

## CHAPTER I

### INTRODUCTION

#### 1.1 Statement and Significance of the Problem

Articular cartilage is a highly specialized tissue that provides low friction and allows for efficient load bearing and distribution (1). Articular cartilage damage is a major problem in osteoarthritis (OA), a cause of morbidity and loss of athletic performance not only in humans but also in horses (2). Joint pain and loss of mobility caused by OA are common causes of poor performance and early retirement in equine athletes. Development of OA in horses is often associated with the stresses of racing and training (3). Cartilage tissue is different from other tissue. It has a limited capacity for self-repair, because chondrocytes are bound in lacunae, they cannot migrate to damaged areas. Also because hyaline cartilage does not have a blood supply, the deposition of new matrix is slow. Therefore, the demand for effective treatment strategies of cartilage defects is continuously increasing. Recently, tissue engineering concepts have been applied to the development of a cell-based repair material for articular cartilage. A number of growth factors like TGF- $\beta$ , FGF, BMP, and IGF, have been explored for their effects on cartilage tissue engineering, for the regeneration of cartilage tissue damaged due to disease or trauma (4). Transforming growth factor- $\beta$  family (TGF- $\beta$ ) have been shown to play a major role in cartilage development, are commonly used to induce chondrogenesis in embryonic (5) and adult MSCs to increase cartilage ECM synthesis (6). TGF- $\beta$  is a multifunctional protein that

regulates many aspects of cellular activity, including cell proliferation, differentiation, and ECM metabolism in a time and concentration-dependent manner (7). More over several studies have shown that TGF- $\beta$  isoforms differ in their effects on various cell types and culture condition (8). In equine articular cartilage the expression of TGF- $\beta$ 1, 2 and 3 mRNA was measured and the level of TGF- $\beta$ 1 was the highest. It was 6-fold higher than TGF- $\beta$ 2 and TGF- $\beta$ 3 (9).

HA, a nonsulfated-glycosaminoglycan, is a major component of the extracellular matrix and has various physiological and biological functions. HA binds to chondrocytes via CD44 receptor (10). Which CD44 adhesion to HA has also been shown to mediate chondrocyte proliferation (11) and stimulate chondrogenic differentiation of embryonic mesenchymal cells (12). HA has been shown to increase proteoglycan synthesis in equine articular cartilage and rabbit chondrocytes (13). A study using cultured chondrocyte within gelatin based scaffold showed that extrinsic HA enhanced chondrocytes proliferation 2 fold and also significantly enhanced matrix production (14). Therefore, HA have important role in extracellular matrix of chondrocytes which may encourage tissue engineering applications.

HA synthesis can be regulated by many growth factors and cytokines, including transforming growth factor-beta. Effect of TGF- $\beta$ 1 on HA anabolism have been studied, in synovial membrane fibroblast HA synthesis were enhanced by TGF- $\beta$ 1 stimulation. HAS2 mRNA expression was enhanced 13-fold after six-hour stimulation with TGF- $\beta$ 1 (10 ng/ml), whereas HAS3 mRNA expression was not changed significantly (15). HAS2 mRNA appears to be constitutively expressed in bovine chondrocytes (42). It is primarily responsible for hyaluronan synthesis in

articular chondrocytes (51). Regulation of HA synthesis by means of the HA synthases differs between cell types because given cell types express distinct profiles of one or more HAS genes.

However, the effects of TGF- $\beta$ 1 on the expression of HAS2 mRNAs and the relevant HA synthesis have not been clarified in equine chondrocytes. We hypothesized that TGF- $\beta$ 1 may enhance HAS2 mRNA expression and contribute to HA synthesis in equine chondrocyte which encourage tissue engineering applications. Therefore, we conducted study to investigate the effects of TGF- $\beta$ 1 on the HAS2 mRNA expression and HA synthesis in monolayer culture and 3-D culture of equine chondrocytes.

## **1.2 Literature Reviews**

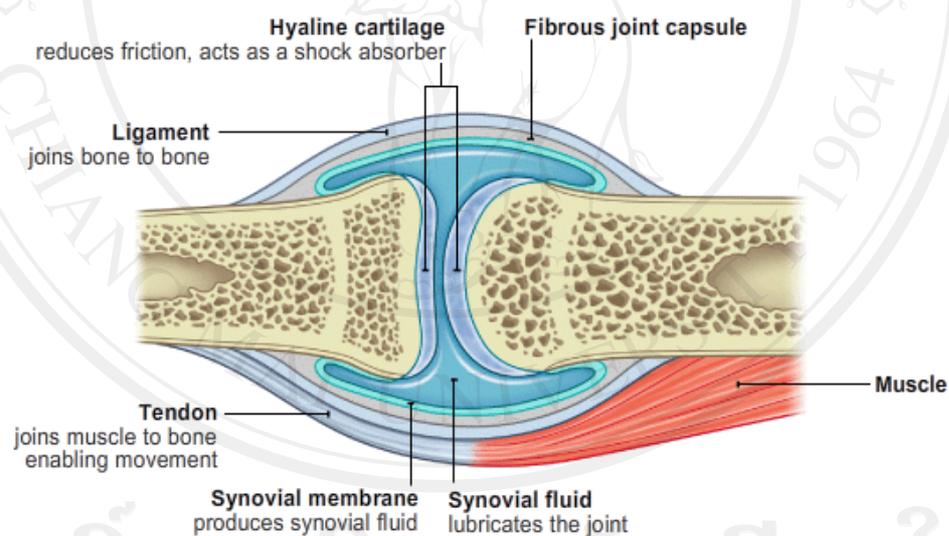
### **1.2.1 Articular cartilage**

Cartilage is a type of dense connective tissue. Cartilage is classified in three types, elastic cartilage, hyaline cartilage and fibrocartilage, which differ in the relative amounts of ECM composition. Cartilage is found in many areas in the body, including the articular surface of the bones, the rib cartilage, the ear, the nose, the bronchial tubes and the intervertebral discs. Its mechanical properties are intermediate between bone and dense connective tissue like tendon (16).

Fibrocartilage contains abundant thick bundles of mostly type I collagen, which can be seen with the light microscope. It is a white, very tough material that provides high tensile strength and support. It contains more collagen and less proteoglycan than hyaline cartilage. Thus, its properties are closer to those of tendons

than hyaline cartilage. This type of cartilage is found in ligament and tendon that insert into bone, in menisci, intervertebral discs, and the symphysis pubis, temporomandibular and sternoclavicular joints. Fibrocartilage provides good resistance to shear and compression forces (16).

