

CHAPTER 2

LITERATURE REVIEW

2.1 Bone and cartilage structure

Bone tissue, the major constituent of animal skeleton has structural, protective and metabolic functions. Bone is formed out of organic and inorganic matrix. The inorganic matrix consists primarily of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$), which is covered by a layer of calcium carbonate (Glimcher, 1992). The mineral deposit of the bone is responsible for the compressional strength of the bone. The organic matrix consists of collagen (90%), fat, glycoproteins and proteoglycans (PGs) (Robey, 1988). Bone is developed either by intramembranous or endochondral ossification. Former means direct ossification of embryonic connective tissue and is responsible for formation of initial bone tissue in the flat bones of the skull and face, mandible and clavicle. Endochondral ossification is the processes characteristic for the vertebrate and long bones development. Both the intramembranous and endochondral ossification begin with the proliferation and aggregation of mesenchymal cells at the site of the future bone. The only distinction between these two processes is the differentiation of mesenchymal cells into chondroblasts before calcification in the endochondral ossification.

Cartilage is classified into three types, elastic cartilage, hyaline cartilage and fibrocartilage, which differ in the relative amounts of these three main components. The most abundant type is the hyaline cartilage or articular cartilage. There is specialized connective tissue that covers the ends of bones within diarthrodial joints (Figure 2.1, 2.2). The primary functions of articular cartilage are to support and distribute forces generated during joint loading, stabilize and guide joint motions and contribute to joints lubrication (Mow, 1997). The biomechanical properties of articular cartilage result from a complex interaction of genetic, environment and growth leading to a heterogeneous. Disruption of the cartilage framework results in alternations of its properties and leads to perception of pain, loss of motion, strength or instability.

During skeletal development cartilage provides a framework for endochondral ossification.

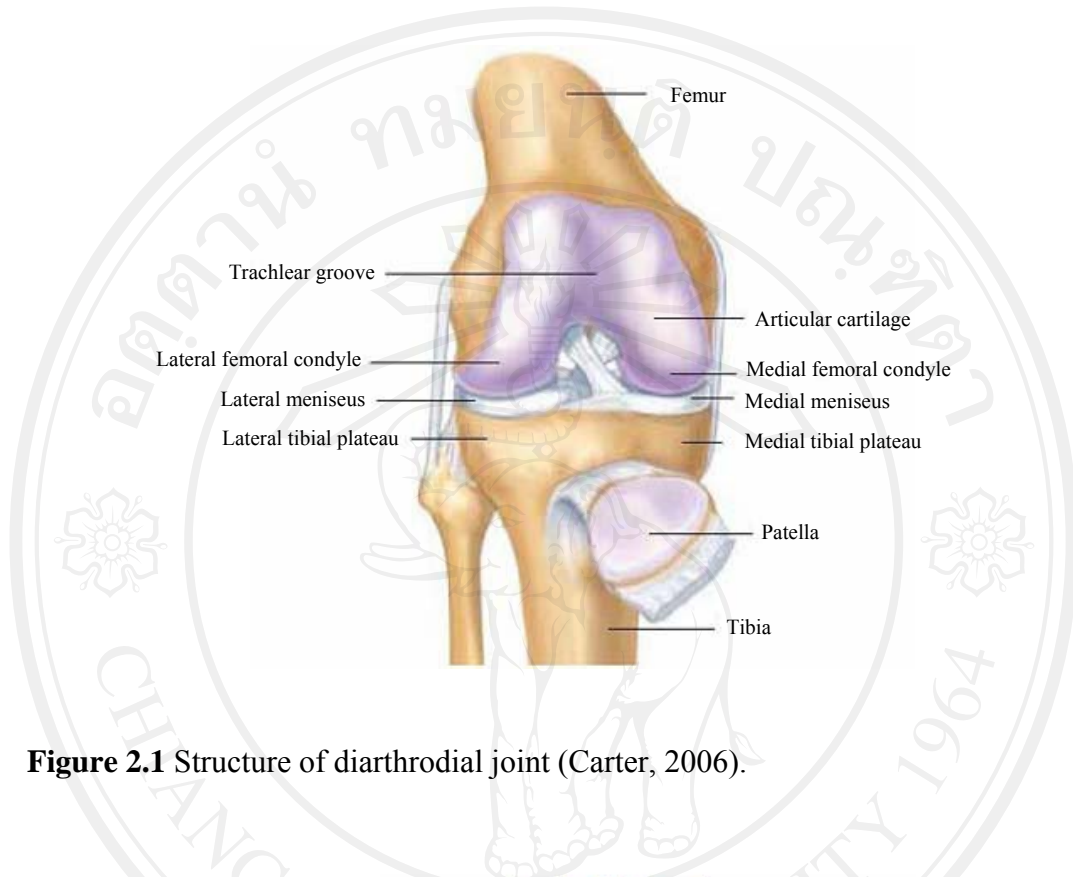


Figure 2.1 Structure of diarthrodial joint (Carter, 2006).

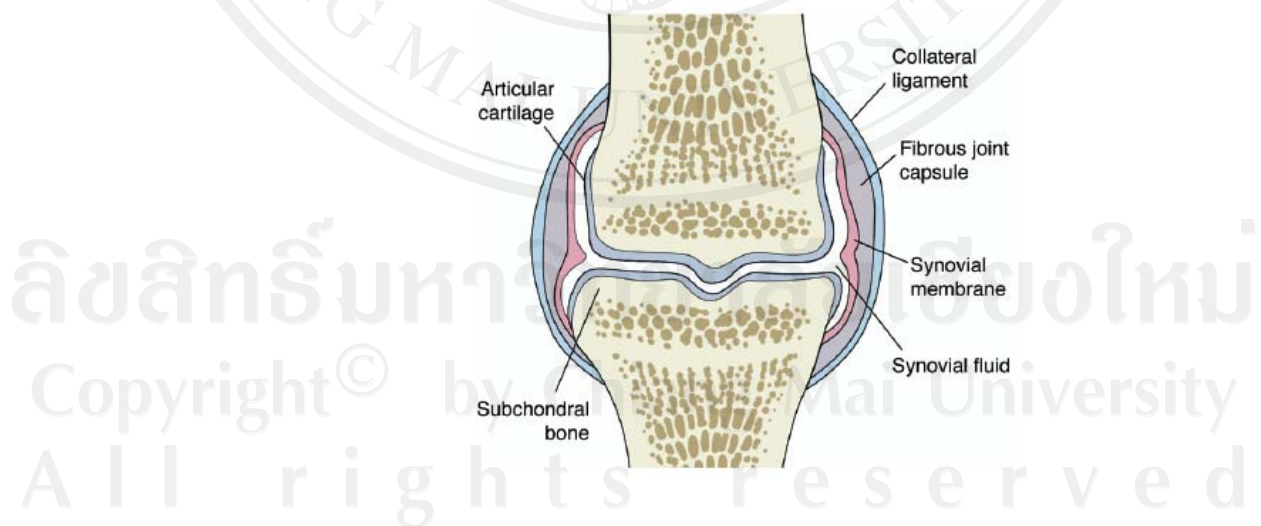


Figure 2.2 Diagrammatic section of a diarthrodial joint (Krane, 2006).

