## **CHAPTER 4**

## **RESULTS AND DISCUSSION**

## 4.1 Characterization of porcine MGP gene

The nucleotide sequence of porcine *MGP* gene was determined by genomic DNA sequencing. The partial sequence of *MGP* gene (Figure 4.1), spans 4060 bp of genomic DNA from the start site of transcription and contains four exons (Genbank Accession no. NC010447.1) separated by three large intervening sequences. There comprise a 5' untranslated region (5' UTR) of 50 bp followed by coding region for 103 amino acids from the start codon (ATG) to the stop codon (TAA) and 226 bp of 3' UTR. Exons are ranging in length from 33 to 287 bp. All the exons-introns boundaries have the consensus GT-AG splicing sequences. For the last exon, the transcription termination is shown in bold italics instead of a splice donor (Table 4.1).

-	Exon	Exon size (bp)	Splicing donor	Intron size (bp)	Splicing acceptor
	1	60	CCTGTGCTAT <b>gt</b> tgag	1437	ttcgagAATCTCATGA
	2	33	TATGAAATCAgtaagt	1091	ttaaagATCCCTTCCT
	3	78	CCCAAGAGAG <b>gt</b> gggt	972	ttctagAATCCGAGAA
	4	287 C	GGCCAAATAA <b>G</b> accag		
Δ		ri	σhts	r e s	erved

Table 4.1 Exon-intron ju	nctions of the	porcine MGP gene
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				~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~		Start	codon
		CGTGAAGCCA Exon 1	CTGCACGAGA	CCCTGAGAGC	AACCTCAGGA	CGCAGGGACC	ATGAAGAGCC
61	-	TGCTCCTTCT	CTCCGTCCTG	GCTGCCTTGG	CCGTGGCAGC	CCTGTGCTAT	GTTGAGAACT
12	21	CCCGCGTGTG	CTTTCTCTGT	TTTTTTTTCC	TCTGGCTTTA	GTTTACTTGA	ATTTTTCCTC
18	31	TCCCTCCCCG	TCTTCCCCCT	TTCTCTACAA	TAACCTTAAA	GTACCATTGA	TTTAATATTT
24	1	CCCAAACCCC	TGGGACACTG	ATGTGTTCCA	TCAAGTCTTT	TTTAGATCCT	CGATATCCAG
30	)1	ACTCTTGCAT	GTATCTTCAT	GGGAAATTTC	TTTCTATGGT	AAAGCCTTCT	ATACCTGGAA
36	51	ACTTTGCTAT	TTCCGAAAGA	AGATATCAAC	TTCCCAGCTC	TCCTTTCCTA	AGGATGTTTA
42	21	TTTAGATTTT	GGTAACAGCT	GGTTAAGGGT	ATGGGTTGAA	TCACTCACCT	TTAAGAAAAT
48	31	CAGTTTTCAC	ACAAAGGATT	AAGCACAGCC	CTCTCCAGGG	AGAGGACTGT	CTCACAAACT
54	1	TAGGACATTT	CTTCCTGAAT	GAATTTAAAA	ATAAAGTTGT	ACACTTAAAA	AATAAAGTTG
60	)1	TAACCTCAGA	GTAAAATCGA	TTTCATAGTT	TCTGTTGTTA	AAAGATAAGT	TTTGCCTGGT
66	51	GTCTATATGT	TGTTAAATAT	ATGTGTGTCT	GTTCAAACTT	ATAAAAGGAA	ATCCGAAGCA
72	21	ΔΔΔΥΥΔΥΥΥΔ	CATCATTCAG	CCTTAAGCTT	CAATGATCTG	GAGCTTCTAG	GTATTCTTGA
78	21	GCCTGAATGA	GATTAACGAC	САТАТСТАТТ	TTGCTTCTCC	CTTTTGAAAT	TATTCACAGG
84	11	TTTTCTCCCC	AATCGGAGGA	TTTCATTTC	TTCATTTCT	TTTTAACTTT	TCAGGCATAT
90		TCACTTCAAA	CATGAGOTTC	TTATTCCCAC	ATTTACCTCC	TGTGCTCAGG	ACAGTCGTCT
96	1	AAACAACAAA	ACCTTTCCTT	TIATIGCCAC	TOTTOTOCOT	CTCAACTCCC	AUAGIGGICI
10	) 1 ) 2 1	CACTCACATC	TCACCTCCAC	CCACTCCTTT	CTCTCCCCT	CIGAACIGGC	CACCTTCTTA
	121	GAGIGAGAIG	ICAGCIGGAG	CCAGIGGIII	CIGIGGCIGI	CAATTIAACA	CAGGIICIIA
10				1011100000	(mmm.c.cm) ) )	CIIZ4A	
10	181	AGAGGCTTTC	AGAACCTCTT	AGAAACCTGT	CITTIGGTAAA	GCCCAGCTGA	ACCAGCIGCC
						C11851	
11	.41	CAGCCTGCCT	GGAGAAAACT	GCCTGTTTTC	CAGACCCTTT	GTTC <mark>C</mark> TTCTT	TGATCCTGCT
12	201	CTCAGACAGG	TCCCAAATCA	ATCAAGGAGG	GCTCTAGGGA	GGTTTGTTCA	CAGCACTTAA
12	261	ATTGTCATAT	TTCACAAGCC	ТААААССТАА	AGGCTTTGGG	AAATTTTAGC	TAACATCATG
13	321	GCCACTAGCT	CCTTCAACAC	CAGACAAACT	CAAGTGTAGC	GATATTCAAG	CAAAGACAGA
13	881	GCTAGAGAAA	TAGCTCACAA	ACACTCAATG	CCTGAGATCT	GTAATAACCC	TACTGCTAAA
14	41	TATATTCCTC	CCATCTTTTT	GATTCCCCCC	CTTCCTTCCC	TATATTTAGA	CCAGGGTGGT
						Exor	n 2
15	501	GAGTGAAAAG	TAATGACAAA	ATGGATCTTT	TTTTTTTTTC	ATTTCGAGAA	TCTCATGAGA
15	661	GCTTGGAATC	CTATGAAATC	AGTAAGTAAA	TATGTGACTT	CCTTATTTAA	ATCTCAAATA
16	521	TTAAAGAATC	TCTCCCAATT	CTCAAAGAGA	CAAAATGACA	ATGCCTATAC	CAGGGAGGGA
16	581	AAAGAGGGTC	TCCTTTGGGA	AATGAAAATA	AATTTTAAAA	TTCTTTTTAA	AAAATGTTTT
17	/41	ATTAAAGAAG	AGTTGCTTTA	CAATGTTGTA	TCAATTTCGG	CTGCCCAGCA	AAGTGACTCA
18	301	GAGATATATA	TCTATATATT	CTTTTTCTCA	TATTATCTTT	CATCATATTC	TATCACGAGA
18	861	GACTGGGTAT	AGCACCCTGT	GCTCTGCAGT	AGGGCCTCAT	TGCTTATCCA	TTCTAAATGT
19	921	GATAGTTTAC	ATCTACTAAC	CCCAAGCTCC	CAGTCCATCC	CACTCCCTTA	GGGCTAAAAA
19	81	AATCACCAGA	TGAGCAATTT	TTTAAGTTTG	AAATGGTGTA	AGATGACAAG	TATGCTCTCA
20	)41	ACATACTTAG	GAACCATAAA	ACCTCATCTG	GAAATTTTAA	GTTGACACCA	CATCAACCAC
21	01	ACAGATGGAA	AATGAGGAGT	AAGGACAAAT	GACCAAGGAC	ATATTGAAGG	CCAAGTCAGC
21	61	TGGGAGACCA	CAGACATCAA	CAGTCTGTTG	GATCCTAGCA	AATATCTGAA	CTTACAATGA
22	221	CTGAGCAGAG	GAGGAAATAA	CACATCATTC	GCATCTTTCA	TTTGTTCATT	ATTTTTATGA
22	281	CGTTTAAACC	ATTTCATTCA	CTGGAAATCT	TAAATGGTGC	TTTACAAGTC	CTCAGGACAG
23	341	AGAGAATTTC	TCCTTCCAGG	AATAAAGAAC	ACACTGAGGA	AGGGTACCAA	AACCAAGAAA
24	101	CCCCAACTCA	TCCCCCAAAC	CCAAAATAAA	TAACCCGTAA	CTTTTAACCT	CCATCTCACA
24	161	CCTTTCTN	A CHC AHCHCH	λααττλάτασα		TCCCCCACCT	TTOTONATO
24	201	TCOTTAR	CCCACTTAN	TOTTOTTOT	COTOCALOTO	I COGGCAGGI	
25	21	CTCTTTAICI		CTCCCACACA	ATTCATCON		AIGAAIGGAI
	DQT	GIGITITICAA	ATATATTTGT	GICGGAGAGI	Exon 3	ATATTTTCAA	CIACIGITIC
26	541	CCTAAAATGG	CACCTGTATT	TTCTGTTTTA	AGATCCCTTC	CTTAACAGGA	GAAATGCTAA
27	01	TACCTTTATA	TCGCCACAGC	AGAGATGGAG	AGCGAAAGCC	CAAGAGAGGT	GGGTGACAGA
			200				