Elastic cartilage is characterized by the presence a large amount of elastic fibers within the matrix which increases elasticity of tissues. It is stiff yet elastic, and is important to prevent tubular structures from collapsing. This type of cartilage is found in the lobe of the ear, the epiglottis and in parts of the larynx.



(<http://www.bbc.co.uk/schools/gcsebiteize/pe/images/synovial>; January 4, 2011)

**Figure 1.1** Articular cartilage or hyaline cartilage covers the joint surfaces.

Articular cartilage is hyaline cartilage, which smooth, glistening white tissue that covers the articular surface. The figure of articular cartilage covers the joint

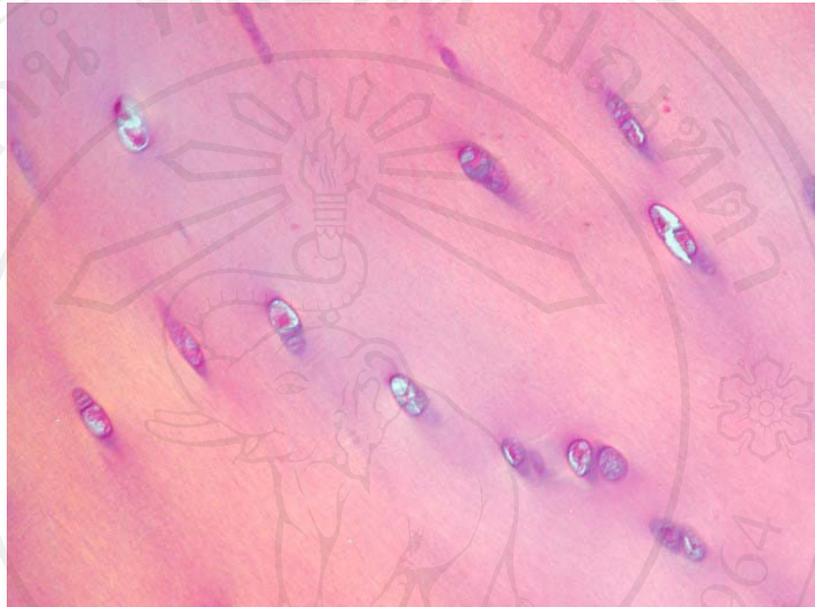
surfaces was shown in figure 1.1. Articular cartilage is a highly specialized tissue that reduces joint friction at the extremities of long bones and allows for efficient load bearing and distribution. It consists of specialized cells called chondrocytes that produce a large amount of extracellular matrix.

Chondrocytes embedded within an extracellular matrix made up of a macromolecular framework and water as shown in figure 1.2. Cartilage is relatively a hypocellular tissue, because it composes of chondrocytes about 2-5% of the tissue volume or contains approximately  $100 \times 10^6$  cells/cm<sup>3</sup> on average (17), throughout the full thickness of mature cartilage. Chondrocytes serve diverse functions during development and postnatal life and are the single cellular component of the articular cartilage of diarthrodial joints. Chondrocytes are relatively inactive metabolically due to the absence of a vascular supply and innervation in the tissue, although they can respond to mechanical stimuli, growth factors and cytokines that influence normal homeostasis in a positive or negative manner.

These cells display a unique palisade architectural pattern with round, single or columnar cells within lacunae (17). Under physiological conditions, chondrocytes mainly maintain the homeostasis of the hyaline cartilage (18). It maintains and remodels cartilage matrix tissue by a careful balance of catabolic and anabolic processes involving matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs). This constant process is dependent of several factors, including growth factors, cytokines, mechanical loading, aging and injury.

Unlike other connective tissues, articular cartilage has no blood vessels, neural, and is hypocellular tissue (18). Thus, articular cartilage has a limited capacity

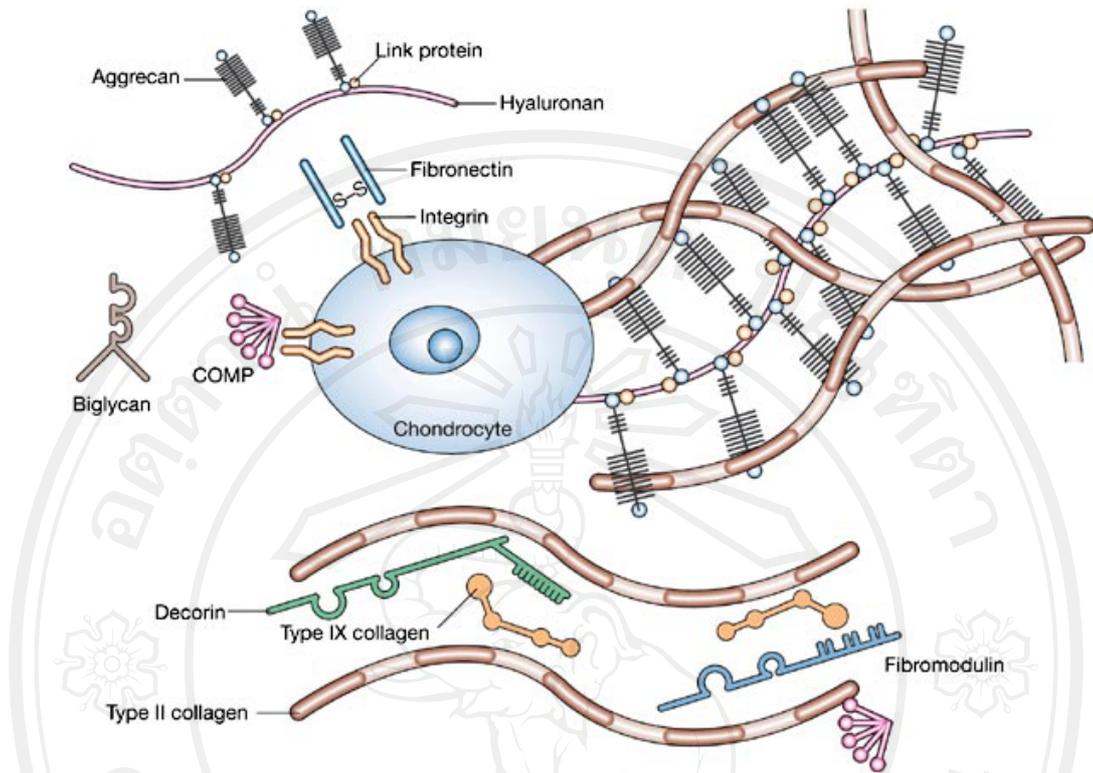
for repair due to the absence of vasculature, which would allow progenitor cells from the blood or the bone marrow to enter the damage area.