2.1.1 Articular cartilage composition

Articular cartilage is composed of chondrocytes and the extracellular matrix component (Figure 2.3). Chondrocytes are the only one cell type in articular cartilage. These cells are highly differentiated cells. They produce and maintain the extracellular matrix through the formation of collagens, non-collagenous protein and proteoglycans. They also produce degradative enzymes that are responsible for the normal turnover of these macromolecules. Adult articular cartilage has a very limited supply of blood vessels. Nutrients and cytokines have to diffuse through the extracellular matrix to reach the chondrocytes. There are no nerves in articular cartilage; the weight-bearing surfaces of the joint depend on nerve endings in the capsule, muscles, and subchondral bone for appreciation of pain and proprioception. As a result, the structure and composition of the extracellular matrix play a significant role in determining the transport properties of the tissue.

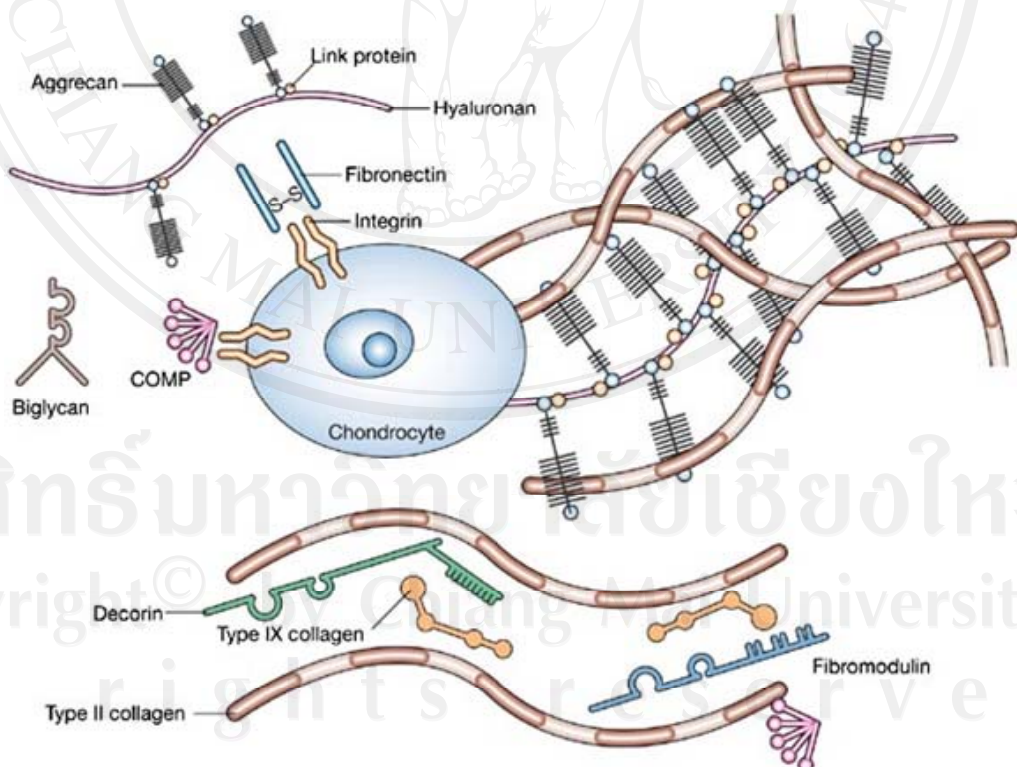


Figure 2.3 Components of articular cartilage (Chen *et al.*, 2006).

Chondrocytes

The chondrocytes are pivotal to the maintenance of cartilage function as they control the synthesis and breakdown of the matrix molecules. Chondrocytes are known to be surrounded by a pericellular matrix made up mainly of proteoglycans and some collagen molecules which separate plasma membrane from extracellular matrix. Although the role of pericellular matrix is unclear, it is thought to be significant in matrix biomechanics and in the transport of molecules to the chondrocytes. Chondrocytes are organized into four different zones of articular cartilage through the tissue depth (Figure 2.4) (Lepine, 2000; Ytrehus *et al.*, 2007). Zone I, the superficial zone characterized by flattened disc-shaped chondrocytes. It has low cellularity and little proteoglycan content. Collagen fibrils are oriented parallel to the articular surface. The orientation resists the swelling pressure exerted by proteoglycans of the deeper zones. Within zone II, the transitional zone is more cellular and has more proteoglycan content than zone I. The chondrocytes are rounded and separated from each other. Collagen fibrils are obliquely oriented. Chondrocytes in zone III, the radiate zone, characterized by round cells arranged in columns. It is the major portion of the cartilage matrix. Chondrocyte density and proteoglycan content are greater than zone II, and cells tend to be arranged in radially aligned vertical columns. The high concentration of proteoglycan in zone III allows it to withstand compressive loads. The region farthest removed from articular surface is zone IV, also referred to as calcified zone. The tidemark delineates zones III and IV. Few chondrocytes are observed in this zone. Those that are present are necrotic chondrocytes due to the calcified matrix. The deep zone forms an interlocking network that anchors the cartilage to the bony substrate.