**Figure 4.1** Nucleotide sequences of porcine *MGP* gene with 4060 bp genomic DNA, the start and stop codon and polymorphisms position.

2821	CAGTTTTTTT	TTTTTTTTTCT	CCATCATATG	ACTTATTTCT	ACTTACATTC	CAAATCAAAT
2881	AATTTTCAAA	CTAAGTACAG	AAAACAAATG	AACTTCTATT	TTAGGAAAGG	CCCAGAAAAG
2941	AGAGAAAGGA	GGAGAAAAGG	GAGGACAGAA	AGAAAATTTC	TATGTTCATA	TTTTTAAAAA
3001	ААААААААА	GGGAATTTGC	CCTTGTGACT	CAGTGGTAAT	GAACCCAACT	AGTATCTATG
3061	AGAATCCAGG	TTCGATCCCA	GGCCTCGCTC	ACTGGGTTAA	GGATCTGGCA	TTACAGTGAG
3121	TTGTGGTATA	GGTCACAGAC	TTGGTTCATC	TCCTACATTG	GCTATGGTGT	AGGCTGGCAG
3181	CTGCAGCTCA	GATTCGGCCC	CTAGCCAGGG	AAATTCCATA	TGCCACAGGT	GCGGCCCTAA
3241	AAAAAGCAAA	АСАААСАААС	АААСАААААА	TAATAACTCC	CCCTCTTCCA	CCTTCCACCC
3301	CTGTTCTGAA	TATCTCTTCA	TATTGGGGAT	GAATGGGATC	TCCCTATGAC	ATTTTCTTGT
3361	TCTTTATTTT	TCTTTCTCTT	TTTATTTCTC	TGCTTTCTTT	GTTCTTTTGG	TTTTTGTTTT
3421	TTGGGTTTTT	TTGCTTCTTA	GGGCCATACC	CACAGCATAT	GGACATTCCC	AGGCTAGGGG
3481	TTTAATTGGA	GCTATAGCTG	CCAGCCTACG	CCACAGCCAC	AGCAACGCCA	GATCCAAGTA
3541	GCGTGTGCAG	CCTACACCAC	AGCTCATGGC	AACGCCGGAT	CCTTAACCCA	CTGAGTGAGG
3601	CCAGGGATCA	AAACCACAAC	CTCATGGTTC	CTAGTCAGAT	TTTTTTCCAC	TGTGCCACGA
3661	TGAAGAACTC	CTCTTTATGC	TTCTTAACTT	GGGCCTCTTT	TTTTTTTTTC	CACTTTCTAG
	Exon 4					
3721	AATCCGAGAA	CTCAACAAGC	CTCCCTATGA	GTTAAACCGG	GAAGCTTGCG	ATGACTACAA
			run -	C3817	Τ	
3781	ACTTTGCGAA	CGCTATGCCA	TGGTTTATGG	ATACAACGCC	GCCTACAATC	GTTATTTCCG
		St	top codon			
3841	GCAGCGCCCA	GGGGCCAAAT	AAGACCAGAA	AAAGTGTCTC	TCTCCAGACC	CCAGTGGCTG
3901	GTTTTGTAAT	CCCTTGCAGT	AGCATCACTG	AACTATATAG	ACACAGACAA	ATTGCTTGTT
3961	TCTTCAATGT	CCTTGTCTGG	CCTCATCCCC	TTTCCTGACC	CAGGTTGATA	AGGAATGAAA
4021	GTGCCATGGA	GTGAAGGTCA	AAAGAGTTAA	ACATGTGATT	•	
1 M			- C . T			

**Figure 4.1** Nucleotide sequences of porcine *MGP* gene with 4060 bp genomic DNA, the start and stop codon and polymorphisms position (continued).

