CHAPTER I

INTRODUCTION

1.1 Statement of the problem

The extracellular matrix (ECM) is built up from the heterogeneity complex of collagens, elastic fibers, glycoproteins, hyaluronan and proteoglycans. In the ECM, whereas fiber components support cells and form the structure of tissues and organs, proteoglycans, hyaluronan, and other glycoproteins confer biophysical moiety characteristic of individual tissues. These molecules form a complex that regulates cell behavior, by storing growth factors and cytokines, forming gradients of their concentrations, and presenting these molecules to target cells (1). Of proteoglycans in the ECM, members of large aggregating chondroitin sulfate proteoglycans (CSPGs) (2, 3) are the most prevalent type expressed in mammalian tissues, including versican, aggrecan, neurocan, brevican, neuroglycan D, NG2, the receptor-type protein tyrosine phosphatase and its splice variant phosphacan (4).

Versican was originally isolated as PG-M (medium-sized chondroitin sulfate proteoglycan) from the mesenchymal condensation areas of chick embryonic limb bud at stage 23 (5). Its core protein consists of an N-terminal G1 and a C-terminal G3 globular domains, and two chondroitin sulfate (6) attachment domains CS-α and CS-β between the two domains. The N-terminal G1 comprises A, B, and B' loops and binds to both hyaluronan (HA) and link protein (LP) (7). The C-terminal G3 domain binds fibulin-1 and -2 (8, 9), tenascins (10), and heparan sulfate proteoglycans (11). Versican exhibits four spliced variants: V0, V1, V2 and V3 (9, 10, 11, 12), with

different CS domains. The V0 variant contains all the domains G1, CS-α, CS-β, and G3; V1 contains G1, CS-β, and G3; V2 contains G1, CS-α, and G3; and V3 contains only G1 and G3. Interestingly, V0 and V1 are expressed widely, V2 is restricted to the nervous systems, and V3 has not been detected as a protein, although mRNA is detected. Thus, the number of CS chains required for the function of versican may vary among tissues. Versican is distributed in a wide variety of tissues during development and characterized by two distinct expression patterns. It is constitutively expressed in some adult tissues, such as heart, blood vessels, and brain, serving as a structural macromolecule of the ECM. It transiently expressed in various developing tissues (13), including brain, hair follicles, developing heart, and mesenchymal condensation areas of cartilage primordium. Several studies have indicated that versican is transiently expressed at high level within the mesenchymal condensation areas, the pre-cartilage limb bud (8, 9), the limb core, and other sites undergoing cartilage differentiation in both chick (5, 10, 11) and mouse (12, 14). However its expression is rapidly faded during cartilage development (13, 14). Thus, the temporary expression of versican in highly aggregation areas of cells such as limb bud implies its important role during cartilage development. To address this aspect, null mutation of versican gene in mouse called mouse line heart defect (*hdf*) was supposed to provide the unhidden role of versican. Result from this mutation, *hdf* homozygous died from embryonic lethality at 10.5 days post-coitum because of severe segmental heart defects (15), while the pre-chondrogenic condensations have been starting. Therefore, the role of versican in this aspect has still been incomprehensible. An *in vitro* analysis of limb mesenchyme from gene-trapped *hdf* mice by micromass indicated that versican is necessary for chondrocyte differentiation of mesenchymal

cells (16) and the study of N1511 chondrocytic cells demonstrated that versican is required for the mesenchymal matrix formation toward chondrocyte differentiation during chondrogenesis (17). However, the signaling pathways affected by versican have not been identified, and the mechanisms by which versican regulates their signal transduction toward cartilage development remain to be elucidated.

Chondrogenesis initiates from the condensation of mesenchymal cells that further differentiate into chondrocytes. Chondrocytes then undergo maturation, hypertrophy, and mineralization, which allow cartilage to be replaced with bone through endochondral ossification. During these processes, synovial joints are generated with packing and lining of mesenchymal cells at the future joint location termed interzone (18, 19, 20). Further of development, physical separation of the adjacent skeletal elements occurs via a process of cavitation within the interzone that will lead to formation of a liquid-filled synovial space. The synovial membrane lines the joint capsule and secretes synovial fluid which provides the lubrication, and nutrition for the articular cartilage that contributes the functional ability of the joint for pain-free movement. From a clinical perspective resulting from trauma, joint are also the target of diseases. Rheumatoid arthritis (RA) is the inflammatory disease marked by hyperplasia and chronic inflammation of the synovial membranes, activated SFs in the lining layer of the synovial membrane invade deeply into the articular cartilage and bone that contribute to cartilage deterioration and joint destruction (21, 22, 23, 24). Several cytokines or MMPs released from immune cells and synovial fibroblasts are the crucial factors to motivate inflammatory process leading to joint destruction (25, 26).

Phytochemical is known as the chemicals produced by plant to protect itself. The various phytochemicals found in plant and herbal sources all have different properties in clinical usage. *Alpinia galanga* (*A. galanga*) (B.L. Burtt), has the traditional uses in treatment of flatulence, dyspepsia, vomiting, high blood pressure, and gastrointestinal complaints. Its anti-rheumatic effect has been suggested to suppress prostaglandin synthesis through the inhibition of cyclooxygenase-1 and cyclooxygenase-2 (27). Previous study has been indicated that *A. galanga* hexane extract suppresses MMPs expression in SW1353 chondrosarcoma, synovial fibroblasts, and cartilage explants induced by IL-1β (28), suggesting its anti-inflammatory effect on these models.

Taken together, the aim of this study is to investigate the *in vivo* role of versican in mesenchymal condensations toward cartilage and joint development by genetically ablated versican expression with *Prx1*-Cre/*loxP* technique, and to investigate the effects of hexane extracts from *A. galanga* on IL-1β-induced human synovial fibroblasts as an inflammatory model.

1.2 Literature reviews

1.2.1 Cre/*loxP* and *Prx1* gene

The Cre recombinase belongs to the integrase family of site-specific recombibases. It is 38 kDa protein that catalyzes the recombination between two of its recognition sites, called *loxP* (29). *LoxP* is a 34 bp consensus sequence, consisting of a core spacer sequence of 8 bp and two 13 bp palindromic flanking sequences. The asymmetric core sequence defines an orientation to the *loxP* site. Concerning the molecular mechanism of recombination, a single recombinase molecule binds to each palindromic half of a *loxP* site, then the recombinase molecules form a tetramer, thus bringing two *loxP* sites together (30). The recombination occurs within the spacer area of the *loxP* sites. The postrecombination *loxP* sites are formed from the two complementary halves of the prerecombination sites, as demonstrated in figure 1.1. Utilization of the Cre/*loxP* system for gene targeting *in vivo* involves two lines of mice. The first mouse line is generated by ES cell technology, in which gene of interest is flanked by two *loxP* sites, called floxed. The other mouse line is generated by oocyte injection techniques. These express Cre driving of specific promoter. As illustrated in figure 1.2, mating of the two mouse lines should result in Cre-mediated gene excision in the cells or tissues where the promoter is active. Therefore, it is feasible to restrict gene ablation using Cre-*loxP* system to study the role of genes at developmental time points by specific promoters driving Cre expression. Several promoters have been used to achieve tissue-specific Cre expression in mice, including *Prx1*. *Prx1* is the member of Homeobox genes family which play a role in many different aspects of development. Numerous studies indicated that *Prx1* is expressed in the early limb bud mesenchyme in both mouse (6) and chick (31, 32) embryos.

The studies of *Prx1*-Cre mice have revealed the activity of Cre recombinase in developing mouse limb bud in several patterns (33). Taken together, these should facilitate analysis of the *in vivo* function of interested gene during the development.

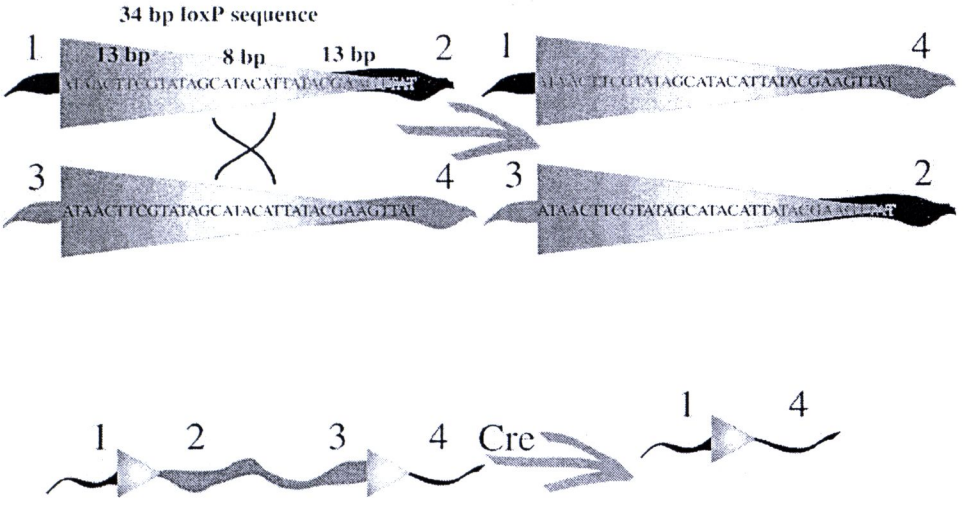

Figure 1.1 Schemetic of Cre recombinase-mediated recombination between two 34 bp *loxP* sites (34). A single recombinase molecule binds to each palindromic half of a *loxP* site, then the recombinase molecules form a tetramer, thus bringing two *loxP* sites together. The recombination occurs within the spacer area of the *loxP* sites. The postrecombination *loxP* sites are formed from the two complementary halves of the prerecombination sites, leading to excision of any DNA sequence (2 and 3) flanked by two *loxP* sites.