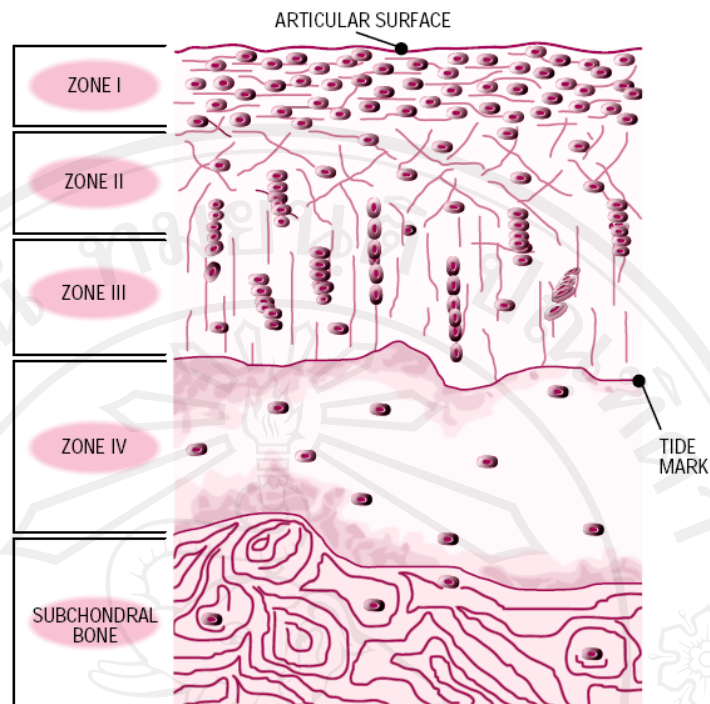


Figure 2.4 Schematic cross-section of an articular epiphyseal cartilage complex (Lepine, 2000).

Collagen

Collagen fibrils impart tensile strength to articular cartilage and provide a framework for embedded proteoglycans and chondrocytes. Collagen molecules are composed of a triple helix of polypeptide α -chains of about 1000 amino acids each. These polypeptides are arranged as a framework in which the collagen fibrils are stabilized by covalent intermolecular cross-links. The high proportions of hydroxylysine and hydroxyproline residues in the polypeptide chains of collagen are unique relative to other proteins of animal origin (Lepine, 2000). There are several different variants of collagen. Approximately 90–95% of the collagen in articular cartilage is type II collagen. The remainder are types V, VI, IX, and XI collagens. It is suggested that the incorporation of these minor amounts of collagen types into the collagen matrix may be important in physically organizing the matrix (Hardingham and Bayliss, 1990; Kuettner *et al.*, 1991). The collagen fibrils are arranged according to a specific architecture, which is crucial for the mechanical properties of articular cartilage.

The collagen fibril network itself also shows a typical architecture in healthy adult cartilage. Three zones can be distinguished, from top to bottom: a superficial zone where the fibrils are highly organised and have a preferential direction parallel to the articular surface; a transitional zone with very low fibril organisation and (therefore) no real preferential direction; and finally, the deep zone where fibrils are again highly organised and prefer a direction perpendicular to the subchondral bone (Irrechukwu, 2007).

Proteoglycan and Glycosaminoglycan

Proteoglycans is the major solid components of articular cartilage. Proteoglycans play an important role in resistance to compressive forces as they are interspersed between the collagen fibrils and through their hydrophilic character, generating a swelling pressure that provides the collagen network with an intrinsic tension. It consists of one or more glycosaminoglycans (GAGs) chains covalently attached to a protein core. Proteoglycans affect the cartilage mechanical and physical properties, such as compressive stiffness, shear stiffness, osmotic pressure and regulation of hydration (Buckwalter *et al.*, 1994).

The large aggregating proteoglycan, which is the most abundantly found in articular cartilage, is called “aggrecan”. The aggrecan structure consists of a core protein with GAGs side chains covalently attached. The attachment to the core protein is at a serine residue and is stabilized by a link tetrasaccharide. These GAGs are linear repeating disaccharide chains, which are highly negatively charged by carboxylate and sulphate groups.

2.1.2 Metabolism of normal articular cartilage

The metabolism of cartilage is very low compared to other tissues. Chondrocytes cell functions as maintenance of matrix homeostasis. Normal tissue function requires that normal architecture of this matrix be maintained. This is controlled through the regulation of synthetic and degradative events. In normal tissue, matrix homeostasis is balanced with the rate of new matrix synthesis being equal to the rate of matrix degradation, so that there is neither net tissue gain nor loss. Both processes are controlled by a variety of extracellular proteins such as growth

factor and cytokines. Growth factor, like insulin-like growth factor-I (*IGF-I*) and transforming growth factor- β (*TGF β*) are most commonly associated with the stimulation of connective tissue formation, whereas cytokines, such as interleukin-1 (*IL-1*) and tumor necrosis factor (*TNF*) are associated with the stimulation of matrix degradation (Semevolos *et al.*, 2004).

2.1.3 Mechanical properties of bone diseases

Mechanical properties of bone are affected by bone quality, geometry and micro-architecture (Sahlman 2007). Bone mineral density (BMD) refers to the amount of mineralized bone tissue in a given area, usually calculated as grams per square centimeter. Volumetric BMD (vBMD) (calculated as grams per cubic centimeter) corrects the BMD for bone size. Thus, the thickness of the bone does not correlate with vBMD as it does with BMD. As the bone grows, the volume increases at a faster rate than the area, so the areal BMD will increase even if the vBMD remains stable. Degenerative factors (osteophytes, osteochondrosis, scoliosis and vascular calcification) were evaluated from plain lumbar radiographs, their estimated probability was analyzed as a function of age, and their influence on BMD measured by DXA was determined (Rand *et al.*, 1997). Decrease in BMD has been demonstrated in animals and humans under conditions of weightlessness or immobilization (Barlet *et al.*, 1995). In human medicine, determination of these variables is critical for the early detection of osteoporosis and other bone diseases (Fürst *et al.*, 2008). Moreover, reported in warm blood foals, BMD was low in the sires group known to have high radiographic evidence of OC (Firth *et al.*, 1999).

In pig, dual energy X-ray absorptiometry (DXA) technique for the measurement of BMD and bone mineral content (BMC) have been validated independently by multiple investigators (Koo *et al.*, 2004). The BMD and BMC parameters are most often considered as the major indicator of bone strength, also the collagen content and fibril organization have been shown to correlate positively with the mechanical strength of bone.

Genes account for about 70 - 80% of the BMD variability (Brown *et al.*, 1997; Deng *et al.*, 2000; Hunter *et al.*, 2001; Ferrari and Rizzoli, 2005). Genes encoding for the proteins that are responsible for the coordination of the osteoclast and osteoblast

function are obviously suspected to be key factors in the genetic variance of BMD, such as genes encoding for vitamin D receptor (Kim, 2002; Grundberg *et al.*, 2003), estrogen receptor alpha, calcitonin receptor, osteoprotegerin, interleukin 6 and interleukin 10. Also, there is evidence for the BMD variation associated to combined polymorphism of several genetic loci (Sahlman, 2007).

2.2 Alterations in articular cartilage

Alterations in articular cartilage are found most frequently in pigs with degenerative joint disease, especially osteochondrosis. Articular cartilage injuries are characterized by the degree of involvement of the extracellular matrix composition and resultant damage to the chondrocytes (Hill, 1990a; Buckwalter *et al.*, 1994). The pathophysiological response of articular cartilage surrounding the injury results in chondrocyte proliferation and synthesis of extracellular matrix protein. Unfortunately, since chondrocytes cannot migrate to the lesion, these efforts do not result in complete repair (Lepine, 2000).