Sequence analysis of the genomic region upstream of the putative transcription start site indicated the absence of TATA and CCAAT boxes. The translation start codon was assigned based on the homology to the human ortholog. The polyadenylation signal AATAAA is located approximately 205 bp downstream of the stop codon resulting in the DNA sequence of porcine *MGP* gene (Genbank Accession no: NC010447) approximately 4088 bp. The porcine *MGP* amino acid sequence shows identities of 85, 92, 87 and 84% to human (Genbank Accession no: NP000891), bovine (Genbank Accession no: NP777132), mouse (Genbank Accession no: NP032623) and rat (Genbank Accession no: NP036994), respectively. Comparison between porcine and mammalian *MGP* genes indicates that all introns are located at conserved sites within the human and bovine *MGP* coding sequence (Figure 4.2). Moreover, the characteristic motifs previously identified in other mammalian *MGP* are also present in the porcine protein. These include a highly conserved domain at the N-terminus which may be subject to serine phosphorylation (motif SxESxESxE) and five glutamic acid residues which are usually modified by  $\gamma$ - carboxylase that are carboxylated in a vitamin K dependent process (Price *et al.*, 1994). The fact that this site is so highly conserved suggests that it is required for the correct regulation of *MGP* function (Cancela *et al.*, 2001).

		010	912		
-	1 0				
Human	MKSLILLAIL	AALAVVTLCY	E <u>SHESMESYE</u>	LNPFINRRNA	NTFISPQQRW
Bovine	MKSLLLLSIL	AALAVAALCY	E <u>SHESLESYE</u>	INPFINRRNA	NSFISPQQRW
Mouse	MKSLLPLAIL	AALAVATLCY	E <mark>SHESMESYE</mark>	ISPFINRRNA	NTFMSPQQRW
Rat	MKSLLPLAIL	AALAVAALCY	E <u>SHESMESYE</u>	VSPFTTRRNA	NTFISPQQRW
Pig	MKSLLLLSVL	AALAVAALCY	ESHESLESYE	INPFLNRRNA	NTFISPQQRW
	51				
Human	RAKVQERIRE	RSKPVH <mark>E</mark> LNR	FACDDYRLCE	RYAMVYGYNA	AYNRYFRKRR
Bovine	RAKAQERIRE	lnkpqy <mark>e</mark> lnr	FACDDFKLCE	RYAMVYGYNA	AYDRYFRQRR
Mouse	rakaqkrvqe	RNKPAYEINR	FACDDYKLCE	RYAMVYGYNA	AYNRYFRQRR
Rat	HAKAQERVRE	LNKPAQEINR	FACDDYKLCE	RYALIYGYNA	AYNRYFRQRR
Pig	RAKAQERIRE	LNKPPY <mark>E</mark> LNR	<b>F</b> ACDDYKLCE	RYAMVYGYNA	AYNRYFRQRP
	101				
Human	GTK.				
Bovine	GAK.				
Mouse	GAKY				
Rat	GAK.				
Pig	GAK.				

Figure 4.2 Multiple alignment of different mammalian *MGP* amino acid sequences.
 Conserved sites of intron insertions in porcine and mammalian *MGP* are boxed. The γ-carboxyglutamate residues are shown in black boxes.
 Motifs previously identified in mammalian *MGP* are underlined.

# 4.2 Identification of polymorphisms in porcine MGP gene

The search for sequence variations by comparative sequencing within the *MGP* gene revealed a total of 3 SNPs shown in figure 4.1. The PCR fragments were amplified with primers (Table 3.3) that covers the polymorphic sites. Two SNPs were located in intron 1, a transversion from cytosine (C) to adenine (A) at position 1124

and transition from cytosine (C) to thymine (T) at 1185 (Figure 4.3). Another SNP at position 3817 in exon 4 was transition from cytosine (C) to thymine (T). These SNP was a synonymous substitution on amino acid of porcine *MGP* gene.



**Figure 4.3** A representative image showing SNP by comparative sequencing. Column show SNP position. A) sequence with homozygous T, B) sequence with homozygous C, and C) sequence with heterozygote TC.

## 4.3 Expression profile of MGP gene in articular cartilage

In this study, RT-PCR analysis indicated that the porcine MGP gene was shown no difference (P>0.05) between healthy and OC lesions samples of articular cartilage (Figure 4.4). MGP was presented in all articular cartilages tested and was shown the lowest level in muscle. Quantitative real time PCR results showed no differentiation levels of expression in both groups. It should be noted that the expression of MGP gene also found no differences in healthy and OC pigs, this may due to a number of samples, only 12 pigs, were examined in this experiment. However, other studies have found evidence of MGP expression in proliferative and late hypertrophic chondrocytes in chondrogenic in vitro model. MGP overexpression delayed chondrocyte maturation and blocked endochondral ossification of cartilage (Yagami et al., 1999; Newman et al., 2001). Several features in MGP deficiency suggest involvement of vascular endothelium that may result from a failure in angiogenesis or vessel fusion and arterial calcification (Lou et at., 1997; Munroe et al., 1999). These may relate to endothelial dysfunction during vascular maturation and loss of architecture and hypertrophic chondrocytes in the bone growth plate that may relate to disturbed vascularization (Warburton et al., 2000).



Figure 4.4 Relative levels of MGP gene expression in total mRNA from healthy (lane 1-6) and OC lesions (lane 7-12) articular cartilage in pig. Lane 13 is RNA from meat and lane 14 is negative control reactions.

### 4.4 Identification of polymorphisms in candidate genes

Polymorphisms in candidate genes were detected in pool DNA from 8 individual experimental animals by comparative sequencing. Primers were designed from corresponding gene sequence and used to amplify overlapping fragments covering exon and intron parts. The sequences of DNA fragments were compared using the BLAST software. In order to confirm the polymorphisms each individual animals in pool DNA were sequenced that revealed a potential polymorphic site after the first analysis.

