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Signaling mechanisms regulating tissue inhibitor of metalloproteinases-3 (TIMP-3) gene in chondrocytes

Par

Hamid Yaqoob Qureshi

Département de Sciences Biomédicales

Faculté de médecine

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présentée par:

Hamid Yaqoob Qureshi

a été évaluée par un jury composé des personnes suivantes:

Dr. Jiangping Wu	, president-rapporteur
Dr. Muhammad Zafarullah	, directeur de recherche
Dr. Hassan Fahmi	, member du jury
Dr. Anie Philip	, examinateur externe
Dr. Daniel Lajeunesse	, représentant du doyen de la FES

Thèse acceptée le:

Summary

The balance between matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) is very important in the normal turn over and integrity of the cartilage extracellular matrix. In diseases such as rheumatoid arthritis and osteoarthritis, this balance is altered in favour of MMPs leading to the destruction of cartilage matrix. In cartilage, TIMPs are produced by chondrocytes and are specific inhibitors of MMPs. The balance between TIMPs and MMPs is regulated by cytokines and growth factors. Transforming growth factor beta (TGF-β) induces TIMP-3 in chondrocytes and promotes matrix synthesis. In this study, we have studied different mechanisms involved in TGF-β-induced TIMP-3 gene expression in chondrocytes.

1) In the first study, we have demonstrated the role of extracellular-signal-regulated kinase (ERK)-mitogen-activated protein kinases (MAPK) in TGF-beta-induced TIMP-3 gene expression in chondrocytes and chondrosarcoma cells. TGF-β time-dependently induced phosphorylation of ERK-MAPKs in primary human or bovine chondrocytes. PD98059 and U0126, specific inhibitors of this pathway downregulated the TGF-β-induced expression of TIMP-3 RNA and protein. Additionally, we have investigated the role of Sp1 which is a down stream target of ERK by using mithramycin and WP631 which inhibited the binding of Sp1 to its consensus site resulting in the decreased expression of TIMP-3. Transfection of cytomegalovirus (CMV) promoter-Sp1 plasmid increased human TIMP-3 promoter (-940 to +376)-driven luciferase activity. Transfection of an antisense phosphorothioate oligonucleotide for Sp1 suppressed TGF-beta-induced TIMP-3 protein expression while sense control had no effect.These results show the involvement of ERK-MAPK pathway and Sp1 transcription factor in the induction of TIMP-3 by TGF-beta in chondrocytes.

2) In the second study, we have investigated the role of AKT/protein kinase B signaling pathway for TGF-β-induced expression of TIMP-3 in human articular chondrocytes. TGF-β stimulated the phosphorylation of AKT in a delayed and sustained fashion which correlated with TIMP-3 mRNA induction.

Phosphatidylinositol kinase (PI3K) inhibitors, Wortmannin, LY294002 and AKT inhibitor (NL-71-101) significantly inhibited TGF-β-induced AKT phosphorylation and TIMP-3 expression. PI3K, AKT and Sp1 small interfering RNA (siRNA)-mediated knockdown of target genes significantly reduced the expression of TGF-β-induced expression of TIMP-3. Wortmannin, LY294002, NL-71-101 and siRNA specific for AKT also inhibited the binding of transcription factor Sp1 and TIMP-3 promoter (-940 to +376)-driven luciferase activity. TGF-β induced phosphorylation of p70S6 Kinase and TIMP-3 protein induction, which was inhibited by rapamycin. The study shows the partial involvement of PI3K/AKT pathway and Sp1 transcription factor. Furthermore, TGF-β-induced TIMP-3 gene expression is regulated by a translational mechanism involving mammalian target of rapamycin (mTOR) signaling. TGF-β-induced pro-survival AKT and ERK cascades and TIMP-3 expression can be related to strengthening and maintenence of cartilage extracellular matrix and joint tissue integrity.

3) Finally, the role of Smad pathway in TIMP-3 regulation was investigated. TGF-β stimulated the phosphorylation of Smad2 and TIMP-3 protein expression, which is inhibited by the Smad2 inhibitors, PD169316 and SB203580 and not by their inactive analogue SB202474. The inhibitor of Smad3, SIS-3 and Smad2, Smad3, Smad4 small interfering RNA mediated knockdown of target genes significantly reduced the expression of TGF-β-induced expression of TIMP-3. Transfection of the Smad2 and Smad3 small interfering RNA also inhibited the TIMP-3 promoter (-940 to +376)-driven luciferase activity. Deletion and transfection analysis of TIMP-3 promotor revealed that most of the TGF-β responsive elements were present within the –167 base pairs with respect to the transcription start site. Chromatin Immunoprecipitation (ChIP) analysis confirmed binding of Smad2 and Smad4 with the –940 and –333, whereas Sp1 binds with the –940, -333 and -167 promoter sequences.

Overall this research showed that ERK, AKT and Smad pathways are involved in TGF-β-induced expression of TIMP-3 in chondrocytes and together are

responsible for maintenance, strengthening and promotion of cartilage extracellular matrix synthesis.

Résumé

L'équilibre entre les métalloprotéinases de la matrice (MMPs) et les inhibiteurs tissulaires des métalloprotéinase (TIMPs) est très important pour l'entretien et l'intégrité de la matrice extracellulaire du cartilage. Dans les maladies, telles que l'arthrite rhumatoïde et l'ostéoarthrite, cet équilibre est altéré en faveur des MMPs, menant à la destruction de la matrice du cartilage. Dans le cartilage, les TIMPs sont produits par les chondrocytes; ils sont les inhibiteurs spécifiques des MMPs. L'équilibre entre les TIMPs et les MMPs est régie par les cytokines et les facteurs de croissance. Le facteur de croissance transformant TGF-β induit le TIMP-3 dans les chondrocytes et favorise la synthèse de la matrice. Dans cette étude, nous avons examiné différents mécanismes impliqués dans l'induction de l'expression du gène TIMP-3 par TGF-β, dans les chondrocytes.

1) Dans la première étude, nous avons démontré les rôles d'ERK (extracellular-signal-regulated-kinase), un des MAPK (mitogen-activated-proteine-kinases) dans l'expression du gène TIMP-3, induite par TGF-β, dans les chondrocytes humains. L'induction de la phosphorylation d'ERK-MAPKs par TGF-β, dans les chondrocytes primaires humains ou bovins, est dépendante du temps d'incubation. Les inhibiteurs spécifiques de cette voie, le PD98059 ainsi que le U0126 diminuent l'expression de la protéine et de l'ARN de TIMP-3. Nous avons également examiné le rôle de Sp1, cible en aval d'ERK, en utilisant les inhibiteurs mitramycine et WP 631. Ces deux produits empêchent la liaison de Sp1 à son site de consensus et il en résulte une diminution dans l'expression de TIMP-3. La transfection du promoteur de cytomegalovirus (CMV), attaché avec le gène Sp1, a augmenté l'activité de la luciférase régulée par le fragment promoteur de TIMP-3 (-940 à +376). La transfection d'un oligonucleotide antisense phosphorothioate pour Sp1 a supprimé l'expression de la protéine de TIMP-3 induite par TGF-β, alors que le contrôle '' sense'' n'avait aucun effet. Ces résultats montrent l'implication de la voie ERK-MAPK et celle du facteur de transcription Sp1 dans l'induction de TIMP-3 par TGF-β, dans les chondrocytes.

2) Dans la deuxième étude, nous avons examiné le rôle de la voie de signalisation d'AKT/protéine kinase B dans l'expression de TIMP-3 induite par TGF-β, dans les

chondrocytes articulaires. Le TGF-β a stimulé la phosphorylation d'AKT d'une manière soutenue et retardée, en corrélation avec l'induction d'ARNm de TIMP-3. Les inhibiteurs de phosphatidylinositol kinase (P13K), Wortmannin, LY294002 et l'inhibiteur d'AKT, le NL-71-101, suppriment d'une manière significative la phosphorylation d'AKT, ainsi que l'expression de TIMP-3 induite par TGF-β. L'ARNsi contre P13K, AKT et Sp1 réduisent d'une manière significative l'expression de TIMP-3, induite par TGF-béta. Wortmannin, LY294002, Nl-71-101 et AKT ont également supprimé la liaison du facteur de transcription Sp1 et l'activité de la luciférase actionnée par le promoteur de TIMP-3 (-940 à +376). La phosphorylation de p70S6 et de TIMP-3 induite par TGF-β, a été inhibée par le rapamycin. L'étude montre l'implication partielle de la voie de P13K /AKT ainsi que celle du facteur de transcription Sp1. De plus, l'expression du gène TIMP-3 induite par TGF-β est contrôlée par un mécanisme traductionnel impliquant le cible mammifère de rampamycine (TORm). Les cascades pro-survie AKT et ERK induites par TGF-β, peuvent se lier pour renforcer et maintenir l'intégrité de la matrice extracellulaire du cartilage ainsi que celle du tissu de l'articulation.

3) Finalement, nous avons étudié le rôle de la voie de SMAD dans la régulation de TIMP-3. TGF-β stimule la phosphorylation de SMAD2 et de la protéine TIMP-3, qui sont inhibées par les inhibiteurs de SMAD2, (PD169316 et SB203580) et non par leur analogue inactif, le SB202474. L'inhibiteur de SMAD3, le SIS-3 et l'inhibition des SMAD2, SMAD3, SMAD4 par ARNsi, réduisent d'une manière significative l'expression de TIMP-3 induite par TGF-β. La transfection d'ARNsi de SMAD2 et de SMAD3 a également inhibé l'activité de la luciférase actionnée par le promoteur de TIMP-3 (-940 à +376). La transfection des mutants du promoteur de TIMP-3 a révélé que la plupart des éléments réagissant à l'action de TGF-β étaient présents dans les paires de base -167, en tenant compte du début du site de transfection. Une analyse de l'immunoprécipitation de la chromatine (ChIP) a confirmé la liaison de Smad2 et Smad4 avec les séquences -940 et -333, et de Sp1 avec les séquences -940, -333 et -167 due promoteur.

Dans l'ensemble, ces recherches ont démontré que les voies d'ERK, d'AKT et de SMAD sont impliquées dans l'expression de TIMP-3 induite par TGF-β et ce, dans les chondrocytes . Ensemble, ils sont responsables pour l'entretien, la fortification et la promotion du synthèse de la matrice extracellulaire du cartilage.

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List of abbreviations

ADAM	a disintegrin and metalloproteinase
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motif
aFGF	acidic fibroblast growth factor
ALK	activin receptor-like kinase
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
C/EBP	CAATT/enhancer binding protein
CIA	collagen-induced arthritis
COL1A1	collagen type 1 alpha-1
COL1A2	collagen type 1 alpha-2
COL3A1	collagen type 3 alpha-1
COL6A1	collagen type 6 alpha-1
COL6A3	collagen type 6 alpha-3
COX2	cyclooxygenase-2
cPML	cytoplasmic promyelocytic leukaemia
CS	chondroitin sulfate
ECM	extracellular matrix
ELISA	enzyme linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
FGF	fibroblast growth factor
GAG	glycosaminoglycan

GH	pituitary growth harmone
GPCRs	G-protein-coupled receptors
G-protein	GTP-binding protein
HASM	human airways smooth muscle cells
HC-gp39	human cartilage glycoprotein 39
HMC	human mesangial cells
HRS/HGS	hepatocytes growth factor-regulated tyrosine kinase substrate
IFN-γ	interferon gamma
IGD	interglobular domain
IGF	insulin-like growth factor
IGFBP	IGF-binding protein
iNOS	inducible nitric oxide synthase
IL-1	interleukin-1
IL-1R	interleukin-1 receptor
IL-1Ra	interleukin-1 receptor antagonist
I-Smads	inhibitory Smads
JNK	c-Jun N-terminal kinase
KS	keratan sulfate
LIF	leukemia inhibitory factor
LRP-1	low density lipoprotein receptor-related protein-1
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MAPKK	MAPK kinase

MAPKKK	MAPK kinase kinase
MMPs	matrix metalloproteinases
MSCs	mesenchymal stem cells
MTT	methyl thiazole tetrazolium
NSAIDS	nonsteroidal anti-inflammatory drugs
OA	osteoarthritis
ODN	oligonucleotide
OSM	oncostatin M
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet-derived growth factor
RA	rheumatoid arthritis
RASFs	rheumatoid arthritis synovial fibroblasts
RISC	RNA induced silencing protein complex
ROS	reactive oxygen species
RNAi	RNA interference
SARA	Smad anchor for receptor activation
SBE	Smad binding elements
Sp1	specificity protein 1
siRNA	small interfering RNA
SF	synovial fluid
TAB	TAK binding protein
TACE	TNF-α converting enzyme
TAK	TGF-β-activated kinase

TGF-β	transforming growth factor beta
TIMPs	tissue inhibitors of metalloproteinases
TNF-α	tumor necrosis factor alpha
TNF-R	tumor necrosis factor alpha receptor

Dedications

I Dedicate this humble effort, the fruit of my thoughts and study to my affectionate parents, who inspired me to the higher ideals of life.

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1. Introduction

1.1 Facts about arthritis in Canada

According to Canadian health statistics (1)

- Approximately four million Canadian adults over the age of 15 years have arthritis. Two-third of them are women.
- Three out of five people with the disease are of working age (under 65).
- In comparison with the people having other chronic conditions, people with arthritis experiences moderate to severe pain, restricted activity in their daily life and long-term disability. These people suffer from sleep disorders and depression, and frequently visit the health care professionals.
- Musculoskeletal disorders including arthritis cost 16.4 billion dollars in Canada.
- The estimated cost of arthritis is 4.4 billion dollars annually.
- Long-term disability accounts for 80 per cent of arthritis-related costs and 70 per cent of these costs is for the individuals between the ages of 35-64.
- The musculoskeletal disorders accounted for 10.3 per cent of the total economic burden of all illnesses, but receive only 1.3 per cent of health science research funding.

1.2 Osteoarthritis

Osteoarthritis (OA) is the most common form of arthritis and affects 10% of the population especially the elderly. OA generally leads to disabilities due to its common occurence in knee and hip joints. It limits the daily activity of the patient and is a major cause of hip replacement surgery. OA is influenced by many factors such as gender and genetic factors including inherited disorders of type II collagen (COL2A1) mutations and ethnicity; non-genetic factors are, aging, obesity, depletion of sex hormones in female and previous surgery. Environmental factors include the nature of daily work, leisure and sports activities (2). In OA the whole joint is involved in the disease and there is a gradual loss of the cartilage, bone underneath the cartilage, osteophyte formation, increased thickness of the subchondral bone and bridging muscle around the joint becomes weak. The synovium become relatively thick with an increase number of lining cells and an infiltrate of macrophages. Proinflamatory cytokines such as IL-1β and TNF-α secreted by the synovial macrophages are the key factors responsible for OA. IL-1β and TNF-α affects the other matrix degrading enzymes, such as metalloproteinases 1 and 3 produced by synovial fibroblast cells (3). Other cytokines like IL-17 and IL-18 are also responsible for the induction of MMP-3 and inducible nitric oxide synthase (iNOS) in chondrocytes (4,5). The cytokines and metalloproteinase alter the metabolism in such a way that leads to the destruction of cartilage and joints.

1.3 Rheumatoid arthritis

Rheumatoid arthritis (RA) is common throughout the world and approximately 1% of the population suffers from RA. RA is an autoimmune disease of synovial joints, with chronic inflammation leading to the destruction of cartilage and bone. RA risk is associated with major histocompatibility complex class II. Type II collagen and human cartilage glycoprotein 39 (HC-gp39) are the target proteins of autoimmunity (6). RA is characterized by the excessive growth of synovial membrane leading to the formation of pannus tissue and increased infilteration of inflammatory cells. Cytokines, growth factors, chemokines and metalloproteinases are involved in the development of disease (7). T cells play an important role in the inflammation of RA. The activated T cell can induce IL-1, TNF-α and metalloproteinases, which are responsible for the degradation of collagen and proteoglycan in extracellular matrix (6).

The potential targets for therapies are TNF-α and IL-1. A number of anti-TNF-α drugs are available in the market. The most important drugs, which are in use in US and Europe for RA treatment, are TNF-α antibody (infliximab Remicade^{TM}) and TNF-receptor-immunoglobulin fusion protein (etanercept, Enbrel^{TM}). These inhibitors relieve symptoms of pain and swelling of joints (8). A new drug Arcoxia, a chemical relative of Vioxx from Merck & Co is available in the market. Nonsteroidal anti-inflammatory drugs (NSAIDS) like cyclooxygenase-2-specific (COX-2) inhibitors block an enzyme cyclooxygenase that is involved in prostaglandin synthesis leading to arthritic pain. Celebrex is another COX-2

inhibitor available in market. Some of these inhibitors have cardiovascular side effects. Merck has withdrawn Vioxx from the market voluntarily due to claim by the people that their loved ones have suffered from heart attack due to the use of Vioxx.

1.4 Chondrocytes and their functions

Chondroblasts and chondrocytes originate from mesenchymal chondroprogenitor cells during the development of embryo. Morphologically, these cells are spindle-shaped, having large, spherical nuclei and small amount of cytoplasm. The mesenchymal cells have the potential to develop into cartilage both in vivo (9) and in cell culture (10), and with advancing cellular aggregation start to show the presence of cartilage transcription factor, Sox9, which is a marker of chondrocytes. Type II collagen mRNA level also increases and the level of mRNA of the core protein of proteoglycan also becomes detectable (11).

Chondrocytes represent the cell type present in the hyaline cartilage. They are responsible for the synthesis of extracellular matrix during development and growth and maintain tissue homeostasis. They produce cytokines, growth factors and proteases, which are responsible for the normal turnover of the tissue. An upset in the balance of these factors, particularly an increase in proteinases results in complications and development of arthritis (4).

1.5 Extracellular Matrix (ECM)

The extracellular matrix is composed of 65 to 80% water. Water content is maximum in superficial zone and decreases progressively with increase in depth. Collagen II accounts for 15 to 25% of the wet weight and about half the dry weight except in the superficial zone where it accounts for most of the dry weight and its concentration is progressively reduced with increasing depth from the articular surface. The proteoglycan aggrecan content constitute up to 30% of the dry weight of proteins. Aggrecan degradation products, which accumulate with age consist mainly of the G1 globular domain and G1, G2 along with keratan sulfate-rich region account for a significant part of the remaining dry weight (12). The fibrous structure is mainly composed of type II collagen made up of tropocollagen molecules having a triple helix of three identical α chains, with non-helical amino and carboxyl-terminal domains. These collagen molecules are linked through hydroxypyridinium cross-links (13). These cross-links strengthen the collagen and render it more resistant to proteolysis. The turnover of collagen in healthy human articular cartilage is probably very low as it has the half life of over 100 years (14). However in pericellular region there is some evidence for ongoing type II collagen cleavage (15). Type IX collagen is bound with type II collagen by cross-linking with its N-terminal and C-terminal nonhelical domain in an antiparallel manner (16,17) and distributed periodically at a distance of 67nm. Its N-terminal globular NC4 domain extends out of the fibril and is basic in nature, so it might act as a binding site for glycosaminoglycans such as hyaluronic acid and chondroitin

sulfate. Type XI is also found within type II collagen and restrict the lateral growth of type II collagen fibrils (18). Type VI collagen is a shorter chain collagen that consist of α chains having no cross-links with other matrix molecules. They form filamentous network in connective tissues. Type VI collagen is found throughout the cartilage matrix but mainly present in pericellular matrix. (19). Type XII and XIV collagens are structurally similar to type IX collagen and are found in skin and cartilage. Type XII is involved in early chondrocytes development (20).

The proteoglycan is another important structure of cartilage extracellular matrix and binds to hyaluronic acid and is known as aggrecan. The core protein of aggrecan consists of three globular domains, G1, G2 and G3, and the region between G2 and G3 is attributed for the glycosaminoglycan attachment. Aggrecan binds to the hyaluronic acid through its N-terminal G1 globular domain. The function of G2 domain is not clear. The link protein binds directly to the G1 domain of aggrecan and to hyaluronan and stabilizes the binding between aggrecan and hyaluronan. The region adjacent to the G2 domain towards the G3 domain contains 30 keratan sulfate chains. Each disaccharide contains at least one sulfate group. After this is the chondroitin sulfate attachment region, and approximately 100 chondroitin sulfate chains are present in this region. Each disaccharide of chondroitin sulfate contains both carboxyl and sulfate groups. So aggrecans are highly negatively charged and are responsible for the high degree of hydration of proteoglycan. The ability of hydration and swelling is restricted by the collagen fibrillar network, which leads to the development of swelling pressure which

contributes to its compressive stiffness and ability to resist deformation under mechanical load (21, 12). The C-terminal G3 domain prevents the core protein from degradation and is responsible for processing and interacellular trafficking of the protein (22).

Apart from this, there are lot of other molecules such as versican, structurally similar to aggrecan and present at a low level in the interterritorial region (23). Decorin, biglycan and fibromodulin are smaller proteoglycans with fewer glycosaminoglycan side chains, and are thought to play some role in the organization of matrix. There are also some noncollagenous proteins with a smaller amount of oligosaccharides, such as anchorin CII, cartilage oligomeric protein, fibronectin and tenascin, all these molecules play role in stabilizing matrix and chondrocytes-matrix interaction (24). Remodeling of the cartilage ECM during health and disease conditions takes place by a family of enzymes to be discussed in the next section.

1.6 Matrix metalloproteinases (MMPs), aggrecanases and TACE

The matrix metalloproteinases consist of a large family of proteolytic enzymes and their over activity plays an important role in tissue destruction in RA, OA and in many other diseases (25). They are produced in inactive proenzyme form, in which the N-terminal cysteine residue binds with the Zn atom in the active site. The removal of a 10 kDa N-terminal fragment leads to the activation of enzyme (26). Some active MMPs are also responsible for the activation of other Pro-MMPs, suggesting a carefully controlled activation cascade in vivo involving different members of the MMP family. The membrane-type MMPs have a conserved sequence of 10-20 amino acids between the propeptide and the N-terminal domain recognized by the furin family of serine proteinases (27). These enzymes are activated within the Golgi apparatus and arrive at the cell surface in an active form and are thought to be responsible for the activation of other MMPs. MT1-MMP and MT2-MMP activate proteolytically proMMP-2 at the cell surface (28). Plasmin also activates some Pro-MMPs (29). Other mechanisms may involve the activation of Pro-MMPs by reactive oxygen species released from inflammatory cells (30).

Many types of cells, such as chondrocytes, synovial cells and polymorphonuclear leukocytes, produce MMPs. Their synthesis is influenced by many factors such as cytokines, growth factors, cellular transformation and physical stimuli (31). Some of the MMPs take active part in the degradation of aggrecan and collagen in cartilage. Collagenases (MMP-1, MMP-8 and MMP-13), gelatinase A (MMP-2)

and gelatinase B (MMP-9), stromelysin 1 (MMP-3), matrilysin 1 (MMP-7) and membrane-type MT1-MMP (MMP-14) are found in cartilage and showed elevated levels in the synovium and in the cartilage from patients suffering from RA and OA (32, 33). All of these MMPs cleave the aggrecan core protein at various sites, but the critical site is the Asn341–Phe342 bond located in the interglobular domain located between the two globular domains G1 and G2, resulting in the release of aggrecan molecules from the cartilage (34). On the other hand, Sandy *et al.* (35) reported the cleavage of core protein at the Glu373–Ala374 bond instead of Asn341–Phe342 bond, when bovine cartilage explants in culture were treated with IL-1. The products resulting from this cleavage accumulate in the synovial fluids of patients with OA (36). The enzymes responsible for this cleavage have been purified and cloned. They are referred to as aggrecanase 1 and aggrecanase 2 (also ADAMTS-4 and ADAMTS-5, members of the ADAM protein family, respectively) (37, 38). The degradation of type II collagen occurs slower than aggrecan degradations in arthritis. This is all due to the action of MMPs. Potential collagenolytic enzymes are MMP-1, MMP-2, MMP-8, MMP-13 and MMP-14. Some MMPs also act on non-matrix proteins substrate resulting in their activation, such as the activation of insulin-like growth factor-binding protein (39), cleavage of the Fas ligand by MMP-7 resulting in soluble Fas important in apoptosis (40) and cleavage of laminin-5 by MT1-MMP resulting in the migration of variety of cells (41). MMP activity also regulates cell attachment and migration by cleaving cell-cell and cell-matrix receptors. Examples are the cleavage of β4 integrin by

MMP-7 (42) and removal of E-cadherin by MMPs (43). TNF-alpha converting enzyme (TACE) is responsible for the proteolytic activation of TNF-α (44), which inhibits the synthesis of link protein and type II collagen, and is a major factor responsible for the onset of arthritis (45).

1.7 Tissue inhibitors of metalloproteinases (TIMPs) and arthritis

MMPs can be inhibited by tissue inhibitors of metalloproteinases, which bind to all known active MMPs with a 1:1 stoichiometry. All connective tissues produce members of the TIMP family (46) and these molecules play an important role in controlling connective tissue breakdown by blocking the action of active MMPs. Four members of the TIMP family have been described and these share similar secondary and tertiary structures, and are able to inhibit MMPs, although with different potencies. Dean *et al* (47) reported higher levels of TIMP and MMP in OA cartilage. Although the level of MMP was only slightly higher as compared to TIMP in normal cartilage, this difference in ratio is responsible for the cartilage matrix degradation. TIMPs consist of a N-terminal inhibitory domain and a C-terminal regulatory domain. TIMPs blocks MMP activities by interacting with the zinc-binding region in the catalytic domain of MMPs.

TIMP-1 is produced in response to a variety of external stimuli, such as growth factors and cytokines, whereas expression of TIMP-2 is mostly constitutive (48). TIMP-1 inhibits aggrecanase-1 (49) and MMPs, and inhibitory activity resides between the cysteine-3 and cysteine-13 in the N-terminal domain (50). The TIMP-

1 level was reported higher in synovial fluids (51) and in serum (52, 53) of RA patients, but not in the sera of OA patients (54). TIMP-1 overexpression by adenovirus-based gene delivery reduced destruction of the joints of TNF-α transgenic mice (55). On the other hand the overexpression of TIMP-1 did not prevent osteochondral injury in the mouse model of collagen-induced arthritis (56). TIMP-2 interaction with MMP-2 is strong as compared to MMP-9. The C-terminal domain of TIMP-2 binds with the C-terminal domain of progelatinase A (proMMP-2) on cell surface to activate proMMP-2 by membrane-bound membrane type 1 matrix metalloproteinase (MT1-MMP) (57). TIMP-3 is different from other TIMPs in many ways. TIMP-1 and TIMP-2 are in soluble forms, where as TIMP-3 is secreted and binds to the ECM. TIMP-3 protein binds to the ECM components, heparan sulphate and chondroitin sulphate through the N-terminal domain of human TIMP-3 rich in lysine and arginine (58). Mutations in the TIMP-3 gene are implicated in a human disease called Sorby's fundus dystrophy, a degenerative eye disease (59). TIMP-3 has also shown proapoptotic role in both normal and cancer cell lines (60, 61). TIMP-3-deficient mice developed alveolar air space enlargement resulting in impaired capacity for gas exchange (62), increased mammary gland apoptosis (63), abnormal cardiac function (64), and increased susceptibility to hepatic inflammation (65). TIMP-3 knockout mice have shown increased aggrecan and collagen degradation with aging, in a manner similar to OA (66).

The above findings suggest that TIMP-3 plays an important roles in physiological and pathological processes. The special characteristics of TIMP-3 invite focus of studies in its biological role. TIMP-3 is known to inhibits MMP-3, collagenase-3, tumor necrosis factor-α converting enzyme (ADAM-17), aggrecanase-1 (ADAMTS-4) and aggrecanase-2 (ADAMTS-5) in vivo. This is also based on experiments where TIMP-3 null mice have shown increased inflammatory response on intra-articular injection of methylated bovine serum albumin as compared to wild type mice (67). The loss of aggrecan is the initial and key step in the pathogenesis of arthritis (68). Therefore, TIMP-3 might play a key preventive role in arthritis development.

1.8 Growth factors and Arthritis

Cytokines and growth factors can be divided into two groups, anti-inflammatory and pro-inflammatory, exerting anabolic or catabolic effects respectively on cartilage in arthritis. The cytokines and growth factors that affect the matrix synthesis and degradation are shown in Figure A. The growth factors oppose the effects of the pro-inflammatory counterparts and promote the synthesis of matrix and repair, induce the protective enzyme inhibitors such as TIMPs, down regulate the destructive enzymes and reduce the levels of pro-inflammatory cytokines (69).

TGF-β is well-known for its chondroprotective effects and for its role in the promotion of matrix synthesis. Cartilage contains vast stores of TGF-β, up to 300–500 ng /g of cartilage mass, which is mostly inactive and requires proteolytic activation. The presence of such reserves strongly indicates the role of TGF-β in

cartilage metabolism (70). There are three isoforms of TGF-β (TGF-β 1, 2, and 3) in mammals, all of them are expressed in articular chondrocytes, (71) as well as in other cells. Basic fibroblast growth factor (bFGF) also stimulates the production of TGFβ (72). TGFβ and bFGF exhibit synergism, resulting in up to a 73-fold increase in chondrocyte replication (73). TGF-β stimulates differentiation of mesenchymal stem cells to chondrogenic phenotype in vitro, and these cells start to express type II collagen, aggrecan, fibromodulin and cartilage oligomeric protein (74). TGF-β counteracts the effects of IL-1-mediated destructive effects in chondrocytes in vitro (75), and in mice with induced inflammatory arthritis (76). This effect may be due to its ability to inhibit IL-1 receptor expression (77). The transgenic mice with blocked TGF-β signal, either due to inactive TGF-β type II receptor (78) or due to interference in intracellular signaling by Smad3 (79), has shown the development of degenerative joint disease similar to OA in humans. These observations suggest that impaired TGF-β function could be a possible cause of OA. On the other hand, it has also been reported that TGF-β induces more PG synthesis in human OA cartilage as compared to normal cartilage in vitro (80). TGF-β levels are relatively elevated in the synovial fluid of OA and rheumatoid arthritis patients (81). Excessive injections of TGF-β into mouse knee joints leads to the osteophytes formation on the articular surface and cartilage lesions due to PG depletion as in OA (82), which indicates that excessive TGF-β activity is responsible for some of the pathological changes in OA.

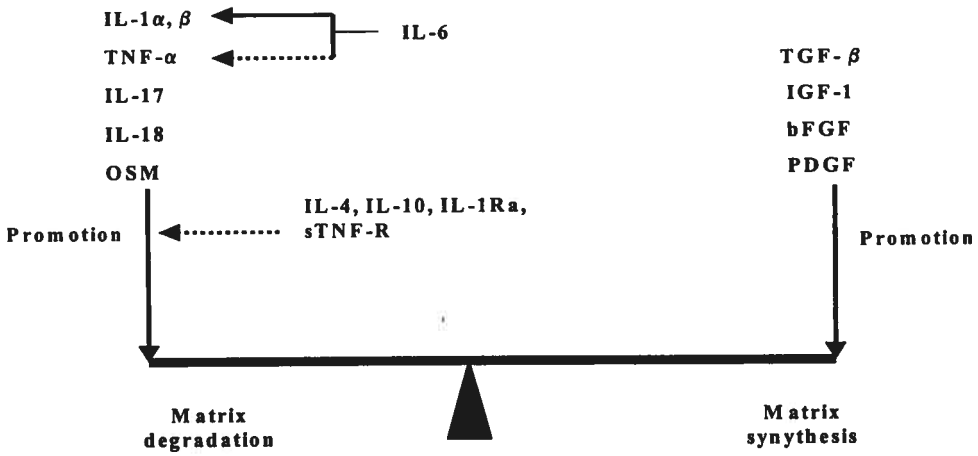

Figure A. Cytokines and growth factors effecting cartilage matrix degradation or synthesis. TGF-β, transforming growth factor beta; IGF-1, insulin like growth factor 1; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; IL, interleukin; IL-1Ra, interleukin-1 receptor antagonist; sTNF-R, soluble tumor necrosis factor-α receptor; TNF-α, tumor necrosis factor-α; OSM, oncostatin M; ——, stimulatory effect, ·····, inhibitory effect.

