200). The fragment analysis was performed using the OneDScan program (Scanalytics) to identify the length of the different amplified alleles. Allele and genotyping errors were checked using Pedcheck software (v 1.1).

3.2.14 Western blot

The proteins extracted from 3 OC and 3 healthy cartilages from condylus medialis humerus of 3 full-sib pairs were separated by SDS-PAGE (gradient 8-18%). Subsequently the proteins were transferred onto a nitrocellulose membrane (Protran®, Schleicher & Schuell Bioscience). After blocking in blocking buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-20 and 1% polyvinylpyrolidone) at room temperature for 1 h, the membrane was incubated with the polyclonal anti-MGP antibody purified from goat (Cat.Sc32820; Santa Cruz). The horseradish peroxidase-conjugated donkey anti-goat IgG (Cat.Sc2020; Santa Cruz) was used as the secondary antibody. The chemiluminescence was detected by using the ECL plus Western blotting detection system (Amersham Biosciences) and visualized by using Kodak BioMax XAR film (Kodak). GAPDH was used as a loading control and for normalizing. The membrane

was stripped by incubation in 2% SDS, 100 mM Tris–HCl, 0.1% beta-mercaptoethanol for 30 min at 60 °C and re-probed with goat polyclonal GAPDH antibody (Cat.Sc20357; Santa Cruz). Relative band intensities were compared by determining the ratio of the area densities of MGP to GAPDH bands for each lane using Image-J software (National Institute of Mental Health, Bethesda, Maryland, USA).

3.2.15 Immunofluorescence

Expression of the MGP protein was detected by immunofluoresence at three different ages of healthy animals. Due to the limitation of fresh sample availability for OC cartilage, we localized the MGP protein in cartilages collected from one day, 2 months and 6 months old healthy pigs. The animals used in this study were three half siblings (all are males, the 2 months and 6 months old were castrated) of the Duroc and Pietrain cross breeds that were born and raised in the same herd and under similar standard conditions. Immediately after collection, tissues were fixed in 4% paraformaldehyde for 24 h and cryoprotected with Tissue-tek® (Sakura, Netherlands) and cut using a cryotome. All sections were kept in -80 ºC for further analysis. Cartilage cryosections (8-12 µm-thick) were fixed for 15 min in freshly prepared 4% paraformaldehyde. Sections were washed in PBS and blocked for 1 h in PBS with 0.3% donkey serum followed by incubation for 90 min at 37 ºC with goat polyclonal anti-MGP (Cat.sc-32820, Santa Cruz) primary antibody (diluted 1:100 in PBS containing 0.3% goat serum). After three washes in PBS, the sections were blocked for 30 min with PBS containing 0.3% normal goat serum. The sections were then incubated for 1 h at room temperature with secondary donkey anti-goat antibody conjugated with fluorescein isothiocyante (FITC) (Cat.sc-2024, Santa Cruz). After several washes in PBS, the sections were mounted in Vectashield (Vector Laboratories) containing 40,6-diamidino-2-phenyl indole (DAPI). For negative controls, the primary antibody was substituted by PBS. Images were obtained by an apotome microscope (Carl Zeiss, Germany).

3.2.16 Promoter prediction and CpG island identification for the porcine MGP gene

The web based Primer3 (Rozen and Skaletsky 2000) program was used for designing primers (Table 5). Genomic DNA was amplified by PCR and sequencing of amplified PCR fragments was performed by the CEQ8000 sequencer system (Beckman Coulter). A sequence of transcription start site (TSS) was predicted using the TSSP program. The published sequences (NC_010447) of the 5´-flanking regions which contained the promoters of the *MGP* gene were submitted to the CpGProd software (Ponger and Mouchiroud 2002) in order to identify the CpG islands.

3.3 Statistical analysis

Relative means of gene expressions were compared between normal and OC groups by the student t-test. The data were analyzed using the software package SAS (v 9.2, SAS Inc., CA, USA) for a detailed description of the data structure. Generalized linear models (PROC GLM) were used to identify any possible obvious effect of sire, dam, age, sex, birth weight, daily weight gain, litter size, parity, season, slaughter weight and carcass length on the investigated traits.

3.3.1 QTL mapping

F_2 QTL interval mapping was performed using the web-based program QTL Express (Seaton et al. 2002) available at http://qtl.cap.ed.ac.uk/ based on a least square method. The analyses were carried out at chromosome and genome wide level with a single QTL model. The basic QTL regression model used in the present study was:

$$y_i = \mu + F_i + C_j + c_{ai}a + c_{di}d + e_i$$

where:

y_i = phenotype of the ith offspring;

μ = overall mean;

F_i = fixed effect of parity;

C_j= regression coefficient on the covariate; covariate (average daily gain for leg and feet score; age for OC; slaughter weight and carcass length for DXA);

c_{ai} = additive coefficient of the ith individual at a putative QTL in the genome;

c_{di} = dominant coefficient of the ith individual at a putative QTL in the genome;

a = additive effects of a putative QTL;

d = dominant effects of a putative QTL; and

e_i = residual error

The regression model was fitted at 1-cM intervals along each chromosome and the F-value for the QTL effect was calculated at each point. Significance thresholds were determined by data permutation (Churchill and Doerge 1994) at 1000 iterations. The chromosome-wide significance level was used as the suggestive significance level (Ding et al. 2009) and ranged from 3.8 to 5.2 for different chromosomes. The empirical 95% confidence intervals (CI) and flanking markers for the QTL positions were obtained by applying the bootstrapping approach with 1000 re-sampling. With two linked QTL, the 95% CI for each was determined by fitting one of them as a cofactor for the other. The phenotype variation that was explained by a QTL was calculated by the following equation.

$$Var\% = \frac{MS_R - MS_F}{MS_R} \times 100$$

Where, MS_R was the mean of square of the reduced model; MS_F was the mean of square of the full model.

3.3.2 Association analysis

All association analyses were performed with SAS software version 9.2 using PROC freq, PROC logistic and PROC GLM (SAS Inc., Cary, NC, USA). Genotype frequency deviation from Hardy-Weinberg equilibrium was analyzed by χ^2 test. The association between SNPs and phenotypic variation was analyzed using a GLM and logistic procedure of the SAS package. The logistic model was used for all the ordinary traits. Odds ratio (OR) and 95% confidence interval (CI) were calculated as an estimate of risk. A general linear model was fitted for the DXA traits. The analyses were done for each gene separately within each of the pig populations. Apart from the fixed effect of the genotype, the model included the fixed effect of parity for all traits in the DuPi population. Average daily gain was used as covariate for leg and feet score and age was used as covariate for OC score. Slaughter weight and carcass length were used as covariate for DXA traits. The association model for the commercial population included the fixed effect of sex and slaughter weight as covariate. The model for association analysis was:

$$Y_{ijkl} = \mu + GENO_j + Fix_k + Cov_l + e_{ijkl}$$

Where: Y_{ijkl} = the phenotype traits measured on the individual i

μ = the overall mean of the trait

$GENO_j$ = the fixed effects of SNP genotype for each gene j

FIX_k = the fixed effects for traits k

COV_l = the covariance for traits

e_{ijkl} = the residual error associated with the observation

In addition, a principal component analysis (PCA) was conducted with the PRINCOMP procedure of the SAS package. The first component of PCA is the mathematical combination of measurements explaining the largest amount of variability in the data, and the association analyses between the SNPs and principal components (PC1, PC2 and PC3) in this study were performed using the GLM model as described above.

4 Results

4.1 Phenotype characteristic of the DuPi resource population

The descriptive statistics of leg weakness related traits are given in Table 7 and 8. The results showed that parity, carcass length, slaughter weight, age and average daily gain had an effect on the measured traits (Table 9). Parity, carcass length and average daily gain showed an effect on FLS but only average daily gain (ADG) had an effect on RLS. Parity showed effects on FFS, HH, CMH and HF. Moreover, age was also found to have an effect on HF. Parity, carcass length and slaughter weight affected all DXA traits (Table 10). BMD and BMC were highly correlated ($P < 0.01$) with the slaughter weight of the animals ($r = 0.54$ and 0.71, respectively).

Principle component (PC) analysis was performed on leg and feet scores and OC scores separately because of the low phenotypic correlations between the traits. For leg and feet scores, the cumulative proportion of the first three principal components (PC1, PC2 and PC3) reached 86%. The PC1 was mainly comprised of fore and rear leg scores, which explained 63% of total variation. For OC traits, PC1, PC2 and PC3 accounted for 80% of total variation. The PC1 mainly included OC scores at HH and HF obtained around 57% of total variation (Table 11).

4.1.1 Leg and feet scores

The highest percentage of the animals showed moderate fore feet score (FFS) (79.35%) and good rear feet scores (RFS) (54.52%). Only 9.03% and 1.29% animals showed poor feet scores for fore and rear feet, respectively. For the fore leg score (FFS), the highest numbers of animals were in score 2 (42.26%). In case of rear leg scores (RLS), the highest number of animals were in score 3 (54.84%). Only a low percentage of animals had very poor leg score (4.84% for fore leg and 0.32% for rear leg). The phenotypic correlations (Table 8) among FLS, RLS, FFS and RLS were low to medium, ranging from 0.19 to 0.44.

4.1.2 Osteochondrosis score

Head of humerus (278) of shoulder joint and condylous medialis humeri (CMH) (279) of elbow joint of pig left fore limb and head of femur (274) of hip joint and condylous medilais femori (CMF) (277) of knee joint of pig left hind or rear limb were considered for the present study. OC lesions observed were defined 13.99, 7.94, 46.30 and 31.77% with score 1, 2, 3 and 4, respectively. However, CMF of knee joint was found to be exposed highest to severe OC score (score 1) than other bones. HH of shoulder joint was exposed lowest to score 1. On the other hand, CMH and HH of fore limb showed higher healthy score (score 4) than that of CMF and HF. This observation suggested that fore limb is less susceptible to OC than the rear limb. However, the distribution, shown in Table 13, indicated that the majority of the animals had some problems. The phenotypic correlations (Table 8) among OC score were very low, ranging from -0.13 to 0.12.

4.1.3 DXA traits

The difference of DXA traits between sexes is given in Table 9. BMD and BMC were higher in castrated males than in females. However, BMD and BMC were not significantly different between castrated males and females pigs in this study. A strong positive phenotypic correlation between BMD and BMC was found (r = 0.70, $P<0.01$). BMC showed significant ($P \leq 0.01$) and strong correlations with BMD (r = 0.7) and BMA (r = 0.6). On the contrary, BMA showed significant ($P \leq 0.01$) weak and negative correlation with BMD (r=-0.2).

Table 7: Numbers and percentage of animals at different leg and feet and osteochondrosis scores[1]

Trait[1] (n)	Number (%)				
Leg score[2]	**5**	**4**	**3**	**2**	**1**
FLS (310)	64(20.65)	131(42.26)	64(20.65)	36(11.61)	15(4.84)
RLS (310)	170(54.84)	107(34.52)	25(8.06)	7(2.26)	1(0.32)
Feet score[3]			**3**	**2**	**1**
FFS (310)			36(11.61)	246(79.35)	28(9.03)
RFS (310)			169(54.52)	137(44.19)	4(1.29)
OC score[4]		**4**	**3**	**2**	**1**
HH (278)		109 (39.21)	136 (48.92)	19 (6.83)	4 (5.04)
CMH (279)		125 (44.80)	109 (39.07)	15 (5.38)	0 (10.75)
HF (274)		74 (27.01)	156 (56.93)	19 (6.93)	5 (9.12)
CMF (277)		44 (15.88)	112 (40.43)	35 (12.65)	6(31.05)
Total (1108)		352 (31.77)	513 (46.30)	88 (7.94)	55 (13.99)

[1]FLS : front leg score, RLS : rear leg score, FFS : front feet score, RFS : rear feet score, HH : Osteochondrosis (OC) at head of humerus, HF: OC at head of femur, CMH: OC at condylus medialis humeri, CMF: OC at condylus medialis femoris

[2] on a scale 1 (poor, very blukled), 3 (very good) and 5 (poor, flexing)

[3]on a scale 1 (poor) to 3 (good)

[4]on a scale 1 (poor) to 4 (good)

Table 8: Statistics and phenotype correlation between leg and feet and OC scores

Traits[1]	N	Mean	SD	Min	Max	Phenotype correlation FLS	RLS	FFS	RFS	HH	CMH	HF	CMF
FLS	310	2.65	1.08	1	5		0.28	0.23	0.22	0.08	-0.02	0.02	-0.04
RLS	310	2.70	0.66	1	5			0.23	0.19	-0.08	-0.003	-0.003	-0.05
FFS	310	2.02	0.45	1	3				0.44	-0.08	-0.12	0.01	-0.07
RFS	310	2.53	0.53	1	3					-0.01	-0.06	0.04	-0.13
HH	278	1.78	0.78	1	4						0.12	0.07	-0.007
CMH	279	1.82	0.95	1	4							0.11	-0.07
HF	274	1.98	0.84	1	4								-0.10
CMF	277	2.59	1.09	1	4								

[1]FLS=fore leg score; RLS=rear leg score; FFS=fore feet score; RFS=rear feet score; HH= OC score at head of humerus;

CMH= OC score at condylus medials humeri; HF= score OC at head of femur; CMF= score OC at condylus medialis femori.