## 4.4.1 Polymorphism in the porcine *TGFβ1*

Primers were designed from the published  $TGF\beta I$  gene sequence (GenBank Accession no: AF461809 and AF461808) and used to amplify overlapping fragment of about 218 and 1083 bp in size covering part of exon 5 – intron 6. One polymorphism was a transition from Adenine (A) to Guanine (G) within exon 5 at position 797 of coding sequence. A forward primer located in intron 4 (Figure 4.5) and a reverse primer position located in exon 5 were derived to produce a 218 bp PCR product from porcine genomic DNA. Theses SNP has no effect on amino acid sequence of porcine  $TGF\beta I$ . Using PCR with primers encompassing the intron 6 (Figure 4.6), sequence comparison of these fragment was found two SNPs. First, a transition from adenine to guanine (A > G) at position 179, another transition (C > T) was found at position 1042 of the intron 6.

1 TGAGGCCCGC CTCCCTGGCC CAGCCTGTG CCCAGCAGTG ACTCTGTGCG 51 TGTGTGTGCA CTCCCTGGCC CAGCCTGTG CCCAGCAGTG GGTTCTTCCC  $TGF\beta1$  F1 101 CTGCCCACCC CCCTGAC CATCTGAGTG TGTGTGTGTA TGTCTCCCCCC 151 AACCCTATCC GCTCCCTGAC TCGTAAACCA AAGCAG

Figure 4.5 Sequence of intron 4 of  $TGF\beta I$  gene (Accession number AF461809).

#### 48

1 ATGCCGCCTT CGGGGCTGCG GCTCTTGCCG CTGCTGCTGC CGCTGCTGTG 51 GCTGCTAGTG CTGACGCCTG GCCGGCCGGC CGCCGGACTG TCCACCTGCA 101 AGACCATCGA CATGGAGCTG GTGAAGCGGA AGCGCATCGA GGCCATTCGC 151 GGCCAGATTC TGTCCAAGCT TCGGCTCGCC AGCCCCCCGA GCCAGGGGGA 201 CGTGCCGCCC GGCCCGCTGC CTGAGGCCGT ACTGGCTCTT TACAACAGTA 251 CCCGCGACCG GGTAGCCGGG GAAAGTGTCG AACCGGAGCC CGAGCCAGAG 301 GCGGACTACT ACGCCAAGGA GGTCACCCGC GTGCTAATGG TGGAAAGCGG 351 CAACCAAATC TATGATAAAT TCAAGGGCAC CCCCCACAGC TTATATATGC 401 TGTTCAACAC GTCGGAGCTC CGGGAAGCGG TGCCGGAACC TGTATTGCTC 451 TCTCGGGCAG AGCTGCGCCT GCTGAGGCTC AAGTTAAAAG TGGAGCAGCA 501 CGTGGAGCTA TACCAGAAAT ACAGCAATGA TTCCTGGCGC TACCTCAGCA 551 ACCGGCTGCT GGCCCCCAGT GACTCACCGG AGTGGCTGTC CTTTGATGTC 601 ACCGGAGTTG TGCGGCAGTG GCTGACCCGC AGAGAGGCTA TAGAGGGTTT 651 TCGCCTCAGT GCCCACTGTT CCTGTGACAG CAAAGATAAC ACACTCCACG 701 TGGAAATTAA CGGGTTCAAT TCTGGCCGCC GGGGTGACCT GGCCACCATT A797G 751 CACGGCATGA ACCGGCCCTT CCTGCTCCTC ATGGCCACCC CGCTGGAGAG  $TGF\beta 1$  R1 801 GGCCCAGCAC CTGCACAGCT CCCGGCACCG CCGAGCCCTG GATACCAACT *TGFβ1*In6F 851 ACTGCTTCAG CTCCACGGAG AAGAACTGCT GCGTGCGGCA GCTCTACATT 901 GACTTCCGGA AGGACCTGGG CTGGAAGTGG ATTCATGAAC CCAAGGGCTA 951 CCATGCCAAT TTCTGCCTGG GGCCCTGTCC CTACATCTGG AGCCTAGACA 1001 CTCAGTACAG CAAGGTCCTG GCTCTGTACA ACCAGCACAA CCCGGGCGCG *TGFβ1*In6R 1051 TCGGCGGCGC CGTGCTGCGT GCCGCAGGCG CTGGAGCCAC TGCCCATCGT 1101 GTACTACGTG GGCCGCAAGC CCAAGGTGGA GCAGCTGTCC AACATGATCG 1151 TGCGTTCCTG CAAGTGCAGC TGAGGCCCCA AGCCCACTTG GGATCGATTA 1201 AAGGTGGAGA GAGGACTGGG TCTCCGTGTG TTGGGCACCT GACTGGGGTC 1251 TTCTTCGGAC GTTACCGGAC CCCCACTCCC AGCCTCCGCC TGCCTCCGCC 1301 TGTGTCTGTC CACCATTCAT TTGTTCCTCC TCCTCATGCA AACGCGTCCT 1351 GAGCAGGTAC TCCTGGTGAA CTCTACTTAG ATTTACTTAC TGAGCATCTT 1401 GGACCTTATC CTGAATGCCT TATATTAATT AACTCATTTA ACCACCATAA 1451 CAAAGCTAAA AGGGACTCTG ATAACACCCA CTTTAAAAAG GAAACGGAAG 1501 CTGGAGTTTC CATTGTGGCT CAGTGGTAAC CTACCCGACT GGTATCCTTG 1551 AAGACACAGG TTCAATCCCT GGCCCTGTTC TGTAAGTTAA AGGTCCGGCT 1601 GTGGCGGCTG TGGTATAGGC CGGCAGCTGC AGCTCCGATT TAACCCCTAG 1651 CCTGGGAACT TCCATATGTC TCAGGTGCGG CCCTAAAAAG ACAAAAAGAA 1701 AGGAAAAGGA AGCCCATAGT GGTTAAGGGA ATAATTCCTG CCCACCAAGA 1751 ACCTGCTTTC GGCTTTCTGG TGGGGAGACA GACATAGCAA AGTTGTGTGA 1801 AAACAGGAAG GCAGTGTGGG TCAGAGAGGG CTTTGGGAGG TGGGAGGGCT 1851 TCTTGGAGGA GGTGGCACCT GGGCCTTGAA GGAAGCCAAG AAAGCAGCCT 1901 AGGGGAGCAT GGGGGAGGGT GTTCATGGTA GGAGGACAAA AGCAAAGTCC 1951 TGGAAGTGAA GATGAATTTG GGGTGAGCTA CACCGGCGGG AAAGAGGCCA 2001 GTGCGGTTGG AAGGGAGGGG CAAGGGGAAA AGTGTTGGGA TCTGAGTCAG 2051 AAAGTAACAC TCACAGGCCA GAGAGTAATA ACAGTTCTCC AGGCTAGGTA 2101 TGGAGCTACT AGCTCAAGGC ATTCTTCCCA CAGCCCAGCA GAGCAGAGGT 2151 TGTTAAACTA TTGCCTGCAG GCACATTCTG ACCCGCTGCC TGTTTCTGTA 2201 AATAAAGTTT TATTGGAGAA C