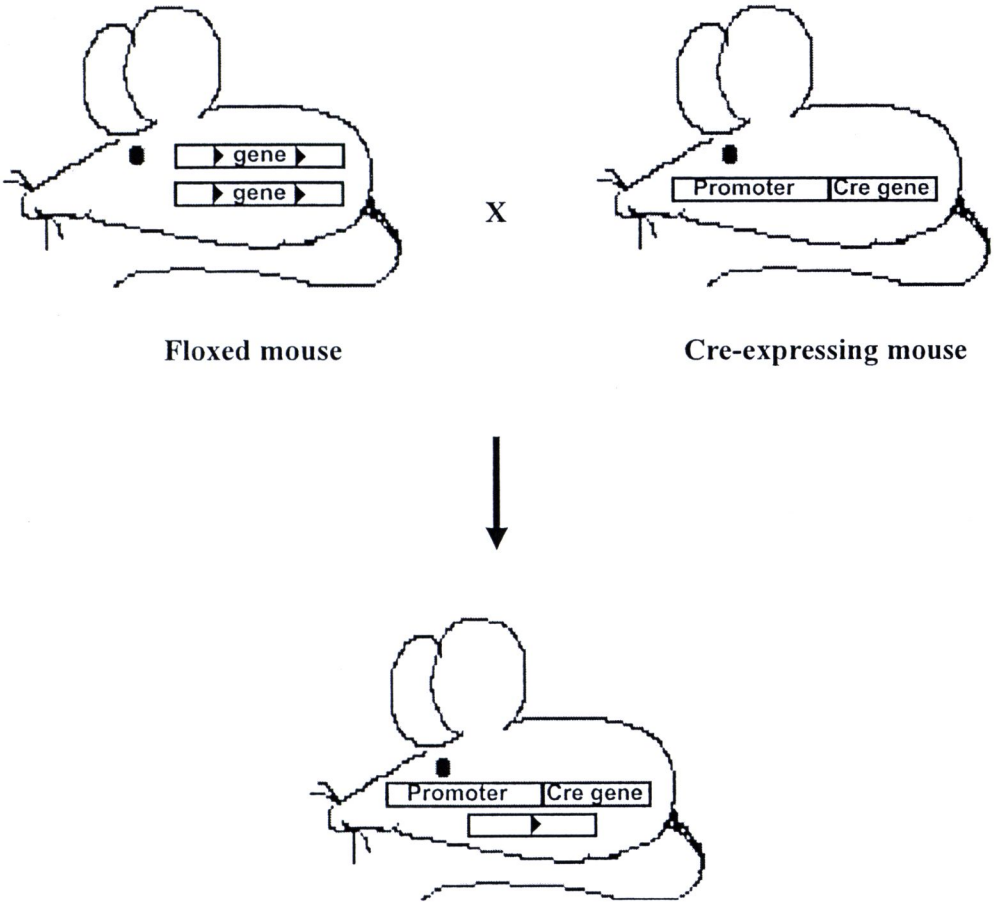

Figure 1.2 Schematic of cell-specific Cre-mediated gene targeting in mice. Mating of the two mice lines results in deletion of the floxed gene segment.

1.2.2 Proteoglycan

Proteoglycans are composed of glycosaminoglycans (GAGs) covalently attached to the core proteins. The protein component of proteoglycans is a core protein which directs the biosynthesis of proteoglycans to different molecular constructions and functions. GAGs forming the proteoglycans are long unbranched molecules containing a repeating disaccharide unit. Usually, one sugar is an uronic acid (either D-glucuronic or L-iduronic) and the other is either *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc). One or both sugars contain sulfate groups (the only exception is hyaluronic acid). Proteoglycans can be categorized depending upon the nature of their glycosaminoglycan chains. These chains may be: chondroitin sulfate (6) and dermatan sulfate (DS), heparin and heparan sulfate (35), keratan sulfate (KS). It was realized that the production of proteoglycans was a general property of animal cells and that proteoglycans and glycosaminoglycans were present on the cell surface, inside the cell, and in the ECM. The major classes of proteoglycans can be defined by distribution, homologies, and function (36).

1.2.2.1 Proteoglycan classification

1.2.2.1.1 Interstitial proteoglycans and the Aggrecan family

A large number of proteoglycans are present in the ECM, and their distribution depends on the nature of the ECM. The interstitial proteoglycans represent a diverse class of molecules, differing in size and glycosaminoglycan composition. At least nine members of this family are known and they carry CS, DS, or KS chains. These proteoglycans help to stabilize and organize collagen fibers, for example, in tendons. In the cornea, KS proteoglycans maintain the register of collagen fibers required for

transparency. Decorin also can bind transforming growth factor β (TGF-β), serving as a sink to keep the growth factor sequestered in the matrix surrounding most cells. The aggrecan or chondroitin sulfate proteoglycan (CSPG) family consists of aggrecan, versican, brevican, and neurocan. In all four members, the protein moiety contains an amino-terminal domain capable of binding hyaluronan, a central region that contains covalently bound CS chains, and a carboxy-terminal domain containing a C-type lectin domain. Aggrecan represents the major proteoglycan in cartilage. It contains as many as 100 CS chains and, in humans, it contains KS chains as well. Versican, which is produced predominantly by connective tissue cells, undergoes alternative splicing events that generate a family of proteins of differing complexity that may have a role in neural crest cell and axonal migration. Neurocan is expressed in the late embryonic central nervous system (CNS) and can inhibit neurite outgrowth. Brevican is expressed in the terminally differentiated CNS, particularly in perineuronal nets.

Table 1.1 Examples of chondrotin sulfate proteoglycans (CSPGs) (36).

Proteoglycan	Core protein (kD)	Number of chondroitin sulfate chains	Tissue distribution
Aggrecan family			
Aggrecan	208–220	~100	secreted; cartilage
Versican/PG-M	265	12–15	secreted; connective tissue cells; aorta; brain
Neurocan	145	1–2	secreted; brain
Brevican	96	0–4	secreted; brain
SLRPs			
Decorin	36	1	secreted; connective tissue cells
Biglycan	38	1–2	secreted; connective tissue cells
Other examples			
Leprecan	82	1–2	secreted; basement membranes
Type IX collagen, α2 chain	68	1	secreted; cartilage; vitreous humor
Phosphacan	175	2–5	membrane bound; brain
Thrombomodulin	58	1	membrane bound; endothelial cells
CD44	37	1–4	membrane bound; lymphocytes
NG2	251	2–3	membrane bound; neural cells
Invariant chain	31	1	membrane bound; antigen-processing cells
Serglycin	10–19	10–15	intracellular granules; myeloid cells

1.2.2.1.2 Secretory granule proteoglycans

Serglycin is the major proteoglycan present in cytoplasmic secretory granules in endothelial, endocrine, and hematopoietic cells. Depending on the species, it has a variable number of glycosaminoglycan attachment sites that can carry CS or heparin chains. Heparin is a highly sulfated form of HS and is made exclusively on serglycin present in connective-tissue-type mast cells. Other granular proteoglycans may exist as well, such as chromogranin A, but the extent of glycosaminoglycan substitution appears to be substoichiometric, making them part-time proteoglycans.

1.2.2.1.3 Basement membrane proteoglycans

The basement membrane is an organized layer of the ECM that lies flush against epithelial cells and consists largely of laminin, nidogen, collagens, and proteoglycans. Basement membranes contain at least four types of proteoglycans depending on tissue type: perlecan, agrin, and collagen type XVIII, which carry HS chains (although perlecan has been shown to carry CS in cartilage), and leprecan, which carries CS chains. Perlecan has a mass of 400 kD and consists of multiple domains that have numerous functions. It has a role in embryogenesis and tissue morphogenesis and a particularly important role in cartilage development. Agrin acts in neuromuscular junctions (where it aggregates acetylcholine receptors) and in renal tubules (where it has an important role in determining the filtration properties of the glomerulus).

1.2.2.1.4 Membrane-bound proteoglycans

The membrane proteoglycans are diverse. The syndecan family consists of four members, each with a short hydrophobic domain that spans the membrane, linking the

larger extracellular domain containing the glycosaminoglycan attachment sites to a smaller intracellular cytoplasmic domain. Syndecan-1 and syndecan-3 carry CS chains on the membrane proximal regions and HS chains at the more distal sites further away from the membrane. In contrast, syndecan-2 and syndecan-4 carry only HS chains. These are expressed in a tissue-specific manner and facilitate cellular interactions with a wide range of extracellular ligands, such as growth factors and matrix molecules. Because of their membrane-spanning properties, the syndecans can transmit signals from the extracellular environment to the intracellular cytoskeleton via their cytoplasmic tails. For example, binding of a ligand to the HS chain can induce oligomerization of syndecans at the cell surface, which leads to recruitment of factors at their cytoplasmic tails, such as kinases (e.g., c-Src), PDZ-domain proteins, or cytoskeletal proteins. The recruitment of cytoplasmic proteins in turn triggers a signal that affects actin assembly. Proteolytic cleavage of the syndecans occurs by matrix metalloproteases, resulting in shedding of the ectodomains bearing the glycosaminoglycan chains. These ectodomains can have potent biological activity as well, for example, by binding the same ligands as cell-surface proteoglycans. Another family is glypican family which each member of cell-surface proteoglycans has a GPI anchor attached at the carboxyl terminus, that embeds these proteoglycans in the outer leaflet of the plasma membrane. Thus, the glypicans do not have a cytoplasmic tail like the syndecans. The amino-terminal portion of the protein has multiple cysteine residues and a globular shape that distinguishes the glypicans from the syndecan ectodomains, which tend to be extended structures. Glypicans carry only HS chains, which can bind a wide array of factors essential for development and morphogenesis. Six glypican family members exist in mammals, two in *D.*

melanogaster and one in *C. elegans*. Glypican-3 (GPC3) is the best-studied member of the family in vertebrates. Humans lacking functional GPC3 exhibit Simpson-Golabi-Behmel syndrome, characterized as an overgrowth disorder. The overgrowth phenotype suggests that GPC3 normally functions to inhibit cell proliferation, but the mechanism by which this occurs is unknown.

In addition to these two families, a number of other membrane proteoglycans are expressed on the surface of many different cell types. The CS proteoglycan NG2 is a surface marker expressed on stem cell populations, cartilage chondroblasts, myoblasts, endothelial cells of the brain, and glial progenitors. CD44, a transmembrane cell-surface receptor present on leukocytes and other cells, has a role in processes as diverse as immune cell trafficking and function, axon guidance, and organ development. Phosphacan is expressed as three different splice variants in the CNS, and, depending on the isoform, it can carry KS or CS chains. One splice variant is present in the ECM, whereas two other forms represent short-and full-length versions of a protein-tyrosine-phosphatase type of transmembrane receptor.

1.2.2.2 Proteoglycan functions

Biological functions of proteoglycans derive primarily from those of the glycosaminoglycan and protein component of the molecule. Several functions generalized regarding to proteoglycan are as follows:

- A common property of the interstitial proteoglycans containing CS and DS chains is their capacity to bind water and form hydrated matrices. Thus, these molecules fill the space between cells. For example, the aggregates of proteoglycans and hyaluronan provide a stable matrix capable of absorbing

high compressive loads by water desorption and resorption in cartilage. Interstitial proteoglycans can interact with collagen, thus aiding in the structural organization of most tissues.