IGF-1 play anabolic role in matrix production (83) and inhibits the activity of IL-1, especially the degradation of proteoglycan by IL-1. The inhibition of IL-1 activity by IGF-1 is of potential importance in decreasing cartilage degradation and the progression of OA (84). IGF-1 and TGF-β have shown synergistic effect in inducing type II collagen and aggrecan proteins in adult articular chondrocytes (85). IGF-I expression is significantly higher in OA cartilage (86), which also has increased number of IGF receptors (87) even then the cells did not show full response to exogenous IGF-I (88). The loss of IGF-1 activity and effects in OA might be due to upregulation of IGF-binding proteins (IGFBPs) (89). IGF-1 is sequestered from its cell-surface receptor by IGFBPs and fibronectin, affecting the bioavailability of IGF-1. IGFBP-3 expression increases with advancing age and affects matrix synthesis in inverse proportion. These proteins are also overexpressed in OA cartilage and cause metabolic disturbances (90). The rats deficient in pituitary growth hormone (GH) and IGF-1 has shown OA like lesions as compared to the normal rats. The decrease of the level of the GH and IGF-1 with advancing age may be the contributory cause of OA (91).

FGF-2 along with IGF-1 synergistically enhanced the proliferation of human articular chondrocytes 7.5- fold in two weeks as compared to their individual effect (92). FGF-2 has a very important role in modulating cartilage repair. In an intrinsic damage-repair response in articular cartilage explant treated with FGF-2, the repaired chondrocytes have shown increased expression of type II, IX, XI collagen but not type I collagen (93). IGF-1, TGF-β, FGF-I, II, and platelet derived growth

factor (PDGF) all have shown to augment chondrocyte proliferation in vitro (94). The intra-articular administration of bFGF in the form of gelatin hydrogel increased the synthesis of ptoteoglycan core protein and had shown therapeutic effect on OA in rabbit knee joint (95). However the higher concentration of bFGF inhibit the anabolic effect of IGF-1 by decreasing the proteoglycan synthesis in chondrocytes (96). The synoviocytes of RA patient produced more acidic fibroblast growth factor, which may be responsible for angiogenesis and fibroblast proliferation (97).

The bone morphogenetic protein BMP family consists of 13 proteins and all belong to the TGF-β superfamily except BMP-1 (98). BMPs promote chondrogenesis. BMP-2 (99), BMP-3, BMP-4 (100) and BMP-7 (101) stimulate the PG synthesis in vitro in articular cartilage. BMP-7 also counters the effects of the IL-1-induced downregulation of PG biosynthesis of articular chondrocytes in vitro (102).

1.9 Cytokines and Arthritis

Proinflammatory cytokines include the TNFs, ILs, and colony-stimulating factors. TNF-α and IL-1β are key cytokines responsible for the pathology of OA (103). They also affect the activity of other cytokines, e.g., IL-6, IL-8, and leukemia inhibitory factor (LIF), which are synthesized in increased amounts upon stimulation of chondrocytes with TNF-α or IL-1β (104). IL-1β and TNF-α also upregulate COX-2 expression in articular tissue, which is responsible for elevated

prostaglandin E2 (PGE2) synthesis, responsible for inflammation in arthritis (105). IL-1 is the important catabolic cytokine produced by the chondrocytes and its level is upregulated in the synovial fluid of RA, OA and cartilage tissue (106). IL-1 upregulates matrix degrading enzymes, MMP-1, MMP-3, MMP-13 and ADAMTS-4 and decreases the amount of mRNA for link protein in the articular chondrocytes (107). IL-1 downregulates proteoglycans synthesis as well as type II collagen (108). TNF-α also downregulates the synthesis of proteoglycans and type II collagen and shows synergistic effect with IL-1 on the inhibition of matrix synthesis (109). IL-1 induced the production of nitric oxide (NO), which inhibits proteoglycan synthesis and may be the primary mediator of inhibition of matrix synthesis (110). IL-17 is a proinflamatory cytokine that increases the synthesis of inducible nitric oxide synthase (iNOS), which is responsible for the generation of NO causing cytotoxic response in RA. IL-18 induces MMP-1, MMP-3, MMP-13 and aggrecanase-2 in a monolayer culture of human articular chondrocytes and seems to have some roles in degradation of cartilage in arthritis. IL-17 and TNF-α exert synergistic effects in degrading cartilage and also inhibit the synthesis of proteoglycan in vito. IL-17 and IL-18 induce IL-1β, TNF-α, MMP-3 and iNOS in human articular chondrocytes (111,112,113).

The interaction of cytokines and their role in the metabolism of chondrocytes is very complex. The exact roles of inflammatory cytokines other than IL-1β and TNF-α in OA are not very clear. Studies in animal models are under way to further reveal the pathogenesis of OA. Uptill now, it is established that the cartilage

destructive process in OA is mainly IL-1 driven, whereas TNF-α is involved in the onset of RA, and that direct generation of IL-1 may also occur in the complete absence of TNF-α (114). OA cartilage shows strong intra- and extracellular presence of IL-1β in the superficial zone by immunohistochemistry (115). The biological activity of IL-1β in OA tissue is also increased due to an increase in the number of IL-1 type 1 receptors on chondrocytes (116), decrease in the synthesis of the natural receptor antagonist IL-1Ra (117), and an increase in IL-1β-converting enzyme responsible for the proteolytic activation of IL-1β (118).

Interferon-γ inhibits the synthesis of type II collagen (119), however the role of interferon-γ is dual. Interferon-γ inhibits the IL-1β-induced MMP-13 in chondrocytes (120). Interferon-γ also downregulates the expression of IL-17 and confers resistance to collagen induced arthritis (CIA) in mice (121).

Cytokines like IL-4, IL-10, and IL-13 are classified as anti-inflammatory, because they reduce the activity of the proinflammatory cytokines in vitro (122). IL-4 induces TIMP-1 and inhibits MMP-3 in human chondrocytes (123). It has been shown that IL-10 upregulates TIMP-1 while downregulates MMP-1 and MMP-3 in human monocytes (124). IL-13 reduces the production of MMP-1 stimulated by IL-1 or IL-17 in cultured RA synoviocytes (125).

Recent studies indicate that some of the cytokines considered to be anti-inflammatory might not always be so. IL-6 cytokine family members, including IL-6 and oncostatin M (OSM), induce TIMP production in chondrocytes and fibroblasts as well as increase matrix synthesis, and are considered as

chondroprotective. However, in combination with IL-1, these cytokines induces a marked catabolic effect by promoting matrix degradation (126). Our laboratory has shown that OSM induces MMPs, aggrecanase and TIMP-3 (127,128). Overall, a balance between proinflammatory and antiinflammatory cytokines is necessary for maintaining cartilage integrity.

1.10 TGF-β Signaling

TGF-β and related growth factors play a prominent role in the development, homeostasis and repair of virtually all tissues in organisms, from fruitfly to human. Collectively, these factors account for a substantial portion of the intracellular signals governing cell fate. The first member of TGF-β superfamily was discovered 20 years ago. Since then the family has grown, and it consists of over thirty members of structurally and functionally related proteins (129).

TGF-β family signals via a transmembrane receptors complex that contains intracellular serine-threonine kinase domains. This serine-threonine kinase receptor complex consists of two different types of transmembrane proteins, known as type I and type II receptors. The binding of TGF-β to its receptor leads to the association of type I and type II receptors. Type II receptor then phosphorylates type I receptor, leading to the activation of its kinase domain. Type II receptors phosphorylate themselves and type I receptors on serine and threonine residues but not tyrosine residues (130). There are seven different types of type I receptors, termed ALK1-7 (activin receptor-like kinase) and five type II receptors in mammalian cells. TGF-β generally uses ALK5 and TGF-β type II receptor in most of the cell types (131). There are also two additional receptors, TGF-β type III and TGF-β type V. TGF-β type III receptor is a proteoglycan, also known as betaglycan and acts as a co-receptor by presenting ligand to TGF-β type II receptor. The cytoplasmic domain of TGF-β type III receptor binds with the autophosphorylated type II receptor and promotes the complex formation between

type II and type I receptors, and then dissociates from this activated signaling complex. (132). TGF-β type V receptor, also known as low density lipoprotein receptor-related protein-1 (LRP-1) and IGFBP-3 is a TGF-β and IGFBP-3 receptor and is involved in growth inhibitory response to TGF-β (133). Glycosylphosphatidylinositol (GPI)- anchored proteins have been reported to regulate TGF-β signaling in certain cell lines. These proteins can bind with the TGF-β type I and II receptors to form heteromeric complex. Mutant human keratinocytes (HaCaT) cells deficient in GPI protein biosynthesis have shown increased Smad2 and Smad3 activation in response to TGF-β treatment, and thus GPI-anchored proteins are responsible for inhibiting TGF-β signaling (134). A variant of TGF-β type II receptor-B (TβRII-B), which contains a 26-amino acids insert instead of Val32 of the type II receptor, is expressed in normal and osteoarthritic human chondrocytes. TβRII-B can combine with TGF-β type I, II, betaglycan and endoglin leading to modulation of TGF-β signaling. Overexpression of TβRII-B in chondrocytes has shown enhanced TGF-β responses in chondrocytes (135).

Once the type I receptor is activated, it transmits signal to the SMADs which are intracellular mediators. Type I receptor phosphorylates R-Smad on the serine residues in the C-terminal region, Ser-Ser-X-Ser motif. Smad anchor for receptor activation (SARA) and hepatocytes growth factor-regulated tyrosine kinase substrate (HRS/HGS) both facilitate the phosphorylation of R-Smads by type I receptor. These proteins contain the phospholipid binding FYVE domain, which

helps in recruitment of Smad2 and Smad3 to the TGF-β receptor complex. Cytoplasmic promyelocytic leukaemia (cPML) is an adapter protein that promotes ALK5 mediated R-Smad phosphorylation (136). Three of type I receptors are responsible for the phosphorylation of R-Smads, Smad2 and Smad 3, and mediate TGF-β-like signal, whereas other four receptors activate R-Smads, Smad1, Smad5 and Smad8 to transduce signals initiated by bone morphogenetic proteins. Upon phosphorylation, these R-Smads dissociate from the receptor/SARA complex and combine with Co-Smad, Smad4 to form a heteromeric complex and translocate to the nucleus. In the nucleus, this complex associates with one of various transcriptional coactivators or corepressors, and interact with Smad binding elements (SBE) or GC rich sequences in the promoter region of various genes, thereby positively or negatively regulating gene expression. Direct binding of Smads with DNA is very weak and their interaction depends on various DNA binding proteins to target specific genes for transcription. TGF-β-regulated Smad2 and Smad3 directly bind to DNA-binding partners, such as FoxH1 (FAST), AP-1, TFE3, Sp1, Mixer, Runx2, and Miz1, whereas the BMP-regulated Smads bind to OAZ, Runx2 and Hoxc-8/9. After binding to their target promoters, Smads can regulate transcriptional activity positively or negatively by recruiting coactivators, such as CBP/p300, or corepressors, such as TGIF and Ski/Sno (137). A schematic diagram of smad signaling is shown in figure B.

In human skin fibroblasts, TGF-β induction of COL1A2 is mediated by Smad3 binding to the COL1A2 promoter (138), whereas Smad3 and Smad4 are important

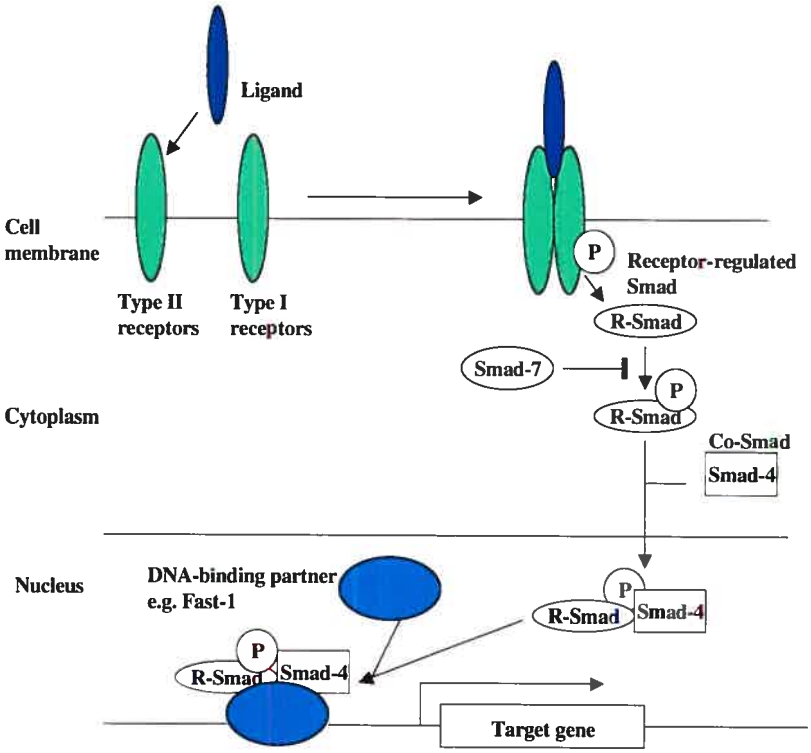

Figure B. The transforming growth factor β (TGF-β)/SMAD pathway. Binding of TGF-β to the type II receptor bring the type II receptor close to type I receptor leading to the phosphorylation of type I receptor. The activated type I receptor subsequently phosphorylates a receptor-regulated SMAD (R-Smad) allowing this protein to associate with Smad4 and move into the nucleus. In the nucleus, the SMAD complex associates with a DNA-binding partner, such as Fast-1 and this complex binds to specific enhancers in targets genes activating transcription.

in COL7A1 gene expression induced by TGF-β in human dermal fibroblast (139). The cooperation between Smads and Sp1 has been reported in the upregulation of PAI-1 and COL1A2 in response to TGF-β stimulation (140,141). In mammalian epithelial cells, Smads 2 and 3 mediate growth inhibition and transcriptional activation of TGF-β and activin reporter genes (142,143).

Another class of Smads known as inhibitory Smads (I-Smads), Smad6 and Smad7 lack the C-terminal sites for phosphorylation by the type I receptor and antagonize TGF-β signaling. Smad6 and Smad7 negatively regulate the TGF-*β* signaling via distinct mechanisms. Smad6 inhibits only the BMP pathway whereas Smad7 inhibits TGF-*β*/activin and BMP pathways. Smad6 competes with Smad4 for binding with phosphorylated Smad1 forming Smad1/6 complex, which is unable to regulate transcription (144). Smad7 competes with R-Smads and binds with type I receptor (145). Smad7 binds with the Smurf2, a nuclear protein, and causes its export and recruitment to the TGF-*β* receptor causing latter's degradation (146).

In certain mammalian cells, the transfected TAK1 expression is increased on stimulation with TGF-β and BMP4. Overexpressing the kinase-defective TAK1 mutant (147) or the truncated form of the TAK1 activator, TAB1, abolished the TGF-β response of a reporter gene construct that contains an AP-1 site, suggesting the involvement of TAK1 (148). TGF-β activation of the MAP-kinase JNK, is also involved in a similar transcriptional response and is downstream of TAK1 (149).

However, the JNK-kinase response to TGF-β is in a delayed manner, indicating that JNK is not a primary transducer of TGF-β signals in these cells.

TGF-β also signals through mitogen-activated protein kinases (MAPKs), which transduce external signals into the cell, resulting in a wide range of cellular responses, including growth, differentiation and development (150). There are three different types of MAPKs. In our study, we have found the involvement of extracellular signal-regulated kinases (ERKs). ERK1 and ERK2 are isoforms and collectively referred to as ERK1/2. ERK1/2 are activated by MEK1/2, which are in turn activated by MAPKKK mediated phosphorylation. MEK1/2 phosphorylates threonine and tyrosine residues in the kinase domain of ERK1/2 leading to the activation of ERK1/2. The activated ERK1/2 then phosphorylates many transcription factors, such as C-Myc, Elk1 and protein kinases resulting in the induction of many genes (151). The detail of MAPKs pathways is given in section 1.11.

Phosphatidylinositol 3-kinases (PI3K) pathway is also involved in TGF-β signaling and plays important role in cell physiology. PI3K pathway is activated by the receptors tyrosine kinase and Ras, which in turn generates lipid second messenger phosphatidyl inositol-3,4,5-triphosphate (PtdIns(3,4,5)P3). The PtdIns (3,4,5)P3 phosphorylates AKT/PKB. The activated AKT phosphorylates downstream a number of proteins resulting in the induction or repression of target genes (158,159,160,161). The detail of PI3Ks pathway is given in section 1.12.

1.11 MAPK signaling

MAPKs are activated due to threonine and tyrosine phosphorylation within the activation loop of the kinase domain. MAPKs are activated by a family of dual-specificity kinases, known as MAPK kinases or MAPK/ERK kinases (MAP2Ks/MKKs or MEKs). MAP2Ks, in turn, are activated by Ser/Thr phosphorylation catalyzed by any of several protein kinase families collectively known as MAPK kinase kinases (MAP3Ks). So far, three major groups of MAPKs have been found in mammals: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs) and the p38 kinases. The JNKs and p38 kinases are also known as stress-activated protein kinases (SAPKs).

The ERKs are very important members within the MAPKs. They consist of ERK1/2, ERK3/4 and ERK5/ BMK and new members, ERK7 and ERK8 (151, 152, 153). ERK1 and ERK2 are the best characterized ERKs and are co-ordinately regulated. During inflammation, the ERK1/2 pathway is triggerd by some cytokine receptors, such as the interleukin-6 (IL-6) receptor, the T-cell receptor, and the B-cell receptor. Enhanced ERK1/2 activity results in the activation of many transcription factors, e.g., the activator protein-1 (AP-1) components, c-Fos and c-Jun (151, 154).

The JNK and p38 kinase pathways are activated by environmental stress and inflammatory cytokines (155). The JNK family of MAPKs consists of JNK1 and JNK2 that are expressed ubiquitously, and JNK3 is mainly expressed in the heart, brain and testis (156). Inflammatory cytokines, such as TNF-α and IL-1, strongly

activate JNKs (151). JNKs play a major role in many apoptotic processes and mostly target transcription factors, such as AP-1 component c-Jun and activating transcription factor-2 (ATF-2). The mammalian p38 family consists of four isoforms, and can be activated by LPS, IL-1 and TNF-α (154, 157). The activation of these pathways leads to increase or decrease in the expression of certain target genes. The MAPK cascades are summarized in Figure C.

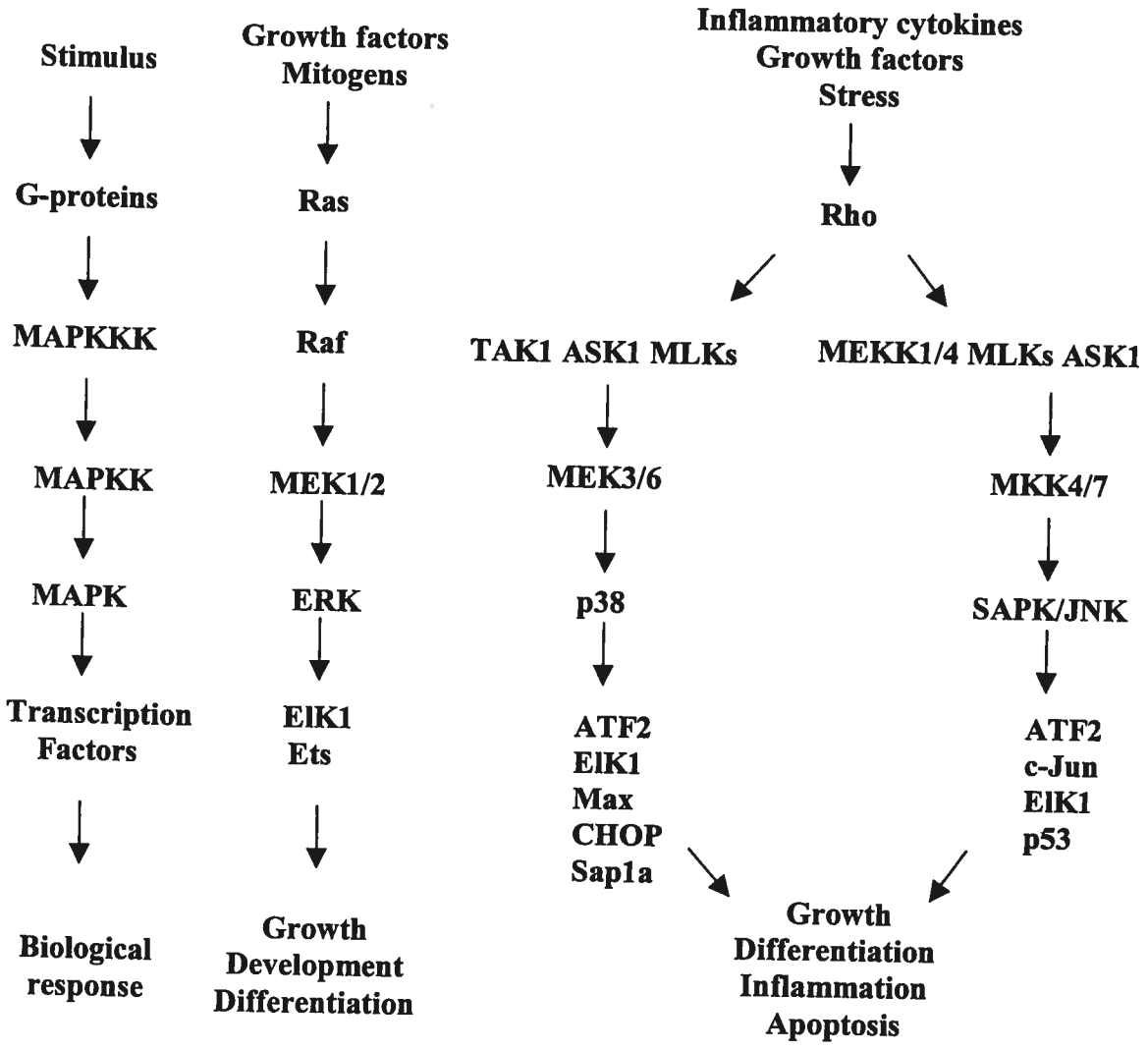

Figure C. Mitogen-activated protein kinase (MAPK) signaling. Upon stimulation, a kinase cascade is triggered that ultimately lead to gene expression. The three main kinases are the MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK. Abbreviations: CHOP, CREB homologous protein; G-protein, GTP-binding proteins; ASK, apoptosis signal-regulating kinase; ERK, extracellular signal-regulated kinsae; JNK, c-Jun N-terminal kinase; Max, Myc-associated factor X; MEK, MAPK/ERK kinase; MEKK, MEK kinase; MKK, MAPK kinase; MLK, mixed lineage kinase; Ras, rat sarcoma virus; Rho, Ras homologous; Sap1a, serum response factor accessory protein-1a; SAPK, stress-activated protein kinase; TAK, transforming-growth-factor-β-activated kinase.

1.12 AKT signaling

In many cellular processes, phosphatidylinositol 3-kinases (PI3Ks) has a key regulatory function. The PI3Ks are a family of enzymes that produce lipid second messengers by phosphorylation of plasma membrane phosphoinositol. They are heterodimeric proteins consisting of a catalytic subunit (110 kDa, p110) and an adaptor/regulatory subunit p85. They are activated by G-protein-coupled receptors (GPCRs) or receptors, which are tyrosine phosphorylated in response to extracellular stimuli (158). Phosphatidylinositol 3-kinases are also activated by direct interaction with the small GTPase Ras (159,160). Upon activation, these enzymes phosphorylate inositol lipids at various positions of the inositol ring to generate the 3-phosphoinositides, phosphatidylinositol 3-phosphate [PtdIns (3) P], phosphatidylinositol 3,4-bisphosphate [PtdIns (3,4) P2] and phosphatidylinositol 3,4,5-trisphosphate [PtdIns (3,4,5) P3].

Phosphoinositides serve as binding targets for proteins containing pleckstrin-homology domains such as AKT/PKB (161). These phosphatidylinositol 3-kinase lipid products bind to the pleckstrin-homology domain of Akt/PKB, while PDK1 and PDK2 are also recruited and activated. Subsequently, Akt/PKB is activated by PDK1 and PDK2 through phosphorylation of the T308 and S473 residues of Akt/PKB, respectively. The latter activated kinase phosphorylates several proteins like S6 kinase (162), glycogen synthase kinase 3 (163) and forkhead-related transcription factors that regulate the expression of p27Kip or cyclin D1. The

tumor suppressor protein PTEN negatively regulates phosphatidylinositol 3-kinase lipid formation by its lipid phosphatase activity. Moreover, several studies have shown the involvement of phosphatidylinositol 3-kinases in cell proliferation (164), differentiation (165), apoptosis (166) and sugar metabolism (167). AKT cascade is shown in figure D.

Mechanisms of signal transduction can be studied by using tools, such as pharmacological inhibitors or by specific inhibition with nucleic acids.

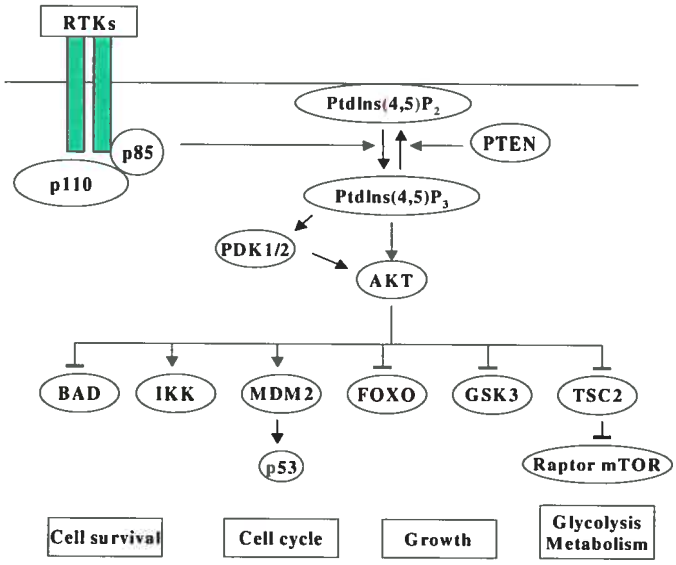

Figure D. A schematic diagram of AKT pathway. Binding of the ligand with the receptors tyrosine kinase results in the dimerization of the receptors and activation of its kinase domain in cytoplasm, which lead to the activation of PI3K (p110-p85) associated with the receptors. The activated PI3K then phosphorylates phosphatidylinositol 3,4-bisphosphate [PtdIns (3,4)P2] to phosphatidylinositol 3,4,5-trisphosphate [PtdIns (3,4,5)P3]. The phosphatidylinositol 3 kinase bind with the pleckstrin homology domain of AKT while PDK1 and PDK2 are also recruited and activated. Subsequently, AKT is phosphorylated by PDKI and PDK2 on the T308 and S473. The activated AKT then affect a number of downstream effectors regulating various processes.

1.13 Antisense and RNA interference strategies, novel tools in research

Oligodeoxynucleotides and RNA interference are part of the antisense technology, a new tool, which seems to be promising in overcoming various diseases like viral infection, cancer, cardiovascular and arthritis disease. The antisense oligos exert a very strong and specific silencing effect and the only requirement is that the sequence of the target RNA should be known. Antisense oligodeoxynucleotides (AS-ODNs) are made synthetically and range from 15-20 bases long. They are designed to hybridize with the target complementary mRNA sequences to block the protein synthesis by that mRNA. The specific silencing of gene product in a relatively short time by this technology has made it very important for the functional analysis of different genes. After entering into the cells, the oligonucleotide must remain intact, should not be destroyed by various endo- and exonucleases and must be hybridized to the target mRNA to trigger a mechanism leading to inhibition of the synthesis of protein (168).

After hybridization with the target mRNA, an endonuclease RNAse H recognizes DNA-RNA duplex and cleave the RNA strand selectively, liberating the AS-ODN, which can combine with another mRNA and ultimately lead to the depletion of that particular mRNA. To increase the stability of AS-ODNs against the nucleases and RNase H, they are generally modified to phosphorothioate ODN and 2-O-methyle ODNs. In phosphorothioate ODNs, one of the non-bridging oxygen atom is replaced by sulfur. The AS-ODNs are synthesized to target sequences, which are scattered throughout the mRNA (169). To demonstrate the true effect of AS-

ODNs, control ODNs synthesized against mismatch sequence are used. Few sequences should be avoided in AS-ODN design, four G residue in a row generate a secondary structure which prevent the hybridization with mRNA (170) and sequences like GACGTC, AGCGCT and AACGTT are known to induce interferon (171). The AS technology has been demonstrated in many cell types and in animal models. In the case of arthritis, the transfection of an oligonucleotides for nuclear factor kappaB (NF-κB) has been reported to reduce the collagen-induced arthritis in mouse model (172). Another study reported the down-regulation of the expression of cathepsin B gene in chondrocytes by antisense DNA (173). Retroviral mediated transfection of an antisense construct for Membrane Type 1 Matrix Metalloproteinase (MT1-MMP) in rheumatoid arthritis synovial fibroblast (RASFs) has significantly reduced the cartilage degradation (174).

The use of double-stranded RNA is another important tool for the functional analysis of the gene and it has revolutionized the drug discovery research. RNA interference (RNAi) is a naturally occuring biological process found in multicellular organisms such as yeast, plants and humans. It consist of two steps interacellular processing in which the precursor double stranded RNA molecules present in the cell are processed by an enzyme Dicer endonuclease, which cleaves it in to fragments of 20-24 base pair called double stranded small interfering RNA (siRNA). The siRNA molecule then becomes the part of RNA-induced silencing protein complex (RISC), where it unwinds and the complementary sequence to the target mRNA combines with the mRNA leading to its cleavage. Importantly, when

the siRNA molecules are exogenously introduced into the mammalian cells, they become incorporated into the RISC complex. The ability of siRNA to incorporate into the RISC pathway was recognized as a great advance in biotechnology and this technology was named as " breakthrough of the year" in 2002 by the Science magazine. The technique is highly specific in identifying its target gene and suppresses the gene expression by targeting the mRNA. In nature it is also considered as important for the development of organism and physiological function (175). Local injection of siRNA for the treatment of macular disease is underway as a clinical trial (176). Other possible local application tested in mouse model are intranasal application by inhaling (177) and direct delivery to the central nervous system by injections (178) Viral vectors are also in use for systemic delivery of siRNA (179) however the use of vectors is risky as vector DNA can incorporate in genome. Other non-specific effects include activation of interferons indicating that siRNA technique also has some complications. Due to the availability of the genetic information about human genome, this technique seems to be more promising in future for the treatment of diseases. Schiffelers et al reported the inhibition of TNF-α in collagen-induced arthritis (CIA) after injecting siRNA against TNF-α in the joint tissue of mice (180). In 2006 the Noble prize in Physiology and Medicine was given to Professor Andrew Z-Fire and Craig C Mello for their work on siRNA.

Overall, the antisense RNA technology is very promising and is likely to play a major role in future research and therapies.