Figure 4.6 Nucleotide sequence of *TGFβ1* gene mRNA (Accession number: AF461808).

## 4.4.2 Polymorphism in the porcine *MMP3*

One polymorphism was found within porcine *MMP3* gene. The *MMP3* primer formed a 430 bp long amplicon (spanning intron 2) in pig (GenBank Accession no: AB044413). The SNP was a transition from C to T at position 158 of amplified fragment in intron 2.

	MMP3F1 →					
1	GACGGGAAAG	CTGGATTCTA	ACACTCTGGA	GGTGATGCAT	AAACCCAGAT	GTGGAGTTCC
61	TGATGTTGGT	TACTTCAGCA	CCTTTCCTGG	CCTGCCCAAG	TGGAGAAAAA	ATGACCTCAC
				C1587	C	
121	TTACAGGTAA	TGGGTCCAAA	GAGATGTTGA	TATAATA <mark>C</mark> TG	AGATTTTATT	AATTACTATC
181	ACAGGAGAAA	TATTATCTCC	AATTTTTTTT	CATACAGGAT	TGTCAATTAT	ACACTGGATT
241	TGCCAAGAAG	TGTTATTGAT	TCTACCATTG	AGAAAGCTCT	GAAAATCTGG	GAGGAAGTGA
301	CTCCGCTTAC	ATTCTCCAAG	ATTTCTGAAG	GAGAGGCTGA	CATAATGATC	ACTTTTGCAG
361	TTCGAGAACA	CGGAGACTTC	AGCCCTTTTG	ACGGACCTGG	AAAATTTTTG	GCTCATGCCT
421	ATGCACCTGG					
	← MMP3R1					

Figure 4.7 MMP3 sequence, showing primer sequences and polymorphism in intron 2.

## 4.4.3 Polymorphism in the porcine COL2A1

Primers derived from the published *COL2A1* gene sequence (GenBank Accession no: AF201724) were used to amplified. The PCR fragments of 397 bp were amplified with primer (Table 3.3) that covers two polymorphic sites at position 81 ('C/T' transition) and 156 ('G/A' transition) in intron 29 of porcine *COL2A1* (Figure 4.8).

	COL2A1 F1 -	→				
1	CAAGATGGCG	AGACAGGTGC	TGCAGGTCCT	CCTGGACCTG	CCGTAAGTAC	CTGCCCAGTC
	right		C81T			
61	TGTCCAGGTG	GCCAACGGGC	CGGGGGCTCGG	TGGGCACCGG	TGGGGAGCAA	CGGAGCTGAC
			_	G156 <i>I</i>	ł	
121	ACATGTGGAT	CTGTGTTGAT	CTGGGATGCC	TGAGCC <mark>G</mark> AGG	CAGGGTATCC	CAGGCAGCAG
181	TGTGAACCCC	ACCCTAATTT	TCAGGAGTGT	GGGTCACCAG	TGGGAGGACA	GCGAGGCACG
241	CACTTCCTTG	ACTTGGGCAG	TGTGGGAAGT	CCCCAGACCC	TTCTAGTCCT	AGATGAGGCT
321	CCCGGGTTCT	CTGGGGTGCT	GGCTCAGCTC	ACCTTTGTCC	TTCTGCTGCA	GGGACCTGCT
381	GGTGAACGAG	GCGAGCAGG	TGCTCCTGGG	CCATGAG		
			<b>←</b> (	<i>COL2A1</i> R1		

Figure 4.8 COL2A1 sequence, showing primer sequences and polymorphism in intron 29.

### 4.4.4 Polymorphism in the porcine COL10A1

Screening for polymorphisms in porcine *COL10A1* gene revealed one SNP at position 103 in exon 1. The SNP was a transition from A to G (Figure 4.9). The comparison of amino acid sequences derived for the sequence revealed that the polymorphism has no effect.

	COL2A1 F4 -	→				
1	TTCAGCCTAC	CTCCATATGC	ATTGTGTGGG	GCGGGGTGGA	GCTGGGGAAC	ACCTTTTCAT
61	GCAGATTTAT	ATTAGCTGGG	CAGGAATGGG	CTCACATTGG	AGCCACTAGG	AATCCTGAGA
121	AAGAGGAGTG	GACGTACTCA	GAGGAGTACA	GCCCGTTCGA	CCCAGCGTTG	GGCAGCTGGA
		A103G				
181	GCCATACCTG	GTC <b>A</b> TTATCT	GTGAGATCAA	GGATGGCACT	CCCTGAAGCC	TGATCCAGGT
241	AGCCTTTGAC	ATATTCATCA	TAGGTGTACA	TGACAGGGGT	GCCATTTTTA	TACAGGCCCA
321	CCCAAGCATG	GGTCCCCTTC	ACGTGAATGT	GGTAGGAGAA	GTAATTGATT	CCAGGTATCC
381	TGCAGGTAAA	GATTCCAGTT	TTTGGGTCGT	AGTGCTGTTG		
				<i>COL2A1</i> R4		

Figure 4.9 COL10A1 sequence, showing primer sequences and polymorphism in exon 1.