Table 9: Descriptive statistics of BMD, BMC and BMA of the DuPi resource population

Traits[1]	Total Mean ±SD	Min	Max	N	Female Mean ±SD	Min	Max	n	Castrated male Mean ±SD	Min	Max	Mean ±SD	n
BMD (g/cm^2)	0.96 ±0.08	0.69	1.25	275	0.95 ±0.07	0.79	1.172	145	0.96 ±0.09	0.69	1.25	0.96 ±0.09	130
BMC (g)	66.72 ±7.07	45.53	87.36	275	66.38 ±6.03	45.53	83.29	145	67.02 ±7.69	48.42	87.36	67.02 ±7.69	130
BMA (cm^2)	69.67 ±5.26	55.91	84.64	275	69.75 ±5.22	55.91	84.64	145	69.62 ±5.36	57.36	83.29	69.62 ±5.36	130

[1]BMD : Bone mineral density, BMC : Bone mineral content, BMA: Bone area.

Table 10: Effect of production and carcass traits on leg and feet scores, OC score and DXA traits[1]

Traits		n	Mean ±SE	Mini/max	Model	R^2	Dam	Sire	Age	ADG	Slaughter weight	Carcass length	Parity
Leg score	FLS	310	2.65±0.94	1/5	***	0.38	***	*	ns	**	ns	*	***
	RLS	310	3.17±0.55	1/5	*	0.02	ns	ns	ns	*	ns	ns	ns
Feet score	FFS	310	2.02±0.44	1/3	**	0.18	*	ns	ns	ns	ns	ns	*
	RFS	310	2.53±0.48	1/3	***	0.21	***	ns	ns	ns	ns	ns	ns
OC	HH	278	1.78±0.74	1/4	**	0.21	*	ns	ns	ns	ns	ns	**
	CMH	279	1.82±0.89	1/4	***	0.24	**	ns	ns	ns	ns	ns	***
	HF	274	1.98±0.81	1/4	*	0.20	*	ns	**	ns	ns	ns	***
	CMF	277	2.59±1.08	1/4	*	0.02	*	ns	ns	ns	ns	ns	ns
DXA	BMD	275	0.96±0.07	0.69/1.25	***	0.42	*	ns	ns	ns	***	ns	***
	BMC	275	66.72±4.31	45.53/87.36	***	0.68	***	ns	ns	ns	***	***	**
	BMA	275	69.67±3.86	55.91/84.64	***	0.54	***	ns	ns	ns	***	***	**

[1]FLS=fore leg score; RLS=rear leg score; FFS=fore feet score; RFS=rear feet score; HH= OC score at head of humerus; CMH= OC score at condylus medials humeri; HF= score OC at head of femur; CMF= score OC at condylus medialis femori, BMD : Bone mineral density, BMC : Bone mineral content, BMA: Bone area. ADG= average daily gain

Table 11: Rotated factor pattern and final communality[1] estimates from principal component analysis of leg weakness-related traits[2]

Traits	h^2	Component		
		1	2	3
HH	0.72	0.87	0.17	0.01
HF	0.32	0.05	-0.04	0.99
CMH	0.52	0.61	-0.47	0.12
CMF	0.75	0.07	0.91	-0.02
FLS	0.81	-0.08	0.04	0.99
RLS	0.33	-0.10	0.99	0.04
FFS	0.72	0.86	-0.03	-0.03
RFS	0.70	0.83	-0.11	-0.08

[1] Communality estimates appear in column headed h^2

[2] FLS=fore leg score; RLS=rear leg score; FFS=fore feet score; RFS=rear feet score; HH= OC score at head of humerus; CMH= OC score at condylus medials humeri; HF= score OC at head of femur; CMF= score OC at condylus medialis femori

4.2 Phenotype characteristic of the animals used for association analysis

The distribution of osteochondrosis scores for the commercial population is shown in Table 12. Among all the investigated regions, head of humerus (HH) and head of femur (HF) showed litter affected by OC, while the most affected regions were distal epiphysis (DEU), condylus medialis femoris (CMF) and condylus medialis humeri (CMH), respectively.

Table 12: Number of the animals at different OC scores[1] of the commercial population

	n (scores)					
Male	1	2	3	4	5	6
Head of humerus	130	-	-	-	-	-
Condylus medialis humeri	110	8	12	-	-	-
Condylus lateral humeri	125	4	1	-	-	-
Radius/Ulna prox	119	11		-	-	-
Distal epiphys. Fug Ulna	15	62	6	14	32	1
Head of femur	129	1	-	-	-	-
Condylus medialis femoris	85	37	7	1	-	-
Female	1	2	3	4	5	6
Head of humerus	166	-	2	1	-	-
Condylus medialis humeri	130	23	16	-	-	-
Condylus lateral humeri	166	-	2	4	-	-
Radius/Ulna prox	155	14	-	-	-	-
Distal epiphys. Fug Ulna	16	72	9	33	38	1
Head of femur	169	-	-	-	-	-
Condylus medialis femoris	104	50	14	1	-	-
Total	1	2	3	4	5	6
Head of humerus	296	-	2	1	-	-
Condylus medialis humeri	240	31	28	-	-	-
Condylus lateral humeri	291	4	3	1	-	-
Radius/Ulna prox	274	25	-	-	-	-
Distal epiphys. Fug Ulna	31	134	15	47	70	2
Head of femur	298	1	-	-	-	-
Condylus medialis femoris	189	87	21	2	-	-

[1] Lesions are scored from 1 to 6 with score 1= normal and score 2 to 6 = mild to severe lesions

4.3 QTL for leg weakness related traits

A total of 16 chromosomal QTL regions (Table 13) were identified for leg weakness related traits on ten porcine autosomes in this study. All QTL reached the 5% chromosome-wide significance level. In this study, most estimated QTL effects appeared to be isolated effects on one individual trait showing no relation to other traits. There were however some chromosomal regions, which influenced more than one of the traits, notably on SSC2 and SSC5.

4.3.1 QTL for leg and feet score

Two chromosomal regions were identified for FLS ($P \leq 0.01$, CW), at 54 cM on SSC5 and at 16 cM on SSC13. One QTL at 0 cM ($P \leq 0.01$, CW) on SSC14 was identified for RLS. Three QTL were identified for fore feet score ($P \leq 0.05$, CW) at 166 cM, 60 cM and 33 cM on SSC1, SSC5 and SSC16, respectively (Table 14). No QTL was found for rear feet score.

4.3.2 QTL for osteochondrosis score

The QTL for OC were located on SSC3, 5, 6, 9 and 17 (Table 14). OC score of HH was influenced by three QTL regions on SSC3, 6 and 9 at 7, 57 and 60 cM, respectively. Among them, a QTL for HH on SSC3 reached 5% genome-wide significance level, the remaining QTL were suggestive. A QTL for CMH was identified at 159 cM on SSC5. Two chromosomal regions affecting OC score of HF were identified on each of SSC9 and 17 at 26 and 125 cM, respectively. However, no suggestive QTL was found for OC score of CMF.

4.3.3 QTL fore bone density traits

Four chromosomal regions were identified affecting bone mineral-related traits, of which three were for BMD and one was for BMC. QTL influencing BMD were found on SSC2, 3 and 9 at positions 7, 82 and 33 cM, respectively. Only one QTL affecting BMC was found at 0 cM on SSC2 which reached 5% GW in this study.

Table 13: Location and estimated effected of the identified QTL for leg weakness-related traits

SSC[a]	Trait[b]	POS[c]	F[d]	A±se[e]	D±se[f]	Var%[g]	CI95[h]	Closest markers[i]
1	FFS	166	6.55*	-0.13±0.05	0.23±0.08	3.98	35 - 203	S0155
2	BMC	0	9.36***	-2.53±0.64	4.37±2.06	6.11	0 - 103	SW2443
2	BMD	7	4.91*	-0.03±0.01	0.08±0.04	4.00	0 - 101.5	SW2443
3	BMD	82	7.00**	-0.03±0.01	-0.05±0.02	4.00	0 - 96	S0002-SW2570
3	HH	7	8.21***	0.07±0.09	0.71±0.18	5.24	0 - 33	SW72
5	FFS	60	6.16*	-0.00±0.05	0.22±0.09	5.50	0 - 195.5	SWR453
5	CMH	159	4.89*	-0.30±0.10	0.48±0.25	4.30	0 - 168	IGF1
5	FLS	54	7.29**	0.04±0.09	-0.59±0.15	4.11	43 - 183	SW1482
6	HH	57	6.17*	-0.28±0.09	-0.32±0.19	3.80	19.5 - 150	S0087
9	BMD	33	5.28*	0.05±0.02	0.19±0.08	4.00	0 - 167	SW21-MMP3
9	HH	60	5.26*	-0.31±0.17	-1.65±0.57	3.17	36 - 158	MMP3
9	HF	26	4.98*	-0.20±0.20	-0.22±0.90	4.40	0 - 167	SW21-MMP3
13	FLS	16	9.16**	-0.31±0.10	0.60±0.22	5.26	0 - 74	S0219
14	RLS	0	6.62**	0.12±0.09	-0.44±0.13	3.69	0 - 142	SW857
16	FFS	33	4.87*	-0.15±0.28	-2.09±0.69	3.18	0 - 156	S0111-S0026
17	HF	125	6.42*	-0.71±0.20	0.92±0.54	4.06	41 - 140	SW2431

[a] Sus scrofa chromosome.
[b]Trait abbreviations: FLS= fore leg score; RLS= rear leg score; FFS = fore feet score; RFS = rear feet score; OC=osteochondrosis; HH= OC at head of humerus; CMH= OC at condylus medials humeri; HF= OC at head of femur; CMF= OC at condylus medialis femori; BMD = bone mineral density; BMC = bone mineral contents.
[c]chromosomal position in Kosambi cM.
[d]Significance of the QTL: *, significant on a chromosome-wide level with $P \leq 0.05$; **, significant on a chromosome-wide level with $P \leq 0.01$; ***, significant on a genome-wide level with $P \leq 0.05$.
[e]Additive effect and standard error. Positive values indicate the Duroc alleles result in higher values than Pietrain alleles; negative values indicate that Duroc alleles result in lower values than Pietrain alleles.
[f]Dominance effect and standard error.
[g]The percentage of phenotypic variance explained by the QTL
[h]95% confidence interval.
[i]The marker closest to the QTL peak as close as possible.