- Proteoglycans help to organize basement membranes, thus providing a scaffold for epithelial cell migration, proliferation, and differentiation. They can regulate the permeability properties of specialized basement membranes.
- Proteoglycans in secretory vesicles have a role in packaging granular contents, maintaining proteases in an active state, and regulating various biological activities after secretion, such as coagulation, host defense, and wound repair.
- Proteoglycans in the ECM can bind cytokines, chemokines, growth factors, and morphogens, protecting them against proteolysis. These interactions provide a depot of regulatory factors that can be liberated by selective degradation of the matrix. They also facilitate the formation of morphogen gradients essential for cell specification during development.
- Proteoglycans can act as receptors for proteases and protease inhibitors regulating their spatial distribution and activity.
- Membrane proteoglycans can act as coreceptors for various tyrosine-kinase-type growth factor receptors, lowering the threshold or changing the duration of signaling reactions.
- Membrane proteoglycans can act as endocytic receptors for clearance of bound ligands.
- Membrane proteoglycans cooperate with integrins and other cell adhesion receptors to facilitate cell attachment, cell-cell interactions, and cell motility as well as proteoglycans in the ECM can regulate cell migration.

1.2.3 Gene and protein structure of versican

Versican belongs to a large aggregating chondroitin sulfate proteoglycan (CSPG) family (37), termed aggrecan family or lectican family, (2). It is widely expressed in various tissues and organs. Versican gene (*Vcan*) has been localized to chromosome 5 in the human genome (38, 39) and chromosome 13 in the murine genome (40). As demonstrated in figure 1.3, versican consists of a core protein and CS chains attached to the core protein. Its core protein is composed of an N-terminal G1 and a C-terminal G3 globular domain, and two CS-attachment domains CS-α and CS-β between G1 and G3 domains. The G1 domain is composed of an Ig-like motif, followed by two proteoglycan tandem repeats which are known as hyaluronan-binding region. The G3 domain of versican consists of two EGF-like motifs, and a complement regulatory protein-like motif (41). Versican core protein is encoded by 15 exons extending over 90-100 kb of continuous DNA. The placement and size of each exon scaled relative to the restriction-mapped almost 100-kb region is shown in figure 1.4. The structure of the mouse versican gene indicated a correlation between exon-intron architecture and the domain structure of the versican core protein. These exons encode five protein domains, including hyaluronan-binding region (HBR), GAG-α domain, GAG-β domain, EGF-like domain, lectin-like and complement regulatory protein (CRP)-like domains.

- Exon I contains part of the 5′-untranslated sequence.
- Exon II contains the translation initiation sitem (ATG).
- Exons III-VI encode the hyaluronan-binding region, whose structure is consists of three loop-like subdomains, loops A, B, and B′. The loop A structure shows homology to an immunoglobulin fold and is encoded by exon III. The loop B forms a

tandem homologous repeat and is encoded by IV and V. The B′ subdomain also forms a tandem homologous repeat, and is encoded by exon VI.

- Exons VII and VIII are very large in size and encode two different chondroitin sulfate attachment domains, CS-α and CS-β, respectively (9). These two exons are differently expressed in a tissue-dependent manner by alternative splicing (9, 42).

- Exons IX and X are encoded for two epidermal growth factor-like tandem repeats.

The carboxyl-terminal region, which has been shown to possess sugar-binding activity (43), is encoded by seven exons. This region consists of two epidermal growth factor-like domains, one C-type lectin-like domain, and one complement regulatory protein-like domain.

- Exons XI-XIII are encoded the C-type lectin-like motif.

- Exon XIV is encoded a complement regulatory protein-like motif.

- Exon XV is encoded the rest of the carboxyl-terminal coding region and the whole 3′-untranslated region.

Exon organization of the gene tightly follows the domain structure of the core protein. Alternative mRNA-splicing occurs in the two large exons encoding the GAG attachment sites of versican mRNA, giving rise to 4 splice-variants; V0, V1, V2 and V3 (8, 9, 42). The differences among the variants are found in the central part of the core protein. In V0 variant, the CS-α and CS-β domains are present whereas the V1 and V2 variants, CS-β and CS-α domains are absent, respectively. There are no CS-attachment domains in V3 variant. Estimated numbers of GAG chains are 17-23, 12-15, and 5-8 in versican isoforms V0, V1, and V2, respectively (8). Depending upon the number of CS chains, the function of versican may vary among tissues.

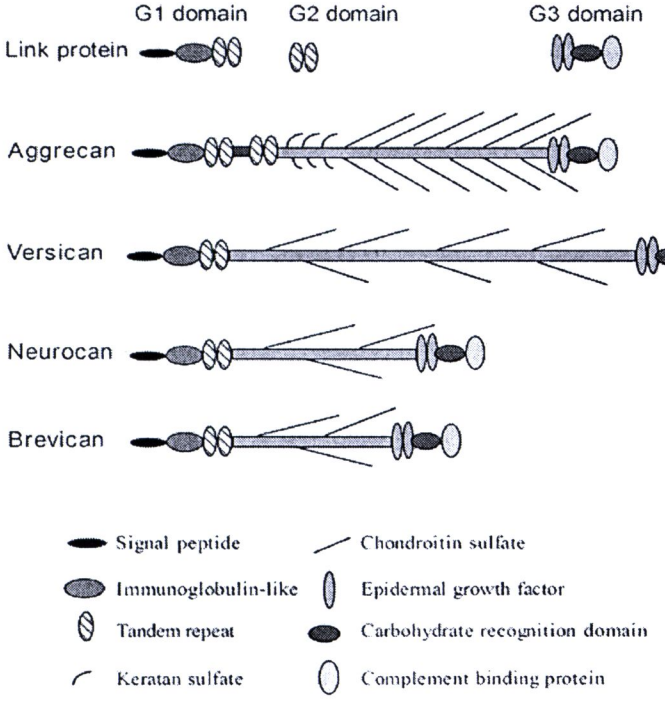

Figure 1.3 Protein structure of versican. SP, signal peptide; Ig, immunoglobulin-like; TR, tandem repeat; EGF, epidermal growth factor; CS, chondroitin sulfate; CRD, carbohydrate recognition domain; CBP, complement binding protein (44).

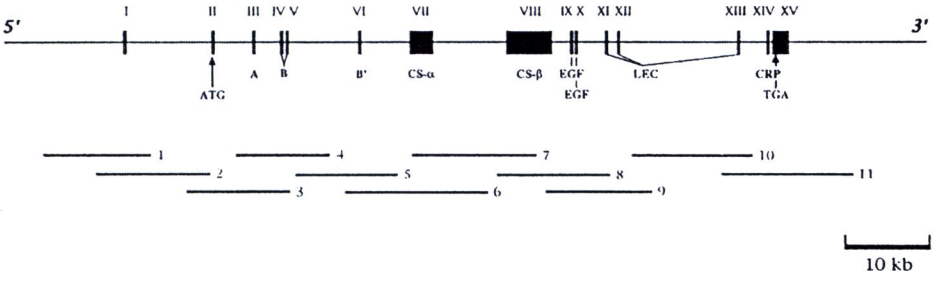

Figure 1.4 Oganization of the mouse PG-M gene and alignment of isolated genomic DNA clones. Eleven phage clones representing an ~100-kb genomic section are shown as numbered horizontal lines. Exons are indicated by *closed boxes* and are numbered from I to XV. The corresponding domains of the PG-M core protein are also shown. Introns as well as 5′- and 3′-flanking regions are indicated by lines. The initiation (ATG) and termination (TGA) codons are indicated by arrows. The scale for 10 kb is indicated. EGF, epidermal growth factor; LEC, C-type lectin; CRP, complement regulatory protein (45).

1.2.4 The role of versican in cell behavior

Versican is widespread in the ECM where it provides hygroscopic properties to create a loose and hydrated matrix that is necessary to support key events in development and disease. Previous *in vitro* studies have revealed various effects of versican on cell behavior, including cell adhesion and survival, cell proliferation, cell migration, and ECM assembly (41). It can inhibit cell adhesion of MG63 osteosarcoma cells and aggravates their malignant phenotype (46). In addition, early studies also show that the anti-adhesive property of versican appears to reside in the G1 domain of versican (46, 47, 48). In contrast, the carboxyl-terminal domain which

resides at the G3 domain of versican interacts with the β1 integrin of glioma cells, and activates focal adhesion kinase (FAK), promoting cell adhesion and preventing apoptosis in this cell type (49).

Versican is expressed along neural crest pathways and influences neural cell migration (50) by inhibition of neural crest cells migration and the outgrowth of motor and sensory axons (51). The *in vivo* studies of Splotch mice, characterized by mutations in the Pax3 gene, exhibited neural crest related abnormalities, including the failure of neural crest cells to colonize target tissues. However, neural crest cells derived from these mutant mice migrated that of controls *in vitro*, so it has been suggested that the defect may not reside in the neural crest cell themselves, but rather in the ECM environment through which they migrate (51, 52). Indeed, previous studies (52) demonstrated that versican was markedly overexpressed in Splotch mutant in neural crest cell migration pathways, suggesting that versican may be responsible for defective cell migration in this species. Versican also appears to plays a role in the migration of embryonic cells in hair follicle formation and heart development (53). It is expressed in dermal papilla, a dense aggregate of dermis-derived stromal cells, suggesting its involvement in hair follicle formation (54). It is expressed in a chamber-specific manner, with high levels in trabeculations of the right ventricle, and in the endocardial cushion of the atrioventricular, semilunar and venous valves in the heart. The essential role of versican in heart development has been demonstrated by the identification of an insertional transgene mutation in the versican gene in the heart-defect (*hdf*) mouse resulted in embryonic lethality at 10.5 days post-coitum because of severe segmental heart defects (15). An *in vitro* analysis of limb mesenchyme from (*hdf*) mice by micromass indicated that versican is necessary for

chondrocyte differentiation of mesenchymal cells (16), and the study of N1511 chondrocytic cells demonstrated that versican is required for the mesenchymal matrix formation toward chondrocyte differentiation during chondrogenesis (17). In addition, versican influences the migration of a variety of other cell types. For example, in the nervous system and in axonal growth, the V2 splice variant inhibits axonal outgrowth and migration (55). This inhibiting activity of versican can be reduced, but not eliminated, by removing CS chains, indicating that multiple domains of versican are involved in controlling axon regeneration. The finding that both the GAGs and core protein domains of the molecule are involved in the inhibitory activity suggests a direct interaction with the cells, or modification of the surrounding matrix to form exclusionary boundaries. Versican also is involved in cell proliferation. For example, versican is upregulated in arterial smooth muscle cells by platelet-derived growth factor (PDGF), contributing to the expansion of the pericellular ECM that is required for the proliferation and migration of these cells (56, 57, 58). It is often found in tissues exhibiting elevated proliferation, such as in development and in a variety of tumors, including melanoma (59, 60, 61), and in early-stage of prostate cancer (60). Cell culture studies suggest that elevated levels of versican result from the prostate tumor cells inducing host stem cells to increase the synthesis of versican via a paracrine mechanism involving transforming growth factor beta 1 (TGF-β1) (62).