1.14 Transcriptional regulation of gene and TIMP-3 promoter structure

Transcriptional regulation is a process in which information in a gene is transcribed into mRNA in response to signals. In eukaryotic cells, only some of the genes are transcribed at any given moment and their proportion and composition changes considerably during the life cycle, different types of cells and in response to changing physiological and environmental conditions (181). A number of mechanisms are involved in the regulation of gene expression in eukaryotes. The most common control is the transcriptional initiation (182). For every eukaryotic gene, transcriptional initiation determines the overall gene expression profile.

Transcription is regulated by proteins known as transcription factors that bind to specific DNA sequences in the promoter region of a gene resulting in the activation or inhibition of transcription. A promoter always possesses two basic features. One is a basal promoter, where the RNA polymerase II holoenzyme complex attaches itself and the other feature is the collection of different binding sites, which are specific for transcription factors and confer specifity to the transcription. A basic promoter alone cannot initiate transcription at a significant level, however the bindings of transcription factors produce stronger response (183).

The activity of transcription factors is regulated by various environmental factors (e.g. stress, heat shock and UV light), cytokines and growth factors. In most cases the transcription factor activity is due to phosphorylation or dephosphorylation induced by protein kinases and phosphatases. Protein phosphorylation and

dephosphorylation alter transcription factors activity in many different ways: cellular location, protein stability, protein-protein interaction and DNA binding (184).

Many transcription factors already have nuclear localization, whereas others are in cytoplasm, and shuttle between cytoplasm and nucleus, and this translocation is regulated by phosphorylation. TGF-β-stimulated phosphorylation of R-Smads result in the dimerization with the co-Smad (Smad-4) and translocation in the nucleus (137). In the nucleus, this Smad complex binds with the Smad binding elements (SBE) CAGA. SBE-like sequences have been identified in the promoters of many TGF-β responsive genes, including plasminogen activator inhibitor-1 (PAI-1), junB, type VII collagen and the germline immunoglobulin Iα region. Mutation of the SBEs in the PAI-1 promoter has been shown to decrease significantly TGF-β inducibility (185).

The binding affinity of Smads for DNA is relatively low (186) and requires other sequence-specific binding partners for efficient binding to the promoters of responsive genes. In ligand-responsive promoter regions, SBE sites are generally located in close proximity to recognition sequences for other transcription factors, for example FAST-1 in the Mix2 promoter (187); AP-1, TFE3 and ATF2 in the PAI-1 promoter (188,189). The binding of these transcription factors enhanced the TGF-β-induced response where as the mutation in these sites for the transcription factors attenuates TGF-β-induced response. The interaction between Smad and a transcription factor can be direct, as in the case for Smad3 and AP-1 family

members (190) or indirect as the Stat3 and Smad1 brought together through their association with the CBP/p300 co-activator upon stimulation by BMP and leukemia inhibitory factor (191).

In certain responses, Smad do not bind to the DNA directly, but by an indirect way as in the case of TGF-β-mediated upregulation of CDK inhibitor p21, where the DNA binding of Smads via interaction with Sp1 bound to the promoter (192). Furthermore there are some proteins that act as Smad co-activators and co-repressors. CBP/p300 act as co-activators (193) where as Ski, SnoN and TGIF act as co-repressors (194).

The transcription of every gene is regulated by transcription factors and cofactors that interact with its promoter. The region of 1.2 Kb immediately upstream with respect to transcription start site in human TIMP-3 coding gene shows TIMP-3 promoter activity, as we have determined in our lab also. This region contains multiple sequences, which are binding sites for known transcription factors (195,196). Among these are four Sp1, one C/EBP, one NF1, one c-Myb and several AP-2 sites in the region between +1 to –600. This region also contains several potential Smad binding elements (SBE) or CAGA sequences which are involved in the induction of plasminogen activator inhibitor-1 (PAI-1) gene promoter in response to TGF-β (140). In this study, we have demonstrated the involvement of Smads and Sp1 transcription factor in TGF-β-induced TIMP-3 gene in chondrocytes. The TIMP-3 promoter structural detail is shown in figure E.

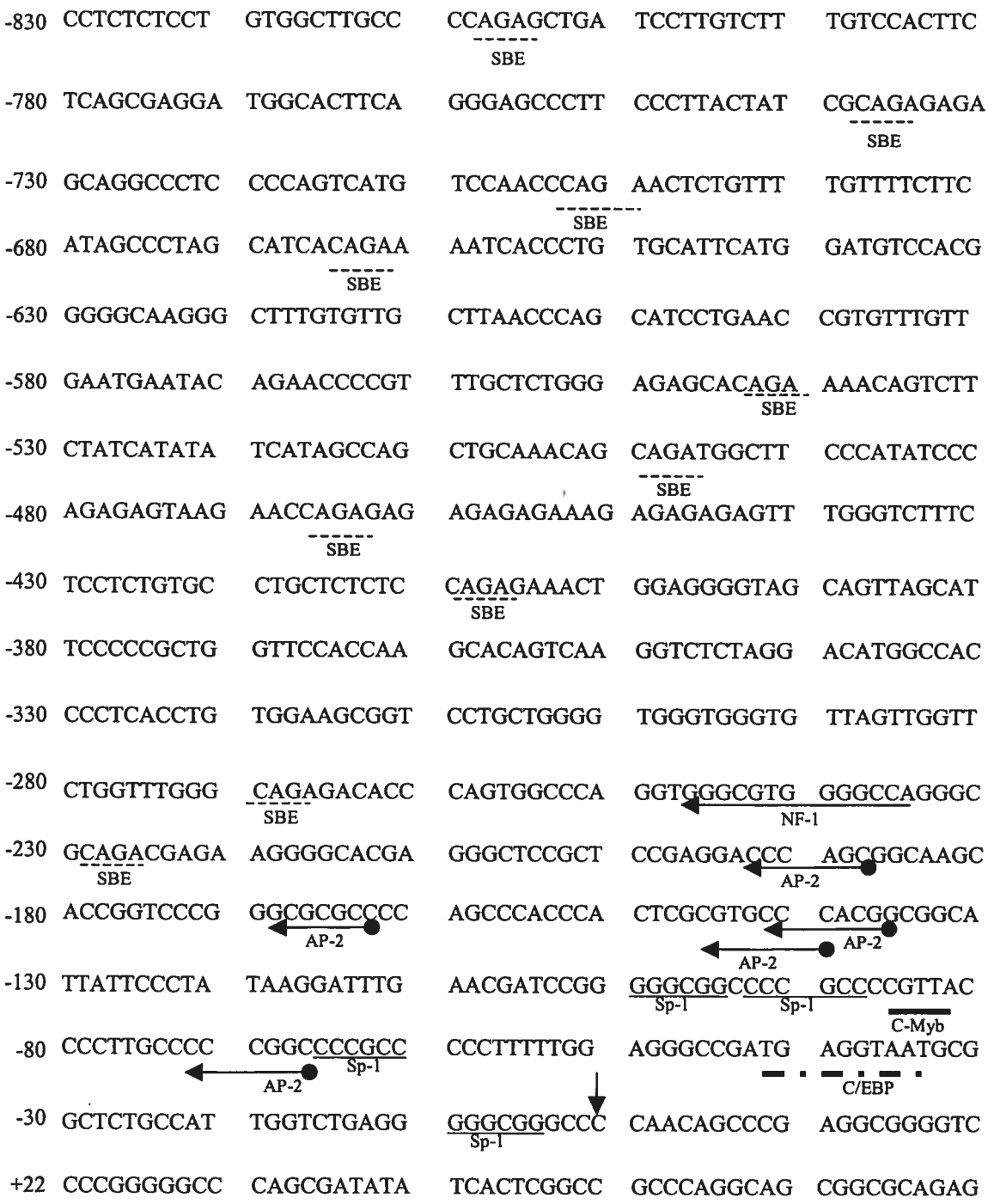

Figure E. Transcription factor binding motifs in the human TIMP-3 promoter. Nucleotide sequence of human TIMP-3 promoter in the region proximal to the

transcription start site (-830 to +1) and 71 nucleotide of the 5 transcribed region. Numbering indicates the nucleotide distance relative to the transcription start point with +1. Different motifs are underlined. C/EBP, CAATT/enhancer-binding protein; SBE, Smad binding element. ▼: transcription start site. (Ref 195, 196)

1.15 Research hypothesis and experimental approach

Tissue inhibitor of metalloproteinases-3 (TIMP-3) inhibits matrix metalloproteinases and aggrecanases implicated in arthritic cartilage loss. The regulation of TIMP-3 gene by transforming growth factor beta (TGF-β) family members in cartilage is poorly understood. This work focuses on examining signaling pathways involved in TGF-β induced TIMP-3 induction in chondrocytes. Based on earlier work, we hypothesize that Smad signaling, ERK and possibly AKT pathways mediate TIMP-3 induction by TGF-β. These signaling cascades could activate certain transcription factors that could bind and induce TIMP-3 promoter. We analyzed these hypotheses in the *vitro* model systems consisting of cultures of human chondrosarcoma SW1353 cells and primary or early-passage cultures of bovine and human chondrocytes.

Specific aims of this study were 1) To examine the stimulation of different signaling pathways (ERK, AKT and Smad) by TGF-β using Western blotting to determine the activation of signaling mediators using phospho-specific antibodies. Specific pharmacological inhibitors as well as genetic inhibitors, such as sense and antisense phosphorothioate oligonucleotides were used to examine the involvement of various mediators of these pathways by Western and Northern blot analysis. The EMSA was used to examine the impact of mithramycin on Sp1 binding activity in SW1353 cells. Co-transfection of cytomegalovirus (CMV) promoter-Sp1 expression vector along with TIMP-3 promoter-luciferase plasmid

was performed to explore the effect of Sp1 on TIMP-3 promoter luciferase expression (article 1).

2) To study the involvement of AKT pathway, specific inhibitors of this pathway were used, and TIMP-3 protein and RNA was measured by Western and Northern blot analysis. Specific siRNAs were transfected to knock-down (PI3K and AKT) genes and respective proteins of down-regulated genes and TIMP-3 were analyzed by Western blot. Effects of TGF-β and inhibitors were also studied on TIMP-3 promoter activity by luciferase assays (article 2).

3) To characterize distinct Smads activated by TGF-β by Western blotting. To identify specific Smads involved in the induction of TIMP-3 gene by using specific inhibitors and gene knockdown by siRNA. Additionally, deletion mutants were made of TIMP-3-luciferase promoter-reporter plasmid to identify the responsive region for TGF-β. Their activities were analyzed after transient transfection and luciferase assays (article 3).

II. Articles

1. Article I

Qureshi HY, Sylvester J, El Mabrouk M, Zafarullah M. **TGF-β-induced expression of tissue inhibitor of metalloproteinases in chondrocytes is mediated by extracellular signal-regulated kinase pathway and Sp1 transcription factor. J Cell Physiol. 203(2): 345-52, 2005.**

JOURNAL OF CELLULAR PHYSIOLOGY 203:345–352 (2005)

TGF-β-Induced Expression of Tissue Inhibitor of Metalloproteinases-3 Gene in Chondrocytes Is Mediated by Extracellular Signal-Regulated Kinase Pathway and Sp1 Transcription Factor

HAMID YAQOOB QURESHI, JUDITH SYLVESTER, MOHAMMED EL MABROUK, AND MUHAMMAD ZAFARULLAH*

Department of Medicine and Centre de Recherche du Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, Quebec, Canada

Transforming growth factor (TGF-β1) is a potent inducer of chondrogenesis and stimulant of cartilage extracellular matrix (ECM) synthesis. Tissue inhibitor of metalloproteinases-3 (TIMP-3) is located in ECM and is the major inhibitor of matrix metalloproteinases (MMPs) and aggrecanase, the principal enzymes implicated in collagen and aggrecan degradation in arthritis. We investigated the role of extracellular-signal-regulated kinase (ERK)-mitogen-activated protein kinases (MAPK) and Sp1 transcription factor in TGF-β-induced TIMP-3 gene in chondrocytes and chondrosarcoma cells. TGF-β time-dependently induced a sustained phosphorylation of ERK-MAPKs in primary human or bovine chondrocytes. Inhibitors of this pathway, PD98059 and U0126, downregulated TGF-β-induced expression of TIMP-3 RNA and protein. Since the ERKs can phosphorylate Sp1, and the promoter of human TIMP-3 gene contains four Sp1-binding sites, we investigated whether Sp1 is a downstream target of this pathway. Mithramycin and WP631, the agents that prevent binding of Sp1 to its consensus site, downregulated TGF-β-inducible TIMP-3 expression. Indeed, mithramycin blocked TGF-β-stimulated Sp1 binding activity. Transfection of cytomegalovirus (CMV) promoter-Sp1 plasmid increased TIMP-3 promoter (−940 to +376)-driven luciferase activity. Depletion of Sp1 by transfection of an antisense phosphorothioate oligonucleotide suppressed TGF-β-induced TIMP-3 protein expression, while its sense homolog had no effect. These results suggest that activation of ERK-MAPK pathway and Sp1 transcription factor play a pivotal role in the induction of TIMP-3 by TGF-β in chondrocytes. J. Cell. Physiol. 203: 345–352, 2005. © 2004 Wiley-Liss, Inc.

Resorption of cartilage extracellular matrix (ECM) in patients with rheumatoid arthritis (RA) and osteoarthritis (OA) occurs by failure of endogenous repair processes induced by an imbalance between anabolic growth factors and catabolic proinflammatory cytokines, interleukin-1 (IL-1) and tumor necrosis factor (TNF-α), which inhibit the ECM synthesis and induce matrix metalloproteinases (MMPs) production (Goldring, 2000; Firestein, 2003). Adult human cartilage has limited capacity to regenerate, and growth factors, such as transforming growth factor beta (TGF-β) family members, are of particular interest in this regard. TGF-β, a multifunctional growth factor, is produced by monocytes-macrophages, platelets, and chondrocytes, and is a potent inducer of chondrogenesis during development and cartilage ECM synthesis (Joyce et al., 1990). As an attempt to repair tissue damage, TGF-β1 is elevated in human RA synovial fluid (Lotz et al., 1990), and is a major growth factor for stimulating cartilage regeneration (Lotz et al., 1995). It suppresses acute and chronic arthritis by counteracting the effects of IL-1 (Brandes et al., 1991). Excessive TGF-β is also implicated in the formation of bony joint outgrowths called osteophytes in OA (van Beuningen et al., 1994), synovial hyperplasia, and inflammation in RA (Hamilton et al., 1991) by inducing or suppressing a battery of genes. Inhibition of endogenous TGF-β in a murine arthritis model resulted in prevention of osteophyte formation and impaired cartilage repair, suggesting its role in these pathological and physiological processes (Scharstuhl et al., 2002a).

MMP and aggrecanase (ADAMTS) families of proteases have the capacity to digest all components of cartilage ECM (Caterson et al., 2000; Mengshol et al., 2002; Nagase and Kashiwagi, 2003). Tissue inhibitors of metalloproteinases (TIMPs) are natural inhibitors of MMPs and consist of four members (Brew et al., 2000; Baker et al., 2002; Mohammed et al., 2003). Activated MMPs in excess of TIMPs are implicated in damage to articular cartilage. TGF-β inhibits the expression of MMPs but induces TIMP-1 and TIMP-3 in articular chondrocytes (Gunther et al., 1994; Su et al., 1996). TIMP-3 is uniquely located in ECM where it binds to sulfated glycosaminoglycans such as chondroitin and heparan sulfate (Yu et al., 2000) and is the major inhibitor of MMP-13 and ADAM-TS4, the principal enzymes implicated in cartilage collagen and aggrecan degradation in arthritis (Hashimoto et al., 2001; Kashiwagi et al., 2001). It also inhibits TNF-α converting enzyme (TACE or ADAM-17), which activates proarthritic cytokine, TNF-α (Amour et al., 1998; Lee et al., 2001). TIMP-3 can thus regulate inflammation in RA. TIMP-3 inhibits angiogenesis (a prominent feature of RA and tumors) by blocking the binding of VEGF to its

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*Correspondence to: Dr. Muhammad Zafarullah, K-5255 Mailloux, Hôpital Notre-Dame du CHUM, 1560 Sherbrooke est, Montréal, Québec, Canada H2L 4M1.

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receptor (Anand-Apte et al., 1997; Qi et al., 2003). These distinct features make TIMP-3 a potentially important therapeutic protein in arthritis (Lee et al., 2002; Nagase and Brew, 2002; Mohammed et al., 2003). Indeed, TIMP-3 overexpression prevents invasion of cartilage by human rheumatoid synovial fibroblasts in a mouse model (van der Laan et al., 2003). The mechanisms by which TGF-β augments TIMP-3 expression are poorly understood. In other systems, TGF-β first binds with type II receptors and then phosphorylates and activates type I receptor on serine and threonine, leading to transcription of the target genes via inhibitory or stimulatory Smads and other pathways (Shi and Massague, 2003). We previously showed the involvement of serine/threonine kinases in TGF-β induction of TIMP-3 in chondrocytes (Su et al., 1998), however, precise signaling pathways and target transcription factors implicated in this induction are unknown. In the present study, we utilized chondrocyte model systems, namely bovine and human primary articular chondrocyte and chondrosarcoma cells, and showed the crucial role of extracellular-signal-regulated kinase (ERK1/2)-mitogen-activated protein kinases (MAPK) and their target, Sp1 transcription factor in TGF-β-stimulated increase of TIMP-3.

MATERIALS AND METHODS

Culture of chondrocytic cells and treatments

Normal bovine articular cartilage was obtained from the knee and hip joints of normal adult, 30-month-old animals from a local abattoir. Human cartilage was acquired from the femoral heads of OA patients who underwent hip-replacement surgery at the Notre-Dame Hospital. Chondrocytes were released by 90 min pronase and collagenase (type II) (Sigma Chemical Company, St. Louis, MO) digestion for 9 h in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Sciences, Inc., Burlington, ON). The cells were washed with phosphate buffered saline (PBS) and grown in DMEM with 10% fetal calf serum (FCS) as high-density primary monolayer cultures until confluent growth. Cells were distributed in 6-well plates (Becton Dickinson, Franklin Lakes, NJ), grown to confluence, washed with PBS, and kept in serum-free DMEM for 24 h. ERK1/2-specific inhibitors, PD98059 (2′-amino-3′-methoxyflavone) (Dudley et al., 1995) and U0126 (1,4-Diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene) (Favata et al., 1998) from Calbiochem (La Jolla, CA) were dissolved in dimethyl sulfoxide (DMSO) and added at the concentration of 15–30 and 2.5–5 μM, respectively, 30 min before treatment with human platelet TGF-β (10 ng/ml) (R&D systems, Minneapolis, MN) for 24 h. In other experiments, cells were pretreated with 100–150 nM mithramycin or 25–50 nM of bisanthracycline WP631 (Martin et al., 1999). TGF-β1 was reconstituted in 0.1% fraction V of bovine serum albumin (BSA) (Sigma) and 4 mM HCl as recommended. A TGF-β-responsive human chondrosarcoma cell line, SW1353, was obtained from American Type Culture Collection (ATCC, Manassas, VA) and treated as described for primary chondrocytes.

RNA extraction and Northern hybridization analysis

Total RNA was extracted by a rapid procedure (Chomczynski and Sacchi, 1987) and aliquots of 3–5 μg analyzed by electrophoretic fractionation in 1.2% formaldehyde-agarose gels. The integrity and quantity of applied RNA were verified by ethidium bromide staining of the gels and photography of the 28S and 18S ribosomal RNA bands. The RNA was electroblotted completely onto Zetaprobe nylon membrane (Bio-Rad, Mississauga, ON) using a Bio-Rad Transblot in the presence of 1× TAE buffer at a current of 40 mA overnight. Northern blots were hybridized with a bovine (Su et al., 1996) or human TIMP-3 (Apte et al., 1994) cDNA probe (latter generously provided by Dr. Suneel Apte, Cleveland, OH). The bovine TIMP-3 probe was a 2.0 Kbp *Eco*RI cDNA fragment cloned in the plasmid pGEM-4Z (Promega Biotech., Madison, WI). The vector was linearized with *Nar*I, and an RNA probe was synthesized using T7 polymerase. The human 28S ribosomal RNA plasmid (from ATCC, Manassas, VA) was digested with *Xba*I and a probe synthesized using T7 polymerase. All probes were labeled to high-specific activity (1×10^8 cpm/μg) with [α-^{32}P] CTP (3,000 Ci/mmol, Perkin Elmer Canada, Inc., Woodbridge, ON) with the RNA labeling kit from Promega Biotech., according to their protocols.

Western blot analysis

The activation of ERK-MAP kinases was analyzed by Western immunoblotting using antibodies against phosphorylated or total proteins (Cell Signaling Technology, Beverley, MA). For TIMP-3 protein levels, total cellular protein extracts from bovine or human chondrocytes (10–20 μg) were subjected to fractionation by a 15% SDS–PAGE mini gel (Bio-Rad system), transferred to PVDF (Pall Corporation, Anne Arbor, MI) by electroblotting, and reacted with the 1:400 dilution of human TIMP-3 polyclonal antibody (Chemicon International, Temecula, CA) as described before (Li and Zafarullah, 1998). Subsequently, membranes were incubated with the anti-rabbit secondary horseradish peroxidase-conjugated antibody (Promega, Madison WI) and the TIMP-3 protein bands revealed with the chemiluminescence detection system of Roche Biochemicals (Laval, Qc, Canada) with their protocols. Control proteins that served as loading control were unaffected proteins from the same cellular extracts obtained by the Coomassie blue staining of the gels. For measuring Sp1 levels, protein extracts were fractionated by 10% SDS–PAGE, reacted with 1:500 dilution of human Sp1 polyclonal (rabbit) antibody (Geneka Biotechnology, Inc., Carlsbad, CA), and reacted with secondary antibody as above, which yielded a 97-kDa band.

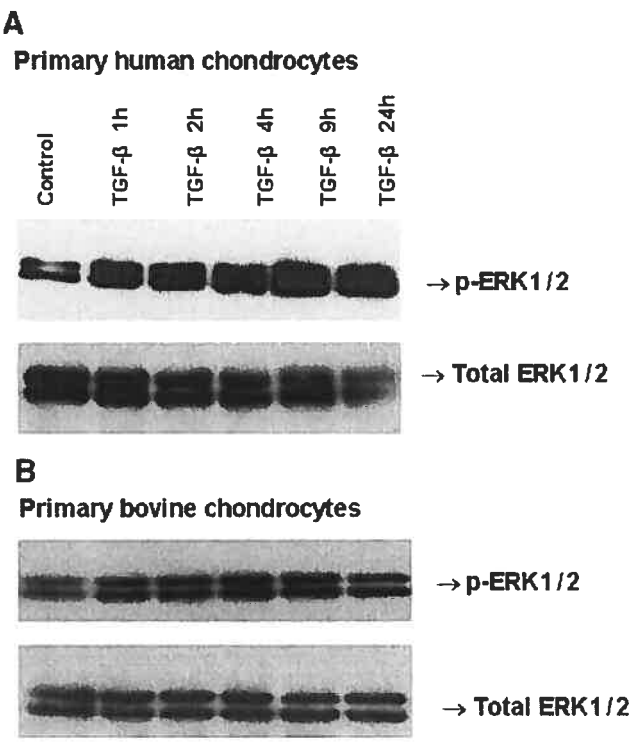

Fig. 1. Induction of extracellular-signal regulated kinase (ERK)-mitogen-activated protein kinases (MAPK) phosphorylation by TGF-β in articular chondrocytes. Cells were treated with 10 ng/ml of TGF-β for the indicated time periods. Cellular extracts (10 μg) were subjected to immunoblotting with phosphorylation-state-specific ERK1 and -2 antibodies, the respective 44 and 42 kDa bands detected by chemiluminescence and identified by their sizes. The blots were stripped and reprobed with regular antibodies, which detected total amount of the respective proteins.

Electrophoretic mobility shift assay (EMSA)

SW1353 cells in 6-well plates were grown to 80% confluence, washed with PBS, and kept in serum-free DMEM for 24 h. Mithramycin was added at a concentration of 100–150 nM, respectively 30 min before treatment with human platelet TGF-β1 (10 ng/ml). Nuclear extracts were prepared as described (Andrews and Faller, 1991) with some modifications. After 24 h, the cells were washed twice with cold PBS (pH 7.4) and detached by scraping, recovered by centrifugation at 10,000 rpm for 5 min at 4°C. The cells were lysed in ice-cold buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 1 mM PMSF, 1% NP 40, 1 mM NaF, and 10 μg/ml protease inhibitors leupeptin A and pepstatin] and mixed by pipetting. After incubation on ice for 10 min, the contents were centrifuged at 4,000 rpm for 2 min. The nuclear pellets were suspended in 60 μl of ice-cold buffer B [20 mM HEPES (pH 7.9), 420 mM NaCl, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM NaF, 0.5 mM DTT, 0.5 mM PMSF, and 10 μg/ml protease inhibitors, leupeptin, and pepstatin A] and kept on ice for 30 min with gentle shaking and then centrifuged at 12,000 rpm for 15 min at 4°C. The nuclear protein supernatants were recovered and protein concentrations were determined by Bio-Rad protein assay.

The double-stranded oligonucleotide containing the Sp1 consensus (Promega Corporation, Madison, WI) sequence was end labeled with [γ-p^{32}] ATP by T4 polynucleotide kinase. Nuclear extracts (5 μg) were incubated with 20,000 cpm of labeled probe for 20 min at room temperature in binding buffer [10 mM Tris-HCl (pH 7.5), 4% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, and 50 μg/ml poly (dI. dC)] and DNA protein complexes were resolved on a 5% nondenaturing polyacrylamide gel at room temperature. Competition assay was performed by incubating 200-fold molar excess of cold probe for 15 min at room temperature before the addition of the p^{32}-labeled oligonucleotide.

Transient transfections

Cells were transfected with Sp1 sense 5′-CCA TGG ATG AAA TGA CAG CTG TGG TG-3′ and antisense 5′-CAC CAC AGC TGT CAT TTC ATC CAT GG-3′ phosphorothioate (at all nucleotides) oligonucleotides (ODN) via calcium phosphate precipitation method (Graham and van der Eb, 1973) for adherent cells in suspension as follows. Cells were detached by trypsinization, trypsin removed by centrifugation and suspension incubated with ODN-calcium phosphate precipitate for

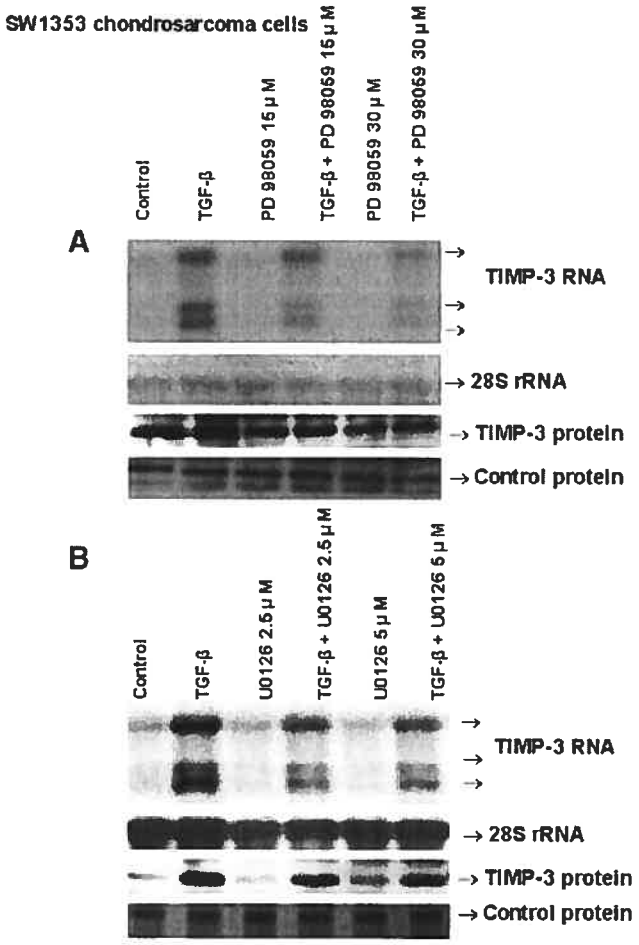

Fig. 2. Impact of ERK/MAPK pathway inhibitors on TGF-β-stimulated expression of TIMP-3 in SW1353 cells. Chondrosarcoma SW1353 cells were pretreated with the indicated doses of (**A**) PD98059 or (**B**) U0126 for 30 min and stimulated with TGF-β for 24 h. TIMP-3 RNA (5 μg) and protein (10 μg) from cellular extracts were analyzed by Northern hybridization with TIMP-3 and 28S ribosomal RNA probes and Western blotting with TIMP-3 antibody. Arrows indicate three different transcripts of TIMP-3. The 28S rRNA serving as loading control is shown at the bottom. TGF-β-inducible TIMP-3 protein band (24 kDa) and a constitutively expressed control or Coomassie blue-stained protein band from the same extracts are shown.

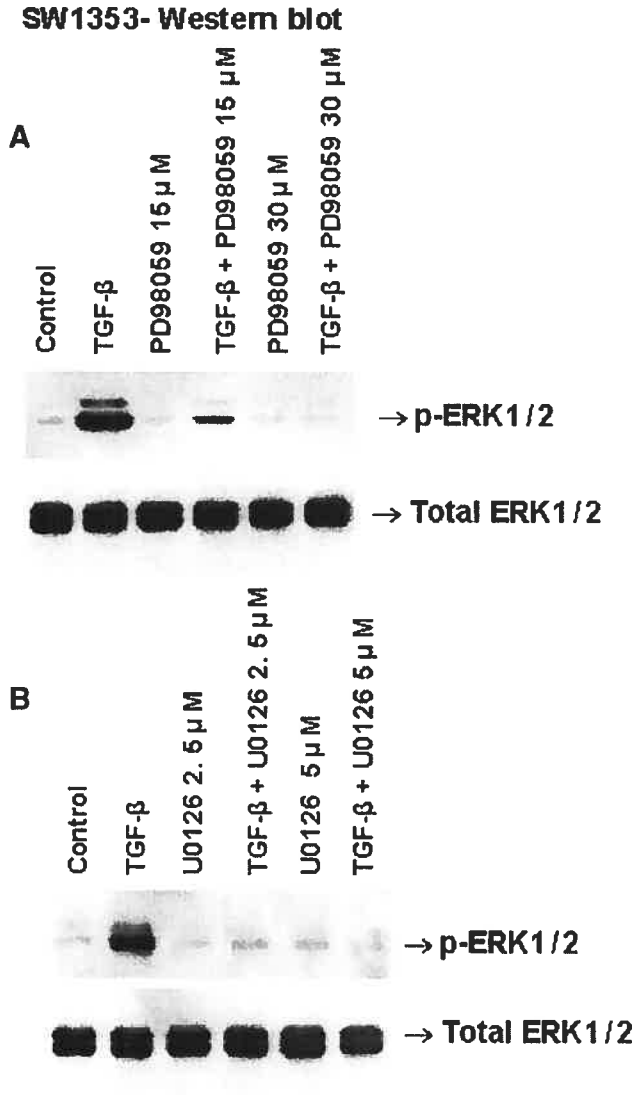

Fig. 3. Inhibition of ERK-1/2 phosphorylation by PD98059 and U0126. SW1353 cells were pretreated with the indicated doses of PD98059 (**A**) or U0126 (**B**) for 30 min, stimulated with TGF-β for 1 h and protein extracts subjected to Western immunoblotting with phosphorylation-state-specific ERK1/2 and total ERK antibodies. Arrows indicate the respective bands.