(<http://en.wikipedia.org/wiki/Cartilage>; January 4, 2011)

**Figure 1.2** Histological image of hyaline cartilage stained with haematoxylin & eosin, under polarized light.

Articular cartilage is composed mainly of water (70 – 80% by wet weight). The solid phase of articular cartilage is composed of a network of type II collagen, proteoglycan and other proteins or glycoproteins represent only about 20 % of the tissue wet weigh.

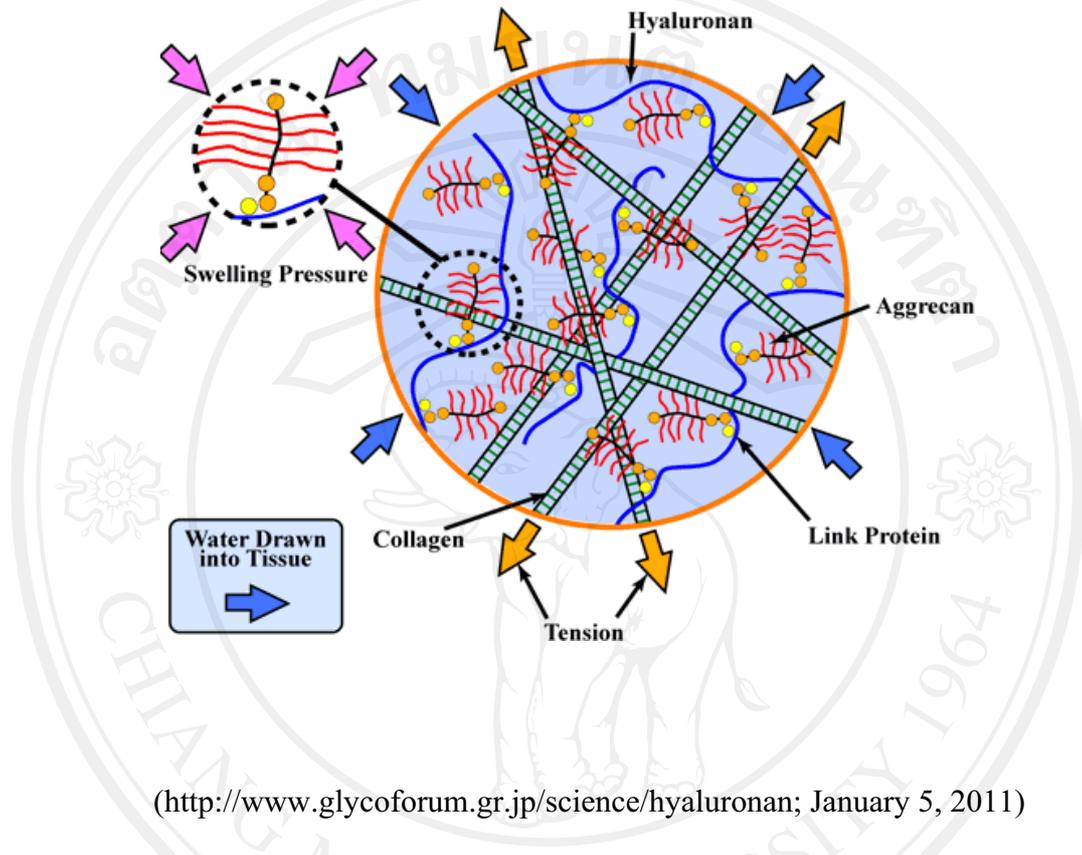


([http://www.nature.com/nrrheum/journal/v2/n7/fig\\_tab/ncprheum](http://www.nature.com/nrrheum/journal/v2/n7/fig_tab/ncprheum); January 4, 2011)

**Figure 1.3** Extracellular matrix of cartilage.

Three classes of proteins exist in articular cartilage as shown in figure 1.3: collagens (mostly type II collagen); proteoglycans (primarily aggrecan); and other noncollagenous proteins (including link protein, fibronectin, cartilage oligomeric matrix protein, hyaluronan) and the smaller proteoglycans (biglycan, decorin and fibromodulin). The interaction between highly negatively charged cartilage proteoglycans and type II collagen fibrils is responsible for the compressive and tensile strength of the tissue, which resists load *in vivo*. Abbreviation: COMP, cartilage oligomeric matrix protein (19).

### Combined Functions of Collagen Fibers and Proteoglycans in Cartilage



**Figure 1.4** Combined functions of collagen fibers and proteoglycans in cartilage

The mechanical behavior of the cartilage depends on the interactions between collagen, proteoglycans, and interstitial fluid components.

A network of type II collagen provides the tensile and shearing properties of the cartilage and immobilizes the proteoglycans within the matrix, proteoglycans give resistance to mechanical stress as shown in figure 1.4 (20). Type II collagen is specific to cartilage and is the primary collagen in this tissue (90-98% of the total

tissue collagen). Type II collagen fibrils is also interacts with types IX and XI collagens.

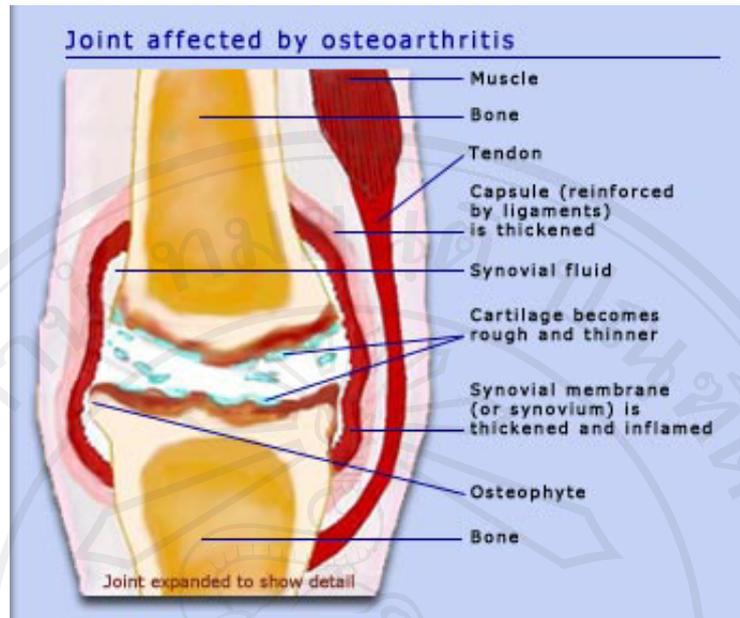
The large aggregating proteoglycan aggrecan, attached to hyaluronan (HA) polymers via a link protein. Because of their numerous negatively charged sulfate groups, these proteoglycan aggregates attract cations, which in turn bring in water to minimize differences in osmotic pressure. Thus, type II collagen and proteoglycan create a swollen, hydrated tissue that resists compression.