Versican interacts with several different ECM molecules and in part, plays a central role in ECM assembly. The domain structure of versican lends itself to multiple types of interactions through either protein-protein or protein-carbohydrate interactions. Perhaps the best known of these interactions involves a specific interaction between the amino-terminal domain of versican (G1) and HA (7). The

binding of versican to HA involves a tandem double-loop sequence in the G1 domain of versican and a stretch of five repeat disaccharides in HA. This interaction is stabilized by another protein-linked protein which exhibits selective binding specificity for both HA and versican. In addition to HA, versican interacts with other ECM molecules such as tenascins (63, 64) through the lectin-binding domain within G3 domain of versican core protein, and involves protein-carbohydrate interactions. The lectin-binding domain participates in other ligand interactions as well. For example, versican interacts with fibulin-1 and fibulin-2 (63, 65) which are a growing family of ECM proteins that are expressed in particularly high levels in the developing heart valve. In adults, however, fibulin-1 and -2 are found associated with microfibrils that are part of elastic fibers. Moreover, versican can interact with the elastic fiber-associated protein; fibrillin-1 (66), and has shown to co-localize with elastic fibers in skin (67). Fibrillins bind fibulin-2, and fibulin is preferentially localized to the elastin/microfibril interface in some tissues, but not in others (68). It is possible that fibulin serves as a bridge between versican and fibrillin, forming high-ordered multi-molecular structure important in the assembly of elastic fibers.

1.2.5 The interaction of versican with its extracellular matrix binding and cell surface molecules

Versican interacts with several ECM molecules, including HA, fibronectin, fibulins, fibrillin, tenascin, selectins, CD44, integrin, and EGFR through its N-terminal and C-terminal globular domain as well as CS-attachment domains as demonstrated in figure 1.5 (44). These multiple binding interactions play important roles in cell and tissue behavior. Versican has been found to be co-localized with HA,

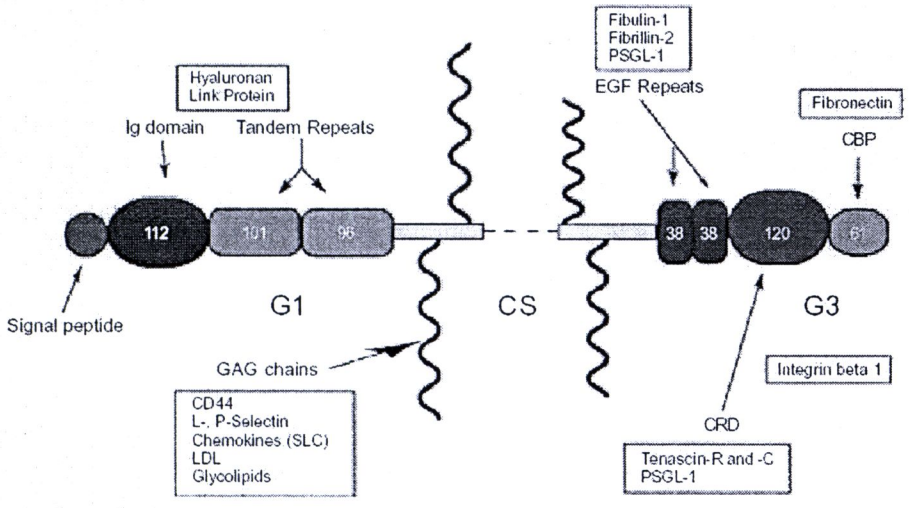

Figure 1.5 The interaction of versican with other molecules. The locations of versican motifs that interact with other molecules are shown (44).

CD44, and tenascin in the pericellular matrix of cultured fibroblasts (46), and in epidermal keratinocyte tumors (69). The association of versican with HA is mediated by BB′ stretches of versican G1 domain to both HA and LP.

Versican appears to interact with selectins via GAGs chains, and may only do so when they are oversulfated. In addition, versican is also found to bind certain chemokines and regulate their functions (70). In particular, the secondary lymphoid tissue chemokine (SLC) was shown to bind to dermatan sulfate with strong specificity. SLC assists in the binding of integrin α4β7, on leukocytes, with its ligand, MAdCAM-1, found on the endothelium (71). Although no consensus sequence has been identified in versican-binding chemokines, it appears that versican generally interacts with those that attract mononuclear lymphocytes. A specific layer of binding specificity is the particular CS chain expressed by versican, either through isoform

expression or carbohydrate modification. As the same GAG chains also bind L-selectin, and as some versican populations do not bind to this leukocyte cell surface adhesion molecule, versican expression patterns may be important in certain *in vivo* situations such as in inflammatory responses, where L-selectin and chemokines are concomitantly involved. Versican has been found to be upregulated in many inflammatory conditions, such as arthritis, asthma, and granulomatous lung diseases (72). Therefore, versican might be involved in inflammation by regulating interaction with selectins and chemokine activities.

GAG chains of versican bind to CD44, and competitive inhibition assays showed that versican, HA, and GAG chains all bind to the link module of CD44, with HA showing a substantially higher binding affinity. As CD44 binds to both hyaluronan and versican, it is possible that the three may form complexes together. However, the binding between versican and CD44 is followed by hyaluronidase treatment, the complex association therefore does not depend on the presence of HA. The fact that GAG chains alone can bind the proteins without any assistance from versican core proteins, suggests that other proteoglycans that contain the proper side chains may also be involved in complexes. Although, the oversulfation of GAGs is critical for binding to L- and P-selectins, GAG modifications do not appear to be important in CD44 binding. Interestingly, the binding of either L- or P-selectin to versican was inhibited with the addition of the solubilized other, but CD44 binding was not affected in the presence of selectins. This suggests that different GAG sites are responsible for these interactions (73). At C-terminal G3 domain of versican core protein, it binds to tenascin-R through protein-protein interaction. In addition, tenascin binds to many other members of the HABP family, such as brevican, neurocan, phosphocan, and

syndecan through lectin-like motif (63, 74). The tenascin family of large ECM glycoproteins comprises five distinct genes coding for (in a chronological order) TN-C, TN-R, TN-X, TN-Y and TN-W (75). Of these, only TN-R and TN-C are expressed in the central nervous system, and have been found to interfere with β1 integrin-dependent cell adhesion and neurite outgrowth on fibronectin. The defined spatiotemporal expression of CSPGs in the ECM or at the cell membrane of glial and neuronal cells is believed to play a role in biological phenomena as diverse as neural cell adhesion and migration; axon path finding, synaptogenesis and plasticity; growth factor and cytokine action; neuronal survival; and structural organization of the ECM (76). Similar in distribution to chondroitin sulfate proteoglycans in the brain, TN-R is also implicated in the same in such phenomena and therefore likely to interfere with chondroitin sulfate proteoglycan action or vice versa. TN-R has been suggested to participate in the macromolecular organization of perineuronal nets, along with HA and HABP, based on its divalent cation-dependent homophilic binding properties (77). This large HA matrix may create a local physical barrier, preventing other cells from entering the microenvironment. TN-R binds to the C-terminal G3 domain of versican by protein-protein interactions, involving at least two fibronectin III domains of TN-R, but is not mediated by carbohydrate moieties of TN-R (63). These results suggest that some of many molecules in the ECM may also interact with versican, which are functionally complementary.

Fibulin-1 and fibulin-2 are ECM proteins that expressed in extracellular fibrils, basement membranes, and elastic fibers (78). Versican binds to fibulin-2 and fibrillin-1 through its C-terminal G3 domain in a calcium-dependent manner. Fibulin *may serve as a bridge between versican and fibrillin, forming highly ordered*

multimolecular structures important in the assembly of elastic fibers. This organization may be particularly meaningful in blood vessels and skin, where these molecules are co-localized (41). The binding site for versican in microfibrils is most likely within a region of fibrillin-1 between its calcium-binding epidermal growth factor 11 and 21 (cbEGF)-like domains (66). Mutations in this region of the human gene, which potentially disrupt versican or other protein binding, can result in severe forms of Marfan syndrome ("neonatal" Marfan syndrome), with significant cardiovascular disease. Therefore, the connection between versican and fibrillin microfibrils may be functionally significant, particularly in cardiovascular tissues. In addition to versican, aggrecan and brevican also interact with fibulin-2 by their lectin-like motif, whereas neurocan does not (65).

Fibronectin and collagen are integrin ligands which play a role in enhancing cell adhesion. The studies have shown that versican interacts with fibronectin, as well as collagen type I (79), and these interactions are responsible for reduced cell adhesion in melanoma cells (61). In several cell types of central nervous system, versican prevents cell binding to fibronectin, but not collagen type I, (80). Conditioned medium from prostate cancer fibroblasts containing versican V0 and V1 can reduce the adhesion of prostate cancer cell lines to fibronectin (81), however, after adding RGD peptide, an integrin-binding sequence, to the medium, it can increase cell attachment to fibronectin. Thus, versican may prevent fibronectin from cell binding by sequestering fibronectin through its RGD domain. Recently, it was demonstrated that versican G3 domain enhances tumor growth and angiogenesis, perhaps through enhancement of fibronectin expression (82). Furthermore, versican G3 domain can form complexes with fibronectin and vascular endothelial growth factor. The

complex was found to stimulate endothelial cell adhesion, proliferation and migration. Removal of this complex with anti-fibronectin antibody reversed the enhancing activities of G3 domain on endothelial cells (83).

Versican interacts with integrin β1 through its C-terminal G3 domain. This interaction can also modulates cell behavior. For example, astrocytoma cells expressing a construct containing versican G3 domains exhibited enhanced spreading and adhesion, with increased phosphorylation of FAK and reduced H_2O_2-induced apoptosis. Exogenous addition of purified versican G3 product (containing two EGF-like motifs, lectin-like motif and CRP motif) was also able to induce FAK phosphorylation and increased cell adhesion (49).

EGFR, another important cell surface receptor, mediates many cellular responses in both normal biological processes and pathological states. Like all receptor tyrosine kinases (RTKs), the EGFR family members comprise an extracellular domain containing a ligand-binding site, a single hydrophobic transmembrane helix, and a cytosolic domain that includes a conserved protein tyrosine kinase core, flanked by regulatory sequences. The ligands of EGFR, such as EGF, bind to their receptors and induce conformational changes in the receptor monomers that promote their activation. Integrins are able to form clusters with EGF receptors, and this physical interaction frequently affects the intensity of EGFR-induced down-stream signal to extracellular signal-regulated kinase. The collaborative pathways derived from these two signals are crucial in regulating a range of cell activities, such as proliferation, differentiation, apoptosis, adhesion, and migration (84). The effects of versican on integrin and EGFR activation were demonstrated in versican V1-transfected PC12 cells, a phaeochromocytoma cell line. Expression of versican V1 in PC12 cells resulted in

upregulation of EGFR and integrin expression and induced NGF-independent PC12 cell neuronal differentiation and neurite outgrowth. Blockade of integrin β1, EGFR, or Src, a downstream signal transduction kinase, significantly blocked this differentiation. This suggested that the differentiation requires signals mediated by versican derived from both integrin and EGFR (85). Versican appears to not only present or recruit molecules to the cell surface, but also to modulate the expression levels of genes and coordinate complex signal pathways.