OC is defined as a degenerative joint disease and continued progression of OC leads to severe joint inflammation and the joint can subsequently develop osteoarthritis (OA) (Crenshaw, 2006). It is characterized by degeneration of endochondral ossification (Nakano and Aherne, 1994). Endochondral ossification is an ordered process, including cartilage proliferation, maturation and calcification followed by osseous replacement (Junqueira *et al.*, 1998). It is responsible for longitudinal bone growth and enlargement of the epiphysis (Olsson, 1987). Necrosis in areas of endochondral ossification may be due to a disruption of capillary vessels that extend from the subchondral bone and irrigate the epiphyseal cartilage. The hypertrophic areas of cartilage mentioned above may impose an abnormally longer passageway of diffusion for nutrients from the synovial into the deeper layers of cartilage, leading to necrosis. The areas of defective cartilage may remain in the deep layer or dissecting lesions may occur, leading to the formation of a cartilaginous fragment. These flaps of cartilage can be avulsed and remain loosely attached to the articular cartilage.

Normally, chondrocytes are able to synthesize the collagen, proteoglycans and other components needed to maintain articular cartilage homeostasis, integrity and

breakdown of extracellular matrix macromolecules. Within limits, connective tissue cells respond to mechanical forces and may adapt and remodel the extracellular matrix by altering this balance. The degeneration or progressive loss of normal structure and function of articular cartilage is the fundamental tenant of OC. Chondrocyte necrosis is evident as osteoarthritis progresses. The synthesis of extracellular matrix ceases while degradative activity remains elevated. The collagen network becomes increasingly disorganized and disintegrated. The content of several extracellular components including collagen and proteoglycans are progressively reduced (Dijkgraaf *et al.*, 1995). The removal of functional proteoglycans from the extracellular matrix results in decreased water content of the cartilage and a subsequent loss of biomechanical properties, such as resilience and elasticity. As a result the chondrocytes are subjected to increasing mechanical stress and trauma, thereby accelerating the osteochondrotic process (Frenkel and Cesare, 1999).

Concurrent with the increased synthetic response by the chondrocyte is matrix degradation, often proceeding at a rate exceeding that of synthesis. Proteoglycan and collagen breakdown is mediated by an increase in matrix metalloproteinases (*MMPs*), aggrecanase and other proteases at the articular surface early in the degenerative process. These proteases are responsible for the extensive matrix degradation associated with osteoarthritis (Figure 2.5). Cytokines such as interleukin-1 (*IL-1*) and *IL-17* induce a switch in the synthesis pattern of chondrocytes from matrix molecules to matrix-degrading enzymes. In addition, synovial fibroblasts start producing matrix degrading enzymes and invade cartilage when activated by cytokines such as *TNF* and *IL-1*. Chondrocyte death is another feature of cartilage damage. It leads to the formation of empty lacunae and deprives cartilage from the ability to replenish matrix (Malemud and Goldberg, 1999). In healthy articular cartilage the activity of the metalloproteinase is low. Enzyme activity is regulated by the presence of tissue inhibitors of metalloproteinases (*TIMPs*) resulting in a low turnover of extracellular matrix proteins. During the osteoarthritic disease process, the production of metalloproteinase greatly exceeds the ability of heightened release of *TIMPs* to maintain homeostasis (Muir, 1978).

Studies on changes of collagens in OC indicate a very slow turnover of collagen type II *in vivo* in adult humans, and the small amounts of the minor collagens

present in the matrix. As mentioned earlier, collagen turnover time is extremely slow in mature individuals, but the presence of type II collagen pro-peptides in adult articular cartilage shows that remodeling does take place, albeit slowly. Type X collagen is normally present only in sites near cartilage mineralization where chondrocytes are hypertrophic (Kim, 2002). In OC, several investigators have reported a decreased expression of collagen type II and increased of type X at sites of osteophytes and in clusters of chondrocytes in cartilage matrix (Jefferies *et al.*, 2002). The destruction of joint cartilage matrix in OC is caused by the action of proteases, which are released by the chondrocytes themselves, by cells in the synovial fluid such as leukocytes and macrophages, or by cells in the synovium (Ohata *et al.*, 2002).

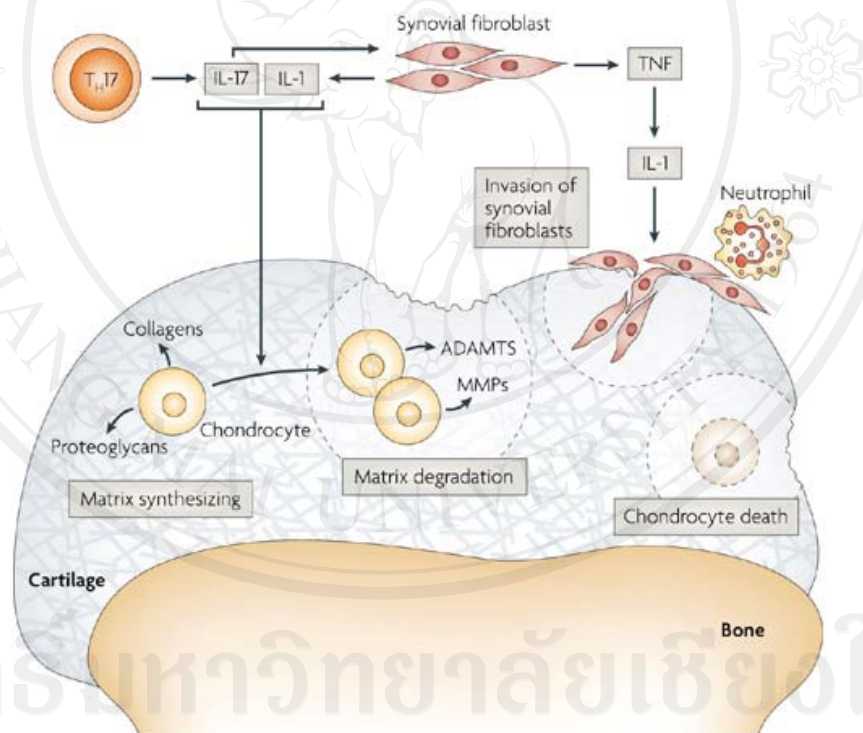


Figure 2.5 Schematic relation of cartilage degradation (McInnes and Schett, 2007).