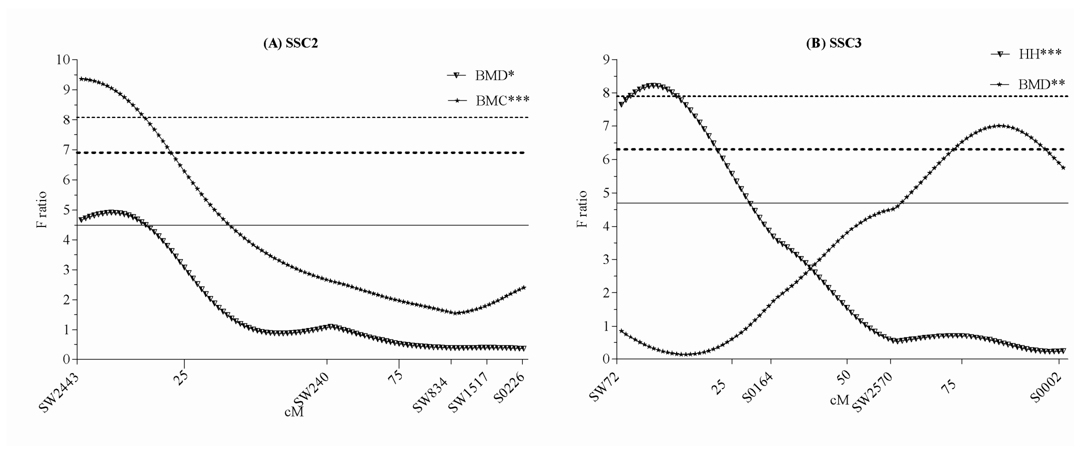

Figure 11: Evidence of QTL for leg weakness related traits on SSC2 and SSC3. Marker positions along each chromosome are indicated in cM on the x-axis, the y-axis showed *F* values. The 5% chromosome-wide, 1% chromosome-wide, 5% genome-wide were indicated by solid, horizontal dashed, and dotted-line, respectively

4.4 Gene characterization and detection of polymorphisms

4.4.1 Collagen type II alpha I (*COL2A1*)

The porcine *COL2A1* mRNA sequence (GenBank: XM_001925959.1) consists of 2550 bp. The porcine COL2A1 protein consists of 849 amino acids which show 95% homology to the human and canine sequences (GenBank: AAC41772.1 and NP_001006952.1, respectively). Porcine *COL2A1* consist of 38 exons with the sequence range from 6 to 143 nt (Figure 12). The comparative sequencing analysis revealed 8 SNP in the intron 29 (Table 14). One silent SNP (GenBank: FJ788665 g.80C>T) was selected for genotyping.

Table 14: List of detected SNP in porcine *COL2A1* gene

Acc No, SNP site	F Primer	R Primer	Position	SNP
FJ788665 g.80 C>T	COL2A1-F1	COL2A1-R1	I-29	C-T
FJ788665 g.85 C>T	COL2A1-F1	COL2A1-R1	I-29	C-T
FJ788665 g.97 C>T	COL2A1-F1	COL2A1-R1	I-29	C-T
FJ788665 g.156 G>A	COL2A1-F1	COL2A1-R1	I-29	G-A
FJ788665 g.184 C>T	COL2A1-F1	COL2A1-R1	I-29	C-T
FJ788665 g.244 G>A	COL2A1-F1	COL2A1-R1	I-29	G-A
FJ788665 g.273 G>A	COL2A1-F1	COL2A1-R1	I-29	G-A
FJ788665 g.335 C>T	COL2A1-F1	COL2A1-R1	I-29	C-T

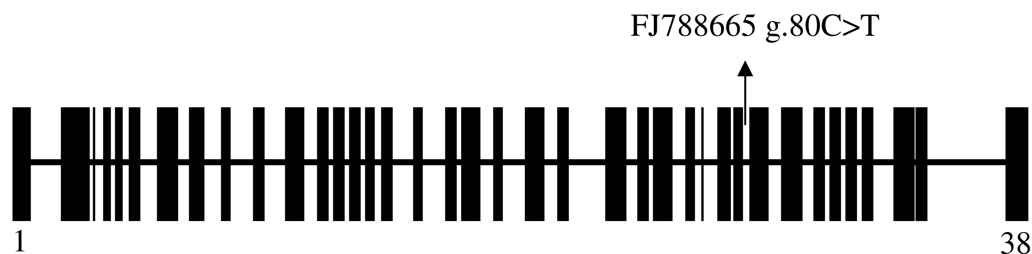

Figure 12: Porcine *COL2A1* gene structure and position of the identified SNP

4.4.2 Collagen type X alpha I (*COL10A1*)

The porcine *COL10A1* mRNA sequence (GenBank: NM_214392.1) consists of 2125 bp including 97 bp of 5′ untranslated region (UTR) and 2028 bp of the coding region. The gene consists of 3 exons ranging from 82 to 1870 nt. The gene encodes a 675 amino acid protein which shows 100% homology to the bovine (GenBank: NP_777059.1), 100% to the human (GenBank: NP_000484.2) and 77% to the murine sequence (GenBank: CAA44741.1). The comparative analysis between porcine mRNA sequences (GenBank: NM_001005153.1) and the genomic DNA sequence (GenBank: NC_010443.1) revealed that the structure of porcine *COL10A1* gene contains 3 exons. Comparative sequencing showed 1 SNP within 3′ UTR (g.72 C>T, GenBank: AF222861).

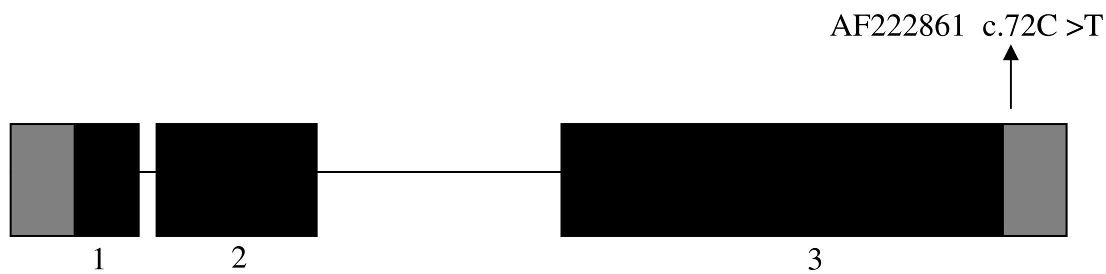

Figure 13: Porcine *COL10A1* gene structure and position of the identified SNP (gray colour indicated the UTR regions)

4.4.3 Matrix gla protein (*MGP*)

The porcine *MGP* mRNA sequence (GenBank: GU391766.1) consists of 606 bp including 69 bp of 5´UTR, 312 bp of coding region and 225 bp of 3´UTR. The coding region encodes 103 amino acids (GenBank: ADD22717) which show 100% homology to the human (GenBank: CAA37418.1), 100% to the bovine (GenBank: NP_777132.1) and also 100% to the murine sequences (GenBank: BAE35492.1). None of the polymorphisms was identified at the 5´UTR of the porcine *MGP*. Three SNP (g.115A>G, g.1073C>T and g.1135C>A; all positions refer to Genbank accession number NC_010447: region 22960152 to 22964239) were located in intron 1 and one SNP was located at 3´UTR (g.3767C >T). For association analysis, a SNP g.3767C >T was selected for genotyping.

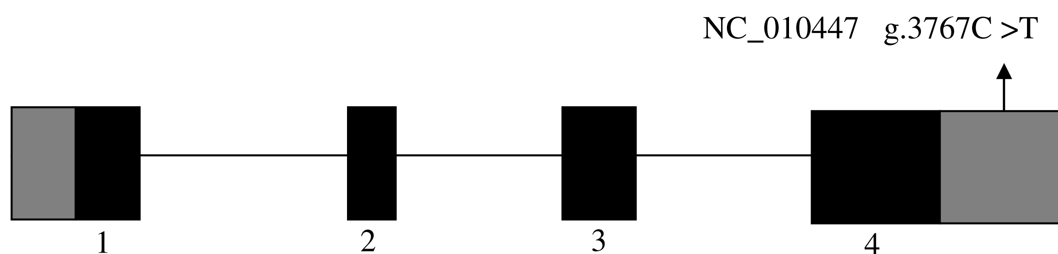

Figure 14: Porcine *MGP* gene structure and position of the identified SNP (gray colour indicated the UTR regions)

4.4.4 Matrix metallopeptidase 3 (*MMP3*)

The porcine *MMP3* mRNA sequence (GenBank: NM_001166308) consists of 1640 bp. The coding region encodes 477 amino acids (GenBank: NP_001159780). The structure of the porcine *MMP3* gene was deducted by comparison of the porcine *MMP3* mRNA sequence, human *MMP3* mRNA sequence (GenBank: NM_002422) and human genomic DNA sequence (GenBank: NC_000011.9) and revealed 10 exons. Screening for SNP showed one SNP in the intron 2 (GenBank: FJ788664 g.158 C>T) which was selected for genotyping.

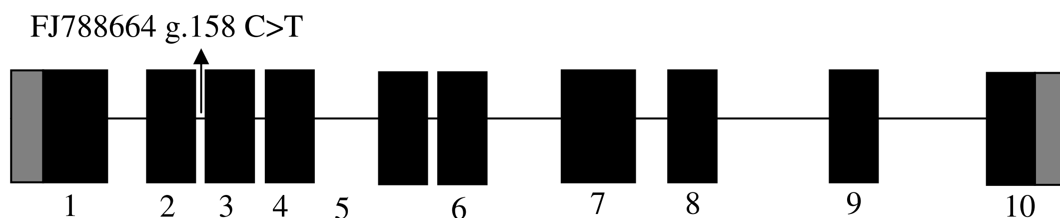

Figure 15: Porcine *MMP3* gene structure and position of the identified SNP (gray colour indicated the UTR regions)

4.4.5 Transforming growth factor beta 1 (*TGFβ1*)

The porcine TG*Fβ1* mRNA sequence (GenBank: NM_214015) consists of 3206 nt. The coding region encodes 390 amino acids (GenBank: NP_999180.1). The structure of porcine *TGFβ1* gene was deducted by comparison of the porcine *TGFβ1* mRNA sequence, the human *TGFβ1* mRNA sequence (GenBank: NM_000660) and the human genomic DNA sequence (GenBank: NC_000019.9) and revealed 7 exons. The SNP identified in the intron 6 (GenBank: g.797 G>A, GenBank: AF461808) was used for genotyping.

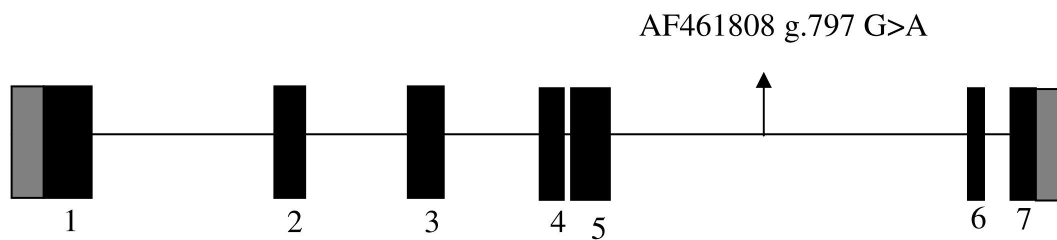

Figure 16: Porcine *TGFβ1* gene structure and position of the identified SNP (gray colour indicated the UTR regions)

4.5 Association of candidate genes with leg weakness related traits

In this study, only *MMP3, COL10A1,* and *TGFβ1* were genotyped in both populations. *COL2A1* and *MGP* were successful genotyped only in the DuPi population due to the lack of DNA from the commercial population. Genotype and allele frequencies of each SNP are presented in Table 15. Single SNPs were identified in each of *MMP3* (g.158 C>T, GenBank: FJ788664), *COL10A1* (g.72 C>T, GenBank: AF222861) and *TGFβ1* (g.180 G>A, GenBank: AJ621785). The SNP genotype frequencies of *MMP3* g.158 C>T and *COL10A1* g.72 C>T in the DuPi and of *TGFβ1* g.180 G>A in the commercial population deviated from Hardy Weinberg equilibrium. In contrast, genotype frequencies of *MMP3* g.158 C>T and *COL10A1* g.72 C>T in the commercial population and *TGFβ1* g.180 G>A in DuPi population were in Hardy Weinberg equilibrium ($P \leq 0.05$).