## 4.5 Development of multiplex SNP genotyping

In total of 10 SNPs were identified in this study. The multiplex SNP were established to facilitate large scale genotyping by using SNP primer extension technique. The primer anneals one base short of the target SNP. The length of the gene-complementary portion of the primer is selected to give a Tm of 69°– 75°C. The genomic DNA containing the SNPs were amplified by multiplexed PCR and used as template for genotyping. Genomic DNA was amplified to multiplex PCR in the presence of 7 primers pairs (Table 3.3) and confirmed each fragment by individual PCR (Figures 4.10). Primer extension step was attempted via the addition of labelled ddNTPs (CEQ SNP-Primer Extension Kit, Beckman Coulter) to SNP-specific oligonucleotide primers, prior to allele identification by analysis on a DNA sequencer (Beckmann Coulter CEQ8000) (Figure 4.11). The SNP primers extension included in the speciation assay range in size from 21 bp to 79 bp, although migration does not exactly correspond to primer size (Table 3.5). SNP primers were all separated by a migration gap of at least 4 bp to ensure ease of identification. As recommended by Beckman, the annealing temperature used for the generation of the primer extension

product at 64°C was selected to be at least 5°C below the lowest primer midpoint temperature.

All SNPs were genotyped in 310 animals of the F2-DUPI resource population including the animals of the grandparental generation (Duroc and Pietrain pig) and the F1 parental generations (data show in the appendix). Genotype frequencies are shown in Table 4.3. Multiplexed SNP genotyping using the CEQ8000 genetic analysis system was explored further by determining 10 different SNP genotypes for 96 genomic DNA samples. The result demonstrates an efficient process of producing for 960 SNP genotypes in total and was able to call the correct genotypes with high degree of accuracy approximately 96%.



Figure 4.10 Agarose gel analysis of normal PCR and multiplex PCR products after amplification. Lane:1-2, *MGP*WF1/WR1 primer (1088 bp), Lane:3-4, *MGP*F2/R1 primer (1161 bp), Lane:5-6, *MMP3*F1/R1 primer (434 bp), Lane:7-8, *COL2A1*F1/R1 primer (397bp), Lane:9-10, *TGFβ1*F1/R1 primer (218 bp), Lane:11-12, *COL10A1*F4/R4 primer (400 bp), Lane:13-14, *TGFβ1*In6F/R primer (1083 bp), and Lane: 15-17, all primers that used in normal PCR.



**Figure 4.11** Example of the genotype identified by multiplex SNP assay. The first and last peaks represent size standards of 13 and 88 bp, respectively. The ten peaks in assay relate to  $MGP_{C1124T}$ ,  $MGP_{C1185A}$ ,  $MGP_{C3817T}$ ,  $MMP3_{C158T}$ ,  $COL2A_{C81T}$ ,  $COL2A1_{G156A}$ ,  $COL10A1_{G103A}$ ,  $TGF\beta1_{A797G}$ ,  $TGF\beta1_B$ ,  $TGF\beta1_C$  moving from the smallest to largest fragment. The colors of the peaks reflect different bases, where T is blue, C is black, A is red and G is green.

### 4.6 Association analysis

Four of the candidate genes were associated with osteochondrosis and DXA traits, and only one gene (*COL10A1*) was not significantly associated in either trait. Individual polymorphisms in genes *MGP*, *TGF* $\beta$ 1, *MMP3* and *COL2A1* were found to be significantly associated (Table 4.2). Haplotype frequencies were estimated by hbat option of FBAT program. Haplotype TAA and TAG of *MGP* gene were associated with OC as well as DXA traits. Whereas haplotype CA of *COL2A1* was associated with OC in knee joint. However, no association of *TGF* $\beta$ 1 gene and OC and DXA traits were found in this study.

Either of individual polymorphism or haplotype at MGP gene was associated with osteochondrosis and DXA traits. This result suggested the function of MGP protein on this bone related traits. These results are in agreement with association analysis in human that human MGP gene had shown to be significantly associated with BMD trait (Tsukamoto et al., 2000, Zebboudj et al., 2002). The mechanisms of inhibits mineralization by MGP remain unclear. However, at least two mechanisms could account for the inhibitory function of MGP on calcification. First, MGP is a member of the mineral-binding Gla protein family (Price et al., 1998), which includes osteocalcin. MGP binds calcium ions and hydroxyapatite via its five  $\gamma$ -carboxylated glutamic acid (Gla) residues. Inhibition of the  $\gamma$ -carboxylation of Gla residues results in increased matrix mineralization, suggesting that the mineral-binding Gla residues are crucial for regulation of matrix mineralization. Second, MGP has been shown to modulate the biological activity of the  $TGF\beta$  superfamily members such as bone morphogenetic proteins (BMPs) (Boström et al., 2004)). It was thereafter reported that MGP modulates BMP activity in mesenchymal cell differentiation (Price et al., 1998). Moreover, deficiency of MGP results in multiple developmental defects including abnormal vascularization of the bone growth plate and induced apoptosis, suggesting involvement of a role in cell differentiation (Newmann et al., 2001, Zebboudj et al., 2004). Another interesting notion, it has been demonstrated that MGP protein could be identified in a synovial fluid sample from an osteoarthritis patient but not in a healthy cartilage (Kamphorst et al., 2007).