Glycosaminoglycans and other cartilage matrix constituents, including biglycan, decorin, cartilage oligomeric matrix protein, tenascins and matrilins may also be synthesised by chondrocytes under low turnover conditions (21).

### **1.2.2 Osteoarthritis (OA)**

Cartilage has a very limited intrinsic reparative capacity. As a consequence, cartilage damage results in progressive disease (22). Osteoarthritis (OA) is the most common form of arthritis, involving cartilage, synovium and bone as shown in figure 1.5. OA can occur in any joint, but it is most common in the hand, knee, hip and foot.

Severe pathological changes can be observed radiologically as a loss of joint space, subchondral bone sclerosis and presence of osteophytes, which are bony spurs located at joint margins. Clinical manifestations include pain, stiffness and impairment of joint motion, which lead to disability and the need for joint replacement.



(<http://www.mydr.com.au/arthritis/arthritis-how-osteoarthritis-and-rheumatoid-arthritis-affect-joints>; January 5, 2011)

**Figure 1.5** Joint affected by osteoarthritis.

In normal cartilage there is constant degradation as well as synthesis of cartilage matrix molecules, controlled by the chondrocytes. A high degradation rate does not necessarily implicate OA as long as there is enough compensation by synthesis. Besides catabolic factors OA chondrocytes also express anabolic factors, like insulin-like growth factor-1 and transforming growth factor beta (TGF- $\beta$ ) that stimulate extracellular matrix (ECM) production (23). Cartilage damage in OA has several hallmarks. Initially, in contrast to what is expected during damage, an increased synthesis of matrix molecules is observed. However, in time cartilage matrix degradation exceeds matrix deposition resulting in net matrix loss. In early OA the cartilage surface is still intact, but shows some focal edema or even minor

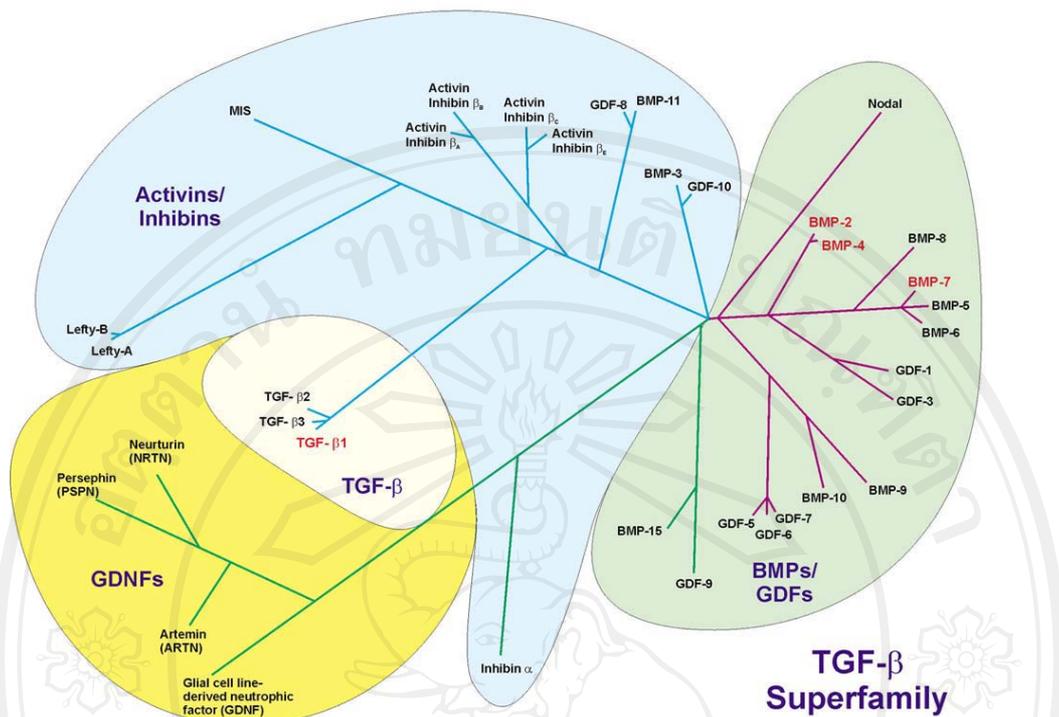
fibrillations (22). Chondrocytes can be stimulated by catabolic cytokines to release cartilage degradation products, ultimately leading to damage. Now termed interleukin-1 (IL-1), was discovered to play a role in OA. Several groups described its capacity to induce metalloproteinases in cartilage and its ability to stimulate chondrocytes to degrade both proteoglycan and collagen (24, 25). A breakdown of this cartilage matrix leads to the development of fibrillation and fissures and the appearance of ulcerations, together with the disappearance of the full thickness of the joint surface.

### **1.2.3 Cartilage tissue engineering**

Articular cartilage has a limited capacity for tissue repairing and these limitations have prompted researchers and clinicians to develop surgical methods to restore cartilage surfaces. Although many, mostly surgical techniques, are currently employed to treat cartilage lesions, none has given satisfactory results in the long term. Recent advances in biology and material science have brought tissue engineering to the forefront of new cartilage repair techniques. The combination of autologous cells, specifically designed scaffolds, bioreactors, mechanical stimulations and growth factors together with the knowledge that underlies the principles of cell biology offers promising avenues for cartilage tissue regeneration (1). The goal of cartilage tissue engineering is to provide living biological, physiological substitutes that could replace tissue loss due to disease, congenital abnormalities, or trauma. Ideally, the biological substitute should structurally and morphologically resemble native tissue and be able to perform similar biological functions.

#### 1.2.4 Transforming growth factor beta

TGF- $\beta$  family consists of over 35 members and includes, besides TGF- $\beta$ s, activins, bone morphogenetic proteins (BMPs) (26), and Nodals (27). Members of the TGF- $\beta$  family have crucial roles in development and in tissue homeostasis. They play vital roles in development and homeostasis of various tissues. They regulate cell proliferation, differentiation, apoptosis and migration, as well as control ECM synthesis and degradation. Moreover, these factors mediate cell and tissue responses to injury and modulate immune functions (8). Perturbation of their signalling has been implicated in several developmental disorders and in various human diseases including cancer, fibrosis and auto-immune disease (28).