The associations between polymorphisms of *TGFβ1, MMP3* and *COL10A1* genes and leg, feet and OC scores were estimated by calculating OR and 95% confidence intervals (CI). The results (Table 17) showed that scores of OC at the HF region in the DuPi population increased 2.003–fold (95% CI>0.920-4.362) with at least one homozygous genotype *MMP3* g.158 CC compared with another homozygous genotype g.158 TT. A significant association was found between *TGFβ1* g.180 G>A and RLS in the DuPi population, with the decrease of 0.377-fold (AA-genotype is reference, 95% CI>0.164-0.867). However, no significant results were found in genotype and allele frequencies of

TGFβ1 g.180 G>A, *MMP3* g.158 C>T, and *COL10A1* g.72 C>T with the OC traits in the commercial population.

Considering the effects of *TGFβ1* g.180 G>A, *MMP3* g.158 C>T, and *COL10A1* g.72 C>T SNPs on bone mineral status of the DuPi population, a significant association was found between *MMP3* g.158 C>T genotype with BMD, BMC and BMA traits (Table 16). Comparing the different genotypes, *MMP3* g.158 CC genotype had a higher least square mean of BMD compared with g.158 TT genotype (0.976 ± 0.008 g/cm2 vs 0.943 ± 0.01 g/cm2), with a significant additive effect for allele C (0.016 ± 0.007 g/cm2 , *P* =0.07). The heterozygous genotype *MMP3* g.158CT showed a higher BMC and BMA than both of the homozygous genotypes. No significant differences were observed for *COL10A1* g.72 C>T and *TGFβ1* g.180 G>A with any of the DXA traits (data not shown).

For the association between SNPs and PCs, the SNP *TGFβ1* g.180 G>A showed a significant ($P < 0.05$) association with PC2 of feet and legs scores and was almost ($P = 0.054$) associated with PC3 of OC scores (Table 18). The SNPs of the genes *COL10A1* and *MMP3* were not association with any of the PCs. From the results of association analyses for single traits and PCs, the *TGFβ1* gene was significantly ($P < 0.05$) associated with leg score traits.

Table 15: Allele and genotype frequencies and Hardy Weinberg equilibrium of the three SNPs in the *MMP3*, *COL10A1* and *TGFβ1* genes

SNP	**Allele**	**DuPi** [1] **(Frequency, %)**	**CP**[2] **(Frequency, %)**	**Genotype**	**DuPi (Frequency, %)**	**HWE**[3] **χ2 (p)**	**CP (Frequency, %)**	**HWE χ2 (p)**
MMP3	C	58.85	45.89	CC	29.50	4.49 (0.03)	19.64	0.91 (0.34)
	T	41.15	54.11	CT	58.70		52.5	
				TT	11.80		27.86	
COL10A1	C	53.05	83.96	CC	35.26	8.17 (0.004)	70.42	0.01 (0.89)
	T	46.96	16.04	CT	35.58		27.11	
				TT	29.17		2.46	
TGFβ1	A	67.64	83.21	AA	47.95	1.01 (0.31)	71.32	5.91 (0.02)
	G	32.36	16.79	AG	39.38		23.77	
				GG	12.67		0	

[1] DuPi: Duroc x Pietrain population; Pi : Pietrain population, [2]CP : Commercial population, [3]HWE : Hardy weinberg equilibrium test

Table 16: Association of *MMP3* g.158 C>T with bone density traits in the DuPi[1] resource population

Traits[2]	**Genotype (Lsmean ± SE)**[3]			**Effect (Lsmean ± SE)**[4]	
	CC	**CT**	**TT**	**Additive**	**Dominance**
BMD	0.976 (0.008)[a]	0.956 (0.006)[a,b]	0.943 (0.01)[b]	0.016 (0.007)*	0.0039 (0.01)
BMC	66.75 (0.56)[a,b]	67.11 (0.45)[a]	64.88 (0.89)[b]	0.935 (0.523)	-1.295 (0.688)
BMA	68.48 (0.54)[a]	70.31 (0.43)[b]	68.78 (0.85)[a,b]	-0.149 (0.502)	-1.679 (0.659)*

[1]DuPi: Duroc x Pietrain population; Pi : Pietrain population
[2]BMD : Bone mineral density, BMC : Bone mineral content , BMA; Bone area.
[3]Least square mean values with superscripts a and b are different at $P < 0.05$.
[4]Least square mean values of additive (a) and dominance (d) effects of *MMP3* alleles: * $P \leq 0.05$

Table 17: Association of SNP in the *MMP3*, *COL10A1* and *TGFβ1* genes with leg weakness related traits in the DuPi[1] and commercial populations

Population	Genes	Traits[2]	OR[3]	95% CI[4]	*P*[5]
DuPi	*MMP3*	HH	0.797	0.369-1.722	0.79
		HF	2.003	0.920-4.362	0.04**
		CMH	0.831	0.386-1.790	0.68
		CMF	0.926	0.444-1.932	0.77
		FLS	1.898	0.785-4.590	0.12
		RLS	0.734	0.345-1.562	0.23
		FFS	0.828	0.322-2.127	0.47
		RLS	1.011	0.473-2.159	0.56
	COL10A1	HH	1.521	0.851-2.718	0.36
		HF	1.191	0.662-2.144	0.72
		CMH	0.91	0.522-1.587	0.06*
		CMF	0.963	0.555-1.673	0.15
		FLS	1.907	1.040-3.499	0.10
		RLS	0.973	0.554-1.708	0.98
		FFS	1.116	0.550-2.262	0.76
		RLS	0.871	0.496-1.529	0.86
	TGFβ1	HH	0.738	0.350-1.555	0.53
		HF	0.679	0.315-1.462	0.14
		CMH	0.854	0.399-1.828	0.85
		CMF	0.771	0.373-1.596	0.68
		FLS	1.491	0.678-3.283	0.61
		RLS	0.377	0.164-0.867	0.03**
		FFS	2.699	1.054-6.913	0.08*
		RLS	1.606	0.746-3.457	0.10*
Commercial population	*MMP3*	CMH	1.53	0.63-3.73	0.63
		DEU	1.07	0.56-2.03	0.93
		CMF	0.96	0.47-1.96	0.95
	COL10A1	CMH	na	Na	na
		DEU	1.1	0.27-4.37	0.99
		CMF	2.16	0.52-8.98	0.56
	TGFβ1	CMH	0.39	0.05-2.92	0.6
		DEU	0.41	0.23-3.03	0.19
		CMF	2.07	0.71-6.02	0.36

[1]DuPi: Duroc x Pietrain population. [2]Estimates odds ratio test between homozygous genotype.

[3]95% Wald confidence limits. [4]Significant level*P≤0.10, **P≤0.05, ***≤0.01.

Table 18: The association analyses between SNP of *TGFβ1* and principal components of the Dupi[1] population

Traits[2]	LSM[3]			*P* value
	AA	AG	GG	
OC PC1	0.042	-0.022	-0.107	0.79
OC PC2	0.050	-0.069	-0.062	0.70
OC PC3	0.203[a]	-0.172[b]	0.009	0.05
FL PC1	-0.115	-0.071	0.228	0.17
FL PC2	-0.014	0.081[a]	-0.411[b]	0.04
FL PC3	-0.012	0.018	0.019	0.57

[1] DuPi: Duroc x Pietrain population

[2]OC: Osteochondrosis scores, PC: Principle component, FL: Feet and legs scores

[3] Least square mean values with superscripts [a] and [b] are different at $P < 0.05$.

4.6 Expression of candidate genes in OC cartilage

Initial studies were performed to investigate the expression of two internal controls *GAPDH* and *TBP* in normal and OC cartilage in order to justify them as internal control. The results showed that expression of *GAPDH* and *TBP* were not different between OC and normal cartilage, this means that the expressions of both genes are accurate to be used as the internal control (Figure 18). Relative expression levels of 6 genes known to be important in the regulation and involved in the endochondral ossification were examined in OC lesions and healthy cartilage in this study. The mRNA expression of *TGFβ1* and *RUNX2* were significantly higher ($P \leq 0.05$) in the OC cartilages compared with healthy cartilage (Figure 18). There were no significant differences in mRNA expression of *MMP3*, *SOX9*, *MMP9* and *COL10A1* genes (Figure 17 and Figure 18). However, *MMP3* tended to be expressed lower in OC cartilage while *SOX9* tended to be up regulated in OC lesions. *MGP* was lower expressed in OC cartilage (Figure 20). Among the genes examined, the transcription factor *SOX9* gene was highest expressed where *MMP9* was lowest expressed in articular cartilage compared with the other genes.

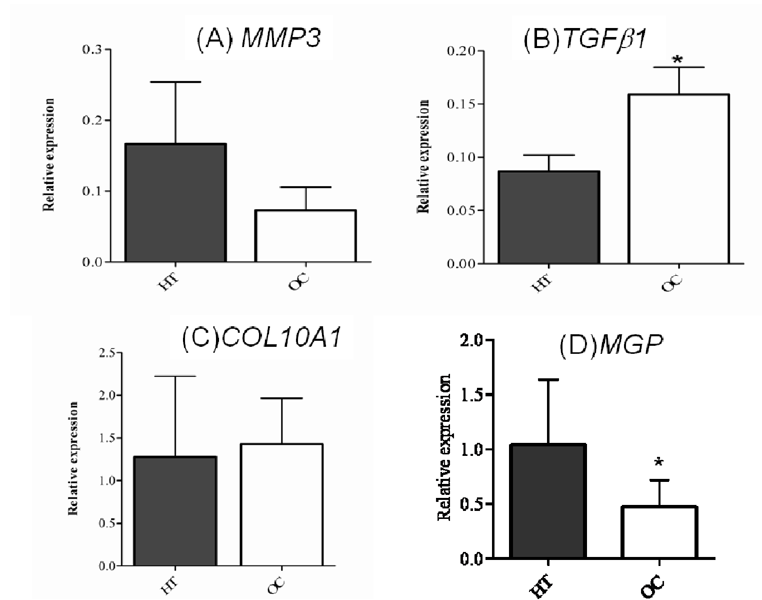

Figure 17: Relative expression of (A) *MMP3,* (B) *TGFβ1,* (C) *COL10A1,* (D) *MGP* genes in healthy (HT) and osteochondrosis (OC) cartilage. Results presented as mean ± SD

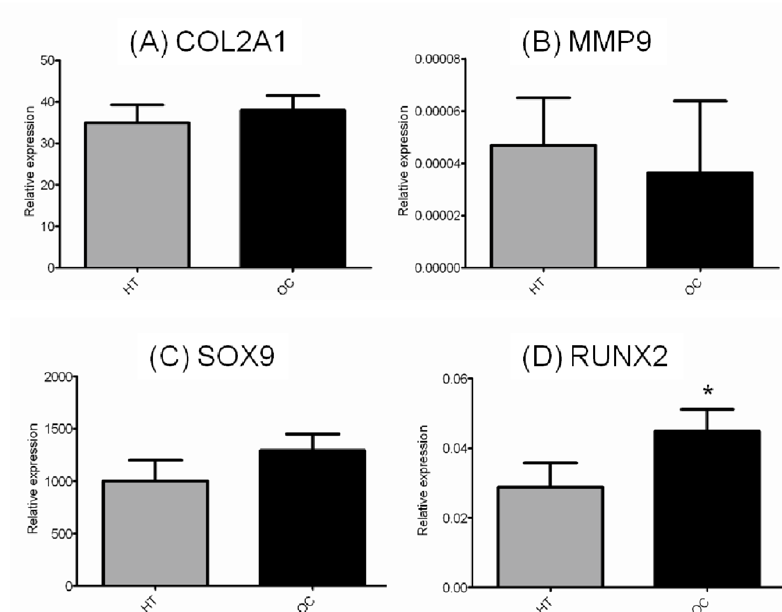

Figure 18: Relative expression of (A) *COL2A1*, (B) *MMP9*, (C) *SOX9* and (D) *RUNX2* in healthy (HT) and osteochondrosis (OC) cartilage. Results presented as mean ± SD, *$P<0.05$

4.7 Molecular characterization of the *MGP* gene

4.7.1 Characterization of the porcine *MGP* gene

The genomic sequence of *MGP* was PCR amplified and sequenced using oligonucleotide primers designed from the *MGP* sequence (GenBank NC_010447, region 22960152 to 22964239). The coding region of the porcine *MGP* contains four exons and three introns. The intron-exon organization of the genomic *MGP* sequence is shown in Table 19. Comparison of the porcine with the human *MGP* gene revealed that the lengths of the exons and introns were almost identical. The full-length *MGP* cDNA isolated from articular cartilage consists of 606 bp with a 69-bp 5′ UTR, a 312-bp open reading frame with a start codon, and a 225-bp 3′ UTR (GenBank accession GU391766). The amino acid sequence similarity between porcine MGP and MGP proteins from man, cattle, rat and mouse was determined by the Clustal method (Figure 19). The alignment showed that the porcine MGP shares common structures and functional elements with MGP orthologous from other species. Completely conserved regions of protein sequence are present in almost all of the aligned mammalian sequences at the glutamic acid (E) residues.