2.3 Osteochondrosis in pig

Osteochondrosis (OC) is a common disease characterized by disturbance of endochondral ossification resulting in retention of cartilage foci within subchondral bone (Figure 2.6). It occurs in joint cartilage of growing animals and human (Nakano

and Aherne, 1994). OC is a problem of economic losses mainly due to culling of pigs at many levels in the pig industry. It is one of the important causes of leg weakness and can reduce longevity in sow and boar herds (Hill, 1990b). OC has been shown to correlate with decreased meat yield of finishing pig (Nakano and Aherne, 1988). OC is usually detectable in animal less than 6 months old by radiographic examination. As growing cartilage is a tissue undergoing complex processes of proliferation and maturation especially during the first few months of life, it is very important to distinguish between the times of the first detection of an osteochondrotic lesion. Early lesions of OC in pig have been associated with abnormalities in cartilage canal blood vessels that are hypothesized to result in local ischemia (Carlson *et al.*, 1991; Ytrehus *et al.*, 2007). It has been suggested that focal failure of blood supply in the growth cartilage causes local ischemia, which in turn leads to focal necrosis cartilage. Cartilage canals, channels that contain blood vessels, nerves, and possibly lymphatics, are also confined to epiphyseal cartilage and may assist in the nutrition of this cartilage (Ytrehus *et al.*, 2004).

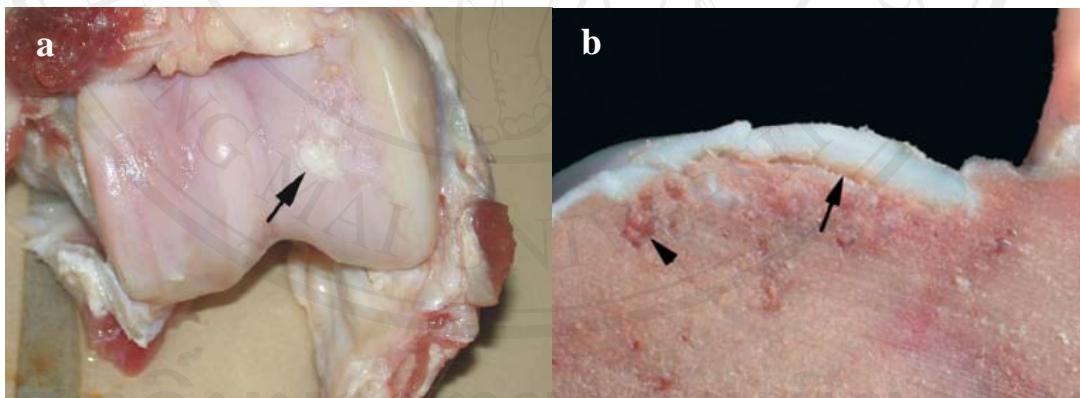


Figure 2.6 Gross lesion of osteochondrosis (a) cartilage repair (arrow) of the medial femoral condyle. (b) OC Section through (a) showing osteochondritis dissecans (arrow) and subchondral cyst formation (arrowhead) (Kirk *et al.*, 2008).

Some studies have been reported on the molecular event of articular cartilage in OC lesions. These studies suggested that disruption of the integrity of cartilage extracellular matrix play a role in the disease development (Nakano *et al.*, 1984;

Wardale and Duance, 1994). Degradation of extracellular matrix may also contribute to alterations in the biochemical characteristics of articular cartilage affected by OC. Cell and matrix components of the growth plate have been studied in order to determine the causes of the premature arrest of chondrocyte differentiation and retention of prehypertrophic chondrocytes observed in OC (Farquharson and Jefferies, 2000). These chondrocytes actively establish an extensive extracellular cartilage matrix (Figure 2.7).

2.4 Genetics background of osteochondrosis in pig

The genetic background of osteochondrosis, which resembles alterations in tissues in aging and degeneration, has been examined and identified. OC is a multifactorial disorder resulted from a combination of small variations in different genes, as well as environmental factors (Hill, 1990a). OC may be assumed to be a quantitatively inherited trait, as it is likely that its development is influenced by different genes and environmental factors. This disease is frequently seen in pigs, horses, and dogs, and has also been described in man and poultry (Persson, 2007). These OC lesions have a genetic basis and are inheritable by progeny generations with the polygenic heritability of 0.1 – 0.5. Unfavorable associations among production traits (growth, feed efficiency, meat quality traits) and OC lesions were detected, implying that selection for production traits will inadvertently result in selection for OC lesions (Lundeheim, 1987; Stern *et al.*, 1995; Kadarmideen *et al.*, 2004; Serenius *et al.*, 2004). Further, OC lesions showing a mixed inheritance with a segregating major gene for a rapid growth may play important role in determining expression of whether any of these developmental diseases (Kadarmideen and Janss, 2005).