(<http://www.bioscience.org/2009/v14/af/3583/figures.htm>; January 4, 2011)

**Figure 1.6** The TGF-beta superfamily.

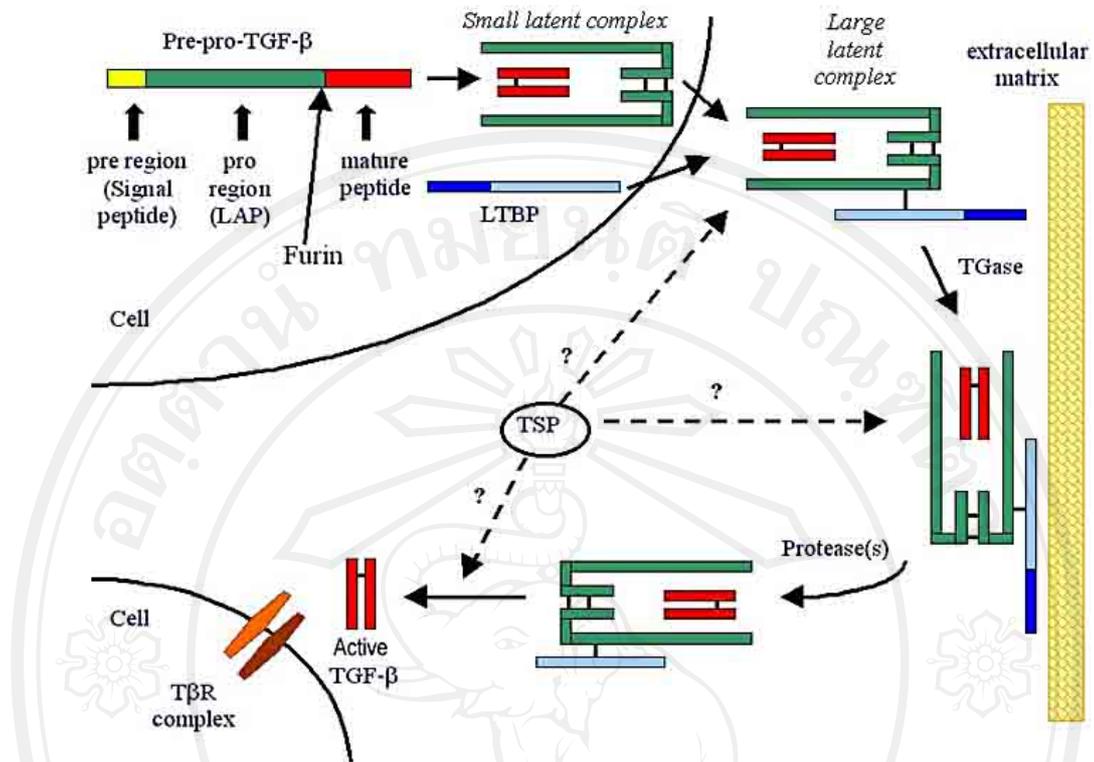
Based on their structural features as shown in figure 1.6, the 35 mammalian members of the TGF-beta family are subdivided into (i) TGF-betas, (ii) activins/inhibins, (iii) bone morphogenetic proteins (BMPs)/growth and differentiation factors (GDFs), and (iv) the more distantly related group of GDNF ligands (29).

TGF- $\beta$  responses can be cell-type specific and are dependent on both the concentration of TGF- $\beta$  signalling components and the activity of other signal

transduction pathways, which can either synergize with or antagonize the TGF- $\beta$  pathway (27).

In mammals three isoforms of TGF- $\beta$  have been identified that is TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3. All isoforms show a high degree of homology of 84-92%. The expression of the three isoforms is differently regulated at the transcriptional level due to the different promoter sequences (8, 30-32). TGF- $\beta$ 1 is the prototypic member of a family of over 35 structurally related pleiotropic cytokines in vertebrates (27).

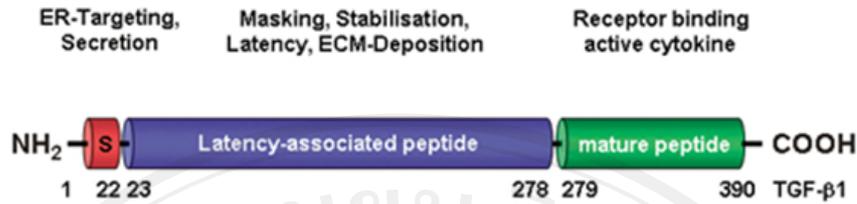
TGF- $\beta$  is secreted as an inactive complex formed by the non-covalent association of a TGF- $\beta$  homodimer, its propeptide LAP (latency associated peptide) and LTBP (latent TGF- $\beta$  binding proteins). Therefore, the secreted TGF- $\beta$  requires activation before it can bind to its receptor as shown in figure 1.7 (33, 34). Activation of TGF- $\beta$  results from proteolytic activity or extreme pH values, among other possible stimuli. TGF- $\beta$  was first described as “cartilage-inducing factor-A” (CIF-A), because it triggered chondrogenic differentiation of embryonic rat muscle cells (35).



**Figure 1.7** Mechanisms regulating biologic activity of TGF- $\beta$ .

(<http://www.bioscience.org/2002/v7/d/gressner/figures.htm>; September 20, 2009)

The peptide structures of the three members of the TGF- $\beta$  family are highly similar. They are all encoded as large protein precursors as shown in figure 1.8 ; TGF- $\beta$ 1 contains 390 amino acids and TGF- $\beta$ 2 and TGF- $\beta$ 3 each contain 412 amino acids. They each have an N-terminal signal peptide of 20-30 amino acids that they require for secretion from a cell, a pro-region (called latency associated peptide or LAP), and a 112-114 amino acid C-terminal region that becomes the mature TGF- $\beta$  molecule following its release from the pro-region by proteolytic cleavage.