Table 19: Discription of exon and intron boundary of the porcine *MGP* gene

Exon	**bp**	**Splicing donor**	**Intron**	**Splicing acceptor**
1	60	CCTGTGCTATgttgag	1437	tcgagAATCTCATGA
2	33	TATGAAATCAgtaagt	1091	ttaaagATCCCTTCCT
3	78	CCCAAGAGAGgtgggt	972	ttctagAATCCGAGAA
4	287	GGCCAAATAAGaccag		

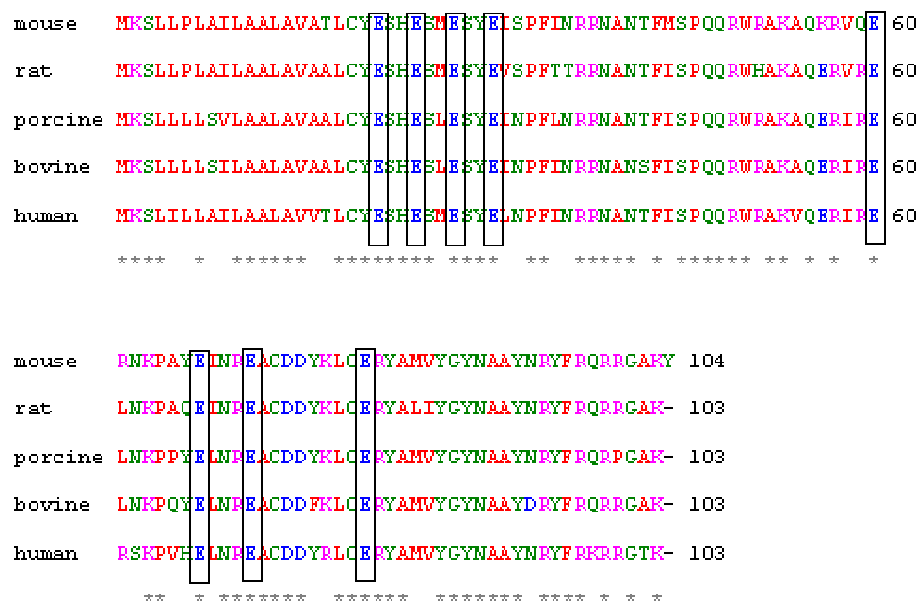

Figure 19: A comparison of the peptide sequence of the porcine MGP (GenBank accession NP_999281.1) protein with the peptide of the human (GenBank accession NP_000891.2), bovine (GenBank accession NP_777132.1), rat (GenBank accession NP_036994.1) and mouse (GenBank accession NP_032623.1). Identical amino acid residues are indicated by asterisk. Conserved glutamic acid (E) residues are bounded

4.7.2 MGP expression in healthy and osteochondrosis cartilage

The Western blot result showed that the MGP protein expression was lower ($P < 0.05$) in OC compared with normal cartilage. This appeared to be consistent with results from the transcription level. The bar graph depicts the means (+SD) ratio of intensity of MGP-to-GAPDH bands showed in figure 21B and 21C.

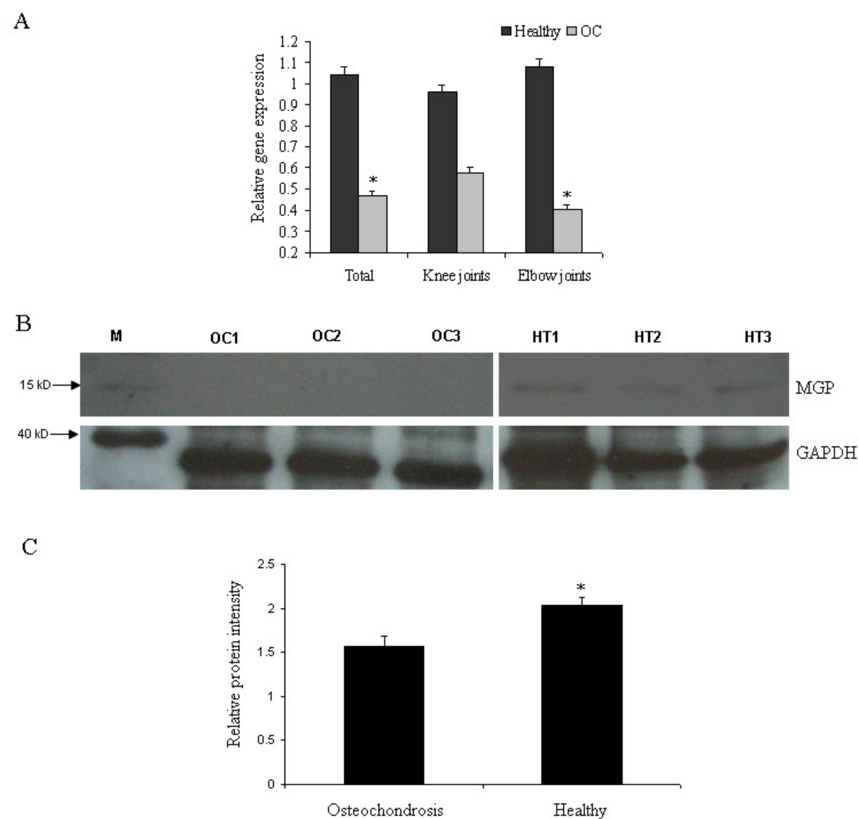

Figure 20: Expression analysis of the *MGP* gene and protein in articular cartilage of pig (A) Quantitative real-time RT-PCR analysis of porcine *MGP* mRNA in healthy and osteochondrosis cartilage of all investigated animals, condylus medialis humeri (elbow joint) and condylus medialis femuris (knee joint). The presented values are the means of duplicate determinations with standard errors. (B) Western blot analysis of MGP and GAPDH protein expression in osteochondrosis (OC) and healthy articular cartilage (HT) (M=Protein marker). (C) The relative intensity graph normalized by GAPDH band. The presented values are the intensity of the means with standard deviation of three different biological experiments. $^*P<0.05$

4.7.3 MGP localization by immunofluorescence

The distribution of the MGP protein was detected throughout different zones of articular cartilage. Positive immunostaining for MGP was detected in all layers of articular cartilage (Figure 22). The staining was mainly seen in the extracellular matrix, although weak immunoreactivity was found in the cytoplasm of chondrocytes. In the articular cartilage of newborn and 2 month-old animals, almost all cartilage canals were surrounded by an immunopositive signal of MGP. The staining intensity tended to decrease with age. The control cartilage specimens showed no immunoreactivity to MGP.

4.7.4 DNA methylation pattern of the *MGP* promoter

We analyzed the methylation status of the computationally predicted CpG islands using genomic DNA isolated from the OC and healthy cartilage of the full-sib pairs from F_2 of the DuPi resource population. The use of DNA from these animals allowed us to minimize possible effects of genetic background except OC on the methylation status of the analyzed animals. The MethPrimer program predicted that the CpG number was 10 in the CpG islands which was validated in the sequence of PCR products of *MGP* derived from bisulfite-converted DNA. There are 4 CG sites that include putative transcription factor binding sites for SRY, CDP-CR, Est-1 and T1 at the CG number 2, 4, 5 and 10 respectively (Figure 23A). The bisulfite sequencing analysis revealed that the overall methylation percentages were not differed between OC and healthy, but it tended to be higher in OC samples. However, the methylation percentages of 3 specific CpG sites (CpG2, CpG7 and CpG9) were higher in OC samples (Figure 23B) than in healthy (CpG2: 5 vs 32.5%; CpG7: 34.58 vs 78.33%; CpG9: 10 vs 48.33%) samples.

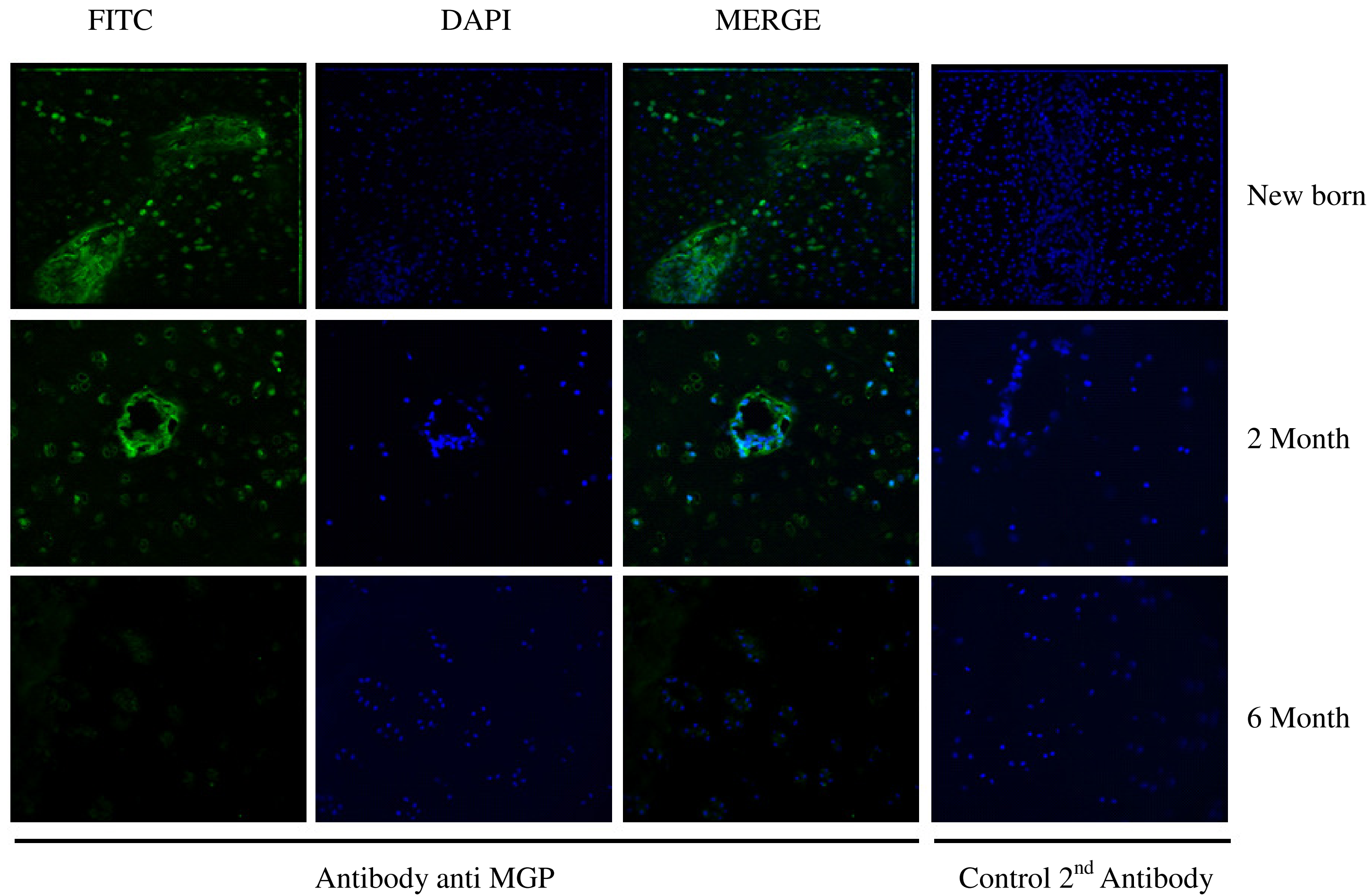

Figure 21: Immunolocalization of MGP in articular cartilage of 1-day, 2-month and 6-month olds pigs. Staining for MGP was detected around cartilage canals of 2-month and 6-month olds pigs (20X)