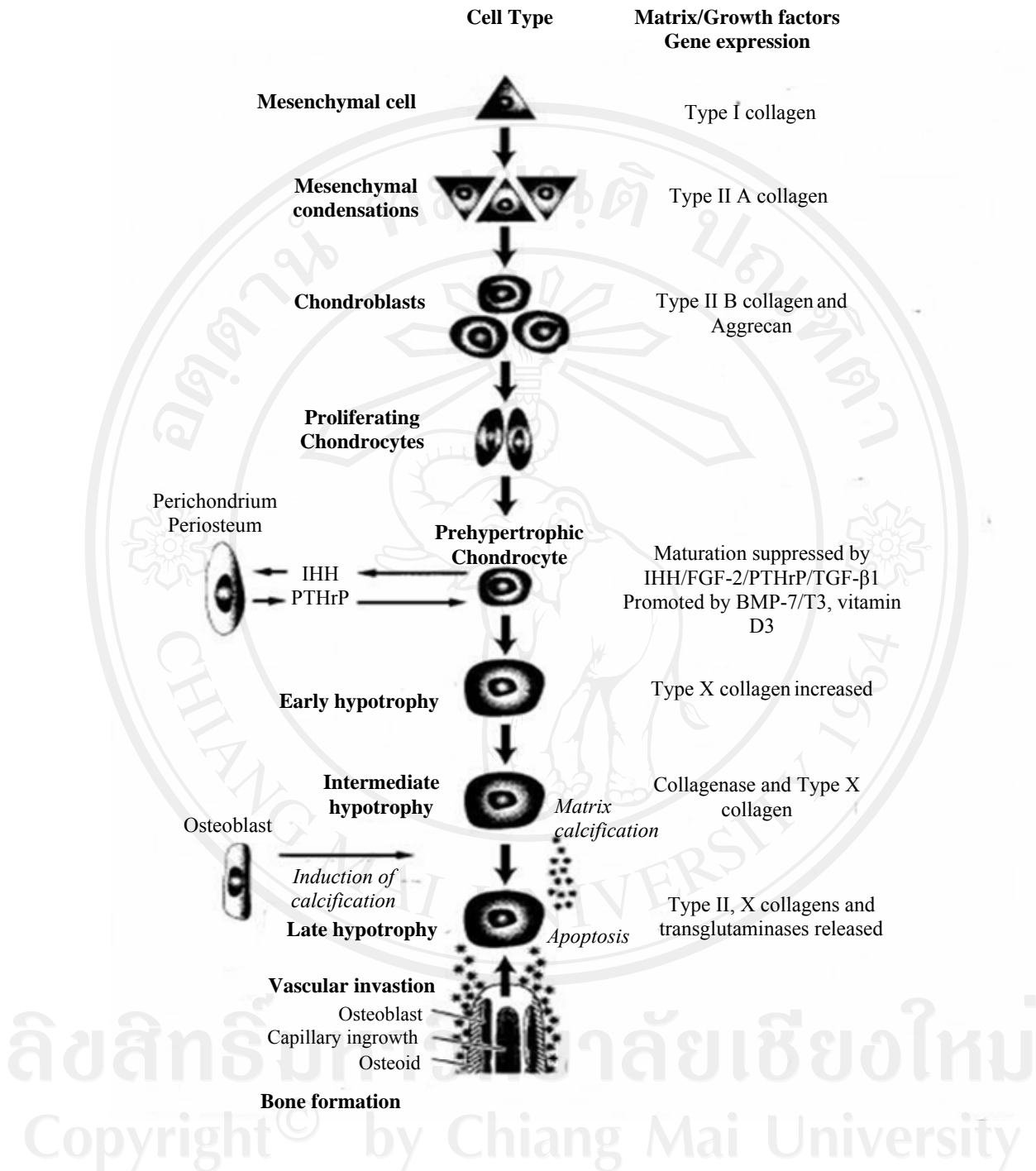


Figure 2.7 A summary of the differentiation and maturation of chondrocytes, the synthesis of extracellular matrix, its remodeling and calcification and regulation of these processes. The stages from chondroblasts to hypertrophic chondrocytes and vascular invasion of cartilage are represented in early development of the diaphysis as well as in the growth plates (Poole *et al.*, 2000).

2.4.1 The role of matrix Gla protein in osteochondrosis

2.4.1.1 Structure and function

The matrix Gla protein (*MGP*) gene belongs to the growing family of vitamin K-dependent proteins, the members of which is involved in function of skeletogenesis and bone formation. *MGP* is an 84 amino acid (approximately 14 kDa) protein that contains five γ -carboxyglutamic acid (Gla) residues (Figure 2.8). These Gla residues bind and incorporate calcium into hydroxyapatite crystals (Newman *et al.*, 2001). The human *MGP* gene is present as a single copy on the short arm of chromosome 12 (12p13.2 - p12.3). The gene spanned approximately 3.9 kb long from the transcriptional start site (major 5' cap site) to the AATAAA polyadenylation signal (Cancela *et al.*, 2000). Four exons contribute the 615 nucleotides of the *MGP* mRNA and 103 amino acids comprise the initial translation product. Between mouse and man the intron-exon structure is highly conserved, as in the 5' flanking nucleotide sequence.

MGP is predominantly expressed in cartilage and blood vessels. In cells of the chondrocytic lineage, expression occurs both in areas that will become ossified and in areas that will remain cartilaginous. Skeleton expression of *MGP* occurs in resting, proliferative and late hypertrophic chondrocytes but not in early hypertrophic chondrocytes or osteoblasts. Primary cultures of osteoblasts have been shown to synthesize and secrete *MGP*. During osteoblast differentiation *in vitro*, *MGP* expression is maximal during maturation of extracellular collagenous matrix which follows the down regulation of cellular proliferation (Barone *et al.*, 1991). It is a component of the extracellular matrix that is generally expressed by chondrocytes and vascular smooth muscle cells (VMSC) (Zebboudj *et al.*, 2002). Thus, *MGP* functions as a calcification inhibitor; however, its molecular mechanism is incompletely understood in OC lesion.

The vitamin K-dependent bone proteins normally inhibit the seeded crystal growth of hydroxyapatite from the fully mineralized metaphysis into growth plate cartilage. When the action of vitamin K is antagonized by warfarin, the under γ -carboxylated bone proteins now secreted are unable to retard seeded crystal growth, and mineralization consequently engulfs the longitudinal septa of growth cartilage. This eventually causes growth plate fusion. *MGP* is currently the best candidate for

the vitamin-K dependent protein that normally inhibits growth plate mineralization, since *MGP* is the only vitamin K-dependent protein known to be synthesized in cartilage. However, because *MGP* is water insoluble, it has not yet been possible to evaluate the ability of *MGP* actually to inhibit hydroxyapatite crystal growth. A moderate literature has arisen on this subject based on spontaneous or warfarin-induced vitamin K deficiency, whereby undercarboxylated osteocalcin with low mineral binding was demonstrated in the circulation and may signify low BMD and a risk factor for fragility fractures. Indeed, administration of vitamin K retards femoral neck bone loss in postmenopausal women (Tsukamoto *et al.*, 2000).

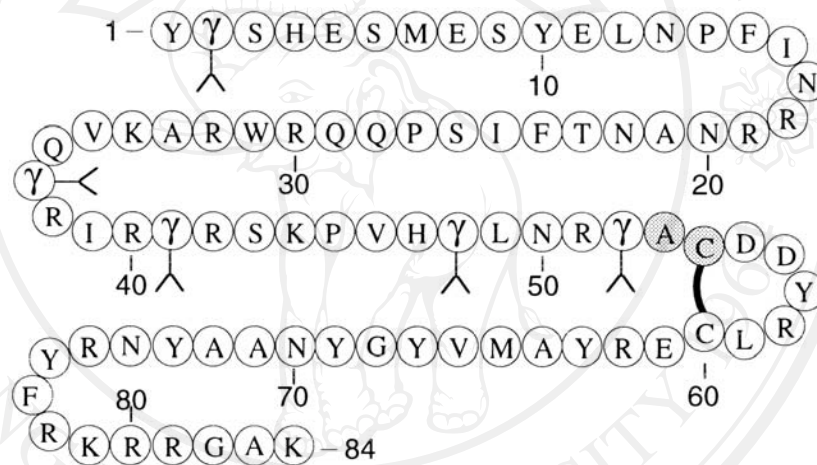


Figure 2.8 Amino acid sequence for human matrix Gla protein. The gray residues at position 53–54 represent the alanine-cysteine native chemical ligation site.