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		9	312		<i>P</i> -value			
Allele	Frequencies	DMD	PMC		Oste	ochondrosis l	esion	
		BNID	DIVIC	Shoulder	Elbow	Нір	Knee	Total
С	0.36	<0.001**	<0.001**	<0.001**	<0.001**	0.002**	<0.001**	<0 001*;
Т	0.64	20.001			_0.001	0.002	20.001	_0.001
G	0.41	0.007**	0 004**	0.006**	0.008**	0.019*	0 009**	0.011*
Α	0.59	0.007	0.001	0.000	0.000	0.017	0.005	0.011
G	0.35	<0 001**	<0.001**	0.003**	<0 001**	0.013*	0 004**	<0.001**
A	0.65	_0.001	_0.001	0.005	_0.001	0.010	0.004	_0.001
C	0.39	<0.001**	<0.001**	<0.001**	<0.001**	≤0.001**	≤0.001**	<b>≤0.001</b> **
Т	0.61	_0.001	20.001	_0.001	_0.001			
G	0.36	0 178	0 184	0.280	0 242	0 369	0.538	0 219
Α	0.64	0.170	0.101	0.200	0.212	0.507	0.550	0.217
С	0.34	0 257	0.237	0.089	0.105	0.542	0.022*	0 037*
Т	0.66	0.237	0.251	0.007	0.105	0.512	0.022	0.007
G	0.49	0.988	0.945	0.466	0.651	0.543	0 569	0.662
Α	0.51	0.900	0.9 18	0.100	0.051	0.515	0.50)	0.002
С	0.47	<0.001**	<0.001**	<0.001**	<0.001**	<0.001**	0 007**	0 001**
Α	0.53	_0.001	_0.001	_0.001	_0.001	_0.001	0.007	0.001
G	0.53	0.011*	0.013*	0.064	0.013*	0 006**	0 077*	0 006**
A	0.47	0.011	0.015	0.004	0.015	0.000	0.022	0.000
C	0.58	0.442	0.359	0.48	0 335	0.538	0.831	0.972
Т	0.42	0.442	0.557	0.70	0.555	0.550	0.051	0.972
	Allele           C           T           G           A           G           A           C           T           G           A           C           T           G           A           C           T           G           A           C           A           C           A           G           A           C           A           C           A           C           T	AlleleFrequenciesC $0.36$ T $0.64$ G $0.41$ A $0.59$ G $0.35$ A $0.65$ C $0.39$ T $0.61$ G $0.36$ A $0.64$ C $0.34$ T $0.66$ G $0.49$ A $0.51$ C $0.47$ A $0.53$ G $0.53$ A $0.47$ C $0.58$ T $0.42$	AlleleFrequenciesBMDC $0.36$ T $0.001^{**}$ G $0.41$ 0.007** $0.007^{**}$ G $0.35$ A $0.001^{**}$ C $0.39$ T $0.001^{**}$ G $0.36$ 0.01** $0.001^{**}$ G $0.36$ 0.39 $0.001^{**}$ G $0.36$ 0.36 $0.178$ G $0.36$ 0.64 $0.178$ C $0.34$ 0.64 $0.257$ G $0.49$ 0.988 $0.988$ C $0.47$ A $0.001^{**}$ G $0.53$ A $0.011^{**}$ G $0.53$ A $0.477$ C $0.58$ T $0.442$	AlleleFrequenciesBMDBMCC $0.36$ T $0.001^{**}$ $0.001^{**}$ G $0.41$ O.59 $0.007^{**}$ $0.004^{**}$ G $0.35$ A $0.001^{**}$ $0.001^{**}$ C $0.35$ C $0.001^{**}$ $\leq 0.001^{**}$ G $0.35$ C $\leq 0.001^{**}$ $\leq 0.001^{**}$ G $0.35$ C $\leq 0.001^{**}$ $\leq 0.001^{**}$ G $0.36$ C $0.178$ $0.184$ C $0.34$ C $0.257$ $0.237$ G $0.49$ C $0.988$ $0.945$ C $0.47$ A $\leq 0.001^{**}$ $\leq 0.001^{**}$ G $0.53$ A $0.011^{**}$ $0.013^{**}$ C $0.58$ T $0.442$ $0.359$	AlleleFrequenciesBMDBMCShoulderC $0.36$ T $0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ G $0.41$ A $0.007^{**}$ $0.004^{**}$ $0.006^{**}$ G $0.35$ A $0.001^{**}$ $\leq 0.001^{**}$ $0.003^{**}$ C $0.39$ T $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ G $0.36$ C $0.178$ $0.184$ $0.280$ C $0.34$ C $0.257$ $0.237$ $0.089$ G $0.49$ A $0.988$ $0.945$ $0.466$ C $0.47$ A $\leq 0.001^{**}$ $\leq 0.001^{**}$ G $0.53$ A $0.011^{**}$ $\leq 0.001^{**}$ G $0.53$ A $0.011^{**}$ $\leq 0.001^{**}$ G $0.53$ A $0.011^{**}$ $0.48$	Allele         Frequencies         BMD         BMC         Shoulder         Elbow           C         0.36 $\leq 0.001^{**}$ $\leq 0.008^{**}$ G         0.35 $\leq 0.001^{**}$ $\leq 0.001^{**}$ $= 0.008^{**}$ $= 0.008^{**}$ G         0.35 $\leq 0.001^{**}$ $\leq 0.001^{**}$ $= 0.001^{**}$ $\leq 0.001^{**}$ C         0.39 $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ G         0.36         0.178         0.184         0.280         0.242           C         0.34         0.257         0.237         0.089         0.105           G         0.49         0.988         0.945         0.466         0.651           C         0.47 $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ G         0.53         0.011^{**} $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^$	Allele         Frequencies         BMD         BMC         Shoulder         Elbow         Hip           C         0.36 T $0.001^{**}$ $\leq 0.001^{**}$ $0.002^{**}$ G         0.41 A         0.59         0.007^{**}         0.004^{**} $0.006^{**}$ $0.008^{**}$ $0.002^{**}$ G         0.35 A $0.007^{**}$ $0.004^{**}$ $0.006^{**}$ $0.008^{**}$ $0.019^{*}$ G         0.35 A $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ C         0.39 T $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ G         0.36 A $0.178$ $0.184$ $0.280$ $0.242$ $0.369$ C         0.34 A $0.257$ $0.237$ $0.089$ $0.105$ $0.543$ C $0.47$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ $\leq 0.001^{**}$ <t< td=""><td>Allele         Frequencies         BMD         BMC         Shoulder         Elbow         Hip         Knee           C         0.36 T         <math>\leq 0.001^{**}</math> <math>\leq 0.001^{**}</math></td></t<>	Allele         Frequencies         BMD         BMC         Shoulder         Elbow         Hip         Knee           C         0.36 T $\leq 0.001^{**}$