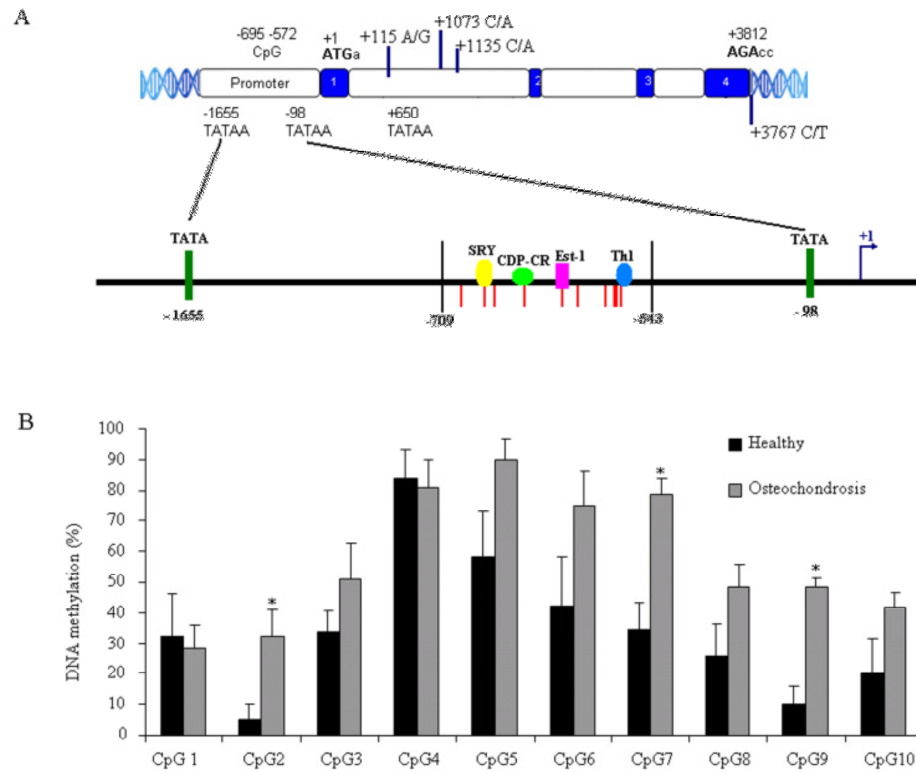

Figure 22: *MGP* gene structure and methylation of *MGP* promoter. (A) Diagrammatic representation of the *MGP* gene and its promoter region which contains the identified SNP, putative binding sites of TATA box and CpG region. Exons are shown as boxes. The CG sites are indicated (-543 to -709). Putative binding sites for transcription factors located in the CpG site are indicated by name. (B) Methylation percentage of normal and OC cartilage derived DNA. *$P<0.05$

5 Discussion

5.1 Characterization of phenotype related to leg weakness traits

As a result of genetic improvement, modern pigs have a high growth rate and increased weight load on immature bone and cartilage, which contributes to a higher incidence of OC (Jørgensen 2000, Kadarmideen et al. 2004). The long bones are formed through an endochondral ossification process, where cartilage cells are replaced gradually by bone matrix. Therefore, the disorders of bone and cartilage are thought to be connected. However, both BMD and OC are reported to have a genetic background and are directly related to each other through genetic factors (Jørgensen and Nielsen 2005, Kadarmideen and Janss 2005b, Mao et al. 2008). No studies are found in pigs to address the relationship between BMD and OC. In this study we found a negative correlation between BMD and OC which is in good agreement with a study in horses in which a correlation between the high degree of OC with a low level of BMD is reported (Firth et al. 1999). Clear understanding of OC pathogenesis is important to prevent this disease and to improve the disease resistance in pigs, which is important for animal welfare. Moreover, reducing the losses due to the disease is an excellent way of improving production efficiency in pigs. OC has mainly been studied in chronic stages because a definitive histological diagnosis is difficult in affected animals, therefore the clinical or acute OC is mostly diagnosed using radiography (Jørgensen et al. 1995).

In this study we found that the frequency of OC is high (31.05%) in the CMF region of the knee joints, which is supported by previous reports (Kadarmideen et al. 2004). Moreover, the results showed that OC was higher in castrated male pigs than in females which also coincides with previous results found in Norwegian Landrace (Ytrehus et al. 2004c). It was observed that the degree of OC in the fore legs of Duroc breed showed the highest incidence compared to other European pig breeds (Pietrain, Dutch Landrace and Great Yorkshire) (Van der Wal et al. 1978). In contrast, the boar femur was less affected by OC in the Duroc compared to Pietrain breeds in the same study. Edwards et al. (2006) reported that Duroc-sired progeny were heavier than Pietrain-sired at 26 wk of age.

In addition to OC, bone mineral density (BMD) is generally regarded as important parameter to assess bone growth and is associated with risk of bone fracture and with structural soundness in pigs (Mitchell et al, 2000, Mitchell et al, 2001). Studies in human have shown that BMD variation can be explained by genetic factors (Amin et al. 2004, Xu et al. 2009). In this study, BMD and BMC were not significantly different between castrated males and females pigs. Strong positive phenotype correlation among BMD and BMC was found ($r^2 = 0.70$, $P<0.01$), indicating that causal genes might have effects on both BMD and BMC traits. BMD and BMC are also strongly correlated ($P < 0.01$) with the slaughter weight of animals ($r^2 = 0.54$ and 0.71, respectively).

5.2 QTL for leg weakness related traits

The genetics of leg weakness related traits such as osteochondrosis in pigs is complex and multiple variables might contribute to the development of the disease. Due to this complexity, identification of the genomic loci affecting leg weakness traits is critical and important. A number of factors are known to influence the development of leg weakness or OC (Jorgensen and Nielsen 2005), specially body and leg structure of the animal, growth rate, mechanical stress and body weight of the animals. Performing QTL studies using exotic breeds crossed with the European breed may not be accurate. Using this type of animals will undoubtedly lower the incidence of leg weakness or OC and the too low frequency of the disease might influenced the existence of QTL. Moreover, it has not been clear that identification of QTL regions in pig using crosses between divergent populations has any significance for the selected populations used in commercial agriculture (Evans et al. 2003). In addition, it has been reported that the degree of leg weakness and OC of one sex in a breed cannot be translated to the other sex within that breed, nor to the same sex of another breed (Van der Wal et al. 1978). Therefore, using both European pig breeds for producing F_2 for the QTL study might be more accurate for study leg weakness related traits.

Several QTL for leg score (Guo et al. 2009), feet score (Lee et al. 2003), OC score (Andersson-Eklund et al. 2000, Christensen et al. 2009) and femur BMD (Mao et al. 2008) were previously identified in White Duroc x Erhualian, Wild boar x Large White, Large White x Meishan and Danish Duroc x Danish Large White intercrosses. However no study was carried out in Duroc x Pietrain intercross. This is the first study to map QTL for leg weakness related traits in Duroc and Pietrain intercross. Different QTL for leg and feet scores were observed, implying that these traits are controlled by multiple gene action.

Previously, QTL analysis for leg weakness and bone-related traits were performed in different pig breeds including Landrace purebred (Uemoto et al. 2010), White Duroc × Erhualian (Guo et al. 2009, Mao et al. 2008), Large white × Meishan (Lee et al. 2003), Duroc × Landrace and Duroc × Large white crossbred (Christensen et al. 2009), and Wild boar × Large white (Andersson-Eklund et al. 2000). To the best of our knowledge this is the first study to map QTL for leg weakness related traits in a Duroc and Pietrain intercross. A total of 16 QTL were identified. Among them, some of the identified QTL are novel and some are supported by the previous studies (Andersson-Eklund et al. 2000, Christensen et al. 2009, Guo et al. 2009, Lee et al. 2003, Mao et al. 2008, Uemoto et al. 2010).

The QTL for FLS found on the SSC13 at 16 cM in the present study and QTL for the same traits is reported at 76 cM in the White Duroc x Erhualian intercross (Guo et al. 2009) and at 58 cM in the Large White x Meishan (Lee et al. 2003) were on the same chromosome. The QTL results for RLS at SSC14 in our study at 0 cM are supported by QTL at 38 cM in Large White x Meishan pigs (Lee et al. 2003). A QTL for rear leg score observed on SSC6 close to marker *SW193* (SSC6q2.1). At this region a positional candidate gene transforming growth factor-beta 1 (*TGFβ1*) is mapped (Yerle et al. 1990). This gene might be one important candidate for LW related traits since *TGFβ1* is a potent regulator of cell proliferation and contributes to the differences in size and shape of the limb (Thorp et al. 1995). The QTL for FFS was found on SSC16 at 33 cM close to one of the previously reported QTL (27 cM) for rear leg score (Guo et al. 2009). In addition, the QTL for FFS on SSC16 was described previously at 61 cM (Lee

et al. 2003). We found QTL for OC score at CMH on SSC5 at 159 cM but Andersson-Eklund et al. (2000) reported QTL for OC at 51 cM on the same chromosome. The functional candidate gene matrix gla protein (*MGP*) for OC (Laenoi et al. 2010) is located in this chromosome. The QTL on SSC2 at 0 cM close to the marker *SW2443* (SSC2p17) was the most significant and the only QTL detected for BMC. Close to this chromosomal region at 7 cM a QTL was also found for BMD, providing a promising chromosomal region to identify genes responsible for bone mineral-related traits. Since BMD and BMC is highly correlated, this QTL could be pleiotropic influencing both of these traits. One of the highest linkage to reach the 5% genome-wide level significance for BMD was found on SSC3 at 82 cM. This QTL is novel and does not overlap with QTL reported by previous studies. A potential candidate gene in this chromosomal region includes the follicle-stimulating hormone receptor (*FSHR*) - a direct regulator of bone mass (Xu et al. 2009). However, large confidence regions in this experiment is a problem in this QTL study, which hampered the interpretation of QTL results since this region could contain many of potential candidate genes (de Koning et al. 2005).

Most of the identified QTL showed larger dominant effects rather than additive affects. In the same population of this study, 31 QTL out of 71 QTL for growth, fatness, leanness and meat quality traits showed also higher dominant effects (Liu et al. 2007). Lee et al. (2003) also reported QTL for leg weakness-related traits mostly with dominant effects in Large white × Meishan pigs. In addition, by using principal components analysis, Andersson-Eklund et al. (2000) also identified QTL regions for OC with significant and large effect of overdominance. The explanation might be that maternal or common environment effects can be partially confounding (Rowe et al. 2008). The dominance effect is an important source of variation in complex traits but dominance has sometimes been ignored (de Koning et al. 2005, Zhang et al. 2008). Moreover, most of the traits or diseasess related to skeletal system such as osteoporosis (Little et al. 2002), hypophosphatasia (Mornet 2007) and osteoarthritis (Meulenbelt et al. 1997, Stock et al. 1999), are inpart inherited in an autosomal dominant pattern. The results from this study and previously reported suggest that dominance plays a role in the genetic control of leg weakness related traits. Therefore, identification of dominance QTL effects in our study supported the mode of inheritance for the leg weakness related

traits and is valuable for the further detection of candidate genes. However, it is important to note that the low heritability of these traits also revealed that they are complex and may be under polygenetic control primarily by non-additive gene action or importantly affected by a major gene with Mendelian transmission (Kadarmideen and Janss 2005). In this study most of the QTL were identified as a single-trait region for each of the traits and were few in number. This could explain the low phenotypic correlations between the traits observed in the population. Different QTL for leg and feet scores were observed in this study, implying that these traits are controlled by multiple gene action. Our results showed novel linkage regions and also support some of the linkage regions previously reported. Though, the confidence intervals were large, these results will help for fine mapping and future identification of candidate genes in these QTL regions using additional markers or polymorphisms of genes located in the identified regions regarding leg weakness-related traits in pigs.