The solid line represents the disulfide-bond. γ -denotes, γ -carboxyglutamic acid (Hackeng *et al.*, 2001).

2.4.1.2 *MGP* associated disorders

MGP synthesis is stimulated by developmental and pathological events. Thus, many hormones, vitamins and growth factors are able to regulate synthesis of *MGP*. Control of the gene is influenced by multiple signals that include vitamin K availability and hormone or morphogenetic factor such as 1,25 dihydroxyvitamin D₃ and transformation growth factor β (*TGF β*). Few substances have a universal effect on *MGP* synthesis due in part to the multiple cell types that make up *MGP*. 1,25

dihydroxyvitamin D₃ stimulates *MGP* synthesis in normal osteoblasts, chondrocytes and osteosarcoma cells. In preosteoblasts the effect is specific for *TGFβ1*, as the closely related *MGP* synthesis (Barone *et al.*, 1991).

In mammal growth plate, *MGP* gene is expressed by proliferative and late hypertrophic chondrocytes, but not by the intervening chondrocytes. *MGP* deficiency in mice results from premature calcification in bone, calcification of normally noncalcifying cartilage, such as the trachea, and severe vascular calcification leading to premature death (Lou *et al.*, 1997; Boström *et al.*, 2004). In the artery wall of the *MGP* knockout mouse, medial smooth muscle cells are replaced by chondrocyte like cells undergoing endochondral ossification, and in the growth plate of growing bones, hypertrophic chondrocytes are lacking. Further support for an effect of *MGP* on cell differentiation comes from the work of Yagami *et al.* (1999) who showed that overexpression of *MGP* in developing limbs delays chondrocyte maturation and blocks endochondral ossification. In addition, *MGP* inactivation triggers mineralization in cultured hypertrophic chondrocytes but not in immature chondrocytes. This is consistent with data from Newman *et al.* (2001) demonstrating that overexpression of *MGP* in hypertrophic chondrocytes reduces mineralization. These also show that *MGP* expression is biphasic and stage-specific during chondrocyte differentiation and that *MGP* has an effect on chondrocyte viability.

As well as a role in matrix calcification, it has been proposed that *MGP* may have additional functions. Evidence in support of this hypothesis includes the arteries in the *MGP*-deficient mouse undergoing chondrocyte metaplasia, which indicates that *MGP* may have a role in cellular differentiation.

2.4.2 Candidate genes for osteochondrosis

The development of osteochondrosis is influenced by both environmental and genetics factors. Much is known about the factors that regulate bone turnover and about the proteins that make up bone matrix. This has facilitated studies of candidate genes responsible for OC. Positional candidate genes should be coded for hormones, enzymes, metabolic factors and/or their receptors involved in the complex of cartilage differentiation and maturation during endochondral ossification or in growth processes. Genes causing or linking to osteoarthritis (OA) that have been identified in

man could be taken for consideration as candidate genes for OC in pig. In a review of the genetic basis of OA (Holderbaum *et al.*, 1999) they stated that a single gene mutation was unlikely to explain all the interactions of protein mutations that could result in OA disorders. However, a number of genes associations with OA have been reported in many publications.

Studies of candidate genes for OC and OA have focused on cytokines, growth factors that regulate bone turnover, genes that encode components of bone matrix, and genes that encode receptors for calcitropic hormones (Ralston, 2002), yielding more than 200 potential candidates. The most important candidate genes are presented below (Table 2.1).

Table 2.1 Candidate genes in osteoarthritis and functions of the gene products (Hartikka, 2005)

Category	Candidate gene	Protein	Function
Calcitropic hormones and receptors	<i>VDR</i>	VDR	Bone cell differentiation, bone turnover, calcium absorption
	<i>PTH</i>	Parathyroid hormone	Calcium homeostasis
	<i>PTHRI</i>	Parathyroid hormone	Chondrocyte differentiation
Cytokines, growth factors and receptors	<i>TGFB1</i>	TGF β	Osteoclast-osteoblast activity
	<i>IGF1</i>	IGF-1	Growth-promoting effects
	<i>IL6</i>	IL-6	Osteoclast differentiation and activity
Bone matrix	<i>COL1A1</i>	Type I collagen	Matrix component
	<i>COL2A1</i>	Type II collagen	Matrix component
	<i>COL10A1</i>	Type X collagen	Matrix component
	<i>BGLAP</i>	Osteocalcin	Matrix component
	<i>MMP</i>	Collagenase	Resorption of bone matrix
	<i>MGP</i>	Matrix Gla protein	Calcification inhibitor

In pig, three quantitative trait loci (QTLs) which involved in the inheritance of OC of F₂ European wild boar and Swedish large white inter-cross were identified on chromosomes 5, 13 and 15 (Andersson-Eklund *et al.*, 2000). They suggested QTL located on chromosomes 5 reached genome-wide significance level for the trait. At this chromosome 5, QTL affecting OC located to a position between the interferon (*IFNG*) and the insulin-like growth factor-1 (*IGF1*) genes. This region is homologous to human chromosome 12q14-24 where a potential candidate gene, cartilage homeoprotein 1 (*CART1*), is located. At pig chromosome 13, they identified a QTL with negative additive effect of the wild boar allele on the prevalence of OC in the region homologous to human chromosome 3p, where *PIT1* locus, which codes for a transcriptional factor of growth hormone and the parathyroid hormone receptor (*PTHHR*) genes are located. But at chromosome 15 the QTL for OC is not very well defined.

Genes encoding type I, II and X collagen (*COL1A1*, *COL2A1* and *COL10A1*) are important candidates for the pathogenesis of OC, since collagens are the main structural protein of bone. Polymorphisms in the coding regions of these genes are rare and do not appear to be associated with osteoporosis (Spotila *et al.*, 1994). The most interesting polymorphism of the *COL1A1* gene is located in intron 1, at the binding site for the Sp1 transcription factor. It has been reported to account for part of the variance in BMD in the normal population and to be associated with fragility fractures (Uitterlinden *et al.*, 2004). BMD increases the risk of osteochondrosis fractures, particularly of the vertebrae. Polymorphisms of several other candidate genes, including those encoding bone structural proteins and regulatory proteins or hormones involved in bone metabolism, have been associated with bone mass or osteochondrotic fractures. Jefferies *et al.* (2002) investigated the difference of gene expression which related to OC/OA in pig fed 25-hydroxyvitamin D₃. They showed that two genes, collagen types II and X, showed consistently altered levels of abundance in OA lesion and near lesion samples, as compared to controls. Collagen type II was downregulated ($P < 0.01$) and collagen type X was upregulated ($P < 0.01$) in lesion and near lesion samples of articular cartilage.