10 min and plated in serum containing medium for 3 h. Medium was removed, cells washed with PBS, allowed them to recover for 16 h, maintained in serum-free medium for 36 h and then stimulated with TGF-β for 24 h. Equal amount (20 μg) of protein was analyzed for TIMP-3 protein levels as above. In other experiments, 2 μg of TIMP-3 promoter luciferase (−940 to +376 region; Zeng et al., 1998), cytomegalovirus (CMV)-Renilla luciferase (0.2 μg, transfection control), CMV-Sp1 expression vector (0–1.2 μg) (from Dr. Stephen Smale, UCLA), and different amounts of mock DNA (pGL3 basic, Promega) were cotransfected by the modified calcium phosphate procedure described above and luciferase activity analyzed with Promega Dual-Luciferase Reporter assay System and Turner Designs Luminometer TD-20/20 according to their recommended procedures.

All the experiments were performed at least three times and the results were reproducible.

RESULTS

TGF-β induces activation of ERK-MAPK pathway in chondrocytes

To investigate the mechanisms of TGF-β signaling in chondrocytes and to examine whether TGF-β activates ERK-MAPK pathway, quiescent human or bovine articular chondrocytes were treated with TGF-β for the time periods of 1–24 h. As shown in Figure 1, TGF-β induced phosphorylation of ERK-1 and -2 within 1 h in both human (Fig. 1A) and bovine (Fig. 1B) chondrocytes that remained elevated up to 24 h (human) or 4–9 h (bovine). The total levels of these proteins remained constant.

ERK-MAPK pathway inhibitors downregulate TGF-β-stimulated expression of TIMP-3

To explore the requirement of ERK pathway stimulation for TGF-β-induced TIMP-3 gene expression, human chondrocytic SW1353 cells were pretreated with the specific inhibitors of this pathway, PD98059 (Dudley et al., 1995) or U0126 (Favata et al., 1998) and then stimulated with TGF-β. As shown in the Northern and Western blots of Figure 2A,B , TGF-β induced TIMP-3 RNA and protein whose levels were downregulated by the both inhibitors. The TIMP-3 protein levels mimicked the pattern of RNA expression. The levels of the loading control, 28S rRNA and constitutively expressed Coomassie blue-stained control proteins from the same

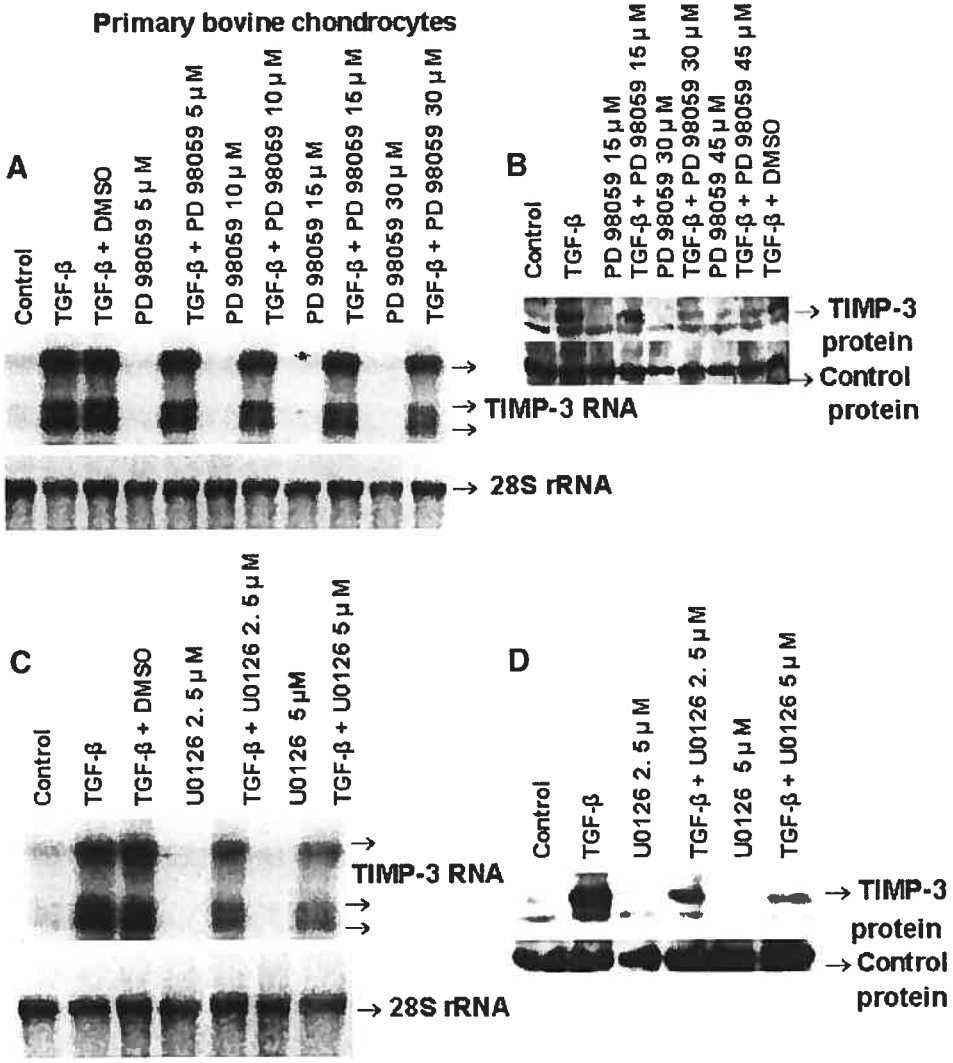

Fig. 4. Effect of ERK/MAPK pathway inhibitors on TGF-β-stimulated expression of TIMP-3 in primary bovine chondrocytes. Cells were pretreated with DMSO (solvent for inhibitors), the indicated doses of PD98059 (**A, B**) or U0126 (**C, D**) for 30 min and stimulated with TGF-β for 24 h. TIMP-3 RNA (5 μg) (A, C) and protein (10 μg) (B, D) from cellular extracts were analyzed by Northern hybridization with TIMP-3 and 28S ribosomal RNA probes and Western blotting with TIMP-3 antibody. Arrows indicate three different transcripts of TIMP-3. The 28S rRNA serving as loading control is shown at the bottom. TGF-β-inducible TIMP-3 protein band (24 kDa) and a constitutively expressed control protein band from the same extracts are shown.

extracts remained consistent. To ascertain if inhibitors indeed affect ERK phosphorylation, SW1353 cells were pretreated with TIMP-3 inhibitory doses of PD98059 or U0126 and then stimulated with TGF-β. These agents completely blocked TGF-β-stimulated phosphorylation of ERK1/2 (Fig. 3A,B). Both inhibitors (and not their vehicle, DMSO) also downregulated TGF-β-induced TIMP-3 RNA (Fig. 4A,C) and protein (Fig. 4B,D) expression in primary bovine chondrocytes without affecting the constitutively expressed 28S rRNA and control proteins.

Sp1 transcription factor inhibitors, mithramyin and WP631, inhibit TGF-β-stimulated TIMP-3 RNA and protein expression

Since p42/p44 (Erk1/2) MAPKs can phosphorylate Sp1 in vitro and in vivo (Merchant et al., 1999; Milanini-Mongiat et al., 2002) and the promoter region of human TIMP-3 gene contains four Sp1 transcription factor-binding sites (Wick et al., 1995), we investigated the role of Sp1 as a possible downstream target of the ERK pathway. Different lines of chondrocytes were pre-treated for 30 min with a pharmacological inhibitor of Sp1 transcription factor, mithramycin, which at 100–150 nM doses prevents the binding of Sp1 to its consensus DNA site (Datta et al., 2000) and then stimulated with TGF-β. Mithramycin dose-dependently inhibited TGF-β-inducible TIMP-3 gene expression in primary human chondrocytes (Fig. 5A), SW1353 chondrosarcoma cells (Fig. 5B), and bovine articular chondrocytes (Fig. 5C) without affecting constitutively expressed 28S rRNA and control proteins. Similarly, an other inhibitor of Sp1-activated transcription, bisanthracycline WP631 (Martin et al., 1999), suppressed TIMP-3 RNA and protein induction in human chondrocytes (Fig. 6).

To study the effect of mithramycin on TGF-β-induced Sp1 binding to its consensus sequence, SW1353 cell nuclear extracts were analyzed by EMSA. As shown in Figure 7, Sp1 activity was increased by TGF-β treatment and completely suppressed by mithramycin. Pre-incubation with molar excess of cold Sp1 oligonucleotide drastically reduced Sp1 binding. Thus this agent does inhibit Sp1 activity.

Transient expression of exogenous Sp1 increases human TIMP-3 promoter activity

To further investigate the role of Sp1 transcription factor in TIMP-3 regulation, human SW1353 cells were transiently cotransfected with the constant amounts of human TIMP-3 promoter-firefly luciferase vector (5′-flanking region from −940 to +376, Zeng et al., 1998), pRL-CMV, an internal control plasmid expressing Renilla luciferase, different amounts of CMV-Sp1 (expressing Sp1 under the CMV promoter) and mock (pGL3 basic) DNA to keep equal amount of DNA. As shown in Figure 8, increasing amounts of Sp1 significantly enhanced TIMP-3 promoter-driven luciferase activity compared to basal levels.

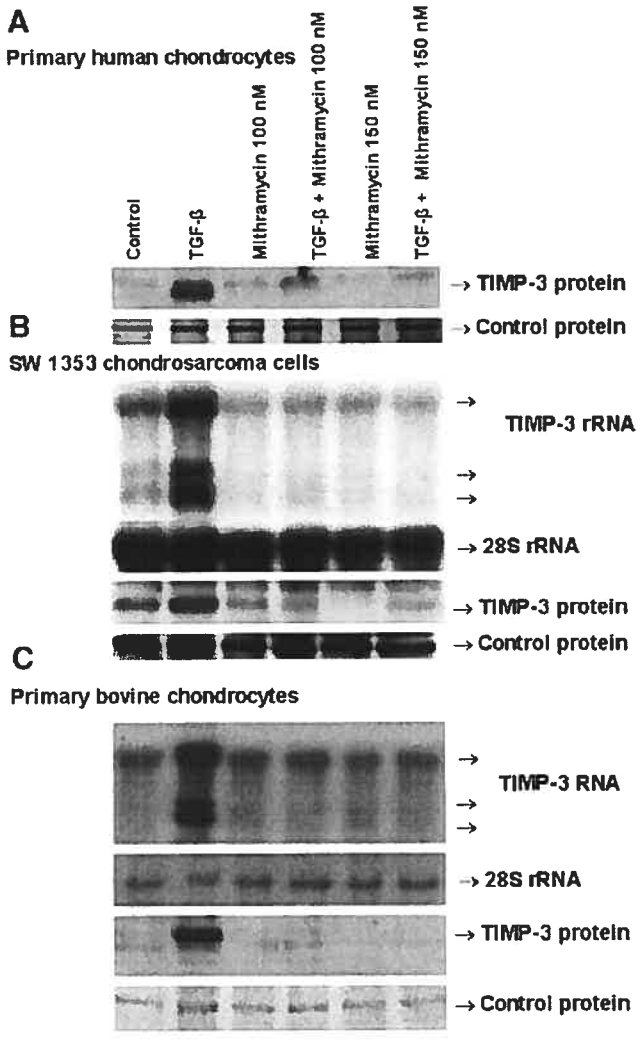

Fig. 5. Inhibition of TGF-β-induced TIMP-3 gene expression by mithramycin in chondrocytes. Primary human chondrocytes (**A**), SW1353 (**B**), and bovine (**C**) chondrocytes were pretreated for 30 min with mithramycin (which at 100–150 nM doses prevents the binding of Sp1 to its consensus DNA site) and stimulated with TGF-β. RNA and protein were analyzed as in Figure 2.

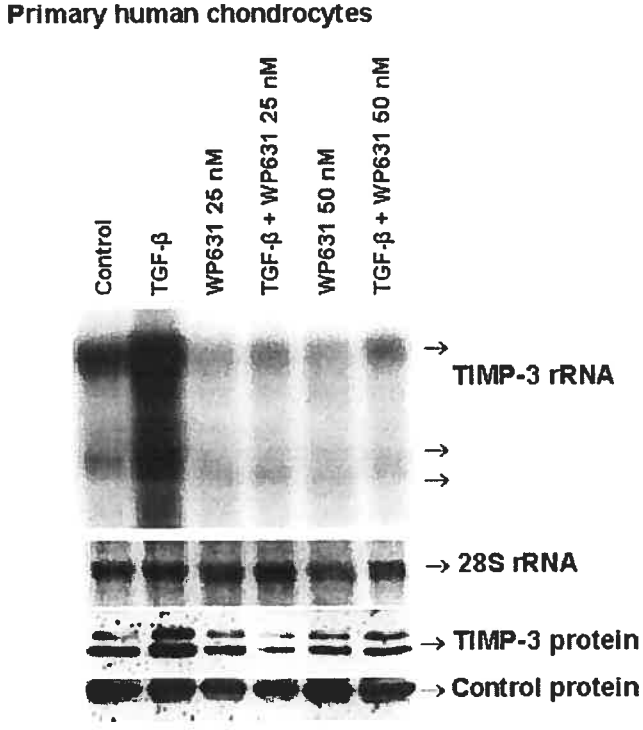

Fig. 6. Suppression of TGF-β-induced TIMP-3 gene expression by WP631 in human chondrocytes. Primary cells were pretreated for 30 min with WP631 (25–50 nM) and stimulated with TGF-β. RNA and protein were analyzed as in Figure 2.

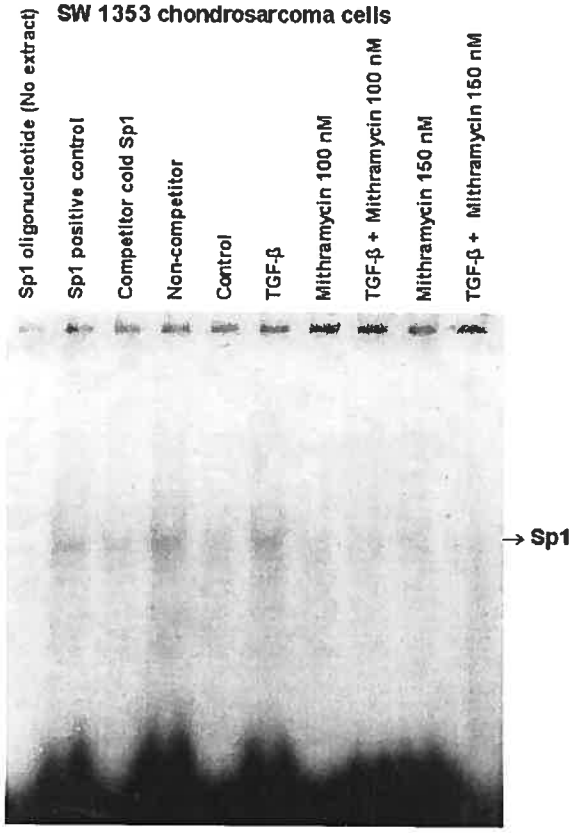

Fig. 7. Impact of mithramycin on Sp1 binding activity in SW1353 cells. Chondrosarcoma cells maintained in serum-free medium were pretreated with mithramycin, stimulated with TGF-β, and nuclear extracts subjected to EMSA. From left to right: Lane 1, labeled Sp1 probe only without extract; lane 2, probe with TGF-β-stimulated Sp1-positive extract; lane 3 (competitor), extract preincubated with 200-fold molar excess of cold Sp1 consensus oligonucleotide and then incubated with labeled probe; lane 4 (non-competitor), extract incubated with non-competitive unlabeled AP2 oligo and labeled Sp1 probe. Other lanes contained labeled Sp1 oligo with nuclear extracts from cells subjected to different treatments.

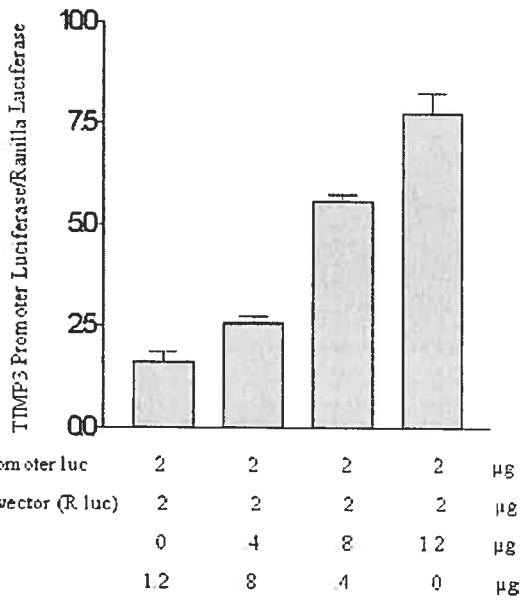

Fig. 8. Influence of Sp1 expression on human TIMP-3 promoter activity. Human SW1353 cells were transiently cotransfected with the indicated constant amounts of human TIMP-3 promoter-firefly luciferase vector (from −940 to +376), a plasmid expressing Renilla luciferase (internal control) and different amounts of vector expressing Sp1 under CMV promoter. To keep the amount of DNA constant, mock DNA (pGL3 basic, Promega) was used. The values of firefly luciferase to Renilla luciferase ratios plotted with Prism 3.0 software. Data are presented as the mean ± SEM of three independent experiments. $P < 0.05$ was considered significant.

Antisense Sp1 oligonucleotide blocks TGF-β-enhanced induction of TIMP-3 expression in human chondrocytic cells

To further evaluate the requirement of Sp1 transcription factor for induction of TIMP-3 induction by TGF-β, primary human chondrocytes or SW1353 cells were transiently transfected with a sense and antisense phosphorothioate oligonucleotide (the latter previously shown to effectively inhibit Sp1 gene expression, Pang et al., 2003) and TIMP-3 protein levels analyzed by Western blotting. As shown in Figure 9A,B, Sp1 and TIMP-3 protein induction by TGF-β was unaffected by the sense oligonucleotide but was significantly inhibited by the antisense oligonucleotide. Similarly, the levels of a constitutively expressed protein (not induced by TGF-β and that served as loading control) were not affected by the ODN.

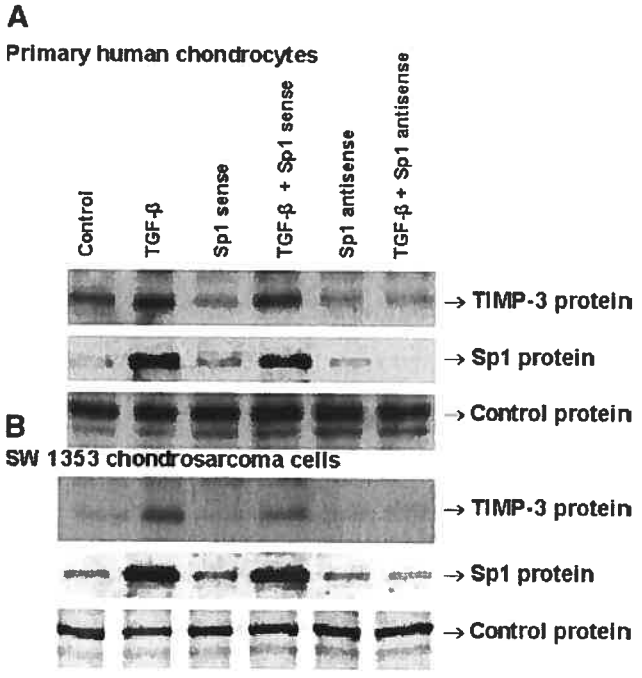

Fig. 9. Effect of antisense Sp1 oligonucleotide transfection on TGF-β-induced Sp1 and TIMP-3 gene expression in chondrocytes. Primary human chondrocytes (**A**) or SW1353 cells (**B**) were transiently transfected with a sense and antisense phosphorothioate oligonucleotide and stimulated with TGF-β for 24 h. Protein extracts (20 μg) were analyzed by Western blotting for Sp1 and TIMP-3 protein expression and results are presented. Sp1 Western blot yields a 97-kDa band. The control unaffected proteins from the same extract are also shown.

DISCUSSION

Due to their ability to inhibit cartilage digesting MMPs, both TGF-β and its target, TIMP-3, are potentially important therapeutic proteins for cartilage maintenance, prevention of cartilage degradation, and its repair in arthritis. By means of several pharmacologic and genetic approaches, we have shown for the first

time that besides well-known Smad pathway, ERK-MAPK cascade and Sp1 transcription factor are needed for induction of the TIMP-3 gene expression by TGF-β in articular chondrocytes.

Increased phosphorylation of ERKs in response to TGF-β treatment in bovine and human chondrocytes suggests conservation of this TGF-β signal transduction mechanism in two different species. TGF-β also activates this pathway in rat articular chondrocytes (Yonekura et al., 1999), during chondrogenesis of human mesenchymal progenitor (Tuli et al., 2003) and prior to aggrecan induction in chondrogenic, ATDC5 cells (Watanabe et al., 2001). Since ERK-MAPK pathway is associated with maintenance and increased survival of chondrocytes (Shakibaei et al., 2001), sustained activation of this pathway over several hours could have profound impact on these processes and TGF-β target genes such as TIMP-3. Since increased apoptosis is one of the mechanisms of cartilage loss in OA (Hashimoto et al., 1998), stimulation of the pro-survival ERK pathway by TGF-β may have an anti-apoptotic effect in chondrocytes. Downregulation of TGF-β-induced TIMP-3 RNA and protein by pharmacological inhibitors, PD98059 and U0126, suggest that ERK-MAPK is partly involved in TGF-β-induced TIMP-3 expression in human chondrocytes, a process that is also conserved in bovine chondrocytes. Similar pattern of TIMP-3 RNA and protein induction and inhibition points to a transcriptional rather than a post-translational mechanism of regulation.

Sp1 was previously considered to be a factor responsible for basal expression of a number of genes. However, several recent studies implicate this factor in inducible expression and fine-tuning of gene regulation. For instance, its phosphorylation at C-terminus is increased during the progression of cell cycle and growth where ERK-MAPK pathway is also turned-on (Black et al., 2001). TGF-β stimulates increased serine phosphorylation of Sp1 in fibroblasts from Scleroderma patients (Ihn and Tamaki, 2000). Phosphorylation of Sp1 at threonine 453 and 739 enhances binding of Sp1 with its target sequence (Milanini-Mongiat et al., 2002). Human TIMP-3 promoter region between −112 to +15′-flanking region has at least 4 Sp1 consensus sites (Wick et al., 1995). Potent inhibition of endogenous TIMP-3 RNA and protein induction by the pharmacological agents, mithramycin and WP631, suggest that this transcription factor is required for TGF-β-induced TIMP-3 expression in chondrocytes. These results were reinforced by the alternative genetic approach of Sp1 transcription factor antisense ODN-driven inhibition of Sp1 and TIMP-3 and by Sp1-enhanced TIMP-3 promoter activity. Our results are in contrast with a gene array analysis of antisense Sp1 vector-transfected NIH3T3 mouse fibroblasts, where basal expression of several ECM-related genes was inhibited but no effect on TIMP-3 was observed (Verrecchia et al., 2001). This may be due to differences in mouse and human TIMP-3 promoters and fibroblast-versus chondrocyte-specific mechanisms of TIMP-3 regulation. Our results with TIMP-3 gene support the recent concept that Sp1 is an important link between increased activation of ERK pathway and its target genes (Milanini-Mongiat et al., 2002). Alternatively, Sp1 sites may also be involved in the interaction with Smads. Indeed, TGF-β was shown to stimulate Sp1-Smad3 interaction prior to induction of plasminogen activator inhibitor and α2(I) collagen genes, which were inhibited by mithramycin (Datta et al., 2000; Poncelet and Schnaper, 2001).

TGF-β is an important growth factor that promotes ECM synthesis. It counteracts the catabolic effects of IL-1 such as inhibition of ECM synthesis and increased production of MMPs and aggrecanases in arthritic cartilage by inducing TIMP-1 and TIMP-3. The process of antagonization by TGF-β was recently shown to be impaired in older mice (Scharstuhl et al., 2002b). It is possible that TGF-β signal transduction and expression of its target genes, such as TIMP-3, are disturbed in older animals and humans, making them more susceptible to OA. Indeed, skeletal tissues of transgenic mice with defective type II receptor kinase have human OA-like degenerative changes due to defect in signaling (Serra et al., 1997). Excessive amounts of active TGF-β stimulate osteophyte formation in OA joints, which is a bony adaptive outgrowth produced as a result of OA (Bakker et al., 2001). Inhibition of endogenous TGF-β via a scavenging TGF-β-RII receptor increased cartilage damage by MMPs and resulted in prevention of osteophyte formation but also impaired cartilage repair (Scharstuhl et al., 2002a). Thus TGF-β in moderation may be useful for maintaining cartilage homeostasis and in restoring proinflammatory cytokine-stimulated alterations in chondrocyte phenotype. However, this factor has also been shown to inhibit type II collagen (the major cartilage collagen) gene in primary rabbit chondrocytes by repressing the synthesis and activity of Sp1 (Chadjichristos et al., 2002).

In summary, based on our results, we propose that TGF-β activates ERK-MAPK pathway, which in turn could increase Sp1 phosphorylation and its DNA binding activity leading to TIMP-3 induction. Overexpression of TIMP-3 in response to TGF-β could potentially reduce TACE activity and diminish TNF-α-driven inflammation in joints. This signaling cascade may have a pivotal role in the maintenance of chondrocyte differentiation, increased survival and their proliferation, the features highly desirable for maintaining the integrity of cartilage.

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2. Article 2

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Requirement of phosphatidylinositol 3-kinase/Akt signaling pathway for regulation of tissue inhibitor of metalloproteinases-3 gene expression by TGF-β in human chondrocytes

Hamid Yaqoob Qureshi, Rasheed Ahmad, Judith Sylvester, Muhammad Zafarullah *

Department of Medicine and Research Centre of Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, Quebec, Canada H2L 4M1

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Abstract

Transforming growth factor beta (TGF-β1) induces cartilage extracellular matrix synthesis and tissue inhibitor of metalloproteinases-3 (TIMP-3), an important natural inhibitor of matrix metalloproteinases, aggrecanases and TNF-alpha-converting enzyme, which are implicated in cartilage degradation and joint inflammation. This study tested the hypothesis that Akt/protein kinase B signaling pathway could mediate TGF-β1 induction of TIMP-3 in human articular chondrocytes. TGF-β activated phosphorylation of Akt in a delayed and sustained fashion that correlated with TIMP-3 mRNA induction. Phosphatidylinositol kinase (PI3K) inhibitors, Wortmannin and LY294002 and Akt inhibitor (NL-71-101) significantly inhibited TGF-β-induced Akt phosphorylation, TIMP-3 expression, TIMP-3 promoter (−940 to +376)-driven luciferase activity and Sp1 transcription factor binding. PI3K p85, Akt and Sp1 small interfering RNA (siRNA)-driven knockdown of the respective gene products significantly suppressed TGF-β-induced TIMP-3 gene expression. TGF-β-stimulated phosphorylation of p70S6 Kinase and TIMP-3 protein induction was inhibited by rapamycin. Thus TGF-β induces TIMP-3 gene expression in human chondrocytes partly through PI3K/Akt pathway and Sp1 transcription factor and by translational mechanisms via mammalian target of rapamycin (mTOR) signaling. TGF-β induction of pro-survival Akt cascade and TIMP-3 may be related to strengthening of cartilage extracellular matrix, increased chondrocyte viability and maintenance of joint tissue integrity.

Keywords: Arthritis; Cartilage; Transforming growth factor beta; Signal transduction; Akt; Transcription factors; TIMP-3; Gene regulation

1. Introduction

A hallmark of rheumatoid arthritis (RA) and osteoarthritis (OA) is resorption of cartilage extracellular matrix (ECM). This is partly due to impaired endogenous repair processes induced by an imbalance between anabolic growth factors and catabolic proinflammatory cytokines, interleukin-1 (IL-1), IL-17 and tumor necrosis factor (TNF-α), which inhibit the ECM synthesis and induce matrix metalloproteinases (MMPs) production [1,2]. Adult cartilage has limited capacity to regenerate and transforming growth factor beta (TGF-β) family members have the potential to stimulate its repair. Human OA cartilage responds poorly to TGF-β due to decreased receptor II [3]. Inhibition of endogenous TGF-β causes impaired cartilage repair and excessive TGF-β leads to the formation of osteophytes in OA [4]. TGF-β, a multi-functional factor produced by monocytes–macrophages, platelets and chondrocytes, induces chondrogenesis and ECM synthesis [5]. TGF-β1 is elevated in human RA synovial fluid and tissue, has immunosuppressive properties, [6] and is a major growth factor for maintaining chondrocyte phenotype and homeostasis [7]. It suppresses inflammatory cell infiltration, pannus formation and joint erosion during acute and chronic arthritis by counteracting the effects of IL-1 [8].

MMPs and aggrecanases (ADAMTS, a disintegrin and metalloproteinase with thrombospondin motif) digest major cartilage ECM components including type II collagen and aggrecan as well as several non-ECM substrates during physiological and pathological remodeling [9,10]. Tissue inhibitors

* Corresponding author. K-5255 Mailloux, Hôpital Notre-Dame du CHUM, 1560 Sherbrooke est, Montréal, Québec, Canada H2L 4M1. Tel.: +1 514 890 8000x25690; fax: +1 514 412 7612.
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of metalloproteinases (TIMPs) are 4 natural inhibitors of MMPs with growth promoting, pro-apoptotic, anti-apoptotic and anti-angiogenic functions [11,12]. Excessive MMPs and ADAMTSs over TIMPs cause loss of articular cartilage. TGF-β inhibits the expression of most MMPs but induces TIMP-1 and TIMP-3 in chondrocytes [7]. TIMP-3 is uniquely located in ECM where its N-terminal domain binds to chondroitin- and heparan sulfate [13] and also inhibits MMP-13, ADAMTS4 and ADAMTS5, the principal cartilage-degrading enzymes [14,15]. It blocks aggrecan degradation in cartilage explants [16] and inhibits pro-inflammatory, TNF-α converting enzyme (TACE/ADAM-17) activity [17]. TIMP-3 can thus reduce inflammation in arthritis. TIMP-3 inhibits angiogenesis by blocking the binding of VEGF to its receptor and could reduce rheumatoid pannus formation [reviewed in [11]]. Such unique features make TIMP-3 a potentially therapeutic protein in arthritis [11,17]. Indeed, TIMP-3 overexpression in proliferating rheumatoid synovial fibroblasts induces apoptosis [18] and prevents invasion of cartilage by pannus [19]. TIMP-3 knockout mice display an increased initial inflammation and serum TNF-α level in antigen-induced arthritis, supporting its protective function against inflammatory arthritis [20]. In other systems, TGF-β binding to cell surface associates types I and II receptors leading to phosphorylation of type I receptor kinase domain, transmission of signal via stimulatory Smads and transcription of the target genes [21]. In chondrocytes, Smad, PKA, PKC and Wnt pathways are induced by TGF-β relative to various cartilage functions [22]. We previously showed the involvement of Smad and extracellular-signal-regulated kinase (ERK1/2)-mitogen-activated protein pathways in TGF-β-induced TIMP-3 in chondrocytes [23,24], however, role of phosphoinositide 3-kinase (PI3K/Akt) pathway and its target transcription factors implicated in this induction are unknown. PI3K-Akt/protein kinase B (PKB) pathway is stimulated by insulin-like growth factor leading to cell proliferation, survival and inhibition of apoptosis [25]. Although PI3K/Akt pathway is activated by TGF-β in human rheumatoid synovial fibroblasts in association with their proliferation [26], its role in chondrocytes and regulation of specific genes is not known. Here, we show the previously unknown and critical role of PI3K/Akt pathway and Sp1 transcription factor in TGF-β-stimulated increase of TIMP-3 in human knee articular chondrocytes.