P-selectin glycoprotein ligand-1 (PSGL-1) is a homodimeric glycoprotein held together by disulfide-bonds expressed on the cell surface of leukocytes and mediates leukocyte rolling on the vascular endothelium (86). Leukocyte rolling from the blood stream into tissues in response to inflammatory stimuli is a critical component of immune response, which involves the activities of a range of adhesion and signaling molecules. PSGL-1 binds to selectins (87) through its N-terminal fragment, and mediates leukocyte rolling on the vascular endothelium. Moreover, it also binds to the G3 domain of versican, cells transfected with PSGL-1 or a shorter form of PSGL-1 containing the G3-binding site, or cells expressing endogenous PSGL-1, aggregate in the presence of versican or G3 product. The aggregation appears to be induced by G3 multimers that bind to PSGL-1 and form a network. Endogenous versican and/or G3-containing fragments also bind to PSGL-1 in human plasma. Removal of the endogenous G3-containing fragments reduces the effect of plasma on leukocyte aggregation. These data display a physiologically relevant role for PSGL-1/versican binding and may have implications in the immune response (88).

Through direct or indirect interactions with these binding molecules, cell surface proteins or growth factors, are believed to provide structural integrity to tissues and regulate cell behavior and tissue morphogenesis.

1.2.6 Cartilage development

Cartilage development or chondrogenesis begins with mesenchymal condensations where mesenchymal or chondroprogenitor cells are aggregated and further differentiated into chondrocyte to form a future cartilage anlagen, as demonstrated in figure 1.6. Following early chondrocyte differentiation, the cells rapidly proliferate, enlarging the cartilage template to form the individual skeletal elements. Chondrocytes then undergo maturation, hypertrophy, and mineralization, which allow cartilage to be replaced with bone via endochondral ossification, a process that requires extracellular matrix remodeling and angiogenesis (89).

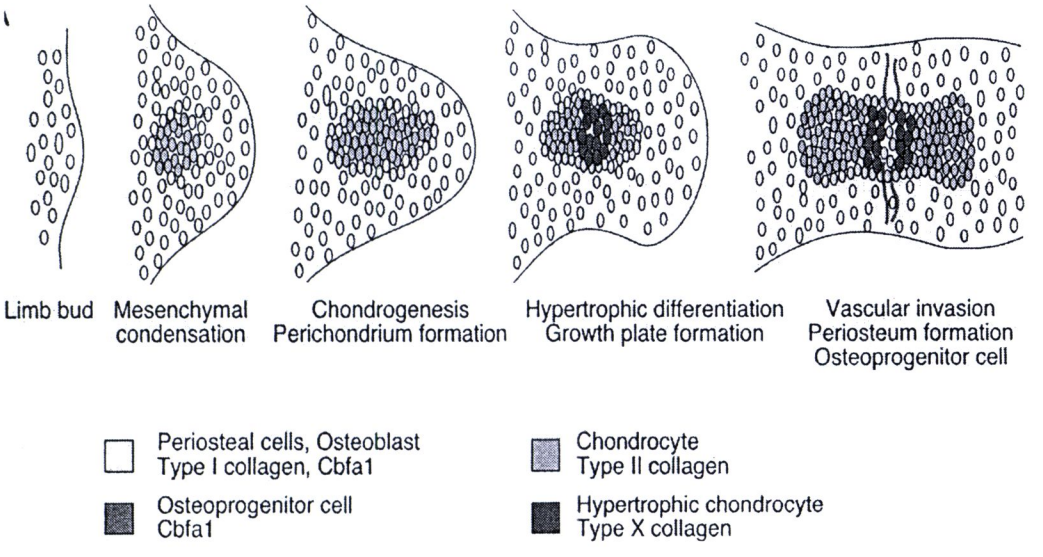

Figure 1.6 The illustration of cartilage development (90).

1.2.6.1 Mesenchymal condensations

Cartilage development can be devided into two interdependent processes: patterning; and cell fate determination. Patterning is the process during which number, size, and shape of the cartilage mold is delineated and established. Cell fate determination is the process by which the orchestral interactions of genetic and environmental factors serve to guide the developmental progression of the cell lineage. Both two interdependent processes are governed by a series of tissue interactions, including the interactions between adjacent components of segmental structures, or between juxtaposed epithelium and mesenchyme. In the early of cartilage development, it is initiated by the differentiation of mesenchymal cells that arise from three sources; (1) neural crest cells of the neural ectoderm that gives rise to craniofacial bones, (2) the sclerotome of the paraxial mesoderm, or somite compartment, which forms the axial skeleton, and (3) the somatopleure of the lateral plate mesoderm, which yields the skeleton of the limbs (91). There are four steps involved in the development. The first is the migration of cells to the site of future skeletogenesis; the second is the aggragation by mesenchymal-epithelial cell interactions; the third is the condensations forming; and the fourth is chondrocyte differentiation (92, 93). Cell condensation is the third of four primary phases of cartilage development, which may start with one or a combination of three processes: enhanced mitotic activity; aggregation of cells toward a center; and failure of cells to disperse from a center (93). This process is dependent on signals initiated by cell-matrix and cell-cell adhesion, and these signals are modified by response of the cells to growth and differentiation factors in the extracellular environment. Condensations are hallmark by changes in cell adhesion and cytoskeletal architecture (93, 94). These

also include categories of extracellular ligands, their cognate receptors, and cytoplasmic transducers (95), nuclear receptors (96), transcription factors or DNA-binding proteins (97) and the matrix glycoproteins. It has known that craniofacial and limb development is the best illustrates for the complexity and hierarchy of regulatory mechanisms underlying cartilage development. Limb buds develop from the lateral plate mesoderm (98). The patterning of limb mesenchyme is due to interactions between the mesenchyme and the overlying epithelium (99). The embryonic limb possesses two signaling centers, the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA), which generate signals accountable for directing the proximal-distal outgrowth and anteriorposterior patterning, respectively (91, 94). Prior to condensations, the prechondrogenic mesenchymal cells express cell surface molecules that bind peanut agglutinin lectin and allow condensations to be visualised; (100, 101, 102) the affinity of prechondrogenic cells in condensations for peanut agglutinin (PNA) is utilised in a technique to isolate and characterize PNA-positive cells. During mesenchymal condensations, factors such as transforming growth factor-β (TGF-β) (103), bone morphogenetic proteins (BMPs) (104), and growth differentiation factor (GDF)-5 (18) are known to play critical roles in the compaction of mesenchymal cells of the condensation areas. As shown in table 1.2, the condensing mesenchyme also expresses various ECM and cell adhesion molecules, such as versican, tenascin, fibronectin, syndecan, neural cadherin (N-cadherin), neural cell adhesion molecule (N-CAM) (105). It is evident that condensations result from epithelial-mesenchymal interactions controlled by TGF-β, BMP-2, *Msx-1*, and tenascin (93). TGF-β is one of the earliest signals in chondrogenic condensation, and is indicated to stimulate the synthesis of fibronectin, which in turn regulates N-CAM.

Then, syndecan binds to fibronectin and downregulates N-CAM, thereby setting the condensation boundaries. The other extracellular matrix molecules, which also include tenascins, thrombospondins, cartilage oligomeric protein (COMP), interact with the cell adhesion molecules to activate intracellular signaling pathways involving focal adhesion kinase and paxillin, lead to the transition from chondroprogenitor cells to a fully committed chondrocyte (94). Other growth and differentiation factors such as fibroblast growth factor (FGF), Sonic hedgehog (Shh), bone morphogenetic protein (BMP), and Wnt pathways coordinate signaling along the three axes of the limb to insure correct patterning along the dorso-ventral and anteroposterior axes (106). Wnt signals, including Wnt2a and Wnt2c, are required to induce FGFs, such as FGF-10 and FGF-8, which act in positive feedback loops (107). Hence, FGF-10 then induces Wnt3a, which acts via β-catenin to increase FGF-8, which then maintains FGF-10 expression (98). Wnt7a is expressed early during limb bud development, and acts to maintain Shh expression (108). Shh signaling is required for early limb patterning, and mediated by the Shh receptor Patched (Ptc1), which activates another transmembrane protein Smoothened and inhibits processing of the Gli3 transcription factor to a transcriptional repressor (107, 109). The hedgehog pathways are regulated by intraflagellar transport proteins which control both the activator and repressor functions of Gli family members (110). Some other signals are BMPs, that are not only set the stage for bone morphogenesis by initiating chondroprogenitor cell determination and differentiation, but also regulate the later stages of chondrocyte maturation and terminal differentiation to the hypertrophic phenotype. BMP-2, -4, and -7 coordinately regulate limb-patterned elements within the condensations depending upon the temporal and spatial expression of BMP receptors and BMP

antagonists, such as noggin and chordin (108, 111, 112). The previous *in vitro* and *in vivo* studies have shown that BMP signaling is required both for the formation of precartilaginous condensations and for the differentiation of precursors into chondrocytes (113, 114). With synchronizing through cell-cell or cell-matrix interactions of these molecules, lead mesenchymal cells to commitment into the chondrocytic lineage, and synthesize the ECM molecules specific to cartilage, such as type II collagen and aggrecan.

Table 1.2 The major classes of genes and gene products associated with skeletogenic condensations along with their functions and stages of action (93).