Several candidate genes have been proposed to be causative for the osteochondrosis. Identification and characterization of specific loci or genes involved

in determining OC and its associated phenotypes will contribute to a greater understanding of the pathogenesis of OC. Polymorphisms of several other candidate genes, including those encoding bone and cartilage structure proteins and regulatory proteins or hormones involved in metabolism, have been associated with bone mass and osteochodrosis lesion (Table 2.1). Other genes, such as those of the cartilage homeoprotein1 (*CART1*), cartilage oligomeric matrix protein (*COMP*) and vitamin D receptor (*VDR*) associated with OC have been reported (Böneker, 2006).

2.5 Single nucleotide polymorphism (SNP)

SNPs are major contributors to genetic variation, comprising some 80% of all known polymorphisms, and their density in the human genome is estimated to be on average 1 per 1,000 base pairs. SNPs are mostly biallelic, more frequent, mutational and more stable, making them suitable for association studies in which linkage disequilibrium (LD) between markers and an unknown variant is used to map disease-causing mutations. In addition, since SNPs have only two alleles, they can be genotyped by a simple plus/minus assay rather than a length measurement, making them more amenable to automation.

Individual SNP is less informative than other marker systems, especially as the widely used microsatellites, but it is more abundant and has a great potential for automation (Wang *et al.*, 1998). Markers used for genome scans should allow the polymorphisms to be typed quickly, accurately and inexpensively. SNP markers possess the properties that fulfill these requirements. First, DNA sequence variations, based on point mutations are estimated to occur once every 500 to 1000 bp when any two chromosomes are compared. Second, SNPs are diallelic in populations, and their allele frequencies can be estimated easily in any population through a variety of techniques (Kwok, 2001). Third, the mutation rate per generation of SNPs is low compared to tandem repeat markers where the high mutation rates can confound genetic analysis in population. Fourth, many of the mutations of the trait result from single nucleotide changes in genes; it is likely that a subset of SNPs is functionally important in complex traits. Last, promising high-throughput genotyping methods are now available for efficient genotyping of SNPs (Wang *et al.*, 1998). Genotyping of

SNPs will likely be a major part of every genetic association study, and the appropriate genotyping method is critical to the success of the study.

The methods for genotyping of single nucleotide polymorphisms include gel-based and non-gel-based approaches. The gel-based genotyping approaches are for example DNA sequencing, restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) analysis (Orita *et al.*, 1989). All non-gel-based genotyping approaches achieve allelic discrimination by one of four mechanisms: allele-specific hybridization, allele-specific primer extension, allele-specific oligonucleotide ligation and allele-specific cleavage of a flap probe. By combining one of these allelic discrimination mechanisms with either a homogeneous or solid-phase reaction format or a detection method such as fluorescence intensity, fluorescence polarization or mass spectrometry fast, reliable, automated and large-scale genotyping is warranted (Kwok, 2000). To date, no such ideal genotyping method exists, further development efforts are needed to create more broadly applicable SNP genotyping methods that permit accurate allelic determination, allow genotyping of multiple SNPs in parallel and are cost-effective. Increasingly efficient methods for typing SNPs open new possibilities in genetic studies. In association studies, more suitable to study complex disorders in which many loci contribute to the risk of developing disease, for more markers could be tested profitably (Risch and Merikangas, 1996). In this study, the single base primer extension (SBE) was used in multiplex genotyping reaction.

2.5.1 Single base primer extension assay

SBE is a method of sequencing the precise location of a SNP site. It utilizes the inherent accuracy of DNA polymerase to determine the presence or absence of the specific nucleotide at the SNP site (Figure 2.9). A specially synthesized DNA primer is used to anneal to the SNP site of interest. The primer anneals one base short of the target SNP. DNA polymerase inserts the complementary dideoxynucleotide terminator or dideoxynucleoside triphosphates (ddNTP) to the SNP site. Primer selection and assay design are simple and allow for detection of multiple SNPs simultaneously; hence, a large number of commercial systems employ primer extension for SNP genotyping. This technology provides a simple multiplex SNP

genotyping solution with high accuracy and reproducibility, which is applicable to multiple platforms including capillary electrophoresis, mass spectroscopy, flow cytometry, HPLC, microarray, etc. The multiplex SNP reaction requires the SBE or SNP primer for each SNP marker and involves the following three steps: (1) multiplex PCR amplification of the sequence surrounding a SNP from genomic DNA, (2) multiplex single nucleotide cycling primer extension using SNP primer and ddNTP, (3) detection SNPs site on capillary electrophoresis (Scott *et al.*, 2007).

SBE approaches that use fluorescence-based detection involve SBE of primer with fluorescently labeled ddNTPs (Figure 2.9). The GenomeLab™ SNP (Applied Beckman Coulter, USA) is a homogeneous reaction and detection procedure that uses polymerase chain reaction (PCR) amplicons for SBE of primers with fluorescently labeled ddNTPs. This leads to the generation of fluorescently labeled extension products that are detected by capillary array electrophoresis and used for genotype determination (Sanchez *et al.*, 2003).

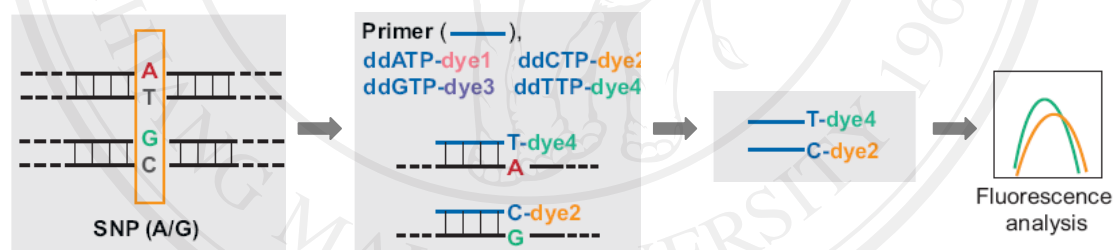


Figure 2.9 Primer extension approaches for SNP genotyping. Fluorescence detection using capillary electrophoresis. This technique uses a primer that anneals one base upstream of the SNP site followed by its extension with ddNTPs that are labeled with different fluorescent tags. Products are detected by fluorescence after capillary electrophoresis and the color of dye indicates incorporated base(s) and hence the SNP genotype.