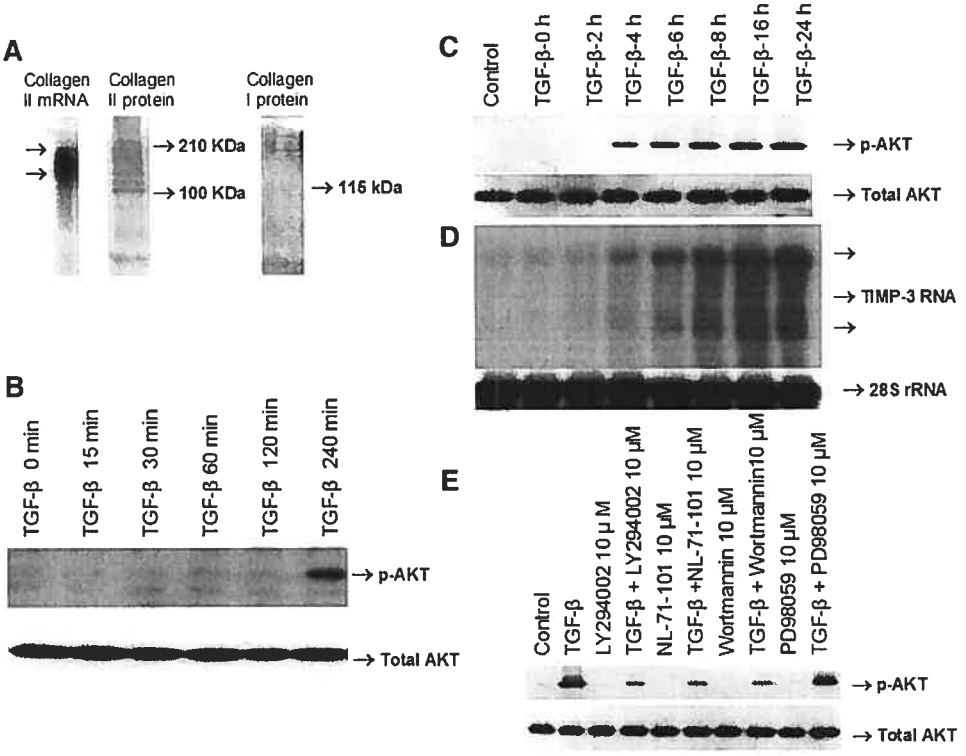

Fig. 1. Monitoring the phenotype of human articular chondrocytes and time-dependent induction of Akt phosphorylation followed by TIMP-3 gene expression in response to TGF-β. In (A), RNA (left panel) and protein (middle and right panels) from passage 3 confluent chondrocytes was extracted and analyzed by Northern or Western blotting for type II and type I collagen expression. Arrows indicate the positions of bands. They do not express 115 kilodalton (kDa) type I collagen but do express 100–210 kDa type II collagen protein and its mRNA. Confluent chondrocytes maintained in serum-free medium were left untreated (Control) or stimulated with TGF-β1 (10 ng/ml) for different time periods up to 4 h (B) or 24 h (C and D). Equal amounts of protein extracts were analyzed by Western blotting (B and C) with anti-phospho Akt (upper panels) and Akt (lower panels) antibodies. Duplicate samples were also analyzed by Northern hybridization of 5 μg of RNA with human TIMP-3 (D, upper panel) and 28S ribosomal RNA (D, lower panel) probes. The three arrows in the Northern blot indicate 3 TIMP-3 transcripts produced due to alternative usage of polyadenylation sites. E) Downregulation of TGF-β-induced Akt phosphorylation by different pharmacological inhibitors. Human chondrocytes were either treated with vehicle (DMSO and PBS with 0.1% BSA and 4 mM HCl) as control or pretreated with 10 μM of the indicated inhibitors and then stimulated with TGF-β for 24 h. Western blots show the levels of phosphorylated and total Akt proteins in cell extracts. PI3K/Akt inhibitors suppress Akt phosphorylation while PD98059 has no effect.

2. Materials and methods

2.1. Culture of chondrocytes and treatments

The normal human knee articular chondrocytes (Cambrex; Walkerville, MD) were grown to confluence as high-density passage 2 monolayer cultures in Differentiation Bullekit medium for maintaining their differentiated phenotype (Cambrex). These cells do not express type I collagen but express type II collagen, a marker of differentiated chondrocytes as determined by Northern and Western blot analysis (Fig. 1A). After trypsinization, the cells were grown in 6-well plates in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Sciences Inc. Burlington, ON) with 10% fetal calf serum (FCS). Cells were washed with phosphate buffered saline (PBS) and kept in serum-free DMEM for 24 h. PI3 Kinase-specific inhibitors, Wortmannin [27], LY294002 (2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one) [28] and Akt-specific inhibitor, NL-71-101 [29] from Calbiochem (La Jolla, CA) were dissolved in dimethyl sulfoxide (DMSO) and added in the same medium at the concentration of 10–20 μM respectively 30 min before treatment with human platelet TGF-β1 (10 ng/ml) (R&D systems, Minneapolis, MN) for 24 h. In some cases, rapamycin dissolved in DMSO was added at 20 and 100 ng/ml concentrations 1 h prior to TGF-β treatment for 24 h. TGF-β1 was reconstituted in 0.1% fraction V of bovine serum albumin (BSA) (Sigma) and 4 mM HCl as recommended.

2.2. RNA extraction and Northern hybridization analysis

Total RNA was extracted by a rapid procedure [30] and aliquots of 5 μg analyzed by electrophoretic fractionation in 1.2% formaldehyde-agarose gels. The RNA was electroblotted onto Zetaprobe nylon membrane (Bio-Rad, Mississauga, ON) using a Bio-Rad Transblot in the presence of 1X TAE buffer at a current of 40 mA overnight. Northern blots were hybridized with a human TIMP-3 [31] RNA probe made with T3 polymerase from plasmid pBlueScript. The human 28S ribosomal RNA plasmid (from ATCC, Manassas, VA) was digested with XbaI and a probe synthesized using T7 polymerase. The type II collagen (kindly provided by Dr Eero Vuorio, Turku, Finland) RNA probe was made with SP6 polymerase. All probes were labeled to high-specific activity (1×10^8 cpm/μg) with [α-^{32}P] CTP (3000 Ci/mmol, Perkin Elmer Canada Inc, Woodbridge, ON) with the RNA labeling kit from Promega Biotech (Madison, WI) according to their protocols.

2.3. Western blot analysis

Type II and type I collagen rabbit polyclonal antibodies (#234187 and 234168, Calbiochem) were used to monitor the phenotype of chondrocytes. PI3 kinase p85 (Cat no. 4292) (Cell Signaling) antibody was used to verify knockdown by siRNA. The activation of Akt kinase was analyzed by Western immunoblotting using monoclonal antibody against Phospho-Akt Ser473 or with polyclonal antibody against all Akt proteins in rabbit (Cell Signaling Technology, Beverley, MA). An anti-phospho-p70 S6 kinase (Thr) rabbit polyclonal IgG (Cell Signaling) was used to analyze the extracts treated with rapamycin. Anti-Sp1 rabbit polyclonal antibody (Active Motif Carlsbad, CA) was used to verify knockdown by RNA interference. For TIMP-3 protein levels, total cellular extracts from human chondrocytes (10–20 μg) were subjected to fractionation by a 15% SDS-PAGE mini gel (Bio-Rad system), transferred to PVDF (Pall Corporation, Anne arbor, MI) by electroblotting and reacted with the 1:400 dilution of human TIMP-3 polyclonal antibody (Chemicon International, Temecula, CA). Subsequently, membranes were incubated with the anti-rabbit secondary horseradish peroxidase-conjugated antibody (Promega, Madison WI) and the TIMP-3 protein bands revealed with the chemiluminescence detection system of Roche Biochemicals (Laval, QC) with their protocols. Control proteins that served as loading control were unaffected proteins from the Western blots of same cellular extracts. In some cases, Western blots were reprobed with monoclonal anti beta actin antibody (Sigma). Protein and RNA bands from multiple blots were scanned by using Alpha Imager 2000 (Alpha Innotech Corporation). The recorded densitometric values were converted into percentage values and presented as bar graphs using GraphPad Prism3 software (San Diego, CA).

2.4. Transient transfections

Cells were transfected with PI3K p85α SMARTpool and control siRNA (Dharmacon, Inc./Upstate Catalog # M-003020 and D-001206-13-05 respectively) or Akt siRNA and its negative control (SignalSilence Akt siRNA kit, Cell Signaling #6210) or Sp1 Validated Stealth RNAi DuoPak and its medium-GC content siRNA control (Invitrogen #12936-62, 12935-300) via calcium phosphate precipitation method for adherent cells in suspension as follows. Cells were detached by trypsinization, trypsin removed by centrifugation and 2.5×10^5 cell suspension aliquots incubated with 70 μl siRNA (200–250 nM)-calcium phosphate precipitate for 25 min with gentle rocking every 5 min, 1 ml of 10% FCS-DMEM added and plated in serum-containing medium for 3–4 h at 37 °C for adherence. Medium was removed, cells washed with PBS, allowed them to recover for 24 h, maintained in serum-free medium for 24 h and then stimulated with TGF-β for 24 h. Due to absence of matrix during transfection in suspended chondrocytes, this method results in 80% transfection efficiency as determined with a fluorescent double-stranded RNA oligonucleotide. Equal amount (20 μg) of protein was analyzed for measuring TIMP-3 protein levels as above. In other experiments, 2 μg of TIMP-3 promoter luciferase (−940 to +376 region) [32], cytomegalovirus (CMV)-*Renilla* luciferase (0.2 μg, transfection control) and Akt siRNA (200 nM) were cotransfected by the modified calcium phosphate procedure described above and after recovery, treated with inhibitors or stimulated with TGF-β and luciferase activity measured with Promega Dual-Luciferase Reporter assay System and Turner Designs Luminometer TD-20/20 according to their recommended procedures.

2.5. Measurement of Sp1 transcription factor activities

Human knee chondrocytes were either transfected with Akt siRNA or pretreated with different PI3K/Akt inhibitors and stimulated with TGF-β for 24 h. Nuclear proteins were extracted as described [33]. Equal amounts of nuclear extracts (10 μg) were used to measure Sp1 activity by using TransAM kit (Active Motif Carlsbad, CA), which is an ELISA-based colorimetric assay for measuring the binding of transcription factors with their consensus sites. After incubation of extracts with immobilized Sp1 consensus DNA for 1 h, anti-Sp1 antibody was added for 1 h followed by incubation with HRP conjugated-anti IgG, color development, stoppage of color development and measurement at OD450 by Fluostar Optima ELISA reader (BMG Lab Technologies).

All the experiments were performed at least 3 times and the results were reproducible.

3. Results

3.1. Induction of Akt phosphorylation and TIMP-3 mRNA by TGF-β1 in human articular chondrocytes

We first examined the differentiated phenotype of human chondrocytes under our experimental conditions. As determined by Western blotting, these cells at passage 3 do not express 115 kDa type I collagen band but do express high levels of Collagen II mRNA and 100–210 kDa type II collagen bands, a chondrocyte-specific marker (Fig. 1A). To examine if TGF-β1 stimulates Akt phosphorylation in human chondrocytes, quiescent cells were exposed to this factor for different time periods. TGF-β induced Akt phosphorylation above basal levels at 4 h that remained elevated up to 24 h without affecting the total Akt levels (Fig. 1B and C). TIMP-3 mRNA was also concomitantly induced by 4 h that continued to increase up to 24 h. The levels of 28S rRNA that served as loading control remained constant (Fig. 1D).

To explore the mechanism of this regulation, we first tested the ability of different PI3K and Akt pharmacological inhibitors to suppress the downstream Akt phosphorylation in human chondrocytes. ERK pathway inhibitor, PD98059 had no effect while all PI3K (Wortmannin, LY294002) and Akt (NL-71-101) inhibitors at 10 μM concentration significantly down-regulated TGF-β1-induced Akt phosphorylation demonstrating their

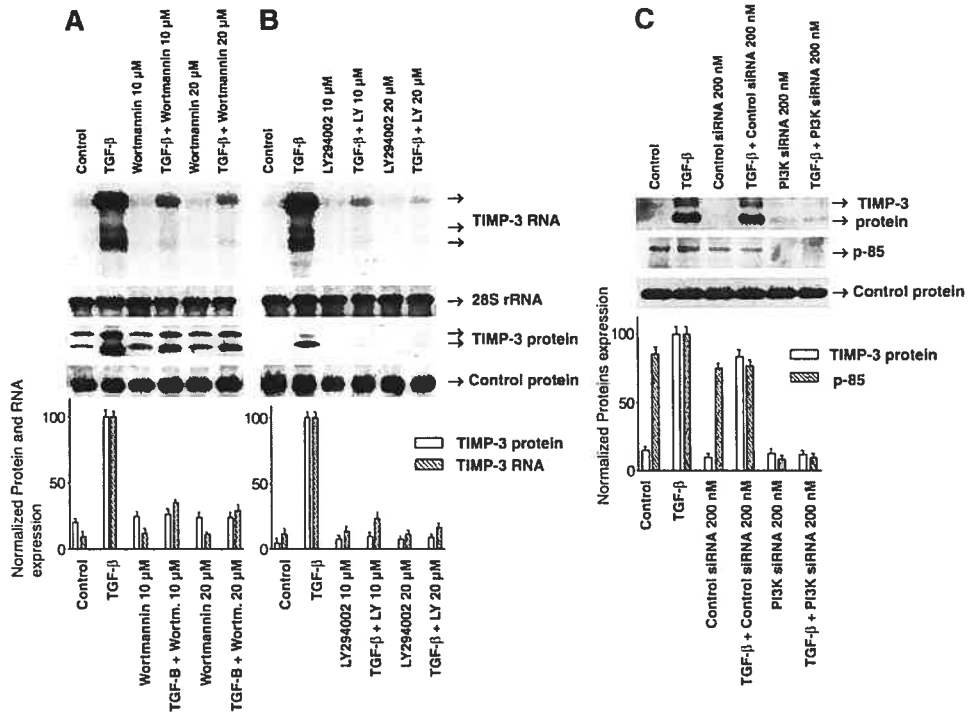

Fig. 2. PI3 kinase inhibitors, Wortmannin, LY294002 and PI3K siRNA inhibit TGF-β-induced TIMP-3 gene expression in human articular chondrocytes. Quiescent and confluent chondrocytes were either treated with vehicle (DMSO and PBS with 0.1% BSA and 4 mM HCl) as control or pretreated with 10 or 20 μM of A) Wortmannin B) LY294002 C) Or transfected with negative control siRNA and PI3K p85α SMARTpool siRNA alone for 30 min and then stimulated with TGF-β for 24 h. In A and B, upper two panels are TIMP-3 and 28S rRNA Northern blots and two lower panels represent TIMP-3 protein Western blot and control protein serving as loading control. The two TIMP-3 bands represent glycosylated (upper) and unglycosylated (lower) forms of TIMP-3 protein. In C, levels of TIMP-3 and PI3K p85 proteins measured by Western blotting are shown. Graphs A and B show densitometric normalized values of TIMP-3 protein and RNA in percentage where as graph C shows TIMP-3 protein and p85 PI3K. Data are presented as the mean±SEM of three independent experiments. $P<0.05$ was considered significant.

specificity (Fig. 1E). These and other inhibitors were used in the subsequent studies.

3.2. Suppression of TIMP-3 induction by phosphatidylinositol kinase (PI3K) inhibitors and siRNA

To investigate if PI3K is involved in TGF-β-induced TIMP-3 gene expression, human chondrocytes were pretreated with the inhibitor of PI3 kinase, Wortmannin or relatively more specific inhibitor, LY294002 for 1 h and then stimulated with TGF-β for 24 h. Both agents (Fig. 2A and B) partially or completely inhibited TGF-β-induced TIMP-3 mRNA and protein expression without affecting the constant levels of 28S ribosomal RNA and unrelated control proteins.

To further verify these results by genetic means, chondrocytes were transfected with p85α PI3K siRNA and negative control siRNA. After transfection and recovery, chondrocytes were stimulated with TGF-β for 24 h and endogenous PI3K p85 (regulatory unit) and TIMP-3 protein levels analyzed. Negative control siRNA did not affect PI3K p85 levels and TGF-β-enhanced TIMP-3 protein expression while PI3K-specific siRNA suppressed expression of both proteins. The levels of control protein remained constant (Fig. 2C). None of the above inhibitors or siRNAs significantly affected the viability of chondrocytes (results not shown).

3.3. Inhibition of TGF-β-induced TIMP3 expression by Akt/PKB inhibitor and Akt siRNA

We subsequently investigated the role of Akt in TGF-β-induced TIMP-3 increase by pretreating the chondrocytes with the specific pharmacological inhibitor of Akt, NL-71-101 (see above) and stimulating with TGF-β. NL-71-101 diminished TIMP-3 mRNA and protein induction by TGF-β without affecting the levels of control RNA and protein (Fig. 3A).

To further evaluate the validity of these results by genetic tools, chondrocytes were transfected with negative control siRNA and Akt-specific siRNA [34] and then stimulated with TGF-β. Negative control siRNA had no effect while Akt siRNA transfection drastically knocked down Akt protein levels and induction of TIMP-3 protein by TGF-β. The levels of p42 MAPK protein that served as loading control did not change (Fig. 3B). These doses of inhibitor or siRNAs did not significantly affect the viability of chondrocytes (not shown).

3.4. Influence of PI3 kinase inhibitors and Akt siRNA on human TIMP-3 promoter activity

To investigate the mechanism of PI3/Akt modulated TIMP-3 gene expression, 5′-flanking region (−940 to +376) of human TIMP-3 promoter placed upstream of luciferase gene [32] along

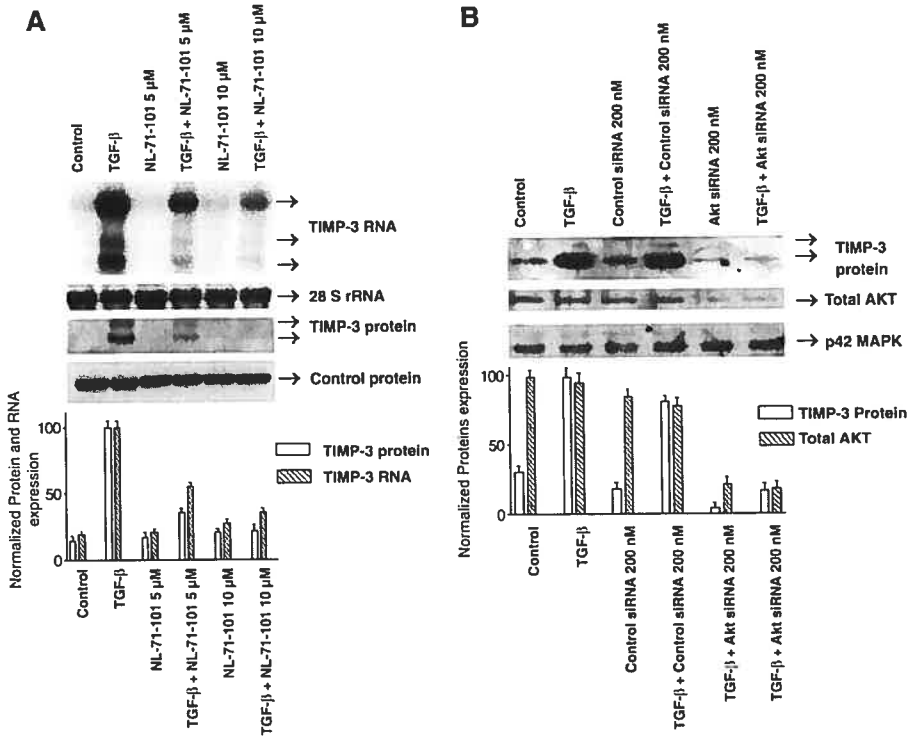

Fig. 3. Akt inhibitor, NL-71-101 and Akt siRNA inhibit TGF-β-induced TIMP-3 gene expression in human articular chondrocytes. A) Quiescent chondrocytes were either treated with vehicles as control or pretreated with 5 or 10 μM of NL-71-101 alone for 30 min and then stimulated with TGF-β for 24 h. Upper two panels depict TIMP-3 and 28S rRNA Northern blots and two lower panels represent TIMP-3 protein Western blot and control protein serving as unaffected loading control. The two TIMP-3 bands represent glycosylated (upper) and unglycosylated (lower) forms of TIMP-3 protein. B) Knee chondrocytes were either treated with vehicles (Control) or transfected with 200 nM of negative control or Akt small interfering RNA (siRNA) and then exposed to TGF-β for 24 h. The panels represent TIMP-3 protein Western blot (upper) and total Akt control protein (middle) and p42 MAPK serving as unaffected loading control (lower). Graph A shows densitometric normalized values of TIMP-3 protein and mRNA in percentage where as graph B shows TIMP-3 protein and total AKT. Data are presented as the mean±SEM of three independent experiments. $P<0.05$ was considered significant.

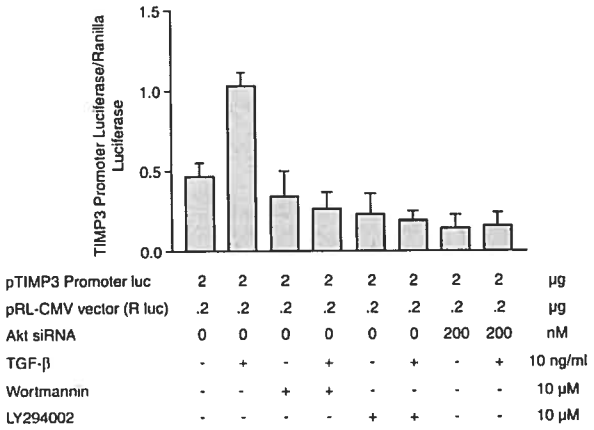

Fig. 4. PI3K/Akt inhibitors and Akt siRNA down-regulate human TIMP-3 promoter-luciferase activity in human chondrocytes. Human knee chondrocytes were transiently cotransfected with 2 μg of human TIMP-3 promoter-firefly luciferase vector, 0.2 μg of a plasmid expressing *Renilla* luciferase (internal control) and 200 nM Akt siRNA as indicated. In other cases, cells were treated with Wortmannin, LY294002 alone or with TGF-β for 24 h. Extracts were analyzed for luciferase activity. The values of firefly luciferase to *Renilla* luciferase ratios were plotted with Prism 3.0 software. Data are presented as the mean±SEM of three independent experiments. $P<0.05$ was considered significant.

with a CMV-driven *Renilla* luciferase-driven vector or Akt siRNA was transfected in human knee chondrocytes. After recovery and confluent growth, cells were also treated with PI3K inhibitors and then stimulated with TGF-β. Analysis of luciferase activity revealed induction of TIMP-3 promoter by TGF-β that was inhibited by Wortmannin, LY294002 and transfection of Akt siRNA, suggesting the role of promoter elements in PI3K/Akt-mediated TIMP-3 induction (Fig. 4).

3.5. Involvement of Sp1 transcription factor in TIMP-3 induction and decrease in TGF-β-induced Sp1 binding activity by PI3 kinase inhibitors and Akt siRNA

Human TIMP-3 promoter contains 4 Sp1 binding sites [32,35]. We have previously shown that Sp1 overexpression and pharmacological/antisense inhibition respectively increases or suppresses TIMP-3 gene induction [24]. To further explore the importance of Sp1 in TIMP-3 induction, Sp1 levels were knocked down by RNA interference with Sp1 siRNA (Fig. 5A, middle panel). Negative control siRNA did not inhibit Sp1 expression. Sp1 siRNA transfection drastically reduced the TGF-β induction of TIMP-3 (Fig. 5A, upper panel) and did not affect beta actin levels (Fig. 5A, lower panel). Viability of chondrocytes was not affected significantly by these treatments (not shown).

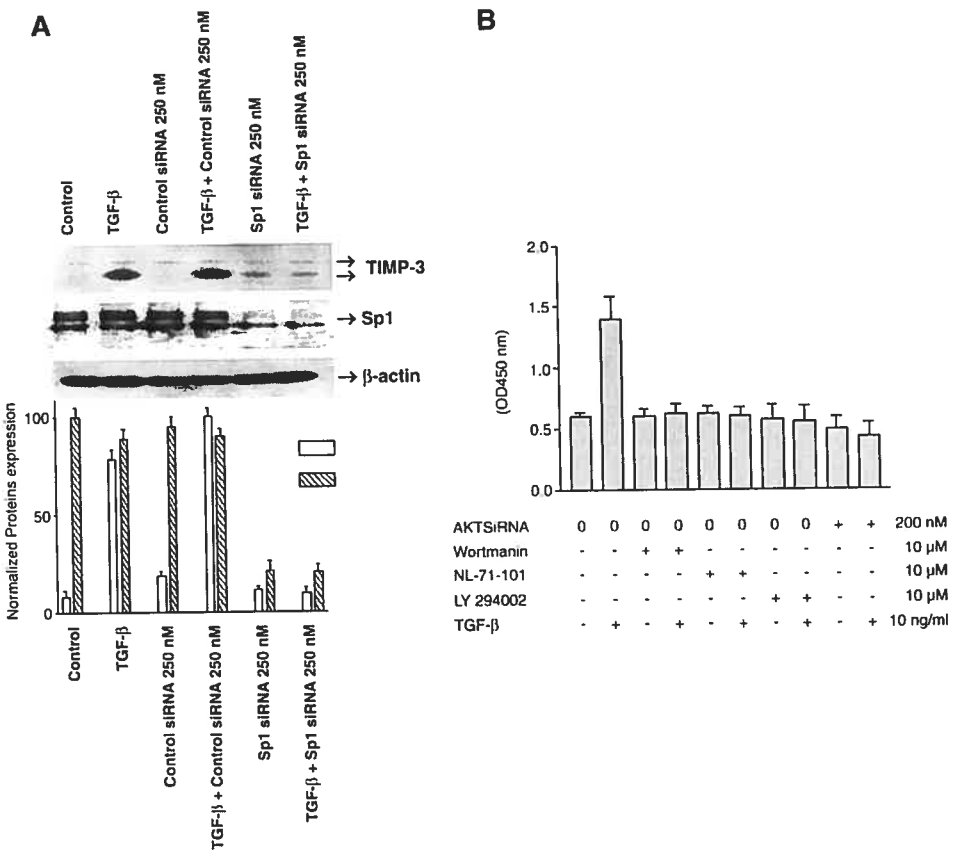

Fig. 5. A) Sp1 knockdown with Sp1 small interference RNA (siRNA) inhibits TGF-β-induced TIMP-3 gene expression in human articular chondrocytes. Quiescent chondrocytes were either treated with vehicles (Control) or transfected with 250 nM of negative control or Sp1 siRNA and then exposed to TGF-β for 24 h. The panels represent TIMP-3 protein Western blot (upper), total Sp1 protein (middle) and beta actin serving as unaffected loading control (lower). The graph shows densitometric, normalized values of TIMP-3 protein and SP1 in percentage. Data are presented as the mean±SEM of three independent experiments. $P<0.05$ was considered significant. B) Effect of Wortmannin, NL-71-101, LY294002 and Akt siRNA on Sp1 activity in chondrocytes. The human knee chondrocytes were treated as indicated and equal amount of nuclear extracts were assayed for Sp1 activity by using TransAM Sp1 Kit with an ELISA reader. Data are presented as the optical density (OD 450) mean±SEM values of three independent experiments. $P<0.05$ was considered significant.

Due to the importance of Sp1 in human TIMP-3 regulation, we explored if this transcription factor may be a possible target of PI3K/Akt pathway. To this end, we transfected human knee chondrocytes with Akt siRNA or treated with different inhibitors followed by stimulation with TGF-β. Sp1 binding activity from nuclear extracts was monitored with an Sp1 transcription factor ELISA. TGF-β increased Sp1 activity and pharmacological inhibitors as well as Akt siRNA significantly diminished binding of Sp1 with its consensus sequence (Fig. 5B).

3.6. Down-regulation of TGF-β-induced p70S6 kinase and TIMP-3 by rapamycin

Since Akt/PKB pathway stimulates multiple downstream signaling events, we investigated whether TIMP-3 induction is sensitive to rapamycin and is modulated by mammalian target of rapamycin (mTOR) [36] and p70S6K. TGF-β enhanced the phosphorylation of p70S6K and induced TIMP-3 protein levels while rapamycin (20–100 ng/ml) dose dependently reduced expression of these proteins. The control beta actin levels remained relatively constant. However, rapamycin did not diminish the induction of TIMP-3 RNA expression (Fig. 6A).

4. Discussion

TGF-β is an important pleiotropic factor involved in chondrogenesis, cartilage repair and matrix synthesis. Due to the versatile ability of TIMP-3 to inhibit cartilage-degrading MMPs and ADAMTS, and TNF-α-activating ADAM-17, it is an important therapeutic protein for arthritis. We have shown here for the first time by several pharmacological and genetic approaches that PI3K/Akt pathway mediates TGF-β-induced TIMP-3 gene expression primarily at the promoter level via Sp1 transcription factor activity. We further demonstrate that downstream of Akt, TIMP-3 is also regulated at the translation level via mammalian target of rapamycin.

Time-course studies demonstrated that TGF-β does not induce Akt phosphorylation rapidly but in a rather delayed fashion after 4 h. This is in contrast with Smad2 and ERK phosphorylation that is induced by TGF-β rapidly within 20 min to 1 h [23,24]. This suggests early events such as cross talk with other pathways or intermediate factors. However, TGF-β did not induce insulin-like growth factor (IGF-1) at any time point between 20 min to 24 h and IGF-1 also did not enhance TIMP-3 expression at various doses. Furthermore,

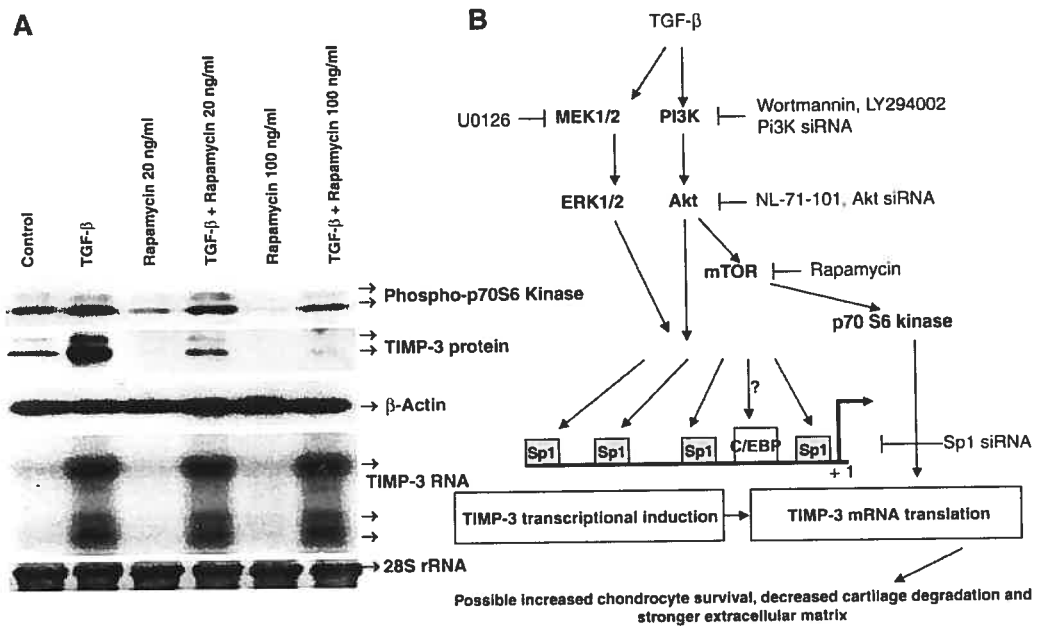

Fig. 6. A) Rapamycin down-regulates TGF-β-induced phosphorylation of p70S6 kinase and TIMP-3 protein but not its RNA expression in human articular chondrocytes. Quiescent chondrocytes were either treated with vehicle (DMSO and PBS with 0.1% BSA and 4 mM HCl) as control or pretreated with 20 or 100 ng/ml of rapamycin alone for 1 h and then stimulated with TGF-β for 24 h. The upper three panels depict phosphorylated p70S6 kinase, TIMP-3 protein and beta actin Western blots. The lower two panels are TIMP-3 Northern blot and 28S ribosomal RNA loading control. B) Proposed model for TIMP-3 induction by TGF-β via activation of PI3K/Akt and ERK pathways. The activation steps are indicated by arrows, which could culminate in increased chondrocyte survival and stronger extracellular matrix. The inhibitors which down-regulate TIMP-3 mRNA or protein expression are also depicted. mTOR, mammalian target of transcription. The model does not rule out the possible cross talk between the two pathways. Sp1 and CCAAT/enhancer binding protein (C/EBP) sites in the human TIMP-3 promoter and transcription initiation site (+1) are also shown.