Gene/ gene product	Function	Stage
	Growth factors	
BMPs	regulate Hox gene (*Hoxa-2, Hoxd-11, Pax-2*) in response to Shh, regulate *Msx-1*, *Msx-2*	growth, transition to differentiation
FGF-2	regulate N-CAM	initiation, proliferation, growth
TGF-β	regulate fibronectin	initiation
	Cell surface, cell adhesion, and extracellular matrix molecules	
Fibronectin (115)	an extracellular glycoprotein regulated by TGF-β; regulate N-CAM	initiation, proliferation
N-cadherin	a cell adhesion molecule	adhesion
N-CAM	a cell adhesion molecule regulated by FN, *Prx-1*, *Prx-2* and FGF	initiation, adhesion
Noggin	a secreted protein that binds and activates BMP-2, BMP-4, BMP-7	slows or stops growth
Syndecan	a receptor that binds to tenascin; binds to FN to activate N-CAM	sets boundary
Tenascin	an extracellular glycoprotein that bind to syndecan	stops condensation growth, sets boundary
	***Hox* genes**	
Hoxa-2	regulated by BMPs, downregulated cbfa-1	sets boundary, growth, prevents differentiation
Hoxa-13	alters adhesive properties	adhesion
Hoxd-11	regulated by BMPs	proliferation, growth
Hoxd-11-13	transcriptional activation	transition to differentiation
	Transcription factors	
cbfa-1	transcriptional activating protein inhibited by *Hoxa-2*	differentiation of chondroblasts
CFKH-1	a chicken forkhead-Helix transcription factor that regulated TGF-β and interacts with Smad transcription factor	initiation, proliferation
MFH-1	mesenchymal transcription factor	proliferation
Osf-2	transcriptional activating protein regulated by BMP-7 and Vimentin	switches cells onto the osteoblastic pathway
Pax-1, Pax-9	encode nuclear transcription factors, regulated by BMP-7	growth
Prx-1, Prx-2	upstream regulation of N-CAM	initiation
Scleraxis	a basic helix-loop-helix protein	proliferation
Sox-9	regulated the collagen2α1 gene	proliferation

1.2.6.2 Chondrocyte differentiation and proliferation

The differentiation of chondroprogenitor cells is required for subsequent stages of cartilage development, and is characterized by the deposition of cartilage matrix containing collagens II, IX, and XI and aggrecan. The transcription factor Sox9, one of earliest markers expressed in cells undergoing condensation, is required for the expression of the type II collagen gene (Col2α1) and certain other cartilage-specific matrix proteins, including Col11α2 and CD-RAP, prior to matrix deposition in the cartilage anlagen (115, 116, 117). In addition, there are two Sox family members, L-Sox5 and Sox6, which are not present in early mesenchymal condensations, but are co-expressed with Sox9 during chondrocyte differentiation (118). The expression of SOX proteins depend upon BMP signaling via BMPR1A and BMPR1B, which are functionally redundant and active in chondrocyte condensations but not in the perichondrium (114).

In the growth plate of cartilage, it is composed of different zones of individual chondrocytes; (1) periarticular proliferating or reserved chondrocytes zone, (2) columnar proliferating zone, (3) prehypertrophic zone, and (4) hypertrophic zone. Throughout cartilage developement, the balance of signaling by BMPs and FGFs determines the rate of proliferation, by which adjusting the pace of the differentiation (119). In the long bones, long after condensation, BMP-2, -3, -4, -5, and -7 are expressed primarily in the perichondrium and only BMP-7 is expressed in the proliferating chondrocytes (119). BMP-6 is found exclusively later in hypertrophic chondrocytes along with BMP-2. Proliferation of chondrocytes in the embryonic and postnatal growth plate is regulated by multiple mitogenic stimuli, including FGFs, which converge on the cyclin D1 gene (120). The proliferation of chondrocytes in the

lower proliferative and the prehypertrophic zones is under the control of a local negative feedback loop involving signaling by PTHrP and Indian hedgehog (Ihh), although Ihh is restricted to the prehypertrophic zone whereas, PTHrP receptor is expressed in the distal zone of periarticular chondrocytes. Early studies indicate that Ihh induces expression of PTHrP in the perichondrium (121) and that PTHrP signaling then stimulates cell proliferation via its receptor expressed in the periarticular chondrocytes (122). Recent evidence indicates that Ihh acts independently of PTHrP on periarticular chondrocytes to stimulate differentiation of columnar chondrocytes in the proliferative zone, whereas PTHrP acts by preventing premature differentiation into prehypertrophic and hypertrophic chondrocytes, thereby suppressing premature expression of Ihh (123). Therefore, Ihh and PTHrP, by transiently inducing proliferation markers and repressing differentiation markers, function in a spatiotemporal manner to determine the number of cells that remain in the chondrogenic lineage against those that enter the endochondral ossification pathway.

1.2.6.3 Chondrocyte maturation

In the epiphyseal growth plate of the embryo as well as the metaphyseal growth plate of the post-natal animal, endochondral ossification involves terminal differentiation of chondrocytes to hypertrophy (chondrocytes maturation), calcification of cartilage matrix, vascular invasion, and ossification (124, 125, 126, 127). During chondrocyte hypertrophy, it is notable increase in cell size by almost 20-fold of its initial resting size. At this time, prehypertrophic chondrocytes as they exit the proliferative phase, and enter the hypertrophic phase begin to express markers

of hypertrophic chondrocytes such as type X collagen (COL10α1) and alkaline phosphatase (128). Other ECM proteins, comprising osteocalcin and osteopontin, are known to play functional roles in cell-matrix interactions during endochondral ossification. The ECM remodeling that accompanies chondrocyte maturation is thought to induce an alteration in the environmental stress experienced by hypertrophic chondrocytes, which eventually endure apoptosis (124, 129, 130). Angiogenesis is a process by which perichondrium and hypertrophic zone are invaded by blood vessels, and is required for the replacement of cartilage by bone (131, 132). VEGF, the angionic factor, is released from the ECM by MMPs such as MMP- 9 expressing endothelial cells that migrate into the central region of the hypertrophic cartilage coincident with Flk-positive cells, promotes the invasion of blood vessels through specifically localized receptors, including Flk expressed in endothelial cells in the perichondrium or surrounding soft tissues, neuropilin (Npn) 1 expressed in late hypertrophic chondrocytes, or Npn 2 expressed restricted in the perichondrium (125). These events of cartilage matrix remodeling and vascular invasion are requried for migration and differentiation of osteoclasts and osteoblasts, which remove mineralized cartilage matrix and replace it with bone.

1.2.7 Joint formation and deterioration

1.2.7.1 Joint specification

During cartilage development, within developing condensations, the cartilagenous interzone specifies the future joint, following by segmenting the developing cartilage template into individual skeletal components. For example, in long bone formation, it initiates as uninterrupted mesenchymal condensations in the

early limb buds, which undergo differentiation to chondrocytes (133, 134). The proximal region of the mesenchyme condensations gives rise to the humerus or femur with the more distal regions forming the radius/ulna or tibia/fibula and digits. As displayed in figure 1.7, synovial joints are begins with packing and lining of mesenchymal cells at the future joint location termed interzone which provides a clear demarcation between the cartilaginous elements (19, 20, 135). At the sites of joint formation, the resident cells flatten and lose their chondrogenic property (136, 137). Nevertheless, the interzone appears to vary in morphology between joints and among species. For example, in the chick phalangeal joints, the interzone can vary between a distinct three-layered structure comprising two chondrogenous layers separated by a flattened cell central layer (137). In many mammalian joints, the interzone consists a thin flattened cell layer (two to three cells) closely compacted between the developing articular surfaces (138). Previous works suggested that the interzone forms as a result of differential matrix synthesis and cell proliferation in the cartilage mold (19, 139, 140). However, there appear to be no detailed studies on the mechanisms of interzone formation, which remains to be elucidated. Several studies have focused on Hox genes, homeodomain-containing transcription factors that pattern the body axis of animal embryos, could also be responsible for specification of segmentation boundaries in developing limbs. Hox genes are organized in clusters and are expressed along the body axis in a manner corresponding to their position within the cluster (141). In higher vertebrates, there are 39 Hox genes found on 4 clusters (a, b, c, and d) on different chromosomes (142). All four Hox groups play a role in limb

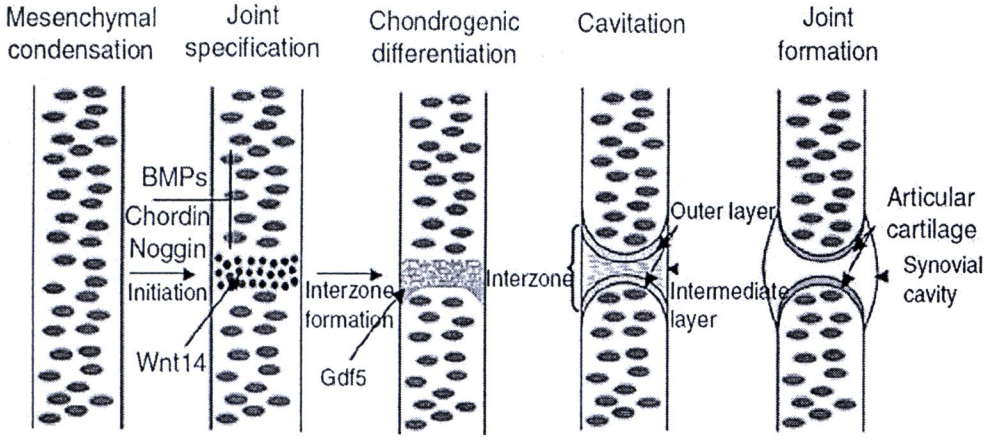

Figure 1.7 The illustration of joint formation, showing the joint interzone specification, cavitation and joint morphogenesis (143).

development, however, the gene expression patterns of Hoxa9-13 and Hoxd9-13 imply preponderant roles in limb development. Experimental evidence shows that expression of Hoxa9 and its paralog. Hoxd9 specify the scapula, paralogs 10 specify the humerus, 11 the radius and ulna,12 the carpals, and 13 specify metacarpals and phalanges (144). Null mutations of paralogs can result in the fusion of some bones, suggesting a role in joint formation, but such analyses are complicated by the fact that there is functional cooperation not only between paralogous but also nonparalogous Hox genes (145). Other skeletal defects due to null mutations of Hox genes are not attributable to changes in patterning but, rather, specifically to changes in cell adhesion and proliferation during chondrocyte condensations (93). Several signaling molecules, growth factors, transcription factors, and other regulatory molecules expressed by the interzone are implicated in the molecular specification of the joint interzone. These include: BMP-2, BMP-4, GDF-5, GDF-6 , Wnts, *Chordin, Noggin,*

FGFs, Cux-1, ERG, *Autotoxin*, and *Stanniocalcin* (146). For example, Wnt pathways mediated by Wnt-4, Wnt-14 and Wnt-16 (143, 147) have been shown to participate in joint formation, and another study indicates that the Wnt/β-catenin canonical signaling pathway is necessary and sufficient for the induction of synovial joints in the limb (148). In deed, in the early studies, it revealed that GDF-5 is expressed in the early interzone in mouse and chick embryo limb joints (149). The rationale for this focus was that in the *brachypod* mouse mutant due to lack of GDF-5 (18), which has appendicular skeletal defects (including the absence of joints in digit, wrist, ankle), and the compound ablation of GDF-5 and GDF-6 genes causes widespread joint defects and skeletal growth retardation (150). Some of these defects are found by patients with mutations in GDF-5 (also know as CDMP1) (151). However, it is now known that GDF-5 is also expressed in condensing mesenchyme, additionally to the joint interzone, and that GDF-5 does not specify joint formation. Instead, GDF-5 has at least three functions during joint and skeletal development. First, it promotes initiation of chondrogenesis. Second, it promotes chondrocyte proliferation, increasing the size of the final skeletal elements (152, 153). Finally, GDF-5 maintains the early joint (154).