(<http://www.bioscience.org/2009/v14/af/3583/figures.htm>; January 8, 2011)

**Figure 1.8** Structural features of TGF- $\beta$ 1.

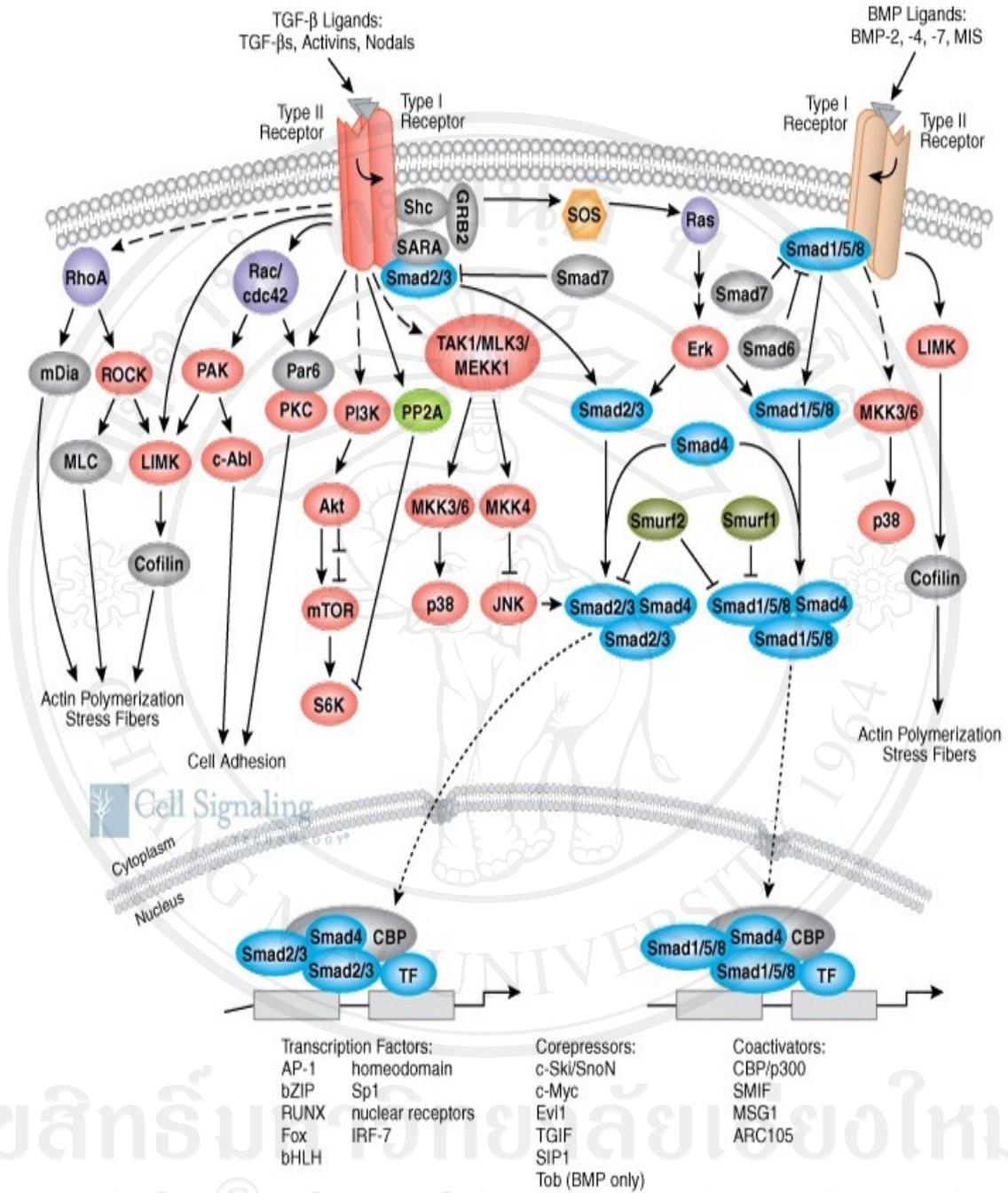
The mature TGF- $\beta$  protein dimerizes to produce a 25 KDa active molecule with many conserved structural motifs. TGF- $\beta$  has nine cysteine residues that are conserved among its family; eight form disulfide bonds within the molecule to create a cysteine knot structure characteristic of the TGF- $\beta$  superfamily while the ninth cysteine forms a bond with the ninth cysteine of another TGF- $\beta$  molecule to produce the dimer. Many other conserved residues in TGF- $\beta$  are thought to form secondary structure through hydrophobic interactions. The region between the fifth and sixth conserved cysteines houses the most divergent area of TGF- $\beta$  molecules that is exposed at the surface of the molecule and is implicated in receptor binding and specificity of TGF- $\beta$ .

TGF- $\beta$ , secreted proteins are anabolic morphogens and growth factors with critical functions for the articular joint (36). The TGF- $\beta$  family controls proliferation, extracellular matrix and/or differentiation in articular chondrocytes, and the molecular mechanisms governing ligand binding, receptor oligomerization and signal transduction begin to be elucidated. These molecules initiate signalling from the cell

surface by interacting with type I and type II serine/threonine kinase receptors, depending on the ligands they bind. Upon ligand binding, the type II receptor activates the type I receptor, which phosphorylates the downstream mediators: Smads 1, 5 and 8 after BMP activation and Smads 2 and 3 after TGF- $\beta$  and activin-binding, respectively. The phosphorylated Smads associate with Smad 4 and translocate into the nucleus and regulate the transcription of target genes as shown in figure 1.9 (37).

TGF- $\beta$ 1, 2 and 3 are generally considered to be potent stimulators of proteoglycans and of type II collagen synthesis in chondrocytes and are able to induce the chondrogenic differentiation of MSCs in vitro (38). In vivo, TGF- $\beta$ 1 can induce the chondral differentiation of MSCs to form ectopic cartilage and was able to repair a full-thickness cartilage defect by improving chondrocyte integration into the endogenous tissue (39).

Transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1) is a secreted homodimeric protein that regulates numerous cellular responses, such as proliferation, differentiation, migration and apoptosis.



(<http://www.cellsignal.com/pathways/tgf-beta-smad.jsp> ; January 8, 2011)

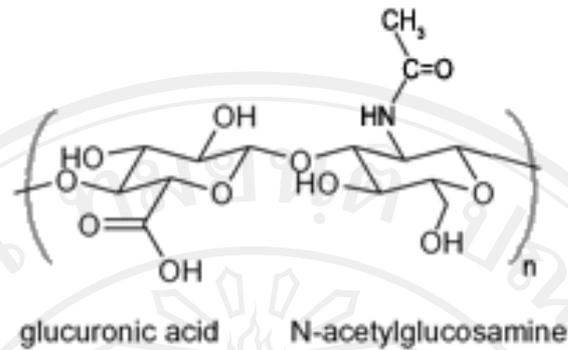
**Figure 1.9** TGF-β signalling pathway

### 1.2.5 Hyaluronan and Hyaluronan synthase

In articular cartilage, hyaluronan (HA) serves as the core filament of the proteoglycan aggregate; these macromolecular aggregates composed of HA, link protein and the major cartilage proteoglycan, aggrecan, establish essential biomechanical properties of cartilage (40, 41).

HA, a nonsulfated-glycosaminoglycan with repeated disaccharide units of D-glucuronic acid and N-acetyl-D-glucosamine as shown in figure 1.10.