IGF-1 receptor blockade did not affect induction of TIMP-3 by TGF-β. Thus TGF-β effects do not seem to be mediated by IGF-1 (results not shown). TIMP-3 and TGF-β are multifunctional proteins. Both have been shown to induce apoptosis in different cell-types [37]. In contrast with these observations, stimulation of PI3K/Akt by TGF-β in chondrocytes demonstrated here suggests a pro-survival role for this factor in chondrocytes. Indeed, TGF-β is considered as a cartilage growth-promoting factor [5]. It has been shown that binding of Akt with nonphosphorylated Smad3 blocks TGF-β-induced Smad3 phosphorylation, its interaction with Smad4, nuclear translocation and apoptosis in certain cell-types [38]. However, in chondrocytes, TGF-β-stimulated Smad2 and ERK phosphorylation is followed by Akt phosphorylation and these pathways or their interactions are needed for TIMP-3 induction [this work, [23,24]]. Treatment of porcine chondrocytes with N-terminal human TIMP-3 did not show any significant apoptosis [16]. Similarly, TIMP-3 may also be anti-apoptotic, as knockout of this gene results in increased apoptosis in mammary gland [39]. This is further supported by another study where TIMP-3 promoted proliferation of non-transformed cells under low-serum condition [40]. TIMP-1 has also been shown to induce cell survival by PI3K/Akt pathway [41]. Whether these apparently paradoxical results are due to differences in endogenous and exogenous TIMP-3 expression levels, remains to be studied further. It is possible that physiological levels of TIMP-3 promote survival and ectopic overexpression at non-physiological levels induces apoptosis.

Concomitant induction of Akt phosphorylation and TIMP-3 suggests direct correlation between the two events as demonstrated by subsequent experiments. Inhibition of TIMP-3 mRNA and protein by two different pharmacological PI3K inhibitors and siRNA strongly support the role of PI3K in TIMP-3 induction. Similarly, suppression of TGF-β-induced TIMP-3 mRNA and protein expression by Akt/PKB inhibitor and Akt siRNA reinforces the involvement of Akt/PKB in TGF-β signal transduction leading to TIMP-3 induction. Based upon inhibition by Wortmannin and LY294002, PI3K/Akt pathway requirement was shown in IGF-1-induced proteoglycan synthesis [42]. It is likely that several growth factors utilize this pathway to promote ECM synthesis, growth and survival. TIMP-3 promoter-reporter transfection studies suggest that the 5′ flanking region spanning −940 to +376 contains most of the TGF-β-responsive sequences. This is in agreement with a previous study where region from −463 to +1 was shown to be responsible for serum-stimulated cell cycle progression; serum contains a heterogeneous mixture of growth factors [35]. It has been previously shown that TIMP-3 is a cell cycle-regulated gene [43]. Mimicry of exogenous TIMP-3 promoter regulation with that of endogenous TIMP-3 gene suggests that inhibition most likely takes place at the transcription level. Inhibition of TIMP-3 promoter-driven luciferase activity by the pharmacologic inhibitors and Akt siRNA also supports the

notion that this promoter region may be the target of TGF-β-stimulated PI3K/Akt pathway. Human TIMP-3 promoter has several putative transcription factor binding sites that may be the targets of this cascade. It has 4 Sp1 binding sites between −112 to +1 region [35], which are important mediators of TIMP-3 expression [24]. The latter results were reconfirmed here by the newly developed RNA interference-mediated Sp1 knockdown approaches. Increased Sp1 activity by TGF-β and its decrease by the inhibitors and Akt siRNA further support Sp1 as a possible target of Akt pathway. Sp1 is a pivotal factor for the expression of various genes related to ECM synthesis, cell cycle and growth [44,45]. In other systems, increased expression of vascular endothelial growth factor by Akt required Sp1 as demonstrated by Sp1 siRNA-driven knockdown [46]. Similarly, a gene array profiling study showed Sp1 binding site in the promoter of *Fra-1* gene as the target of PI3K pathway activation [47]. Other potential targets include CCAAT enhancer binding protein (C/EBP) site at −40 region of human TIMP-3 promoter.

Multiple targets downstream of Akt/PKB involved in growth, proliferation, survival and protein synthesis are activated. Phosphorylation of p70S6 kinase by TGF-β and its dose-dependent inhibition by rapamycin suggest that TIMP-3 induction occurs in part via mTOR and p70S6 kinase. This mechanism appeared to be specific, as the levels of β-actin were not affected. Since this pathway is involved in cell growth, survival, mRNA translation and ribosome biogenesis [48], TIMP-3 protein inhibition may be due to decrease in its translation by rapamycin as this drug did not affect TIMP-3 mRNA induction levels. Alternatively, rapamycin may inhibit cell cycle-regulated TIMP-3 by interfering with cell-cycle progression. These results also suggest TIMP-3 as a target of this immunosuppressant drug. Interestingly, IGF-1-induced proteoglycan synthesis in chondrocytes is also inhibited by rapamycin possibly at the translation level [42].

5. Conclusions

In conclusion, TGF-β1 could exert its growth-promoting effects on chondrocytes by activating PI3 kinase/Akt pathway and TIMP-3 expression, which by binding to heparan sulfate and chondroitin sulfate may result in strengthening of cartilage ECM, increased chondrocyte viability and maintenance of joint tissue integrity (Fig. 6B). The abilities of TIMP-3 to block MMP and ADAMTS activities could be an added benefit. It is also interesting to note that in contrast with the cell proliferation and survival-associated PI3Kα and β isoforms, knockout and specific pharmacological inhibition of PI3Kγ isoform has recently been shown to reduce both synovial inflammation and cartilage erosion in RA-like mouse models [49]. Thus, specific isoforms of this pathway have different functions and can be targeted for reducing inflammation or enhancing survival. Further, observed suppression of arthritic inflammation by TGF-β could in part be due to increased TIMP-3 and reduced TACE and TNF-α activities. Thus, a better understanding of the mechanisms of TIMP-3 induction by TGF-β can lead to novel therapies for inhibiting synovial hyperplasia/inflammation and for stimulating cartilage regeneration, the two important goals in treating arthritis.

Acknowledgements

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3. Article 3

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Smad signaling pathway is a pivotal component of tissue inhibitor of metalloproteinases-3 regulation by transforming growth factor beta in human chondrocytes

Hamid Yaqoob Qureshi and Muhammad Zafarullah

From the Department of Medicine, University of Montreal and Research Centre of Centre Hospitalier de l'Université de Montréal (CRCHUM), Montreal, Quebec, Canada, H2L 4M1

*To whom corresponding should be addressed: K-5255 Mailloux, Hôpital Notre-Dame du CHUM, 1560 Sherbrooke est, Montréal, Québec, Canada H2L 4M1. Phone: 1-514-890-8000 ext. 25690; Fax: 1-514-412-7612;

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Running Title: TGF-β regulation of TIMP-3 by Smad pathway

ABSTRACT

Transforming growth factor beta (TGF-β1) promotes cartilage extracellular matrix synthesis and induces tissue inhibitor of metalloproteinases-3 (TIMP-3), a natural inhibitor of matrix metalloproteinases, aggrecanases and tumor necrosis factor alpha converting enzyme, which are implicated in articular cartilage degradation and joint inflammation. TGF-β1 activates Akt, ERK and Smad pathways in chondrocytes. However, specific role of Smad pathway in TIMP-3 induction by TGF-β1 has not been investigated which was explored here by pharmacological and genetic knockdown approaches. TGF-β1-enhanced Smad2 phosphorylation and TIMP-3 protein expression could be inhibited by the Smad2/3 phosphorylation inhibitors, PD169316 and SB203580 but not by an inactive homologue, SB202474. Similarly, Smad2-specific siRNA-driven Smad2 deficiency also down-regulated TIMP-3 induction. Specific inhibitor of Smad3 (SIS3) and Smad3 siRNA abolished TGF-β induction of TIMP-3. Negative control siRNAs had no effect. Smad2 and 3 siRNAs also down regulated TIMP-3 promoter-driven luciferase activities, suggesting transcriptional regulation. SiRNA-driven co-Smad4 knockdown abrogated TIMP-3 augmentation by TGF-β. TIMP-3 promoter deletion analysis revealed that –828 deletion retains the original promoter activity while –333 and –167 deletions display somewhat reduced activity suggesting that most of the TGF-β-responsive, cis-acting elements are found in the –333 fragment. Chromatin Immunoprecipitation (ChIP) analysis confirmed binding of Smad2 and Smad4 with the –940 and –333 promoter sequences. These results

suggest that receptor-activated Smad2 and Smad3 and co-Smad4 mediate TGF-β-stimulated TIMP-3 gene expression in human chondrocytes and TIMP-3 gene is a target of Smad signaling pathway.

INTRODUCTION

Degradation of cartilage extracellular matrix (ECM) by proinflammatory cytokine-stimulated matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS or A Dystrophin And Metalloproteinase with ThromboSpondin motif) (1,2) is observed in inflammatory rheumatoid arthritis (RA) and aging-associated osteoarthritis (OA), the most prominent forms of arthritis in clinic (3,4). Cartilage from older patients has limited capacity to regenerate possibly due to reduced transforming growth factor beta (TGF-β), its type II receptor and resulting chondrocyte hyporesponsiveness (5). TGF-β superfamily members stimulate chondrogenesis during development and maintain cartilage integrity in adults (6,7). In rat models, it suppresses acute and chronic arthritis by counteracting the effects of the proinflammatory, interleukin-1 (IL-1) (8); an observation not reproduced in older mice (9). Elevated TGF-β levels in human rheumatoid synovial fluid activates synovial fibroblasts, which ultimately invade and destroy cartilage (10-12). Excessive TGF-β induces formation of joint outgrowths called osteophytes, which limit joint movement and cause pain (13). Inhibition of endogenous TGF-β

by scavenging type II receptor or by its signaling antagonist, Smad7, in a papain-induced murine arthritis model prevented osteophyte formation, synovial thickening and impaired cartilage repair, suggesting its crucial role in these pathological and physiological processes (14,15). In a rabbit model, TGF-β failed to repair cartilage and induced adverse effects such as cartilage degradation, muscle edema, fibrogenesis and chondrogenesis in synovial lining (16). Multiple TGF-β injections into the murine knee joints induce OA-like changes in the articular cartilage and surrounding tissue, suggesting a role of TGF-β in OA (17). Thus, in small amounts, TGF-β is implicated in suppression of arthritis and cartilage remodeling. TGF-β1 is also a favorite stimulus for regenerating high-quality cartilage (18).

TGF-β exerts its effects by inducing multiple genes including tissue inhibitor of metalloproteinase-3 (TIMP-3), a member of TIMP family of proteins (19,20). TIMP-3 protein is uniquely located in ECM where its N- and C-terminal domains (21) bind to chondroitin- and heparan sulfate (22). In cartilage explants, TIMP-3 inhibits MMP-13 and ADAMTS4, the principal proteases causing collagen and aggrecan degradation in arthritis (23-25). It also inhibits through different mechanism (26), the TNF-α converting enzyme (TACE or ADAM-17) that activates membrane-bound precursor of proarthritic cytokine, TNF-α into soluble secreted form (27,28). Thus, TIMP-3 can regulate TNF-α-driven inflammation. TIMP-3 knockout mice display an increased initial inflammation and TNF-α level in antigen-induced inflammatory arthritis, supporting its protective function. These

mice are more susceptible to age-dependent cartilage degradation (29,30). These distinct features make TIMP-3 a potentially important therapeutic protein in arthritis (31,32). Indeed, TIMP-3 overexpression prevents invasion of cartilage by human rheumatoid synovial fibroblasts in a mouse model (33).

Due to therapeutic potential of TIMP-3, it is important to understand mechanisms of TGF-β induction of TIMP-3. In other cell types, TGF-β first binds with type II receptors and then phosphorylates and activates type I receptor on serine and threonine, leading to transcription of the target genes via stimulatory Smad2/3 and co-Smad4 (34,35). Recently, TβRII-B a type II receptor variant was found to complex with other TGF-β receptors such as betaglycan and endoglin to enhance signal transduction (36). Smad2 and -3 are found in association with proliferating and maturing chondrocytes respectively (37). Smad3 knockout mice display OA-like symptoms (38). We previously showed Smad2 phosphorylation by TGF-β in bovine and human chondrocytes (39). Subsequently, we demonstrated the essential role of extracellular-signal-regulated kinase (ERK)-mitogen-activated protein kinases (MAPK), PI3K/Akt pathway and Sp1 transcription factor in TGF-β-induced TIMP-3 gene in chondrocytes (40,41). Here we show for the first time that Smad pathway is critically involved in TGF-β-enhanced TIMP-3 gene expression.

EXPERIMENTAL PROCEDURES

Cell culture and treatments-Normal human knee chondrocytes (Cambrex, Walkerville, MD) were grown in Differentiation Bullekit medium as high-density short-term monolayer cultures in 6-well plates up to passages 2 and 3. Under these culture conditions, they continue to express collagen II, a differentiated chondrocyte marker (41). Following confluent growth, chondrocytes were maintained in serum-deficient Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Life Sciences Inc, Burlington, ON) for 24 h and subjected to different treatments. In some cases, chondrocytes were pretreated in serum-free medium with the Smad2/3 pharmacological inhibitors, PD169316, SB203580, SB202474 or SIS3 (Calbiochem, La Jolla, CA) for 60 min and then stimulated with 10 ng/ml of TGF-β1 (R & D Systems, Minneapolis MN) for 20 min (for Smad phosphorylation) or 24 h (for TIMP-3 protein expression). Control cells received equivalent amounts of the vehicle, dimethyl sulfoxide (DMSO) and 0.1% fraction V of bovine serum albumin (BSA) and 4 mM HCl.

Western blotting-Rabbit polyclonal anti-human antibodies for phosphorylated Smad2 (#3101S) and Smad3 (#9514S) and total Smad2 (#51-1300, Zymed laboratories), Smad3 (#AB3817, Chemicon International, Temecula, CA), Smad4 (# 9515, Cell Signaling Technology, Beverly MA, 1:400 dilution) and Smad7 (H-79, Santa Cruz Biotechnology Inc., Santa Cruz CA) were used as before at dilutions (mostly 1:400) recommended by the manufacturers. For TIMP-3 protein levels, total cellular extracts from human chondrocytes (20-30 μg) were

fractionated by a 15% SDS-PAGE mini gel system (Bio-Rad Laboratories Canada Ltd., Mississauga ON), transferred to PVDF (Pall Corporation, Anne arbor, MI) by electroblotting and reacted with the 1:400 dilution of human TIMP-3 polyclonal antibody (Chemicon International, Temecula, CA). Subsequently, membranes were incubated with the anti-rabbit secondary horseradish peroxidase-conjugated antibody (Promega, Madison WI) and the TIMP-3 protein bands revealed with the chemiluminescence detection system of Roche Biochemicals (Laval, QC) with their protocols. In some cases, Western blots were reprobed with monoclonal anti beta actin antibody (Clone AC-15, 1: 10000 dilution, Sigma-Aldrich, Oakville ON).

Transfections and luciferase assays-Validated Stealth DuoPak Smad2 (#12937-09), Smad3 (#12937-10) and Smad4 (#12938-126) siRNAs and medium-GC content negative control siRNA (#12935-300) were transfected by calcium phosphate precipitation method for adherent cells in suspension as described before (41). Briefly, chondrocytes were released by trypsinization and 2.5 X 10^5 cell suspension aliquots incubated with 70 μl siRNA (200-250 nM)-calcium phosphate precipitate for 25 min with gentle agitation every 5 min, 1 ml of 10% FCS-DMEM added and plated in serum-containing medium for 3-4 h at 37°C for adherence. Medium was removed, cells washed with PBS, allowed them to recover for 24 h, maintained in serum-free medium for 24 h and then stimulated with TGF-β for 24 h. Due to absence of matrix during transfection in suspended chondrocytes, this method results in 80% transfection efficiency as determined with a fluorescent

double-stranded RNA oligonucleotide. In other experiments, 2 μg of TIMP-3 promoter luciferase (-940 to +376 region) (42), cytomegalovirus (CMV)-driven Renilla luciferase (0.2 μg, transfection control) and rerspective siRNA (200 nM) were cotransfected by the modified calcium phosphate procedure and after recovery, stimulated with TGF-β and luciferase activity measured with Promega Dual-Luciferase Reporter assay System (Promega, Madison WI) and Turner Designs Luminometer TD-20/20 as recommended.

Construction of promoter deletions and analysis-The original TIMP-3 promoter plasmid obtained from Dr Jean Bennet (42) was a Sac I-Sac I fragment from –940 to +376 regions. To generate shorter 5' fragments, the sequence was entered in the http://rebase.neb.com (New England Biolabs) database to find unique restriction sites. Three unique sites including Asc I (at position –167), MSc I (at position –333) and Kpn I (at position –828) were found and confirmed by double restriction enzyme digestion (each of the three enzymes and Hind III site in the polylinker region of pGL-3 basic vector) and 1.5% agarose gel analysis. The Asc I-Hind III (-167 to +376), MSc I-Hind III (-333 to +376) and Kpn I-Hind III (-828 to +376) were eluted and cloned into polylinker sites of pGL3-Basic. Cloning of the three fragments was confirmed by rapid plasmid extraction and double digestion with Kpn I-Hind III analysis of numerous clones, along with the original TIMP-3 promoter plasmid with the Hind III and respective enzymes. These constructs were purified by Qiagen QIAfilter Plasmid Midi Kit (Qiagen Inc. Canada, Mississauga ON) and transfected as described above.

Chromatin Immunoprecpitation (ChIP) analysis-Control and TGF-β stimulated chondrocytes were fixed with 1% formaldehyde treatment, cross-linked chromatin prepared with ChIP-IT and sheared with enzymatic shearing kit (Active Motif Cat#53006 and 53007) and DNA-protein complexes immunoprecipitated with negative control IgG or with Smad2, Smad3, Smad4 and SP1 antibodies. After proteinase K digestion, extraction and purification of DNA, TIMP-3 promoter fragments were amplified by PCR with the TIMP-3 promoter-specific primers from the –167, -333 and –940 and +1 regions and fragments detected by 2% agarose gel analysis.

All the experiments were performed at least 3 times and the results were reproducible.

RESULTS

Role of Smad2 phosphorylation in TGF-β-stimulated TIMP-3 induction in human chondrocytes-We previously showed that Smad2 is phosphorylated in response to TGF-β1 treatment (39). As reconfirmed in Fig 1, this observation is highly reproducible. To investigate the possible involvement of Smad2 in TGF-β-induced TIMP-3 gene expression, chondrocytes were pretreated with previously known inhibitors of Smad2/3 phosphorylation (43), PD169316 and SB203580 as well as their inactive analogue, SB202474 for 1 h and then stimulated with TGF-β for 20 min. As shown in Fig 1A, TGF-β-stimulated Smad2 phosphorylation was

inhibited by the 10-μM dose of PD169316 and SB203580 but not by SB202474. The levels of total smad2 remained relatively constant (Fig 1A, lower panel). Thus, the role of Smad2/3 phosphorylation could be tested with these pharmacological inhibitors.

To further investigate the role of Smad2/3 in TIMP-3 gene regulation, chondrocytes were pretreated for 60 min with the above-tested pharmacological inhibitors and then stimulated with TGF-β for 24 h. Analogous to the results of Smad2, TGF-β-induced TIMP-3 protein expression could be inhibited by the Smad2/3 phosphorylation inhibitors, PD169316 and SB203580 but not by its inactive homologue, SB202474. The levels of constitutively expressed β-actin levels remained unaffected (Fig 1B). As these inhibitors are also known to block p38 phosphorylation, role of Smad2 was further explored by the more specific genetic approach of RNA interference (RNAi).

Blockade of TGF-β-stimulated TIMP-3 increase by Smad2 siRNA-mediated knockdown-We recently modified the classical Calcium phosphate transfection procedure to conduct highly efficient transfections in chondrocytes by first releasing them from their matrix and then transfecting with nucleic acids in cell suspension followed by their re-adherence in monolayers (41). Transfection of Smad2-specific siRNA resulted in Smad2 knockdown while negative control siRNA did not block Smad2 expression. Smad2-deficient chondrocytes displayed severe down-regulation of TIMP-3 induction. Non-specific siRNA did not affect

TIMP-3 induction thus demonstrating specific effects of Smad2 siRNA. The levels of β-actin were not affected by any of the treatments (Fig 2).

Suppression of TGF-β-induced TIMP-3 expression by specific inhibitor of Smad3 (SIS3)-We investigated the role of the other stimulatory Smad, Smad3, in TIMP-3 induction. Jinnin et al have recently described the specific inhibitor of Smad3 (SIS3), which selectively abrogates Smad3 phosphorylation without affecting Smad2 activation (44). Pretreatment with SIS3 dose-dependently inhibited TGF-β-induced Smad3 phosphorylation (Fig3A) and TIMP-3 protein (Fig 3B) expression without affecting the constitutive Smad3 or β-actin levels.

Abrogation of TGF-β-induced TIMP-3 expression by Smad3 siRNA-To further validate the role of Smad3 in TIMP-3 induction by the recently introduced genetic tool of RNA interference, chondrocytes were transfected with Smad3 siRNA or its negative control and then induced with TGF-β. Smad3 siRNA but not its negative control siRNA drastically inhibited Smad3 phosphorylation and total Smad3 expression (Fig 4A). Induction of TIMP-3 was similarly abolished by Smad3 siRNA but not by the negative control siRNA (Fig 4B). The levels of β-actin in these extracts remained constant.

Inhibition of human TIMP-3 promoter activity by Smad2 and Smad3 siRNAs-To gain further insight into the mechanism of Smad-mediated TIMP-3 regulation, we cotransfected chondrocytes with Smad2 or Smad3 siRNA along with the human TIMP-3 promoter (-940 to +376)-luciferase construct (42) followed by induction with TGF-β. As reported previously, TGF-β induces TIMP-3

promoter-driven luciferase-activity (40). Negative control siRNA had very little effect while Smad2 and Smad3 siRNAs significantly inhibited promoter activity (Fig 5). These results with exogenous TIMP-3 promoter mimic the endogenous pattern of TIMP-3 protein expression.

Inhibition of TGF-β-stimulated TIMP-3 enhancement by Smad4 siRNA-Since stimulatory Smad2 and Smad3 interact with co-Smad4 for TGF-β signal transduction, we investigated the role of Smad4 in TIMP-3 induction. Transfection of Smad4 siRNA resulted in abrogation of the corresponding protein expression that was not affected by the negative control siRNA. Smad4-specific siRNA and not its negative control siRNA diminished TGF-β-induced TIMP-3 protein expression in human knee chondrocytes without affecting the constitutive β-actin levels (Fig 6).

Identification of minimal TGF-β responsive TIMP-3 promoter-Considering the responsiveness of the larger (-940 to +376) human TIMP-3 promoter to TGF-β, we investigated the identity of minimal region responsible for TIMP-3 induction by this growth factor. Using the convenient restriction enzyme sites, we constructed a series of TIMP-3 promoter deletion mutants. They span –828 to +376 (named Δ–828), –333 to +376 (named Δ–333) and –167 to +376 (called Δ–167) regions (Fig 7A). Functional significance of these promoter mutants was tested by transient transfections and analysis of luciferase activity, which revealed that –828 deletion retained the original promoter activity while –333 and –167 deletions displayed somewhat reduced activity. Thus, most of the TGF-β-responsive region is localized

within the -333 region and additional cis-acting elements may be present in the upstream regions (Fig 7B).

Confirmation of Smad interaction with the TIMP-3 promoter by ChIP analysis-To investigate whether Smads indeed bind with the TIMP-3 promoter region in vivo, chromatin from untreated and TGF-β-treated chondrocytes was immunoprecipitated with different Smad antibodies. Analysis of DNA in the DNA-protein complex with specific primers (-940 to +1, -333 to +1 and –176 to +1) revealed that Smad2 and Smad4 antibodies were able to immunoprecipitate –940 and –333 TIMP-3 promoter fragments, thus confirming binding. The expected Smad3 binding could not be demonstrated possibly due to lack of ChIP-quality antibody (Fig. 8). The –167 fragment which contains Sp1 binding sites immunoprecipitated with the Sp1 antibody.

DISCUSSION

TGF-β is an important growth factor for cartilage development (chondrogenesis), its maintenance and regeneration. Similarly, TIMP-3 is a major natural inhibitor of MMPs, aggrecanases and TACE, the enzymes implicated in cartilage degradation and joint inflammation. By several pharmacological and genetic approaches, we have demonstrated for the first time that Smad pathway is a pivotal mediator of TIMP-3 regulation by TGF-β and TIMP-3 gene is a Smad pathway target. We have further shown that the regulation is primarily at the

promoter level and most of the TGF-β responsive elements could be found within the –333 to +1 region of the promoter where Smads bind in vivo.

Inhibition of TIMP-3 induction by pharmacological inhibition of Smad2 and Smad3 phosphorylation strongly suggested the implication of Smad pathway in TIMP-3 regulation. This inhibition was specific, as the equivalent amount of an inactive inhibitor did not suppress Smad2 phosphorylation and TIMP-3 induction. These results were reinforced by the alternative and more powerful genetic approach of Smad2 and Smad3 siRNA-mediated knockdown. Similar induction of endogenous TIMP-3 and exogenous TIMP-3 promoter-luciferase activity by TGF-β and their inhibition by Smad2 and Smad3 siRNA suggests that TIMP-3 promoter is responsive to these agents and regulation most probably occurs at the level of promoter as Smad2 and possibly Smad3 bind with the TIMP-3 promoter sequences. Lack of Smad3 binding in ChIP anlaysis may be possibly due to quality of the available Smad3 antibody which is good enough for Western blotting but not for the ChIP analysis. Thus both receptor-activated stimulatory Smad2 and Smad3 may be required for TGF-β-induction of TIMP-3. This is in contrast with Smad2 and Smad3 knockout murine embryonic fibroblasts where selective requirement of Smad2 for MMP-2 and that of Smad3 for c-fos, Smad7 and TGF-β was observed (45).

Smad2 phosphorylation is reduced during the progression of murine OA (46). Smad2 deficiency delays TGF-β-mediated Meckel's cartilage development showing its strong association with different types of cartilage (47). In this respect,

it is interesting to note that Smad3 knockout mice display enhanced chondrocyte maturation and develop osteoarthritis-like degenerative symptoms, suggesting that Smad3 is required for the maintenance of articular cartilage (38,48). Smad3 overexpression in mesenchymal stem cells leads to primary chondrogenesis (49). TGF-β binding of an ECM protein, asporin, with aspartic acid repeats polymorphism leads to greater inhibition of TGF-β signaling and increased susceptibility of certain Japanese patients to hip and knee OA (50). Diminished TGF-β receptor in older mice may be one of the causes of OA development (51). Thus lack or alteration in TGF-β signaling and its target genes could severely impair the cartilage homeostasis. Indeed, TIMP-3 knockout mice display increased collagen and aggrecan degradation with aging in a manner similar to osteoarthritic patients (30). In a gene array study, TIMP-1 was found to be a target of Smad3 in human dermal fibroblasts (52). In contrast, TIMP-1 is still induced by TGF-β in Smad knockout cells (53). Smads were also shown to bind with the rat TIMP-1 promoter (54). Smad signaling is also required for TGF-β induction of MMP-1 and MMP-13 in chondrocytes, squamous carcinoma cells and dermal fibroblasts (55-57).

Smad4 interaction with Smad2/3 is required for TGF-β signal transmission (58). Constitutive Smad4 protein expression is in agreement with a previous study where Smad4 mRNA expression was shown in both normal and OA chondrocytes without any major differences (59). Coordinate inhibition of TIMP-3 in Smad4-depleted cells strongly support the requirement of Smad4 for induction of TIMP-3

by TGF-β in articular chondrocytes. This is in contrast with the well-known TGF-β responsive plasminogen activator inhibitor (PAI) gene, which is induced in the absence of Smad4 (60), though both Smad3 and Smad4 bind with the promoter of the PAI gene (61). Smad4 knockout animals are dwarf and have disorganized growth plate suggesting its requirement for hypertrophic chondrocyte differentiation and proper growth plate organization (62). Smad7 overexpression downregulated chondrocyte proliferation, PAI gene induction and proteoglycan synthesis (63). Smad7 overexpression can also block synovial fibrosis while maintaining TGF-β-dependent cartilage repair (64). However, its partial inhibition by siRNA had no effect in our system (results not shown).

The TGF-β responsive elements in human TIMP-3 promoter have not been defined. Sp1 sites in this fragment are important for its regulation by ERK and Akt pathways (40, 41). By constructing serial 5' deletions of the larger TIMP-3 promoter fragment and by conducting transfections of the deletion mutants, we were able to define minimal 333 bp TGF-β-responsive region. Additional upstream sequences may also be important for full responsiveness to TGF-β. This region contains Sp1 and some partial Smad binding elements (SBEs). The –167 fragment binds with Sp1 only and not with Smads. One possibility is that Smad2/3/4 complex bind directly with SBEs in the TIMP-3 promoter region. ChIP analysis supported this view. Alternatively, Smads may interact with Sp1 to activate TIMP-3 promoter. Smad3-Sp1 interaction occurs in the TGF-β-induced tenascin-C promoter (65). Smad3-Sp1 interaction is needed for full erythropoietin promoter

activity (66) and the well-characterized, TGF-β-induced PAI (67) and collagen (68) promoters.

We have previously shown the requirement of Erk and Akt pathways for TGF-β induction of TIMP-3. Induction of multiple pathways by TGF-β in association with TIMP-3 has also been observed in other systems. Both Smad and p38 pathways are known to independently and additively regulate excessive deposition of collagen I in hepatic stellate cells during liver fibrosis (69). In TGF-β induction of biglycan in pancreatic cells, p38 was activated downstream of Smad pathway (70). Aggrecan induction by TGF-β in chondrocytic cells occurs by cross talk of Smad, ERK and p38 pathways (71). Both ERK and Smad2 are activated during TGF-β-induced chondrogenesis (72). Thus multiple pathways and their interactions may activate the human TIMP-3 gene.

In summary, we have shown by pharmacological inhibition and RNA interference-mediated knockdown approaches that TGF-β receptor-activated Smad2 and Smad3 and co-Smad4 are required for induction of TIMP-3 expression and promoter activity by TGF-β. We have also identified a minimal TIMP-3 promoter fragment needed for this TGF-β response where Smad2 and Smad4 bind in vivo. Thus TIMP-3 gene is a target of the Smad pathway. These mechanisms of TIMP-3 regulation may be pivotal in TGF-β-stimulated cartilage ECM synthesis, repair and maintenance.