5.3 Association of candidate genes with leg weakness related traits

In this study we tested whether polymorphisms in the genes controlling chondrocyte metabolism may explain parts of the observed phenotype variation in susceptibility to LW. Sequence comparisons among three pig breeds revealed one SNP in 3´UTR of the porcine *COL10A1* gene which was reported previously by Madsen et al. (2003) as well as the SNP found in intron 6 of the porcine *TGFβ1* (Wimmers et al. 2002). Statistically significant ($P < 0.01$) association was found between *TGFβ1* g.180 G>A polymorphism and rear leg score. Recently, a SNP at intron 9 of *TGFβ1* was reported to be associated with body length in pigs (Fan et al. 2009). This indicates that *TGFβ1* might be associated with the structural soundness in pigs, which is consistent with its major functions in the development and maintenance of both cartilage and bone metabolism (Janssens et al. 2005). Moreover, we found positive association of the *MMP3* g.158 C>T SNP with some of the OC scores and BMD in the DuPi population. To our knowledge, this is the first association study of the *MMP3* polymorphism with leg weakness related traits in pigs that makes it difficult to compare our result with the other findings. However in humans, *MMP3* was found to be linked with different types of joint disease such as osteoarthritis (Kubota et al. 1997), suggesting that the association

of the *MMP3* with the joint disease can occur also in pigs. In this study we found a significant additive effect of *MMP3* g.158 C>T SNP on BMD, which indicated that the C allele of *MMP3* gene was associated with the increase in bone mineral density. It is important to note that BMD can significantly contribute to the progression of osteoarthritis in humans (Betancourt et al. 2009, Hunter et al. 2009, Richette et al. 2009).

No significant association between SNP of *COL10A1* and the LW-related traits in pigs could be detected in this study, suggesting that the investigated *COL10A1* g.72 C>T SNP can be excluded as genetic marker for the LW-related traits at least in our population. Since this is the first study on the association of *COL10A1* gene with leg weakness related traits in pigs, it is difficult to compare our results with literature data in pig. Previous study in dogs showed that a deletion in the *COL10A1* gene was not associated with skeletal changes (Young et al. 2006). But association between *COL10A1* polymorphism and osteoarthritis has been recently reported in humans (Lamas et al. 2010). Some of the SNP tested in this study deviated from HWE and this could have modestly affected the type I error of the analysis. Screening of genes with departure from HWE in data sets of affected individuals has been proposed as a relatively efficient method for detecting gene–disease associations (Salanti et al. 2005). Therefore, the generality of our finding could be limited. However, the departure from HWE for some SNP in our study may be due to the intensive selection of the used populations.

In conclusion for the association results, this study demonstrated that *TGFβ1* g.180 G>A and *MMP3* g.158 C>T polymorphisms may contribute to the susceptibility to traits related with leg weakness. The lack of consistence between results in DuPi and CP suggests that the association between SNP and LW-related traits can be depending on other causative mutations in linkage disequilibrium with the analyzed SNPs. Further confirming the involvement of this gene function involved in OC or LW development is needed. Association of *TGFβ1* and *MMP3* genes polymorphisms and their impact on leg weakness related traits, should be further validated in other populations.

5.4 Gene expression in porcine articular cartilage with and without OC lesion

To further explore the relationship between candidate genes and OC occurrence in pigs, we investigated the gene expression between OC and healthy cartilage. Relative expression levels of genes known to be important in the regulation and involved in the endochondral ossification were examined. There was a significant increase of *TGFβ1* and *RUNX2* in OC lesions compared to healthy cartilage, while *MGP* was significant decreased in OC cartilage. *SOX9* showing non significant (but trend to) up regulation in OC lesions. Among the genes examined, the transcription factor *SOX9* was highest expressed where *MMP9* was lowest expressed in articular cartilage compared with the other genes. No changes were detected in the expression of *COL2A1* and *MMP9*. The expression of these genes has been previously correlated with the variation of the OC lesion in pigs (Ohata et al. 2002, Thorp et al. 1995, Wardale and Duance 1994). The significant up regulation of *TGFβ1* at OC lesions found in this study was in contrast with a previous report in pig (Thorp et al. 1995) in which this gene was found to be deficient in the chondrocytes at the sites of OC. However, it was in agreement with studies in horses (Henson et al. 1997, Semevolos et al. 2001) in which the expression of this gene was higher in OC lesions. *TGFβ1* is generally considered as a stimulator of type II collagen. It is known as the main cellular component of articular cartilage. Therefore, down regulation of the gene encoding type II collagen can partially result in low quality of articular cartilage. In a previous study, the proportion of type II collagen in OC lesions from both osteochondrotic articular and growth plate cartilages was substantially decreased (Wardale and Duance 1994). Moreover, genetic variations of the gene *COL2A1* encoding type II collagen have been reported to be associated with several types of joint diseases including OA (Uitterlinden et al. 2000, Valdes et al. 2007). However, in this study, the expression of *COL2A1* was not different between OC and healthy cartilage. Our result is also supported by a study in horses but not in pigs. The reason for this may be that the expression of structural genes such as *COL2A1* and *COL10A1* may not change in the early development of OC lesion and may not different from the normal cartilage. Regarding to a key transcription factor in cartilage formation, sex-determining region Y-type high mobility group box 9 (*SOX9*) was reported as a potent activator of type II collagen expression (Kypriotou et al. 2003, Ushita et al. 2009). As recently reported, expression of *SOX9* mRNA was decreased in osteoarthritic

chondrocytes compared to healthy cartilage (Orfanidou et al. 2009). However, in this study there was no difference in expression of *SOX9* mRNA in OC lesions compared with healthy cartilage. In addition to *SOX9*, *RUNX2* is another bone and cartilage specific transcription factor that has been intensively studied and might play an important role in the OC development. It is one of the important key transcription factors controling skeletogenesis (Karsenty 2008). *RUNX2* gene expression and protein function are regulated at multiple levels, including transcription, translation and post-translational modification (Jonason et al. 2009). It was hypothesized in this study that there may be an increase in *RUNX2* gene expression in OC lesions. The fact that we observed the high expression of *RUNX2* in OC lesions is consistent with findings in horses (Mirams et al. 2009), and also in human OA (Orfanidou et al. 2009). *RUNX2* acts as a transcription factor for genes expressed in hypertrophic chondrocytes (Jonason et al. 2009); this means that in the formation of OC lesions, chondrocyte hypertrophy might be involved. Expression of *MMP3* and *COL10A1* genes in healthy versus OC cartilage were not statistically different in our study. The expression of MMP3 has been previously investigated in regard to cartilage degradation in human (Kubota et al. 1997b). However, differential expression of the *MMP3* gene between OC and healthy cartilage could not be confirmed in our study. We found that the expression of *COL10A1* is consistent with the results of a previous report in horses (Semevolos et al. 2001), but in contrast with results previously reported in pigs (Wardale and Duance 1994) and in horses (Mirams et al. 2009). Collagen type X function may be related to the development of OC; according to the association and expression results of this study, *COL10A1* gene expression is not associated with any of the leg weakness traits at least in our populations.

Further research is therefore required to elucidate the precise mechanisms of chondrocyte hypertrophy on OC lesion development. Although, most of the previous studies both in human and animal have focused on OC lesions that cause clinical signs or the chronic stages of the disease. Since it has been well known that the matrix metalloproteinases (MMPs) are responsible for cartilage collagen breakdown (Davidson et al. 2006), it has been recently reported in dogs with OA that the expression of *MMP9* mRNA was increased in OA lesions (Clements et al. 2009). This means that if OC

progresses to OA, the expression of those enzymes must be up regulated. However, in this study we could detected a very low expression of *MMP9* in our sample and there was not differential expression between the OC and the healthy groups. The reason may be that the articular cartilage samples used in this study are not rich the stage of OA.

5.5 Molecular characterization of *MGP* as a candidate gene for OC

Genes involved in maintaining the normal structure of the articular cartilage might be important factors in the pathogenesis of OC. Abnormal expression of genes causing disturbances in cartilage metabolism may lead to OC. *MGP* is regarded as one of the potent inhibitors of extracellular matrix calcification, indicating that it can disrupt bone and cartilage metabolism. However, we found that the staining intensity of MGP tend to decrease with age as the pattern of immunostaining was lower in the articular cartilage of 6 month-old pigs in comparison to new born and 2 month-old pigs. We found that cartilage canals regressed with age. Our immunoreactivity result showed that MGP is localized within/around the cartilage canal suggesting that it may be released either from chondrocytes or directly from the cells of the cartilage canals. With respect to the developing skeleton and during endochondral bone formation, *MGP* is found to be expressed during this process (Tuckermann et al. 2000), indicating that, a slight decrease of *MGP* expression might be normal in growing pigs. In this study, we speculated that the abnormal changes in the *MGP* expression might play a role in the process of cartilage canal regression during endochondral ossification leading to the development of OC. We found that the *MGP* at mRNA and protein levels was down-regulated in articular cartilage with OC lesions compared with healthy cartilage. Our result is supported by the previous study in human, using an immunosorbent assay. Schurgers et al. (2005) found that the circulating MGP concentration in persons with disease of the cartilage (osteoarthritis) was significantly lower. In mammals, chondrocytes are responsible for maintaining the cartilage homeostasis. The change in *MGP* gene expression in maturing chondrocytes and the lower expression of *MGP* in proliferative and hypertrophic chondrocytes are reported to induce chondrocyte apoptosis (Newman et al. 2001). As a result, failure of chondrocytes to maintain the cartilage matrix could lead to the development of cartilage disease.

During animal growth, the extracellular matrix and the chondrocytes of cartilage undergo a maturation process. However, many growth factors and matrix molecules also take part in this process. Transforming growth factor-beta1 (*TGFβ1*) and parathyroid hormone related peptide (*PTHrP*) decrease the rate of chondrocyte maturation, while bone morphogenetic proteins (*BMPs*), thyroid hormone, and retinoic acid stimulate terminal differentiation of chondrocytes. *MGP* has been identified as an inhibitor of bone morphogenetic protein-2 and 4 (Yao et al. 2006). Furthermore, during bone growth, the invasion of cartilage by blood vessels is required and vascular endothelial growth factor (*VEGF*) plays important role in vasculogenesis (Ytrehus et al. 2004a). Decreased levels of *VEGF* in the cartilage canals are reported to promote endothelial cell apoptosis, which leads to the disruption of blood supply to the growth cartilage and finally the development of OC (Ytrehus et al. 2004b). *MGP* has been shown to increase *VEGF* expression through increased *TGFβ1* in bovine endothelial cells (Boström et al. 2004). *VEGF* plays an important role in the growth of new blood vessels which may explain the pathogenesis of the vascular abnormalities that are commonly found in OC. On the other hand, *TGFβ1* is reported to stimulate both *VEGF* and *MGP* expression (Zhou et al. 1993). *TGFβ1* is a secreted protein that performs many cellular functions, including the control of cell growth, proliferation and differentiation. Therefore, it can be speculated that down regulation of *MGP* decreases the level of *VEGF* which may contribute in the process of cartilage canal vessels regression and the development of OC. Moreover, *TGFβ1* is found to be deficient in chondrocytes at the sites of porcine osteochondrosis (Thorp et al. 1995), suggesting that *MGP* expression differences are one of the important factors contributing to the development of OC in pigs. The expression of *MGP* may be required for reducing the cartilage canals as previously found in endothelial cells that MGP protein stimulates *VEGF* expression through enhancing *TGFβ1* (Boström et al. 2004). Therefore, this result will improve our understanding how *MGP* contributes to OC development.