HA is a major component of the extracellular matrix, is a major molecule in joint fluid and play a crucial role in joint motion and the maintenance of joint homeostasis. HA has been shown to play an important role in a number of cellular functions including mitosis, cell migration, joint cavitation in development and interaction with cell surface receptors (42). CD44 serves as a primary trans-membrane receptor for HA, providing cells a mechanism for matrix attachment or for sensing changes in the ECM (43). HA has various physiological and biological functions. It physically acts as a viscous lubricant for slow joint movements, such as walking, and as an elastic shock absorber during rapid movements, such as running (44). The ability of HA to influence cell behavior is due in part to its role in the organization of the extracellular matrix (ECM) and the capacity of HA to interact directly with cells (45).

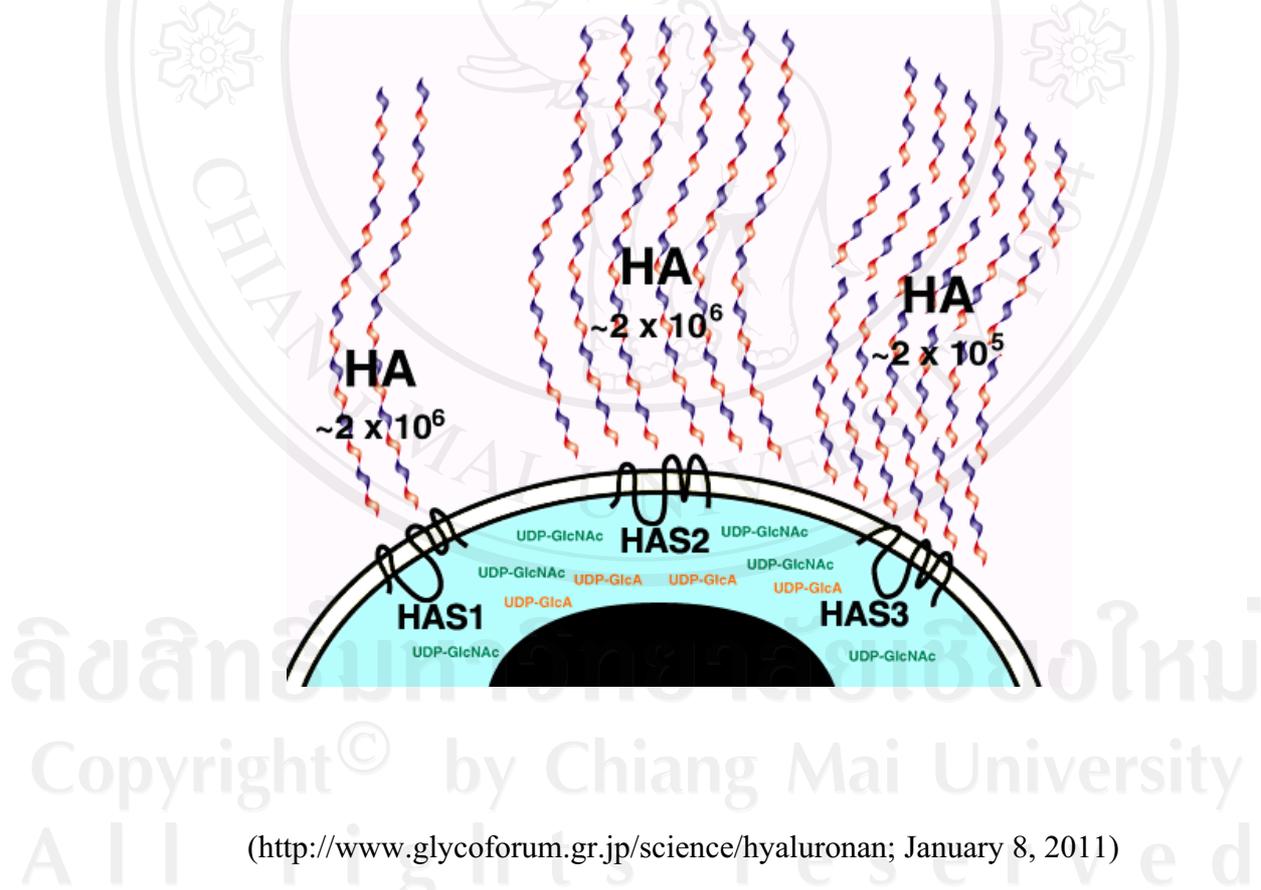


([http://www.glycosan.com/ha\\_science/what\\_hyaluronan.html](http://www.glycosan.com/ha_science/what_hyaluronan.html); January 10, 2011)

**Figure 1.10** Structure of hyaluronan; composed of two monosaccharides:  $\beta(1,4)$ -N-acetyl-D- glucosamine and  $\beta(1,3)$ -D-glucuronic acid

Unlike the other component molecules of extracellular matrix which are synthesized intracellularly, HA is produced and extruded at the cell surface, thereby enabling unconstrained assembly of very long polymers (46). HA was synthesized by hyaluronan synthases (HAS), are membrane-bound enzymes. In mammalian was found three isoform that is HAS1, HAS2 and HAS3, which encoded by different gene and the three HAS enzymes are found on different chromosomes (47). The chromosomal localization of the three HAS genes have been identified in human and in mouse. The genes localized to three different positions within both the human and the mouse genomes. HAS1 was localized to the human chromosome 19q13.3-q13.4 boundary and HAS1 to mouse Chr 17. HAS2 was localized to human chromosome 8q24.12 and HAS2 to mouse Chr 15. HAS3 was localized to human chromosome 16q22.1 and HAS3 to mouse Chr 8 (47), thereby implicating additional mechanisms

for the control of HAS activity (e.g. at the level of mRNA transcription) (42). All members of the HAS gene family are proposed to have a common domain structure possessing multiple membrane-spanning regions and a conserved central (intracellular) domain which is likely to contain the catalytic site within the enzymes (48). Interestingly, the three HAS isoforms have been shown to exhibit different activities in vitro, with each enzyme generating HA chains of varying length as shown in figure 1.11.



(<http://www.glycoforum.gr.jp/science/hyaluronan>; January 8, 2011)

**Figure 1.11** Regulation of hyaluronan amount and chain length by expression of a specific HAS protein.

HAS1 produces small amounts of high-molecular-weight HA (up to 2000 kDa). HAS2 produces significantly more high-molecular-weight HA (up to 2000 kDa). HAS3 is the most active of the hyaluronan synthases, yet produces low-molecular-weight HA chains (from 200 to 300 kDa). Biochemical analyses revealed that the three encoded polypeptides have differential stability (half-life) and enzymatic activities. Overall, HAS3 is most stable, HAS2 is less, and HAS1 is the least stable (49). HA synthesis can be regulated by many growth factors and cytokines, including transforming growth factor-beta/ bone morphogenetic protein superfamily members, interleukin-1 beta, tumor necrosis factor-alpha, epidermal growth factor, and keratinocyte growth factor, depending on the cell types (50). Regulation of HA synthesis by means of the HA synthases differs between cell types in part because given cell types express distinct profiles of one or more HAS genes. HAS2 is primarily responsible for hyaluronan synthesis in articular chondrocytes (51).