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Figure Legends

Figure 1. PD169316 and SB203580 but not the inactive analogue, SB202474 inhibit TGF-β-induced Smad2 phosphorylation and TIMP-3 protein expression without affecting constitutive beta-actin levels in human articular chondrocytes.

Quiescent confluent chondrocytes were either treated with vehicle (DMSO and PBS with 0.1% BSA and 4 mM HCl) as control or pretreated with 5 or 10 μM of PD169316, SB203580 and SB202474 alone for 60 min and then stimulated with TGF-β for 20 min (Smad2) or 24 h (TIMP-3). **A)** Upper two panels are phospho- (p-Smad2) and total Smad2 Western blots while **B)** lower two panels represent TIMP-3 and beta-actin Western blots. The lower graphs shows densitometric normalized value of pSmad-2 and TIMP-3 proteins in percentage. Data are presented as the mean ± SEM of three independent experiments. $P< 0.05$ was considered significant.

Figure 2. Smad2-specific siRNA and not its negative control siRNA suppresses Smad2 expression and TGF-β-induced TIMP-3 protein expression in human knee chondrocytes without affecting constitutive beta-actin levels. Articular chondrocytes were either treated with vehicles (Control) or transfected with 200 nM of negative control or Smad2 small interfering RNA (siRNA) and then exposed to TGF-β for 24 h. The panels represent Smad2 (upper), TIMP-3 and beta actin (lower) western blots. The lower graph shows densitometric normalized value of Smad-2 and TIMP-3 proteins in percentage. Data are presented as the mean ± SEM of three independent experiments. $P< 0.05$ was considered significant.

Figure 3. Specific inhibitor of Smad3 (SIS3) dose-dependently inhibits TGF-β-induced Smad3 activation and TIMP-3 protein expression in human knee chondrocytes. Quiescent chondrocytes were either treated with vehicle (control) or pretreated with 5 or 10 μM of SIS3 alone for 60 min and then stimulated with

TGF-β for 20 min (A) or 24 h (B). In A) upper and lower panels represent phosphorylated and total Smad3 levels respectively. In B, TIMP-3 and beta-actin Western blots are shown. Dose-dependent Smad3 phosphorylation and TIMP-3 inhibition by SIS3 without affecting constitutive beta actin can be noticed. Graphs A shows densitometric normalized value of pSmad-3 in percentage where as graph B shows TIMP-3 proteins. Data are presented as the mean ± SEM of three independent experiments. $P< 0.05$ was considered significant.

Figure 4. Smad3-specific siRNA and not its negative control siRNA suppresses TGF-β-induced Smad3 phosphorylation, Smad3 and TIMP-3 protein expression in human knee chondrocytes. Articular chondrocytes were either treated with vehicles (Control) or transfected with 200 nM of negative control or Smad3 small interfering RNA (siRNA) and then exposed to TGF-β for 24 h. In A) specific inhibition of phosphorylated (p Smad3) and total Smad3 is shown without any affect on beta actin level. In B), besides Smad3 (upper panel) and TIMP-3 (middle panel) suppression by Smad3 siRNA, the unaffected beta actin (lower panel) Western blots are depicted. Graphs A shows densitometric normalized value of pSmad-3 and total Smad-3 in percentage where as graph B shows Smad-3 and TIMP-3 proteins. Data are presented as the mean ± SEM of three independent experiments. $P< 0.05$ was considered significant.

Figure 5. Smad2- and Smad3-specific siRNAs down-regulate TGF-β-induced TIMP-3 promoter-luciferase activity in human chondrocytes. Human knee

chondrocytes were transiently cotransfected with the 2 μg of human TIMP-3 promoter-firefly luciferase vector, 0.2 μg of a plasmid expressing Renilla luciferase (internal control) and 200 nM Smad2 or Smad3 siRNA as indicated. Cells were then treated with TGF-β for 24 h. Extracts were analyzed for luciferase activity. The values of firefly luciferase to Renilla luciferase ratios were plotted with Prism 3.0 software. Data are presented as the mean ± SEM of three independent experiments. $P< 0.05$ was considered significant.

Figure 6. Smad4-specific siRNA diminishes Smad4 expression and TGF-β-induced TIMP-3 protein expression in human chondrocytes. Articular chondrocytes were either treated with vehicles (Control) or transfected with 200 nM of negative control or Smad4 small interfering RNA (siRNA) and then exposed to TGF-β for 24 h. Specific inhibition of Smad4 (upper panel) and TIMP-3 (middle panel) and unaffected expression of beta actin (lower panel) by Western immunoblotting are depicted. Graphs shows densitometric normalized value of Smad-4 and TIMP-3 proteins in percentage. Data are presented as the mean ± SEM of three independent experiments. $P< 0.05$ was considered significant.

Figure 7. Design of TIMP-3 promoter deletion mutants and their impact on TGF-β-induced luciferase activity in human knee chondrocytes. A) The starting TIMP-3 promoter fragment is shown in the top panel. By using Kpn I, MSc I and Asc I in combination with Hind III sites (inverted arrows), TIMP-3 promoter segments were cloned in pGL-3 basic upstream of luciferase (LUC) gene. +1 and long arrow indicates transcription start site. Δ, deletion. B) Human chondrocytes were

transiently cotransfected with the 2 μg of original or deleted human TIMP-3 promoter-firefly luciferase vector and 0.2 μg of a plasmid expressing Renilla luciferase (internal control) as indicated. Cells were then treated with TGF-β for 24 h. Extracts were analyzed for luciferase activity. The values of firefly luciferase to Renilla luciferase ratios were plotted with Prism 3.0 software. Data are presented as the mean ± SEM of three independent experiments. $P< 0.05$ was considered significant.

Figure 8. Binding of Smads and Sp1 with the human TIMP-3 promoter by Chromatin Immunoprecipitation (ChIP). Human articular chondrocytes were either treated with vehicle (-) or with TGF-β (+) for 60 min, fixed with formaldehyde, sheared enzymatically and precipitated with the control (IgG) and indicated test antibodies. Following purification of DNA, genomic promoter fragments were amplified by PCR with the primers from the indicated (left of the figure) regions and products analyzed by agarose gel. Input DNA is an aliquot of DNA purified prior to immunoprecpitation.

Figure 1

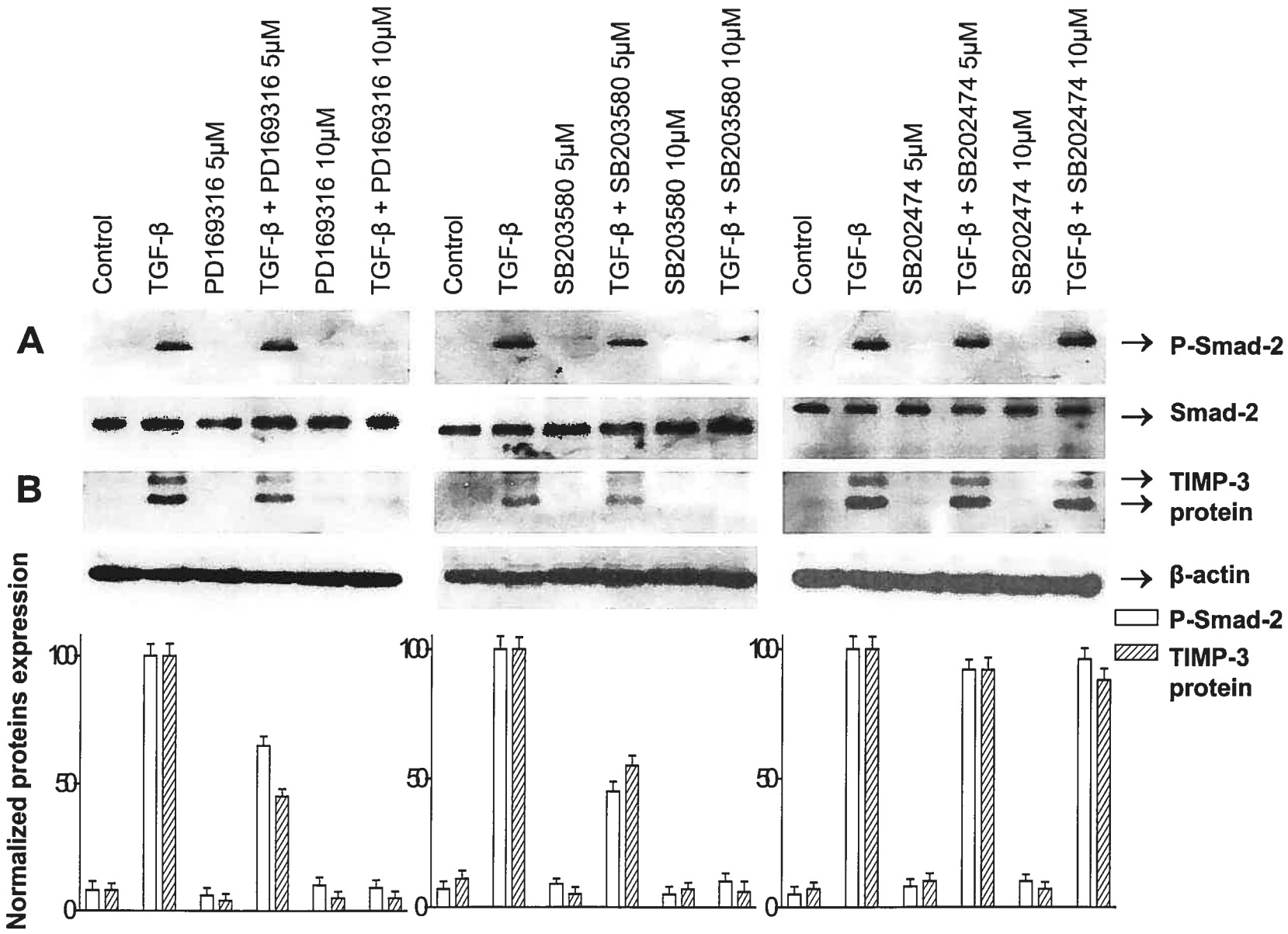

Control
TGF-β
PD169316 5μM
TGF-β + PD169316 5μM
PD169316 10μM
TGF-β + PD169316 10μM
Control
TGF-β
SB203580 5μM
TGF-β + SB203580 5μM
SB203580 10μM
TGF-β + SB203580 10μM
Control
TGF-β
SB202474 5μM
TGF-β + SB202474 5μM
SB202474 10μM
TGF-β + SB202474 10μM
A
B
→ P-Smad-2
→ Smad-2
→ TIMP-3 protein
→ β-actin
P-Smad-2
TIMP-3 protein
Normalized proteins expression
100
50
0

Figure 2

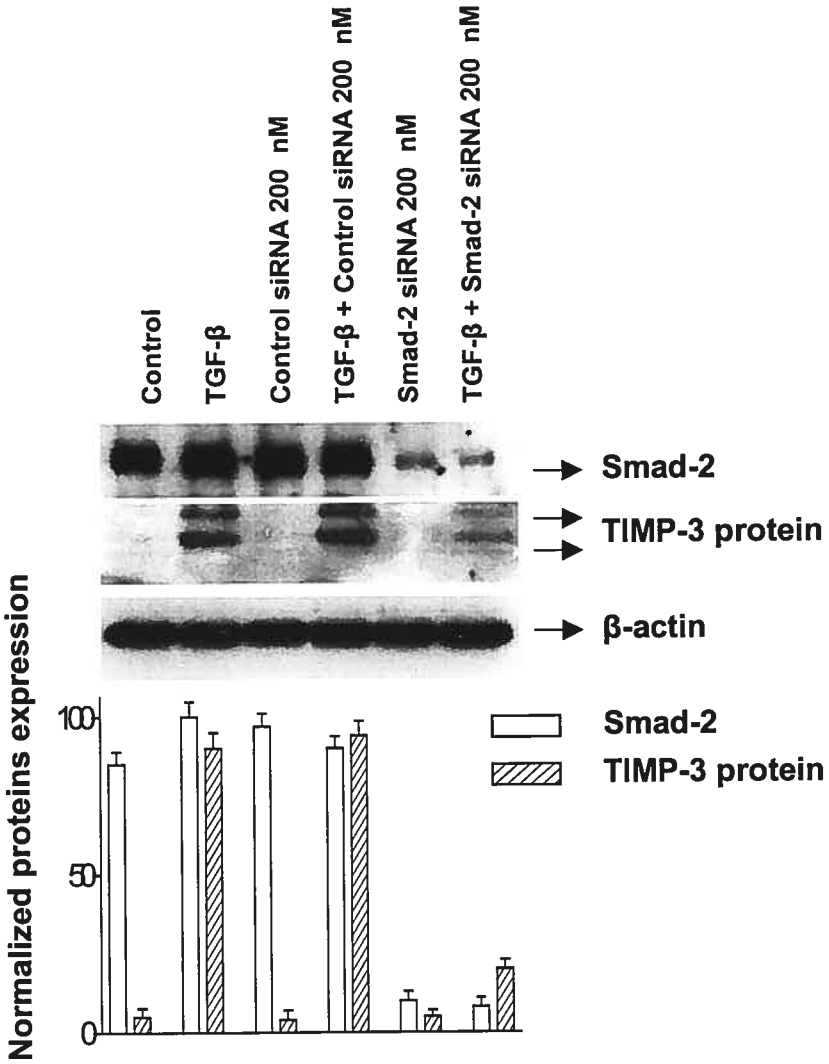

Control
TGF-β
Control siRNA 200 nM
TGF-β + Control siRNA 200 nM
Smad-2 siRNA 200 nM
TGF-β + Smad-2 siRNA 200 nM
Smad-2
TIMP-3 protein
β-actin
Normalized proteins expression
100
50
0
Smad-2
TIMP-3 protein

Figure 3

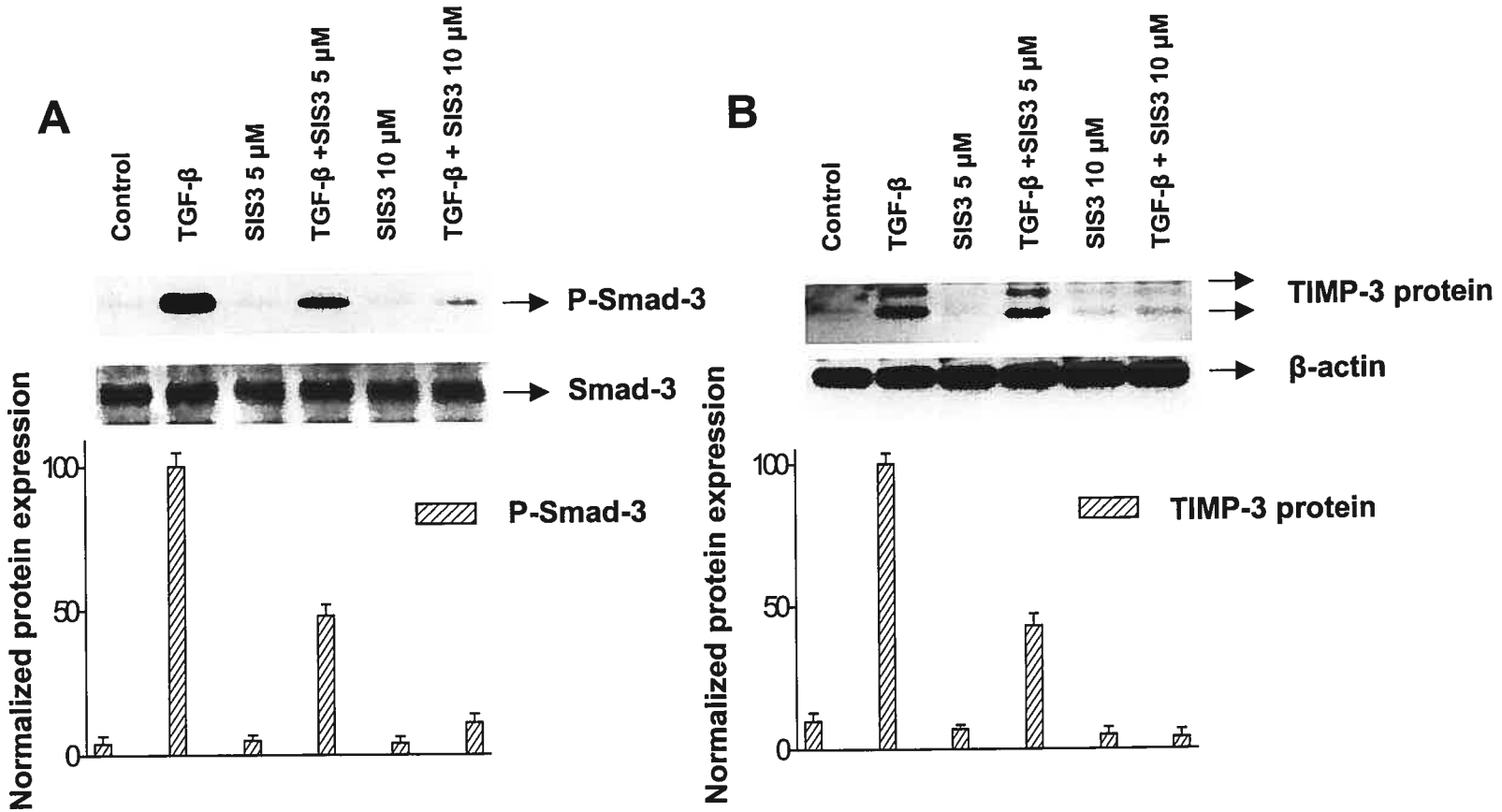
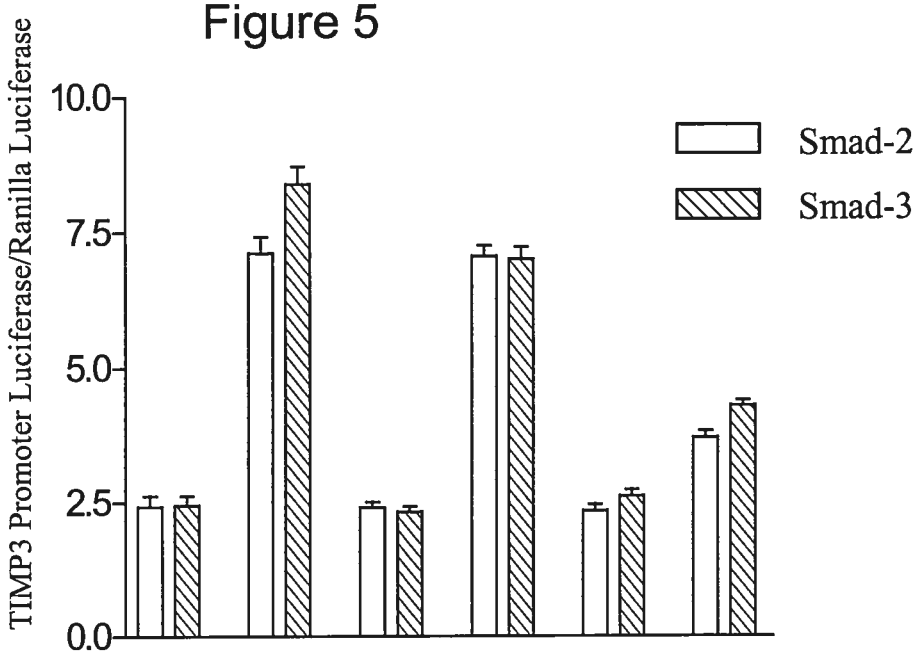

A
Control
TGF-β
SIS3 5 μM
TGF-β +SIS3 5 μM
SIS3 10 μM
TGF-β + SIS3 10 μM
P-Smad-3
Smad-3
Normalized protein expression
100
50
0
P-Smad-3
B
Control
TGF-β
SIS3 5 μM
TGF-β +SIS3 5 μM
SIS3 10 μM
TGF-β + SIS3 10 μM
TIMP-3 protein
β-actin
Normalized protein expression
100
50
0
TIMP-3 protein

Figure 4

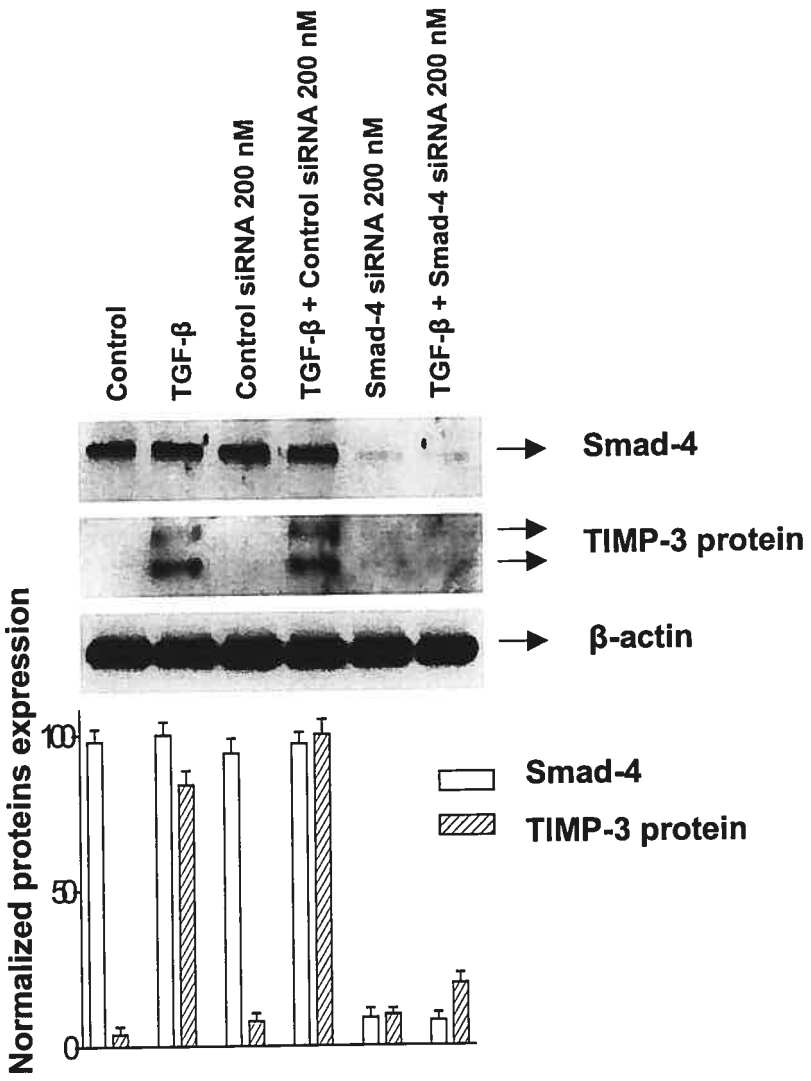
A
Control
TGF-β
Control siRNA 200 nM
TGF-β + Control siRNA 200 nM
Smad-3 siRNA 200 nM
TGF-β + Smad-3 siRNA 200 nM
p-Smad-3
Smad-3
β-actin
Normalized proteins expression
100
50
0
p-Smad-3
Smad-3
B
Control
TGF-β
Control siRNA 200 nM
TGF-β + Control siRNA 200 nM
Smad-3 siRNA 200 nM
TGF-β + Smad-3 siRNA 200 nM
Smad-3
TIMP-3 protein
β-actin
Normalized proteins expression
100
50
0
Smad-3
TIMP-3 protein

Figure 5



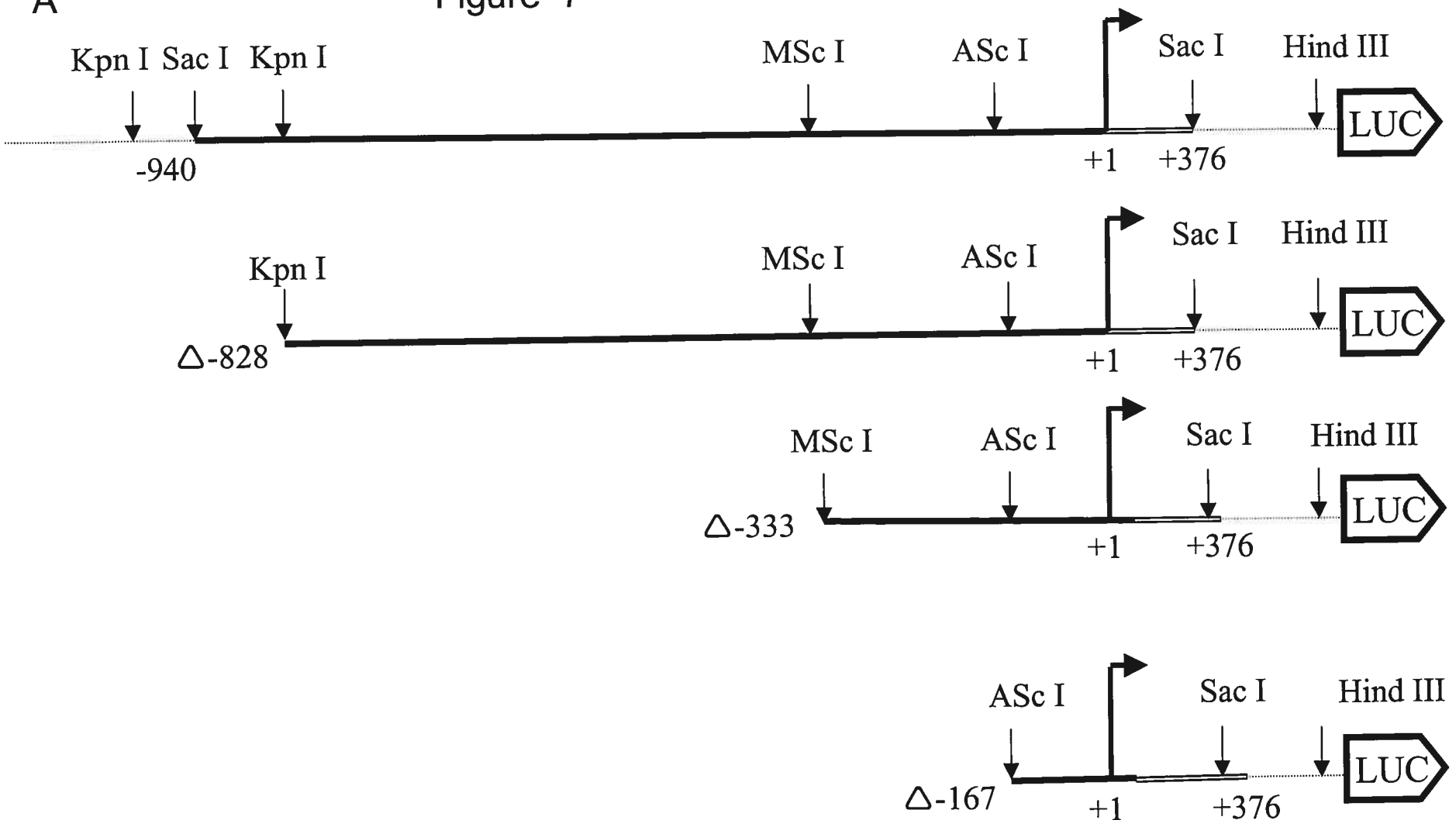
pTIMP3 Promoter luc	2	2	2	2	2	2		µg
pRL-CMV vector (R luc)	.2	.2	.2	.2	.2	.2		µg
SiRNA (negative control)	-	-	+	+	-	-	200	nM
SiRNA (Smad-2/Smad-3)	-	-	-	-	+	+	200	nM
TGF-ß	-	+	-	+	-	+	10	ng/ml

Figure 6


Control
TGF-β
Control siRNA 200 nM
TGF-β + Control siRNA 200 nM
Smad-4 siRNA 200 nM
TGF-β + Smad-4 siRNA 200 nM
Smad-4
TIMP-3 protein
β-actin
Normalized proteins expression
100
50
0
Smad-4
TIMP-3 protein

A

Figure 7



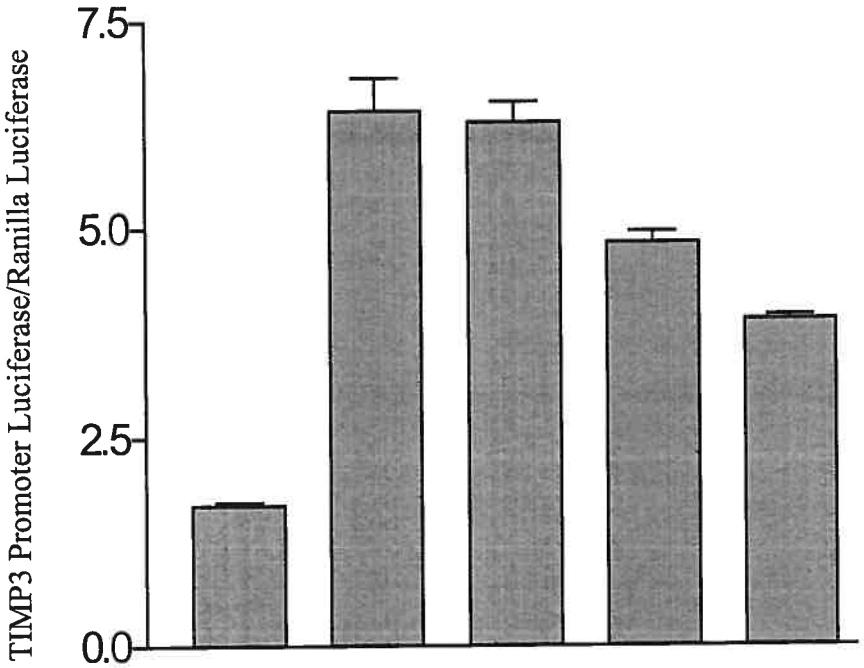
TIMP-3 promoter region

5'Untranslated region of TIMP-3 gene

Polylinker region of pGL-3 Basic

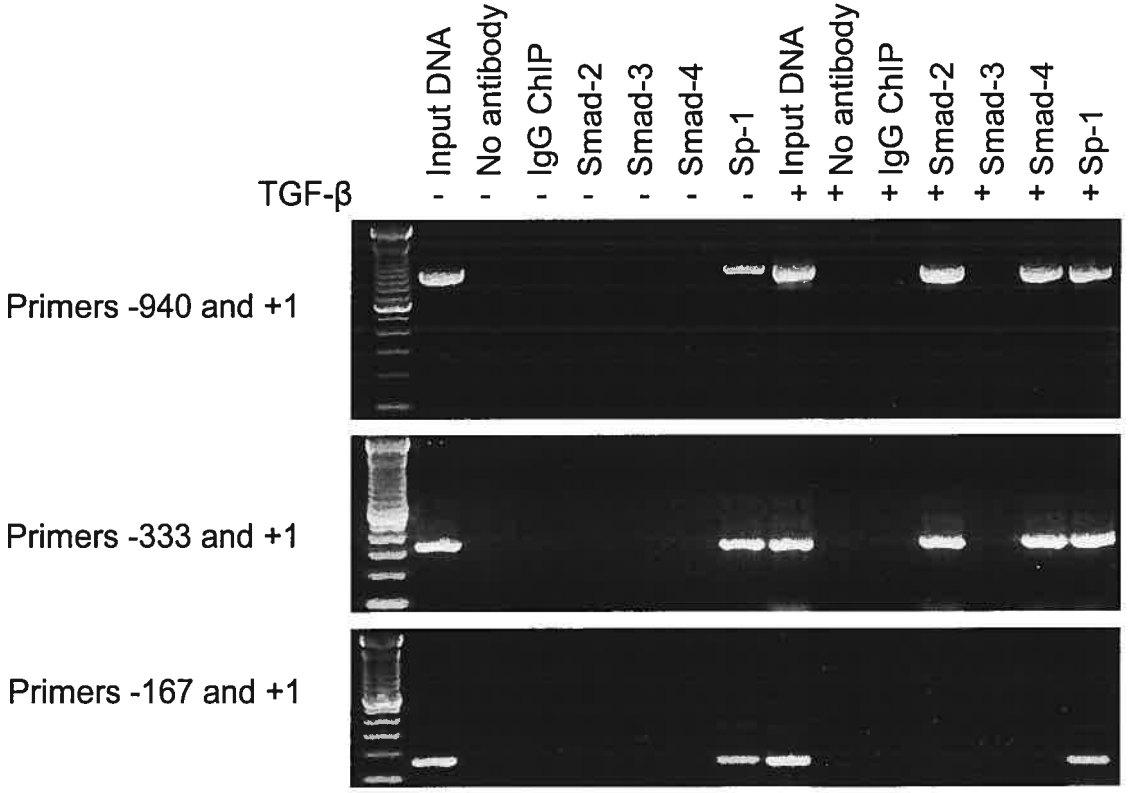
Figure 7

B



pTIMP3 Promoter luc (-940 to +376)	-	2	-	-	-	µg
pTIMP-3 Promoter luc (-828 to +376)	-	-	2	-	-	µg
pTIMP-3 Promoter luc (-333 to +376)	-	-	-	2	-	µg
pTIMP-3 Promoter luc (-167 to +376)	-	-	-	-	2	µg
pRL-CMV vector (R luc)	.2	.2	.2	.2	.2	µg
Mock DNA	2	-	-	-	-	µg
TGF-ß	-	+	+	+	+	10 ng/ml

Figure 8


TGF-β
- Input DNA
- No antibody
- IgG ChIP
- Smad-2
- Smad-3
- Smad-4
- Sp-1
+ Input DNA
+ No antibody
+ IgG ChIP
+ Smad-2
+ Smad-3
+ Smad-4
+ Sp-1
Primers -940 and +1
Primers -333 and +1
Primers -167 and +1

GENERAL DISCUSSION

Extracellular matrix is degraded by MMPs, which are inhibited by TIMPs. There are four types of TIMPs. TIMP-1, TIMP-2 and TIMP-4 are secreted in soluble form, where as TIMP-3 is associated with ECM and is an important protein for cartilage maintenance and repair in arthritis. TGF-β has an important role in cell proliferation, differentiation and apoptosis in different types of cells (197).