Three SNPs in the porcine *MGP* were detected, but none was in the promoter. Thus, we questioned whether DNA methylation at different CG sites within the CpG islands might be responsible for the lower *MGP* expression. DNA methylation has been suggested to play a role in maintaining the stability of cell differentiation and in

regulating gene transcription. An extensive review on the role of epigenetics in the pathogenesis of osteoarthritis is described (Roach and Aigner 2007). DNA methylation levels of CpG-rich promoters of genes related to chondrocyte phenotype have been reported recently in humans (Ezura et al. 2009). We have analyzed the methylation patterns of CpG regions at the *MGP* promoter using genomic sequencing of bisulfite-treated genomic DNA. To the best of our knowledge, this is the first report regarding DNA methylation patterns of one gene specifically related to the OC in pigs by using samples from healthy and OC articular cartilage of full-sib animals. DNA methylation differences were found between individuals. This difference was also found in different clones from the same animal which might be the cause of inter-individual variation. Consistent with the pattern of *MGP* expression, the result showed that the second, seventh, and ninth CG sites were highly methylated in OC compared with normal cartilage. No study has been dedicated to determine whether the methylation of *MGP* is responsible for permitting aberrant *MGP* gene transcription. It was stated earlier that methylation generally prevents binding of transcription factors and other DNA binding proteins (Roach and Aigner 2007). We found that, when the CG locations were compared with the transcription factor biding sites, the second CG is within a binding site of the SRY-related transcription factor whereas the fourth, fifth and tenth CG are located within the *CDP-CR*, *est-1* and *Th1* biding sites, respectively. One of *SRY*-related transcription factor that has been reported to play an important role in endochondral ossification is *SOX9*, which is a cartilage specific transcription factor (Tew et al. 2005). *MGP* mRNA and protein are down-regulated in the OC cartilage which might be due to the hyper methylation of its promoter. All these results suggested that *MGP* might play role in the pathogenesis of OC in pigs.

6 Summary

The present study has been carried out to identify quantitative trait loci and candidate genes associated with leg weakness related traits in pigs, as well as to identify the differentially expressed genes in articular cartilage with osteochondrosis. For QTL analysis, a total of 310 F_2 pigs from a Duroc x Pietrain resource population were genotyped with 82 genetic markers in this study. Fore and rear leg (FLS, RLS) and feet (FFS, RFS) were scored following a set of attributes in live animals. Osteochondrosis (OC) was histologically scored at the head and condylus medialis of the left femur (HF, CMF) and humerus (HH, CMH). Bone mineral density (BMD), bone mineral content (BMC) and bone mineral area (BMA) were measured in the whole ulna and radius bones using dual energy X-ray absorptiometry (DXA). Parity was found to have an effect on most of the traits and average daily gain was affecting the leg score. Parity, carcass length and slaughter weight were found to have effect on DXA traits, whereas parity and age were affecting OC score. The DXA traits were moderately to highly correlated with each other. OC was observed mostly in the CMF. A total of 16 chromosomal QTL regions were identified for leg weakness related traits on ten porcine autosomes in this study. Our results confirmed some of previous studies and provided information on new QTL for leg weakness related traits in pigs. All QTL reached the 5% chromosome-wide significance level. A QTL for BMD reached 1% chromosome-wide significance level. QTL on SSC2 and SSC3 for BMC and HH, respectively, reached 5% genome-wide significance level, hence are worthwhile for further investigation. The knowledge of QTL affecting these traits will contribute towards a more understanding of the genetic background of leg weakness in pigs.

Association studies for SNPs in the genes *TGFβ1, MMP3 and COL10A1* were carried out using 310 F_2 DuPi and additional 299-pigs of a commercial population. Association ($P < 0.01$) was found between SNP of *TGFβ1* (g.180 G>A) with rear leg score in DuPi. Significant association was found between SNP of *MMP3* (g.158 C>T) with OC at head of femur ($P < 0.05$) and bone mineral density ($P < 0.05$) in the DuPi population. However, the associations found in DuPi could not be confirmed in the commercial population. On the basis of histological results, six healthy and diseased castrated males were used for gene expression studies. Genes known to play a significant role in

endochondral ossification were analyzed. *TGFβ1* and *RUNX2* gene expression was higher in OC samples ($P < 0.05$) compared to healthy cartilage, while *MGP* expression was lower in OC cartilage. Accordingly, the intensity of MGP protein band was lower ($P < 0.05$) in the OC group when quantified by Western blot. Furthermore, one CpG region was identified in the *MGP* promoter and DNA methylation of three CpG sites was higher in OC compared with normal cartilage. This suggested that the high DNA methylation at specific CpG sites in the *MGP* promoter might be involved in the down regulation of *MGP* in OC. The expression of *COL2A1*, *MMP9* and *SOX9* mRNA was not different between OC and healthy cartilage. Immunofluorescence of normal cartilage collected from pigs of different ages revealed that MGP signals were higher in younger pigs and decreased in older pigs. The MGP protein was expressed closer to the cartilage canals. These results suggest that *MGP, TGFβ1* and *RUNX2* genes might be candidate genes for the development of OC in pigs.

In conclusion, this study identified QTL regions underlying leg weakness related traits in a fast growing crossbreeds between Duroc and Pietrain pigs. These results will support the fine mapping and the future identification of candidate genes within these QTL regions. Some of the proposed candidate genes were found differentially expressed in healthy and OC cartilage, and associated with leg weakness traits. However, association results were not confirmed between populations. Taken together, these results will be helpful for the future understanding of the genetic and biological events that underly traits related with leg weakness in pigs.

7 Zusammenfassung

Die Studie wurde durchgeführt, um QTLs und Kandidatengene, die mit den Beinschwäche-Merkmalen beim Schwein in Verbindung stehen, zu identifizieren, so wie unterschiedlich exprimierte Gene in Gelenkknorpeln mit Osteochondrose aufzuzeigen. Für die QTL Analyse wurden in dieser Studie insgesamt 310 F_2 Schweine aus einer Duroc×Piétrain Ressourcen Population mit Hilfe von 82 genetischen Markern genotypisiert. Mit Hilfe der linearen Fundamentbeurteilung war es möglich sowohl die Vorder- und Hinterbeine (FLS, RLS) als auch die Füße (FFS, RFS) zu bewerten. Der Osteochondrose (OC) Status wurde histologisch am Kopf und Condylus medialis des linken Femurs (HF, CMF) und Humerus (HH, CMH) bewertet. Die Knochenmineraldichte (BMD), Knochenmineralgehalt (BMC) und die Knochenmineralfläche wurden an Ulna und Radius mittels Dual-Röntgen-Absorptiometrie (DXA) gemessen. Die Trächtigkeit hatte einen Effekt auf die meisten Merkmale. Ebenfalls hatte die durchschnittliche tägliche Zunahme einen Einfluss auf die Fundamentbewertung. Außerdem hatten die Trächtigkeit, die Schlachtkörperlänge und das Schlachtgewicht einen Effekt auf die DXA-Merkmale, wohingegen die Trächtigkeit und das Alter den OC Status beeinflussten. Auch korrelieren die DXA Merkmale mittel bis stark untereinander. OC wurde am meisten bei den CMF beobachtet. In dieser Studie wurden insgesamt 16 chromosomale QTL Regionen auf 10 Autosomen für die Beinschwäche-Merkmale identifiziert. Unsere Ergebnisse bestärken einige vorherige Studien und liefern Informationen zu neuen QTLs für Beinschwäche-Merkmale beim Schwein. Alle QTLs erreichten ein chromosomweites Signifikanzniveau von 5%. Ein QTL für das BMD-Merkmal hatte ein chromosomweites Signifikanzniveau von 1% beziehungsweise erreichten die QTLs auf SSC2 und 3 für BMC und HH ein genomweites Signifikanzniveau von 5%. Diese Ergebnisse eignen sich für weitere Untersuchungen. Die Erkenntnisse über die QTLs, die diese Merkmale beeinflussen, könnte zu einem besseren Verständnis über den genetischen Hintergrund von Beinschwäche-Merkmalen beim Schwein führen.

Die Assoziationsstudie zu den Genen *TGFβ1*, *MMP3* und *COL10A1* wurden bei den DuPi Tieren und 299 zusätzlichen Schweinen aus einer kommerziellen Population durchgeführt. Eine Assoziation (P<0,01) wurde zwischen dem SNP von *TGFβ1* (g.180 G>A) und der Fundamentbeurteilung der Hinterbeine bei den DuPi Tieren festgestellt. Signifikante Assoziationen wurden zwischen dem SNP von *MMP3* (g.158 C>T) und der OC Gruppe am

Femurkopf (P<0,05) sowie der BMD Gruppe (P<0,05) in der DuPi Population festgestellt. Allerdings konnte keine Assoziation zwischen den DuPi Tieren und der kommerziellen Population bestätigt werden. Auf der Basis von histologischen Ergebnissen, wurden 6 gesunde und 6 kranke Börge für eine Expressionsstudie genutzt. Gene, die bekannt dafür sind, während der endochondralen Ossifikation eine signifikante Rolle zu spielen, wurden analysiert. Dabei wurde festgestellt, dass die Genexpression von *TGFβ1* und *RUNX2* höher in den OC-Proben (P<0,05) war als in den gesunden Knorpeln, während die Expression von *MGP* in den OC-Knorpeln geringer war. Zusätzlich wurde beim Western Blot eine geringere Intensität der *MGP*-Protein Bande bei der OC Gruppe (P<0,05) festgestellt. Weiterhin konnte für *MGP* eine CpG-Region im Promotor identifiziert werden. Dabei zeigte sich, dass die DNA-Methylierung von drei CpG Bindungsstellen in der OC Gruppe im Vergleich zu der Gruppe mit den gesunden Knorpeln höher war. Diese Ergebnisse zeigen, dass die erhöhte DNA-Methylierung an der spezifischen CpG Bindungsstelle im MGP Promotor für die Down-Regulierung von *MGP* bei OC verantwortlich sein könnte. Bei der Expression von *COL2A1*, *MMP9,* und *SOX9* wurden keine Unterschiede zwischen den OC-Knorpeln und den gesunden Knorpeln festgestellt. Bei der Immunfluoreszenzuntersuchung von normalen Gelenkknorpeln, die von Schweinen unterschiedlichen Alters stammten, war das MGP-Signal am stärksten bei jungen Schweinen und nahm mit zunehmendem Alter ab. Die MGP Proteinexpression war um die Knorpel Kanäle am höchsten. Diese Ergebnisse zeigen, dass die Gene *MGP*, *TGFβ1* und *RUNX2* potentielle Kandidatengene für die Entwicklung von OC bei Schweinen sein könnten.

Abschließend lässt sich sagen, dass die Studie erstmals QTL-Regionen beschreibt, denen Beinschwäche-merkmale bei schnellwachsenden Kreuzungen aus Duroc und Piétrain Schweinen zu Grunde liegen. Diese Ergebnisse könnten bei der zukünftigen Feinkartierung sowie Identifikation von Kandidatengenen innerhalb dieser QTL-Regionen helfen. Manche der vorgegebenen Kandidatengene wurden unterschiedlich in gesunden und OC-Knorpeln exprimiert und waren mit den Beinschwäche-Merkmalen assoziiert. Dennoch konnten die Assoziationsstudien zwischen den Populationen nicht bestätigt werden. Zusammengefasst lässt sich sagen, dass diese Ergebnisse in der Zukunft hilfreich bei dem Verständnis der genetischen und biologischen Ereignisse sein könnten, denen die Beinschwäche-Merkmale beim Schwein zu Grunde liegen.

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