HA chains of different lengths have different effects on cell behavior. Very short HA chains have been demonstrated to stimulate cell proliferation and to initiate signalling cascades. High-molecular-weight HA chains have the opposite effect, inhibiting cell proliferation. High-molecular-weight HA has a variety of biologic effects on cells in vitro, such as: the inhibition of prostaglandin E2 synthesis and the release of arachidonic acid induced by interleukin-1 from cultured fibroblasts (52, 53) and protection against proteoglycan depletion and cytotoxicity induced by oxygen-derived free radicals, interleukin-1 (54, 55). Short HA chains can also be generated by degradation of extracellular HA (49).

In cartilage, HA is synthesized by the resident chondrocytes (56-58) and incorporated into the extracellular matrix where it binds to aggrecan and link protein, thereby providing a critical role in enabling and promoting the maintenance of multimolecular hydrophilic aggregates which facilitate the load bearing properties of the tissue (42).

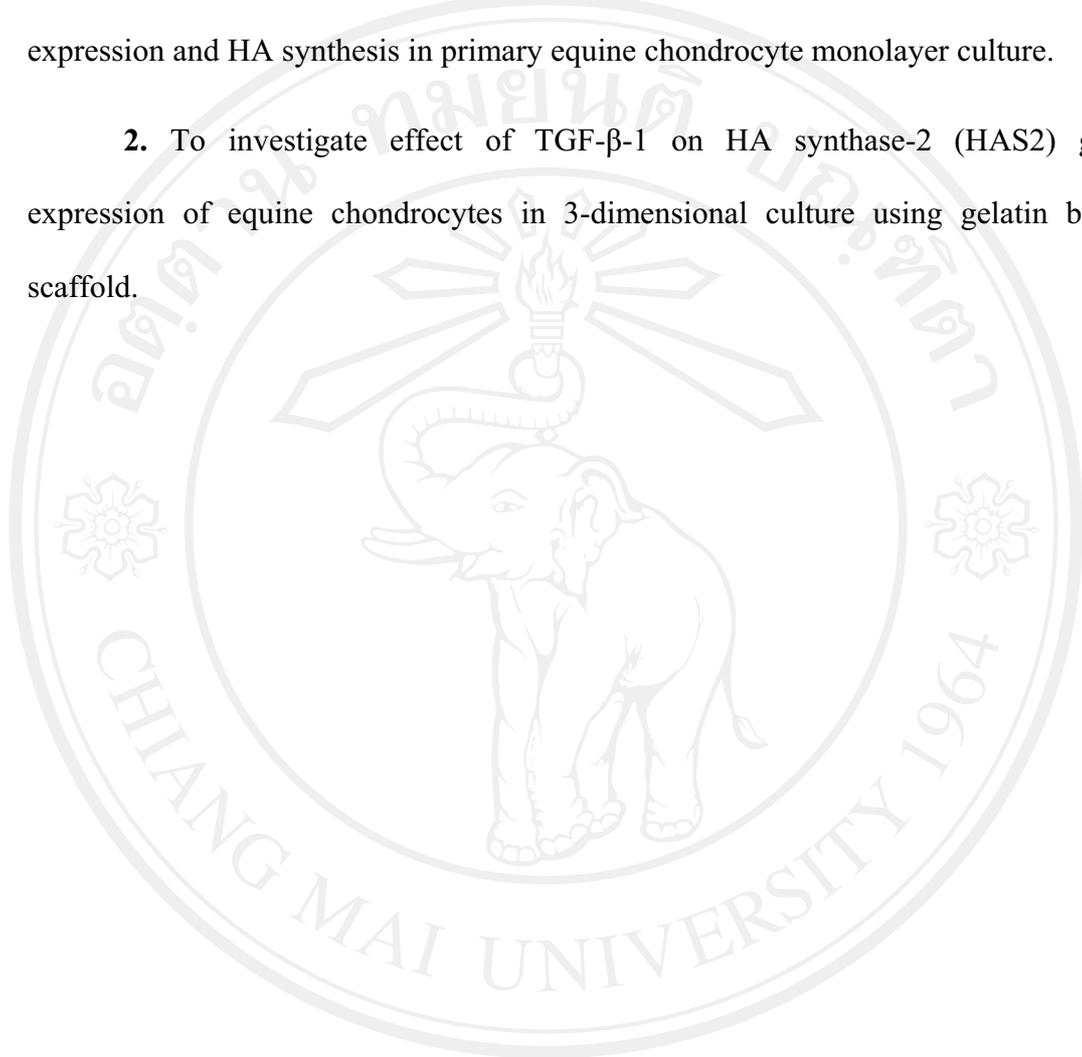
During embryogenesis, the extracellular and pericellular matrix surrounding migrating and proliferating cells is rich in HA (59). In the embryo, HAS2 is widely expressed at high levels throughout gestation, whereas HAS1 and HAS3 have restricted expression at early and later stages, respectively (60). Loss-of-function studies in the mouse established that HAS2-dependent HA synthesis is critical for normal embryonic development (61). Of interest, HAS1 and HAS3 homozygous null animals are viable and fertile. Thus, HAS2 is the major HA synthase during embryogenesis (59).

As a molecule, that closely associates with cells and has the ability to bind to multiple extracellular proteins. HA is an effective support structure in the extracellular matrix, particularly in supporting tissues of vertebrates, including cartilage, skin, smooth muscle, and other connective tissues (50). As a signalling molecule, HA is able to activate several pathways through binding of HA receptors such as CD44 and receptor for HA-mediated motility (62-66).

Among the isoform of TGF- $\beta$ , TGF- $\beta$ 1 is known to be a potent mediator of HA synthesis in fibroblast. We are therefore interest study in the effect of TGF- $\beta$ 1 on HAS2 gene expression and HA synthesis.

### 1.3 Objectives

1. To investigate effect of TGF- $\beta$ -1 on HA synthase-2 (HAS2) gene expression and HA synthesis in primary equine chondrocyte monolayer culture.
2. To investigate effect of TGF- $\beta$ -1 on HA synthase-2 (HAS2) gene expression of equine chondrocytes in 3-dimensional culture using gelatin based scaffold.



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