In the first part of this study (article 1), we have shown for the first time involvement of ERK/MAPK pathway and that transcription factor Sp1 is required for TGF-β-induced TIMP-3 expression in bovine chondrocytes and human SW1353 chondrosarcoma cells. TGF-β-stimulated phosphorylation of ERKs in both bovine and human chondrocytes (article 1, Fig 1) suggests the involvement of this pathway in two different species. The activated ERKs have been implicated in many studies associated with chondrocytes survival. TGF-β reduced the lipopolysaccharide (LPS)-induced catabolic effects and increased pERK2 levels in human articular chondrocytes (198). Dexamethasone (DEX) suppressed TGF-β-induced proliferation of cultured rat articular chondrocytes (CRAC) and collagen II by inhibiting ERK and AP-1, which suggests the involvement of ERK pathway in CRAC growth (199). ERK pathway is also involved in aggrecan induction by TGF-β in chondrogenic, ATDC5 cells (200). The ERK activation by IGF-1 plays important role in maintaining chondrocytic phenotype (201). As the ERK-MAPK pathway is associated with increased proliferation and maintenance of chondrocytes, prolonged activation of this pathway observed in this study, could

have profound impact on these processes and TGF-β target genes, such as TIMP-3. Apoptosis is one of the mechanisms of cartilage loss in OA (202) and stimulation of the prosurvival ERK pathway by TGF-β may have an antiapoptotic effect in chondrocytes. Lei et al (203) reported that TGF-β signaling enhance the survival of breast carcinoma MCF cells through the activation of ERK (phosphorylated form). Abrogation of TGF-β signaling with dominant negative type II receptors (DNRII) or treating with type I receptor inhibitor increased apoptosis in MCF cells. Downregulation of TGF-β-induced pERK, TIMP-3 protein and RNA by pharmacological inhibitors, PD98059 and U0126, (Article 1, Fig. 2, 3,4) suggest that ERK-MAPK is partly involved in TGF-β-induced TIMP-3 expression in human chondrocytes, a process that is also conserved in bovine chondrocytes. Kwak et al reported similar results for TGF-β-induced TIMP-1 in human fibrosarcoma cells (HT1080). Treatment of cells with TGF-β stimulated the phosphorylation of all three mitogen-activated protein kinases (ERK1/2, p38, JNK) and AKT, but the inhibition of only ERK1/2 pathway by PD98059 and transfection of dominant negative MEK1 inhibited the TIMP-1 induction by TGF-β suggesting the major role of ERK1/2 pathway (204).

Sp1 is an important transcription factor, which is involved in the inducible expression of a number of genes. Sp1 is the member of Sp family of proteins, which are structurally closely related to each other. Structurally, Sp1 protein consists of several domains, N-terminal inhibitory domain, serine/threonine (Ser/Thr)-rich domain, glutamine-rich domain and C-terminal zinc finger DNA

binding domain (ZFDBD). The ZFDBD is highly conserved region among the Sp family proteins and play important role in binding to DNA. The phosphorylation of Ser/Thr is important for the regulation of Sp1 activity (205). TGF-β stimulates serine phosphorylation of Sp1 in fibroblasts from Scleroderma patients (206). Phosphorylation of Sp1 at threonine 453 and 739 enhances binding of Sp1 with its target sequence (207). Sp1 binds to the GC boxes in the promoter regions (205) and human TIMP-3 promoter contains at least 4 Sp1 consensus sites between -112 to +15 flanking region (196). Inhibition of endogenous TIMP-3 RNA and protein induction by the pharmacological agents, mithramycin and WP631, (article 1, Fig. 5, 6) suggest that this transcription factor is required for TGF-β-induced TIMP-3 expression in chondrocytes. Electrophoretic mobility shift assay (EMSA) showed that TGF-β enhanced the Sp1 DNA binding activity while mithramycin downregulated this binding (article 1, Fig. 7). These results were reinforced by the alternative genetic approach. Transfection of Sp1 transcription factor antisense ODN completely knocked down the Sp1 protein leading to inhibition of TIMP-3 protein expression (article 1, Fig, 9), where as the overexpression of Sp1 enhanced TIMP-3 promoter activity (article 1, Fig. 8). The involvment of ERK1/2 followed by Sp1 transcription factor is also reported in TGF-β stimulated TIMP-1 expression in HT1080 cell for anti-invasive and anti-migration activities (204). BMP2 induced osteoblast differentiation in mesenchymal cells (C3H10T1/2) through up-regulation of ERK and Sp1 mediated transcription mechanism (208).

Our results with TIMP-3 gene support the concept that Sp1 is an important link between increased activation of ERK pathway and its target genes (207).

The ERK pathway is a linear cascade of protein kinases, including Ras, Raf, MEK and ERK. ERK pathway is activated by growth factors and is constitutively activated in many cell lines. During the activation of ERK pathway Raf is recruited to the membrane and binds to Ras-GTP. This recruitment of Raf to Ras-GTP is necessary but not strong enough for the full activation of Raf (209). Previous studies have shown that other factors feed into the ERK pathway at Raf and some of the candidate molecules are Src, member of the protein kinase C (PKC) and PI3K (210).

Our results have shown that the inhibitor of ERK pathway, PD98059, does not inhibit the TGF-β-induced activation of PI3K pathway (Article 2, Fig. 1E), whereas the inhibitors of PI3K pathway, LY294002, wortmannin, and NL-71-101 inhibit the PI3K pathway. Based on these results, it appears that TGF-β-induced PI3K signaling is independent of the ERK pathway.

The modulation of ERK pathway by PI3K cascade is controversial. In COS-7 cells, the expression of activated form of p110γ type of PI3-kinase has been reported to stimulate the ERK pathway, but p110α could not. The ERK activity was abolished by wortmannin indicating that PI3Kγ can mediate wortmannin sensitive activation of ERK (211). Wortmannin also inhibits PDGF-induced activation of Raf in CHO cells, indicating that PI3K is required for the activation of Raf in these cells (212). Conversely in the human breast cancer cell line MCF-7,

the IGF-1-induced AKT directly phosphorylated Raf-1 on serine-259 and decreased the Raf-1 activity leading to proliferation. High Raf-1 activity in MCF-7 cells induced growth arrest by activating cell cycle inhibitor $p21^{cip1}$. PI3K inhibitor LY294002 inactivates the cross talk between the two pathways and switches the biological response from proliferation to growth arrest (213).

In our study we have used TGF-β, and secondly IGF-1 does not induced TIMP-3 protein (data not shown), so the downregulation of ERK pathway by PI3K pathway cannot be expected. However, there may be some sort of interaction between the two pathways.

In the second part of this study, we have investigated the involvment of PI3K pathway in TGF-β-induced TIMP-3 expression in human chondrocytes. The involvment of TGF-β-induced PI3K/AKT pathway is well known for its prosurvival role in many cell types i.e. TGF-β-induced PI3K/AKT dependent differentiation of epithelial to mesenchymal cells (214). PI3K/ AKT pathway is also involved in BMP2-induced anti-apoptotic effect in chondrocytic cell line, N1511 (215). Krymskaya et al demonstrated the co-immunoprecipitation of the p85 subunit of PI3K along with both TGF-β receptor I and II, and showed that TGF-β receptor I can modulate the EGF-stimulated PI3K activity in the human airways smooth muscle cells (hASM) (216).

We have shown here by several pharmacological and genetic approaches that PI3K/AKT pathway mediates TGF-β-induced TIMP-3 gene expression through

Sp1 transcription factor activity. We also found that TIMP-3 gene expression is regulated at the translation level via mammalian target of rapamycin. TGF-β induces phosphorylation of PKB/AKT time-dependently in a delayed manner after 4 hours (article 2, Fig.1 B, C, D) as compared to Smad2 and ERK phosphorylation that is induced by TGF-β rapidly within 20 min to 1 hour (217,218). This suggests early events such as cross talk with other pathways or intermediate early factors. Previous studies have reported a link between PI3K and p38 MAPK pathway. TGF-β induces rapid activation of p38 MAPK, which is followed by a delayed activation (12 hours) of PI3K/AKT pathway. p38 MAPK-dependent production of growth factors (possibly FGF-2 or IGF-1) that function in an autocrine manner were responsible for the activation of PI3K/AKT pathway, which confer resistance to apoptosis in human lung fibroblast cells (219). In our study we have tried to find this intermediate growth factor and hypothesized that it may be insulin-like growth factor (IGF-1) but our results showed that TGF-β did not induce IGF-1 at any time point between 20 min to 24 h. IGF-1 also did not induced TIMP-3 expression at various doses in human chondrocytes. Furthermore, blockade of IGF-1 receptor did not affect induction of TIMP-3 by TGF-β. Thus TGF-β effects do not seem to be mediated by IGF-1 (results not shown). TIMP-3 has been shown to induce apoptosis in rheumatoid arthritis synovial fibroblast and in many different cell-types (220,221). However our study suggests the pro-survival role of TGF-β-inducd PI3K/AKT in chondrocytes. Indeed, TGF-β is a cartilage growth-

promoting factor and plays anabolic role in matrix synthesis, cell proliferation and osteochondrogenesis (222).

The TGF-β-induced PI3K survival pathway has been demonstrated in many other cell types. TGF-β induces elastin mRNA through PI3K/AKT pathway in human embryonic lung fibroblast that is inhibited by inhibitor LY294002 (223). TGF-β exerts its growth and antiapoptotic effects through the PI3-kinase/AKT pathway in human RA synovial and normal fibroblasts (224). Insulin induces chondrogenesis of ATDC5 cells mainly by PI3K pathway (225). In chondrocytes, TGF-β stimulated Smad2 and ERK phosphorylation is followed by AKT phosphorylation and these pathways or their interactions are needed for TIMP-3 induction (this work, 217,218). N-terminal human TIMP-3 inhibits the cleavage of glycosaminoglycan release in bovine nasal and porcine articular cartilage explants, treated with IL-1α and very little apoptosis was observed in porcine chondrocytes. (226). Similarly, TIMP-3 may also be anti-apoptotic, as knockout of this gene results in increased apoptosis in mammary gland (63). This is further supported by another study where TIMP-3 promoted proliferation of non-transformed cells under low-serum condition (227). TIMP-1 has also been shown to exert its anti-apoptotic effects through PI3K/AKT pathway in erythroid cells. Bad is a member of Bcl-2 family, which is pro-apoptotic. Bad phosphorylation by AKT leads to its sequestration by 14-3-3 protein and inhibits its heterodimerization with Bcl-X_L, a pro-survival factor with anti-apoptotic activity (228). TIMP-3 role seems to be pro-apoptotic as well as anti-apoptotic and it might be possible that physiological

levels of TIMP-3 promote survival and non-physiological levels induce apoptosis. TGF-β-induced AKT phosphorylation and TIMP-3 protein induction suggests a direct relation between the two events, as demonstrated in our subsequent experiments. Inhibition of TIMP-3 mRNA and protein by two different pharmacological PI3K inhibitors Wortmannin, LY294002 and by siRNA for PI3K (article 2, Fig. 2 A, B, C) strongly support the role of PI3K in TIMP-3 induction. Similarly, suppression of TGF-β-induced TIMP-3 mRNA and protein expression by AKT/PKB inhibitor, Nl-71-101 and AKT siRNA (article 2, Fig. 3 A, B) reinforces the involvement of AKT/PKB in TGF-β signal transduction leading to TIMP-3 induction. It is likely that several growth factors utilize this pathway to promote ECM synthesis, growth and survival.

TIMP-3 promoter-reporter transfection studies suggest that the 5' flanking region spanning –940 to +376 contains most of the TGF-β responsive sequences. This is in agreement with a previous study where region from –463 to +1 was shown to be responsible for serum-stimulated cell cycle progression; serum contains a heterogeneous mixture of growth factors (196). We have also demonstrated the inhibition of TGF-β-induced TIMP-3 promoter-driven luciferase activity by the pharmacologic inhibitors, wortmannin, LY294002 and AKT siRNA (article 2, Fig.4), which showed that this promoter region is the target of TGF-β stimulated PI3K/AKT pathway. Human TIMP-3 promoter contains Sp1 binding sequences, which are important mediators of TIMP-3 expression (218). The involvement of Sp1 transcription factor was reconfirmed by the newly developed RNA

interference-mediated Sp1 knockdown, which resulted in inhibition of TGF-β-induced TIMP-3 gene expression (article 2, Fig. 5A). Sp1 activity was measured by a newly developed ELISA based assay. The inhibition of TGF-β-induced Sp1 activity by the inhibitors Wortmannin Y294002, NL-71-101, and siRNA for AKT further support Sp1 (article 2, Fig. 5B) as a possible target of AKT pathway. Sp1 is a pivotal factor for the expression of various genes related to ECM synthesis, cell cycle and growth (141,229). Sp1 has an important role in the induction of TIMP-1 as demonstrated by mutation analysis and cotransfection of anti-sense oligonucleotide (230). In other systems, increased expression of vascular endothelial growth factor by AKT required Sp1 as demonstrated by Sp1 siRNA-driven knockdown (231). Through the phosphorylation of various substrate, the activated AKT/PKB regulates various biological processes such as cell survival, cell cycle, growth, glucose metabolism and transcription factors that regulate gene expression.

The inhibition of PI3K pathway with wortmannin, LY294002, PI3K siRNA, AKT inhibitor, NL71-101, and AKT siRNA (Article 2, Fig. 2A, B, C, 3A, B) all have shown decreased expression of TIMP-3 protein indicating the blockade of PI3K pathway. This effect clearly seems to be due to the downregulation of mTOR, a downstream effector of AKT. The inactive AKT cannot activate mTOR, which regulates the protein translational machinery through the phosphorylation and activation of p70S6 kinase. The inhibition of mTOR by rapamycin (Article 2, Fig. 6A) decreased the expression of p70S6 kinase and TIMP-3 protein. This

mechanism appeared to be specific, as the levels of β-actin were not affected. The inhibition of TIMP-3 protein expression by rapamycin seems to be due to decrease in its translation as this drug did not affect TIMP-3 mRNA induction levels. The inactivation of p70S6 kinase simultaneously results in the activation of eukaryotic initiation factor 4E binding protein 1 (4E-BP1), which inhibits the initiation of translation (232,233). The synthesis of the signaling intermediates of ERK and Smad pathways might be under the control of this translational machinery and their expression decreases on inhibition of PI3K pathway leading to complete inhibition of TIMP-3 protein expression. The same type of translational control was also observed in IGF-I stimulated synthesis of proteoglycan in chondrocytes, which requires involvement of the PI 3-kinase pathway and was inhibited by rapamycin (234).

In the third part of this study, by using pharmacological and genetic approaches, we have investigated the involvement of Smad pathway in TGF-β-induced TIMP-3 induction in human chondrocytes. The deletion mutant analysis of TIMP-3 promoter has further revealed that the region between −167 to +376 contains sequences which are responsive for the minimal expression of TGF-β-induced TIMP-3 expression where as the upstream region is also responsible for enhanced expression in chondrocytes.

Inhibition of TIMP-3 induction and Smad2 phosphorylation by pharmacological inhibitors, PD169316 and SB203580 strongly suggested the involvement of Smad

pathway in TIMP-3 regulation whereas the equivalent amount of an inactive inhibitor SB202474 did not suppress Smad2 phosphorylation and TIMP-3 induction (article 3, Fig. 1). The involvement of Smad is further confirmed by using a Smad3 inhibitor, SIS3, which decreases the TGF-β-induced TIMP-3 expression (article 3, Fig. 3). These results were reinforced by the alternative and more powerful genetic approach. The transfection of siRNA for Smad2, Smad3 and Smad4 completely knockdown (article 3, Fig. 2, 4, 6) the respective genes and TIMP-3 expression, indicating a direct correlation between Smad proteins and TIMP-3 gene expression. A slight increase in the total Smad2/3 expression (article 3, Fig. 2, 4) on treatment with TGF-β and control siRNA might be due to induction of these proteins by TGF-β and control siRNA. Similar induction of TIMP-3 promoter-luciferase activity by TGF-β and their inhibition by Smad2 and Smad3 siRNA suggests that TIMP-3 promoter is responsive to these agents and regulation most probably occurs at the level of promoter (article 3, Fig. 5) Thus both receptor-activated stimulatory Smad2 and Smad3 are required for TGF-β-induction of TIMP-3.

The significance of Smad proteins association with chondrocytes survival has been reported in many studies. Immunohistochemical studies in murine OA model have shown that the loss of proteoglycan was associated with reduced level of TGF-β and Smad2 phosphorylation. Severely damaged articular cartilage was completely negative for pSmad2. The reduced TGF-β and pSmad2 expression in damaged cartilage suggest the involvement of Smad pathway and protective role of TGF-β

in cartilage. TGF-β and pSmad2 are upregulated in chondrogenesis (235). In the perichondrium, Smads-mediated TGF-β signaling is well documented in the development of long bones. The infection of organ culture with adenoviral vector containing dominant negative mutant of Smad2 and Smad3 showed that Smad3 is partially required for the proper hypertrophic differentiation of chondrocytes, as the Smad2 can compensate for the loss of Smad3. However, Smad3 is required for regulating chondrocyte proliferation (236). Smad2 mutant mice showed abnormalities in craniofacial development (237) where as Smad3 knockout mice displayed enhanced chondrocyte maturation, developed osteoarthritis, skeletal abnormalities and died within 10 months (79,238,239).

The knockdown of Smad3 decreased proteoglycan synthesis and COL2A1 expression while double kockdown of Smad2/3 resulted in strong inhibition of chondrogeneic differentiation in human mesenchymal cells (240). In fibroblast, COL1A2, COL3A1, COL6A1, COL6A3 and tissue inhibitor of metalloproteases-1 are Smad3-mediated TGF-β target genes (241). Smad3 maintained articular cartilage by inhibiting chondrocyte hypertrophic differentiation and targeted disruption of Smad3 resulted in degenerative joint disease resembling human osteoarthritis (79). A single base mutation from A to T resulting in the change of amino acid asparagines to isoleucine in the Smad3 protein was found in an osteoarthritic patient indicating that TGF-β/Smad3 signaling disturbance is involved in human osteoarthritis (242).

Our study further showed that knockdown of Smad4 resulted in inhibition of TGF-β-induced TIMP-3 expression (Article 3, Fig. 6) in chondrocytes suggesting the involvement of Smad4 in signal transduction. This is in contrast with plasminogen activator inhibitor (PAI) gene, which is induced in the absence of Smad4 (243), though both Smad3 and Smad4 bind with the promoter of the PAI gene (244). Smad4 knockout animals are dwarf and have disorganized growth plate suggesting its role in hypertrophic chondrocyte differentiation and requirement for proper growth plate organization (245). Smad7 is an inhibitory Smad and translocates from nucleus to cytoplasm on TGF-β stimulation, which then competes for binding to the activated type I receptor and inhibits Smad2/3 phosphorylation (246). Our experiments, showed that knockdown of Smad7 by Smad7 siRNA decreased the expression of Smad7 (data not shown). Normally, low Smad7 level could have lead to decreased inhibitory action. This decreased inhibitory action however, could not increase TIMP-3 expression, which might be due to an already stronger TGF-β interaction with TGF-β receptors and Smad2/3.

The TGF-β responsive elements in human TIMP-3 promoter have not been defined. Sp1 sites in this fragment are important for its regulation by ERK and AKT pathways (218,247). In order to see the degree of responsiveness by various regions of human TIMP-3 promoter, we have made serial 5'deletions of the larger TIMP-3 promoter. Transfection studies of these mutants have revealed the requirement of minimal fragment Δ–167 for TGF-β-induced TIMP-3 promoter luciferase activity (article 3, Fig. 7B) Additional upstream sequences may also be

important for full responsiveness to TGF-β. Gene3 software analysis has shown that this fragment contains 4 Sp1 binding sites. The sequences analysis has also shown that upstream fragment in TIMP-3 promoter contains number of Smad binding elements (SBEs) as shown in Fig. E. Our study clearly shows that these SBEs are co-operatively enhancing TIMP-3 promoter luciferase activity.

Chromatin immunoprecipitation analysis revealed that Smad2, Smad4 and Sp1 antibodies were able to immunoprecipitate –940, -333 and –167 TIMP-3 promoter fragments (article 3, Fig. 8) showing the in vivo binding of these proteins with DNA. The expected Smad3 binding was not observed, which may be due to the lack of ability of Smad3 antibody to bind with Smad3 in contact with DNA.

One possibility is that Smad2/3/4 complex bind directly with SBEs in the TIMP-3 promoter region. Another possibility is that Smads may interact with Sp1 directly or may be through some third partner to activate TIMP-3 promoter. Smad-Sp1 interaction has been reported in many studies. Smad3-Sp1 interaction was observed for COL1A2 promoter in the TGF-β-induced normal fibroblasts (248). Smad3-Sp1 interaction is needed for full erythropoietin promoter activity (249). Smad2, Smad3 and Smad4 complex interact with Sp1 in the induction of cyclin-dependent kinase inhibitor [p15(Ink4B)] gene by TGF-β in epithelial cells (250).

A physical interaction between AKT serine threonine kinase and Smad3 modulates the Smad pathway by PI3K pathway stimulated by IGF-1 in hepatocytes. The AKT inhibits Smad3 activation by inhibiting its phosphorylation, complex formation with Smad4 and nuclear translocation. The AKT inhibits the

phosphorylation of Smad3 by inducing the membrane localization of AKT, which directly binds to Smad3 and prevent Smad3 availability to TGF-β receptor I. TGF-β induced pSmad3 does not bind with AKT. High levels of pSmad3 induce apoptosis in hepatocytes, whereas AKT antagonizes this effect and promotes survival (251, 197). In our study the PI3K pathway is activated in a delayed manner by TGF-β, which might have played some anti-apoptotic role and promoted TIMP-3 induction, which has a pro-survival role. Although TIMP-3 has been implicated in apoptosis in other cell types, we have not observed any apoptosis in chondrocytes by TGF-β induction of TIMP-3.

Conversely, another study has shown that PI3K pathway modulates positively Smad3 transcriptional activity. The COL1A2 expression by TGF-β-stimulated Smad3 was partially blocked by LY294002 and AKT dominant-negative construct in the human glomerular mesangial cells. The Smad2 and Smad3 C-terminal phosphorylation was not affected by LY294002, whereas the total serine phosphorylation was decreased. The transcriptional activity of Smad3 mutated at C-terminal site was inhibited by LY294002, suggesting that activation of Smad3 by the PI3K pathway is not dependent on the TGF-β receptor I phosphorylation target sites. The inhibition of PI3K pathway decreased the total serine phosphorylation of Smad2/Smad3 as well as the association between Smad2/Smad3 and Smad4, indicating the contributory role of PI3K pathway in the formation of complex between Smad2/Smad3 and Smad4 (252). In a mammary epithelial cell line, LY294002 decreased the Smad2 C-terminal phosphorylation

suggesting the involvement of PI3K pathway in TGF-β-mediated phosphorylation of Smad2 (214).

The MAPK pathway inhibitors, PD98059 and U0126 also decreased the TGF-β-induced total serine phosphorylation of Smad2/3 without affecting the phosphorylation in its C-terminal region in human mesangial cells (253). There might be some regulatory role of ERK pathway on Smad pathway. PI3K has been shown to activate ERK in certain cell types (211,212), so the phosphorylation of Smad2/3 by PI3K in response to TGF-β could be mediated by ERK. It seems that MAPK and Smad pathways are directly involved in the TGF-β-induced expression of TIMP-3 protein. Interactions of these pathways will be the subject of future research.

The experiments with inhibitors, sense and antisence RNA and siRNAs have certain limitations. The use of higher concentrations can cause some toxic and non-specific effects. However, the concentrations of these reagents used in our experimentation are non-toxic under our experimental conditions. Methyl thiazole tetrazolium (MTT) viability assay for the PI3K inhibitors and siRNA has shown that the percentage decrease in chondrocytes viability is less than 7% of the control value, which is non significant (data not shown). Secondly, after harvesting, equal amount of RNA and protein was loaded for analysis, so clearly the decrease in the mRNA and protein was due to particular inhibiting agent. Additionally, internal control RNAs (28S) or proteins (total respective proteins or β-actin) levels were

not affected. In many cases, negative controls of pharmacological inhibitors or siRNAs were used to demonstrate the specific effects.

Conclusions and future perspectives

In summary, based on our results, we propose that TGF-β exerts its growth promoting effects on chondrocytes by activating ERK-MAPK, PI3K/AKT and Smad pathways, which in turn could increase Sp1 activity and the formation of Smad complex, which binds to the DNA leading to TIMP-3 induction. Overexpression of TIMP-3 in response to TGF-β and versatile ability of TIMP-3 to inhibit cartilage-degrading MMPs, ADAMTS and TNF-α-activating, ADAM-17 gives it a therapeutic value for arthritis. TIMP-3 binding to heparan sulfate and chondroitin sulfate may result in strengthening of cartilage ECM, increased chondrocyte viability and maintenance of joint tissue integrity. It is also interesting to note that in contrast with the cell proliferation and survival-associated PI3Kα and β isoforms, knockout and specific pharmacological inhibition of PI3Kγ isoform has recently been shown to reduce both synovial inflammation and cartilage erosion in RA-like mouse models (254). Thus, specific isoforms of this pathway have different functions and can be targeted for reducing inflammation or enhancing survival. Further, observed suppression of arthritic inflammation by TGF-β could in part be due to increased TIMP-3 and reduced TACE and TNF-α activities. Thus, a better understanding of the mechanisms of TIMP-3 induction by TGF-β can lead to novel therapies for inhibiting synovial hyperplasia/inflammation and for stimulating cartilage regeneration, the two important goals in treating arthritis. The TGF-β-induced ERK-MAPK, PI3K/AKT and Smad pathways appear to be independent of one another. Nevertheless, based

on the literature, there may be synergy and cross talk between these signalling intermediates as the inhibition of any one of the pathways resulted in inhibition of TIMP-3 protein expression. Thus multiple pathways and their interactions are responsible for the activation of TIMP-3 gene. Such studied will be the subject of future research in the laboratory. The pharmacological inhibition and RNA-interference-mediated knockdown approaches showed that TGF-β receptor-activated Smad2 and Smad3 and co-Smad4 are required for induction of TIMP-3 expression and promoter activity by TGF-β. We have also identified a minimal TIMP-3 promoter fragment needed for this TGF-β response. Thus TIMP-3 is a target of the Smad pathway. These signaling cascades involved in TGF-β induced TIMP-3 expression may have a pivotal role in cartilage ECM synthesis, repair and maintenance.

One question of future interest is the order of activation and possible interaction of these pathways, which need additional work. The future work will also be focused on the nature of interaction of Smad and Sp1 with TIMP-3 promoter DNA. Our initial ChIP analysis has shown that Smad2, Smad4 and Sp1 bind directly in the general TIMP-3 promoter region. One possibility is that Smad2/4 complex bind directly with SBEs in the TIMP-3 promoter region or may interact with Sp1 to activate TIMP-3 promoter. Whether Sp1 and Smad bind directly to each other or through a third partner, like some activating proteins still remains to be answered in chondrocytes. Future studies on detailed protein-DNA interactions and composition of Smad/Sp1 transcriptional complex will generate more precise

molecular mechanisms involved in TGF-β-induced TIMP-3 gene expression. This may require additional mutants and in vitro mutagenesis of specific motifs. Our studies also do not rule out the involvement of additional pathways and transcription factors in TGF-β-regulated TIMP-3.

TGF-β has a major role in chondrogenesis during development and the growth of adult chondrocytes, but based on our study, its effects on matrix production and stability appears to be more pronounced. The term growth is generally used in the context of increase in the number of cells by means of cell division. In our study we have treated the serum-starved chondrocytes with TGF-β for 24 hours and this short treatment did not result in any increase in the number of cells. Analysis by MTT cell proliferation assay, for the viability of the cells, has not shown any significant increase or decrease in the number of cells (data not shown). TGF-β induces certain metabolic responses but alone was unable to promote chondrocyte proliferation in 24 hours time period. However, the long-term treatment of TGF-β (one to two weeks) leads to proliferation, and when used in combination with other growth factors resulted in enhanced proliferation. TGF-β and bFGF combination resulted in 136-fold increase in cell number (255). On the other hand TGF-β has stimulatory effect on the expression of TIMP-3 protein, which is inhibitor of cartilage-degrading MMPs, ADAMTS and TNF-α-activating, ADAM-17. In other reports, TGF-β induced three-fold increase in type II collagen expression in chondrocytes in 24 hours (256), whereas the increased aggrecan expression was observed just after three hours of treament of TGF-β in chondrogenic ATDC5 cells

(200). Both collagen type II and aggrecan proteins are important components in the structure of cartilage matrix; thus, we can speculate that the effect of TGF-β may be relatively more pronounced on matrix production and stability. In summary, we have elucidated some of the early TGF-β signalling mechanisms leading to TIMP-3 induction.

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Déclaration des coauteurs d'un article

Identification de l'étudiant et du programme
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[redacted] [redacted]
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Déclaration de tous les coauteurs autres que l'étudiant
À titre de coauteur de l'article identifié ci-dessus, je suis d'accord pour que Hamid Yaqoob Qureshi inclue cet article dans sa thèse de doctorat qui a pour titre »Signaling mechanism regulating tissue inhibitor of metalloproteinases-3 (TIMP-3) gene in chondrocytes».

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Coauteur. Mohammed El Mabrouk Date 17/04/2007

Coauteur. Muhammad Zafarullah Date 30-4-2007

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Coauteur. Judith Sylvester 25/4/2007 Date

Coauteur. Muhammad Zafarullah Date 30-4-2007

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