The involvement of Wnt family members in joint formation has also been studied. Wnt-14 (also known as wnt9a) is fairly expressed as a transverse stripe in the presumptive joint regions of future joints in the autopod of developing chick limbs. In later stages of development, Wnt-14 is observed in parts of the fibrous capsule as well as the synovial lining of the joint capsule. More recent work has indicated that Wnt14 is expressed in the developing joints and is sufficient to direct joint development in the chick, as judged by its ability to induce and/or maintain a panel of

gene markers indicative of joint development, including the secreted phosphodiesterase/pyrophosphatase, *autotaxin*, *chordin*, CD44, and GDF-5 (147). Interestingly, Wnt-14 overexpression in chick limb mesenchymal micromass cultures resulted in the chondrogenic differentiation arrest at the early prechondrogenic step, rather than stimulation as seen after GDF-5 overexpression. This was indicated by the maintenance of peanut agglutinin-stained cells, which are found in early condensations, and the absence of alcian blue staining, which is indicative of chondrogenic differentiation. In addition to Wnt-14, Wnt-4 and Wnt-16 is expressed in overlapping and complementary patterns in the joint interzones of developing limbs, and all signal is mediated through the canonical Wnt-signaling pathway. Wnt ligands bind to receptors encoded by the Frizzled (Fz) gene family and LRP5/6 that result in the stabilization of cytosolic β-catenin which is then translocated to the nucleus, where it activates downstream gene expression by binding LEF/TCF transcription factors. The studies of micromass assays performed in which Wnt-14 adenovirus was added to mouse limb mesenchymal cells that had a conditional targeted allele of β-catenin, demonstrate that the cells were inhibited for chondrogenic differentiation, however, this effect was reversed when cultures were serially infected first with Cre-adenovirus to inactivate β-catenin followed by Wnt-14 adenovirus which led to the absence of joints (148). Furthermore, the ectopic expression of activated β-catenin or Wnt-14 in chondrocytes lead to the ectopic of joint marker. Similarly, the ectopic of GDF-5 expression in transgenic expressing a constitutive-active form of β-catenin and its transcriptional mediator LEF (CA-LEF) was also observed in chondrocyte (155). Collectively, Wnt-14 is sufficient and necessary for joint formation, and would acts as

an upstream regulator of GDF-5, and maintain the mesenchymal nature of the interzone by preventing chondrogenesis (147, 148).

However, limb environmental signals are probably extremely important in determining the length of the different skeletal structures, and inducing the formation of the initial joint. The role of environmental signals is highlighted by the correct joint spacing that occurs in ectopic digits that are formed in the interdigital mesenchyme in response to wounding and/or TGF-β application (156). TGF-βs introduce their signal by binding to TGF-β type II receptor (TβRII) that leads to the phosphorylation of TβRI and TβRII-TβRI complex formation, which then activates the signaling cascade through R-Smad-dependent (Smad-2,-3,-4) and Smad-independent pathways. In human and mouse embryonic cartilage, TGF-βs are expressed in the endochondral template with high expression in the perichondrium (157-161). Genetic manipulation of the TGF-β system genes has revealed their critical roles in skeletogenesis (162, 163, 164) for review see (165), for example, the implantation of TGF-β induces extra digit formation (166). Analysis of conditional null-mice of TGF-β receptor II (TβRII) has demonstrated an essential role of TGF-β signaling in joint morphogenesis (167). Morever, versican is found to be expressed in the joint interzone, in the articular cartilage and synovial tissue, lining the inside margin of the cavity (168, 169). During the process of synovial joint formation and cartilage development, versican exhibits dynamic expression patterns. Its expression initiates at a high level in mesenchymal condensation areas. While the cells differentiate into chondrocytes, it remains in peri-condensation areas. When the joint is formed by accumulation and lining up of mesenchymal cells, versican is expressed in the joint interzone. After the formation of the joint cavity, versican is present in the

articular cartilage and synovial tissue, lining the inside margin of the cavity (168, 169). These characteristic expression patterns of versican and its effects on cell behavior observed in cell culture systems strongly suggest that versican regulates, in the ECM, the function of ligands that mediate signaling toward cartilage development and synovial joint formation. There is limited information on the mechanisms that regulate the complex multistep process that leads to joint interzone formation. In fact, very few genes have been reported to be necessary and/or sufficient to initiate the joint formation process, as mentioned above. The factors that induce the expression of those joint morphogenic molecules are obscure; furthermore, the mechanisms that determine the emergence of joint interzone cells within the chondrogenic condensations are unclear.

1.2.7.2 Joint cavitation

The next stage of joint development is the formation of the fluid-filled joint space separating the opposing skeletal elements, cavitation, followed by the morphogenesis. The cartilage anlagen will be separated to form a functioning synovial cavity. Many factors have been postulated to play a role in this process required for the formation of the synovial cavity between the long bone rudiments. These factors include cell death, enzymatic degradation of the interzone, differential growth of opposing elements, differential matrix synthesis, and mechanical influences (170-175). Cell death has been detected within the interzone prior to cavitation, though in most cases the extent of cell death is minimal or nonexistent (172-178). Thus, the contribution of cell death to the cavitation process is thought to be minimal. Alterations in local fibrillar collagen content during the cavitation process, the loss of

type I collagen and the expression of type II collagen (137, 171), suggest an enzymatic turnover of the matrix. Immunohistochemical studies have shown that matrix-degrading enzymes are barely detected in the interzone and developing cavity (138, 179). However, matrix metalloproteinase activity has been detected postcavitation in articular cartilage (180) , and this enzyme activity is hypothesized to contribute to cartilage matrix turnover during growth. Differential growth of the developing cartilage anlagen of the limb has been demonstrated (138, 178, 181, 182, 183, 184), but the mechanism of differential growth is not clearly understood in terms of the formation of a fluid-filled cavity, since proliferating cells are located immediately beneath the articular surface, and it is unlikely that proliferation contributes directly to the cavitation process. Differential growth, in association with differential cartilage matrix synthesis (175, 185, 186, 187), is more likely implied in shaping specific topographical regions of the growing articular cartilage. To date, evidence points toward movement-induced mechanical stimuli being responsible for altering the synthesis and degradation of ECM components along the joint line resulting in cavitation. There is strong evidence to suggest that HA is one of these components. The experimental data regarding the mechanism of joint cavitation involves differential HA synthesis under the influence of mechanical stimuli. The long-chain glycosaminoglycan HA, and its principal cell surface receptor, CD44, are differentially expressed at the joint interzone and developing articular surfaces (138, 183, 188, 189, 190). It has demonstrated using enzyme histochemistry assay that uridine diphosphoglucose dehydrogenase (UDPGD) activity is essential in providing the precursors for HA synthesis, and is significantly increased just prior to cavitation in the interzone, and during cavitation in the articular surfaces and synovium (189,

191). The interaction between HA and CD44 can induce both cell adhesion and cell separation, depending on the concentration of HA surrounding a cell population through receptor saturation, with increasing HA concentrations leading to cell separation (192). Thus, during limb development, the expression of CD44 in the interzone and at the developing articular surfaces, and the increased HA synthesis associated with cavitation can facilitate tissue separation and create a functional joint cavity. Another important gene involved in HA synthesis is HA synthase (HAS) which exists in three isoforms, derived from three separate genes HAS1, HAS2, and HAS3 (193) which have been shown in vitro to be responsible for synthesizing HA of different chain lengths and at different rates. Differential expression of the three HAS genes suggest that each plays an important role in maintaining joint function. Protein kinase C has been shown to increase HAS activity through phosphorylation. Interestingly, the activation of PKC in rabbit knee joints *in vivo* and in rabbit synoviocytes *in vitro* results in increased HA release, whereas blocking PKC activity results in a decrease in HA release (194, 195, 196). Additionally, the activation of both PKCα and PKCδ by stretch supports a role for mechanical stimuli in HA release. Taking all these data into consideration, it has been suggested that mitogen-activating protein kinase (MAPK) signaling could be involved in the mechanotransduction pathway which results in HA synthesis. There is an evidence suggests that extracellular-signal-related kinases (ERK1/2), downstream of the MAPK-kinase pathway, are important in the mechanotransduction pathway (197, 198). Phosphorylated ERK1/2 has been found to be present at the sites of joint cavitation and is also preserved after cavitation (197, 199).

1.2.7.3 Joint morphogenesis

Following joint cavitation, the skeletal elements undergo morphogenesis. However, the mechanisms underlying morphogenesis of the opposing skeletal elements into the diverse interlocking structures remains to be fully elucidated and is the (146). The morphogenesis of the functional joint organ results in articular cartilage lining the ends of skeletal elements, which are bathed in synovial fluid, produced by a synovial membrane, and encased within a fibrous capsule. A synovial membrane lines the capsule and secretes synovial fluid which provides the lubrication, required for low-friction articulation, and nutrition for the articular cartilage. The surfaces of the two opposing skeletal elements are covered by hyaline articular cartilage that contributes most toward the functional ability of the joint providing a durable load-bearing surface that permits pain-free movement. Consequently, disease or trauma affecting the joint has wide-ranging implications on the health and function of the joint.

1.2.8 Joint deterioration

1.2.8.1 Rheumatoid arthritis and pathology

Arthritis is a group of conditions where there is damage caused to the joints of the body. The most common arthritic diseases are rheumatoid arthritis (RA) and osteoarthritis (OA) which are often referred to as an autoimmune disease and age-related joint disease, respectively. RA is self-directed inflammatory cascade that culminates in joint destruction. The pathogenesis of RA originates in the synovial tissues adjacent to the joints and then spreads the cartilage. RA is also known as the inflammatory disease marked by hyperplasia and chronic inflammation of the

synovial membranes, activated synovial fibroblasts (SFs) in the lining layer of the synovial membrane invade deeply into the articular cartilage and bone that contribute to cartilage deterioration and joint destruction (21, 22, 23, 24). One of the most prevalent events during pathogenic progression of RA is the hyperplasia of SFs in the lining layer, which is caused by vascularization, infiltration of activated T-cells and mononuclear cells, and the hyperproliferation of SFs (24). Indeed, it may be divided the inflamed pannus of synovium into two compartments. As shown in figure 1.8, the first compartment is an immune part comprised by T cells, B cells, macrophages, dendritic cells, and many cellular mediators. In this compartment, antigen-activated $CD4^+$ T cells stimulate monocytes, macrophages, and SFs to produce the cytokines, including interleukin (IL)-1, IL-6, and tumor necrosis factor (TNF)-α. Furthermore, activated $CD4^+$ T cells also stimulate B cells, through cell-surface contact and through the binding of $\alpha 1\beta 2$ integrin, CD154 (CD40 ligand), and CD28, to produce immunoglobulins, including rheumatoid factor. To date, the precise pathogenic role of rheumatoid factor is still undefined, but it may involve the activation of complement through the formation of immune complexes. The perspective that T cells and various cell mediators participated in the etiology and pathogenesis of RA was one of a keys intellectual breakthrough in understanding of the disease. The other compartment of the rheumatoid pannus is composed of cells adjacent to bone and cartilage that contribute directly to the erosion and destruction of those tissues. While several RA cell types contribute to cartilage and bone destruction, including neutrophils (in the joint space itself) and chondrocytes (sequestered deep within the cartilage), the primary cells that propagate articular damage at the pannus's leading edge are osteoclasts and SFs.