Table 4.2 Associations of individual SNP with DXA and osteochodrosis lesions

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			9	312		P-value			
Gene, SNP	Hanlotyne	Frequencies				Oste	ochondrosis l	esion	
Gene, SNP MGP: MGPe4, MGPin1A, MGPin1B TGFβ1: TGFB1ex5, TGFB1in6A, TGFB1in6B			BMD	BMC	Shoulder	Elbow	Нір	Knee	Total
MGP:	TAA	0.368	≤0.01*	≤0.01*	≤0.01*	≤0.01*	≤0.01*	≤0.01*	≤0.01*
MGPe4,	TAG	0.314	<b>≤0.01</b> *	≤0.01*	≤0.01*	<b>≤0.01</b> *	<b>≤0.01</b> *	≤0.01*	<b>≤0.01</b> *
MGPin1A,	CAA	0.101	ns	ns	ns	ns	ns	ns	ns
MGPin1B	CCG	0.062	na	na	ns	ns	ns	ns	ns
	CAG	0.061	ns	ns	ns	ns	ns	ns	ns
	TCG	0.047	ns	ns	ns	ns	ns	ns	ns
	TCA	0.047	ns	ns	ns	ns	ns	ns	ns
TGFβ1:	CGC	0.333	ns	ns	ns	ns	ns	ns	ns
TGFB1ex5,	TGC	0.208	ns	ns	ns	ns	ns	ns	ns
TGFB1in6A,	TGT	0.125	ns	ns	ns	ns	ns	ns	ns
TGFB1in6B	TAC	0.104	ns	ns	ns	ns	ns	ns	ns
	CAT	0.083	na	na	na	na	na	na	na
	CAC	0.062	na	na	ns	ns	ns	ns	ns
	CGT	0.042	ns	ns	ns	ns	ns	ns	ns
	TAT	0.042	ns	ns	ns	ns	ns	ns	ns
COL2A1:	ТА	0.412	ns	ns	ns	ns	ns	ns	ns
COL2A1in29A,	TG	0.305	ns	ns	ns	ns	ns	ns	ns
COL2A1in29B	CG	0.225	ns	ns	ns	ns	ns	ns	ns
	CA	0.058	ns	ns	ns	ns	ns	≤0.05*	ns

Table 4.3 Estimated haplotype frequencies of MGP, TGFβ1 and COL2A1 with DXA and osteochodrosis lesions

ns= no significant (P>0.05),\* P≤0.05, \*\*P≤0.001, na = not available

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 $TGF\beta 1$  promotes type II collagen and proteoglycan synthesis and down regulates cartilage degrading enzymes (Hashimoto et al., 2007). TGF\$1 is secreted by chondrocytes in an inactive form bound to a latency molecule, underlying the importance of defining the factors leading to  $TGF\beta 1$  activation in the growth plate (Pedrozo et al., 1998). It has been implicated as being responsible for regulator of growth cartilage metabolism, particularly in the control of chondrocytes differentiation and hypertrophy, such as those present during osteoclastic bone resorption (Angelo et al., 2001). The result was found significant association in BMD, BMC and OC lesions except lesion on shoulder (P = 0.064) (Table 4.3). In recent years, polymorphisms in  $TGF\beta 1$  have been identified and found to be correlated with spine OA in some studies (Horton et al., 1997). Furthermore, in vitro studies of  $TGF\beta$ 1 are a potent inhibitor of maturation, including cell hypertrophy, type-X collagen expression and alkaline phosphatase (ALP) activity (Ballock et al., 1993; Schipani et al., 1997). ALP is expressed at the cell surface and facilitates the release of inorganic phosphate required for bone formation. ALP activity is elevated in the transitional chondrocytes of chicks with dyschondroplasia, confirming our earlier report (Farquharson et al., 1992c). TGF\$1 is known to inhibit ALP activity as well as regulate proteoglycan production and collagen synthesis within chondrocytes. TGF\$1 on other hand, potently enhances the proliferation of osteoprogenesis, but inhibits their terminal differentiation and expression of OC (Ogata et al., 1997).

The polymorphism at *MMP3* gene had a very large effect on OC lesions. *MMP3* gene encodes a matrix metalloproteinase involved in articular cartilage extracellular matrix. *MMPs* are responsible for catabolism and turnover of the matrix and are induced during the process of chondrocyte hypertrophy (Angelo *et al.*, 2001). Inappropriately high *MMP3* levels would implicate increased protease activity as a cause of abnormal extracellular matrix in cartilage affected by OC (Ohata *et al.*, 2002, Kuroki *et al.*, 2005). *MMP3* is produced by hypertrophic chondrocytes and is secreted in matrix vesicles. Mineralization of the matrix is associated with a marked increase in the cleavage of type II collagen by *MMPs* (Angelo *et al.*, 2001; Mwale *et al.*, 2002). Matrix vesicles also contain *TGF* $\beta$ , which is present in a latent form but is activated by *MMP3*. The increased levels of active *TGF* $\beta$  present in the growth plate at the onset of mineralization are believed to be due, in part, to the presence of *MMPs*  in matrix vesicles. Additionally, *MMPs* are critical for angiogenesis in the growth plate and thus are necessary for normal calcification and bone formation. Mice without *MMPs* have defective angiogenesis, reduced chondrocyte apoptosis, widening of the hypertrophic zone, and decreased mineralization of the matrix (Vu *et al.*, 1999). The effects of the *MMPs* on angiogenesis may be related to a decrease in catabolism of the matrix, release of important growth factors, or other effects, but these relationships have not yet been clarified.

Polymorphisms in the *COL2A1* gene have previously been implicated in the risk of OC as well as in the occurrence of chondrocytes. *COL2A1* gene encoding for type II collagen has been shown to cause a variety of chondrodysplasias and osteoarthrosis. It also plays a role in the pathogenesis of disc degeneration (Sahlman, 2007). Mutated type II collagen has been shown to disturb chondrogenesis and decrease bone strength (Savontaus *et al.*, 1996). Recent studies of animals have demonstrated that uremia, which retards growth in children, is associated with an increase in the width of hypertrophic cartilage as well as a decrease in the deposition of both type II collagen, alterations in collagen fibril architecture, and defective mineralization (Alvarez *et al.*, 2001). The deletions in the mouse *COL2A1* gene result in dysmorphogenesis of the joint leading to poor articulation and biomechanical imbalance in addition to loss of matrix integrity (Helminen *et al.*, 2002). It appears that any mutation in extracellular matrix components that can compromise either matrix integrity or morphogenesis have the potential to predispose a joint to OA.

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