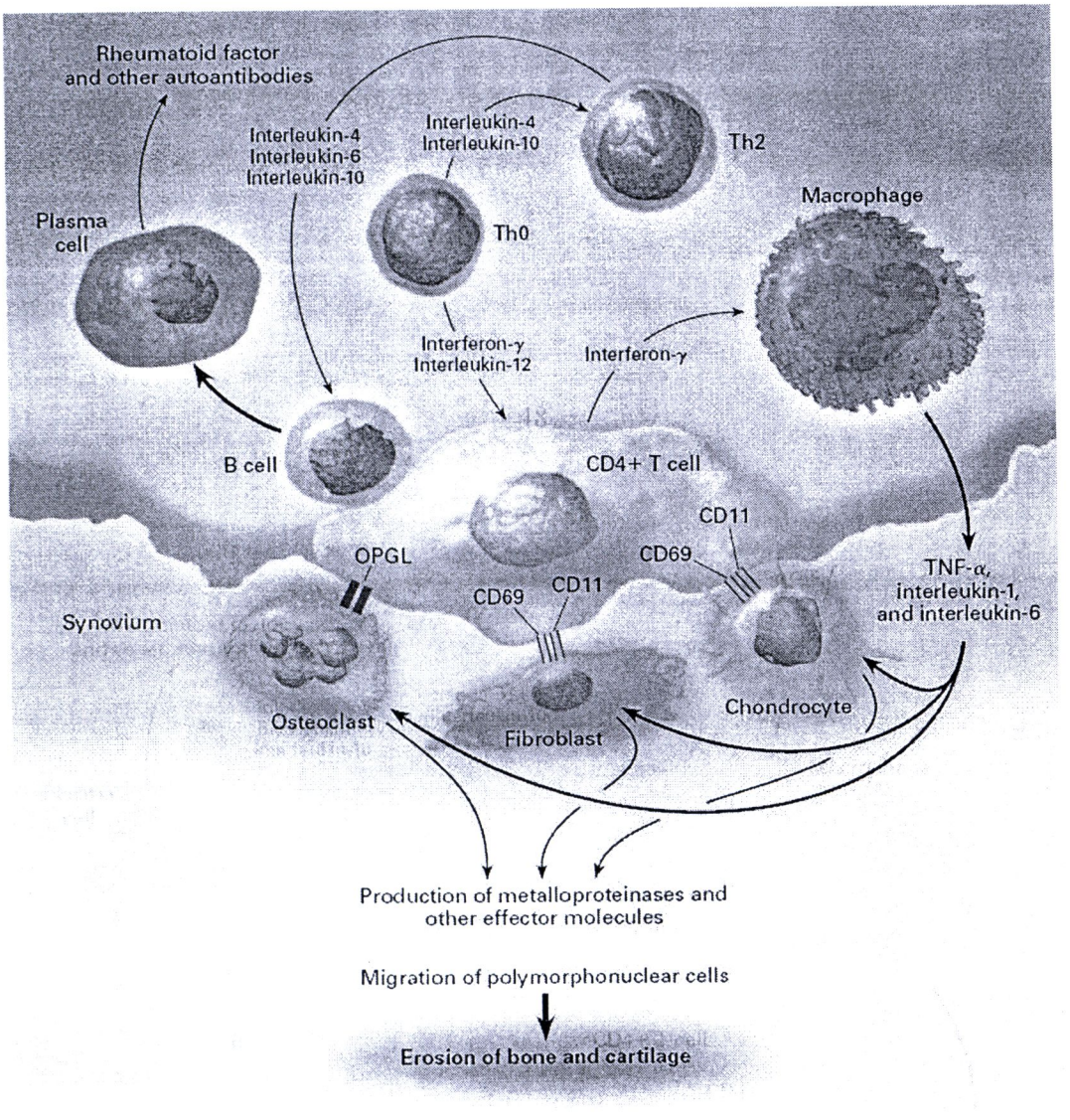

Figure 1.8 The illustration of cytokine signaling pathways involved in RA. The major cell types and cytokine pathways involved in joint destruction mediated by IL-1 and TNF-α are shown (25).

1.2.8.2 Role of synovial fibroblast in rheumatoid arthritis

SFs (in the literature also referred to as synoviocytes or fibroblast-like synovial cells) are in the intimal lining layer of the synovium, and can produce factors that maintain inflammation by activating cells in the local environment. During joint development, SFs form the main element of the nascent joint tissue. And in response to hyaluronic acid and other signals, it begins to define the joint space and capsule. In the healthy adult joint, the SFs perform several functions. As the primary stromal cell of the joint, the SFs are likely to be responsible for the production of collagen and other connective tissue molecules that form and maintain the joint capsule. It is also the major secretor of hyaluronic acid and other molecules into the joint space itself, providing lubrication to the joint surface as well as signaling functions to the joint tissues. Healthy SFs appear to secrete controlled amounts of enzymes, such as MMPs that have the capacity to digest connective tissue, and presumably maintain the structure and pliability of the joint capsule through remodeling (200). However, in RA, SFs acts as a producer of pro-inflammatory, and as a mediators of cartilage erosion by secreting many MMPs. Hence, SF is one of the crucial cell types that regulate multiple processes in the RA joint, including propagation of inflammation, maintenance of pannus architecture, and regulation of other cell types. A broad array of cytokines, including IL-1, IL-6, IL-7, IL-8, IL-15, and many others produced by SFs (201), concurrently with pro-inflammatory cytokines or chemokines produced by monoclytes/macrophages in the arthritic area of the joint (202), induce and/or augment the production of several MMPs (203, 204, 205), leading to matrix degradation and the promotion of joint inflammation. Most of these cytokines, IL-1 and TNF-α can be detected in synovial fluid from patients with rheumatoid arthritis

(206). The serum and synovial concentrations of both cytokines are high in patients with active rheumatoid arthritis (207, 208). Furthermore, IL-1 and TNF-α are potent stimulators of mesenchymal cells, such as synovial fibroblasts, osteoclasts, and chondrocytes, that release tissue-destroying matrix metalloproteinases (MMPs) (209). IL-1 and TNF-α also inhibit the production of tissue inhibitors of metalloproteinases (TIMPs) by synovial fibroblasts (209). These dual actions are thought to lead to joint damage, and both IL-1 and TNF-α are likely to have primary roles in the pathogenesis of rheumatoid arthritis.

Regarding to IL-1, it is a 17-kd protein that is mostly produced by monocytes and macrophages but is also produced by endothelial cells, B cells, activated T cells, and SFs (201, 210). The biologic activity of interleukin-1 depends on the precise quantities of many interacting molecules. Studies of arthritis in animals have strongly implicated IL-1 in joint damage, and injection of IL-1 into the knee joints of rabbits results in the degradation of cartilage (211). The progressive of joint destruction by cytokines such as pro-inflammatory IL-1β, IL-6, and TNF-α released from macrophages, monocytes, and synovial fibroblasts is the crucial mechanism to drive inflammation in RA (25) through induction of cytokines or up-regulation MMPs which are the major enzymes for erosion of bone and cartilage (26).

In RA synovial tissues, MMP-13 has been detected in fibroblasts, chondrocytes, macrophages, vascular endothelial cells (212, 213, 214), and at the sites of joint destruction (215, 216). MMP-2 (gelatinase A) (217, 218) and MMP-9 (gelatinase B) (219, 220, 221) have been found in elevated levels in serum and synovial fluid, and expressed at the junction between the synovial pannus and cartilage or bone (222). These MMPs not only degrade collagens, proteoglycans and other extracellular matrix

(ECM) macromolecules in cartilage, but also activate other MMPs (223). Thus, inhibition of pro-inflammatory cytokine-induced MMPs secretion by synovial fibroblasts could be one of the solutions to alleviate the symptom in RA patients.

1.2.9 Phytochemicals

1.2.9.1 *A. galanga* and its characteristic

Phytochemicals are non-nutritive chemical compounds that occur naturally in plant. It is known that plant produces these chemicals to protect itself but recent research demonstrate that they can protect humans against diseases. There are currently many phytochemicals possibly having medicinal properties in clinical trials for a variety of diseases. The various phytochemicals found in plant and herbal sources all have different properties in reducing inflammation. For instance, spice components, such as curcurmin, turmeric, and capsaicin from red pepper reduce inflammation by influencing arachidonic acid metabolism and also the secretion of lysosomal enzymes by macrophages. Curcumin and capsaicin also inhibited the secretion of collagenase, elastase and hyaluronidase demonstrating that they can control the release of pro-inflammatory mediators such as eicosanoids. Cytokines are chemicals that modulate the immune response. Many phytochemicals have effects on at least one of the following cytokines: IL-1, IL-6, TNF, and IFN (224).

Alpinia galanga (B.L. Burtt), Thai herb, has the traditional uses in treatment of flatulence, dyspepsia, vomiting, high blood pressure, gastrointestinal complaints. The rhizome of *A. galanga*, figure 1.9, is widely used in the Ayurveda (a form of alternative medicine) in the treatment of various inflammatory diseases, diabetes mellitus and obesity. It has been suggested to suppressing prostaglandin synthesis

through the inhibition of cyclooxygenase-1 and cyclooxygenase-2 (27). The aqueous acetone extract from rhizomes of *A. galanga* has shown the inhibitory effects on lipopolysaccharide (LPS)-induced nitric-oxide production (225). Of particular interest, the study of *p*-hydroxycinnamaldehyde, the purified substance of the acetone extract of *A. galanga*, indicates the potential ability of *p*-hydroxycinnamaldehyde in suppression of the catabolic genes expression levels comprising MMP-3 and MMP-13, whereas increase the levels of anabolic genes of collagen II, SOX9 and aggrecan (226). Previous study indicated that MMPs expression induced by IL-1β in SW1353 chondrosarcoma, synovial fibroblasts, and cartilage explants is reduced by hexane extract of *A. galanga* (28), suggesting its anti-inflammatory effect on these models. Collectively, *A. galanga* extracts might be a promising therapeutic agent for arthritis, and the effects of *A. galanga* hexane extracts should further be examined.

Figure 1.9 Photographs of trunk, blossom, and rhizome of *A. galanga* (227).

1.3 Objectives

(1) To investigate the *in vivo* role of versican in mesenchymal condensations toward cartilage and joint development by genetically ablated versican expression with *Prx1*-Cre/*loxP* technique.

(2) To investigate the effects of hexane extracts from *A. galanga* on IL-1β-induced human synovial fibroblasts as an inflammatory model.